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Targeting Transferrin Binding Protein A for Vaccine Development in Neisseria gonorrhoeae

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Targeting Transferrin-Binding Protein A for Vaccine Development in *Neisseria gonorrhoeae*

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

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List of Symbols and Abbreviations

~	approximately
<	less than
>	greater than
±	plus or minus than
%	percent
°C	degrees celsius
α	alpha
β	beta
Δ	delta
Ω	omega
µg	microgram
µl	microliter
µM	micromolar
3D	3 dimensional
ANOVA	analysis of variance
apo-hTf	apo-human transferrin
BSA	bovine serum albumin
C4BP	C4b-binding protein
$CaCl_2$	calcium chloride
CAMP	cationic antimicrobial proteins
CAT	Chloramphenicol marker

CD66	neutrophil marker, carcinoembryoic antigen-related glycoprotein
CDC	Center for Disease Control and Prevention
CDM	chemically defined chelex-treated media
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CO_2	carbon dioxide
CDM	chelex-defined media
CR3	complement receptor 3
DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FbpA	ferric binding protein A
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
$Fe(NO_3)_3$	ferric nitrate
Fe-S	iron- sulfur
fH	factor H
FHBP	factor H binding protein
Fur	ferric uptake regulator
GCB	gonococcal medium base
H_2SO_4	sulfuric acid

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Hb	hemoglobin
Hp	haptoglobin
HpuA	hemoglobin transporter receptor lipoprotein
HpuB	hemoglobin transporter receptor
HIV	human immunodeficiency virus
HmbR	hemoglobin transporter receptor
hTf	human transferrin
HRP	horseradish peroxidase
HS-TBST	high salt tris buffered saline tween20 solution
HSPG	heparin sulfate proteoglycan
kDa	kilodaltons
KU	klett units
ICP-MS	inductively coupled plasma mass spectrometry
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
IMD	invasive meningococcal disease
L	liter
L3H	loop 3 helix
LAMP1	lysosome-associated membrane protein 1
LbpA	lactoferrin-binding protein A

LbpB	lactoferrin-binding protein B
LB	luria-bertani *E. coli* growth media
Lf	lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LS-TBS	low salt-tris buffer saline
M	molar
MeNZB	*Neisseria meningitidis* vaccine against serogroup B
MgCl	magnesium chloride
MIC	minimum inhibitory concentration
MILLIQ	ultrapure water
mL	mililiter
mM	millimolar
mRNA	messenger ribonucleic acid
MrtC-MtrD-MrtE	gonococcal efflux pump
MSM	men who have sex with men
mTncat	mini transposon insertion of chloramphenicol marker
N	Normal
NaCl	sodium chloride
NadA	*Neisseria* adhesin A
$NaHCO_3$	sodium bicarbonate
NANA	N-acetyl neuraminic acid

NaOAc	sodium acetate
NAAT	nucleic acid amplification tests
N-CAM	neural adhesion molecule
NEB	New England Biolabs
NET	neutrophil extracellular trap
NETosis	release of neutrophil extracellular traps
ng	nanograms
nM	nanomolar
OD	optical density
OMV	outer membrane vesicle
Opa	opacity protein
OPTI-C4N	4-chloro-1napthol
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PEA	phosphoethanolamine
PID	pelvic inflammatory disease
pg	picograms
PMF	proton motive force
PMN	polymorphonuclear leukocyte
PorA	porin A
PorB	porin B
RNA	ribonucleic acid

rRNA	ribosomal ribonucleic acid
RPM	revolutions per minute
RT	room temperature
rTbpA	recombinant transferrin-binding protein A
rTbpB	recombinant transferrin-binding protein B
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Supp I	supplement I
Supp II	supplement II
TAE	tris base, acetic acid, ethylenediaminetetraacetic acid
TbpA	transferrin-binding protein A
TbpB	transferrin-binding protein B
TDT	TonB-dependent transporter
TNF	tumor necrosis factor
TMB	3,3',5,5'-tetramethylbenzidine
TFR	transferrin receptor
TFR1	transferrin receptor 1
TFR2	transferrin receptor 2
VA	Virginia
WHO	World Health Organization
WT	wild-type

Abstract

Targeting Transferrin Binding Protein A for Vaccine Development in *Neisseria gonorrhoeae*

By Ashley Greenawalt, B.S.

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

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Neisseria gonorrhoeae, the causative agent of the sexually-transmitted infection gonorrhea, has recently been labeled a superbug. With dwindling treatment options, there is urgent need for a gonococcal vaccine. TonB-dependent transporters (TDTs) are important gonococcal virulence factors that allow the gonococcus to pirate metals directly from host proteins. The TDTs, TbpA and TbpB, are promising vaccine targets because both proteins are expressed and highly conserved in gonococcal strains, and they are not subject to high-frequency antigenic variation. Because the Tbps bind to host protein as their natural function, our immune system does not recognize them as foreign antigens; thus, immunogenicity is weak. Recent work with *Haemophilus parasuis* TbpB has demonstrated that pigs vaccinated with a nonbinding TbpB mutant elicit enhanced immune-cell responses and superior protection against challenge with *H. parasuis* compared to pigs vaccinated with WT TbpB.

The extracellular loop 3 helix (L3H) in TbpA is essential for binding to human transferrin (hTf). Neutral or charge mutations in the L3H are insufficient for abrogating hTf binding; therefore, this study inserted proline mutations in the L3H to further disrupt the helical

structure and abrogate hTf binding. This study generated several single point mutations in TbpA that result in significantly decreased hTf binding. Both TbpA D355P and A356P mutants show significantly reduced hTf-TbpA binding and iron uptake from Fe-loaded hTf. Both TbpA D355P and A356P vaccine candidates will need to undergo structural studies and testing in a hTf transgenic mouse model to characterize their true potential as vaccine antigens.

Chapter 1: Introduction

I. *Neisseriaceae*

A. *Neisseriaceae*

Many changes have affected the classification of the *Neisseriaceae* family (1). The 1933 classification of *Neisseriaceae* included four genera: *Acinetobacter, Kingella, Moraxella,* and *Neisseria* (2, 3). Recent rRNA analysis catalyzed reclassification of *Moraxella* and *Acinetobacter* into the new family, *Moraxellaceae* (2). *Eikenella (4), Simonsiella (4), and Alysiella (5)* have been reclassified into the family *Neisseriaceae*. *Neisseriaceae* is a family in the class of β-proteobacterium and are Gram-negative rods or cocci, often diplococci, are non-motile, and use aerobic respiration (2, 6). *Neisseria* produce oxidase, catalase, and often produce acid from sugars (2, 7).

Neisseria are a part of the normal commensal flora in humans, but a few species are able to act as opportunistic pathogens (8). *Moraxella* resides on the mucosa, and under certain conditions, it can act as an opportunistic pathogen (3). The two neisserial pathogens are *Neisseria meningitidis* and *Neisseria gonorrhoeae (8)*. Human commensal and pathogenic *Neisseria spp.* can reduce nitrate, produce polysaccharide from sucrose, and produce acid from carbohydrates (1).

B. Pathogenic *Neisseria*

i. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is the causative agent for gonorrhea, the sexually-transmitted infection. *N. gonorrhoeae* is a Gram-negative bacterium, which forms diplococci with flattened adjacent sides (3). *N. gonorrhoeae* is an obligate human pathogen with suggestive evidence of

gonococcal infection indicated in the bible (Hebrew Bible; Leviticus 15:1–3 (9)). For thousands of years, *N. gonorrhoeae* has evolved alongside its only host, and recent evidence suggests divergence from *Neisseria meningitidis* (10). This evolution has likely caused pathogenic *Neisseria* to be specific to the human host.

ii. *Neisseria meningitidis*

The main causative agent for bacterial meningitis is *Neisseria meningitidis* (11-13). Like the gonococcus, the meningococcus has diplococcal morphology with flattened adjacent sides (3). Humans are also the only known host for *N. meningitidis.* Contrary to *N. gonorrhoeae*, which is always a pathogen, *N. meningitidis* can be found residing asymptomatically as an upper respiratory tract commensal in approximately 1-25% of healthy humans for days to months (6, 12-15). *N. meningitidis* can produce a capsule that serves many purposes, including aiding in transmission and colonization and providing protection from desiccation, phagocytosis, opsonization, and complement fixation (6, 12, 16). Transformation and horizontal gene transfer allow the meningococcus to switch capsule types as a means of avoiding natural or vaccine-specific protective immunity (6, 17). Meningococcal strains have been grouped into twelve serogroups based on capsule polysaccharides (3). Five serogroups are most commonly associated with meningococcal disease: A, B, C, W, Y (12). Categorization of serogroups is further subdivided into serotypes based on expression of outer membrane proteins, principally porin (3). Some serotypes are more associated with meningococcal disease than others (3). Another meningococcal serotyping is based on antigenicity of LOS antigens (3). While the LOS serotyping is independent of protein serotypes, specific protein and LOS combinations occur together frequency (3).

II. Meningococcal infection

A. Epidemiology and incidence

N. meningitidis is one of the most common causes of bacterial meningitis, and meningococcal infection can also cause sepsis and pneumonia (18). Infants, children, and young adults, especially those living in group settings, are at the highest risk for meningococcal disease (13). The meningococcus colonizes the human nasopharynx in 5 to 30% of people without causing disease (3). The highest incidence of invasive meningococcal disease (IMD) is found in the African meningitis belt, consisting in sub-Saharan countries (18, 19). Other regions in Africa, Europe, and Australia have more moderate levels of IMD incidence, followed by low incidence in the Americas and other regions of Europe (18). Serotypes vary by geographical distribution: serotypes B, C, and Y are more common in the Americans and Europe; whereas, serogroups A,C,W, and X are more prevalent in Africa (18). Serogroup A causes the highest incidence of meningococcal disease and has caused repeated pandemics in the African meningitis belt, as well as Russia and China (6, 20-24). In endemic cases, meningococcal infection in infants is primarily associated with serogroup B, infection in adolescents is commonly associated with serogroup C, and serogroups B and Y are more common in older adults (6, 17, 23, 25-27). Unique to the African meningitis belt, major epidemics of meningococcal disease occur every few years (6). African epidemics usually start at the onset of the dry season and end during the rainy season (6). There is evidence suggesting that several environmental factors such as absolute humidity and dust concentration play roles in the frequency and size of meningococcal disease epidemics in Africa, but other reasons are poorly understood (6, 28).

B. Disease presentation

Transmission of meningococci occurs via respiratory droplets or throat secretions from carriers (3, 13). Sexual transmission of *N. meningitidis* is rare but can occur (6). Inhaled infectious particles attach to mucosal epithelial cells in the nasopharynx or oropharynx, cross the mucosal barrier to invade the bloodstream, and if left untreated, can enter the central nervous system and cause bacterial meningitis (3). Incubation may range between 2 and 10 days; however, death can occur within 24 hours upon dissemination of meningococcal infection (29).

N. meningitidis survives well in the bloodstream, ultimately causing sepsis or meningitis (30). Meningococcal infection can manifest in two disease presentations: meningococcemia or fulminant disease (3). Meningococcemia is characterized by skin lesions and acute bacterial meningitis (3). Skin lesions are key signs of meningococcemia and can rapidly spread in just a few hours (3). Fulminant disease has higher mortality and is characterized by multisystem infection with or without meningitis (3). Symptoms and signs may range in severity, but stiff neck, high fever, light-sensitivity, headaches, confusion, and vomiting may occur (13). Patients deficient in complement are at particularly high risk for systemic infections (3, 31, 32). Meningococcal septicemia is severe, presenting with a hemorrhagic rash and circulatory collapse (13). Even with rapid treatment, 8-15% of patients will die from meningococcal sepsis (13). If patients are not treated, only 50% will survive (13). Survivors of meningitis have a 10-20% chance of developing brain damage, kidney failure, deafness, and may even require limb amputation (13, 33).

C. Detection and treatment

Detection is accomplished using lumbar puncture to collect purulent spinal fluid (13). Initial microscopic detection is confirmed by culturing, agglutination tests, or polymerase chain reaction (PCR) (13). Serotype detection and analysis of antibiotic susceptibility are also performed (13). Currently the WHO recommends treatment with penicillin, ampicillin, or ceftriaxone (13). Ceftriaxone is preferred in epidemic regions in Africa due to limited infrastructure and health resources (13). Treatment should be administered as soon as possible due to rapid onset of severe meningococcal disease (6). Antibiotics immediately halt meningococcal proliferation (34, 35), and intravenous administration can kill all meningococci in cerebrospinal fluid within 3-4 hours (36).

D. Meningococcal vaccines

There are 12 serogroups of *N. meningitidis*, and 5 are the most common: A, B, C, W, Y (12). All serogroups except A contain sialic acid derivatives, and the polysaccharide of serogroup B, alpha (2-8) *N*-acetyl neuraminic acid (NANA), is identical to human N-CAM (neural cell adhesion molecule). Not only does serogroup B polysaccharide elicit poor immunogenicity, there was a concern for safety because the identical polysaccharide poses a risk of cross-reactivity to self-antigens (37, 38). Due to these aforementioned concerns, a protein-based vaccine specific for serogroup B was only just recently developed, despite the high prevalence of serogroup B disease worldwide (12). Between 2004 and 2008, over one million people were vaccinated in New Zealand using an outer-membrane vesicle vaccine against *N. meningitidis* serogroup B (MeNZB) (39). The Bexsero vaccine, replaced MeNZB, as the first multicomponent vaccine to provided protection against meningococcal serotype B strains (12). The Bexsero

vaccine is comprised of four protein antigens: factor H binding protein (FHBP) fused to GNA2091, neisserial adhesion A (NadA), neisserial heparin-binding antigen (NHBA) fused to GNA1030, and the MeNZB outer membrane vesicle (OMV) NZ98/254, which contains PorA and several other antigens (12, 39, 40). Fusion with GNA2091 (41) and GNA030 (42) increase immunogenicity and serum bactericidal titers. NadA is important for adhesion during meningococcal colonization, invasion, and pro-inflammatory cytokine induction (12). NHBA is present in almost all meningococcal strains and inhibits bactericidal activity by binding heparin (12). In MeNZB, PorA is antigenically diverse and is the main target for bactericidal antibodies (39, 43). Recent analysis has demonstrated that Bexsero may provide protection against gonococcal infection (39, 44).

III. Gonococcal infection

A. Epidemiology and incidence

Reported cases of gonorrhea are much lower than actual cases due to lack of testing sites and resources, poor reporting, and high prevalence of asymptomatic infections that cause a patient to not seek out medical treatment (45). The CDC recently released an updated report suggesting that there were an estimated 1.6 million new cases of gonorrhea per year in the United States in 2018 (46). The WHO has estimated that there were approximately 87 million new cases of gonococcal infection worldwide in 2018 (47).

B. Gonorrhea

i. Symptomatic, uncomplicated gonococcal infection

N. gonorrhoeae targets mucosal sites, including the following: urethra, cervix, pharynx, conjunctiva, and rectum (9). Urogenital infections begin as cervicitis in women or urethritis in

men (9). Men initially present with urethral purulent exudate, dysuria, and testicular discomfort (48, 49). In women, the primary site of infection is the endocervical canal, and common symptoms include vaginal discharge, dysuria, and pelvic pain (9, 50-52). In women, gonococci can also infect the Bartholin's glands, situated adjacent to the vaginal introitus, resulting in pain, edema of the labia, and abscess formation (53). Symptomatic infection is considered uncomplicated: symptoms direct the patient to seek treatment, but often infections do not cause symptoms. Gonococcal infection is not protective against reinfection, and may even increase susceptibility (54).

ii. Asymptomatic, complicated gonococcal infection

Complicated gonococcal infection in men may include orchitis, epididymitis, penile lymphangitis or edema, and post-infectious urethral strictures (49). Men are more likely than women to exhibit signs and symptoms of gonococcal infection, resulting in a higher incidence of severe secondary sequelae in women. An estimated 10- 43% of gonococcal infections in men are asymptomatic (9, 48); whereas, approximately 50- 80% of cases in women are asymptomatic (9, 49, 55). If cervicitis is left untreated, the infection can ascend the reproductive tract and cause severe secondary sequelae such as chronic pelvic pain, pelvic inflammatory disease, endometritis, infertility, salpingitis, and ectopic pregnancy (45, 56, 57).

iii. Pharyngeal and rectal gonococcal infection

Gonococcal infection of the rectum and pharynx are often asymptomatic; therefore, infection at these sites are more difficult to detect (49). Rectal gonococcal infections present with pain, bleeding, discharge, and proctitis (51).

iv. Conjunctivitis

Gonococcal infection of the eye causes conjunctivitis and can cause blindness if untreated. Conjunctivitis in adults is primarily caused by autoinoculation. Conjunctivitis is common in neonates, where the eyes of newborns are infected during birth through passage of the birth canal. Conjunctivitis is the leading cause of blindness in neonates. If gonorrhea screening and treatment do not occur during pregnancy, passage through the birth canal exposes the infant to gonococcal infection, causing conjunctivitis. Conjunctivitis can be treated with antimicrobial drops; however, as the incidence of antimicrobial resistance increases, conjunctivitis will be even more difficult to treat.

v. Disseminated gonococcal infection

Disseminated gonococcal infection (DGI) occurs when a localized gonococcal infection invades the bloodstream. DGI is characterized by skin lesions, polyarthralgia, and tenosynovitis (58, 59). Only approximately 0.5% to 3% of gonococcal infections disseminate and cause DGI (60). Clinical manifestations of DGI have been characterized in two stages: the bacteremic stage and a stage where the infection has been localized to the joint with suppurative arthritis (58).

To survive in the bloodstream, gonococcal strains that cause DGI are serum resistant (61-63). Complement is known to be important in managing DGI. Approximately 13% of patients with DGI have deficiencies in complement. Patients deficient in complement have been found to have a high rate of recurrent bacteremic gonococcal infection (58, 64-66). DGI is frequently caused by strains with the porin 1A serotype (58, 67). Porin 1A binds to factor H (fH) and C4b-binding protein (C4BP) to downregulate complement activity (58, 68, 69).

Women are more susceptible to gonococcal infection during menstruation due to changes in hormones and an increase in pH (70, 71). Women are even more susceptible to gonococcal infection during the proliferative stage, due to high estradiol levels, compared to the luteal stage (72, 73). During menstruation, the phenotypes of gonococci change, causing a higher risk for dissemination (58). Approximately 50% of women with DGI observe symptoms within one week of the onset of their menstruation cycle (58). Pregnancy, intrauterine devices, and history of pelvic surgery can also increase risk of dissemination (60).

Strains auxotrophic for arginine, hypoxanthine, and uracil (*Arg*$^{-}$*Hyx*$^{-}$*Ura*$^{-}$) are highly resistant to penicillin G and have been associated with asymptomatic gonococcal infection of the urethra and DGI (74). DGI and asymptomatic urethral infections are less common in men who have sex with men (MSM) (74). Evidence suggests that auxotrophic strains are less common in MSM, which may explain the difference in infection phenotype (74). Strains that are auxotrophic for arginine, hypoxanthine, and uracil appear to be more virulent (61, 75). Pharyngeal infection may also favor dissemination of gonococci, but the reasons for this is unknown (61). Recommended treatment of DGI is intravenous administration of 1 g ceftriaxone, and 1 g oral azithromycin will be added for suspected coinfection with *Chlamydia trachomatis* (60). The CDC has recently made the recommendation to discontinue use of azithromycin for uncomplicated gonococcal infection; however, no recommendations have been made for DGI (76).

IV. Detection, antibiotic resistance, and treatment

A. Detection

Gonococcal infection can be detected in several ways, including direct detection of gonococci from first-catch urine or swab specimens of urogenital, anorectal, pharyngeal, or conjunctival mucosal sites (77). Detection can be performed by culturing bacteria from samples, performing light microscopy of stained smears, or by performing nucleic acid amplification tests (NAATs). Light microscopy of stained smears can be used to visualize gonococci in urethral or cervical mucous, but this technique is more specific and sensitive for detection in urethral discharge (9). Gonococcal detection via NAATs is highly-sensitive: NAATs testing is approximately 95% sensitive and specific in cervical swabs as well as urethral swabs and first-catch urine in men (78). As NAATs become more routine, it has shown that rectal and pharyngeal infections are more common than previously thought (78).

The development of commercially-available point of care assays have allowed for faster treatment (79). While these commercial assays are convenient and highly-specific, the wide range of sensitivity leads to a large range of false negative rates, ranging from 13 to 90% (79). Repeat, follow up screening has shown to be effective in preventing transmission and eliminating infection (79).

B. Antibiotic resistance and treatment

Treatment guidelines for gonococcal infection have changed often since the 1930's **(Table 1)** (49). Sulfonamides were the first antibiotics used to treat gonococcal infection (45). As of December 2020, the CDC released new guidelines that recommend discontinuing the use of dual therapy of 250 mg intramuscular ceftriaxone and 1 g oral azithromycin (76, 80). The new

recommendation is 500 g intramuscular ceftriaxone in a sole therapy; however, if infection with *C. trachomatis* is suspected, 100 mg oral doxycycline is recommended twice a day for 7 days (76). Patients with cephalosporin or β-lactam allergies can be treated with 1 g intravenous aztreonam monotherapy. For pharyngeal or rectal gonococcal infections, 2 g of intravenous aztreonam has shown to be effective (81).

Table 1. Antimicrobial resistance

Effective Years (US)	Antibiotic	Mechanism	Conferred Resistance
1938-1942	Sulfonamides	Folate synthesis	SNPs in *folP* that reduce sulfonamide affinity for DHPS enzymes
1944-1987	B-lactams: penicilliin	Cell wall synthesis	Plasmid-mediated β-lactamase (TEM-1 and TEM-35); *penA* mutations that decrease acylation of PBP2; *ponA* mutations that decrease acylation of PBP1; *mrtR* promoter mutations that increase levels of MtrCDE efflux pump; *pilQ* mutations that decrease influx
~1962-1986	Tetracycline	Inhibits protein synthesis; targets ribosomal 30S subunit	*tetM*; ribosome protection; SNPs in *rpsJ*; reduction of affinity for 30S ribosomal subunit protein S10 (V57M)
~1967-1986	Doxycycline	Inhibits protein synthesis; targets ribosomal 30S subunit	*mtrR*; *porB1B* SNPs
~1962-1986	Spectinomycin	Inhibits protein synthesis; targets ribosomal 30S subunit	16S rRNA SNP (C1192U) reduces spectinomycin binding; *rpsE* mutations (30S ribosomal protein 5) disrupt spectinomycin binding
~1990-2007	Quinolones	Inhibits DNA gyrase	QRDR mutations in *gyrA* and *parC* reduce drug binding to DNA gyrase and topoisomerase IV, respectively; overexpression of NorM efflux pump
Early 1980s-present	Macrolides: azithromycin (used in dual therapy with ceftriaxone)	Inhibits protein synthesis; targets ribosomal 50S subunit	23S rRNA SNPs result in a 23S rRNA target (peptidyltransferase loop of domain V) with a reduced affinity for the 50S ribosomal macrolide target; *mtrR* promoter mutations (see above); *erm* genes (*ermB, ermC*,and *ermF*) encoding rRNA methylases that methylate nucleotides in the 23S rRNA target that block the binding of macrolides; MacAB efflux pump overexpression increases the MICs of macrolides; *mef*-encoded efflux pump exports macrolides out of the bacterial cell and increases the MICs of macrolides
~1980-present	Third-generation Cephalosporins	Inhibits cell wall synthesis; β-lactam	Non-β-lactamase mechanisms described; highly remodeled PBP2 due to mosaic *penA*

V. Gonococcal vaccine challenges

A. High-frequency antigenic variation

Developing a gonococcal vaccine has proven to be challenging due to the ability of the gonococcus to undergo high-frequency antigenic or phase variation on surface antigens (45). High-frequency antigenic variation allows the pathogen to exponentially expand the repertoire of protein expression on the surface; thus, the host is challenged with producing antibodies to the vast, changing antigenic surface components (82). There are two types of high-frequency antigenic variation including RecA-dependent and RecA-independent variation. During DNA replication, RecA-independent high-frequency phase variation occurs during slipped-strand mispairing, resulting in a reversible translational frameshift (83).

B. Human gonococcal vaccine trials

There is increasing evidence that the Bexsero vaccine elicits some level of protection against gonococcal infection. One study characterized antibody production elicited by vaccination of rabbits with Bexsero antigens that were cross-reactive to *N. gonorrhoeae* (39). This is further supported by the sequence conservation between the meningococcal and gonococcal proteins, except NadA, which is not present in the gonococcus (39). While the gonococcus does not have a capsule, many of the meningococcal proteins are highly similar to gonococcal proteins.

VI. Virulence factors

A. Type IV pili

An important virulence factor, located on the gonococci surface, is the type IV pilus **(Figure 1.1)** (84). Pili are hair-like structures, approximately 6 nM in diameter, that extend from the surface of the gonococci and promote adherence to secretory (non-ciliated) epithelial cells

(85-87). The primary component of the type IV pilus is the polypeptide, pilin (12-22 kDa) (84). Biogenesis of the pilus is associated with 23 proteins, and begins with assembly, maturation, counter-retraction, and emergence (88). The main pilin subunit, PilE, assembles to create the helical structure of the type IV pilus (88, 89). The gene, *pilE*, is subject to high-frequency antigenic variation via RecA-dependent recombination, resulting in variations of PilE expression (90, 91). Nonreciprocal gene conversion occurs between *pilE* and the silent *pilS* genes (91, 92). Function of the type IV pilus is modulated by PilC, PilV, and PilX (90). Gonococcal pilin expression is subject to phase variation, turning expression on or off (92). Phase variation often occurs after deletion of *pilE1*, *pilE2* or both pilin genes (92).

Depending on the cell line, pilin may promote or prevent invasion (87, 93). The gonococcus extends and retracts the pilus to promote invasion of cells (85). Pili bind to host complement receptors type 3 (CR3) and allows for invasion into cervical epithelial cells by rearranging host actin to produce membrane ruffling (94-96). Subsequent to membrane ruffling is the formation of macropinosomes, large vacuoles which contain gonococci (95).

B. Opacity proteins

Opacity proteins (Opa) are outer membrane proteins that are able to promote adhesion, and in some cases, invasive phenotypes in gonococci **(Figure 1.1)** (97, 98). Residing in the outer membrane, the Opa structure consists of eight transmembrane domains and four surface-exposed loops that contain hyper-variable and semi-variable regions which allow the gonococcus to change expression of the opas via recombination of Opa proteins intergenically or between strains (99). The gonococcus encodes approximately 11 Opas; whereas, the meningococcus encodes 4 to 5 Opas. Opa undergoes phase variation via slipped-strand

misparing during DNA replication (100, 101). Opa phase variation occurs at the pentameric pyrimidine tandem repeat sequence, located at the 5' end of the signal peptide sequence (100, 101). Slipped-strand mispairing allows for varied Opa expression; even in a single colony, each opa can be turned on or off.

Opa proteins are responsible for essential adhesion, which promotes colonization. Opa proteins recognize and have specific tropism to human carcinoembryonic antigen cell adhesion molecules (CEACAMs) or heparin sulphate proteoglycans (HSPG) (99). Different CEACAMs have select tissue tropism: CEACAM3 is expressed exclusively on neutrophils, and CEACAM1 is expressed on neutrophils, epithelial cells, and lymphocytes (99). CEACAM3 binding triggers engulfment of gonococci by neutrophils and subsequent bactericidal killing (99). Opa binding to CEACAM1 on CD4+ T lymphocyte cells suppresses activation and proliferation (102). Opa binds to CD66 to facilitate transcytosis of *N. gonorrhoeae* across polarized epithelial cells (103).

C. Porin

Of the pathogenic *Neisseria*, the most abundant outer membrane proteins belong to the class of porins, an important virulence factor **(Figure 1.1)** (58, 104). Comprised of a trimeric β-barrel structure, porin serves as an aqueous anion channel (104, 105). Meningococcal and gonococcal porins share approximately 60-70% amino acid similarity with a moderate level of antigenic variability (106-108). *N. meningitidis* expresses both PorA (45 kDa) and PorB (33 kDa) (104, 109); whereas *N. gonorrhoeae* produces one of two types of PorB: PorB1A (35 kDa) or PorB1B (37 kDa) (110).

Porin is not only able to interact with eukaryotic cell membranes (111, 112), but it is also able to insert itself into mammalian cell membranes to create a functional pore, disrupt

membrane potential, and interfere with cell signaling (104, 113-117). PorB1A of *N. gonorrhoeae* is associated with enhanced cell invasion in vitro (111, 118). The role of the meningococcal porin and invasion of cells has not been as closely evaluated as the gonococcal porins, but there is evidence that porins may reorganize host actin (119), and they may bind to or insert into the membrane of host cells (84, 104, 115, 120).

PorB1A allows for DGI by providing higher level serum-resistance to the complement compared to PorB1B (111). Serum resistance is mediated by PorB1A binding to fH, which inhibits the alternative complement cascade (69, 104). Several other gonococcal strains expressing PorB1A can provide serum-resistance to the classical complement pathway by binding of PorB1A to C4BP (68, 121). PorB in the gonococcus has been shown to induce apoptosis (104, 122-124); however, meningococcal porins have not been associated with increased apoptosis (104, 125). Porin is antigenic and has adjuvant-like qualities such as activation of B cells and other antigen-presenting cells (APC (104)). Porin upregulates CD86 (costimulatory molecule B7-2) in a MyD88-dependent and Toll-like receptor 2-dependent manner (104). Bactericidal and opsonophagocytic antibodies have been found to target porin (105).

D. Lipooligosaccharide

N. gonorrhoeae synthesize lipooligosaccharide (LOS). Gonococcal LOS contains three oligosaccharide chains attached to a lipid A core (126). Lipid A is bound to two heptose residues via two 2-keto-deoxy-mannoctulosnic acid molecules, and oligosaccharide branches from the heptose (126). The length of oligosaccharides and number of branches varies among gonococcal strains (126). The LOS glycosyltransferases (*lgtF, lgtE, lgtA, lgtB, lgtD*) genes control

expression of LOS at the surface (126). Of these LOS glycosyltransferase genes, *lgtG, lgtA, lgtC,* and *ltgD* undergo phase variable due to homopolymeric tracts of poly guanine (G) or poly cytosine (C) tracts (126-128). These homopolymeric tracts cause slipped-strand mispairing during DNA replication, resulting in premature termination and, ultimately, truncation of LOS (126, 127). Occurring at a frequency of 10^{-2} to 10^{-3} in culture (127), phase variation allows the gonococcus to change the outer LOS composition to evade an adaptive immune response (126).

The gonococcus can evade all three pathways of complement activity through sialylation of LOS **(Figure 1.1)** (126, 129). Sialylation inhibits antibody binding and reduces C1q interaction to inhibit the classical pathway. Sialylation also inhibits the lectin pathway by reducing mannose binding lectin (MLB), and fH binding is increased, resulting in significant down-regulation of the alternate pathway. LOS sialyltransferase (Lst) allows *Neisseria* to sialylate LOS (130). Additionally, sialylation reduces complement activation and inhibits C3 fragment deposition to inhibit opsonization of gonococci. Sialylation is also linked to Opa-mediated invasion in human epithelial cells. Additionally, LOS binds to sperm cells to promote transmission (131).

E. IgA protease

Neisseria spp. colonizes mucosal sites where IgA is the predominant antibody (132, 133). IgA is important for its neutralization activity (133). *Neisseria spp.* release IgA protease as a virulence factor against IgA antibody activity. IgA protease is classified as an autotransporter protein, which is a modular protein comprised of the secreted passenger domain, an N-terminal signal sequence that is exported via the Sec system, and a C-terminal translocator domain (134-136).

Following secretion, the immature form of IgA protease matures after several endo-proteolytic cleavages (137). The mature IgA protease then cleaves IgA specifically at the proline-rich hinge region of the IgA1 heavy chain (137, 138). Each *Neisseria* strain expresses one of two types of IgA proteases: type 1 protease cleaves at a specific proline/serine bond at position 237, and type 2 cleaves at a proline/threonine bond at position 235 (139, 140). The proteolytic activity of IgA protease is unable to cleave IgA2 subclass because it does not have a duplicated octameric amino acid sequence (137).

IgA protease is also able to cleave lysosome-associated membrane protein 1 (LAMP1), which is an integral glycoprotein found in late endosomes and lysosomes (139). Cleavage of LAMP1 results in accelerated degradation, and further promotes survival of intracellular *Neisseria* (139, 141).

While anti-IgA protease antibodies can be detected in the cervical mucous of infected women, the neutralization effect on IgA protease is not significant and thus, not thought to be a strong defense against gonococcal infection (142, 143). Antibodies to IgA protease are also detected in sera of both meningococcal infected patients and asymptomatic carriers (140). IgA protease is not required for gonococcal colonization of the human male urethra, as shown in an experimental human infection study (132). Evidence suggests that IgA is important for transcytosis: *iga* mutants were defective in transcytosis across the epithelium (141).

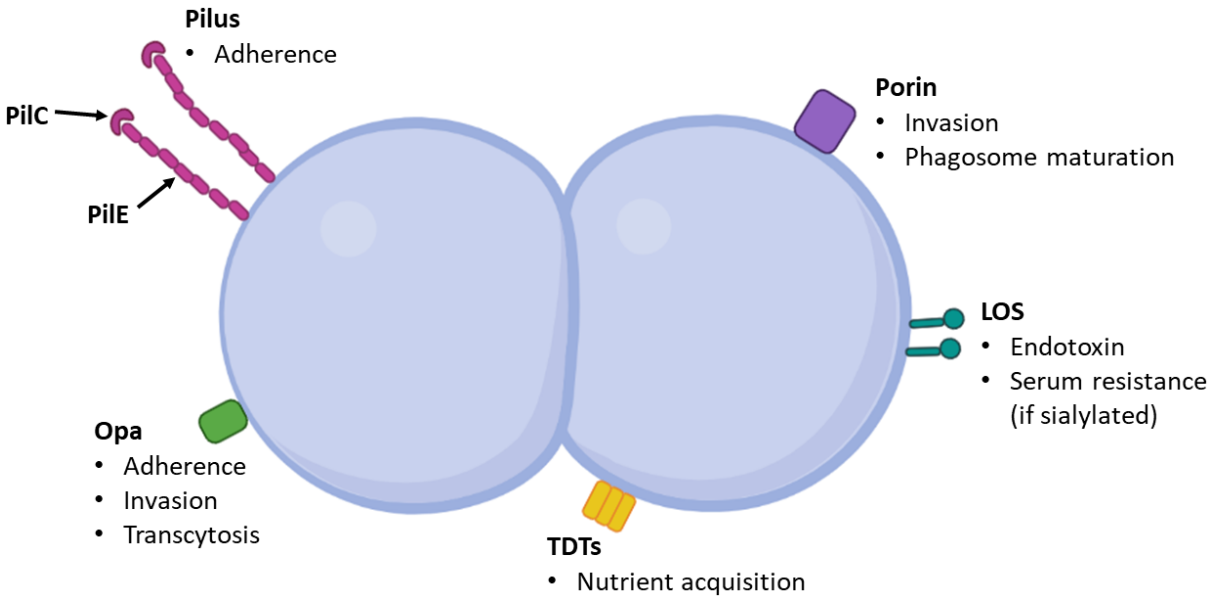

Figure 1.1. Gonococcal virulence factors

Figure 1.1. Gonococcal virulence factors

The gonococcus expresses several important virulence factors. The type IV pilus is important for initial adherence and colonization. Opa proteins bind to host CEACAMs to promote adherence, invasion, and transcytosis. The TDTs are important for acquiring essential nutrients, such as iron. The gonococcus decorates LOS with host sialic acid to evade complement-mediated killing. Porin promotes cell invasion and inhibits complement deposition and intracellular killing. Image made with Biorender.com.

VII. Animal models

A. Animal model challenges

N. gonorrhoeae is an obligate-human pathogen, meaning that humans are the only known reservoir for gonococcal infection. Due to this host specificity, evaluating gonococcal infections in animal models has proven to be challenging. The chimpanzee (*Pan troglodytes*) was the first animal model in which it was possible to initiate genital tract infection (144, 145). Gonococcal urethritis was initiated in three male chimpanzees when human urethral exudates were transferred to the chimpanzee urethras (144, 145). The genital tract model was further characterized by presence of *N. gonorrhoeae* specific complement-fixing antibodies, transmission among the primates, and purulent conjunctivitis was observed in one chimpanzee, presumably caused by auto-inoculation (145). Chimpanzees resemble humans anatomically and physiologically; they share similar blood typing and lysozymes, and chimpanzee IgA can be cleaved by the gonococcal A protease (138, 144). The limited availability of chimpanzees suitable for gonococcal infection studies was further reduced as chimpanzees were reallocated for human immunodeficiency virus (HIV) infection studies in the late 1980s (144). To date, the only other animal model developed to model genital tract gonococcal infection is performed by treating female mice with estradiol (146, 147). Estrus is induced and prolonged following treating with 17β-estradiol; however, antibiotics must be given to reduce the estradiol-induced overgrowth of commensal bacteria that otherwise prevent gonococcal colonization (148). Estrus must be induced because female mice are only able to be colonized transiently by gonococci when mice are in proestrus phase (45). Ovulation in mice initiates robust neutrophil influx; thus, estradiol is used to suppress cytokine production and change expression of Toll-like

receptors and effector cells used in protection from infection (45, 149, 150). The estradiol-treated mouse model has provided much information to the *Neisseria* field: including furthering the understanding of gonococcal colonization and infection, as well as testing vaccination strategies. Recently, an upper-reproductive tract (URT) mouse model has been developed. *N. gonorrhoeae* can be inoculated transcervically directly into the uterine horn of female mice, either in diestrus-stage or treated with progesterone (151).

B. Transgenic mouse model

N. gonorrhoeae is an obligate human pathogen; therefore, it is no surprise that there are host restrictions that prevent effective infection of mice (45). Mice lack several receptors that are key for colonization of gonococci (45). Mice do not express several complement proteins such as fH and C4BP. While chimpanzees express IgA1, mice do not express IgA1 (45). Mice also do not have FcαR (CD89), which serves as the opsonophagocytic receptor for IgA (45).

These host restrictions make modeling gonococcal infection difficult, but these results are also difficult to understand and compare to humans. One of the best ways to make testing in mice more similar to humans is to utilize transgenic mouse models. Transgenic mouse models have been made to express the human transgenes such as human CEACAMs (99); CD46 (152); fH (153); C4BP (154); and transferrin (155). Expression of human transgenes in mice vaccination studies is superior when evaluating host binding proteins, such as TbpA, because this binding is predicted to interfere with the immune response (156, 157). Binding to host may prevent activation of the immune response by disguising foreign antigens as host. By eliminating host binding, antigens are predicted to become exposed and recognized as foreign, thus, eliciting an immune response.

VIII. Innate immune response

A. Cationic antimicrobial peptides

Female genital tract secretions and other mucosa sites contain many antimicrobial components such as fatty acids, progesterone, and cationic antimicrobial peptides (CAMPs (45)). CAMPs are bactericidal and modulate the immune system during infection. Epithelial cells induce expression of CAMPs during infection and phagocytic granules deliver them to the site of infection (45). The first line of defense against host cytotoxins is the gonococcal MtrCDE efflux pump (158). In the rectum, bile salts act to inhibit gonococcal colonization (158). The MtrCDE efflux pump provides enhanced resistance to bile acids, fatty acids, and fecal lipids isolated from rectal cultures (159, 160). Clinically relevant *mtr* mutants confer a range of erythromycin resistance that resembles the same hierarchy of resistance to CAMPS (161). The gonococcal MtrCDE efflux pump captures LL-37 and removes cathelicidin-related antimicrobial peptides (161). Additionally, LOS can be modified with phosphoethanolamine (PEA) substitution on lipid A to inhibit CAMPs from binding to the surface (162).

B. Complement and opsonization

Of the innate immune system, complement is one of the most essential protection systems against *Neisseria* infections (62, 129, 154, 163). Complement is very important for protection against blood infections, and basal levels of complement are naturally high in the female genital tract (45). In the presence of *Neisseria*, pattern recognition receptors (PRR) activate one of two complement systems: the classical pathway or the alternative pathway (133). The classical pathway functions by insertion of the membrane attack complex (MAC) in

the bacterial membrane to cause lysis (62, 63). The alternative pathway amplifies complement component 3 (C3), which allows for opsonization and subsequent intracellular killing (11, 133).

Gonococcal strains range in susceptibility to complement and can be classified as either serum-sensitive or serum-resistant (164). Serum-sensitive strains are generally more susceptible to complement; whereas, serum-resistant strains correlate with complement susceptibility. The ability to resist complement allows for gonococci to invade the blood stream and cause disseminated gonococcal infection (164). Complement-deficient patients are significantly more susceptible to invasive meningococcal infection and DGI, and infections are often recurrent (129, 165, 166). Meningococcal infections are strongly correlated with defects in the alternative pathway, the most common defect being properdin (Factor D), which activates the alternative pathway by cleaving factor B (129). Defects of the MAC are associated almost exclusively with DGI or invasive meningococcal disease (129). Treatment using the complement inhibitor, Eculizumab, which functions by blocking C5 cleavage, is associated with high risk of septic *Neisseria* infections and DGI (129, 167-169). Blocking C5 cleavage prevents activation of neutrophils and production of the oxidative burst, which is important for bacterial clearance (129, 170).

N. gonorrhoeae porin binds to C4BP or fH to inhibit activation of the complement (68, 69, 129). Defense against complement is particularly important in preventing dissemination of both meningococcal and gonococcal infections (31, 58). Complement-dependent killing is also inhibited by LOS sialylation or PEA substitution of lipid A on LOS (162, 171, 172). Additionally, LOS sialylation inhibits opsonophagocytosis (173, 174).

C. Neutrophil killing

Gonococci are resistant to ingestion by polymorphonuclear leukocytes (PMNs) and are adept at surviving intracellular killing (45). To prevent phagocytosis uptake, gonococci sialylate LOS, which inhibits C3b deposition (171, 174-176). Phase variation of surface Opa proteins can generate gonococcal variants that do not bind to neutrophil-specific CEACAMs, allowing the gonococcus to avoid non-opsonic uptake (177, 178). Additionally, gonococcal isolates expressing Opa proteins that bind CEACAM1 can downregulate proliferation of CD4+ T-cells and B-cells to suppress the adaptive immune response (102, 179). Approximately 50% of gonococci are viable inside PMNs, but the reasons for this are still unclear (45). Gonococci are resistant to neutrophil oxidative stress defenses (180-182). Gonococci are susceptible to neutrophil killing despite antioxidant factors including catalase, cytochrome c peroxidase, or quenching via manganese uptake (183-185). Survival of gonococci in PMNs may depend on suppression of the phagocytic respiratory burst (180), the delay phagosome fusion with primary granules (186), or by extracellular release of several peptidoglycans and proteins to promote envelope integrity and resistance to lysozyme (187, 188).

Because Opa proteins are subject to high-frequency phase and antigenic variation, they are not ideal vaccine candidates; however, gonococcal Opa binding to CEACAMs is relevant for discussion in vaccine development (45). The gonococcus binds to CEACAMs depending on differential Opa expression and promotes bacterial adhesion and tissue invasion (99). Gonococci expressing Opas that are specific to non-neutrophil CEACAMs allow the gonococcus to evade phagocytosis; however, human neutrophils constitutively express CEACAM1, CEACAM3, and CEACAM6 and promote effective phagocytosis of Opa-expressing gonococci (99,

189). CEACAM3 is expressed exclusively on neutrophils and upon binding, gonococci are engulfed via non-opsonic phagocytosis and killed via the oxidative burst (99). Because Opa binding to CEACAM3 does not promote bacterial survival, and there is no known nonbacterial ligand for CEACAM3, CEACAM3 is speculated to act as a decoy receptor with the goal of capturing gonococci expressing Opa proteins before they can interact with other cell types (190). If a vaccine is able to induce antibodies to another surface protein, such as TbpA, anti-TbpA antibodies could coat the pathogen and make other receptors on neutrophils, such as CEACAM3, more available for binding to phagocytes. Additionally, induction of opsonic antibodies could promote gonococcal opsonization and opsonic phagocytosis, further boosting intracellular killing of gonococci.

IX. Iron homeostasis

A. Iron regulation in humans

In humans, iron is stored by various proteins including hemoglobin, ferritin, transferrin, lactoferrin, and lipocalin (191). Inside erythrocytes, hemoglobin stores approximately 75% of all the iron in the body, and the remaining 25% is stored by ferritin in liver, spleen, and bone marrow in hepatocytes and macrophages (192, 193). The hormone hepcidin and its receptor ferroportin work together to regulate host iron homeostasis. Ferroportin is a receptor on hepatocytes and macrophages that exports iron into plasma. In iron-replete conditions, hepcidin binds to and causes endocytosis of the ferroportin receptor to prevent cell iron export (194). Hepcidin production is induced in infection and inflammation to decrease available plasma iron (193). Ngo has shown upregulation of hepcidin and downregulation of ferroportin in infection of monocytes and macrophages, resulting in an increase or iron retention (195).

Iron retention in macrophages is advantageous to the gonococcus because it inhibits nitric oxide production, which is important in killing of intracellular bacteria (196). Iron, heme, and hemoglobin retention in macrophages is also induced during inflammation and tissue damage (193, 197).

B. Iron sources in humans

i. Transferrin

Only approximately 0.1% of body iron is found extracellularly and is primarily bound by the glycoprotein transferrin, which acts to chelate and transport iron to cells that require iron (192). Transferrin is an 80 kDa glycoprotein consisting of an N and C-lobe, wherein each lobe binds one Fe^{3+} atom (198, 199). Transferrin is synthesized in the liver, then sent to serum to act as an iron chelator (198). Serum transferrin is found between 12% and 50% saturation of the iron-binding capacity, defined as 60 to 75 µM; therefore, serum iron levels range from 10 to 30 µM (191, 200). Transferrin binds with nanomolar affinity (at pH 7.4) to transferrin receptor 1 (TFR1) or transferrin receptor 2 (TFR2) which are expressed on iron-requiring cells (198). Transferrin binding to TFR triggers clathrin-mediated endocytosis, and endosome acidification releases Fe^{3+} into the cytoplasm (201-203).

ii. Lactoferrin

Lactoferrin, or lactotransferrin, is an 80 kDa glycoprotein that has similar structure and function to the serum glycoprotein transferrin (204-207). Lactoferrin has antimicrobial and anti-inflammatory properties (208, 209). Lactoferrin is synthesized by neutrophils and exocrine glands and is primarily located in human milk and mucosal surfaces (208, 210-216). Lactoferrin has 60% sequence identity with transferrin and shares a similar structure (217). Apolactoferrin,

which has no iron bound, has an open conformation; when iron is bound, the N- and C-lobes close, forming hololactoferrin (218). Like transferrin, lactoferrin binds one ferric iron atom in each lobe (218-220). Lactoferrin binds with many host receptors: CD14, LDL receptor-related protein-1, intelectin-1, TLR4, CXCR4, and heparin sulfate proteoglycans (HSPGs (208, 220)). HSPGs are important for bacterial or viral cell invasion (220-222). Evidence suggests that lactoferrin-mediated cleavage of *Neisseria* heparin binding antigen (NHBA) abrogates binding to HSPGs, thus, inhibiting *N. meningitidis* adhesion to epithelial cells (223).

Neutrophil extracellular traps (NETs) are formed in a process called NETosis (209). NETosis occurs by rupture of neutrophil plasma and nuclear membrane, releasing chromatin fibers and components of neutrophil granules such as lactoferrin, elastase, and myeloperoxidase (224). NETs trap and kill bacteria as well as modulate acute and chronic inflammation (208, 209). NET formation is beneficial for host defense in moderation. NET formation plays a role in massive thrombosis in organs and can trigger or exacerbate autoimmune disorders (209). Lactoferrin suppresses NETs with its positive charge, and is hypothesized to be an endogenous NET inhibitor when NETosis levels are too high (209).

Neisseria are able to pirate iron from lactoferrin using LbpA and LbpB (225, 226). Only 50% of gonococcal strains express LbpA; whereas, all meningococcal strains express a lactoferrin receptor (227, 228). Approximately 30% of *lbpA+* gonococcal strains do not express *lbpB*, implying that LbpB is not essential for the use of the LbpA receptor (225, 229, 230). In a male urethra gonococcal infection model, the lactoferrin receptor is able to support colonization in the absence of the transferrin receptor (225). Together, these findings suggest

that the lactoferrin receptor is not essential for gonococcal infection but does provide an advantage (225).

iii. Ferritin

Ferritin is a hollow globular protein, approximately 450 kDa, that acts as the second largest iron reservoir for the body (231-234). Ferritin stores iron intracellularly in hepatocytes and hepatic macrophages, and ferritin found in serum is derived from the aforementioned sources (194). Serum ferritin is relatively iron poor, and low serum ferritin levels roughly correlate with low iron stored in the body (194, 232). Inflammation, tissue damage, or hyperferritinemia disease can cause increases in ferritin (194, 235). Ferritin holds iron in the cytosol to protect cells from the Fenton reactions involved in iron oxidation and mineralization (236). Iron in ferritin is biologically available and allows for iron to be solubilized (236). Eukaryotic ferritins are highly conserved, and the structure is characterized with two types of subunits, H and L, folding in a 4-helical bundle and assembling into a 24-mer shell (232, 236). Ferritin captures Fe^{2+} in the ferroxidase center where it is oxidized to Fe^{3+} using ferroxidase (236). Iron capture and release from ferritin is still being elucidated (194). During iron-depleted conditions, ferritin binds to nuclear receptor coactivator 4 to import into the cell in a process referred to as ferritinophagy (194, 237-240). Ferritin is sent to the autophagy pathway where ferritin is degraded in lysosomes and iron is released into the cytoplasm (194).

The role of ferritin in *Neisseria* infection has not been evaluated well. Reduced ferritin has been shown to inhibit *N. meningitidis* growth, but no direct interaction has been identified between the pathogen and ferritin (241). *N. meningitidis* has also been shown to accelerate

ferritin degradation to acquire iron in infected host epithelial cells, but the mechanism is not well understood (242).

iv. Heme/hemoproteins

To maintain proper serum iron levels, hemoglobin is responsible for sequestering free heme inside erythrocytes (243). Approximately two-thirds of total body iron is stored in heme groups within hemoglobin, the most abundant protein in blood and erythrocytes (231, 244). The precursor to hemoglobin is heme, a heterocyclic porphyrin ring that binds centrally-coordinated ferrous iron (Fe^{2+}) (231, 244). Heme can be free or bound to hemoproteins, such as hemoglobin, haptoglobin, and hemopexin (231). Hemoproteins bind strongly to heme at one or two of the free iron coordination sites located perpendicularly to the porphyrin ring (231). Imidazole groups on histidine side chains are the most common ligand for coordination of the iron in heme, but methionine, cysteine, and tyrosine are also found (231, 245).

Release of heme from hemoglobin may occur spontaneously or by bacterial proteases (231, 246). Serum proteins, albumin and hemopexin, also rapidly bind and sequester free serum heme (231). Hemolysis, or spontaneous lysis of erythrocytes, releases hemoglobin into serum, where tetrameric hemoglobin then dissociates into dimers that are then rapidly sequestered by haptoglobin (231). Haptoglobin binds hemoglobin almost irreversibly to later be recycled by macrophages (231, 244, 247). Macrophages have an essential role in recycling iron from senescent erythrocytes to newly developing erythrocyte precursors (194).

In bacteremic meningococcal infection in the bloodstream, severe disseminated intravascular coagulation causes erythrocytes to lyse and release hemoglobin in serum (83). Because of the significant amount of free hemoglobin-haptoglobin (Hb-Hp) complexes in serum

during meningococcemia, Hb-Hp could be the primary heme source for the meningococcus (83, 248).

Neisseria spp. acquire iron from heme with the two-component TDT system HbuAB, where HbuB functions as TonB-dependent outer-membrane receptors (249, 250) and HpuA functions as a lipoprotein (83, 247, 251). HpuAB binds to hemoglobin, Hb-Hp, and apo-haptoglobin (83). HpuA and HpuB are required for acquisition of iron in the form of heme from hemoglobin and Hb-Hp complexes (83, 251-254). The meningococcus expresses a second hemoglobin receptor, HmbR, which binds to heme (255). Both *hpuAB* and *hmbR* undergo phase variation due to polyguanine (G) tracts (83, 128, 251).

Meningococcal strains express HpuAB or HmbR or both (256); whereas, *hmbR* is a frameshifted pseudogene in the gonococcus (257, 258). Dissemination into the blood stream is less common with the gonococcus; therefore, acquisition of iron from heme is suggested to be less important than for the meningococcus, which disseminates through the blood stream during infection (258). However, gonococcal strains isolated from the genital tract of women in early menses were more likely to express HpuAB, suggesting that the HpuAB expression is turned phase on by the gonococcus in order to exploit the abundant availability of heme and hemoglobin (128, 259). Some bacteria can use hemopexin as a source of heme as well as secrete hemophores that compete with hemopexin (231). It is still unknown if *Neisseria* are able to extract heme from albumin or hemopexin.

X. Nutritional immunity and iron acquisition

A. Iron and nutritional immunity

Metals such as iron, zinc, and manganese are essential for metabolism in pathogens (192, 260). Concentrations of metals must be regulated as too much will be toxic to both host and pathogen and too little will be deleterious to growth and health (192, 260). Iron is most common metal ion in humans (260, 261). Iron is an essential cofactor for proteins and enzymes because it readily accepts and donates electrons (261). Oxygen and energy metabolism require iron to form iron-sulfur (Fe-S) cluster proteins, heme-binding proteins, and RNA reductases (260, 261). The downstream effects of the aforementioned metabolism allow for heme synthesis, oxygen transport, and DNA synthesis (193, 260). Iron is also important for immune cells such as T-lymphocytes and neutrophils (192, 262). An important part of this complex metal regulation is defined as nutritional immunity. In humans, there are proteins that are used to sequester metals which prevents a nutrient-replete environment for pathogens to flourish and prevents metal toxicity (192). Iron is sequestered by transferrin, ferritin, hemoglobin, haptoglobin, lactoferrin, and lipocalin (191). During inflammation, tissue damage may increase iron sequestration of iron, heme, and hemoglobin by macrophages (193, 197).

B. Siderophores

Siderophores are low-molecular cages (<1 kDa) that bacteria secrete into the environment for the purpose of iron acquisition (263-265). Siderophores chelate iron (Fe^{3+}) with such high specificity and affinity (with a dissociation constant range of 10^{-22} to 10^{-52} M), that iron can be taken directly from host metalloproteins (264, 266). Over five-hundred siderophores have been described and categorized into three main groups: catecholates,

hydroxamates, and hydroxycaboxylates (263, 264, 267). In iron-deplete conditions, iron-regulated genes are derepressed to initiate siderophore synthesis, which occurs in the cytoplasm via non-ribosomal peptide synthetases (264, 267). Following synthesis, siderophores are secreted, scavenge for iron, and transport the iron back to the bacterium (264). Some bacteria, such as *Neisseria,* are unable to produce siderophores; however, they are able to use xenosiderophores, siderophores produced by other bacteria (265, 268, 269). *Neisseria* can utilize salmochelin and enterobactin using the outer membrane receptor FetA (266, 268, 270, 271).

To combat the iron-chelation effects of siderophores, the human host uses the protein siderocalin to sequester siderophores (191, 272). Siderocalin belongs to the lipocalin family of binding proteins (272). Siderocalin is an important defense mechanism against bacterial infection: siderocalin knock-out mice are significantly more susceptible to infection compared to wild-type (WT) mice (273-275). Despite the effectiveness of siderocalins in suppressing infection, bacteria have evolved strategies to inhibit siderocalin binding to siderophores by modifying the siderophore with glycosylation or the addition of glucose molecules (276-278).

C. TonB-dependent transporters

A more reliable mechanism for iron acquisition are the TonB-dependent transporters (TDTs), which are expressed in most Gram-negative bacteria (249, 279). TDTs are highly conserved in *Neisseria* and are present in all strains, suggesting that they are important for survival and virulence **(Figure 1.1)** (280-285). TDTs are characterized by a beta-barrel structure residing in the outer membrane, in which a pore allows for import of essential metals such as iron and zinc into the cytoplasm. Each of these transporters bind to its respective host protein,

such as transferrin and transferrin-binding protein A (TbpA), lactoferrin and lactoferrin-binding protein A (LbpA), hemoglobin and hemoglobin-binding protein B (HpuB), calprotectin and TdfH (286), or S100A7 and TdfJ (287). Under metal duress, pathogens induce expression of these TDTs to allow for sufficient uptake of these essential nutrients **(Figure 1.2)** (192).

TDTs use the proton-motive force (PMF) to power the Ton system, which consists of integral membrane proteins, TonB, ExbB, and ExbD **(Figure 1.3)** (288). As electrons are transported during aerobic respiration, a proton gradient is formed across the inner membrane. This gradient produces the PMF as protons can only cross the cytoplasmic membrane at certain points, including through ATPase complexes (279). ExbB and ExbD complex act as a motor for proton translocation (288). This PMF is capitalized upon by TonB as an energy source. PMF-derived energy enables TDTs to transport ligand into the periplasm against a concentration gradient (288). A periplasmic protein then transports the metal ion to an ABC transporter localized in the inner membrane (279). Any internalized iron is used for essential metabolic processes to allow for infection and pathogenesis (194, 257).

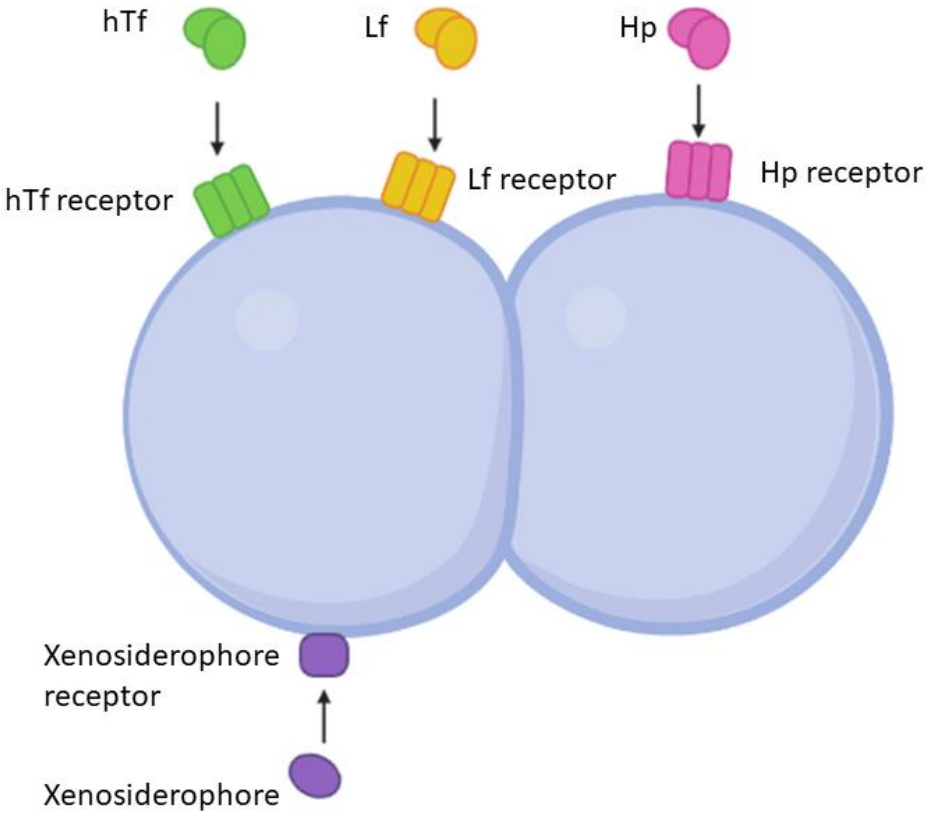

Figure 1.2: Host sources of iron

Figure 1.2: Host sources of iron

The gonococcus expresses outer membrane receptors to directly acquire iron from host proteins including transferrin (hTf), lactoferrin (Lf), and hemoproteins (Hp). The gonococcus also expresses a receptor to recognize xenosiderophores, molecular cages produced by other Gram-negative bacteria. Image made with BioRender.com

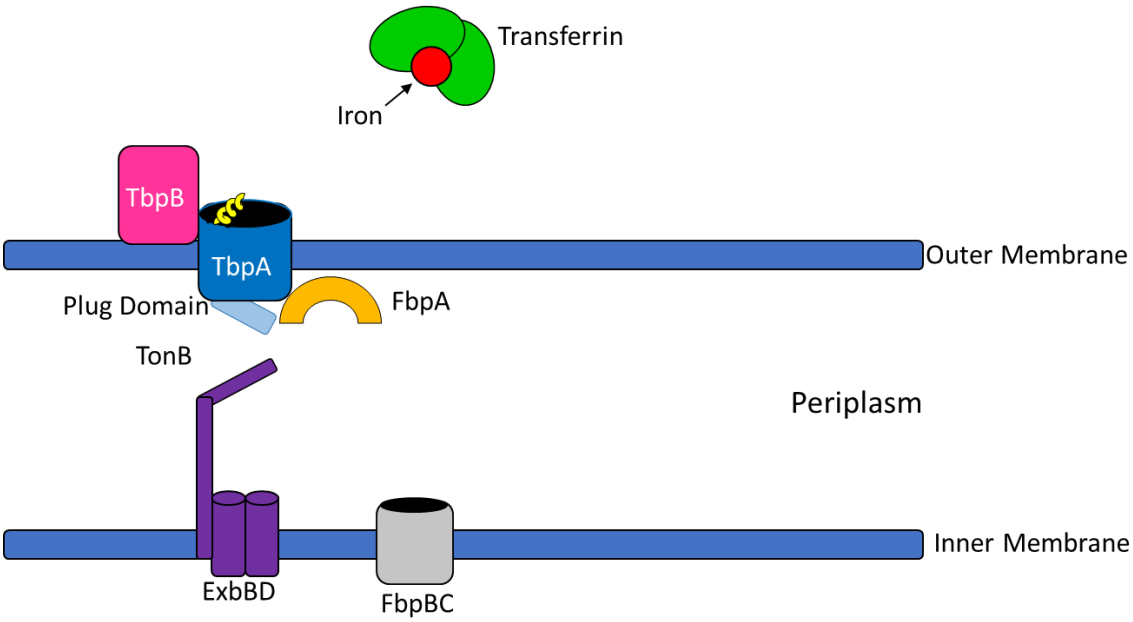

Figure 1.3. The gonococcal outer membrane receptor, TbpA, binds to hTf to hijack and then internalize iron in a TonB-dependent mechanism.

Figure 1.3. The gonococcal outer membrane receptor, TbpA, binds to hTf to hijack and then internalize iron in a TonB-dependent mechanism.

The gonococcal transferrin receptor system includes the outer membrane receptor TbpA and a lipoprotein TbpB, which facilitates but is not required for iron internalization. Upon hTf binding to TbpA, a conformational change leads to iron release, resulting in a higher affinity to the plug domain, located inside the β-barrel of TbpA. TonB, powered by PMF, releases the ligand and pulls the plug domain into the periplasm. Iron exposure to the periplasm allows for FbpA to bind to and shuttle the iron ion to an ABC transporter, FbpBC, in the inner membrane, which internalizes the iron.

D. Iron regulation in *Neisseria*

Like the human host, pathogens need to regulate concentrations of metals to find a complex balance between toxic levels and metal-deplete conditions (289). The Fur regulon mediates transcription of metal-important genes via the ferric uptake regulator (Fur) protein (289, 290). Fur has been best characterized in *Escherichia coli,* but there are known homologs in other Gram-negative bacteria such as *N. gonorrhoeae* (291) and *N. meningitidis* (292, 293). In high iron concentrations, the Fur protein (15 to 17 kDa) complexes with iron and binds to the fur box, located upstream of metal-acquisition genes, to prevent unnecessary transcription (289, 294). In iron-deplete conditions, Fur is derepressed, and RNA polymerase gains access to the promoter to allow for transcription of iron transport genes, as well as siderophore biosynthetic enzymes (289, 295). Fur regulation is important in responding to the environment: if iron is abundant, valuable energy would be lost if these genes were under constitutive expression (290).

XI. Potential vaccine candidates

A. TonB-dependent transporters as vaccine candidates

TDTs are not subject to high-frequency antigenic variation and are highly conserved; therefore, they have been identified as vaccine candidates for several Gram-negative pathogens such as *Neisseria spp*., *Haemophilus spp*., *Moraxella spp*., and *Actintobacillus spp* (296-303). The gonococcal outer membrane protein, TbpA, and the lipoprotein, TbpB, are involved in iron acquisition from hTf in a TonB-dependent mechanism **(Figure 1.3)**. A comparison of TbpA and TbpB can be found in **Table 2**.

Recombinant TbpA (rTbpA) was first shown to provide protection when used as the sole immunogen to vaccinate mice against *N. meningitidis* infection intra-peritoneally, but no bactericidal activity was observed (304). Rabbits vaccinated with either rTbpA or rTbpB demonstrate bactericidal activity, with TbpB eliciting the highest levels of bactericidal activity (304). Intranasal immunization of mice against conjugated TbpA and TbpB to the cholera toxin B subunit TbpA or TbpB showed similar results: TbpB was more immunogenic, but anti-TbpB antibodies were less cross-reactive than anti-TbpA antibodies (305). Immunization of mice using TbpA and TbpB fused to the A2 subunit in cholera and subsequent *N. gonorrhoeae* challenge, showed protection against infection; however, TbpB elicited much higher serum IgG levels compared to TbpA (306). In animals, TbpB has been shown to be more immunogenic than TbpA, but TbpA is more conserved among *Neisseria*; therefore, a combination of TbpA and TbpB is predicted to be more effective than a singular protein vaccine (281, 283, 307).

Table 2: Comparison of TbpA and TbpB

Characteristics	TbpA	TbpB
Protein type	Outer membrane protein; TonB-dependent transporter (308)	Outer membrane lipoprotein (308)
Protein conformation	22-stranded β-barrel (308)	Bilobed with each lobe containing β-barrel domain, side handle domain, lipid-modified (309, 310)
Size	100-103 kDa (280, 281)	78-85 kDa (281, 309)
Species conservation	Present in all gonococcal isolates to date (311)	Present in all gonococcal isolates to date (311)
Susceptible to high-frequency antigenic variability	No (308)	No (308)
Amino acid sequence identity scores	95.4-98.1% identity score among gonococcal isolates and 76.9-96.7% with meningococcal TbpA (283)	69-84% identity score among gonococcal isolates and 64-75% with meningococcal TbpB (281)
Iron repressible	Yes (207)	Yes (308)
hTf binding preference	No hTf binding preference between saturated or unsaturated hTf (312)	Preferentially binds to saturated hTf (312, 313)
hTf utilization	Required for hTf utilization (280, 314)	Not required for hTf utilization, increases efficiency (309, 315)
Growth on hTf plates	TbpA deficient cannot grow on hTf plates (280, 299)	TbpB deficient can grow on hTf plates (309)
Immunogenic	Human: no (316) Mouse: yes (305, 306)	Human: no (316) Mouse: yes (305, 306)
Binding location on hTf	C-lobe (317)	C-lobe (310)

B. Nonbinding mutants as vaccine candidates

Humans with natural gonococcal infection do not elicit TbpA or TbpB antibodies in sera or vaginal secretions, regardless of previous history of gonococcal infection (316). TbpA binds to hTf, and this binding property is predicted to inhibit the generation of the immune response by preventing the recognition of the antigen as foreign. To promote antigen recognition as foreign and development of subsequent protection, the generation of nonbinding mutants has been proposed and predicted to improve the immune response.

Recent vaccination targets have investigated mutant TDTs that are deficient in binding to their associated host ligand. TbpA and TbpB in *Haemophilus parasuis*, the causative agent of Glässer's disease in pigs, have been evaluated as vaccine candidates (297, 298, 302, 318, 319). Vaccination using a WT fragment of rTbpA elicited bactericidal activity (318); however, no protection could be observed in pigs challenged with *H.parasuis* (298). Similar vaccine studies with WT rTbpB from *H. parasuis* also failed to provide protection (298, 320, 321) despite the observation of immunogenicity from WT TbpB in *N. meningitidis* (322) and *A. pleuropneumoniae* (323). Follow up studies compared vaccination with either WT TbpB or a nonbinding TbpB mutant (Y167A) when challenged with *H. parasuis* (303). The nonbinding mutant elicited higher cytokine responses and better protection compared to WT TbpB (303). In fact, pigs vaccinated with the nonbinding mutant demonstrated 100% survival; whereas, pigs vaccinated with WT TbpB only showed 50% survival (303). A nonbinding mutant of FHBP has similary been evaluated as a meningococcal vaccine antigen. A nonbinding mutant produced 4-to-8-fold higher serum bactericidal antibody responses compared to WT fH in a transgenic mouse model (156, 303). In human fH transgenic mice, FHBP mutants with reduced binding to

human complement fH elicited significantly higher IgG titers and serum bactericidal antibody responses compared to WT FHBP (157). These studies serve as preliminary rationale for creating a gonococcal vaccine with a nonbinding TbpA mutant that is unable to bind to host transferrin. The L3H of TbpA is essential for hTf binding and will be targeted for mutagenesis studies (299, 324). Mutations of the opposite charge or to alanine inserted in the L3H region were unable to create fully deficient mutants (299). This study is aimed to target mutagenesis of the L3H region with prolines in an effort to obtain and characterize a nonbinding TbpA mutant, which can eventually be tested in a transgenic transferrin mouse model.

C. Importance of testing nonbinding TbpA mutants in an hTf transgenic mouse model

TbpA and TbpB generate better immunogenicity in mouse studies, whereas, antibodies have not been observed in humans (305, 306, 316). One possible explanation for this observation is the fact that mouse transferrin does not bind to TbpA or TbpB (155, 325), and this fact is hypothesized to explain why Tbp vaccination studies using WT mice elicit a better immune response compared to responses elicited in natural infections (305, 306, 316). Because TbpA and TbpB specifically bind to hTf, the use of hTf transgenic mice will allow for a better simulation the human response when characterizing the true vaccine potential of a nonbinding TbpA mutant. Anti-TbpA or anti-TbpB antibodies have not been observed in serum or secretions in infected humans (316); however, antibodies to both Tbps have been observed in the serum and vaginal secretions in several WT mouse studies (305, 306). In these studies, anti-Tbp antibodies demonstrated bactericidal activity and gonococcal growth inhibition (304-306). The difference in immune response in humans and mice is hypothesized to be a result of the different Tbp-binding capabilities of mouse and hTf. Mouse transferrin does not bind to TbpA or

TbpB; therefore, the immune system is able to recognize the Tbps as foreign (325). In the human, hTf effectively binds to the Tbps and prevents the immune system from recognizing the antigens as foreign. This study proposes the generation of nonbinding TbpA mutants with subsequent vaccination testing in the mouse. Because mouse transferrin does not recognize WT TbpA, it is not expected to recognize a nonbinding mutant; thus, the WT mouse is not a good model for comparing the two vaccine antigens. To accurately test the true vaccine potential of a nonbinding TbpA mutant, expression of hTf is the mouse is essential. Following vaccination with both WT TbpA and the nonbinding mutant, a more accurate comparison of protection and any elicited immune response will be observed in hTf mice.

Heterozygous hTf transgenic mice were generated in C57BL/6 mice by replacing the transferrin gene with human transferrin gene using the Cre-Lox recombination system. Expression of hTf is controlled by the mouse transferrin promotor; thus, hTf expression is expected to be consistent with natural mouse transferrin expression levels and tissue sites. Mice with the inverted hTf gene flanked by two *loxP* (locus of X-over P1) sites were bred with Cre recombinase mice to generate heterozygous mice expressing both functional transferrin mouse and hTf (326). (Unpublished).

Chapter 2: Materials and methods

I. *Escherichia coli* strains: growth and maintenance

Escherichia coli were routinely cultured in Luria-Bertani broth (10 g tryptone, 10 g NaCl, 5 g yeast extract in 1 L of water (327)) in the presence of the appropriate antibiotic as needed; ampicillin/carbenicillin (100 µg/mL) or chloramphenicol (34 µg/mL). Plasmids were propagated in *E.coli* strains (Table 4): Top10 (Invitrogen), XL-10 Gold (Agilent Technologies), or NEB 5-alpha (New England BioLabs, NEB). Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN; 27106) or Zymo Classic Plasmid Miniprep Kit (Zymo Research; D4054). **(Table 4)**

II. Gonococcal strain growth conditions

Gonococcal cells were propagated using GC medium base (GCB; Difco; Lot 91068340) with Kellogg's Supplement I (328) and Supplement II (12 µM $Fe(NO_3)_3$) at 35-37°C with 5% atmospheric CO_2. Gonococcal strains were iron-stressed using deferoxamine (desferal; Sigma). Desferal was reconstituted in sterile MilliQ water at 50 mM and filter sterilized. Gonococcal strains were iron-stressed by passaging approximately three non-pileated colonies onto 10 µM desferal GCB agar plates overnight at 36°C with 5% CO_2. Cultures were inoculated at 15 to 20 Klett unit (KU) into 5 to 10 mL of liquid Chemically-Defined Medium (CDM, (329)); pretreated with Chelex 100 (Bio-Rad) in trace-metal free sidearm flasks (Bellco). Cells were grown at 36°C with 5% CO_2 with shaking at 225 RPM until cells doubled to 40 KU. After the doubling event, cells were typically back diluted to 20 KU and grown an additional 3 to 4 hours.

III. Gonococcal transformation

Two micrograms of plasmid were linearized using SacI (NEB) restriction enzyme in a total volume of 100 µL. After complete digestion, 10 µL 3M NaOAc (pH 5.2) was added to

digested plasmid, followed by 250 µL 100% ethanol. Precipitation occurred overnight at -20°C. Precipitated DNA was centrifuged for 10 minutes at 15,000 RPM, and the pellet was washed once with 500 µL 70% ethanol. The DNA pellet was allowed to air dry before resuspending into 10 µL of Molecular Biology Grade water. Pileated FA19 was grown overnight on GCB (Difco; Lot: 91068340) plates. Approximately half of a plate was resuspended in 1 mL of alternate GCB media (40 mL GCB, 400 µL Supp I, 5.2 µL 1M $CaCl_2$ [130 µM final], and 20 µL 1M $MgCl_2$ [50 µM final]; filter-sterilized with 0.2 µM filter). In a 1.5 mL tube, 10 µL of cell suspension was added to 90 µL alternative GCB media and 5 µL of linearized DNA, as described above. Cells and DNA were incubated at 36°C with 5% CO_2 for 30 minutes. Incubated cells and DNA were then moved to a 6-well dish with 900 µL of alternative GCB media and incubated at 36°C with 5% CO_2. After approximately 5 hours, 10 to 200 µL of cells were plated onto GCB chloramphenicol plates (1 µg/mL). One hundred microliters of cell suspension were flash frozen and stored at -80°C. Plates were incubated at 36°C with 5% CO_2 for 40-50 hours. Transformed gonococci were flash frozen and stored at -80°C prior to single colony purification of *tbpB-/+*.

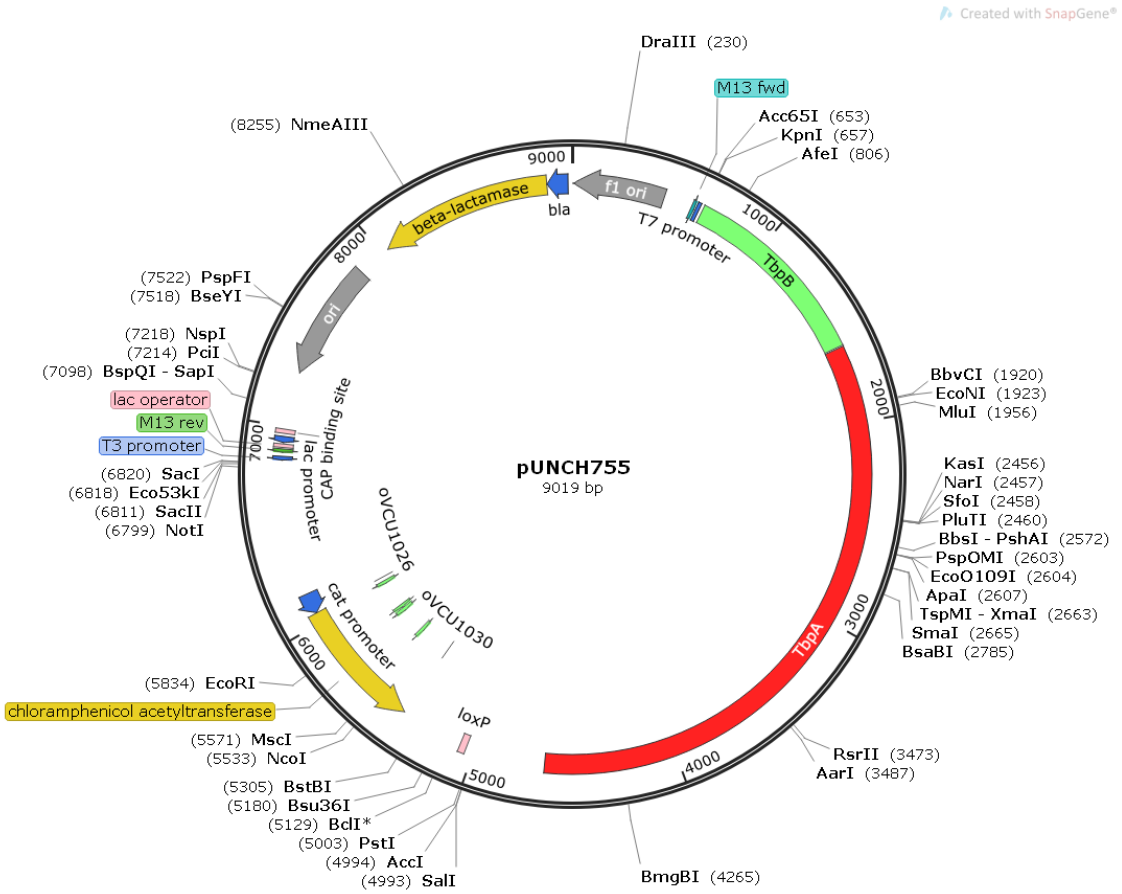

Figure 2.1. Vector map of pUNCH755

Figure 2.1. Vector map of pUNCH755

Vector, pUNCH755, containing truncated and nonfunctional *tbpB* upstream of full length *tbpA,* a mTn3cat insertion was placed downstream of *tbpA.* Image created using SnapGene Viewer 5.2.4.

IV. Site-directed mutagenesis and generation of gonococcal TbpA mutants

Site-directed mutagenesis utilized pUNCH755 (**Figure 2.1)** (299, 309)). Primers used for site-directed mutagenesis and final vector description are located in **Table 3** and **Table 4**. First, a silent StuI (NEB) restriction site was inserted downstream of the *tbpA* L3H region in pUNCH755 by using primers oVCU 905 and oVCU 906, resulting in pGSU001, as described in **Table 3**. pGSU001 was used as template for all downstream mutagenesis of *tbpA*. Because the silent StuI insertion is unique to pGSU001, *tbpA* can be amplified from transformed gonococcal mutants to screen for any WT *tbpA,* which will not cut with StuI.

Using pGSU001, proline mutations were directed to the L3H region of TbpA between D355 and Q360 as described in **Table 3**. Sequences of mutated plasmids were confirmed before being used for FA19 gonococcal transformation using primers described in **Table 5**. Following gonococcal transformation, mutated FA19 strains were purified into *tbpA+/tbpB+* and *tbpA+/tbpB-* strains using polymerase-chain reaction (PCR). Primers described in **Table 5** were used to amplify *tbpA* via PCR, and amplicons were digested with StuI to confirm purity of mutant *tbpA*. WT *tbpA* does not contain the StuI site; therefore, only mutant *tbpA* will cut with StuI. Single colonies were passaged until no WT *tbpA* remained. Colonies were also purified into *tbpB+* and *tbpB-* strains using PCR. The vectors contain truncated *tbpB*; therefore, both *tbpB+* and *tbpB-* strains will arise from homologous recombination in the gonococcus (**Figure 2.2**). These colonies were purified similarly to *tbpA*. Using PCR and primers described in **Table 5**, *tbpB* was amplified. For both *tbpA* and *tbpB*, PCR products were subjected to a 0.7% to 2% agarose gel in 1X TAE buffer to confirm genotyping. Final gonococcal strains are described in **Table 4**.

Table 3: Mutagenesis primers used in this study. Mutations are identified in bold and underline.

Generated Plasmid	Generated TbpA Mutation	Target Plasmid	Primer Name	Direction	5'-3' Sequence	Kit
pGSU001	StuI insert	pUNCH755	oVCU 905	Fwd	GGCAACCACAAATACGG**AGGCCT**GTTTACCAGCGGC	Agilent
			oVCU 906	Rev	GCCGCTGGTAAACAGGCC**TCCGTA**TTTGTGGTTGCC	
pGSU002	D355P	pGSU001	oVCU 927	Fwd	AAGGCGGTTTTT**CCT**GCAAATCAAAAACAGGCG	Agilent
			oVCU 928	Rev	CGCCTGTTTTTGATTTGC**AGG**AAAAACCGCCTT	
pGSU003	A356P	pGSU001	oVCU 929	Fwd	CTGACCAAGGCGGTTTTTGAT**CCA**AATCAAAAACAGGCG	Agilent
			oVCU 930	Rev	CGCCTGTTTTTGATT**TGG**ATCAAAAACCGCCTTGGTCAG	
pGSU004	N357P	pGSU001	oVCU 973	Fwd	TTTTGATGCA**CCT**CAAAAACAGGCGGGTTC	NEB Q5
			oVCU 974	Rev	ACCGCCTTGGTCAGAAAT	
pGSU005	Q358P	pGSU001	oVCU 975	Fwd	TGATGCAAAT**CCA**AAACAGGCGG	NEB Q5
			oVCU 976	Rev	AAAACCGCCTTGGTCAGA	
pGSU006	K359P	pGSU001	oVCU 977	Fwd	TGCAAATCAA**CCA**CAGGCGGGTTC	NEB Q5
			oVCU 978	Rev	TCAAAAACCGCCTTGGTC	
pGSU007	K360P	pGSU001	oVCU 913	Fwd	GATGCAAATCAAAAA**CCG**GCGGGTTCTTTGCGC	Agilent
			oVCU 914	Rev	CTACGTTTAGTTTTT**GGC**CGCCCAAGAAACGCG	
pGSU008	K359P/Q360P	pGSU001	oVCU 917	Fwd	GATGCAAATCAA**CCACCG**GCGGGTTCTTTGCGC	Agilent
			oVCU 918	Rev	CTACGTTTAGTT**GGTGGC**CGCCCAAGAAACGCG	
pGSU009	N357P/Q358P/K359P/Q360P	pGSU008	oVCU 933	Fwd	GCGGTTTTT**CCTCCACCTCCACCACCG**GCGGGTTCTTTGCGC	Agilent
			oVCU 934	Rev	GCGCAAAGAACCCGC**CGGTGGTGGAGGTGGAGG**AAAAACCGC	

Table 4: Bacterial strains used in this study

Strain	Description	Source
E. coli		
Top10	F *mcrA* (*mrr-hsdRMS-mcrBC*) 80*lacZ*M15 *lacX74 recA1 deoR araD139* (*ara-leu*)*7697 galU galK rpsL*(Strr) *endA1 nupG*	Invitrogen
XL10 Gold UltraCompetent	*endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac* Hte (*mcrA*)*183* (*mcrCB-hsdSMR-mrr*)*173* Tetr F= [*proAB lacI*qZM15 Tn*10*(Tetr Amyr Cmr)]	Agilent Technologies
NEB 5-alpha Competent	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs
N. gonorrhoeae		
FA19	wild-type	(330)
FA6747	TbpA- (*tbpA*::mTn3*cat)*	(280, 312)
FA6815	TbpAB- (*ΔtbpB::Ω*)	(309)
FA6905	TbpB- (*ΔtbpB*)	(312)
MCV 192	TbpA L3HΔ (T350-A361)	(299)
RSC 100	TbpA D355P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 101	TbpA D355P	This study
RSC 102	TbpA A356P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 103	TbpA A356P	This study
RSC 104	TbpA N357P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 105	TbpA N357P	This study
RSC 106	TbpA Q358P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 107	TbpA Q358P	This study
RSC 108	TbpA K359P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 109	TbpA K359P	This study
RSC 110	TbpA Q360P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 111	TbpA Q360P	This study
RSC 112	TbpA K369P + Q360P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 113	TbpA K369P + Q360P	This study
RSC 114	TbpA N357P + Q358P + K359P+ Q360P TbpB- (*tbpB*Δ T350-A361)	This study
RSC 115	TbpA N357P + Q358P + K359P + Q360P	This study

Table 5: Amplifying and sequencing primers used in this study

Target	Primer Name	Use	5'-3' Sequence
tbpB	oVCU 750	Fwd: *tbpB* deletion screening	TCGGGCGATGAAGGCGAAACAACTTCCA
	oVCU 752	Rev: *tbpB* deletion screening	CTGCATAAGCGGGCAGCGCAGTCAT
	oVCU 993	Rev: *tbpB* sequencing	GTTCATAACAAACCCGATTC
	oVCU 1002	Fwd: *tbpB* sequencing	CCGGCTGGTTTTACAAACACGC
	oVCU 1003	Rev: *tbpB* sequencing	GCGTGTTTGTAAAACCAGCCGG
	oVCU 1004	Rev: *tbpB* sequencing; starts upstream in *tbpA*	CGGAACAAATGTTGCTGTTGCAT
tbpA	oVCU 08	Fwd: *tbpA* amplification	GACGACGACAAGATGGCGGCATTGGGCGGGACGAGG
	oVCU 830J	Rev: *tbpA* amplification	GACATTGTCCCGGACCGCCGCGTAATAG
	oVCU 1005	Fwd: *tbpA* sequencing	ATGCAACAGCAACATTTGTTCCG
	oVCU 1006	Fwd: *tbpA* sequencing	GAAATCCGCGCCCACGAAG
	oVCU 1007	Fwd: *tbpA* sequencing	CGACCGCGTGATTTACG
	oVCU 1008	Fwd: *tbpA* sequencing	GCCTACCGCGATTTGATTGTCCG
	oVCU 1009	Rev: *tbpA* sequencing	TTAGAACTTCATTTCCAAGCTAAATG

Fwd, Forward; Rev, Reverse

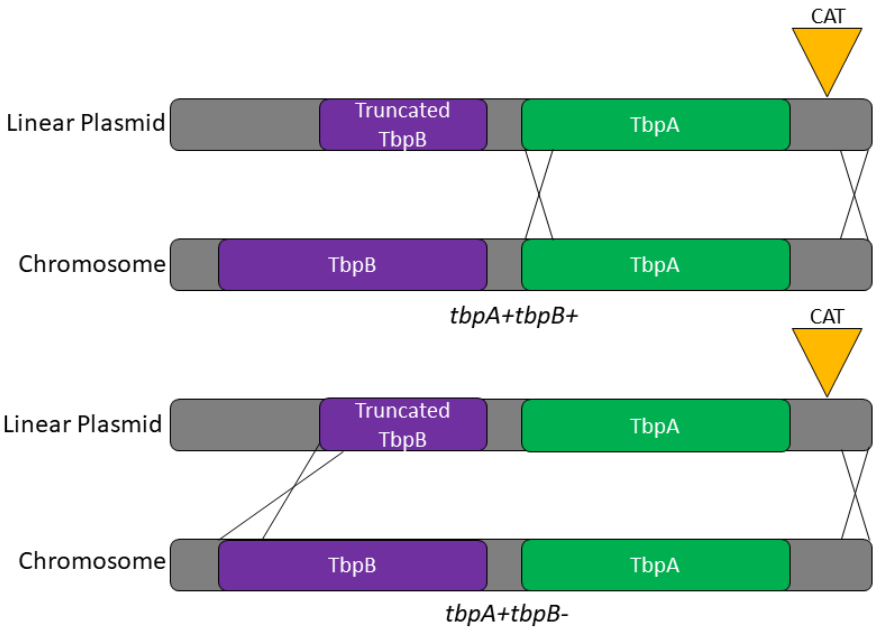

Figure 2.2. Homologous recombination results in *tbpB+* and *tbpB-* gonococcal strains

Figure 2.2. Homologous recombination results in *tbpB+* and *tbpB-* gonococcal strains

Description of homologous recombination events using vector pUNCH755 containing truncated and nonfunctional *tbpB* upstream of full length *tbpA.* Crossover events will result in two genotypes. The top panel shows resulting genotype, *tbpA+/tbpB+,* when crossover occurs at the CAT marker and downstream of *tbpB*. When crossover happens at the CAT marker and upstream of *tbpB*, the resulting genotype is *tbpA+/tbpB-*, as shown in lower panel.

V. Preparing iron-loaded human transferrin

Human transferrin (hTf; Sigma T2036-500 mg; Lot# SLCC6518) was reconstituted at 10 mg/mL or 125 µM in initial buffer (for 100 mL; 1.214 g Tris [100 mM]; 0.87 g NaCl [150 mM]; 0.168 g $NaHCO_3$ [20mM]; pH 8.4; filter-sterilized at 0.2 µM). To achieve 30% Fe-saturation, 75 µL of ferration solution (For 100 mL: 2.94 g Na Citrate [100 mM]; 0.84 g $NaHCO_3$ [100 mM]; 0.135 g 5 mM $FeCl_3$ *6 H20; pH 8.4; filter sterilized at 0.2 µM) was added to 10 mL of 125 µM transferrin + initial buffer and nutated at 4°C for 1 hour. The first dialysis was completed for 4-6 hours at room temperature (RT) using 2 L of dialysis buffer (for 2 L: 9.68 g Tris [40 mM]; 17.4 g NaCl [150 mM]; 3.36 g $NaHCO_3$ [20 mM]; pH 7.4)/10 mL of transferrin. The second dialysis was completed overnight at 4°C, followed by filter sterilizing of hTf using 0.2 µM filter. Transferrin is light-sensitive; thus, it is stored in opaque conical tubes at 4°C.

VI. Protein analysis

A. Whole cell lysates

Whole, iron-stressed gonococcal cell pellets were standardized to 100,000 KU µL and lysed in 100 µL 2x Laemmli solubilizing buffer (Bio-Rad) supplemented with 5% β-Mercaptoethanol. Whole cell lysates were boiled at 100°C for 10 minutes.

B. SDS-PAGE

A total of 10-15 µL of lysate was loaded per well onto a 7.5% SDS-PAGE, and gels were run at 120-150 volts for approximately 1 hour. Proteins were transferred to 0.45 µM nitrocellulose overnight at 28 milliamps at RT.

C. Western blotting

Even protein loading was confirmed via Ponceau S. All blots were blocked for 1 hour at RT with 5% BSA in high salt-tris buffered saline with 0.05% Tween20 (HS-TBST; 20 mM Tris, 500 mM NaCl [pH 7.5], 0.02% NaN_3, 0.05% Tween20). Between antibodies, washes were completed three times with 1x HS-TBST for 10 minutes. TbpA was probed for by using a 1:5,000 dilution of polyclonal rabbit serum (11581-B6) against full-length TbpA (280). A 1:3,000 dilution of goat anti-human transferrin (Sigma #T2027) was used to detect hTf. A 1:15,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Bio-Rad) or a 1:5,000 dilution of rabbit anti-goat IgG HRP (Bio-Rad) were used as secondary antibodies for TbpA and hTf, respectively. OPTI-C4N (Bio-Rad) or West Femto (Thermo) were used as substrates per manufacturers recommendations. Images were taken using Bio-Rad Chemidoc System.

VII. Protease digestion assay

Protease accessibility assays were completed using trypsin as previously described (312). FA19 WT and mutant strains were grown as described above to doubling point, back-diluted, and grown for 3-4 hours at 36°C with 5% CO_2. Whole, iron-stressed gonococci were treated with 12.5 µL reconstituted trypsin (Sigma)/5 mL of culture for 0, 10, 20, and 30 minutes at 36°C with 5% CO_2. Reactions were quenched with 75 µL or 0.6 trypsin-inhibiting units of aprotinin (Sigma). Whole cell lysates were collected and subjected to western blotting for TbpA as described above.

VIII. Growth on CDM plates with 30% Fe-saturated human transferrin as the sole source of iron

CDM agarose plates were made with ultrapure agarose (Invitrogen; catalog# 16500-500; Lot# 004X5P) that had been prepared in advance. All preparation of ultrapure agarose was competed in acid-washed glass. First, 500 g agarose was washed twice with 1 L MilliQ water for 1 hour at RT, washed twice with 1 L 100% ethanol at RT, and washed twice with 1 L of methanol at RT. Centrifugation steps were completed at 3800 x g. After the final wash, agarose was placed in an aluminum foil-covered casserole dish in the biosafety cabinet and stirred occasionally to allow for complete evaporation of alcohols. CDM agarose plates were made using the following recipe for 100 mL: 1 g washed ultrapure agarose, 0.5 g potato starch, and 75 mL VA water were autoclaved. Autoclaved media was allowed to cool before addition of 25 mL of 4X CDM, 25 µL Solution 6, 107 µL Solution 7, 2 mL Solution 8, and 2 mL of 30% Fe-saturated hTf. To solidified CDM agarose plates, approximately equivalent amounts of gonococcal strains were streaked and incubated for 48 hours at 36°C with 5% CO_2. Images were taken with Bio-Rad ChemiDoc.

IX. Growth in liquid CDM using 30% Fe-saturated human transferrin as the sole source of iron

Strains were streaked onto GCB plates supplemented with 10 µM desferal and incubated at 36°C with 5% CO_2 for 12-16 hours. Iron-starved strains were inoculated into liquid CDM as described above. After doubling, cells were back diluted to 2×10^{-5} OD_{600} with 1x CDM, and 100 µL of dilution was plated per well into 96-well plates, pretreated with 5 µM desferal and 5 µM hTf. BioTek Synergy and Cytation plate readers were used to incubate the cells at

36°C with 5% CO_2 with continuous orbital shaking (Frequency: 180 cpm (6 mm). The OD_{600} was plotted over 24 hours. Statistical analysis was performed using GraphPad Prism 9.0. A two-way analysis of variance (ANOVA) with Tukey's *post-hoc* was performed on 3 technical replicates.

X. Transferrin-binding ELISA

Human transferrin was prepared as previously described above. Strains were iron-starved on GCB plates supplemented with 12.5 µM desferal overnight to induce TbpA expression. MaxiSorp microtiter plates (Nunc) were treated with 100 µL 0.01% poly-lysine in H_2O (Sigma P8920-100ML; Lot# SLBZ7200) overnight at 4°C. Cultures were standardized to 1.0 OD_{600} and 100 µL per strain was incubated in sextuplicate wells for 1 hour at RT. Excess cell suspension was removed and cells were blocked with 200 µL 3% bovine serum albumin (BSA) in 1x PBS for 1 hour at RT. Blocking was then followed by addition of 1 µg/mL HRP-conjugated hTf (Jackson ImmunoResearch) in 3% BSA in 1x PBS for 1 hour at RT. To show specificity, 1 µL of 1 mg/mL apo-human transferrin [100 µg/mL] was added to with HRP-hTf Comp wells to be used as a competitor. Wells were washed five times in 200 µL 1x PBS using a BioTek plate washer. Using the BioTek plate reader, 100 µL of Colorimetric 1-Slow-step TMB ELISA Substrate solution (Thermo Fisher; catalog# N301; Lot# UG288102) was applied to each well until sufficient color change was observed after 10-15 minutes. The reaction was quenched with 100 µL 1.8 N H_2SO_4. Results were read at 450 nm using a BioTek Plate Reader. Standard curves were completed for each assay using a range of HRP-hTf between 1 x 10-3 ng and 2 x 10-6 ng. Strains were run in at least triplicate with standard deviation plotted. The Student *t* test was used to determine significance to the positive control FA6905 ($p<0.05$). Pairwise comparisons with a p value of <0.05 were considered statistically significant. Values are shown as the means of multiple

concentration points ± standard error of the mean. Biological replicates were collected in at least triplicate.

XI. Transferrin binding pulldown with hTf or serum

Gonococcal strains were grown in liquid CDM as described above. Cells were back diluted after doubling, grown for 3-4 hours, and 1 mL per strain was allocated per well of a 6-well dish. Cells were first blocked with 100 µL of 10 mg/mL BSA for 5 minutes at 36°C with 5% CO_2 to prevent non-specific protein binding. Three individual samples of human plasma (LEE Biosolutions; catalog # 991-58-PS; Lot 01B3177-10) were used for this study. Three donor samples (Donor # P587039, O+, Female, age 54, Caucasian; Donor# P587070, A+, Male, age 54, Caucasian; Donor# P587041, B+, Female, age 27, Black and Minority Ethnic) were pooled, filter-sterilized with a 0.8 µM filter, and heat-inactivated at 55°C for 15 minutes. After blocking, cells were incubated with either 8 µL of 125 µM hTf or 20 µL of an estimated 50 µM hTf in human plasma (191, 200, 331) for 20 minutes at 36°C with 5% CO_2. Lysates were standardized and subjected to SDS-PAGE and western blotting. Images were taken with Bio-Rad ChemiDoc.

XII. Inductively coupled plasma mass spectrometry (ICP-MS)

FA19 WT and mutant strains were grown under iron-stressing conditions in 10 mL liquid CDM at 36°C with 5% CO_2. After doubling, 10 mL of CDM was added to the flasks, and cells were grown an additional 4 hours. After 4 hours, 800 µL of 125 µM 30% Fe-saturated human transferrin [5 µM] was added to cells and incubated for 1 hour. After final incubation step, cells were standardized to 100,000 Ku, and whole-cell lysates were collected as described above. Remaining cells were pelleted in metal-free tubes at 3570 x g for 5 minutes, washed once in 5 mL of Chelex-treated 10mM HEPES + 1 mM EDTA, pH 7.4 (for 1 L: 2.383 g HEPES and 2 mL of

0.5M EDTA were added up to volume of 1 L MilliQ water; then treated for 2 hours at RT with 50 g Chelex; followed by filter-sterilization with 0.2 µM filter), and washed twice in 5 mL of 10 mM HEPES (pH 7.4) (for 1 L: 2.383 g HEPES was added up to 1 L MilliQ water; then treated for 2 hours at RT with 50 g Chelex; followed by filter-sterilization with 0.2 µM filter). Final pellet masses were recorded for standardization purposes. Pellets were stored at -20°C and sent in one batch for processing. Digestions and analysis of frozen pellets were performed by University of Georgia's Center for Applied Isotope Studies Plasma Chemistry Laboratory. Statistical analysis was completed in GraphPad Prism 9.0. A Student *t* test was performed to determine significance from the positive control, FA19. Pairwise comparisons with a p value of <0.05 were considered statistically significant. Each strain was tested in at least triplicate. Graph shows the mean concentration of iron ± standard error of the mean. FA19 and FA6747 were included in each sample set as controls, and if either showed aberrations to the expected value, the entire data set was excluded from analysis.

XIII. Homology modeling of TbpA and alignment of the Loop 3 Helix (L3H)

The homology model of TbpA from strain FA19 was generated based on the known structure of TbpA from *N. meningitidis* strain K454. Phyre 2.0 was used to generate the PDB file, and Pymol was used to generate **Figure 3.1.** ESPript 3.0 was used to generate the multiple strain alignment and secondary structure prediction of TbpA L3H as previously described (332). GenBank Numbers include the following: *N. gonorrhoeae* FA19: EEZ46093.1; *N. gonorrhoeae* FA1090: WP_010951283.1; *N. gonorrhoeae* DG12: EFE04786.1; *N. meningitidis* K454: AAF81744.1. The Pymol mutagenesis wizard was used to predict the structural effects from each mutant **(Figure 3.10 and data not shown)**.

Chapter 3: The generation and characterization of nonbinding TbpA mutants in the gonococcus

I. Introduction

Neisseria gonorrhoeae is the causative agent of the human-specific sexually-transmitted infection, gonorrhea. The World Health Organization (WHO) reported 87 million new cases globally in 2016 (47), and in 2018 the Centers for Disease Control and Prevention (CDC) has estimated an approximate 1.6 million new cases occurring in the United States alone (46). Uncomplicated gonorrhea presents as urethritis in men and cervicitis in women (9, 55, 282, 333). An estimated 80% of gonococcal infections in women are asymptomatic (48, 55). Asymptomatic cases allow for gonococcal infection to ascend the reproductive track and cause more severe secondary sequalae, such as pelvic inflammatory disease, ectopic pregnancy, and infertility (55). Previous gonococcal infection does not provide protective immunity (333-335), and there has been a rise in antimicrobial resistant strains (47, 336-342). Reports to the WHO in 2018, indicated that 7 out of 65 countries reported isolating gonococcal strains with decreased susceptibility or resistance to extended-spectrum cephalosporins (336). Drastic increases in antimicrobial resistance have caused the CDC to modify recommendations for treatment as of December 2020: uncomplicated gonococcal infection should be treated with a monotherapy of ceftriaxone (500 mg intramuscular dose (76). Availability of effective therapeutics have dwindled to the point that researchers are analyzing older antibiotics for alternative treatments against gonorrhea infection (341). The widespread prevalence of gonococcal infection, increasing incidence of antibiotic resistance, and lack of protective immunity emphasize the demand for an effective vaccine.

Many of the neisserial outer-surface proteins, such as the Opas and pilin, are subject to high-frequency antigenic variation (90, 301, 343, 344), allowing the gonococcus to effectively camouflage itself from an adaptive immune response. This genetic adaptability has presented quite a challenge in vaccine development; however, recently TonB-dependent metal transporters (TDTs) have been the focus of many vaccine studies (296-303). TDTs are ideal vaccine candidates because they are highly conserved, present in all pathogenic *Neisseria* (308), and most are not subject to high-frequency antigenic variation (283, 301). One such TDT system in *Neisseria* acquires iron through the outer-membrane-bound proteins, transferrin-binding protein A (TbpA) (299, 345). With the help of the lipoprotein TbpB, human transferrin (hTf) binds to TbpA, and iron is internalized in a TonB-dependent mechanism powered by proton-motive force (308, 345-348). TbpA is required for iron acquisition from hTf (280, 314). While TbpB increases the efficiency of TonB-dependent iron transport, TbpB is not essential (309, 349). TbpA binds both apo- and saturated transferrin with similar affinity, but TbpB preferentially binds to 30% Fe-saturated transferrin (312, 313). The importance of the *tbpAB* system in gonococcal infection has been demonstrated in a human male colonization study, where a *tbpAB* double knockout gonococcal strain was unable to colonize the male urethra (282).

TbpA and TbpB have previously been evaluated as vaccine candidates for *Neisseria spp.* (304, 305, 316). Overall, TbpA demonstrates some protection and immunogenicity but not enough to be considered a great vaccine candidate (304, 305, 316). Recombinant TbpA (rTbpA) was first shown to provide protection when used as the sole immunogen to vaccinate mice against *N. meningitidis* infection, but no bactericidal activity was observed (304). Consistently,

follow up studies have shown that TbpB is more immunogenic, eliciting higher bactericidal activity and serum IgG levels compared to TbpA (304, 305, 316). The immunogenicity of TbpB has led to increased variability in the amino acid sequence, resulting in less cross-reactive antibodies compared to anti-TbpA antibodies (305). TbpA is not as immunogenic as TbpB, but its amino acid sequence is more conserved among strains compared to TbpB (281, 283). TbpA is also essential for iron acquisition from hTf (280, 314). Overall, TbpB is more immunogenic, but TbpA is more conserved among *Neisseria*; therefore, a combination of TbpA and TbpB or other TDTs is predicted to be more effective than a singular protein vaccine (281, 283, 307).

Tbps have also been targeted as vaccine candidates in several other Gram-negative pathogens including *N. meningitidis* (322, 350, 351), *Moraxella catarrhalis* (352), *Actinobacillus pleuropneumoniae* (323), *Pasteurella haemolytica* (353), and *H. parasuis* (297, 298, 302, 303, 320, 321). *H. parasuis*, the etiological agent of Glässer's disease in pigs, also utilizes TbpA and TbpB for iron acquisition (297, 298, 302, 318, 319). While there is a current vaccine for Glässer's disease, it does not provide the most effective protection (303). A fragment of recombinant TbpA has been shown to elicit bactericidal activity against *H. parasuis* (318), but no protection was observed in challenged pigs (298). TbpB has shown promise as an immunogen in other pathogens (322, 323), but TbpB failed to provide effective protection against *H. parasuis* infection (298, 320, 321). TbpB was still functional and in native conformation and thus, still capable of binding host transferrin. To examine the immunological effects of host-transferrin binding to TbpB, a follow up study vaccinated pigs with either wild-type (WT) TbpB or a nonbinding TbpB mutant (Y167A) and then challenged with *H. parasuis* (303). Colostrum-deprived pigs were vaccinated with WT or a non-host binding TbpB mutant (Y167A), followed

by challenge with the porcine pathogen, *H. parasuis* (303). The TbpB Y167A mutant was characterized with a 300-fold reduction in binding to porcine transferrin compared to the native protein (303). *H. parasusis* challenged pigs demonstrated 100% survival when vaccinated with the Y167A mutant, whereas, pigs vaccinated with WT TbpB only had 50% survival (303). Vaccination with both TbpBs was an improvement compared to the currently available Porcilis Glässer vaccine, which only provided 20% survival (303). Additionally, the TbpB Y167A vaccinated pigs showed a strong induction of B and T helper 2 responses, suggesting a superior immune response compared to WT (303).

Factor H binding protein (FHBP) in *N. meningitidis* has also been evaluated as a vaccine. Like the TDTs, FHBP binds to a host protein, factor H (fH) in this case. In a transgenic mouse model, a mutant that was unable to bind to fH produced 4-to-8-fold higher serum bactericidal antibody responses compared to WT fH (156). Nonbinding TbpB and FHBP vaccination studies serve as guides for conducting parallel non-host binding TbpA (156, 303). Based on these seminal studies, we hypothesize that a non-hTf binding TbpA mutant will elicit better protection and immunogenicity when compared to a WT TbpA antigen in a vaccine study.

The current study aims to expand on previous Tbp structure-function studies by generating nonbinding TbpA mutants by creating proline mutations in the L3H. Mutant TbpA gonococcal strains will be characterized for hTf binding, iron uptake from hTf, and growth using hTf as the sole iron source. The recent generation of a 3 dimensional (3D) crystal structure of TbpA in *N. meningitidis* strain K454 has provided essential knowledge to understanding the mechanism and essential hTf-binding domains (299, 310, 345). Deletion of the L3H prevents the gonococcus from binding to hTf and internalizing iron from hTf (299, 324). A recent study aimed

to identify crucial hTf-binding residues in the L3H using point mutations; neutral, alanine, or the opposite charge mutants failed to demonstrate significantly reduced hTf-TbpA binding (299). Likewise, these mutants did not show significant reduction in iron internalization from hTf or disruption in growth when hTf was used as the sole iron source (299). The current study uses proline-substitution mutations in an effort to generate a TbpA mutant that is unable to bind hTf with minimal protein conformational changes. An ideal vaccine candidate will retain overall 3D protein conformation compared to WT and thus, share the same epitopes as WT. Promising TbpA mutants from this study will be tested for colonization and protection studies in a human transferrin transgenic mouse model.

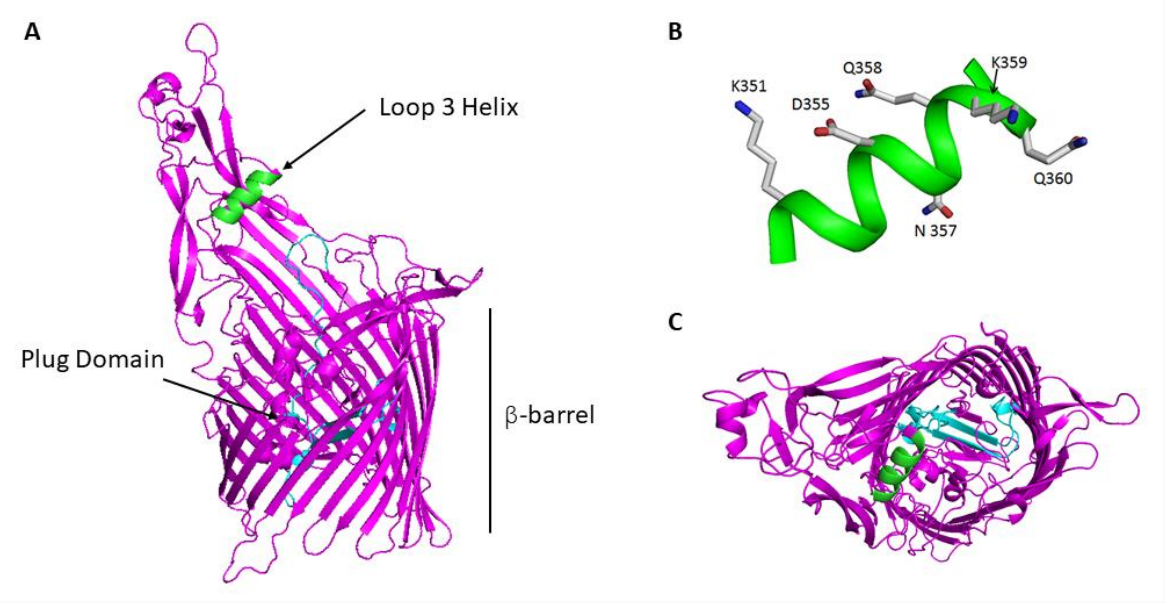

Figure 3.1. TbpA homology model from gonococcal strain FA19 (Adapted from (299))

Figure 3.1. TbpA homology model from gonococcal strain FA19 (Adapted from (299)) **(A)** TbpA homology model predicted structure based on the *N. meningitidis* strain K454 TbpA crystal structure. The TonB-interacting plug domain is shown in cyan. The L3H is shown in green. **(B)** The residues of the L3H targeted for site-directed mutagenesis in this study. **(C)** A top-down view of TbpA, demonstrating the plug domain situated in the beta-barrel pore.

II. Results

A. The TbpA L3H sequence is well conserved at the amino terminal end but residues are variable at the carboxy-terminal end.

TbpA from *N. meningitidis* strain K454 has been previously crystalized (308). *N. gonorrhoeae* strain FA19 shares 94% sequence identity to the TbpA from *N. meningitidis* strain K454 (345). A homology model was generated to demonstrate the predicted structure of gonococcal strain FA19 TbpA **(Figure 3.1).** Key components of the TbpA structure include the outer-membrane embedded beta-barrel motif; key hTf-interacting region, the L3H; and TonB-interacting region, the plug domain **(Figure 3.1A and Figure 3.1C).** A closer look at the L3H **(Figure 3.1B)** shows the key residues that were mutated in this study in order to abrogate hTf binding. **Figure 3.1C** shows TbpA from a top-down perspective to demonstrate the location of the plug domain. Both the FA19 model and the K454 TbpA structure share the L3H, previously shown to be essential for hTf binding (299, 308, 324). Interestingly, the helical structure is well conserved at the amino terminal end but contains variable residues at the carboxy-terminal end, which interacts with the iron ion in hTf during iron acquisition **(Figure 3.2).** To investigate the nature of these residues and impact of hTf binding, site-directed mutagenesis was conducted on several point mutations in the L3H region D355 to Q360, mutating each residue to a proline **(Figure 3.2).**

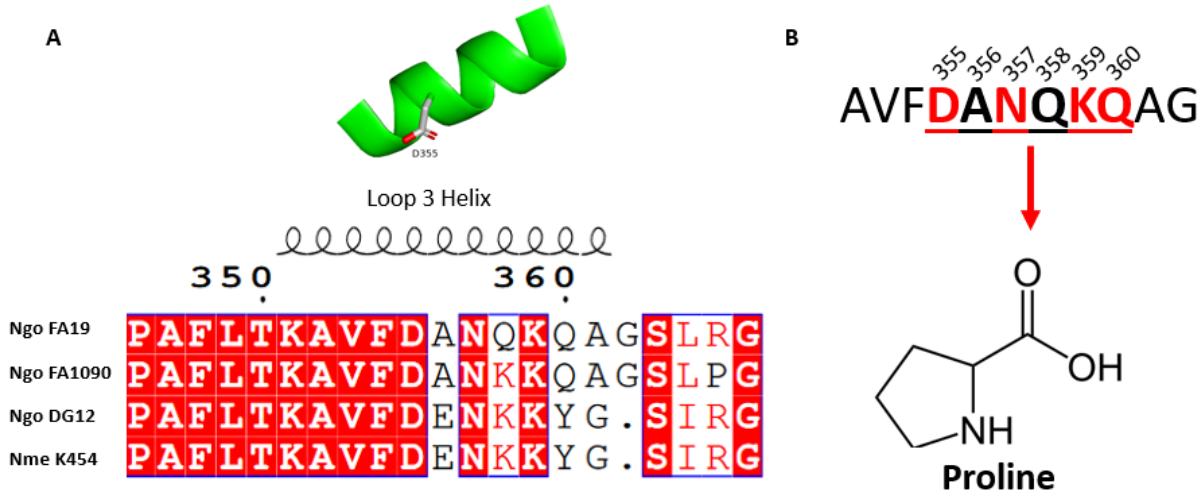

Figure 3.2. L3H structure and amino acid sequence alignment in *Neisseria* strains

Figure 3.2. L3H structure and amino acid sequence alignment in *Neisseria* strains

(A) An alignment of the TbpA L3H domain amino acid sequences for three *N. gonorrhoeae* strains (FA19, FA1090, and DG12) and one *N. meningitidis* strain (K454). Conserved residues are highlighted in red. A secondary structure prediction is shown above the residues. Alignment was generated using EPScript. **(B)** The FA19 L3H sequence residues that were substituted with proline for this study (D355-Q360). Conserved residues are written in red, and variable residues are shown in black.

B. TbpA is surface exposed in L3H proline mutants.

A previous study characterized several FA19 TbpA L3H point mutants and did not identify any single residue essential for hTf binding (299). Prior vaccination studies have shown increased protection when non-host binding mutant proteins are used as an antigen compared to vaccination with the WT protein (297, 303). Therefore, the main focus of this study is to identify a non-hTf binding mutant to be used as a vaccine candidate. Our current study challenges our previous findings by proposing more helix-disruptive proline mutations in leu of alanine or opposite charge mutations.

To ensure proper expression of TbpA in each mutant, strains were grown in iron-stressed conditions and whole-cell lysates were prepared, run on SDS-PAGE, and transferred to nitrocellulose for western blotting using a polyclonal anti-TbpA antibody **(Figure 3.3).** The western blot analysis shows approximately equivalent TbpA protein expression. Because mutagenesis has the potential to disrupt 3D protein conformation and localization to the outer membrane, each strain was evaluated for proper surface exposure of TbpA using protease digestion. Each gonococcal strain was grown in iron-stressed conditions, then subjected to a time course of trypsin digestion before whole-cell lysates were collected for western blot analysis **(Figure 3.4).** As the cells were exposed to trypsin, full length TbpA, approximately 100 kDa, was cleaved into 95 and 55 kDa fragments, as previously described (299, 312). Each mutant showed a similar pattern to FA19 (WT TbpA). Taken together, this data suggests that the mutations are not disrupting TbpA expression or surface exposure.

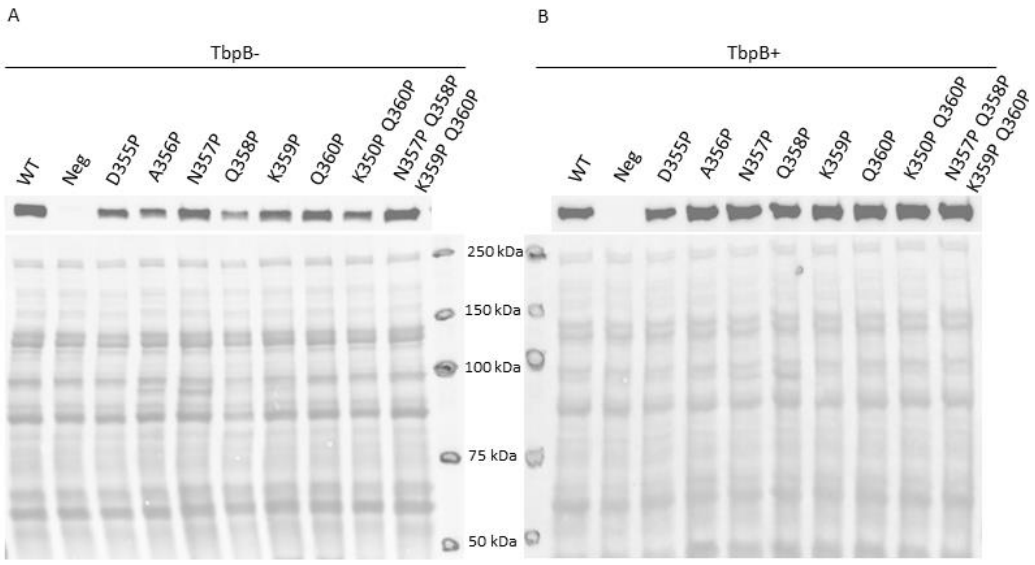

Figure 3.3. TbpA expression in gonococcal mutant strains

Figure 3.3. TbpA expression in gonococcal mutant strains

All mutant strains express even levels of TbpA. Whole, iron-stressed gonococcal cells were standardized, lysed, subjected to SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed with polyclonal TbpA antibody. **(A)** shows a representative image of TbpB- mutants. **(B)** A representative image of TbpA expression in TbpB+ strains. TbpA is 100 kDa. Ponceau S stain was used to confirm equal loading of all wells.

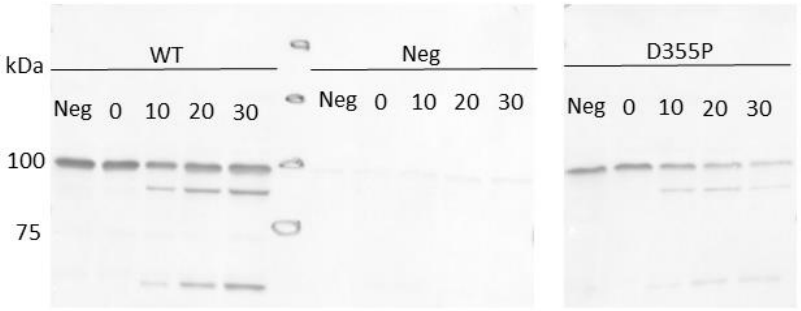

Figure 3.4. Surface exposure of TbpA proline mutants

Figure 3.4. Surface exposure of TbpA proline mutants

Whole iron-stressed gonococcal cells were exposed to trypsin for 0, 10, 20, and 30 minutes. Aprotinin was used to quench the reaction. Lanes “Neg” were not treated with trypsin. Bacteria were lysed, subjected to SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed with polyclonal TbpA antibody. Full length TbpA is 100 kDa, and trypsin cleavage of TbpA results in fragments of approximately 95 and 55 kDa.

C. TbpA L3H proline mutants are deficient in hTf binding.

First, the TbpA mutants were evaluated on ability to bind to 30% saturated hTf. Since TbpB is capable of binding to hTf, and the presence of TbpB was able to rescue iron uptake compared to the TbpB deficient strains (299), only *tbpB*- mutant strains were used for the HRP-hTf binding assays. Whole-cell ELISAs of strains lacking *tbpB* were conducted using HRP conjugated hTf **(Figure 3.5).** In these experiments, FA6905 (*tbpA+/tbpB*-) was used as a positive control and two negative controls were used: FA6815 (*tbpA-/tbpB*-) and the FA6905 competitive inhibition (Comp) condition, which included adding 100x excess unlabeled apo-hTf to HRP-conjugated hTf showing that the HRP-hTf binding is specific to TbpA. A previous study that identified the L3H as essential for hTf-binding showed that deletion of the L3H resulting in 9% of WT TbpA-binding (299). TbpA point mutations of alanine or the opposite charger reduced hTf binding to 40-80% of WT levels (299).

The current study found significantly reduced hTf binding in almost every TbpA proline mutant **(Figure 3.5).** In mutants with significantly-reduced HRP-hTf binding, binding ranged from 0.042 to 0.178 pg or 14-58% of WT binding. Two mutants in particular, D355P (0.042 pg or 14% of WT HRP-hTf binding) and A356P (0.055 pg or 18% of WT HRP-hTf binding) showed very low levels of HRP-hTf binding. Binding levels in the TbpA D355P *tbpB*- and A356P *tbpB*- mutants showed the most significantly decreased hTf binding and were not statistically significant from the negative controls **(Figure 3.5)**.

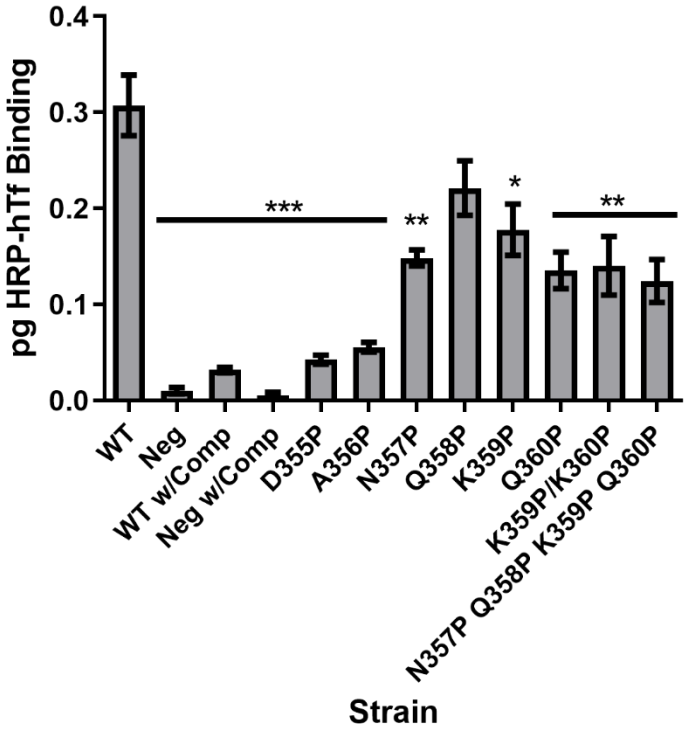

Figure 3.5. TbpA-hTf binding of *tbpB*- strains

Figure 3.5. TbpA-hTf binding of *tbpB*- strains

Whole, iron-stressed gonococcal cells were applied to microtiter dishes for ELISAs using HRP-labeled hTf as ligand. FA6905 (t*bpA+/tbpB*-) served as the positive control, and FA6815 (t*bpA*-*/tbpB*-) and the excess competitor apo-hTf condition (Comp) served as negative controls. The data represent the means ± standard errors of at least three independent experiments. All strains were compared to the WT FA6905 TbpA positive control. Statistics were calculated with the Student *t* test. Significant differences are noted ($p<0.05$ * $p<0.005$ ** $p<0.0005$ ***).

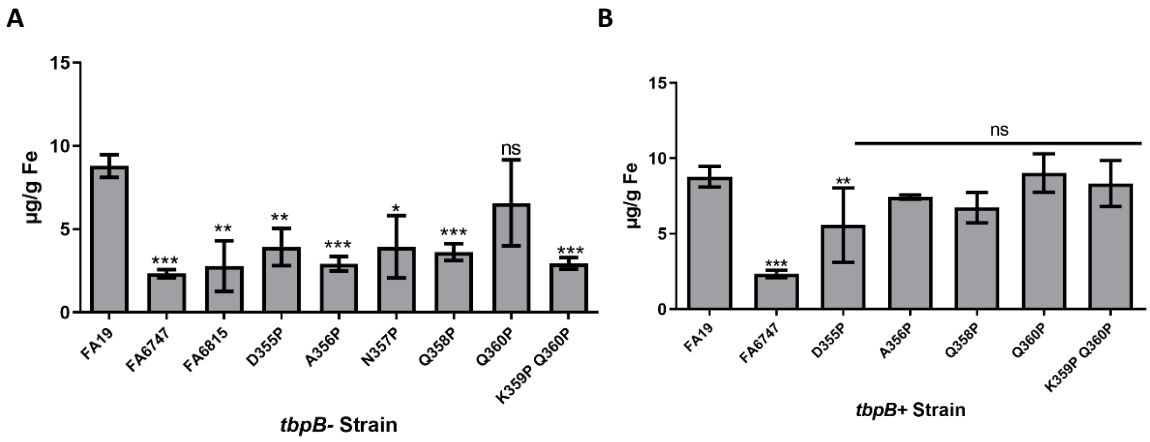

Figure 3.6. Iron internalization by TbpA proline mutants in (A) *tbpB*- and (B) *tbpB+* background strains

Figure 3.6. Iron internalization by TbpA proline mutants in (A) *tbpB-* and (B) *tbpB+* background strains

Gonococcal cells were iron-stressed and allowed to bind to 5 µM 30% Fe-saturated hTf for 1 hour. Bacteria were pelleted and washed prior to being subjected to nitric acid digestion for ICP-MS analysis. Raw µg bacteria/ g iron was plotted for each strain. FA19 (*tbpA+/tbpB+*) served as the positive control. FA6747 (*tbpA-/tbpB+*) served as the negative control. The data represent the means ± standard errors of at least three independent experiments. All strains were compared to the positive control. Statistics were calculated with the Student *t* test. Non-significant (ns) was defined as $p>0.05$. Significant differences are noted ($p<0.05$ * $p<0.005$ ** $p<0.0005$ ***).

D. TbpA L3H proline mutants are deficient in iron internalization from hTf.

Inductively-coupled plasma mass spectrometry (ICP-MS) was used to measure amounts of internalized iron and determine if each proline mutation caused a defect in iron internalization from hTf. The *tbpB-* strains of the D355P, A356P, N357P, Q358P, and double K359P Q360P mutants demonstrated significantly lower levels of iron internalization compared to WT TbpA (FA19) **(Figure 3.6).** These same mutants in a *tbpB+* background did not demonstrate significant reduction in iron internalization, suggesting that expression of TbpB is able to compensate for any proline mutations causing reduced iron internalization in a *tbpB-* background **(Figure 3.6).**

E. TbpA proline mutant strains are unable to grow if hTf is the sole source of iron.

Reduction in transferrin binding and internalization does not necessarily suggest that there will be a reduction in growth when transferrin is used as the sole iron source. Previously, only the L3H deletion mutant in *tbpB+/-* backgrounds showed any defect in growth when using transferrin as a sole iron source (299). Using the same methodology, the growth phenotype of the TbpA mutants was characterized by plating mutant gonococcal strains onto agar plates using hTf as the sole source of iron. Approximately equivalent numbers of colonies were struck out onto Fe-deficient CDM agarose plates supplemented with physiologically-equivalent, 30% saturated hTf as the sole iron source **(Figure 3.7)** FA19 (*tbpA+/tbpB+*) and FA6905 (*tbpA+/tbpB*) served as positive controls and FA6747 (*tbpA-/tbpB+*) and FA6815 (*tbpA-/tbpB-*) served as negative controls. Plates were incubated for 48 hours at 36°C and 5% CO_2. Both the D355P mutant and quadruple N357P/Q358P/K359P/Q360P mutant were unable to grow on solid CDM with hTf as the sole source of iron. As seen previously (299), all *tbpB+* strains were able to grow

like WT **(Figure 3.7).** Interestingly, the quadruple 357P Q358P K359P Q360P *tbpB*- mutant was the only other mutant to show a diminished growth phenotype **(Figure 3.7)**, even though both the D355P and A356P had the least amount of hTf-binding **(Figure 3.5)**. Interestingly, the D355P *tbpB*- and D356P *tbpB*- mutants both had very significant reduction in hTf binding **(Figure 3.5)** and iron internalization **(Figure 3.6)**, but growth phenotypes using hTf as the sole source of iron were different. The A356P *tbpB*- mutant was able to grow using hTf as the sole source of iron; whereas, the D355P *tbpB*- mutant was unable to grow **(Figure 3.7)**.

To assess the growth defect of the D355P *tbpB*- mutant further, a similar assay was performed using liquid CDM. Iron-starved cells were transferred to a 96-well plate and supplemented with 5 μM hTf and 5 μM desferal to chelate free iron. The OD_{600} was plotted over 24 hours. The D355P *tbpB*- strain showed significantly reduced growth when iron-starved gonococci were grown in liquid CDM using hTf as the sole iron source **(Figure 3.8)**.

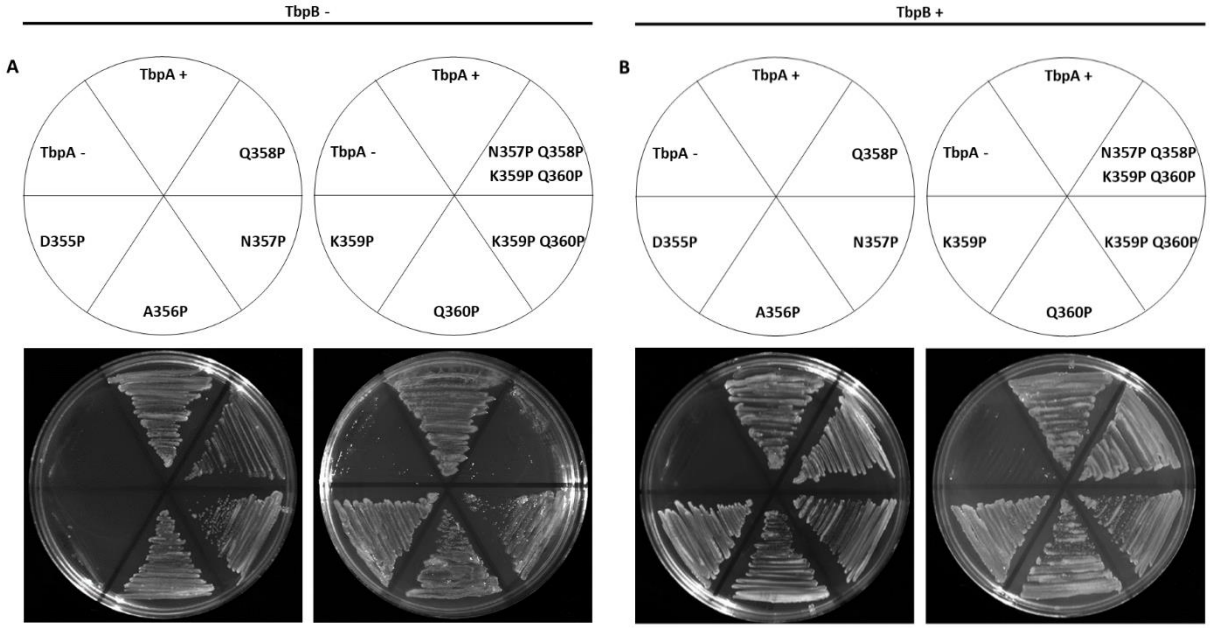

Figure 3.7. Growth of TbpA L3H proline mutants on hTf plates

Figure 3.7. Growth of TbpA L3H proline mutants on hTf plates

Strains were grown on solid CDM agarose plates supplemented with 30% Fe-saturated hTf as the sole source of iron. CDM agar plates containing 2.5 µM 30% saturated hTf and growth phenotype for **(A)** TbpB- and **(B)** TbpB+ mutants. Approximately equivalent amounts of bacteria were streaked onto each plate. **(A)** FA6905 (*tbpA+/tbpB-)* served as the positive control for *tbpB*- mutants, and FA6815 (*tbpA-/tbpB-)* was the negative control for *tbpB*- mutants. **(B)** FA19 (*tbpA+/tbpB+)* was plated as positive control for *tbpB+* mutants, and FA6747 (*tbpA-/tbpB+)* was the negative control for *tbpB+* mutants.

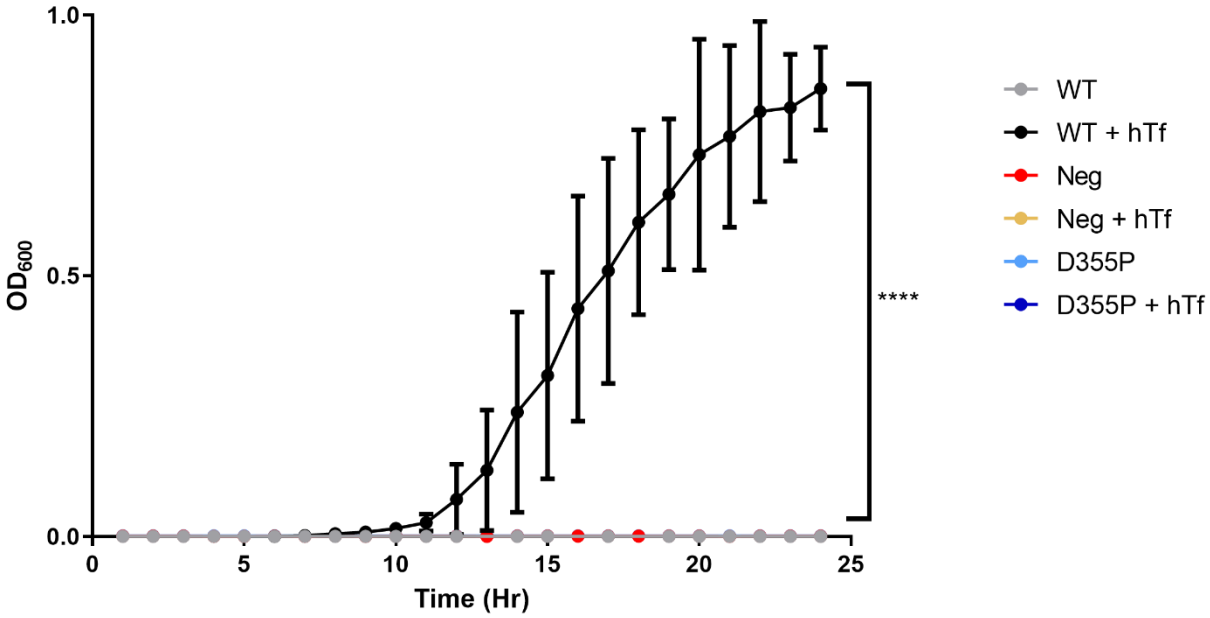

Figure 3.8. Growth of TbpA L3H mutants with hTf as the sole source of iron in liquid CDM

Figure 3.8. Growth of TbpA L3H mutants on hTf as the sole source of iron in liquid CDM

Whole, iron-stressed gonococci were grown in liquid CDM supplemented with 5 µM 30% saturated hTf over 24 hours. FA6905 (*tbpA+/tbpB-*) was used as a positive control and FA6815 (*tbpA-/tbpB-*) served as a negative control. A two-way ANOVA was used to determine significance in comparison to the positive control. Significant differences are noted ($p<0.0005$ ***).

F. The TbpA D355P mutant demonstrates reduced serum hTf binding.

To assess if the binding deficiency in the TbpA D355P mutant could be replicated in a more biologically relevant source of hTf, three samples of human plasma were pooled and used as a source of hTf to assess hTf binding to TbpA. WT FA6905 (*tbpA+/tbpB-*) served as a positive control for hTf binding, and FA6815 (*tbpA-/tbpB-*) served as a negative control for hTf binding. The controls and the D355P *tbpB-* strain were grown in Fe-deplete liquid CDM culture. Cells were grown 3-4 hours after their first doubling and supplementation with fresh Fe-deplete media. Cell cultures were first blocked by adding 10 mg/mL BSA in 1x PBS directly to culture to prevent non-specific protein binding, then either 1 µM hTf or human serum (with approximately 1 µM of hTf) was allowed to bind for 20 minutes at 36°C 5% CO_2. Lysates were immediately collected, and TbpA and transferrin levels were assayed by western blot **(Figure 3.9)**. Approximately equivalent levels of TbpA were seen in all strains expressing TbpA compared to WT, but there was reduced binding in the D355P *tbpB-* mutant in each condition (reconstituted hTf or plasma hTf). Serum hTf concentrations are estimated to be approximately 30 µM based on the literature; however, preliminary tests suggested that hTf is higher than 30 µM. Serum hTf binding experiments were conducted under the assumption that hTf concentration was approximately 50 µM; however, the small amount of background binding in the FA6815 (*tbpA-/tbpB-*) strain suggests the concentration of hTf in this sample set of serum is slightly higher.

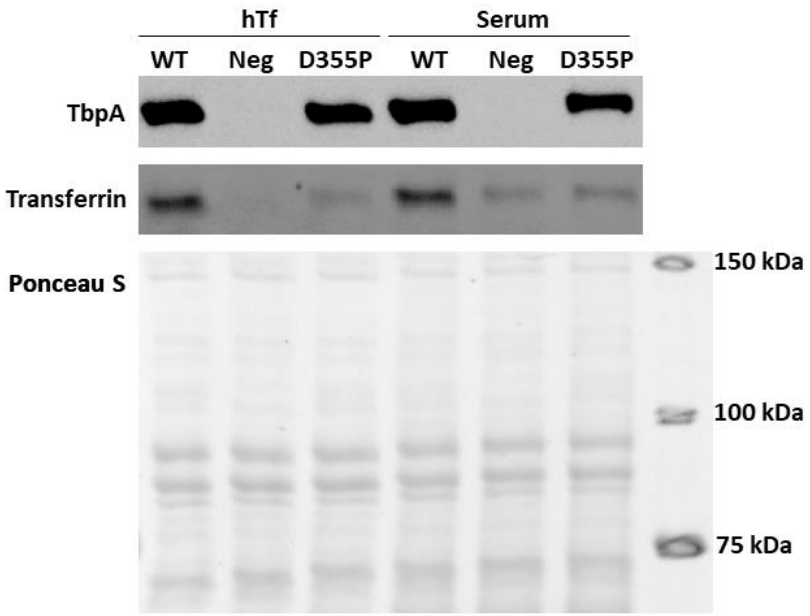

Figure 3.9. The TbpA D355P mutant shows reduced hTf binding compared to WT TbpA.

Figure 3.9. The TbpA D355P mutant shows reduced hTf binding compared to WT TbpA.

Iron-stressed gonococcal cells were incubated with 1 μM of hTf or approximately 1 μM hTf from human plasma for 20 minutes. Lysates were collected and subjected to SDS-PAGE, transferred to nitrocellulose, and probed with polyclonal anti-TbpA antibody and polyclonal anti-hTf antibody. FA6905 (*tbpA+/tbpB-*) was used as a positive control, and FA6815 (*tbpA-/tbpB-*) was used as a negative control. Ponceau S stain was used to show even protein loading. TbpA runs at approximately 100 kDa, and hTf runs at approximately 80 kDa.

G. TbpA D355P and A356P mutants are not predicted to have 3D conformational changes.

The Pymol mutagenesis wizard was used to predict any structural changes caused by proline mutations **(Figure 3.10)**. None of the proline mutations were predicted to cause structural changes **(Figure 3.10 and data not shown)**. Prediction analysis provides evidence that the proline mutations do not affect overall 3D protein structure; however, this does not replace further structural analysis.

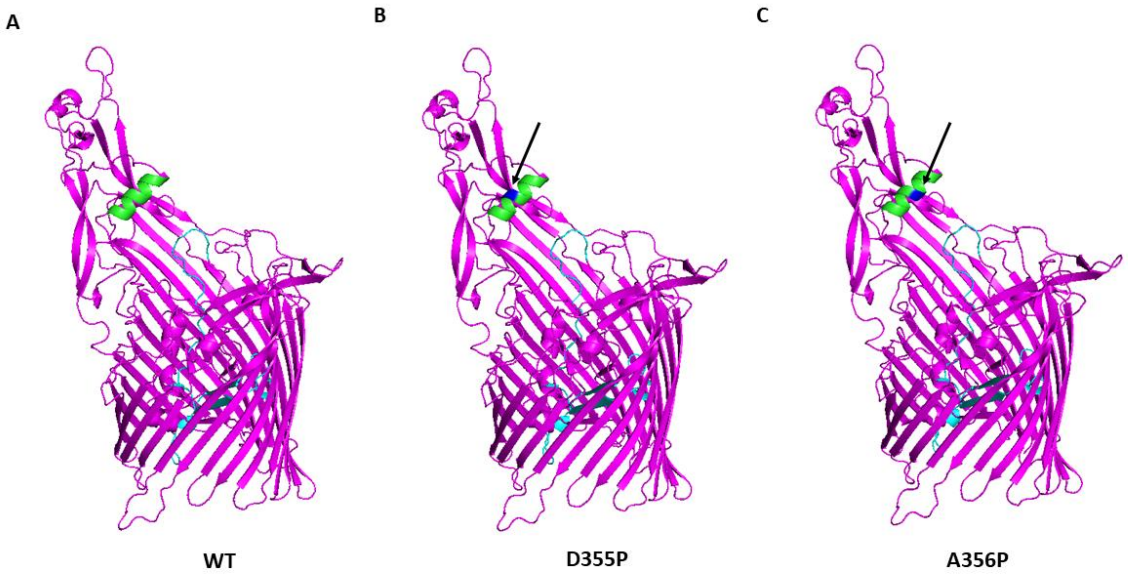

Figure 3.10. TbpA D355P and A356P mutants are not predicted to have 3D conformational changes.

The WT TbpA structure **(A)** is comparable to the TbpA D335P mutant conformation **(B)** and TbpA 356P mutant conformation **(C)** using the Pymol mutagenesis wizard. Proline mutations directed to the L3H (green) are highlighted in blue, as indicated by the arrow.

Figure 3.10. TbpA D355P and A356P mutants are not predicted to have 3D conformational changes.

The WT TbpA structure **(A)** is comparable to the TbpA D335P mutant conformation **(B)** and TbpA 356P mutant conformation **(C)** using the Pymol mutagenesis wizard. Proline mutations directed to the L3H (green) are highlighted in blue, as indicated by the arrow.

III. Discussion

The TDTs, TbpA and TbpB, are highly conserved, essential for infection for *N. gonorrhoeae*, and are not subject to high-frequency antigenic variation; thus, they have been scrutinized as potential vaccine candidates (281-284, 296, 299, 316). Immunogenicity of TbpA has been evaluated, but generation of the immune responses is low (305, 316). The ability of a vaccine antigen to bind to host proteins is hypothesized to inhibit development of a robust immune response and may explain the weak immunogenicity of WT TbpA, WT TbpB, and WT FHBP (281, 283, 299, 308). Creation of nonbinding mutants has already been shown to be a viable option in creating a superior vaccine candidate using TbpB and FHBP (156, 157, 297, 303).

To date, a nonbinding TbpA mutant has yet to be tested as a vaccine candidate to prevent neisserial infection. The generation of nonbinding TbpA mutants has been facilitated by the recently available 3D crystal structure of TbpA from *N. meningitidis* (K454) bound to hTf (299, 310). Because the gonococcal strain FA19 amino acid sequence is 94% identical to the meningococcal K454 strain, important structural inferences can be made to identify crucial binding domains (299). Previously, the L3H was found to be essential for hTf binding to gonococcal TbpA: when the L3H is deleted, hTf binding is completely abrogated (299). The same study attempted to identify crucial residues involved in hTf binding, but point mutations to alanine or the opposite charge did not abrogate hTf-TbpA binding (299). With this is mind, this current study aimed to target the same L3H of TbpA but insert proline mutations in attempt to destroy the helical structure (354, 355). Each mutation was generated in strains with and

without TbpB to evaluate the impact of TbpB on hTf binding, iron internalization, and growth when hTf is used as the sole source of iron.

The purpose of our study is to identify a nonbinding TbpA mutant; therefore, the iron-internalization assay results were primarily used to characterize the mutations and provide more information on a potential mechanism for hTf binding and iron internalization. Our findings suggest that hTf binding does not necessarily correlate with iron internalization or growth phenotype when hTf is used as the sole iron source **(Figure 3.5- Figure 3.7)**.

The Q358P *tbpB*- mutant did not have significantly reduced hTf binding **(Figure 3.5)** but showed significant reduction in iron internalization **(Figure 3.6)**. This data suggests that a Q358P *tbpB*- mutation may inhibit internalization more than hTf binding but not enough to prevent growth using hTf as the sole iron source **(Figure 3.7)**. In regards to the double K359P Q360P *tbpB*- mutant, iron internalization levels **(Figure 3.6)** were significantly reduced and comparable to the D355P *tbpB*- and A356P *tbpB*- mutants; however, the double K359P Q360P *tbpB*- mutant appears to be more capable of binding hTf **(Figure 3.5)**. The double K359P Q360P *tbpB*- mutation inhibits hTf binding and iron internalization, yet this mutant was still able to grow on hTf plates **(Figure 3.7)**.

The mechanism of iron extraction from hTf is still being elucidated; thus, further mutagenesis and structural studies will be key to understanding this mechanism. Iron-internalization data on the L3H TbpA mutants can help us understand the mechanism of iron extraction from hTf; however, the primary focus of our study was to identify a TbpA mutant with significantly reduced binding to hTf for use in a gonococcal vaccine. Each proline mutation at each residue location may cause dissimilar structural changes, and the results of each

mutation are difficult to predict. Initially, we hypothesized that the K359 and Q360 residues would be key hTf-interacting and binding partners based on the positive charge at the end of the L3H and binding results from the previous mutagenesis study (299). However, this current study showed that other mutations (D355P and A356P) resulted in the most significant reduction in hTf binding compared to WT **(Figure 3.5)**.

A reduced growth phenotype **(Figure 3.7)** coupled with the significant reduction in HRP-hTf binding **(Figure 3.5)** and significantly reduced iron internalization **(Figure 3.6)** suggest that the D355P *tbpB*- mutant is unable to properly bind to hTf and internalize iron. Understanding the mechanism of the D355P *tbpB*- mutant seems more straightforward than the A356P *tbpB*- mutant. Both the D355P *tbpB*- and A356P *tbpB*- mutants revealed similar reduced hTf binding, and correlative iron-internalization data **(Figure 3.5 and Figure 3.6)**; however, only A356P *tbpB*- was able to grow on hTf plates **(Figure 3.7)**. The location of the proline mutation in the helix is likely to be a key factor in hTf binding, but what each mutation does structurally and how this affects iron internalization from hTf will need to be further evaluated. The structural comparison between both D355P *tbpB*- and A356P *tbpB*- mutants may reveal that D355P *tbpB*- shows the lowest levels of hTf binding because the 3D structure has been altered to inhibit iron uptake. In this case, the D355P *tbpB*- mutant would not be the preferred vaccine candidate.

Our findings suggest that the D355P *tbpB*- or the A356P *tbpB*- mutants are the best vaccine candidates because both showed the lowest hTf-binding levels **(Figure 3.5)**; however, each point mutation may alter overall protein conformation, biochemical properties, hTf binding, and TonB-dependent iron internalization. Our hTf-binding deficient TbpA mutants show similar protein expression levels (**Figure 3.3)** and protease digestion patterns **(Figure 3.4)**,

which provides a crude evidence that TbpA mutants are being properly folded and presented at the surface. Preliminary analysis using the Pymol mutagenesis wizard did not predict any structural changes to any of the TbpA proline mutants **(Figure 3.10 and data not shown)**. In addition, TbpB has a strong affinity to hTf and has been shown to compensate for defects in hTf binding in gonococcal mutants except when the L3H is deleted (299, 309). With the exception of the L3HΔ strain, all TbpB+ strains were capable of growing like WT; thus, TbpB binding to hTf is sufficient to rescue the growth of any mutant tested in this study **(Figure 3.7)**. The compensation observed in all TbpB+ mutants suggests that the TbpA proline mutants are still functional and able to internalize iron from hTf.

Together these data suggest the TbpA proline mutants have no conformational defects; however, more robust structural and kinetics studies will be necessary in evaluating any nonbinding protein as a potential vaccine candidate. A nonbinding mutant that shares similar structure to WT TbpA is predicted to maintain the same epitopes and thus, elicit a cross-protective immune response during natural infection. Characterization of 3D structure and functional changes is crucial in understanding how each TbpA L3H proline mutation affects hTf binding and iron internalization and importantly, which mutant is ideal for vaccine studies. A nonbinding vaccine candidate that resembles the native protein conformation will share more similar epitopes and is predicted to elicit a more robust immune response, as antibodies are more likely to cross-react to native and the nonbinding mutants.

Incorporation of TbpB in a vaccine will require mutagenesis or modification to inhibit binding to the host, as binding has been shown to reduce immunogenicity (297, 303). Because anti-TbpB antibodies are less cross-reactive and more immunogenic, and anti-TbpA antibodies

are more cross-reactive and less immunogenic, a combination of the two is predicted to enhance protection and the immune response (304, 305, 316). Ideally, nonbinding mutant Tbps would be included in a vaccine cocktail to increase the cross-reactivity and immunogenicity. As more TDTs are characterized, nonbinding mutants can be included in this vaccine cocktail to enhance protection and immunogenicity.

This is the first study to identify a nonbinding TbpA mutant using a single point mutation. Of interest, the TbpA D355P *tbpB*- and A356P *tbpB*- point mutations will be important to evaluate as potential vaccine candidates. Both have significantly reduced hTf-binding; however, the phenotypic differences in growth on hTf plates suggest that these mutations have different functionalities and potentially different effects on 3D protein conformation. Comparison of the 3D protein conformation is expected to be challenging due to the flexibility of the extracellular loops in nonbinding mutants; however, structural analysis is a crucial next step.

Each D355P and A356P TbpA mutant will be tested as vaccine antigens alongside WT TbpA to compare protection against gonococcal colonization in an hTf transgenic mouse model. The use of a hTf transgenic mouse is essential because TbpA binding is specific to hTf (325). Because mouse transferrin does not recognize TbpA or TbpB, this provides an explanation for the enhanced immunogenicity elicited by both proteins when used as vaccine antigens in mouse models compared to human secretions (304-306, 316). This nonbinding mutant characterization study and subsequent structural and vaccination studies will serve as groundwork for future vaccination studies utilizing nonbinding proteins of other TDTs for *Neisseri*a and other pathogens.

Chapter 4: Iron, inflammation, and *Neisseria gonorrhoeae*

I. Abstract

Iron is essential for metabolic functions in humans and most bacteria. Nutritional immunity in humans utilizes iron metalloproteins to sequester iron from pathogens to prevent infection. *Neisseria gonorrhoeae*, the causative agent of the sexually-transmitted infection gonorrhea, is capable of extracting iron directly from these metalloproteins, including hemoglobin, transferrin, and lactoferrin, using specific outer-membrane transporters. Each metalloprotein has its own ecological niche, and this review will summarize the function and expression of each protein at gonococcal infection sites. This review will also discuss how other potential mechanisms for iron acquisition by modulation of host cytokines and proteins could be advantageous to colonization, invasion, and transmission of the gonococcus.

II. Gonorrhea

Neisseria gonorrhoeae is an obligate human pathogen responsible for the sexually-transmitted disease, gonorrhea (356). Gonococcal infections are on the rise: the World Health Organization (WHO) estimates an approximate 87 million worldwide cases of gonorrhea in 2016 (47). The Center for Disease Control (CDC) recently released an update stating that there were an estimated 1.6 million new cases in the United States in 2018 (46). *N. gonorrhoeae* has become a priority to monitor as a drastic increase in antimicrobial resistant strains have been observed (47, 336-342). As recently as December 2020, the CDC discontinued the recommendation for dual therapy with ceftriaxone and azithromycin and instead recommends doubling the dose of ceftriaxone for treatment of uncomplicated gonococcal infection (76). No vaccine is available for gonorrhea, and patients can easily be reinfected with the same strain because prior immunity does not provide any protective immunity against reinfection (333-335).

N. gonorrhoeae initially colonizes mucosal sites such as the genitals, conjunctiva, or oropharynx; gonococcal infections begin as urethritis in men and cervicitis in women (48, 55, 282, 333, 356). Dissemination to the blood stream or disseminated gonococcal infection (DGI) occurs in less than 3% of cases (58, 60, 356). Gonococcal infection is notorious for being asymptomatic, especially in women (9, 49, 55). An estimated 80% of cases in women exhibit no symptoms, and therefore, do not seek treatment, allowing the infection to ascend the reproductive tract (48, 55). As the infection ascends, inflammation and tissue damage garner, causing severe secondary sequalae in men and women (9, 45, 55, 56).

The widespread epidemiology of gonorrhea coupled with limited treatment options have caused the research community to focus on alternative therapeutics and most importantly, a vaccine to prevent infection in the first place (356). Recent research has investigated TonB-dependent transporters (TDTs) as vaccine targets (266, 296, 297, 299, 302, 303, 305-307, 316, 357-361). Many Gram-negatives specifically recognize host metalloproteins, such as transferrin (Tf), lactoferrin (Lf), hemoglobin (Hb) using outer-membrane transporters (231, 243, 266, 345, 348, 362, 363). These host metalloproteins are essential for successful nutritional immunity, as described below. This review will compile the available information of host iron metalloproteins on the roles in regulating iron homeostasis, availability in key gonococcal infection sites, and how the gonococcus obtains iron.

III. Iron and the host

Iron is the most abundant metal ion in humans (260, 261). Metals such as iron are essential for metabolism in most aerobic organisms (192, 196, 260). The soluble and more bioavailable ferrous (Fe^{2+}) iron is oxidized to insoluble ferric (Fe^{3+}) iron in the host aerobic environment (272, 364). In the host metabolism, iron is used as a cofactor to form iron-sulfur (Fe-S) cluster proteins, heme-binding proteins, and RNA reductases, promoting heme synthesis, oxygen transport, and DNA synthesis (193, 260, 261). Iron is also important for proliferation of immune cells such as T-lymphocytes and neutrophils (192, 262). While iron is essential, iron overload is cytotoxic due to generation of reactive oxygen species (ROS) and oxidative stress (192, 260). The delicate balance of iron levels is stringently regulated in the human host. Metalloproteins bind and chelate metals to deplete the environment of free metals. Over 99.9% of mammalian iron is sequestered intracellularly, either via ferritin or heme, and the

extracellular iron is bound to metalloproteins such as Lf and Tf (191, 207). Metalloproteins play a key role in iron homeostasis by solubilizing iron, making iron bioavailable, and protecting the host from damaging ROS. Each host iron-binding protein is described below.

A. Hemoglobin

Hemoglobin (Hb) is a globular protein consisting of α and β-globulin chains (231). Inside erythrocytes, Hb stores approximately 75% of all the iron in the body, and the remaining 25% is stored by ferritin in liver, spleen, and bone marrow (192, 193, 231, 244, 365). Hb is the most abundant protein in blood and erythrocytes, and it sequesters iron in the form of heme inside erythrocytes to prevent high levels of serum free iron (231, 243, 244). Heme is a heterocyclic porphyrin ring that binds centrally-coordinated ferrous iron (Fe^{2+}) and acts as the precursor to Hb (231, 244). Heme is typically bound to hemoproteins, such as Hb, haptoglobin (Hp), and hemopexin, but heme can also be found in the unbound form (231). Hemoproteins bind heme strongly at one or two of the free iron-coordination sites, which are located perpendicularly to the porphyrin ring (231). The most common ligand(s) for coordination of the iron in heme are imidazole groups on histidine side chains; however, methionine, cysteine, and tyrosine have also been observed (231, 245). Hb may release heme spontaneously or as a result of bacterial proteases (231, 246). Erythrocytes naturally spontaneously lyse, releasing up to 3 μM free Hb into serum in healthy patients (231, 246). In serum, the tetrameric Hb dissociates into dimers, which are rapidly sequestered by Hp (231). Almost irreversibly, Hp binds Hb, and the Hb-Hp complex is then recycled by macrophages (231, 244, 247). Other serum glycoproteins, such as albumin and hemopexin, act to rapidly bind and sequester any free serum heme (231).

i. Plasma

Hb is specific to the blood (366). Hb presence in mucosal secretions is thought to be contamination from blood. This fact is unsurprising given that the *hpuAb* system is typically phase off in gonococcal isolates, except when isolated from women in the early stages of menses (259).

B. Transferrin

The 80 kDa glycoprotein Tf is the primary extracellular iron protein (192, 193, 207). Tf is synthesized by hepatocytes and secreted to the serum where it solubilizes ferric iron, inactivates iron to prevent toxicity, and delivers iron into cells via transferrin receptors (TFRs) (194). The structure of Tf contains a C- and N-lobe, in which each lobe can bind a single ferric iron ion (Fe^{3+}). Tf is naturally found at approximately 30% iron-saturation in serum (193, 194). Saturation of Tf is primarily determined by the rate of iron release from macrophages, when erythrocyte recycling occurs at a steady rate (194). While inflammation increases hepcidin concentrations, serum Tf concentrations decrease during inflammation (367).

i. Plasma

Serum Tf is naturally between 12% and 50% (with 30% considered standard) saturation of the iron-binding capacity (60 to 75 µM), and serum iron levels are calculated to be between 10 to 30 µM (191, 193, 194, 200).

ii. Tears

Tf is hypothesized to travel to the tears by leaking from blood vessels, as it has been observed in very low concentrations in tears (206).

iii. Male genital tract

Tf found in seminal plasma is secreted by Sertoli cells, which secrete proteins responsible for regulating spermatogenesis (368, 369). Early studies looking at Tf levels in seminal plasma show a range of 30.3 μg/mL to 163.5 μg/mL Tf (368). One study identified a correlation between low levels of seminal Tf and infertility: levels in vasectomy patients were 13.4 μg/mL compared to 65.6 μg/mL in pregnancy-proven patients (370).

Hormonal regulation was first observed in rat Sertoli cells. Follicule-stimulating hormone and testosterone were used to stimulate Tf secretion (371). These results suggest that iron is important for spermatogenesis. *N. gonorrhoeae* binds to sperm using the asialoglycoprotein receptor, allowing for transport to another site of colonization (131). The high levels of Tf found in seminal plasma could provide for ample Tf levels to allow for colonization. The concentration of Tf in whole ejaculate was 0.07 ± 0.05 mg/mL (with a range of 0.02-0.19 mg/mL) (372). Interestingly, Tf levels are highest in the earlier phases of ejaculation; whereas, Lf originates from the seminal vesicles, or later phases of ejaculation (372).

iv. Female genital tract

Proteolytic analysis shows the presence of Tf in vaginal lavages, but specific concentrations have not been measured (373).

C. Lactoferrin

Lf is an 82 kDa iron-binding glycoprotein similar in structure and function to Tf (205-207). Synthesized by neutrophils and exocrine glands, Lf is primarily located in human milk and mucosal surfaces (208, 210-216). Lf is antimicrobial and anti-inflammatory (206, 208, 209, 374). Lf has been previously referred to as multiple names including the following: red milk protein,

Lf 3, lactotransferrin, and lactosiderophilin (212, 375). Lf shares 60% sequence identity with Tf, and shares a similar structure (217). Like Tf, Lf contains both a C-lobe and N-lobe, where one Fe^{3+} ion binds to highly conserved iron site residues on each lobe (205, 218-220). One notable difference between Lf and Tf is the ability for Lf to maintain high affinity to iron under low pH: Tf loses its iron-binding affinity below pH 6.5; whereas, Lf maintains affinity down to pH 3.0, characteristic of many of its sites of expression (205, 216). When no iron is bound, the N- and C-lobes of Lf have an open conformation referred to as apolactoferrin. When iron is bound to Lf, the N- and C-lobes close to form hololactoferrin (218). Because Lf is present in mucosal surfaces and functional at lower pH values compared to Tf, it is a crucial defense protein in the female genital tract (216, 331).

Lf has long been studied to understand its role in infection and inflammation. Earlier studies indicated it as an iron-binding protein similar to Tf, but more recent studies have indicated it as a regulator of inflammation in some situations (205, 216, 376, 377). Lf is secreted by cervical or epithelial cells and found in secondary granules of human neutrophils (204, 378-385). Fifteen μg of Lf is released from 10^6 neutrophils (216, 376). For instance, in Lf knockout mice, the oxidative burst pathway was impaired after treatment with phorbol myristate-13-acetate (PMA), a powerful stimulator of the NADPH oxidase-dependent respirator burst (386); however, no impairment was observed when the Lf knockout neutrophils were treated with opsonized bacteria (377).

i. Milk

Lf concentrations are highest in colostrum at 7 mg/mL, initially thought to be exclusive to milk. Lf has been isolated from human colostrum and milk between 2 and 197 days after

childbirth (212). Lf concentrations were measured at different stages of milk: colostrum (2-5 days postpartum), transitional milk (6-10 days postpartum), and mature milk (11-60 days). Lf decreased during the first two weeks after childbirth (212). Hormonal regulation of Lf has also been observed in human colostrum and mature milk samples (385). Milk samples were taken on day 1, day 4, and days 57-70 after birth: Lf levels were highest on day 1 at 1650 mg/dl, followed by a sharp decline to 530 mg/dl on day 4, and leveled off to 266 mg/dl on days 57-70 (385).

ii. Plasma

Lf was originally not thought to be present in blood plasma (372, 387), but studies have identified its presence at levels at median concentration 176 ng/mL (range 39 and 312 ng/mL) (384).

iii. Tears

Tear Lf was first identified in 1966 (388). Lf is a large proportion of tear protein, 24-27% in postemenopausal women (388). A study observed a correlation between Lf and epidermal growth factor (EGF) in middle-aged women with Sjögren syndrome, an autoimmune disorder characterized by decreased lacrimal gland function (389).

iv. Male genital tract

Masson et al. in 1966 first discovered the presence of Lf in human seminal plasma. Hekman et al. identified Lf in human seminal plasma, as well as on the surface of spermatozoa; but not in the testis, epididymis extract, or testicular spermatozoa (387). This study suggested that Lf originates in the seminal vesicles: no Lf was detected in seminal plasma in patients without seminal vesicles, and Lf was still detectible in azoospermic semen (387). Lf is secreted

by seminal vesicles (372, 387). Reduced seminal vesicle secretion caused by high-dose testosterone injection, decreased Lf concentrations in seminal plasma (390). The surface-coating Lf on spermatozoa was suggested to also originate from the seminal plasma (387). Lf coating spermatozoa could provide several advantages. The bacteriostatic quality of lactoferrin may provide protection against bacterial infection (387). Additionally, coating of the surface of spermatozoa with non-specific protein, such as Lf, could allow for less production of antibodies against spermatozoa or blocking against sperm-specific antibodies in the female genital tract (387). Buckett et al. compared Lf concentrations among 368 men with varying levels of sperm density, motility, oligoasthenospermia, or azoospermia. There was no significant difference in Lf concentration among the groups, ranging from 11.2 mg/100 mL to 13.4 mg/100 mL (391). Seminal plasma Lf ranges from 0.23 to 2.75 mg/mL with a mean concentration of 1.18± 0.74 mg/mL (372).

Evidence of hormonal regulation of Lf secretion has been observed. Yu and Chen demonstrated that 17β-estradiol could stimulate mouse epididymis Lf mRNA (392). Elevated testicular estradiol has been observed during oligospermia (393, 394).

v. Female genital tract

Hormonal regulation of Lf has been observed in the human vaginal secretions (215, 216, 395). The lowest levels of Lf, 3.8 to 11.4 μg/mg, were observed before menstruation, and the concentrations peaked to 62.9 to 218.0 μg/mg after menstruation (215). Hormonal regulation was not observed in women taking oral contraceptives, where the mean concentration was less than 19.8 μg/mg throughout the menstrual cycle (215). Serum Lf levels were also unchanged throughout the hormonal changes (215). Lf is not considered to be under hormonal regulation

by oral contraceptives and the menstrual cycle. While the source of the extra Lf during menses is unknown, it was hypothesized that Lf could leak from the serum into the vaginal mucus; however, this was not observed in women taking oral contraceptives (215).

vi. Rectum

Fecal Lf is currently being frequently evaluated as a potential biomarker for patients with inflammatory bowel disease (IBD) (396-399). Fecal Lf levels have mostly been characterized in patients with IBD, and not much information is available in healthy patients. Median Lf concentrations in IBD range from 5.7 μg/g to 26.2 μg/g (400).

D. Siderophores

Most Gram-negative bacteria produce siderophores, small molecular weight cages (<1 kDa), that are synthesized and secreted into the environment to scavenge for iron (263-265, 331). Siderophores are a key component that bacteria deploy to combat nutritional immunity. Siderophores have such a high affinity and specificity to iron that they can pirate iron directly from Tf, Lf, but not heme (191). *N. gonorrhoeae* is unable to synthesize siderophores; however, the gonococcus can use xenosiderophores, siderophores produced by other bacteria, such as salmochelin and enterobactin (268, 270).

E. Lipocalin

Siderocalins are in the lipocalin family of binding proteins that chelate and inhibit bacterial siderophores (191, 272, 401). Lipocalin 2 (Lcn2) is a small, 25 kDa protein (402) that binds to the siderophore enterobactin to scavenge iron in an attempt to inhibit iron piracy by pathogens (403, 404). The nomenclature of Lcn2 varies throughout the literature. Recent literature still refers to human Lcn2 as neutrophil gelatinase-associated lipocalin (NGAL), human

neutrophil lipocalin (HNL), 24p3, uterocalin, and siderocalin (275, 405). This review will focus on human Lcn2. Lcn2 was first discovered as a neutrophil granule component (402).

Lcn2 primarily and tightly binds bacterial catecholate ferric siderophores but can also sequester some carboxylates (275). The structure of Lcn2 consists of the standard lipocalin fold and eight-stranded β-barrel, but the binding pocket of Lcn2 is much larger and consists of more polar and positively-charged resides compared to other lipocalins (402).

Lcn2 is produced by neutrophils and macrophages; therefore, it is present at mucosal sites at the initial stages of gonococcal infection and colonization (404). Other cells secrete Lcn2 including hepatocytes, epithelial cells, and adipocytes (405). Lcn2 has been shown to be important for regulating iron during infection. Under inflammatory signals, epithelial cells secrete Lcn2 (402).

i. Plasma

The mean serum Lcn2 concentration for healthy adults was reported to be 63 ng/mL, ranging between 36 and 106 ng/mL (406), 60.06 ng/mL ± 20.30 (407), and another study reported lower serum Lcn2 levels in healthy controls (14.30 ng/mL) (408). Another study reported 30.7 ± 8.1 ng/mL Lcn2 in healthy patients (409). Another study reported significantly raised serum Lcn2 levels in men (72.1 µg/mL) compared to women (57.6 µg/mL) (410).

Elevated serum Lcn2 levels have been observed in several conditions associated with high inflammation levels. Lcn2 is upregulated in human papillomavirus (HPV) positive women compared to HPV negative women (411). Lcn2 concentrations in normal healthy patients were 15,540 pg/mL with a range of 1237-32,872 pg/mL versus HPV positive healthy patients of 39,375 pg/mL with a range of 7778-41,147 pg/mL (411). Patients with IBD demonstrate raised

systemic Lcn2 levels, possible as a result of high levels of inflammation consistent with IBD (406, 407, 412). Patients with psoriasis, characterized by inflammation, show elevated serum and tissue Lcn2 levels (409). Evidence suggests that serum Lcn2 and Lcn2-MMP9 (matrix metalloproteinase 9, with immunomodulatory properties) complex levels are elevated with obesity, a condition characterized by chronic low-grad inflammation (410, 413). There was no significant difference in serum Lcn2 (approximately 75 ng/mL) between lean and obese patients; whereas, Lcn2-MMP9 went from approximately 29 ng/mL to 53 ng/mL (413).

ii. Oropharynx

Bacteria colonization induces expression of nasal Lcn2 in mice (414). There is evidence suggesting that Lcn2 is proinflammatory and promotes iron sequestration in the mucosa after addition of enterobactin (414). Lcn2 binds to enterobactin and induces secretion of IL-8, a neutrophil chemoattractant, in respiratory cells in vitro (414).

iii. Tears

The second most common protein in tears is tear lipocalin, also referred to as lipocalin 1, von Ebner's gland protein, or human tear albumin (401, 415). First discovered in 1956, tear lipocalin protects the conjunctiva by scavenging for destructive lipid peroxidation products and breaking down invading bacterial or viral DNA with catalytic endonuclease activity, and inhibits microbial proteinases and siderophores to inhibit colonization (401, 415). Lipocalin consists of a large portion of tear protein. One study measured 1.2 mg of tear lipocalin isolated from 7.2 mg of total tear protein (415), and another study estimated that 15-33% of tear proteins to be tear lipocalin (388). Tear lipocalin has several functions, including endonuclease activity. In fact, lipocalin isolated from tears comprises over 75% of DNA endonuclease activity (415). Tear

lipocalin is capable of complexing to many tear lipids such as ceramides fatty acids, alcohols, phospholipids, cholesterol, glycolipids, and diacylglycerols (416).

iv. Female genital tract

Lcn2 levels in vaginal secretions were 456 ng/mL in women with no inflammation, and Lcn2 levels increased to 780 ng/mL in women with intense inflammation (417). Lcn2 levels measured in cell-free vaginal lavages are a mean concentration of 1,242 (392-3,432) μg/L (373). In another study, Lnc2 levels were measured in the vaginal secretions of women diagnosed with bacterial vaginosis and vulvovaginal candidiasis (418). Lcn2 levels were as follows: control (561 ng/mL), bacterial vaginosis (402 ng/mL), and vulvovaginal candidiasis (741 ng/mL) (418). Murine lipocalins are not only highly abundant but also demonstrate differential expression throughout the proestrus, estrus, and metestrus phases (419).

v. Rectum

Lcn2 expression is induced in inflammatory sites of colonic and intestinal epithelial cells (402, 420-422). Like Lf, Lcn2 has been proposed to be used as diagnostic biomarker for IBD (397, 422, 423). Inflammation or the diagnosis of such inflammatory bowel diseases, leads to an increase in Lcn2 expression in the gut. In the mouse, restriction of dietary iron intake reduced fecal and serum Lcn2, and inflammatory responses induced by bacteria in the gut (424).

IV. Importance of iron acquisition for *Neisseria gonorrhoeae*

A. Nutritional immunity

Metalloproteins not only protect the host from iron toxicity, but they also play an essential role in nutritional immunity (192). Nutritional immunity is a host defense against infection, where metalloproteins sequester valuable nutrients away from pathogens (196, 207).

Pathogens require metals for essential metabolism and infection, as such, there is a constant battle between host and pathogen to acquire essential metals. Free (unbound) serum iron levels range from 10^{-18} M and 10^{-24}M, which are insufficient levels to allow for bacterial growth: each bacterial cell requires 10^5 and 10^6 M iron (260, 331, 425). Because of the dearth of free environmental iron, pathogens have adapted ways to acquire metals, such as iron and zinc, directly from host metalloproteins (260, 331). *N. gonorrhoeae* acquires iron from several human host metalloproteins: Tf, Hb, Hp, Lf, and a potential TDT ligand, lipocalin (191).

B. TonB-dependent transporters

TonB-dependent transporters (TDTs) are the main methods for iron acquisition in *N. gonorrhoeae*. TDTs are present in most *Neisseria* strains and are highly conserved, suggesting that it is essential to survival (266, 281, 283, 296, 311). TDTs are employed by many Gram-negative bacteria to pirate iron, zinc, and other metals directly from host metalloproteins (257, 286, 287, 426). TDTs are characterized as beta-barrels embedded in the outer membrane of many Gram-negative bacteria (347, 427, 428). A lipoprotein in the outer-membrane often assists in ligand-binding the beta-barrel receptor (266, 346, 347). In the pore of the beta-barrel is the plug domain. With the help of TonB, located in the inner membrane, TDTs pirate metals such as iron and zinc from metalloproteins (286, 287, 299, 345). A metalloprotein, often derived from the host, serves as the ligand for the receptor. Upon ligand binding, TonB, powered by the proton-motive force, imports the metal ion into the cell (299, 308).

The mechanism of TDT metal import is still being characterized, but it is hypothesized that ligand binding creates a conformational change in the receptor, exposing the metal ion to the plug domain that is located in the pore. After the conformational change, the iron then has

higher affinity to the plug domain than the ligand; thus, the metal ion then relocates to the plug domain. TonB is required for release of ligand and pulling the plug domain into the periplasm, where the metal ion is exposed to a transporter protein that will ferry it to an ABC transporter in the inner membrane (429). The ABC transporter then imports the metal ion into the cytoplasm, where it can then be used for essential metabolic processes and ultimately replication and pathogenesis (308, 345-348).

Several outer-membrane receptors have been identified for iron acquisition, and each of these transporters have a specific protein that acts as the ligand. To date, receptors have been described to acquire iron from Tf and Lf, and iron in the form of heme from hemoglobin (430). TbpA is one of the best characterized TDTs, and hTf serves as the ligand for iron-extraction (308). Lactoferrin-binding protein A (LpbA), binds to Lf, and HpuAB binds to Hb and Hb-Hp complex (226, 231, 243, 362, 431). Several TDTs have been identified to import zinc, but this review will focus specifically on TDTs and iron (286, 287, 432). TDTs are not constitutively expressed: pathogens will induce expression of these TDTs exclusively in metal-deplete conditions to conserve energy (192).

TDTs have been the focus of many vaccine studies (296-303) because they are highly conserved and present in all pathogenic *Neisseria* (308). Most TDTs are not subject to high-frequency antigenic variation, which is a mechanism the pathogen employs to evade the adaptive immune response by having the capability to express exponential versions of ever-changing surface antigens (45). For these reasons, the TDTs have been identified as ideal vaccine candidates (283, 301). This review will summarize the important iron metalloproteins and tissue localization involved in gonococcal pathogenesis.

C. The gonococcal hemoglobin receptor system

N. gonorrhoeae acquires iron via heme with the two-component TDT system HbuAB, where HbuB (85 kDa) functions as the outer-membrane receptor (249, 250), and HpuA (35 kDa) is a lipoprotein (83, 247, 251, 254). HpuAB binds to Hb, Hb-Hp, and apo-haptoglobin (83). HpuA and HpuB are both required for acquisition of iron in the form of heme from Hb and Hb-Hp complexes (83, 251-254). The gene *hpuAB* undergoes phase variation due to polyguanine (G) tracts, causing slipped-strand mispairing during DNA replication to cause a functional frameshift (83, 128, 251).

Another similar receptor system called the HmbR system exists in *Neisseria meningitidis*; however, in *N. gonorrhoeae*, *hmbR* exists as a nonfunctional, frameshifted pseudogene (257, 258). Gonococcal infection disseminates to the blood stream in less than 3% of cases; thus, acquisition of iron via heme is likely not as important for the gonococcus than the meningococcus, which characteristically invades the bloodstream (58, 258). In the female genital tract, gonococcal isolates taken from women in early menses are more likely to express HpuAB, suggesting that *N. gonorrhoeae* is utilizing HpuAB expression when Hb and Hp are abundant (128, 259). There is evidence that other bacteria extract heme from hemopexin; however, heme extraction from albumin or hemopexin has yet to be identified in *Neisseria* (231).

D. The gonococcal transferrin receptor system

The TDTs, TbpA and TbpB, extract iron directly from host Tf for the gonococcus (266, 299). Gonococcal TbpA is a 105 kDa, 22-stranded β-barrel outer-membrane receptor, and TbpB is a 75 kDa lipoprotein that facilitates Tf binding to host Tf (266, 299, 433). It has long been

observed that TbpA is highly conserved and present in all gonococcal strains (225). TbpA specifically recognizes hTf (434, 435) and is required for Tf utilization (280). TbpB is not essential for iron acquisition from Tf, but TbpB expression facilitates iron uptake (309). Unlike its lipoprotein TbpB, TbpA does not have a preference for binding iron-saturated Tf or apo-Tf (309, 313, 435, 436). In a study of male subjects, a gonococcal strain deficient in TbpA and TbpB was unable to colonize the male urethra, suggesting that the ability to acquire iron from Tf is essential for colonization and infection (282).

E. The gonococcal lactoferrin receptor system

N. gonorrhoeae utilize lactoferrin-binding protein A (LbpA) and lactoferrin-binding protein B (LbpB) to extract iron from Lf (225). Only 50% of gonococcal strains express LbpA; whereas, all gonococcal strains express the transferrin receptor (TFR) (227, 228, 266, 299). Of *lbpA+ N. gonorrhoeae* strains, approximately 30% do not express LbpB, suggesting that LbpB is not required for the use of the LbpA receptor (225, 229, 230). Additionally, *lbpB* is subject to phase variation (311). The lactoferrin receptor supports male urethra gonococcal colonization if the TFR is nonfunctional (225). Together, these findings suggest that the lactoferrin receptor provides an advantage for gonococcal colonization but is not essential for gonococcal infection (225).

V. Inflammation, iron, and infection

Bacterial infection and inflammation act as signals for the host to deplete iron by activating an acute phase response and/or upregulating nutrient sequestration mechanisms (193, 257, 260, 437). Low blood iron was first noted in 1946 during the first 24 hours of acute

infection in patients (438). Since then, much work has been done to understand the relationship between inflammation, infection, and iron.

A. Host cellular iron export

The receptor ferroportin and the serum hormone hepcidin regulate iron export into the plasma (193). Ferroportin is a receptor expressed by iron-storage cells, including hepatocytes and macrophages, which export iron into serum. Export of iron is tightly-regulated to ensure that serum iron levels do not reach harmful levels. In iron-repleted conditions, hepcidin binds to the ferroportin receptor, which triggers degradation of the receptor, and ensues inhibition of iron efflux (193). The secretion of mediators from erythroid precursors downregulates hepcidin production as serum iron levels increase (193). In hepcidin-deficient mice, Lcn2 levels are upregulated, suggesting that Lcn2 may mediate hypoferremia (low serum iron levels) (439).

B. Hepcidin and iron retention in macrophages

Cytokines and tissue damage from inflammation are known to induce hepcidin production, which promotes iron, heme, and Hb sequestration by macrophages and other iron-storage cells (193, 440). Inflammatory cytokines such as IL-1, IL-6, and IL-22 upregulate hepcidin production in the liver, which increases intracellular iron stores (441, 442). Synthesis of hepcidin is stimulated primarily by IL-6 in infection (442). Endoplasmic reticulum stress and lipopolysaccharide (LPS) have also been found to stimulate hepcidin production (441, 443). Cytokines also modulate hepcidin production. Tumor necrosis factor alpha (TNF-α) inhibits iron absorption in the small intestine (444). Pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and interferon gamma (INF-γ) modulate iron internalization; however, an increase in iron storage seems to be situationally dependent (445-448). Anti-inflammatory cytokines such as IL-

4, IL-10, and IL-13 have also been observed to increase iron storage in monocytes and macrophages (445, 446, 448-451).

C. Recycling of erythrocytes

As serum iron levels dip below 10 to 30 μM, inhibition of erythropoiesis, the synthesis of erythrocytes, is initiated (193). Maturing erythrocytes require approximately 25 mg of iron daily to support Hb production, making erythropoiesis the most iron-expensive process in the body (219, 452). Because dietary iron intake is only an estimated 1 to 2 mg, macrophages have the important role of recycling iron from senescent erythrocytes to newly produced erythrocytes (194, 244).

D. The host transferrin receptor

The TFR is expressed ubiquitously in the host and binds to Tf to obtain iron (452). Iron delivery in erythropoiesis, the synthesis of erythrocytes, is mediated by transferrin receptor 1 (TFR1) (452-455). TFR1 expression is expressed ubiquitously, as all cells require iron, but the highest expression is found in erythrocytes due to the high need for iron in Hb synthesis (452, 455). Upon hTf binding to TFR1, clathrin-mediated endocytosis imports the hTf-TFR1 complex into the cell, followed by endosome acidification to release free iron, and recycling of apo-hTf and TFR1 (452, 456, 457).

TFR2 is ubiquitously expressed, with the highest expression in erythrocytes (erythroblasts) and hepatocytes, where TFR2 participates in iron homeostasis (452). Recently, high TFR2 expression has been observed in macrophages, osteoblasts, and neurons (452). The role of TFR2 in erythropoiesis is still being elucidated, but a role in intracellular iron trafficking directly to the mitochondria has been suggested (452, 458). TFR2 is primarily associated in the

lysosome compartment, has a mitochondrial targeting sequence, and associates with MFN2, a receptor involved in mitochondria-lysosomal contacts (452). Direct iron transfer to the mitochondria would prevent generation of oxidative stress in the cytoplasm (452). A similar iron-delivery mechanism has already been observed in neurons (459).

Evidence suggests that TFR1 and TFR2 act as sensors to extracellular iron concentrations (452). When iron levels drop below the host's preferred physiological levels, erythrocytes are used to redirect iron for the most essential processes such as metabolism, host defense, and neurobehavioral function (193). Iron is stored in hepatocytes and splenic and hepatic macrophages (193). Macrophages recycle 95% of daily metal needs via erythropoiesis of senescent erythrocytes (261, 403, 460). In turn, heme is recovered and degraded by heme oxygenase-1 into iron, biliverdin, and carbon-monoxide (196, 365, 461, 462). Ferritin is highly upregulated after erythrophagocytosis, suggesting an important role in iron storage in macrophages (365, 463).

If a pathogen could modulate the TFR, iron internalization could increase or decrease, depending on the mechanism. Cytokines have been shown to increase iron retention via the TFR. In human monocytic cell lines THP-1 and U937, cells treated with both INF-γ and LPS reduced Tf receptor mRNA, protein expression, and iron uptake; however, IL-10 was observed to stimulate iron acquisition via Tf receptor (445). The same study revealed that combination treatment of INF-γ and LPS induced expression of divalent metal transporter 1 in a dose-dependent fashion, leading to increased uptake of ferrous iron; meanwhile, IFN-γ and LPS treatment downregulated ferroportin transcription (445). INF-γ downregulates expression of TFRs as well as intracellular ferritin levels in monocytes (448, 464).

N. gonorrhoeae can invade cells, including macrophages and neutrophils, which are the first immune cells to arrive at the site of infection (195). Iron retention in macrophages could be particularly beneficial for gonococcal infection, as iron retention in macrophages inhibits nitric oxide formation which normally aids in killing of intracellular bacteria (196). Interestingly, upon infection of monocytes and macrophages, *N. gonorrhoeae* can upregulate hepcidin and downregulate ferroportin, resulting in an overall increase of iron retention (195). *N. gonorrhoeae* and *Neisseria meningitidis* have been shown to reduce expression of the TFR in infected epithelial cells (465), and *N. meningitidis* has shown reduction of mRNA levels of host transferrin receptor 1 (TFR-1) on epithelial cells (466).

VI. Importance of metalloproteins and *N. gonorrhoeae*

The role of Lcn2 in gonococcal infection has not been studied. Lcn2 is crucial in controlling intracellular replication in mycobacteria infection in alveolar macrophages (405). While Lcn2 is considered protective against bacterial infection, *Salmonella* is able to exploit host Lcn2 and calprotectin in the gut (467, 468). No evidence is available on whether *N. gonorrhoeae* is capable of exploiting Lcn2, but the role of Lcn2 in gonococcal infection deserves some investigation. If *N. gonorrhoeae* is able to bind Lcn2 and extract iron from a bound siderophore, there may be an unidentified virulence factor, like the TDTs, which could be used in a vaccine.

If Lcn2 is not exploited by the gonococcus or manipulated to benefit infection, Lcn2 may be a promising therapeutic. One such method involves utilizing Lcn2 as a scaffold to generate an anticalin. Anticalins are a novel synthetic alternative to monoclonal antibodies that recognize specific ligands (469-472). Lcn2 is a small protein (25 kDa), consisting of a simple

polypeptide chain, and is bioactive without posttranslational modification (469). Lcn2 can be utilized as a scaffold and modified in order to generate specificity to a specific target (405, 469). The scaffold structure can be altered to modify plasma half-life, immunogenicity, and tissue penetrance (405, 469). Anticalins have already been shown to be feasible: digoxigenin-binding anticalins are specific to digitalis, a cardiac drug, and reversed digitalis toxicity (471). Anticalins specific to oncofetal fibrinogen (473) and vascular endothelial growth factor receptors (VEGFR) (474) are currently being evaluated. Development of an anticalin for use in bacterial infection has been demonstrated as proof of principle in several studies (470). *N. gonorrhoeae* has already reached "superbug status," ushering the development of new treatments and a vaccine. This review has highlighted several key areas for future therapeutic and vaccine work.

Chapter 5: Implications, perspectives, and future

The sexually-transmitted infection, gonorrhea, is caused by the Gram-negative pathogen *Neisseria gonorrhoeae* (356). Incidence of gonococcal infections is estimated to be 1.6 million cases in the United States as of 2018 (47). Prior gonococcal infection does not provide protective immunity, and there is currently no available vaccine to prevent spread of the disease (333-335). Additionally, antibiotic resistance is on the rise, and *N. gonorrhoeae* has been labeled as a key research priority (47, 336-342). Until December 2020, a dual therapeutic of azithromycin and ceftriaxone was recommended by the CDC for gonorrhea (76). The high incidence of isolates reported to have resistance to azithromycin has led the CDC to discontinue the use of azithromycin for uncomplicated gonococcal infection treatment (76). After discontinuation of azithromycin, the only effective therapeutic remaining is ceftriaxone, of which resistant isolates have already been reported (76, 342, 475-484). Without effective treatment, gonococcal infections can ascend the reproductive tract and cause severe secondary sequelae (9, 45, 55, 56). For these reasons, development of alternative therapeutics and an effective vaccine is imperative.

TonB-dependent transporters (TDTs) are essential for nutrient acquisition in Gram-negative pathogens (257, 286, 287, 426). TDTs are essential virulence factors for pathogenic *Neisseria*. Under metal-deplete conditions, the expression of TDTs is activated, allowing the pathogen to extract metals such as iron or zinc directly from host metalloproteins (192). The receptor is located in the outer-membrane of the pathogen, and often a lipoprotein will assist in ligand binding. Upon binding, the receptor extracts metal from the ligand via TonB, energetically powered by proton motive force (288, 429). A plug domain resides in the β-barrel

of the receptor. Upon ligand-binding, activation of TonB pulls the plug domain into the periplasm. Followed by periplasmic exposure of the metal ion, a metal-binding protein will transport the metal to an ABC transporter in the inner membrane where the metal will be internalized to the cytoplasm and used for essential metabolism **(Figure 1.3)** (266, 345).

Humans require iron for key metabolic processes; however, excess iron generates reactive oxygen species, which are cytotoxic (192, 260). Excess free iron in the host also promotes bacterial infections. To thwart bacterial infection, the host employs metalloproteins to chelate excess free iron (192). *N. gonorrhoeae* steals iron from host Tf or Lf, and iron in the form of heme is taken from Hb using TbpA, LpbA, and HpuAB, respectively **(Figure 1.2)** (226, 231, 243, 362, 431). Tf, Lf, and Hb are found in high abundance at gonococcal infection sites, including the mucosa and bloodstream. The availability of these aforementioned iron-binding proteins promotes iron acquisition and subsequent colonization. The gonococcus is also able to modulate iron regulation in the host by promoting iron retention in several important iron storage cell types, including macrophages (195) and epithelial cells (465). Iron retention in macrophages inhibits intracellular killing of bacteria and may allow for increased survival and ascension of gonococcal infection to the upper reproductive tract (195). Further investigation of modulation of host iron homeostasis will provide a better understanding of gonococcal infections and potential ways to hinder infection. Understanding how the gonococcus exploits host metalloproteins and manipulates host iron homeostasis is relevant for identifying potential targets for alternative therapeutics, which are highly necessary as the occurrence of antibiotic resistance increases.

The siderocalin Lcn2 is also present in high concentrations at key gonococcal infection sites, which suggests that it may play a role in gonococcal infection. *N. gonorrhoeae* may interact or bind Lcn2 for iron acquisition in a similar manner to Tf, Lf, or Hb. Characterizing such a mechanism could reveal another receptor with vaccine candidate qualities. On the other hand, if no interaction between *N. gonorrhoeae* and Lcn2 can be determined, this may suggest that Lcn2 could be used therapeutically.

The TFR system, including TbpA and TbpB, is perhaps the best characterized TDT (308). The receptor TbpA, an integral outer membrane protein, binds to hTf, extracts and internalizes iron for essential metabolic processes (266, 308, 348). The lipoprotein TbpB facilitates transferrin binding, but is not absolutely required for iron-internalization from Tf (266, 309, 346, 347). TbpA and TbpB are considered ideal vaccine candidates because both are highly conserved, not subject to high-frequency antigenic variation, and essential to iron-acquisition from Tf (257, 266, 280-283, 296, 311, 314). Immunogenicity has been characterized for both proteins in *Neisseria* (304, 305, 316). Of the two proteins, TbpB is more immunogenic and elicits more bactericidal activity and serum IgG levels (304, 305, 316). Additionally, the amino acid sequence of TbpB is more variable than TbpA, and anti-TbpB antibodies are typically less cross-reactive than anti-TbpA antibodies (281, 283, 305). In a study using TbpA and TbpB conjugated to cholera toxin B subunit to intranasally vaccinate mice, anti-TbpB antibodies were less cross-reactive but more abundant than anti-TbpA antibodies (305). Vaccination with TbpA and TbpB epitopes fused to the cholera toxin A2 subunit, elicited bactericidal antibodies specific to TbpA and TbpB in mice; however, anti-TbpB serum IgG levels were much higher than anti-TbpA (306).

Sera collected from rabbits vaccinated with intramuscular injection of a rTbpA fragment elicited polyclonal antibodies specific to rTbpA and demonstrated bactericidal activity against *H. parasuis* (318); however, vaccination with rTbpA or rTbpB in colostrum-deprived piglets provided no protective advantage following challenge with *H. parasuis* (298). Immunogenicity of the Tbps has been somewhat inconsistent. Cross-reactive and bactericidal anti-TbpB meningococcal antibodies have been observed in vaccinated rabbit sera (322) yet, rTbpB vaccination failed to provide protection from *H. parasuis* infection in colostrum-derived piglets (298, 320, 321).

This promising but overall lackluster immune response prompted researchers to consider methods of improving immunogenicity. Binding to host proteins is hypothesized to inhibit development of a robust immune response, due in part to the recognition of self-antigen (281, 283, 299, 308). The ability to bind to host is thought to be a key factor in the poor and somewhat inconsistent immune response following vaccination with Tbps. The feasibility of protection using a nonbinding mutant has been shown with TbpB in *H. parasuis*. Colostrum-deprived pigs were vaccinated with an intramuscular injection of TbpB or a nonbinding TbpB mutant (Y167A) (303). Pigs vaccinated with the Y167A TbpB mutant yielded better protection when challenged with *H. parasuis* and superior B- and T-cell responses in sera compared to pigs vaccinated with WT TbpB (303). Fifty percent of pigs vaccinated with WT TbpB survived to the end of the study, whereas, 100% of the pigs vaccinated with the Y167A mutant survived, suggesting that the nonbinding mutant is a better vaccine antigen than WT (303). Only 20% of pigs survived after vaccination with the currently available vaccine (Porcilis Glässer), which implies that vaccination with either TbpB is an improvement (303). This observation of

improved immunity in nonbinding mutants is not exclusive to the TDTs. The meningococcal factor H binding protein (FHBP) binds to human compliment factor H (fH), and is already a component in the Menz4B vaccine. Vaccination with a non-host binding FHBP in a fH transgenic mouse model elicits higher bactericidal antibody levels than naïve, binding FHBP (156).

This current study aimed to apply the aforementioned concepts to gonococcal TbpA, by mutagenizing residues of the loop 3 helix (L3H) domain, which is essential for hTf binding (299, 324). The published, cocrystal structure of the meningococcal TbpA (strain K454) bound to hTf has been instrumental in identifying the L3H as essential for hTf binding (299, 345). Deletion of the TbpA L3H, prevents the gonococcus from binding hTf, internalizing iron from hTf, and growing when hTf is used as the sole iron source (299). Our lab previously aimed to abolish hTf binding by inserting point mutations in the L3H; however, point mutations to alanine or the opposite charge were insufficient in abolishing hTf binding in gonococcal strains expressing mutant TbpA (299). TbpA point mutations also failed to show significant impairment of iron-internalization and growth inhibition when hTf is used as the sole iron source (299). Because alanine or opposite charge mutations were insufficient in generating a nonbinding TbpA mutant, and because the sequence of the helix is not well-conserved, we hypothesized that the structure itself was critical for iron extraction. The current study, therefore, proposed to insert proline mutations on the L3H with the hypothesis being that prolines would be more disruptive to the helical structure.

The goal of this study was to identify a TbpA mutant with abolished hTf binding. The L3H of TbpA was mutagenized in gonococcal strain FA19 to characterize the effects of proline mutations on hTf binding, iron internalization, and growth when hTf is the sole source of iron.

Single proline mutations were inserted at the following residues: D355, A356, N357, Q358, K359, Q360. Single point mutations are preferred, because one residue change is less likely to change the overall protein conformation; however, one residue change may fail to abolish or significantly impair hTf binding. In the event that one residue change was unable to abrogate hTf binding, two additional TbpA mutants were analyzed in this study: the double mutant, K359P/Q360P, and the quadruple mutant, N357P/Q358P/K359P/Q360P. A total of sixteen TbpA proline mutants were generated: eight L3H mutants were generated in both *tbpB+* and *tbpB-* backgrounds. The TbpA D355P and A356P single point mutations demonstrated the most significantly reduced hTf binding and are, therefore, the best vaccine candidates.

Vaccination studies will be conducted on both aforementioned mutants to evaluate the immunogenic response; however, a single-component vaccine is not ideal. A combination vaccine including nonbinding Tbps and any other TDTs is predicted to elicit a broader immune response. Multiple proteins provide diverse epitopes and expand the available antibody repertoire (281, 283, 307). Additionally, the inclusion of TbpA and TbpB in a gonococcal vaccine would combine the immunogenic and cross-reactive properties of each protein. TbpB is more immunogenic and variable (281, 305, 306, 316, 485), and TbpA elicits more cross-protective antibodies (283, 307). Generation of a nonbinding TbpB mutant will require further mutagenesis studies to abrogate hTf binding, like our study with TbpA.

Because of the variability of TbpB, there is a chance that anti-TbpB antibodies will not offer great cross-protection. The diversity of TbpB in the Gram-negative pig pathogens (*Actinobacillus pleuropneumoniae, Actinobacillus suis, and H. parasuis*) can be broken into 3 groups not based on species specificity (486). The highest sequence diversity in TbpB is

observed at the transferrin binding face (N-lobe) (486). The phylogenetic tree of TbpA in the pig pathogens can be broken into more subgroups that primarily follow species divisions, but sequence diversity is the highest at the hTf-interacting face (486). There is little correlation between clustering patterns of the TbpA and TbpB phylogenetic trees, suggesting that horizontal gene exchange occurs independently (486). Phylogenetic analysis of the sequences of TbpA and TbpB in *N. gonorrhoeae* and *N. meningitidis* show the grouping of one subset in regards to TbpA and two subsets of TbpB (307, 433, 487). The sequence of TbpB is substantially more variable than the sequence of TbpA, but the hTf-binding interface contains the most sequence variability in both Tbps (307). Together, sequence analyses suggest that two to three nonbinding porcine TbpB variants may be feasible and sufficient for cross-protection against the pig pathogens (307, 357, 486). These results also suggest that two nonbinding TbpB variants may be necessary for sufficient cross-protection in a neisserial vaccine (307, 433). TbpB of *H. parasuis*, a pig pathogen, shares only 24 to 35 % sequence identity to TbpB in TbpB in *Neisseria* (data not shown). Because the amino acid sequence of TbpB is so different between *Neisseria* and the pig pathogens, extrapolating the use of the Y167A nonbinding mutagenesis studies cannot be applied to neisserial TbpB. In fact, the *H. parasuis* Y167 residue is not conserved at all with *Neisseria* TbpB. Likewise, the TbpA the amino acid sequence and protein structure are so different from TbpB that mutagenesis studies cannot be compared between the two proteins. The highest sequence variability for both Tbps is located at the hTf-interacting region (486). The L3H of TbpA, which is not shared in TbpB, is located at the hTf-interacting region and essential to hTf binding (299). This current study targeted the L3H in the hTf-interacting region of TbpA and identified several mutations that significantly abrogate hTf binding to TbpA.

The primary goal of this study was to identify a nonbinding TbpA mutant. The available structural data and initial mutagenesis studies suggested that the last two residues in the L3H, K359 and Q360, are important for hTf binding, due to their positive charges and vicinity to hTf (299, 488). Our data suggest that the location of the proline mutation may seem subtle between two adjacent residues, but even moving the proline mutation one residue provides a great impact on hTf binding and, presumably, the structure of the helix **(Figure 3.1 and Figure 3.2)**. Further structural analysis will be needed to provide better explanation. The TbpA D355P and A356P mutants resulted in the most significant reduction hTf binding **(Figure 3.5)**, but interestingly, only the A356P mutant was able to grow on hTf-loaded plates **(Figure 3.7)**. Both D355P and A356P *tbpB*- mutants had significantly reduced hTf binding and iron internalization levels **(Figures 3.5 and Figure 3.6)**, which implies that both mutants are unable to properly bind hTf and internalize iron; however, the different growth phenotypes are difficult to interpret **(Figure 3.7)**. These findings suggest that hTf binding does not necessarily correlate with iron uptake or growth phenotype on hTf plates. Further structural and mutagenesis studies will better elucidate the mechanism of iron uptake and relevance of our findings.

This study is the first to abrogate hTf binding by a single point mutation in TbpA. We recommend that both D355P and A356P mutants be tested as vaccine antigens because hTf binding was almost completely abrogated **(Figure 3.5)**. Not only will structural and kinetic studies be essential for identifying the best vaccine candidate, but they may shed light on the mechanism for hTf binding and iron internalization, which is still not fully understood. Obtaining a structure for a TbpA that is not complexed to hTf is expected to be difficult because the extracellular loops are flexible without bound hTf. An ideal nonbinding protein vaccine antigen

will resemble the 3D conformation of the native protein as close as possible in order to preserve epitopes and generate antibodies that are cross-reactive to both WT and mutant. How each D355P and A356P mutation affects the protein conformation of TbpA is not clear; however, preliminary analysis using western blotting and protease-digestion suggest that each mutant in this study has a similar conformation to WT TbpA **(Figure 3.3 and Figure 3.5).** Additionally, because TbpB has such a strong affinity to hTf, it can compensate for abrogation of hTf binding to TbpA **(Figure 3.5- Figure 3.7)**, which suggests that the TbpA proline mutants are functional (299, 309). These data are merely a rudimentary analysis of protein structure; therefore, both mutants are recommended for further analysis.

Despite similar hTf binding levels, each mutant may elicit a different immune response. In the previously mentioned TbpB vaccine study in pigs, colostrum-derived piglets were vaccinated intramuscularly with two mutants (Y167A and W176A) (357). Both mutants targeted the hTf-interacting N-lobe and share similar biochemical properties and porcine transferrin-binding levels (357). Following challenge with *H. parasuis*, the Y167A mutant provided substantial protection, but none of the pigs vaccinated with the W176A mutant survived to the end of the experiment (357). Immunization with the W176A mutant generated a robust immune response, produced antibodies, activated B-cells and T-cells, and activated complement (357). Characterization of the W176A mutant revealed no obvious cause for the lack of protection (485). These studies emphasize the need for robust characterization of both D355P and A356P nonbinding mutants and also suggest that the development of protection using nonbinding mutants is poorly understood.

A gonococcal vaccine using a nonbinding TbpA mutant has several possible mechanisms for effectively protecting against gonococcal infections **(Figure 5.1)**. Development of mucosal IgA antibodies promotes neutralization of gonococci by coating the bacterium with IgA (133). IgA coating could inhibit the gonococcus from adhering to the mucosa or promote phagocytosis (133). Development of antibodies could also result in the blocking of hTf binding to TbpA receptors and subsequent inhibition of iron internalization from hTf. The transferrin receptor system is required for gonococcal colonization in a male urethral infection model (282), highlighting the importance of hTf binding in infection. Blocking of Tbp receptors may slow growth by inhibiting iron uptake; however, the effect on growth and iron internalization will depend on the levels of antibody and abundance of receptor. As demonstrated previously (299) and in this study **(Figure 3.5 and Figure 3.7)**, gonococci can grow even when hTf binding is reduced to approximately 80-90% of WT hTf binding. Anti-Tbp serum antibodies demonstrate reduced hTf binding and meningococcal growth in mice and rabbit models (489, 490). Vaginal anti-Tbp antibodies have also been shown to inhibit growth of the gonococcus (306). Blocking antibodies may facilitate bacterial clearance by slowing gonococcal growth, providing more time for phagocytosis. Complement-mediated killing is the most important mechanism for controlling neisserial infection, and studies have shown that anti-Tbp antibodies demonstrate bactericidal activity in meningococcal (489) and gonococcal infections (305, 306). Lastly, the generation of opsonic antibodies may promote enhanced gonococcal killing by opsonophagocytosis, but this has not been evaluated in Tbp vaccination studies to date. Additionally, Opa-mediated phagocytosis may be affected by anti-Tbp antibodies. Neutrophils express CEACAMs that effectively bind to gonococcal Opa proteins, resulting in non-opsonic

phagocytosis and intracellular killing (189). The gonococcus can evade neutrophil-mediated phagocytosis by recognizing CEACAMs on other tissues to promote adherence and tissue invasion (189). This evasion strategy is particularly helpful with expression of Opas that do not recognize neutrophil-specific CEACAMs (189). Anti-Tbp antibodies could coat the pathogen and make CEACAMs more or less available for Opa-mediated phagocytosis by blocking the Tbp receptor(s). Antibodies could sterically hinder the interaction between Opas and CEACAMS, or the blocking of Tbps could facilitate Opa-CEACAM binding. The effect of this mechanism is dependent on Opa expression. If the gonococcal strain expresses Opas that do not recognize neutrophils, the gonococcus may gain a selective advantage to adherence and tissue invasion. On the other hand, neutrophil-specific Opa-CEACAM interactions could promote non-opsonic phagocytosis and bactericidal killing.

Vaccination studies will be conducted using purified rTbpA as a vaccine antigen in an hTf transgenic mouse model **(Figure 5.2A)**. Vaccination using hTf mice is the best model to compare vaccination with WT TbpA and a nonbinding TbpA mutant. Mouse transferrin does not recognize either Tbp; thus, no comparison can be made between a bound or unbound mutant. Neither the WT or nonbinding TbpA mutant protein is expected to bind to mouse transferrin; therefore, WT mice are not a good model for comparing proteins with different hTf-binding capabilities (325). The ability to express hTf in a mouse will allow us to properly investigate the true vaccine potential of a nonbinding Tbp mutant.

The inability of mouse transferrin to bind to Tbp antigen is speculated to increase the immune response. Serum and secretions collected from infected humans did not elicit antibodies to TbpA or TbpB in serum or secretions (316). Following vaccination with the Tbps,

antibodies were elicited in mouse serum and vaginal secretions and demonstrated bactericidal activity and gonococcal growth inhibition (304-306). A similar study used a fH transgenic mouse model to compare vaccination with WT FHBP or a nonbinding mutant, and the nonbinding mutant elicited significantly higher bactericidal antibodies compared to WT FHBP (156). We propose a similar study to compare WT TbpA, D355P, and A356P mutants in a hTf transgenic mouse model.

Recombinant WT TbpA, D355P, and A356P mutant proteins will be expressed and purified using *E. coli* and used for subsequent vaccination studies. First, a colonization study with determine if either mutant provides superior protection by calculating bacterial clearance, **(Figure 5.2A)**. Mice will be vaccinated intraperitoneally or intranasally with rTbpA 2-3 times, followed by challenge with gonococci in either the lower or upper genital tract infection model. Bacteria will be enumerated from daily vaginal lavages and provide median days of clearance. The duration of colonization will decrease if protection is observed. If protection is observed, there are several assays that can be used to characterize the type of immune response in serum and mucosal secretions: such assays will characterize antibody levels and subclasses, bactericidal activity, cytokine profiles, opsonization activity, and growth inhibition **(Figure 5.2B)**. Adjuvants, amounts of antigen, and location of immunization (intraperitoneal or intranasal) are all known to influence the immune response and will need to be investigated.

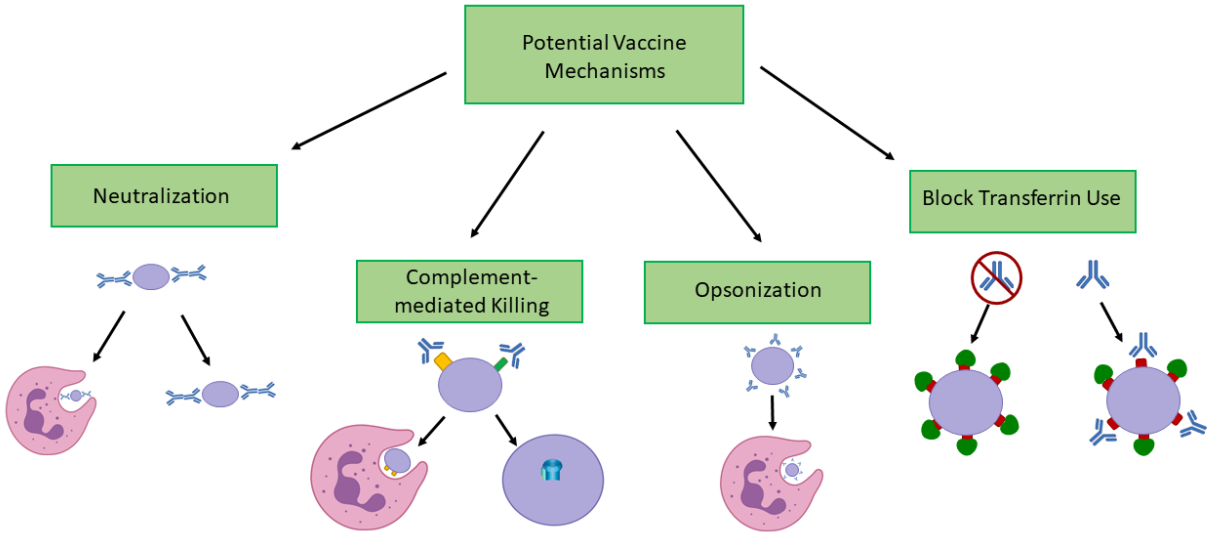

Figure 5.1. Potential vaccine mechanisms

Figure 5.1. Potential vaccine mechanisms

Vaccination with a nonbinding TbpA mutant has four potential paths for protection against gonococcal infection: neutralization, complement, opsonization, and nutritional blocking. Image made with BioRender.com.

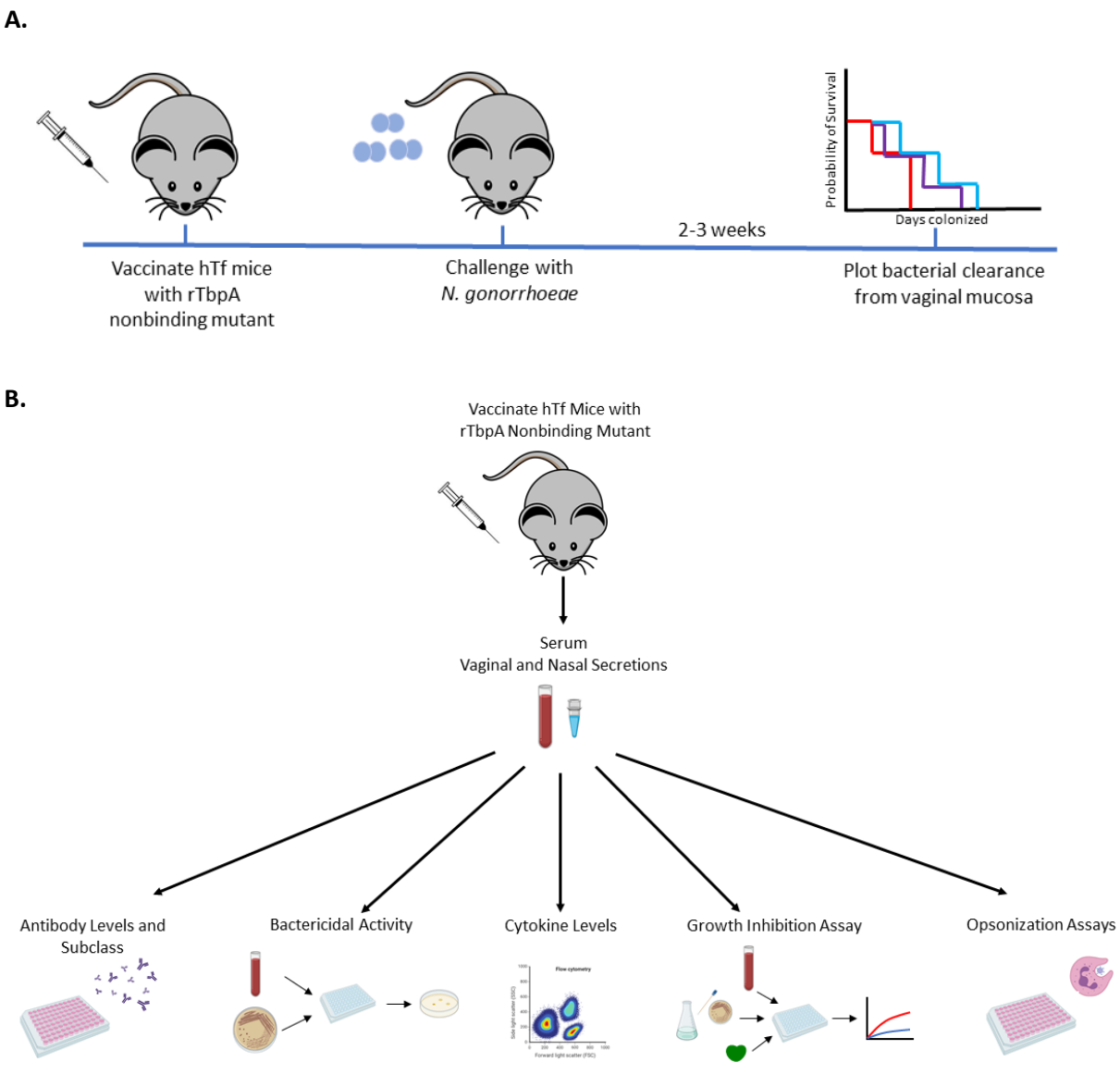

Figure 5.2. Vaccination model

Figure 5.2. Vaccination model

(A) shows the vaccination model using hTf mice. Mice will be vaccinated 2-3 times before being challenged with *N. gonorrhoeae* to determine if vaccination affects bacterial clearance rates. **(B)** If protection is observed, the immunological responses in serum and vaginal secretions can be characterized based on antibody levels and type, bactericidal activity, cytokine profiles, growth inhibition, and opsonization. Image made with BioRender.com.

Delivery of vaccine antigens is an essential step in vaccine research. Production, storage, and administration contribute to the preservation of the antigens. TbpB production and purification in *E. coli* has been established. On the other hand, TbpA is more difficult to produce as it is much larger and yields fewer amounts of protein due to toxicity to *E. coli* (307). The feasibility of mass protein production will need to be considered in development of a TbpA. Administration of vaccines is challenging. Injecting rTbpA or rTbpB directly into the bloodstream does not guarantee that proteins antigens are properly folded, which is essential for development of specific antibodies.

OMVs are a promising avenue for vaccine administration. The Bexsero vaccine provides proof of principle that OMVs can be an effective vaccine platform. Gram-negative bacteria naturally release OMVs, spherical bi-layered membrane structures. (491, 492). Production of OMVs is simple and cost-effective, and the size (diameter 20-50 nm) of OMVs is ideal for cell uptake (491). The natural bacterial components are intrinsically antigenic, a result of LPS and other pathogen-associated molecular patterns (PAMPS); however, modifications of the lipid A structure of LPS can be used to reduce LPS reactogenicity if levels are not tolerable (491). The membrane component allows for antigens to be displayed in their native conformation, which is especially a challenge when using a hydrophobic membrane protein, such as TbpA, as a vaccine. OMVs induce maturation and antigen presentation of antigen-presenting cells (APCs), which are spatiotemporally-dependent (492).

Our lab has already initiated similar mutagenesis studies on other TDTs, such as TdfH, TdfJ, and HpuAB. Efforts are being made to create nonbinding mutants similar to this study with TbpA. Identifying key binding residues is difficult, regardless of the availability of structural

data. No crystal structure has been resolved for TdfH or TdfJ, and the only available structure for full-length HpuAB is the *Kingella dentrificans* homolog, which shares 30% amino acid sequence identity and 48% similarity with *Neisseria* (243, 286, 287). Overall, generating nonbinding mutants has proven to be challenging and difficult to predict, but the combination of the meningococcal TbpA structure and results of this study can be used as a guide for selecting key binding residues for mutagenesis targeting other TDTs with similar structures.

Generation of a nonbinding TbpA gonococcal vaccine may also provide protection against meningococcal infection. Vaccination with the single component MeNZB vaccine has been associated with reduced rates of gonorrhea after mass vaccination and protection was estimated to be 31% (493). The meningococcal vaccine, Bexsero, contains the same MenNZB OMV and three recombinant proteins including NadA, FHBP-GNA2091, and NHBA-GNA1030 (39). Sera immunized with Bexsero elicits antibodies that recognize the gonococcus (39). Most of the MeNZB-like OMV proteins and NHBA share a high level of amino acid and identify with the Ngo homologs (44). Gonococcal NHBA produces antibodies that are bactericidal and opsonophagocytic (44).

The CDC has labeled gonorrhea as a superbug (46, 494). No alternative therapeutic or vaccine is currently available, and worldwide incidence is at 87 million cases per year (47). The lack of protective immunity and severity of secondary sequelae in untreated patients emphasize the urgent need for a vaccine (333-335). The TDTs are highly conserved, not subject to high-frequency antigenic variation, and have been viewed as promising vaccine antigens (257, 296). TDTs also play a vital role in metal acquisition for many Gram-negative pathogens. These studies highlight the importance of iron for the gonococcus and emphasize the

importance of the source of iron, location in the host, and iron acquisition methods. This study demonstrates the feasibility in generating nonbinding mutants in TbpA, similar to studies conducted with TbpB and NHBP (156, 303). This study is the first to identify TbpA mutants that are significantly defective in hTf binding due to a single point mutation. Ultimately, this study validates further analysis of nonbinding TbpA mutants for inclusion in a gonococcal vaccine. These findings can serve as groundwork for future mutagenesis studies in other TDTs, which can be applied to other Gram-negative pathogens including *Neisseria spp*., *Moraxella spp*., and *Haemophilus spp*.

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VITA

Ashley Greenawalt was born on April 27, 1991 in Brookville, Pa. She graduated from Clarion-Limestone Jr. Sr. High School in 2009. In 2013, Ashley graduated with her Bachelor of Science in Biology from Juniata College in Huntingdon, Pa. In 2016, Ashley matriculated into Biomedical Sciences Doctoral Portal at Virginia Commonwealth University School of Medicine and joined the Department of Microbiology and Immunology the spring of 2017. Her accomplishments and are listed below.

AWARDS

Phi Kappa Phi Membership, 2018 Virginia Commonwealth University; Dept. of Microbiology and Immunology

ORAL PRESENTATIONS AND POSTERS:

"Site-Directed Mutagenesis of Transferrin-Binding Protein A (TbpA) Loop 3 Helix to Characterize TbpA as a Potential Vaccine Candidate for *Neisseria gonorrhoeae*"

- U19 International Collaborators Meeting, Atlanta, GA, 2019. (Oral presentation)
- American Society for Microbiology, San Francisco, CA, 2019. (Poster)
- International Pathogenic *Neisseria* Conference, Asilomar Conference Grounds, CA, 2018. (Poster)
- R01 International Collaboration Meeting, Asilomar Conference Grounds, CA, 2018. (Oral presentation)
- Biomedical Sciences Doctoral Portal Colloquium, Virginia Commonwealth University, Richmond, VA, 2017. (Poster)

"Identifying Host Regulators in Ty1 Retrotransposition in *Saccharomyces cerevisiae*"

- Juniata College Liberal Arts Symposium, Huntingdon, PA, 2013. (Oral presentation)
- The Mid-Atlantic Transposable Element Meeting, John Hopkins University, Baltimore, MD, 2013. (Oral presentation)
- Juniata College Liberal Arts Symposium, Huntingdon, PA, 2012. (Poster)
- Third Annual Landmark Summer Research Symposium. Goucher College, July22, Baltimore, MD, 2012. (Poster)

"Developing an in-vivo assay for HOXB13 in an ER+ breast cancer cell line"

- Allegheny Branch of the American Society for Microbiology Annual Meeting, Pennsylvania State University, State College, PA, 2012. (Oral presentation)