Fall 12-8-2024

Characterization of Zinc and Manganese Homeostasis in Neisseria gonorrhoeae

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Characterization of Zinc and Manganese Homeostasis in *Neisseria gonorrhoeae*

by

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the Institute for Biomedical Sciences

Georgia State University

2024

ABSTRACT

Neisseria gonorrhoeae and its obligate human host require transition metals for biological functions including cell signaling and metabolism, gene regulation, enzymatic processing, and oxidative stress resistance. During infection, the host employs two innate immunity mechanisms: either starve the pathogen of metals or overload the pathogen with intoxicating amounts of metals, either resulting in bacterial growth inhibition. The mechanisms by which the host starves or overloads the pathogen with metal nutrients are termed nutritional immunity and metal overload, respectively. In response to nutritional immunity and metal overload, *N. gonorrhoeae* differentially expresses metal transport systems that allow it to maintain homeostatic metal concentrations within the cytoplasm. Such transporters include the high-affinity zinc importer, ZnuABC, which is regulated by the zinc uptake regulator Zur, and the manganese exporter, MntX. As the gonococcus acquires and maintains antibiotic resistance mechanisms, the necessity to develop novel therapeutics and treatments becomes more urgent. Metal transporters are attractive therapeutic targets as they are often required for survival and virulence. However, the precise mechanism by which zinc and manganese are transported, sensed by Zur, and impact the transcriptional response to maintain metal homeostasis have not yet been elucidated. Investigating the mechanism by which zinc and manganese are transported and sensed is crucial to characterizing gonococcal metal homeostasis in the face of host-employed nutritional immunity and metal overload. In this work, I hypothesized that Zur mounts zinc- and manganese-dependent transcriptional responses to metal limitation and metal overload and that this response maintains internal metal concentrations at homeostatic levels. RNA-sequencing, RT-qPCR showed that Zur is a

zinc-dependent regulator of the genes encoding ZnuABC and that manganese-dependent regulation by Zur is strain-specific. ICP-MS, growth assays, and transporter complementation experiments showed that internal homeostatic manganese levels differ between gonococcal strains and that this difference can be attributed to the manganese exporter, MntX. Therefore, novel treatment strategies that target metal transporters as a means of starving or overloading and subsequently killing *N. gonorrhoeae* should be informed by the metal environment sensed by *N. gonorrhoeae* and the intracellular metal pools maintained by different gonococcal strains.

INDEX WORDS: Zur, manganese, zinc, *Neisseria*

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August 2024

DEDICATION

To the one true God (Deut. 4:4, 1 Tim. 2:5), who goes by many names - Yahweh, Jehovah, Adonai, I AM, to the unchanging (Mal. 3:6), eternal (Rev. 1:4,8), and self-existent (John 5:26) Almighty God of the universe, from whom, through whom, and to whom all things flow (Rom. 11:36), I dedicate the first fruits (Neh. 10:35) of this dissertation, this uncovering of the facts God foreordained at the beginning. It is God and God only who established the things to be uncovered through science and it is his unwavering compassion and grace which has provided science as a means of revealing himself and his creation to us. Science is the tool God has given us to discover new ways to heal the sick, feed the many, and serve the helpless. However, science can only reveal that which is seen and observable – things of the flesh and this fallen world and not those things which are unseen – those things which are of the Spirit. Science cannot tell us how things ought to be or how things will ultimately be in the future. It cannot tell that which is good or evil, just or unjust, moral or immoral. To uncover these unseen truths, we must rely on Scripture, which in turn informs our scientific ethics and integrity. The ultimate purpose of science is to increase the quality of this physical life for all that we might turn to God in recognition of who he is and what he has done – to glorify him. However, there is life beyond this physical life, eternal life which is unseen by us now but will be known fully upon death (1 Cor. 13:12). This eternal life is a free gift from God (Rom. 6:23) to those who accept that God accomplished what science cannot – the forgiveness and expiation of sin through the crucifixion and resurrection of his Son, Jesus the Christ (Col. 1:20). It is my prayer that whoever reads this will be saved from a death that science cannot overcome and will come to a knowledge of the

truth (1 Tim. 2:4) - that is salvation from sin and death by the grace of God through faith alone (Eph. 2:8,9).

To my mother, Janet, father, Paul, and sister, Brianna, each of whom have sacrificed time, money, and comfort to help me grow into the God-serving woman and scientist I am today and will be in the future, I dedicate a portion of this dissertation. It was my mother who showed me how to walk with the Lord. It was she who stewarded the whole Armor of God (Eph. 6:10-17) and in doing so passed it down to me that I might carry it through all the battles, seen and unseen, that come with completing a dissertation. It was my father who showed me that God is still doing a sanctifying work in me (Phil. 2:13), which surpasses my work as a scientist. It was my sister who demonstrated unconditional love, abounding generosity, and steadfast grace to all in the midst of and sometimes by the way of her own lament. It was she that God used to reveal the character of his heart that I might extend those same graces to my colleagues and students.

To my church family who took me in with love and grace and demonstrated in action and words the truth of God, I dedicate the remaining portion of this dissertation. You welcomed me when I was a stranger, prayed for me when I was sick, and fed me when I was hungry (Matt. 25:35,36). You, the 99, were standing firm on the Rock when I, the 1, had gone astray and fell into sinking sand that through Christ I might be welcomed into the fold to stand firm with you.

Science has taught me that in this world, there are many troubles, but I take heart because Christ has overcome the world (John 16:33).

ACKNOWLEDGEMENTS

For her dedication and commitment to students and their needs, her scientific integrity and rigor, and her demonstrated values of freedom, collaboration, and curiosity, I would like to acknowledge my mentor, Dr. Cynthia Cornelissen. Thank you for always giving me the independence and freedom to think, learn, and speak. For her sacrificial service to her students, her extraordinary work ethic, and her invaluable friendship, I would like to acknowledge my mentor, Dr. Julie Stoudenmire. Thank you for being a great example of what it means to lead from behind.

Thank you, Dr. Denning for always challenging me and pushing me toward excellence and away from mediocrity. Thank you Dr. Eichenbaum for always supporting me and reminding me to be excited about science.

Thank you, Dr. Pooneh Tavakoley, for being patient with me and for being willing to give up your right to be right for me. Thank you, Natalie for always being there, for listening and being a friend when I needed one. Thank you, Reeham for being a sister to me. Thank you to the many lab members who have come and gone over the past few years.

I would like to acknowledge Ian Liyayi for providing the Ngo1049 antibody, Novogene for providing the RNA-sequencing analysis, and our collaborators, Dr. Alison Criss and Dr. Nick Noinaj, at the University of Virginia and Purdue University.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
%	Percent
~	Approximately
°C	degrees Celsius
α	alpha
β	beta
Δ	deletion
µg	micrograms
µL	microliter
µM	micromolar
ABC	ATP-binding cassette
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochlorid
ANOVA	analysis of variance
AP	alkaline phosphatase
apo-	Not bound to metal
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
C-	carboxy
C3	complement component C3

C4BP	complement component C4b binding protein
Ca^{2+}	Calcium (II)
cDNA	complementary DNA
Cd^{2+}	Cadmium (II)
CDC	Centers for Disease Control and Prevention
CDM	chelex-treated defined media
CEACAM	carcinoembryonic antigen-related cell adhesion molecules
CO_2	carbon dioxide
CP	calprotectin
Cu/Cu^{2+}	copper
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fe	iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
$Fe(NO_3)_3$	ferric nitrate
FPKM	fragments per kilobase of transcript per million mapped reads
Fur	Ferric uptake regulator
g	gram
GCB	gonococcal medium base
GSK	GlaxoSmithKline

h-lamp1	human lysosome/late endosome-associated membrane protein 1
h	hours
Hb	hemoglobin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
ICP-MS	inductively-coupled plasma mass spectrometry
IgA	immunoglobulin A
IgG	immunoglobulin G
IMD	invasive meningococcal disease
IL	interleukin
IPTG	isopropyl ß-D-1-thiogalactopyranoside
LB	Luria-Bertani
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
Mg^{2+}	magnesium (II)
mL	milliliter
mM	millimolar
Mn	manganese
Mn^{2+}	manganese (II)
Mn^{3+}	manganese (III)

Mnt	manganese transport
MSM	men who have sex with men
N-	amino
NaCl	Sodium chloride
NBT	nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
Nel	*Neisseria elongata*
NET	neutrophil extracellular trap
Ni^{2+}	nickle (II)
Nla	*Neisseria lactamica*
nm	nanometer
nM	nanomolar
OD_{600}	Optical density at 600 nanometers
OMV	outer-membrane vesicle
PBS	phosphate buffered saline
PMN	polymorphonuclear leukocyte
q-/PCR	q-polymerase chain reaction/ polymerase chain reaction
RNA	ribonucleic acid
RPM	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
TB	terrific broth

TBS	tris buffered saline
Tdf	TonB-dependent function
Tdt	TonB-dependent transporter
TGF-β	transforming growth factor beta
Th	T helper
TPEN	N,N,N’,N’-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
WHO	World Health Organization
WT	wild type
Zn/Zn2+	zinc
$ZnSO_4$	zinc sulfate
Znu	Zinc uptake
Zur	Zinc uptake regulator

CHAPTER 1: INTRODUCTION

I. The *Neisseria* Genus

The *Neisseria* genus is composed of non-motile, oxidase- and catalase-positive, Gram-negative, β-proteobacteria. The genus is composed of both commensal and pathogenic species with both human and non-human hosts (4). These bacteria are fastidious meaning they require media supplemented with growth factors. Growth for these capnophilic bacteria requires a moist, warm (35-37°C) environment supplemented with ~2-10% carbon dioxide (5, 6). Additionally, the *Neisseria* can utilize only some carbon sources for growth, those being glucose, lactate, pyruvate, and in some species, maltose (7-9). Growth of the *Neisseria* species is optimal under aerobic conditions and suboptimal but supported nonetheless under anaerobic conditions when nitrite serves as a terminal electron acceptor (10, 11).

II. Commensal *Neisseria*

The growth requirements of the commensal and pathogenic *Neisseria* are met within the host niches they occupy. Of the commensal species (spp.), *Neisseria perflava, Neisseria mucosa, Neisseria flava, Neisseria cinera, Neisseria lactamica, and Neisseria sicca* among other commensal *Neisseria* spp. are known to colonize the human nasopharynx (12, 13) and are a part of a healthy oral microbiome (14). Among the pathogenic *Neisseria* is *Neisseria meningitidis* which is carried asymptomatically by ~10% of children and young adults (15) but can cause disease only after crossing the nasopharyngeal barrier. Thus, *N. meningitidis* is both a commensal and a pathogenic species. Although, the transition from the carriage to disease state is poorly understood, limited horizontal recombination between *N. meningitidis* of

different clonal complexes due to clade-associated restriction modification systems (16) results in reduced exchange of virulence genes and thus limits the potential for transition from the commensal to the pathogenic state. Consequently, clonal complexes are reliable indicators of *N. meningitidis* virulence (16, 17). Infection by *Neisseria gonorrhoeae* , the other pathogenic *Neisseria* spp., can be asymptomatic in females. However, in contrast to carriage of N. meningitidis, asymptomatic infection by *N. gonorrhoeae* results in a disease state that often goes undetected; thus *N. gonorrhoeae* is considered strictly pathogenic.

As a consequence of occupying the same niche, commensal and pathogenic *Neisseria* spp. likely compete for space and resources. This is evidenced by reduced epithelial cell association and microcolony formation of *N. meningitidis* in the presence of *Neisseria cinera* (18). When individuals were challenged with *Neisseria lactamica* (Nla)*, N. meningitidis* carriage was reduced in *N. meningitidis* carriers (19). However, the reduction in *N. meningitidis* in the presence of Nla was due in part to cross-reactive antibodies against the pathogen rather than direct competition between bacteria. Similarly, in the presence of *Neisseria elongata* (Nel), *N. gonorrhoeae* exhibits reduced viability and is cleared more rapidly during murine vaginal infection (20). Despite the ability of the commensals to limit pathogenic *Neisseria* viability *ex vivo*, the pathogenic *Neisseria* establish a foothold at the colonization site. Established infection in the presence of commensal killing mechanisms could be in part attributed to the activity of type four secretion system (T4SS), which is present in the pathogenic *Neisseria* spp. but not the commensals and is able to export DNA (21).

Due to their proximity at the site of colonization, natural competence (22) and DNA sequence homology, commensal and pathogenic *Neisseria* readily exchange DNA sequences

(23) which are maintained in the chromosome and contribute to antimicrobial resistance among the pathogenic *Neisseria* spp (24-26). Thus, the commensal *Neisseria* are a genetic reservoir for the pathogenic *Neisseria*. The commensal and pathogenic *Neisseria* species demonstrate a balanced tension between competition and cooperation that allows both groups to persist in a highly specific host niche.

III. Pathogenic *Neisseria*

The pathogenic *Neisseria*, *N. meningitidis* and *N. gonorrhoeae*, are human-specific pathogens with significant public health impacts. Although the DNA sequence similarity between these two strains is 80-90%, they cause quite different diseases (27). *N. meningitidis* is carried asymptomatically in the naso- and oropharynx of ~5-10% of the population (15), but transitions to the disease state once it crosses the epithelial barrier, can result in invasive meningococcal disease, bacterial meningitis, and sepsis (28). *N. gonorrhoeae* is a strictly pathogenic species meaning that infections should always be treated in human host. *N. gonorrhoeae* primarily infects the urogenital and oropharyngeal mucosa but can also infect rectal and ocular tissues. Disseminated gonococcal infections do occur and can result in septic arthritis and endocarditis, although these symptoms are less common.

IV. *Neisseria meningitidis*

A. Meningococcal Disease

B. Etiology

Invasive meningococcal disease (IMD) is caused by *N. meningitidis* and manifests as meningitis and/or septicemia (meningococcemia). *N. meningitidis* infection symptoms can

include fever, headache, stiff neck, nausea, vomiting, photophobia, and confusion. Symptoms of late infection include disseminated intravascular coagulation (29), which can lead to bleeding into the skin and organs, skin necrosis and limb amputation (30). Septic shock due to *N. meningitidis* infection is difficult to manage and can lead to death within hours (29).

N. meningitidis is classified into serogroups based on differences in the capsular polysaccharide (28, 31). There are 12 characterized *N. meningitidis* serogroups, six of which (A, B, C, W135, X and Y) predominantly cause invasive meningococcal disease (31). Unencapsulated *N. meningitidis* is rarely associated with disease (32). Meningococcal meningitis is a rapidly progressing disease with a case fatality rate (CFR) between 10% and 15% (33).

C. Epidemiology

Incidence of *N. meningitidis* infection in the United States had steadily declined since 2006; however, in 2023 incidence reached ~0.06/100,000, a rate not seen since 2011 (34). This upward trend is due to increase in incidence of infection by *N. meningitidis* of serogroup Y. Rates of meningococcal disease are highest among infants and children under 1 year old. *N. meningitidis* infection is a significant public health concern on a national and global scale. In a portion of sub-Saharan Africa known at the “meningitis belt”, incidence rates of 1,000/100,000 persons have been reported (33). Meningococcal meningitis is hyperendemic in the meningitis belt and this region experiences large-scale epidemics every 5-12 years (33). In the past 50 years, 25 countries have experienced a deadly meningococcal meningitis outbreak with case fatality rates as high at 100% (35).

D. Treatment and Prevention

The current recommended treatment for confirmed *N. meningitidis* infection is penicillin G, ampicillin, ceftriaxone or cefotaxime (36). Resistance to these antimicrobials has previously been uncommon. However, resistance to penicillin, rifampicin, cefotaxime, and ciprofloxacin has emerged in recent years (37). The most effective prevention for IMD is vaccination. Meningococcal vaccines have been generated using polysaccharide only, polysaccharide conjugated to proteins, outer membrane vesicles, and protein only antigens (38). Generally, effective *N. meningitidis* vaccines generate high levels of bactericidal antibodies that are maintained in the vaccinated individuals resulting in herd protection (38). Some serogroups however are more difficult to protect against through vaccination due to the sialylation state of the capsule which mimics host antigen (39). Additionally, the incidence of disease associated with certain *N. meningitidis* serogroups impacts the vaccine recommendations for each geographic location (38).

V. *Neisseria gonorrhoeae*

A. Gonococcal Disease

B. Etiology

N. gonorrhoeae is a human-specific pathogen that causes the sexually transmitted infection gonorrhea. *N. gonorrhoeae* infects mucosal membranes including those in the urogenital tract, conjunctiva (40), rectum and oropharynx (41). Gonococcal disease commonly presents as urethritis, cervicitis, proctitis, conjunctivitis, and pharyngitis (42). However, *N. gonorrhoeae* infection commonly presents asymptomatically in women (43), and thus increases

the risk of disseminated gonococcal disease. Infection sequelae is characterized by pelvic inflammatory disease, ectopic pregnancy and infertility (44). Disseminated infection is characterized by septic arthritis, endocarditis and dermatitis (45). *N. gonorrhoeae* lacks a capsule and therefore is not classified according to polysaccharide capsular composition like N. meningitidis. Instead, *N. gonorrhoeae* is serotyped according to the major outer membrane protein, porin (46, 47).

C. Epidemiology

In 2020, The World Health Organization (WHO) estimated over 80 million new cases of gonorrhea globally (48). Additionally, the WHO reported and incidence rate of 19/1,000 women and 23/1,000 men (48). In 2022, the Center for Disease Control and Prevention (CDC) reported over 600,000 cases of gonorrhea in the United States alone (49). Gonorrhea disproportionally affects young adults (15-24 y.o.), men who have sex with men (MSM), pregnant women, and racial/ethnic minority groups; of those minority groups, Black and African American people experience highest morbidity (50, 51). Additionally, individuals infected with *N. gonorrhoeae* are more likely to be infected with human immunodeficiency virus (HIV), and *N. gonorrhoeae* infection significantly increases HIV load and transmission (52).

D. Treatment and Prevention

Gonococcal infection is not associated with high mortality rates. However, as *N. gonorrhoeae* continues to acquire and maintain antimicrobial resistance determinants, morbidity is expected to increase as the infection becomes more challenging to treat. *N. gonorrhoeae* has acquired antimicrobial resistance determinants that protect it from all classes of antimicrobials including sulfonamides (53), penicillins (54, 55), tetracyclines,

fluoroquinolones, and cephalosporins (56). The mechanisms by which *N. gonorrhoeae* resists killing by these antimicrobials has been reviewed previously (57). Briefly, resistance is conferred through three primary mechanisms whereby mutations in the *N. gonorrhoeae* genome result in (a) change of the antimicrobial target, (b) change in the level of intracellular antimicrobials by controlling import or export, and (c) change in the antimicrobial itself that make it no longer harmful to the bacteria. Each of these mechanisms have been observe in *N. gonorrhoeae*. Resistances to sulfonamides, conferred through mutation in the target, *folP* (58, 59) emerged in the 1940s *(60).* Resistance to penicillins emerged in the late 1970s (61) and can be conferred through mutations in *mtrR* that results in upregulation of MtrCDE which effluxes those antimicrobials (62). Additionally, penicillin resistance can be conferred by the non-mobile plasmid, p*bla*, which encodes TEM β-lactamases that degrade β-lactams (61). Fluroquinolone resistance emerged in the late 90s and is confers through mutations in the topoisomerases, *gyrA* and *parC* (63). Resistance to cephalosporins emerged in the late 1980s (64) but increased resistance within the gonococcal population has been observed since the early 2000s (65, 66). Resistance to cephalosporins can be conferred through mutations in genes encoding the penicillin binding proteins (PBPs) (i.e. *penA* (64, 67) and *ponA* (68)), or porin (*penB*) (69), and pilQ (70). Each antimicrobial resistance determinant can be acquired through DNA uptake and maintained through homologous recombination into the gonococcal chromosome. This can result in a step-wise increase in multi-drug resistance as new determinants are shared between gonococci (68). In the past, the proportion of multi-drug resistant gonococci was proportionally lower resulting in the CDC recommendation of a dual antimicrobial treatment which included a dose of cephalosporin (i.e. cefixime or intramuscular ceftriaxone) and another antimicrobial

(i.e. azithromycin) (71). However, in 2012, the CDC ceased recommending cefixime for routine treatment of gonococcal infections due to increased cefixime resistance. Again, multi-drug resistance in the pathogen increased in 2015 causing the CDC to remove azithromycin from the recommended treatment regimen (72). The current recommended treatment is a single dose of intramuscular ceftriaxone (72). As the number of cases of multi-drug-resistant *N. gonorrhoeae* infections increases (73-75), the need for novel therapeutics and effective prophylactic treatments and vaccines becomes more urgent. Currently the only means of preventing gonococcal disease is abstaining from sexual intercourse. Condom usage can limit transmission if used correctly (76), but no prophylactic to prevent infection after exposure exists.

There is currently no effective vaccine against *N. gonorrhoeae* due to high frequency variation of many surface-exposed proteins on the outer membrane of the gonococcus. Recent progress in the development of the meningococcal serogroup B vaccine, 4CMenB (Bexsero, GSK) provides a promising outlook for gonococcal vaccine development. Antigens in the Bexsero® vaccine include outer membrane vesicles (OMV) from MeNZB (GSK), Neisserial Adhesin A (NadA), and two fusion proteins - neisserial heparin binding protein (NHBA) fused to GNA1030 and factor H binding protein (fHbp) fused to GNA2091. The immune response to Bexsero is characterized by production of cross-reactive, anti-*N. gonorrhoeae* antibodies (77). Additionally, retrospective and comparative studies correlated Bexsero vaccination with reduced *N. gonorrhoeae* infection prevalence in young adults (16-23 yo) (1, 78). However, the exact correlates of protection against *N. gonorrhoeae* remain poorly defined, and high bactericidal antibody titers alone are not correlated with protection (79). In spite of this, immunization of mice with Bexsero results in accelerated *N. gonorrhoeae* clearance (80)

suggesting that a protective immune response may be conferred via bactericidal antibody function in addition to another response such a cellular response, although such response remains to be uncovered. Thus, an effective gonorrhea vaccine should include antigens that are not subject to high-frequency variation, exhibit bactericidal activity, and confer long-lasting protection.

Immune response to N. gonorrhoeae

N. gonorrhoeae employs mechanisms of evading the host immune system. For example, sialylated gonococcal lipooligosaccharide (LOS) binds factor H and C4b irrespective of sialylation state. C4b is rapidly converted to iC4b on the gonococcal surface (81), inhibiting the complement cascade. The innate cellular response to *N. gonorrhoeae* is immune suppressive in that macrophages secrete predominately immune inhibitory cytokines such as interleukin-10 (IL-10) rather than activating cytokines interleukin-6 (IL-6) (82). Additionally, presentation of *N. gonorrhoeae* to dendritic cells results in suppressed T cell activation (83). The T cell response that remains is skewed toward a T-helper 17 (Th17) response through production of interleukin-17 (IL-17) and transforming growth factor-β (TGF-β) and away from a T-helper 1 (Th1) and T-helper 2 (Th2) response, resulting in an influx of neutrophils (84, 85). The neutrophil response to *N. gonorrhoeae* is essentially non-productive because *N. gonorrhoeae* suppresses oxidative burst in Polymorphonuclear leukocytes (PMN) (86) and produces catalase and peroxidases that allow it to propagate with PMNs (87). Fortunately, immunization of mice with *N. gonorrhoeae* OMVs in the presence of interleukin-12 (IL-12), which is involved in Th1 response activation, resulted in higher IgG and IgA titers and more rapid clearance in a mouse model of infection (88). Thus, an effective vaccine will need to elicit antibodies that are

bactericidal to even serum-resistant, immune-camouflaged gonococcal strains, and also promote a Th1 and Th2 response.

VI. Gonococcal Virulence Factors

N. gonorrhoeae expresses a number of virulence factors that allow it to attach to and invade host cells while evading the immune system or driving non-productive immunological responses (Figure 1). Virulence factors discussed in this work include pilin, opacity proteins, porin, LOS, reduction modifiable protein (Rmp), and IgA protease.

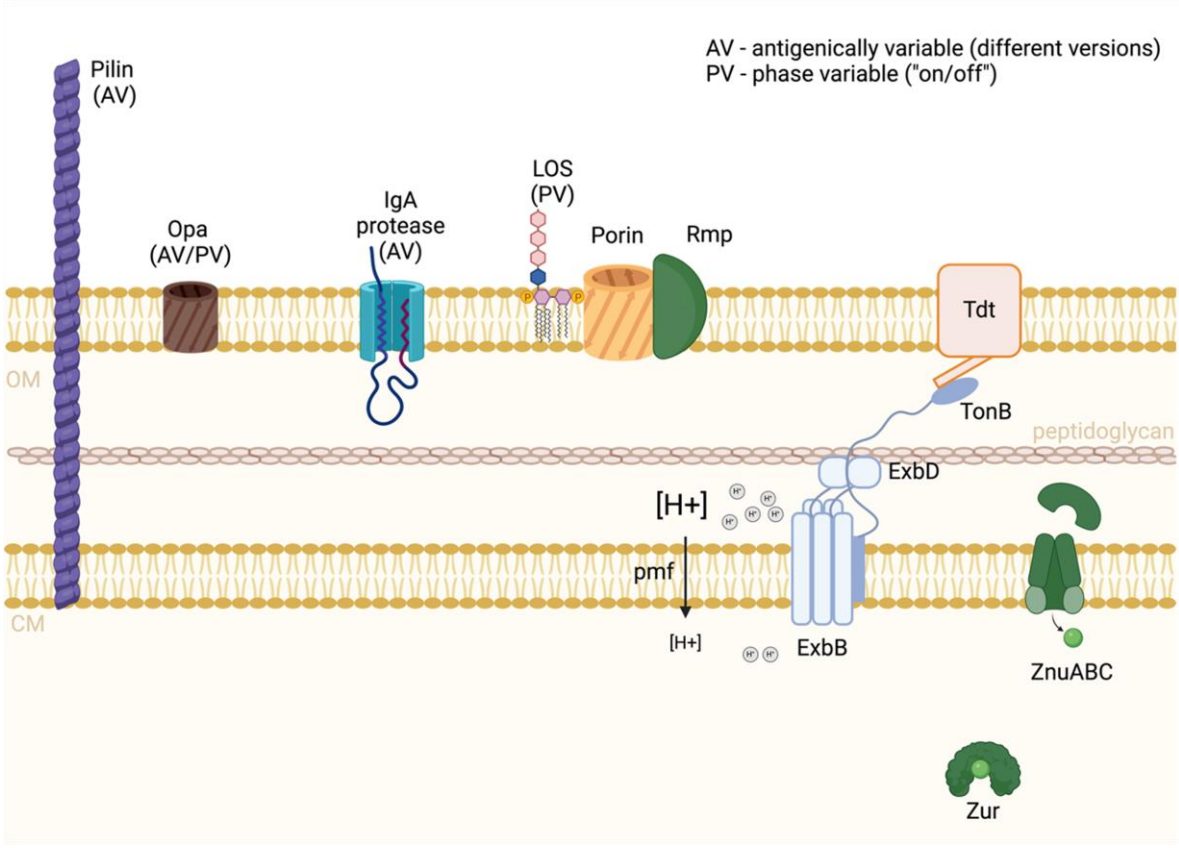

Figure 1. The Surface of N. gonorrhoeae is Highly Variable

N. gonorrhoeae expresses multiple virulence factors in the outer membrane (OM). AV indicates antigenically variable proteins. These proteins are encoded by multiple loci on the gonococcal genome; therefore different versions of the protein can be expressed simultaneously. PV indicates phase variable proteins. Expression of these proteins can be turned "on" or "off." Surface-exposed proteins not subject to AV or PV do not have this denotation (i.e. Porin, Rmp, and the Tdts). TonB and the proton motive force (pmf) which powers it are depicted in the cytoplasmic membrane (CM). Also depicted in the CM is ZnuABC, the zinc ABC transporter. In the cytoplasm is depicted the Zinc uptake regulator, Zur

A. Pilin

The type IV pili (Tfp) of *N. gonorrhoeae* are hair-like, surface exposed projections required for attachment (89), twitching motility (90), and natural competency (91). The Tfp is extended by polymerization of the major pilin protein, PilE. Once in close proximity with host epithelial cells, PilC binds to an unknown host cell receptor on the epithelial surface (92, 93). Depolymerization of PilE helps to bring *N. gonorrhoeae* in close contact with the epithelial surface. Attachment of pilin in conjunction with Opa cause microvilli formation and engulfment of *N. gonorrhoeae* by epithelial cells (94). PilE is antigenically variable in that multiple silent *pilS* loci can recombine into the *pilE* gene (95) result in the presence or absence of pilin on the surface (96). Pilin is also required for natural competence of *N. gonorrhoeae* and therefore plays a role in gene exchange and antimicrobial resistance determinant acquisition (97, 98).

B. Opacity Proteins

The opacity (99) proteins are phase and antigenically variable outer membrane invasins that bind to host carcinoembryonic antigen-related cell adhesin molecules (CEACAMs) on host epithelial cells, endothelial cells and neutrophils (95, 100). Phase variation occurs due to the CTCTT repeat within each *opa* gene, which results in slipped-strand mispairing that turns each gene on or off depending on the number of repeats (101). There are 12 separate *opa* loci in *N. gonorrhoeae* and each gene contains multiple hypervariable regions (101, 102). While pilin helps *N. gonorrhoeae* make initial contact with the host cells, Opa also mediates invasion once at the surface (103). The Opa-CEACAM interaction is particularly important in the neutrophil response to *N. gonorrhoeae*. Expression of different Opa variants impacts neutrophil binding and internalization. When human males were infected with a predominantly Opa- *N.*

gonorrhoeae inoculum, only Opa+ strains were recovered demonstrating that the Opa+ phenotype is selected for during infection in men (104).

C. Porin

Porin is an essential outer membrane protein that acts as an ion and small molecule channel (105, 106). *N. meningitidis* expresses both Porin A (107) (107) and Porin B (PorB), while *N. gonorrhoeae* expresses only PorB. *N. gonorrhoeae* PorB is variable in that it is encoded by two different alleles in the gonococcal population, those being PorB1A, and PorB1B, which is the basis of the gonococcal serotyping system (108, 109). Gonococcal porin has been shown to modulate the host immune response to *N. gonorrhoeae*. Porin binding to C4-binding protein (C4BP) and factor H inhibits the complement cascade and contributes to serum resistance in strains expressing the *porB1A* allele (110, 111). Additionally, LOS sialylation inhibits bactericidal antibody activity directed towards PorB (112). The *porB1A* allele is present more frequently in disseminated strains than urogenital strains (113), suggesting that Porin type may be a determinant of disseminated gonococcal infection (DGI). Complement deficiency is associated with increased risk of *N. gonorrhoeae* infection and recurrent infection (114). The cytotoxic activity of Porin can be attributed to its channel activity. During *N. gonorrhoeae* infection of macrophages, gonococcal PorB is hypothesized to be targeted to the mitochondrial membrane where it disrupts the membrane potential causing apoptosis (105). Porin has also been shown to inhibit dendritic cell-dependent activation of T cells (83).

D. Lipooligosaccharide (LOS)

LOS is anchored in the outer leaflet of the outer membrane by the hydrophobic lipid A moiety, which is endotoxin (115). Lipooligosaccharide also contains a core oligosaccharide and lacks the

distal O-antigen of LPS (115). The core oligosaccharide is composed primarily of glucose, galactose, mannose, N-acetylneuraminic acid (Neu5Ac), 2-keto-3-deoxyoctulosonic acid (KDO), glucosamine, and galactosamine (116). Glycosyltransferase genes, *lgtA*, *lgtC*, *lgtD*, and *lgtG*, are responsible for LOS biogenesis and are themselves phase variable (117, 118). Phase variation in *lgt* genes results in distinct LOS structures on the gonococcal surface depending on the *lgt* genes expressed.

Expression of *lgtA* is critical to *N. gonorrhoeae* invasion of epithelial cells (93) suggesting that *N. gonorrhoeae* strains expressing certain variants of LOS are more invasive than others. LOS also binds to asialoglycoprotein receptor (ASGP-R) on human urethral cells (119) and sperm cells demonstrating a role for LOS in both infection and transmission (107). Additionally, sialylation of LOS is a mechanism of immune evasion by preventing antibody and complement deposition and subsequent opsonic killing of *N. gonorrhoeae* (120). Despite the high levels of variation in LOS due to the phase variability of the *lgt* genes, a glycan epitope identified by the monoclonal antibody, 2C7, is highly conserved across *N. gonorrhoeae* strains (121). Antibodies against this epitope are bactericidal, and sialyation of LOS does not ablate bactericidal activity, although activity is reduced (121). Mice immunized with the GLA-SE-adjuvanted 2C7 epitope produced bactericidal antibodies and cleared *N. gonorrhoeae* infection quicker than mice immunized with the adjuvant alone (122). Taken together, the 2C7 epitope is a promising vaccine and therapeutic candidate.

E. Reduction Modifiable Protein (Rmp)

Rmp is a conserved outer membrane protein found in close association with Porin (123). In the case of most of the outer membrane virulence factors, phase and antigenic variation

protect *N. gonorrhoeae* from an immune memory response. In the case of porin, which is antigenically variable, Rmp acts as a second layer of protection in that it is an antibody sink of sorts. The antibody response to PorB, which promotes complement dependent killing, is blocked by the anti-Rmp response, and depletion of anti-Rmp antibodies restores the bactericidal activity of serum (124, 125). Moreover, antibodies against Rmp are associated with increased gonococcal infection rates (126). Mice actively immunized with Rmp or treated prophylactically with anti-Rmp antibodies remained colonized longer than those mice treated prophylactically with the 2C7 monoclonal antibody (mAb), which has been shown to increase *N. gonorrhoeae* clearance in a mouse model (122, 127). Rmp antibodies in the presence of the 2C7 mAb block C3 complement deposition that would otherwise deposit in the presence of the 2C7 mAb alone (127). Rmp exacerbates infection, distracts the immune system from a generating a productive response and thus confounds vaccine and therapeutic developments.

F. Immunoglobulin A (IgA) Protease

IgA is the predominant Ig isotype secreted by mucosal epithelial cells and is involved in neutralization, compliment activation, and opsonization for phagocytic cell targeting (128). Gonococcal IgA protease is secreted by a type Va secretion system (autotransporter) and cleaves IgA (129). This autotransporter consists of an N-terminal signal sequence, a passenger domain, which in the case of *N. gonorrhoeae* is an IgA protease, and a translocator domain which is the barrel that is inserted into the outer membrane so that the protease can be exported (130). IgA protease is variable in that it is encoded by two different alleles in the population, of which type 2 is antigenically variable in itself (131). Each isolate however only produces one type and the types differ according to the cleavage sequence (132). Type 1 IgA

protease cleaves synaptobrevin (133), a Soluble N-ethylmaleimide-sensitive factor Activating protein Receptor (SNARE) protein which mediates exocytosis and vesicle fusion (134). Type 2 IgA protease cleaves human lysosome/late endosome-associated membrane protein 1 (h-lamp1), which is an integral host membrane protein that is enriched in endosomes and lysosomes (135). *N. gonorrhoeae* IgA proteases inhibit exocytosis by preventing the membrane fusion event required for release of compartment contents (133) and thus promote intracellular survival (136). IgA proteases are not required for attachment and invasion of fallopian tube mucosal cells (137) nor is it required for human male urethral infection (138). Taken together, these data suggest that the functions of IgA proteases are expendable during human infection and that the protective characteristics of IgA protease may be redundant or compensated for by some other gonococcal virulence factor. IgA proteases help *N. gonorrhoeae* escape IgA-dependent neutralization and intracellular killing; however, its function is not critical to infection which is contrasted with the functions of other virulence factors such as Opa or Pilin.

VII. Host Metal Homeostasis and Restriction

The host goes to great lengths to transport and regulate metals for its own biological processes. Host metal-binding proteins discussed in this work include Hemoglobin (Hb), Transferrin (115), Lactoferrin (LF), ferritin (FT), Calprotectin (CP), and psoriasin (PS) (Figure 2). As free metals are highly insoluble and volatile, the host binds these metals with specific proteins to keep them inaccessible without harming the host.

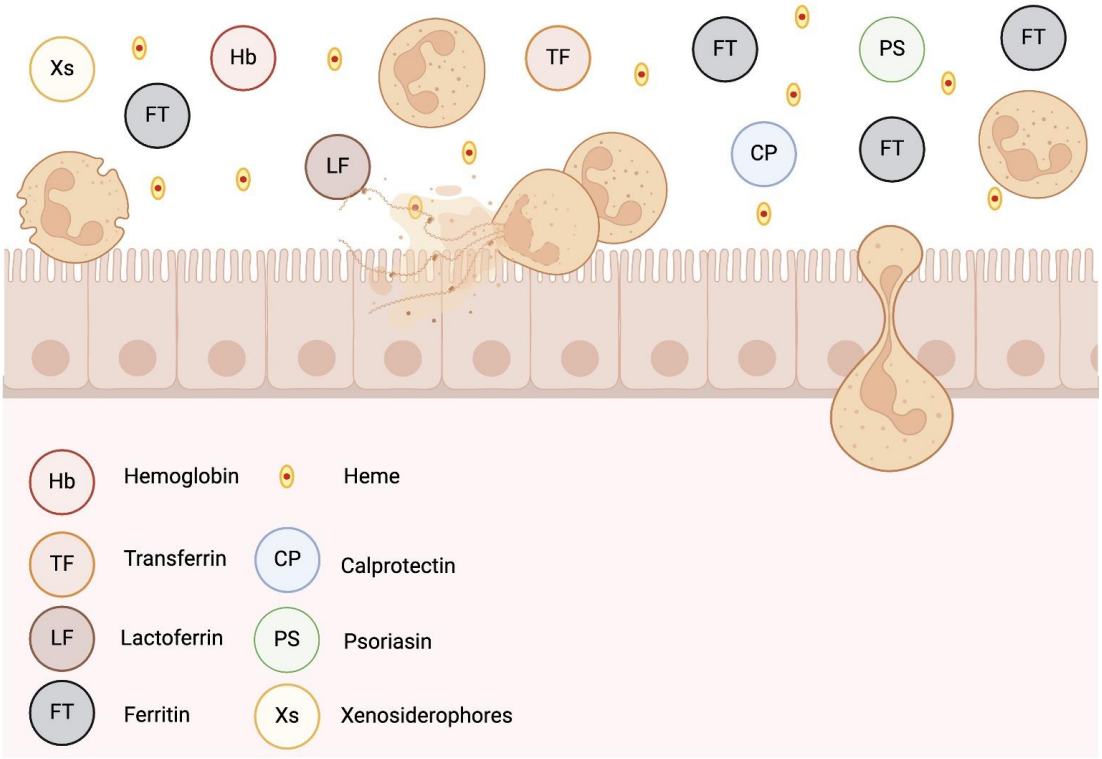

Figure 2. The Mucosal Surface Inhabited by N. gonorrhoeae

At the site of infection, the mucosal membrane, host nutritional immunity (NI) protein sequester metals away from the bacterial nutrient supply. NI proteins are shown as circles with abbreviated names. The key provides the full name of each abbreviated NI protein. The infected mucosal surfaces is depicted with and influx of neutrophils, some of which are degranulating, undergoing NETosis, or traversing the epithelial cell layer.

A. Iron

Iron (Fe) is essential for life in both pathogen and host. The redox potential of Fe makes it highly useful in fundamental host cell processes like respiration, erythrocyte biosynthesis, oxygen transport, cell signaling and host defense (139). Due to its ability to participate in Fenton chemistry resulting in damaging hydroxyl radicals (140), free soluble iron is relatively limited in the host. The host relies on Fe-binding proteins to solubilize and transport Fe safely throughout the organism.

Transferrin (TF)

TF is a bi-lobed Fe-binding protein composed of an N- and C- lobe (141). Each lobe can be subdivided structurally into 2-subdomains connected by a hinge region (142). TF binds two Fe^{3+} ions, one per lobe, with an affinity of 10^{-30} M (143, 144). TF is synthesized in the liver (145) and is 30% saturated in circulation. TF has a primary function of scavenging and transporting Fe to tissues in the body. TF is internalized by cells through binding to the TF receptor, and Fe is released upon exposure to the acidic endosome (146). This function of TF is critical to erythrocyte biogenesis (147). Although the Fe bound to TF only accounts for ~0.1% of total iron in the body, the percentage of Fe-saturated TF is a significant means of estimating plasma or serum Fe levels (148). Additionally, TF in present in cervical-vaginal fluid, a primary gonococcal infection site, and even more so when that fluid also contains PMNs (149).

Lactoferrin

Lf shares about 60% sequence identity with Tf (150) and is similarly composed of N- and C- lobes that bind two Fe^{3+} (151, 152). Consequently, it has been previously referred to as lacto-TF (153). Lf is secreted by mucosal epithelial cells and is found in the associated exocrine fluids

such as vaginal secretions (~1 g/L)(154), milk (1-3 g/L)(155), saliva (~10g/L)(156), sweat (~0.021 g/L)(157), tears (~3 g/L)(158), and digestive fluid (~33 μg/L) (159). The concentration of Lf in the serum is relatively lower (125-880 μg/L) than that found in secretions (160). Lf has an affinity for iron of 10^{-20} M. Unlike Tf, Lf does not release Fe in the low pH conditions of the endosome suggesting a primary role in Fe sequestration from pathogens once secreted as opposed to Fe delivery to cells (161).

Heme and Hemoproteins

Heme is an Fe-binding porphyrin that is essential in structure and function to many proteins such as oxidase, cytochromes, peroxidase and catalase, and Hb (162). The iron in heme binds oxygen, which allows for electron transfer in enzymatic reactions and for the transport of oxygen by Hb through the body (163). Hb is a heterotetramer of two alpha and two β chains. Each polypeptide chain binds one heme group and oxygen is coordinated via the Fe bound to each group (164). Hb in the human body can be found at concentrations of 12-18 g per liter.

Ferritin

FT forms a cage-like complex made of 24 subunits capable of binding 4500 Fe atoms (165). The primary role of FT is to store intracellular iron and maintain it in a redox-inactive state. During iron scarcity in the host cell, FT is targeted to the lysosome for degradation and subsequent Fe release (166). The role of FT in intracellular gonococcal growth and survival deserves further investigation considering FT is abundant in the serum and the male urethral tract (167).

B. Zinc, Manganese, and Copper

Host allocation and homeostasis regarding Zn, Mn, and Cu are reviewed in detail in Chapter 3 of this work.

VIII. Metal Acquisition by Pathogenic *Neisseria*

A. Ton Motor Complex

The outer membrane of Gram-negative bacteria is a barrier between the sensitive cytoplasmic contents of the cell and harmful extracellular molecules such as antimicrobials. However, the OM is not energized like the cytoplasmic membrane. Consequently, energy must be transduced from the cytoplasmic membrane to the outer membrane to allow active transport of vital nutrients across the outer membrane. Energization of the outer membrane is achieved through the Ton motor complex (TonB-ExbB-ExbD or TBD) (Figure 3).

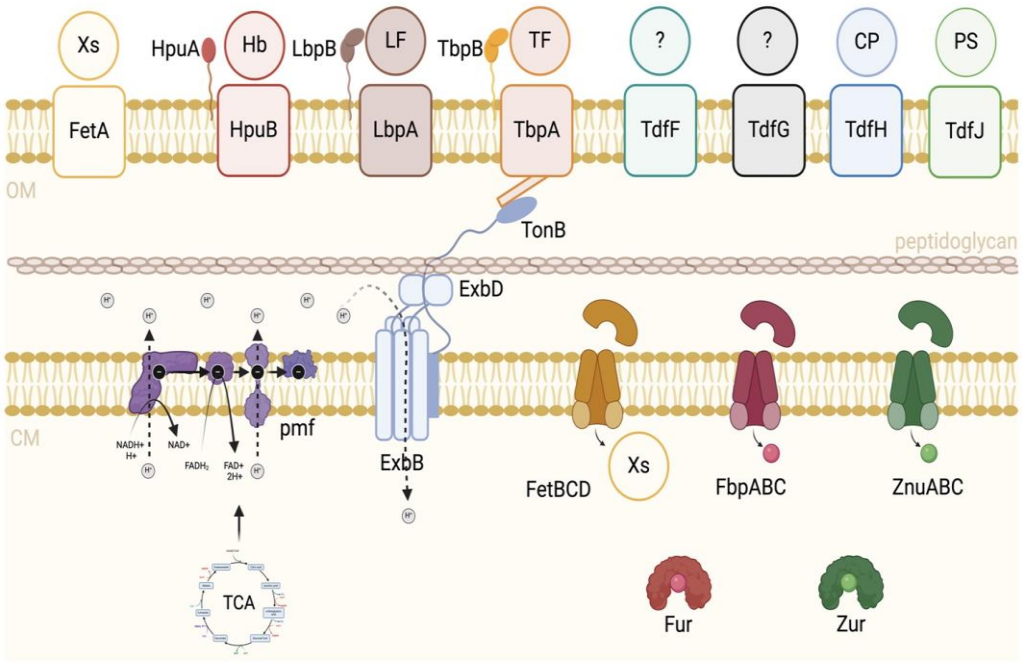

Figure 3. N. gonorrhoeae Expresses High-affinity Metal Transporters

The TonB-dependent transports (Tdts) expressed by *N. gonorrhoeae* are depicted in the outer membrane (OM) of the cell wall below their cognate ligands. Tdts with associated lipoproteins (i.e. HpuBA, LbpAB, and TbpAB) and Tdts without associated lipoproteins (i.e. FetA, TdfF, TdfG, TdfH, and TdfJ) are shown in the OM. Xs is Xenosiderophores. Hb is hemoglobin. LF is lactoferrin. TF is transferrin. CP is calprotectin. Ps is Psoriasin also known as S100A7. Question marks indicated unknown ligands. TonB is shown in the cytoplasmic membrane (CM) and is powered by the proton motive force (pmf) which utilizes the proton gradient across the CM. Electrons from the Tricarboxylic Acid (TCA) Cycle are used to oxidize or reduce complexes I-IV in the CM that are a part of the electron transport chain. The oxidation and reduction reactions result in the release protons into the periplasm which generates the proton gradient. These protons are hypothesized to travel through ExbB causing a ratcheting motion and allowing ExbD to rotate TonB so that it can energize the Tdts in the OM. FetBCD, FbpABC, and ZnuABC are ABC transporters in the CM. The periplasmic binding protein for each transporter in the in the periplasm. The permease is in the CM, and the ATPase is in the cytoplasm. Depicted in the cytoplasm are the Ferric uptake regulation (Fur) which binds iron (pink sphere) and the Zinc uptake regulator (Zur) which binds zinc (green sphere).

ExbB exists as a pentameric, alpha helical channel, within which an ExbD dimer is situated (168). TonB is composed of a folded, C-terminal, periplasmic-associated domain that is disordered and linked by a transmembrane domain within the cytoplasmic membrane (169). The transmembrane domain of TonB is closely associated with ExbB. TonB associates with TonB-dependent transporters (Tdts) through a high-affinity (nanomolar) interaction between the C-terminus of TonB and the TonB box at the N-terminus of the Tdt (170, 171). To achieve transport across the outer membrane, ExbB and ExbD work together to transduce the proton motive force (pmf) of the cytoplasmic membrane to the outer membrane through mechanical work. More frequently than not, ExbD occludes the ExbB channel (172). However, following an unknown regulatory signal, the ExbD "plug" binds to peptidoglycan in the periplasm allowing for protons to flow into the ExbB channel (172). The flow of protons into ExbB powers the ratcheting motion of ExbD within ExbB in a mechanism similar to that of flagella twirling. It is proposed that ExbD, powered by the pmf, pulls on the disordered linker of TonB, which is bound to the TonB Box of the Tdts, as it rotates thereby releasing the imported molecule into the periplasm. The "pulling" mechanical work of ExbB/D on TonB can be likened to a pully system where ExbB/D is the wheel and TonB is the rope that exerts force or "pulls" on the Tdts causing release of the molecule being transported.

B. TonB-dependent Transporters (Tdts)

Tdts are integral outer membrane proteins composed of C-terminal surface exposed loops, 22 transmembrane β-strands, periplasmic turns, and an N-terminal plug domain containing the TonB Box. The TonB Box is required for transport function (173, 174). Tdts transport small molecules such as metals, vitamins, and sugars and it is hypothesized that

binding of these small molecules results in conformational changes in the Tdt structure culminating with the exposure of the TonB Box to the periplasm, making it accessible to TonB (171, 175). In *N. gonorrhoeae*, TonB is required for replication within epithelial cells (176). Additionally, TonB and the eight Tdts (Figure 3) play critical roles in survival of *N. gonorrhoeae* in the face of nutritional immunity (177-179).

C. Tdts with Associated Lipoproteins

The gonococcal TF-binding proteins A and B (TbpAB) are the Tdt and associate lipoprotein responsible for pirating and internalizing Fe from Tf. TbpA is the TonB-dependent component of the Tbp system and binds both holo- and apo-Tf with nanomolar affinity (180). Tf utilization through TbpA requires a structurally intact loop 3 helix which is responsible for stripping the Fe from Tf. The EIEYE motif located within the plug domain chelates iron once extracted from Tf. TbpB is a lipid-modified protein that is anchored to the outer leaflet of the outer membrane and binds specifically to the ferrated form of Tf, increasing the specificity of the receptor for iron-loaded Tf. This specificity results in more efficient iron acquisition and ligand release subsequent to iron extraction. The *tbpBA* operon is Fur- and Fe-repressed as well as MisR-MisS (181) regulated. Although the locus is co-transcribed, twice the amount of *tbpB* transcript is made relative to that of *tbpA* suggesting multiple TbpB proteins service fewer TbpA proteins and further increasing the efficiency of Fe uptake. Ablation of a long noncoding RNA eliminates the difference in expression between the two genes, allowing them to be transcribed in a 1:1 ratio (182). Because TbpAB is required for infection and is not subject to high-frequency antigenic variation like Pilin or Opa proteins, this system is a promising vaccine target. Price et al. showed the portions of TbpA and TbpB when conjugated to cholera toxin

subunit B are immunogenic and produce bactericidal antibodies in mice (183). They also showed that the anti-TbpAB antibodies could block gonococcal growth *in vitro* (183). The same group showed that TbpB is much more immunogenic than TbpA (184, 185). Taken together with the fact that TbpB is much more genetically variable than TbpA (186), the increased immunogenicity of TbpB suggests that it may act as an immune shield for TbpA allowing the antibody response to be directed instead toward TbpB, which can sustain more mutations. Human volunteers infected with the Tbp- mutant exhibited no signs or symptoms of urethritis (187). It is important to note that the strain used in this study was also unable to utilize LF as a sole iron source.

The LF binding proteins, LbpAB, is similar to the Tbp system where LbpA is the Tdt and LbpB is the lipoprotein. LbpA contains the same essential functional motifs as TbpA (i.e. plug domain, TonB box, the Loop 3 helix, and the EIEYE motif), and is Fur- and Fe-repressed. In the absence of the LbpAB system, the TbpAB system can compensate for Fe transport (188). However, LbpB is phase variable and LbpA is produced in only ~50% of gonococcal strains and thus is not a promising vaccine target. Additionally, mutants lacking the Lbp system remain infective in an experimental human infection model (188).

The Hb-binding proteins (HpuAB) are required for Hb utilization as an iron source. The Hpu system binds Hb and the Hb-haptoglobin complex and transports the associated heme. Both binding partners, HpuA and HpuB, are required for binding to Hb. The nomenclature for the Hpu system in reversed in that HpuA is the lipoprotein and HpuB is the Tdt. Interestingly, binding of HpuB to Hb is not human-restricted, meaning HpuB binds porcine and mouse Hb in addition to human Hb (189). The Hb structures are likely not significantly divergent between

species to confer a species-specific binding interaction. The Hpu system is phase-variable and is selected for in females during the first half of their menstrual cycle (190), suggesting that Hb is released during menstruation and selects for gonococcal strains that can use this Fe source.

D. Tdts without Associated Lipoproteins

N. gonorrhoeae does not produce siderophores like other bacteria and instead utilizes the siderophores of other bacteria (i.e. xenosiderophores). *N. gonorrhoeae* can utilize enterobactin, salmochelin, and dihydroxybenzoylserine acid (DHBS). These siderophores are transported through the ferric enterobactin Tdt, FetA (191). FetA forms a trimer in a manner similar to that of porins (192). Trimerization increases stability of the protein in the membrane and enhances pore function (193). FetA is encoded by the Fe-regulated *fet* operon, where *fetA* is the first gene in the operon and the downstream genes encode the associated ABC transport system. The Fet system is not only Fur-regulated but it is also regulated by the AraC-like regulator, MpeR (194). Another layer of regulation occurs through slipped strand mispairing in the promoter of *fetA* allowing for phase variation. This phase variability is not like that of the Hpu system where the system is either “on” or “off” but rather the slipped-strand mispairing event at the promoter results in gradient *fet* expression where expression can be likened to that under the control of a “rheostat”.

TdfF and TdfG remain poorly characterized and the conditions under which these Tdts are expressed has not yet been clearly defined. However, TdfF is expressed in epithelial cells and is Fe-regulated. TdfF is homologous to iron-binding siderophore transporters such as FhuE in *Esherichia coli* (Ec) which binds coprogen, the pseudobactin transporter in *Pseudomonas putida*, and the ferric pyoverdine transporter of *Pseudomonas aeruginosa*. While *tdfF* is Fe-

repressed, differential Fe conditions are not solely sufficient to alter *tdfF* expression in vitro (176). Expression of *tdfF* under Fe-limited conditions also required fetal bovine serum (FBS)(176). The TdfF ligand has not been identified. However, Hagen and Cornelissen showed that intracellular survival of *N. gonorrhoeae* requires both TonB and TdfF (176). Additionally, the intracellular growth defect of the *tdfF* mutant was overcome by excess Fe, suggesting that the ligand for TdfF is expressed in epithelial cells and binds Fe (176). It is not likely that the TdfF ligand is enterobactin or salmochelin because the *tdfF* mutant is able to grow to wild-type levels in the presence of both molecules (195). This evidence is well-aligned with that which shows that FetA, rather than TdfF, transports enterobactin and salmochelin (191, 195). TdfG is the least characterized of all the Tdts. However, we do know that this protein is Fe-regulated (196) and weakly peroxide regulated (197), but as with TdfF, the ligand as not been identified. The Zn-transporting Tdts, are TdfH and TdfJ, which bind CP and PS respectively. TdfH is required for growth when CP is the only available Zn source. While CP chelates Zn at both binding site 1 and site 2, TdfH interacts exclusively near site 1 and acquires Zn only from this site (198). While site 1 binds both Zn and Mn, a role for TdfH in CP-dependent Mn utilization has not been established. ZnuD is able to bind free Zn (199), free Copper (Cu) (200) and PS (179). The affinity of TdfJ for PS is quite high (41 nM) (201). PS utilization in a TdfJ-dependent manner requires the high-affinity Zn ABC transporter, ZnuABC (179). Like TbpA, piracy of metal from the host ligand by TdfJ requires a similar alpha-helix in loop 3 (201). Both TdfH and TdfJ are repressed by the zinc uptake regulator (Zur) in a Zn-dependent manner and neither protein is subject to high-frequency variation. Interestingly, TdfJ is also Fe induced (179) by Fur. The physiological niche in

which Zn stress and Fe replete conditions might be encountered by *N. gonorrhoeae* has been postulated (202) but not supported yet by empirical evidence.

E. ABC Transporters

ATP-Binding Cassette (ABC) transporters are critical to metal ion transport across the periplasm and the cytoplasmic membrane. They are composed of a periplasmic binding protein (PBP), a permease that traverses the cytoplasmic membrane and an ATPase associated with the cytoplasmic face of the inner membrane. The two nucleotide-binding domains of the ATPase allow for ATP hydrolysis while the two transmembrane domains of the permease confer substrate specificity (203). Two ABC transporters in *N. gonorrhoeae* have been well-characterized, FbpABC and ZnuABC. FbpABC transports Fe and xenosiderophores (195), while ZnuA transports Zn and Mn (204, 205). The *fbpABC* operon is co-transcribed (206) and is Fur-repressed in an Fe-dependent manner (207). FbpA is required for survival in the presence of nutritional immunity proteins, TF and LF (208). Unfortunately, the nomenclature for ZnuABC has been muddled in the literature. ZnuABC is encoded by the *znuCBA* locus with the gene tag numbers NGO_0170, NGO_0169, and NGO_0168, respectively. The Znu nomenclature was originally assigned by Chen and Morse in August of 2001 (204). The same locus was named *mntABC* by Wu et al. in December of 2001 when the contribution of these gene products to Mn transport was identified (209). Thus, ZnuABC and MntCBA are different names for the same proteins encoded by the same genetic locus where NGO_170 is named both *znuC* and *mntA* and encodes the ATPase. NGO_0169 is named both *znuB* and *mntB* and encodes the permease, and NGO_0168 is named both *znuA* and *mntC* and encodes the PBP. In this work, the system will be referred to using the Znu nomenclature for simplicity. The *znuCBA* locus is Zur-repressed in a

Zn-dependent manner in *N. meningitidis* (210) and is Mn-repressed in a Zur-dependent manner in *N. gonorrhoeae* strain 1291 (211). However, data presented in the current work show that *znuCBA* is not Mn-regulated in strain FA1090, indicating that Mn-dependent regulation of the locus depends on the *N. gonorrhoeae* strain and genetic background. More work is required to identify whether the Znu system is produced in response to Mn sequestration in multiple strains. This is of particular interest considering that CP binds to Mn in addition to Zn and potentially creates a Mn limited environment t for *N. gonorrhoeae in vivo*. Strains able to mount a Mn-dependent transcriptional response characterized by the upregulation of *znuA* may be more suited to withstand CP-dependent Mn sequestration. Although the role of ZnuA in response to CP-dependent Zn and Mn limitation has not been directly tested, it is known that ZnuA is required for utilization of PS and free Zn (179, 204). Thus, it is feasible that ZnuA is required for utilization of CP as a Zn source as well.

Finally, the *fetBCDE* operon encodes an ABC transport system that is responsible for cytoplasmic membrane transport of xenosiderophores brought across the outer membrane by FetA and potentially TdfF (191). The PBP, FetB, is required for utilization of ferric enterobactin (191).

F. Regulation and Gene Expression

The gonococcal genome encodes two Fur-family regulators, Fur and Zur, which both adopt a similar mechanism of regulation but with respect to Fe and Zn. The gonococcal genome does not encode a manganese uptake regulator, Mur. Fur-family regulators form homodimers containing four metal binding sites per dimer. Under low metal conditions, only two of those sites are occupied resulting in an “open” conformation that is unable to interact with operator

sequences (“Fur boxes”) within the protomer of Fur-regulated genes. When metals are in excess, all four metal-binding sites are occupied resulting in a “closed” conformation capable of interacting with DNA (212).

Fur

Fur is a global regulator involved in transcriptional activation or repression of a variety of processes in *N. gonorrhoeae* such as transport, respiration, regulation, and metabolism in addition to the Fe-utilizing processing (207). Fur activity is Fe-dependent and necessary for cell viability. Fur null mutants have not been successfully isolated; however, a Fur missense mutant has been isolated. This mutation generates a less active Fur protein that cannot fully repress Fe-regulated genes in the presence of excess Fe (213).

Zur

Zur is a global regular similar to Fur but with Zn-dependent regulatory activity. Zur is known to regulate various cellular processes as well including metabolism, transport, and Zn storage (210). Unfortunately, the nomenclature for Zur has been muddled due to its role in Zn, Mn, and peroxide stress regulation (211, 214, 215). Zur, which is encoded by the *zur* gene with gene tag NGO_0542, has been additionally named PerR for its connection to the Mn-dependent response to oxidative stress. For simplicity, the name Zur will be used throughout this work. Zur was shown to repress *znuCBA* in the presence of Mn, a reactive oxygen species (ROS) quencher. When the Zur mutant was grown in the presence of ROS, *znuCBA* was fully de-repressed allowing maximal import of Mn via ZnuABC and Mn-dependent ROS quenching (211). The Zur mutant was less sensitive to ROS-dependent killing than the WT, and growth of the Zur mutant in the presence of ROS was further enhanced by the addition of Mn (211). Thus, strains lacking

Zur are more resistant to ROS killing. In the absence of Zur, Zn import systems such as ZnuABC, TdfH, and TdfJ are derepressed. It would be interesting to know the impact of the Zur null mutation on survival of *N. gonorrhoeae* within neutrophils. It is feasible that a Zur null mutant would be more resistant to ROS-dependent killing in neutrophils. The absence of Zur would result in overexpression of TdfH, which could potentially import Mn from CP, thus quenching ROS and conferring resistance to ROS-dependent killing in neutrophils. To date, the impact of Zur on *N. gonorrhoeae* growth and survival in established cell models of infection has not been tested.

G. Research Objectives

N. gonorrhoeae is the human specific pathogen that causes the sexually transmitted disease, gonorrhea. The CDC reported over 600,000 cases of gonorrhea in 2022 (216) in the United States, while the WHO estimated approximately 80 million new cases globally in 2020 (217). *N. gonorrhoeae* primarily infects the lower urogenital tract resulting in urethritis in men and cervicitis in women. However, infection can also present asymptomatically in women (218). If left untreated, gonococcal infections can ascend into the upper reproductive tract, where it can cause salpingitis, ectopic pregnancy, scarring, and infertility. Further dissemination of the infection to the synovial joints (219), blood stream (220) and meninges (221) can be life-threatening.

The ability of the gonococcus to acquire antimicrobial resistance genes has resulted in a rapid decline in effective antimicrobial treatments for infection (57). Recent development of azithromycin resistance has led the CDC to remove the drug from the recommended treatment regimen (72). The ever-present threat of untreatable gonorrhea calls for the development of

novel therapeutic and vaccine targets. Metal acquisition systems and the transcription factors that regulate them are promising targets for small molecule inhibitor and vaccine development as these import systems are often required for survival and virulence. Understanding the involvement of metal transporters and the proteins that regulate them in the coordinated response to nutritional immunity can inform development of novel therapeutic and vaccine studies.

The overarching goal of this work is to characterize metal homeostasis in N. gonorrhoeae as it pertains to Zn, Mn, and to a lesser extent Cu, as the metal-dependent response of *N. gonorrhoeae* to nutritional immunity is directly related to internal metal homeostasis. Additionally, this work aims to identify the mechanisms through which metal homeostasis is maintained in *N. gonorrhoeae*. Specifically, the research objectives were to (1) characterize Zn, Mn, and Cu homeostasis in *N. gonorrhoeae* and the subsequent response to host-imposed nutritional immunity and metal intoxication through a comprehensive review of the literature, (2) identify the mechanisms by which N. gonorrhoeae senses metals, specifically Zn and Mn, through the transcriptional regulator Zur and alters global gene expression, and (3) identify the Mn conditions within the gonococcus and how the putative Mn efflux protein, MntX, contributes to Mn homeostasis in *N. gonorrhoeae*.

CHAPTER 2: MATERIALS AND METHODS

I. Routine Maintenance of Bacterial Strains

All bacterial strains and plasmids are listed in Table 1. Wild-type and mutant *Neisseria gonorrhoeae* (GC) strains were routinely maintained at 36°C with 5% CO_2 on GC Medium Base (Difco™) agar supplemented with Kellogg's supplements 1 (222)(40% (w/v) glucose, 1% (w/v) glutamine, 0.002% (w/v) thiamine pyrophosphate) and 2 (12.5 µM $Fe(NO_3)_3$) (GCB). When appropriate, GC was incubated on GCB containing antibiotic (1 µg/mL chloramphenicol or 50 µg/mL Kanamycin) *Escherichia coli* strains were routinely maintained at 36°C in Luria-Bertani (LB) agar or broth containing antibiotic (carbenicillin 100 µg/mL, 34 µg/mL chloramphenicol, or 50 µg/mL Kanamycin). For long-term storage, bacterial strains were stored in GC or *E. coli* freezing media containing glycerol at -80°C.

Table 1. Strains and Plasmids Used in this Study

Strain/plasmid	Genotype/relative characteristics	Reference
E. coli strains		
DH5α	F + endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYAargF)U169, hsdR17(rKmK+), λ–	NEB
BL21 (DE3)	F–ompT hsdSB (rB–, mB–) gal dcm (DE3)	Invitrogen
N. gonorrhoeae strains		
FA1090	Wildtype	(223)
FA19	Wildtype (Δ*mntX*)	(224)
1291	Wildtype (Δ*mntX*)	(225)
MCV964	FA1090 *zur::kan* (KanR)	Jean et al. (226)
MCV951	FA19 znuA::Ω	Maurakis et al. (179)
RSC200	1291 *zur::kan* (KanR)	This study, Reeham Victor
RSC201	FA19 + ectopic FA1090 *mntX*	This study

Plasmids		
pET19b-	10x N-terminal His-Tag followed by the enterokinase cleavage sequence and the multiple cloning site; Ampicillin^{R}; T7 promoter/terminator; *lac* operator, *lacI* promoter, and *lacI*	Novagene
pGSU061	10x His-Tagged Zur expression vector	This study
pGSU066	FA1090 mntX inserted into pVCU234	This study
pVCU234	pKH37 + Ribosome Binding Site	Cash (227)
pUC19 *zur*::Kan	pUC19 containing *zur* disrupted by a Kanamycin cassette	Jean et al. (226)

II. Bacterial Mutant Construction

The plasmid used to generate MCV964, the Zur- isogenic mutant in the FA1090 genetic background, was previously generated by kanamycin cassette insertion in the *zur* gene and named pUC19 *zur::Kan* by Jean et al. (214). This plasmid was digested with ScaI and used to transform *N. gonorrhoeae* strain 1291. Colonies were selected on GCB plates containing 50 µg/mL kanamycin, and the resulting strain was named RSC200. The full length *mntX* gene (*ngo1768*) was PCR amplified from the FA1090 genome using oligonucleotides oGSU463 and oGSU464. oGSU463 included 2 nucleotides between the start codon of *mntX* and the XmaI restriction site to generate an 8-nucleotide spacer between the ribosome binding site (RBS) and the start codon. oGSU463 included the XhoI restriction site. Using the In-fusion Cloning Kit (Takara) according to manufactures instructions, the *mntX* gene was inserted between the XmaI and XhoI restriction sites behind an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter in pVCU234 (228). The resulting plasmid, pGSU066, was used to transform DH5α competent *E. coli* cells. Colonies containing pGSU066 were selected on LB agar containing 34 µg/mL chloramphenicol. To generate the *mntX* complement in *N. gonorrhoeae* strain FA19, pGSU066 was first digested with PciI. The linearized plasmid was used to transform strain FA19. Gonococcal colonies that incorporated the pGSU066 DNA were selected on GCB containing 1 µg/mL chloramphenicol and the resulting strain was named RSC201.

To generate a gonococcal Zur-expressing strain of *E. coli*, the gonococcal *zur* gene (*ngo0542*) was PCR-amplified with primer oGSU017 and oGSU018 from the genome of *N. gonorrhoeae* strain FA1090 and inserted between the BamHI and NdeI restriction sites of pET19b-. The resulting plasmid, named pGSU061, encoded an N-terminal, 10x His-tagged

gonococcal Zur protein that was IPTG-inducible and transcribed from the T7 promoter. The *zur* gene was separated from the 10x His-tag by an enterokinase cleavage site. pGSU061 then was used to transform DH5α and BL21 *E. coli* strains. Colonies containing pGSU061 were selected on LB agar containing 100 μg/mL carbenicillin.

III. *N. gonorrhoeae* Growth Conditions for Identifying Differential Gene Regulation

N. gonorrhoeae strains FA1090 and MCV964 (Zur-) were incubated onto GCB agar at 36°C with 5% CO_2 for 14-16 hours before being passaged onto GCB supplemented with 5 μM N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN). Colonies from GCB-TPEN agar plates were used to inoculate 50 mL Chelex® 100-treated chemically defined media (CDM) (229) to an optical density at 600 nm (OD_{600}) between 0.08-0.1. Cultures were incubated at 36°C, 5% CO_2, with shaking at 225 rpm until early log phase (~2 hours). Once cultures reached early log phase, 7 μM TPEN was added to the zinc-deplete condition, and 20 μM $ZnSO_4$ was added to the zinc-replete condition. Cultures were then incubated until late log phase at which point samples were taken for western blotting and RNA isolation. To characterize manganese dependent regulation, strains FA1090, 1291, and the isogenic Zur- mutants (i.e. MCV964 and RSC200) were used to inoculate 10 mL CDM to an OD_{600} between 0.08-0.1. Once in early log phase, cultures were back diluted 2x (1291 and RSC200) or 4x (FA1090 and MCV964). For the excess manganese condition, cultures were supplemented 10 μM (1291 and RSC200) or 15 μM (FA1090 and MCV964) $MnCl_2$. For the manganese-depleted condition, chelator was omitted considering that no manganese-specific chelator is available. Cultures were then allowed to

grow to late log phase at which point culture densities were standardized and pelleted via centrifugation for generation of whole cell lysates.

IV. RNA Isolation

To isolate RNA from *N. gonorrhoeae* for RT-qPCR, RT-PCR, and RNA-sequencing, cultures were pelleted by centrifugation at max speed for 5 minutes at 4°C. Pellets were weighed to determine the appropriate volume of NucleoProtect RNA Reagent (Macher-Nagel) for stabilization and storage of tissues and cells. Pellets were resuspended in of NucleoProtect RNA Reagent and incubated on ice at 4°C for 1 hour. Cell suspensions were then pelleted by centrifugation at max speed for 5 minutes at 4°C. Cell pellets containing stabilized RNA were stored at -80°C until RNA isolation. Between processing steps, cultures and pellets were placed on ice.

RNA was isolated using the epMotion® 5075 liquid handling tool (Eppendorf) and the NucleoMag RNA Isolation Kit (Machery-Nagel) according to manufacturer's instructions, with the following exceptions: half of the suggested volume (175 µL) of Buffer MR1 with the addition of (3 µL) tris(2-carboxyethyl)phosphine (TCEP) was added to each cell pellet. Pellets were then removed from the liquid handling tool and vortexed briefly to homogenize the lysate. Lysates were cleared by centrifugation at max speed for 5 minutes and the supernatants were placed in a new tube which was then placed in the liquid handling robot. The remainder of the protocol proceeded according to manufacturer's instructions. Following elution, 40 U Superase•In™ RNase Inhibitor (Invitrogen) was added to each RNA sample prior to measuring the concentration.

V. DNase-treatment of RNA and cDNA Synthesis

Isolated RNA was DNase I digested using the TURBO DNA-free Kit™ (Invitrogen™) according to manufacturer's instructions for vigorous digestion. DNase-digested RNA was used as template for a quality control PCR to test for DNA contamination. DNA-contaminated RNA was DNase-treated until all DNA contamination was removed. DNA-free RNA was used as template for cDNA (complementary DNA) synthesis using the SuperScript™ IV First-Strand Synthesis System (Invitrogen™) according to manufacturer's instructions. No template and no Reverse Transcriptase (RT) controls were included to ensure DNA-free RNA was synthesized into cDNA.

VI. RNA-sequencing Analysis

RNA sequencing and data analysis was completed using the Illumina large-scale sequencing platform by Novogene. Sequencing image files were transformed into raw sequence files in the form of FASTQ files. Each raw data file contained a sequence identifier and description, raw nucleotide sequences, and read quality values for each base. The sequencing error rate for each nucleotide was less than 0.05% per base. Sequences with higher error rates were filtered out. Raw sequencing reads containing uncertain nucleotides, low-quality sequences, or cDNA adapters were also filtered out. GC/AT content was analyzed for equal distribution of a stranded data set. Sequences that passed the quality controls were mapped to the FA1090 reference genome. Pearsons correlation between samples was used to identify comparable samples. Expression quantification analysis included differential expression analysis and functional analysis. The functional analysis included identification of GO, KEGG, and GSEA

enrichment and analysis of protein-protein interaction (230) networks. The gene structure analysis included operon and antisense transcript prediction, and single nucleotide polymorphism (SNP), untranslated region (UTR) and small RNA (sRNA) identification.

VII. Primer Design and Primer Efficiency Testing for RT-qPCR

Primers used to amplify cDNA of target transcripts were designed according the BIORAD qPCR assay design guide. Primers were designed to have a melt temperature between 50°C and 60°C, GC content between 50 and 60%, and salt and oligonucleotide concentrations of 50 mM and 300 nM respectively. Secondary structures, long repeats (>3) of Gs or Cs and primer dimer formation were avoided. Where the genomic sequence allowed, Gs and Cs were placed at the end of the primer. Amplicons were designed to be between 75 and 200 nucleotides in length. Primers that formed secondary structures had a melt temperature of <40°C. Amplicons of long (>4) repeats of single nucleotides were avoided. GC content was maintained between 50 and 60%. Primer efficiency tests were completed by generating a standard curve using serially diluted genomic DNA from *N. gonorrhoeae* strain FA1090, the SYBR™ SensiFAST®, Hi-ROX kit (Meridian Bioscience), and various primer concentrations. Only primer pairs with an efficiency of 95-100% were used in RT-PCR and RT-qPCR analysis of transcript levels.

VIII. RT-qPCR and RT-PCR for Measuring Gene Expression and Co-transcription

For RT-qPCR reactions, cDNA was diluted to 1 ng/µL so that 2.5 µL (10% of the total reaction volume) could be added to 25 µL reactions. Each reaction contained diluted cDNA, gene-specific primers at various concentrations which had been optimized for efficiency, 2x

SYBR SensiFAST Hi-ROX, and diethylpyrocarbonate (DEPC)-treated water. Reactions were loaded into Hardshell PCR Plates (96-well, thin wall, BIORAD), sealed with the Microseal 'B' seal (BIORAD), and centrifuged briefly to bring the reaction contents to the bottom of the well. Plates were then loaded into the BIORAD 1000 Series Thermal Cycling Platform CFX96 Real-Time System or the BIORAD C1000 Touch Thermal Cycler CFX96 Real-Time System. Cycle threshold (C_T) values were measured by the BIORAD CFX-Maestro system. Target gene expression values were calculated relative to *rmpM* and normalized to the excess Zn condition using the ΔC_T method. RT-PCR reactions were conducted the same as RT-qPCR reactions with the following exceptions. RT-PCR reactions included GoTaq® Green Master Mix (Promega) instead of SYBR, were loaded into individual 0.2 mL PCR tubes, and loaded into the T100 Thermal Cycler. Qualitative expression was imaged by Ethidium bromide staining of samples run on a 1% agarose gel. All primers used in this study are listed in Table 2.

Table 2. Primers Used in this Study

Primer	Sequence	Description
Zur expression vector construction		
oGSU017	CTAGCATATGAAAACAAATTTCAAACAGAAAATTATCGAACA	*zur* forward; NdeI cut site for insertion into pET-19b-; paired with oGSU018 at 55°C annealing
oGSU018	CTAGGGATCCTCACTTCTGACATTTTTTACAGATTCCAGTT	*zur* reverse; BamHI cut site for insertion into pET-19b-; paired with oGSU017 at 55°C annealing
MntX complementation vector construction		
oGSU463	TTAAAAGGAGCCCGGGCTATGAGTCTTTACGCTTTGCTCTTGG	*mntX* forward; In-Fusion cloned with 8 nt space before RBS which includes

		the XmaI site; paired with oGSU464 at °C annealing
oGSU464	CGGGCCCCCCCTCGAGTCATTGAATCAAACCCAAATGCG	*mntX* reverse; In-fusion cloned into XhoI site; paired with oGSU463 at 60°C annealing
<u>RT-qPCR/PCR</u>		
oGSU195	GGAGCAGGCTCCTCAATATG	*rmpM* forward; paired with oGSU196 at 60°C annealing; 95-100% efficient at 40 µM (202)
oGSU196	TAAAGTCGGTATGGCCTTCG	*rmpM* reverse; paired with oGSU195 at 60°C annealing; 95-

		100% efficient at 40 µM (202)
oGSU315	AACAGGCACGCAGAGAGGAC	*zur* forward; paired with oGSU316 at 60°C annealing; 100.1% efficient at 15 µM
oGSU316	TGTAAAACGCCCTGATCCGC	*zur* reverse; paired with oGSU315 at 60°C annealing; 100.1% efficient at 15 µM
oGSU397	TGGAATACGGCGAGGCATTG	*adhP* forward; paired with oGSU398 at 60°C annealing; 95-100% efficient at 10 µM (202)
oGSU398	TCACACCGTCGGCAACTTCT	*adhP* forward; paired with

		oGSU397 at 60°C annealing; 95-100% efficient at 10 µM (202)
oGSU125	ATCGAAGCCAGAAACGGTCC	*tbpB* forward; paired with oGSU126 at 60°C annealing; 95-100% efficient at 20 µM (202)
oGSU126	ATATCTGTCGCCGGACTTCG	*tbpB* reverse; paired with oGSU125 at 60°C annealing; 95-100% efficient at 20 µM (202)
oGSU108	TCCGCAGTGCAAAACTCGTC	*znuA* forward; paired with oGSU109 at 60°C annealing; 95% efficient at 10 µM (202)

oGSU109	TGGTGGTGTCCTTCGTGGTC	*znuA* reverse; paired with oGSU108 at 60°C annealing; 95% efficient at 10 µM (202)
oGSU421	GGGATTGCAGCCTATCGATACGG	*znuC* forward; paired with oGSU422 at 60°C annealing; 90.3% efficient at 20 µM
oGSU422	TCATAGGCTGGGAACGGTCG	*znuC* reverse; paired with oGSU421 at 60°C annealing; 90.3% efficient at 20 µM
oGSU425	CCACCCTGAAAGAAGATGCC	*znuB* forward; paired with oGSU426 at 61.1°C

		annealing; 91.5% efficient at 10 µM
oGSU426	GCAAGCACAGATCCGAAAAG	*znuB* reverse; paired with oGSU425 at 61.1°C annealing; 91.5% efficient at 10 µM
oGSU423	GCAACCGAGACCATTCTGACAG	*znuC-B* intergenic region forward; paired with oGSU424 at 64.3°C annealing
oGSU424	GCAGGTCGTAGAGGTTCATGGTG	*znuC-B* intergenic region reverse; paired with oGSU423 at 64.3°C annealing
oGSU427	ATCCTCTGTTGCAGCGT	*znuB-A* intergenic region

		forward; paired with oGSU428 at 55.7°C annealing
oGSU428	GCCTGTTTGTTCAAGCG	*znuB-A* intergenic region reverse; paired with oGSU427 at 55.7°C annealing

IX. Sodium Dodecyl Sulfate (SDS)-PAGE and Western Blotting

Cultures of *N. gonorrhoeae* were standardized to 100 klett units (KU) per mL of cells and pelleted by centrifugation. Pellets were resuspended in 2x Laemmli sample buffer (BIORAD) containing 0.7 M 2-Mercaptoethanol to make whole cell lysates. Whole cell lysates were boiled for 2 minutes and briefly centrifuged before being loaded into a 4–20% Mini-PROTEAN® TGX™ Precast Protein gel (BIORAD). Gels were run at 135 V for 72 minutes in Laemmli running Buffer (25 mM Tris, 192 mM Glycine, 3.47 mM SDS, pH 8.3). Protein was transferred to a nitrocellulose membrane (Amersham™ Protran®) in transfer buffer (20 mM Tris, 150 mM Glycine, 0.2% v/v Methanol) overnight at 28 mA. Membranes were ponceau stained, blocked with 5% bovine serum albumin (BSA) in high-salt tris buffer saline (TBS) containing Tween20 (20 mM Tris, 500 mM NaCl, 0.1% Tween20, pH 7.5), and probed with guinea pig anti-ZnuA (project 4166, guinea pig #7, diluted 1:1000 in blocker) (generated by Stavros Maurakis of the Cornelissen lab at Virginia Commonwealth University), guinea pig anti-Ngo1049 (supplied by Ian Liyayi of the Criss lab at the University of Virginia), or rabbit anti-TbpB (project NC161-4, diluted 1:2500 in blocker)(231). Blots were washed three-times with high-salt TBS containing 0.1% Tween20 before probing with anti-guinea pig or anti-rabbit antibody conjugated to alkaline phosphatase (AP) and diluted 1:5000 in blocker. Blots were developed with Pierce 1-Step™ NBT/BCIP Substrate Solution (Thermo Scientific) for 5 minutes before being rinsed with water. Blots were dried overnight and imaged.

X. Zur and MntX Protein Structure Modeling and Sequence Alignment

The homology models of Zur and MntX from strain FA1090 were generated based on the open-source software plugin, ColabFold (v. 1.3.0) through the USCF ChimeraX (v. 1.7rc202311302110) platform. The ColabFold: AlphaFold2 using MMseqs notebook was utilized to run AlphaFold. The algorithm employs the predicted Local Distance Difference Test (pLDDT) to rank the five structural models produced, and PYMOL was used for visualization. All Zur sequences used for sequence alignments are listed in Table 3.

Table 3. Zur Protein Sequences Used in this Study

Zur protein sequences

Species	Accession No. or Reference
Neisseria gonorrhoeae	(232)
Vibrio cholerae	BER93739.1
Bacillus subtilis	WP_134991307.1
Mycobacterium tuberculosis	ALB19554.1
Yersinia pestis	TQD67718.1
Pseudomonas aeruginosa	QPV54142.1
Acinetobacter baumannii	SUU48413.1
Corynebacterium diphtheriae	WJY87927.1
Xanthomonas campestris	AAU06119.1
Escherichia coli	CAD6022841.1
Neisseria meningitidis	EJU79121.1

XI. Phylogenetic Tree Analysis of Zinc-dependent Regulators

The phylogenetic trees of characterized Zur and manganese- or peroxide-related regulators were generated from the sequences in Table 3.

The phylogenetic tree was generated using the Jukes-Cantor genetic distance model by the neighbor-joining tree building method with no outgroup. Pairwise distances were obtained from the multiple sequence alignment. The bootstrap resampling method with random seed of 304,621 and 100 replicates was utilized to create a consensus tree with a support threshold of 50%

XII. Zur Protein Expression and Purification

To express the His-tagged gonococcal Zur protein, BL21 *E. coli* containing pGSU061 was grown in LB containing 100 µg/mL carbenicillin overnight at 28°C with shaking at 225 rpm. The overnight culture was diluted 1:200 in Terrific Broth (TB) (24 g/L yeast extract, 20 g/L tryptone, 8 mL/L 50% glycerol) containing 100 µg/mL carbenicillin and incubated at 37°C for 6 hours at 225 rpm. Expression of the His-tagged Zur protein was then induced by the addition of 0.5 mM IPTG, and cultures were incubated at 20°C overnight. Cultures were pelleted by centrifugation for 5 minutes at 4°C and stored at -20°C until purification. To purify the His-tagged Zur protein, cell pellets from 200 mL expression culture was resuspended in 5 mL/g cold Lysis Buffer (50 mM Tris pH 8, 200 mM NaCl, 0.1 µL/mL (100 U/mL) nuclease, 100 µg/mL AEBSF). Cells were incubated in lysis buffer overnight at 4°C with stirring before being subject to three passages through a high-pressure homogenizer (Avestin Emulsiflex) at 17,500 psi. Cell debris was removed by centrifugation at 4°C, and His-tagged Zur was column-purified from the

supernatant by Fast Protein Liquid Chromatography (FPLC) (ÄKTA Go™). Washes were completed by passing 60 column volumes of 5% wash buffer (50 mM Tris, 200 mM NaCl, pH 8) over the column. Zur was eluted in 1 mL fractions with gradient elution buffer (50 mM Tris pH 8, 200 mM NaCl, 1 M Imidazole) where the imidazole percentage increased 1% to 100% over 10 column volumes. Fractions containing purified Zur were dialyzed into 10 mM Tris, 50 mM KCl, pH 8.3 overnight at 4°C with stirring to reduce the imidazole concentration. Reducing agent (i.e. 1 mM Dithiothreitol (DTT) or 2 mM TCEP) was added to the Zur protein just prior to use.

XIII. ICP Measurement of Metal Internalization

For ICP analysis of internal manganese levels, *N. gonorrhoeae* strains FA1090 and FA19 were incubated on GCB agar at 36°C with 5% CO_2 for 14-16 hours. Single, non-piliated colonies were then passaged onto GCB agar containing 7 µM Ethylenediaminetetraacetic Acid (EDTA) and incubated for 14-16 hours. Single, non-piliated colonies were used to inoculate 15 mL CDM to an OD_{600} between 0.08-0.1 for each strain. Cultures were allowed to grow to early log phase at which point 10 mL culture was removed from each flask and divided evenly into 2 flasks. One of the two flasks contained 5 mL CDM only for the manganese-deplete condition and the other flask contained 5 mL CDM supplemented with 40 µM Mn so that the addition of 5 mL culture would dilute the manganese to a final concentration of 20 µM. The resulting cultures were allowed to grow to late log phase for 2-3 hours. Cultures were then pelleted by centrifugation at room temperature and washed with 40 mL of 20 mM Chelex-treated 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) pH 7.4. Pellets were sent to the CAIS Plasma Chemistry Laboratory at the University of Georgia for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to measure internal manganese, iron, copper, and zinc concentrations.

Metal concentrations were reported as micrograms of metal per kilogram (µg/kg) or parts per billion (ppb).

XIV. Growth of *N. gonorrhoeae* at Increasing Manganese Concentrations

N. gonorrhoeae strains FA1090, FA19, the isogenic Zur- mutants (MCV963 and MCV964), the ZnuA- mutant in the FA19 genetic background (MCV951), and the mntX complement in the FA19 background (RSC201) were plated onto GCB agar containing 25 µM $ZnSO_4$ and incubated overnight under routine maintenance conditions. Addition of $ZnSO_4$ was required for growth of the ZnuA- mutant and was therefore included in agar plates for all strains. Strains were passaged onto 25 µM $ZnSO_4$-GCB plates and used to inoculate CDM to an OD_{600} of 0.02 in a 96-well flat bottom polystyrene non-pyrogenic plates (233). CDM contained 7.5 µM 30% iron-saturated human TF (sigma), 25 µM $ZnSO_4$, 5 mM D-mannitol, Kellogg's supplement 1, phosphate buffer saline. For the excess manganese condition, 15 µM or 25 µM $MnCl_2$ was added to the supplemented CDM. RSC201 was grown similarly but in the presence or absence of 1 mM IPTG. Cultures were incubated in the Agilent BioTek BioSpa 8 Automated Incubator for 24 hours with OD_{600} measurements collected by the BioTek Cytation 5 Cell Imaging Multimode Reader (Cytation5) every 30 minutes. At each time-point, the Cytation5 mixed cultures at 225 rpm for 30 seconds. Data was analyzed by plotting OD_{600} over time.

CHAPTER 3: ACCLIMATION TO NUTRITIONAL IMMUNITY AND METAL INTOXICATION REQUIRES ZINC, MANGANESE, AND COPPER HOMEOSTASIS IN THE PATHOGENIC NEISSERIAE

I. Abstract

Neisseria gonorrhoeae and *Neisseria meningitidis* are human-specific pathogens in the Neisseriaceae family that can cause devastating diseases. Although both species inhabit mucosal surfaces, they cause dramatically different diseases. Despite this, they have evolved similar mechanisms to survive and thrive in a metal-restricted host. The human host restricts, or overloads, the bacterial metal nutrient supply within host cell niches to limit pathogenesis and disease progression. Thus, the pathogenic *Neisseria* require appropriate metal homeostasis mechanisms to acclimate to such a hostile and ever-changing host environment. This review discusses the mechanisms by which the host allocates and alters zinc, manganese, and copper levels and the ability of the pathogenic *Neisseria* to sense and respond to such alterations. This review will also discuss integrated metal homeostasis in *N. gonorrhoeae* and the significance of investigating metal interplay.

II. Introduction - Pathogenic *Neisseriae* Cause Devastating yet Distinct Diseases to the Same Host

Neisseria gonorrhoeae and *Neisseria meningitidis* are human-specific pathogens of significant public health concern. Despite high DNA and amino acid sequence identity, *N. gonorrhoeae* and *N. meningitidis* cause significantly different diseases (27, 234).

N. gonorrhoeae is the causative agent of the second most common reportable infectious disease in the United States, gonorrhea, and predominantly colonizes the genital mucosal epithelium and oropharynx (235). Symptomatic gonococcal infection presents as urethritis, cervicitis, salpingitis, pharyngitis or conjunctivitis (235). However, gonorrhea can present asymptomatically as well. Asymptomatic infection in women is of great concern as it enables the pathogen to ascend to the upper reproductive tract, where it can cause pelvic inflammatory disease. Pelvic inflammatory disease can lead to ectopic pregnancy, scarring, infertility, and chronic pelvic pain. In 2018, the incidence of gonococcal disease was 179.1 cases per 100,000, correlating with a total of 583,404 reported cases in the United States (235).

N. meningitidis can inhabit the nasopharynx without eliciting symptoms; this carrier state can be found in 5-10% of the US population (236). The carrier state can transition to symptomatic disseminated infection, sometimes referred to as invasive meningococcal disease (IMD), which is characterized by nausea, vomiting, rash, stiffness of neck, fever, and diarrhea (236). While the incidence of meningococcal disease has decreased dramatically from ~1.50 per 100,000 in 1980 to ~0.2 per 100,000 in 2018, IMD remains a severe threat to infants. Incidence of IMD in children younger than 1 year has averaged around 1.20 per 100,000 in the past 10

years (237). About 12% of infections result in death, and some survivors experience permanent brain damage, loss of limbs or hearing loss (237, 238).

While the pathogenic *Neisseriae* pose a direct threat to human health, they also represent a substantial economic burden in the United States. In 2018, gonorrhea infections resulted in an estimated $85 million in direct medical costs in the United States (239). The estimated total cost of the response to an IMD outbreak at the University of Oregon Hospital (7 cases) and the Oregon State University Hospitals (6 cases) was $12.3 million (238).

During neisserial infection, the host limits bacterial proliferation by modulating metal availability through two related mechanisms: nutritional immunity and metal intoxication. Nutritional immunity is characterized by host sequestration of free metals from the bacterial nutrient supply, limiting metals required for enzymatic and metabolic functions (240). Metal intoxication is the process by which the host overloads pathogens with toxic metal concentrations (241). Metal overload in bacteria contributes to reactive oxygen species (ROS) and reactive nitrogen species (RNS) cycling (242), protein mismetallation (243), and subsequent stalling of respiration (244). Pathogenic bacteria have evolved mechanisms to access restricted metals as well as limit metal overload.

Metal availability within a biological niche dictates pathogen survival and the extent of pathogenesis. Although causing distinct disease presentations, pathogenic *Neisseria* share many mechanisms of responding to metal scarcity and overload to ensure survival and maintain virulence. This review aims to provide an overview of the neisserial response to nutritional immunity and metal intoxication with respect to zinc, manganese, and copper.

III. Zinc, Manganese, and Copper are Allocated to Specific Host Niches

Transition metals, such as zinc, manganese, and copper, are essential to many host processes, including oxidative stress resistance (245-247), cell signaling and metabolism (248), immune modulation (249), post-translational modifications (250, 251), and structural maintenance and enzymatic processing (247, 251). These metals play similar roles in pathogens including *Neisseria meningitidis* (210, 252, 253) and *Escherichia coli (254)*.

Zinc within the human body is primarily localized to the bone and skeletal muscle, with moderate concentrations found in the kidneys and liver (255). Most zinc, however, is metabolically unavailable to the host due to slow zinc turnover with the exception being zinc found in the sperm (256). High levels of zinc- binding metallothioneins (257), which maintain zinc and copper homeostasis and limit heavy toxicity in host cells (258), can be found in male secretions (259). It is feasible that zinc-binding metallothioneins help create a zinc limited environment for the gonococcus during male urethral infection. The majority of zinc within the host is not easily accessible to pathogens due to a limited pool of labile zinc (260), which can be further restricted during infection by production of CP and other S100 proteins that act to sequester free zinc away from the pathogen (178). CP makes up 45% of the protein content in neutrophils (261) and is released following neutrophil death (262) and Neutrophil Extracellular Trap (NET) formation (263). Zinc sequestering protein, S100A7, is enriched in lower genital tract epithelial cells (264). In the case of mucosal infection by *N. gonorrhoeae,* a robust Th17 response results in the influx of neutrophils (84). Thus, S100A7 and CP, which has been released by neutrophils, create a zinc limited environment for *N. gonorrhoeae* (265)**.** The remaining zinc dispersed among the reproductive (256) and immune systems (260).

Within the human host, manganese exists as Mn^{2+} and Mn^{3+} (266). Mn^{2+} is found in the blood bound to albumin, ß-globulin, bicarbonate, and citrate, and in the cytosolic content of neutrophils bound to CP (265, 266). Within the cell, Mn^{2+} is found at the highest concentrations in the endoplasmic reticulum and mitochondria, where it plays an antioxidant role through Mn-dependent superoxide dismutase (MnSOD) (247, 267). In neurons, Mn^{2+} is required for signal transduction and enzymatic function (251, 268). Excess Mn^{2+} accumulates in the liver, kidneys, bone, and pancreas, with higher levels bound to regulatory proteins in the brain and cerebrospinal fluid. Mn^{3+} can be bound to TF, which transports Mn^{3+} to neuronal cells in a mechanism similar to Fe^{3+} transport (268, 269). Many Gram-negative pathogens utilize Mn^{2+} (e.g. *Acinetobacter baumanii, Salmonella enterica, E. coli, Helicobacter pylori,* and *N. gonorrhoeae*), in the face of nutritional immunity, for oxidative stress resistance and metabolism (209, 270-274). During gonococcal infection of macrophages, the manganese transport protein Natural resistance-associated macrophage protein 1 (NRAMP1) (275), is upregulated on the phagosomal membrane (276, 277). NRAMP1 shuttles manganese from the phagosomal compartment to the cell cytosol to restrict pathogen access to manganese. Consequently *N. gonorrhoeae* may experience manganese limitation in the endolysosomal compartment following phagocytosis by macrophages (278).

Copper is found primarily in the liver and plasma in free and ceruloplasmin-bound forms (279). Ceruloplasmin facilitates copper transport through the vasculature and possesses the ferroxidase activity necessary for the oxidation of Fe^{2+} to Fe^{3+} and subsequent iron loading of TF (280, 281). Ceruloplasmin is also transported to the urinary tract during human infection (280). Thus, *N. gonorrhoeae* may experience ceruloplasmin-dependent copper limitation in addition

to S100 protein-dependent zinc limitation at the mucosal surface**.** Copper can also be found within the cytosol of neutrophils, which are recruited to the gonococcal infection site (282, 283), following import by CTR1 on the neutrophil membrane (284, 285).

IV. Metal Acquisition by the Pathogenic *Neisseriae* in Metal-restricted Niches Requires Highly Specific Metal Import

Metal import poses a particular challenge to Gram-negative bacteria, as it requires transport across a two-component cell wall. Outer membrane transport utilizes the proton motive force and requires energy transduction, via the Ton system, from the cytoplasmic membrane. Scarce metals (i.e., zinc, manganese, and copper) are transported into the cytoplasm in an ATP-driven mechanism and is often tightly regulated to avoid metal overload, protein mismetallation, and oxidative stress. Highly specific metal transport is required in a host that uses metal sequestration to restrict microbial growth and pathogenesis.

In the human host, which allocates metals to specific niches, the pathogenic *Neisseria, N. gonorrhoeae and N. meningitidis,* have evolved mechanisms to acquire zinc, manganese, and copper in specific environments. Gonococcal TdfH and TdfJ are zinc-specific TonB-dependent transporters that pirate zinc from CP (198) and S100A7 (179), respectively, to transport that zinc across the outer membrane to the periplasm (Figure 4).

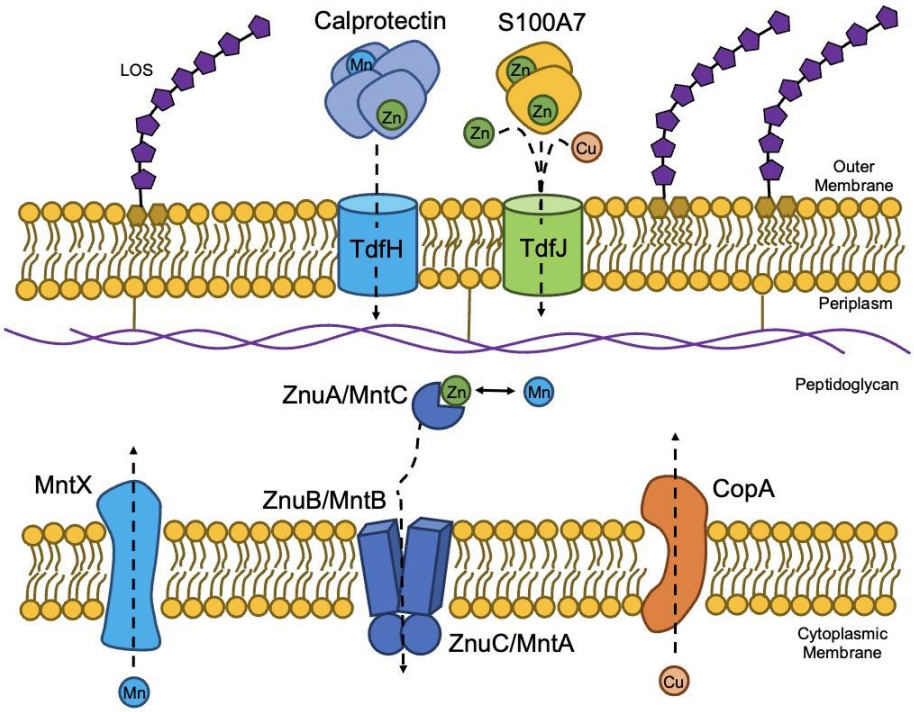

Figure 4. Neisseria gonorrhoeae and Neisseria meningitidis Experience Host-driven Nutritional Immunity and Metal Intoxication.

The host exerts nutritional immunity on the pathogenic *Neisseriae* by exposing the bacteria to calprotectin and S100A7. Calprotectin sequesters both zinc (Zn) and manganese (Mn) from the extracellular environment to limit Zn and Mn availability. *N. gonorrhoeae* in turn expresses TdfH, which has been shown to strip Zn from calprotectin and subsequently import the ion. TdfH is able bind Mn-loaded calprotectin suggesting a role for Mn import across the outer membrane. TdfH is referred to as CbpA in *N. meningitidis.* S100A7 also sequesters Zn from the extracellular environment. Gonococcal TdfJ binds S100A7 to pirate and import the Zn payload. Lipooligosaccharide (LOS) is shown in the outer membrane for reference. Once in the periplasm, Zn and Mn are chaperoned by the periplasmic binding protein, ZnuA (MntC) to the permease in the cytoplasmic membrane, ZnuB (MntB). Transport across the cytoplasmic membrane is energized by the ATPase, ZnuC (MntA). The host also exerts metal intoxication, specifically copper (Cu) intoxication on *N. gonorrhoeae* and Mn intoxication in *N. meningitidis* through unknown mechanisms. In response, these efflux proteins export excess cytoplasmic Cu or Mn from to the periplasmic space.

Gonococcal TdfH binds CP through a high-affinity bimodal interaction. TdfH interacts with a tetramer of CP, which itself is a heterodimer of S100A8 and S100A9 (286). Interestingly, gonococcal growth when CP is the sole zinc source requires the presence of zinc in site 1 of CP, the preferred zinc utilization site by gonococcal TdfH. Mutant CP unable to bind zinc at site 2 fully supports gonococcal growth (198). The cryoEM structure of the CP:TdfH complex has been determined by Bera et al. (286). Since site 1 of CP is capable of binding both zinc and manganese (287) and gonococcal TdfH is able to bind manganese-loaded CP (286), it is feasible that TdfH may also be a manganese importer. The meningococcal TdfH homolog was renamed CP-binding protein A (CbpA); this protein has been shown to bind to zinc- or manganese-loaded CP (Table 4) with higher affinity than it does to unloaded CP, demonstrating a preference for metalated CP over the apo form (Figure 4) (288). Gonococcal TdfJ was shown to bind S100A7 with high specificity and to use the human protein as a zinc source (179). Similarly, the meningococcal homolog of TdfJ is a zinc-specific importer required for zinc import during zinc limitation(289).

After crossing the outer membrane, metals must be escorted across the periplasm to transporters in the cytoplasmic membrane. Metal chaperoning across the periplasm is often accomplished by periplasmic metal-binding proteins (PBP) of the Cluster A-I substrate-binding protein family. PBP transport precedes the ATP-dependent transport step through the cytoplasmic membrane (290, 291). Precise metal transport across the periplasm is required for acclimation to the specific metal environments encountered by Gram-negative pathogens (205, 292-297). PBPs deliver specific metals to permeases at the cytoplasmic membrane, where an ATPase then hydrolyzes ATP to energize metal transport into the cytoplasm.

N. gonorrhoeae express a zinc import system encoded by *znuCBA* (NGO_0170-_0168) (232) where the gene products, ZnuC, -B, and -A, are the ATPase, permease, and PBP, respectively (Figure 5 and Table 4). ZnuCBA transports zinc through the periplasm and across the cytoplasmic membrane. A *znuA* mutant was growth deficient in the presence of all supplemental metals (i.e. Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, and Cd^{2+}) except Zn^{2+}, demonstrating the specificity of this importer for zinc, over other metals, under these conditions (204). Growth only with supplemental zinc suggests that the gonococcus requires specific zinc import via ZnuA for cellular processes that cannot be completed with substituting metals under these conditions.

A manganese-specific outer membrane importer has not been identified in pathogenic *Neisseriae* despite the requirement for manganese, rather than zinc, cobalt, or magnesium, to resist oxidative killing (209). However, the ability of TdfH to bind to manganese-loaded CP suggests the possibility for highly specific manganese import across the outer membrane in the pathogenic *Neisseriae* (Table 4) (286).

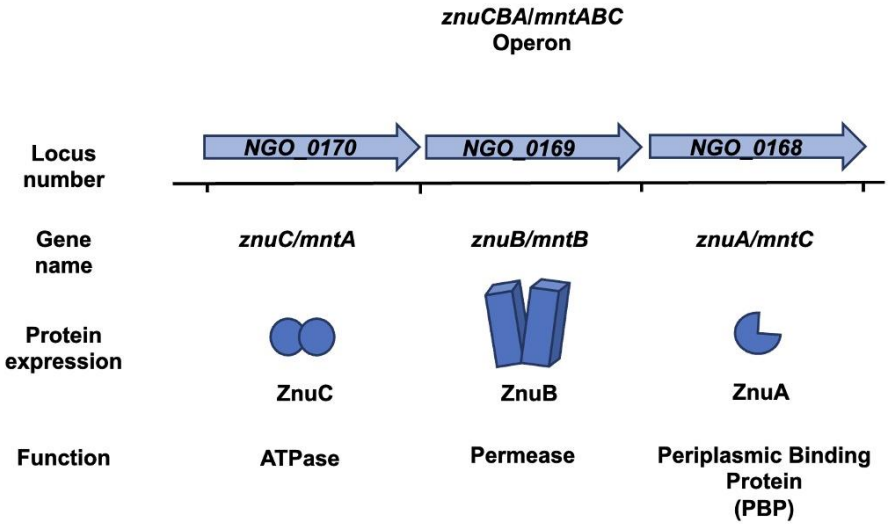

Figure 5. The Genome of Neisseria gonorrhoeae and Neisseria meningitidis Encodes the znuCBA/mntABC Operon

The *znuCBA* operon in *N. gonorrhoeae* is indicated by the locus numbers NGO_0170, NGO_0169, and NGO_0168. The operon is named in order of predicted transcriptional direction. NGO_0170 encodes an ATPase named ZnuC which provides energy from zinc (Zn) transport into the cytoplasm. NGO_0169 encodes a permease named ZnuB which serves as a channel for transporting Zn through the cytoplasmic membrane. NGO_0160 encodes ZnuA, which chaperones Zn from the periplasm and delivers it to ZnuB. A simplified image of the *znuCBA* gene products is depicted. The gene products of the NGO_0170, NGO_0169, and NGO_0168 have been implicated in both Zn and manganese (Mn) binding and transport. Thus, *znuCBA* is also referred to as *mntABC* in reference to Mn transport. *znuCBA* and *mntABC* are two different names to identify the same genetic sequence indicated by the locus numbers NGO_0170, NGO_0169, and NGO_0168. The *znuCBA* operon including the intergenic regions in *N. gonorrhoeae* is 92% identical to that in *N. meningitidis* (Accession number AE002098.2), in which *znuCBA* is also responsible for Zn and Mn import.

Table 4. Neisseria gonorrhoeae (Ng) and Neisseria meningitidis (Nm) Express Proteins Which are Potentially Involved in Integrated Metal Homeostasis

Ng protein (Affinity [ligand])	Nm protein	Integrated metals	Reference
TdfH (4 nM and 35 µM [CP])	CbpA	Zn, Mn	(198, 210, 214, 288)
TdfJ (40 nM, (S100A7))	ZnuD	Zn, Cu, Fe	(179, 200, 201, 214)
TbpB (7.4 nM[TF])	TbpB	Mn, Fe	(298-300)
ZnuCBA/MntABC (100 ± 8 nM [Mn^{2+}]; 104 ± 5 nM [Zn^{2+}])	ZnuCBA/MntABC	Zn, Mn	(204, 209, 211)
MntX (nk, [Mn^{2+}])	MntX	Mn, Fe	(243)
Zur/PerR (nk, [Mn^{2+}])	Zur	Zn, Mn	(210, 211, 214)

nk (not known) indicates the affinity for that ligand is not known

The *znuCBA* operon is also referred to as *mntABC* in the context of manganese transport through the periplasm and across the cytoplasmic membrane (Table 4). MntA, -B, and -C are the ATPase, permease, and PBP, respectively. *znuCBA* and *mntABC* are different names for the same operon. *znuCBA* was used to describe the operon when the gene products were demonstrated to be involved in zinc import; somewhat confusingly, *mntABC* was deployed as the term to describe the operon when the gene products were involved in manganese import (Figure 5). The gene locus is the same for both systems and presumably encodes the proteins required for both manganese and zinc import.

ZnuA (Figure 5), from *N. gonorrhoeae* strain FA1090 (232) shares 96% amino acid identity with a zinc ABC transport PBP encoded by *N. meningitidis*. This high sequence similarity suggests that *N. meningitidis* also requires a PBP for zinc and manganese transport through the periplasm. A gonococcal *mntC* mutant imports 500-fold less manganese than the wild type, demonstrating a dual metal-binding capacity (209). MntC binds manganese and zinc with nearly equal affinity (100 ± 8 nM for Mn^{2+} and 104 ± 5 nM for Zn^{2+}), suggesting that the gonococcus occupies niches during infection that are limited in both metals (204, 205). This import system may be required for growth and pathogenesis in *N. meningitidis* much like it is in *N. gonorrhoeae*.

Calmettes et al. showed that the meningococcal TdfJ homolog, ZnuD, crystalizes with zinc and cadmium at distinct binding sites in the absence of a chelator, suggesting the ability to bind both ions in their free form (199). Hecel et al. defined the metal binding specificity of the flexible loop responsible for ion capture by ZnuD (200), noting that this flexible loop binds copper (Figure 4) with higher affinity and stability than it does zinc and that the loop undergoes

a substantial conformational change upon copper binding (200). The ability of ZnuD and likely TdfJ to bind copper suggests that this transporter may import copper in addition to zinc (Figure 4, Table 4).

A periplasmic copper chaperone has not yet been identified in the pathogenic *Neisseriae*. However, the ability of ZnuD to bind free copper suggests that copper could be imported through the outer membrane to the periplasm and may require a chaperone for delivery to the cytoplasmic membrane.

V. Metal Availability Sensors Regulate Metal Import Genes

Metal-dependent regulation in bacteria is often accomplished by the ferric uptake regulator (3) -family proteins (3). Fur-family metalloregulators include Fur, PerR, Mur, Nur, Zur, and Irr, which are responsible for regulating iron uptake, peroxide stress sensing, manganese uptake, nickel uptake, zinc uptake, and heme-dependent iron uptake (301). These Fur-family regulators are responsible for sensing metal availability within microenvironments inhabited by pathogens and subsequently coordinating a transcriptional response.

Pathogenic *Neisseriae* express two well-characterized metal dependent regulators: Fur and Zur (Zur has also previously been called PerR) (210, 211, 214, 226). In *N. gonorrhoeae*, Zur is hypothesized to repress zinc and manganese import genes in the presence of these metals and to de-repress zinc and manganese import genes in the absence of these metals (Table 4) (204, 211). Production of gonococcal proteins TdfJ and TdfH is zinc-repressed in a Zur-depending manner (214). In the context of infection at mucosal surfaces and in neutrophils, *N. gonorrhoeae* requires a zinc sensor, such as Zur, to mount a transcriptional response to CP- and S100A7- mediated zinc limitation. Zur de-represses expression of high-affinity metal importers,

including *znuCBA, tdfJ*, and *tdfH,* so that the gonococcus can effectively and efficiently import Zn. Meningococcal Zur specifically binds to the promoter of *znuD (P_{znuD})* in the presence of Zn^{2+}, but not Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+}. Zinc-dependent binding of Zur to P_{znuD} is abrogated with the addition of a zinc-specific chelator, N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN) (210), suggesting that Zur is responsible for sensing zinc availability and coordinating a transcriptional response that allows for zinc acquisition. Microarray and RT-qPCR analyses also showed zinc-dependent regulation of 11 other genes, including *cbpA*, *znuCBA,* the high-affinity zinc ABC importer, and genes encoding multiple ribosomal proteins, nitrosative stress resistance proteins, and metabolic proteins (210, 288).

Manganese-dependent regulation by gonococcal Zur (also referred to as PerR) was demonstrated by Tseng et al. and Wu et al. (Table 4) (209, 211, 215). Wu et al. established that *znuCBA (mntABC)* is manganese-repressed in a Zur-dependent manner. Manganese has also been shown to upregulate many ribosomal proteins, pilus assembly proteins, adhesion proteins, outer membrane proteins, the multidrug efflux pump protein channel, MtrE, and many metabolic proteins (298). Interestingly, the iron-repressed TF-binding protein A (TbpA) and the transport protein ExbB were also manganese-repressed (298, 299). These data suggest that gonococcal Zur senses manganese limitation during infection where CP and potentially other manganese-binding proteins sequester manganese.

Copper sensing and regulation in bacteria are often accomplished by CueR, which is absent from the gonococcal genome (232, 302). The genome of *N. meningitidis* encodes a putative CueR regulator (accession number MBF1297094.1) that is 47.62% identical to that found in *E. coli* (accession number NP_415020). Although, it has not yet been empirically

characterized as a copper-dependent regulator. Neisserial MisR (accession number WP_002214312.1), the response regulator of the MisR-MisS two-component regulatory system is involved in cationic antimicrobial peptide resistance (181). Interestingly, MisR is 36% identical and 58% similar to *Pseudomonas aeruginosa* CopR, which is involved in regulation of copper homeostasis (303).

Much work is needed to characterize the ability of pathogenic *Neisseriae* to sense and regulate copper, considering that ceruloplasmin is found in the serum, which is a meningococcal infection site (304). Ceruloplasmin concentrations in the cerebrospinal fluid (0.8-2.2 μg/mL) are 100-500-fold lower than that in the serum (305). The gonococcus may also need to sense copper levels, considering the potential for copper to fluctuate following CTR1 protein expression within neutrophils.

VI. Transition Metals are Required for Survival and Virulence

Bacteria utilize scarce metals for several mechanisms related to survival and virulence, such as resistance to reactive oxygen species (ROS) and reactive nitrogen species (RNS), metabolism, maintenance of cell structural integrity, and proper protein structure and function. Zn contributes to the function of biosynthetic pathways and virulence in *N. gonorrhoeae* and *N. meningitidis* as a cofactor for enzymes, enabling survival and virulence. For example, in both species, biosynthesis of lipid A, a potent immune activator (306, 307), involves a putative zinc-dependent metalloamidase, UDP-3-O-(R-3- hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC) (308-310). Lipid A anchors LOS into the bacterial membrane and can activate the immune system after its release from the bacterial cell wall during cell death (307). Additionally, it can be directly recognized by host Lipid-A binding protein (LPB), which plays a

role in sensing of pathogenic (311) and commensal species (307). Thus, LOS is a key virulence factor in *N. gonorrhoeae* and *N. meningitidis*. Similarly, the *N. meningitidis* protein, Ght, a zinc binding protein involved in LOS biogenesis, (312) is involved in LOS expression and outer membrane integrity (312). These observations implicate zinc in virulence and survival.

Manganese contributes to oxidative stress resistance in the pathogenic *Neisseriae* and thus contributes to survival during infection at highly oxidative sites (209, 211). Manganese in bacteria cycles between the Mn^{2+} and Mn^{3+} states during MnSOD processing of reactive oxygen species (313). Interestingly, the pathogenic *Neisseria* do not express a MnSOD and instead use manganese directly as an ROS quencher (215). Wu et al. demonstrated a role for gonococcal *mntC* in the oxidative stress response under anaerobic rather than aerobic conditions (314). The vagina and cervix are normally oxygen-depleted, making anaerobic gonococcal growth conditions highly relevant (315). *In vitro*, growth of an *mntC* mutant under anaerobic conditions was inhibited by paraquat, an intracellular inducer of ROS, to an extent similar to that of the wild type (314). However, the *mntC* mutant was less competitive than the wild type during *in vivo* infection, which is characterized by both anaerobic and highly oxidative conditions (314). Reduced competition by the *mntC* mutant under anaerobic and oxidative conditions suggests that manganese is critical to gonococcal growth within the cervical niche. In contrast to the gonococcus, growth of the meningococcus in the presence of manganese does not enhance oxidative stress resistance (316).

N. meningitidis is able to grow on manganese concentrations more than 50-100 times higher (>100 mmol/L) than *N. gonorrhoeae*, which suggest that manganese homeostasis differs between these species (209, 316). Despite the non-restorative effect of manganese during

oxidative conditions in the meningococcus, manganese is a vital cofactor in biosynthetic pathways within the bacterium. Meningococcal sialic acid synthase, NeuB, was shown to crystalize best with the addition of manganese, suggesting that this enzyme also requires a manganese cofactor (209, 317). NeuB is involved in sialylated capsule formation (317), and the sialylated surface of *N. meningitidis* has been shown to protect the bacterium from complement deposition (318) through molecular mimicry of host cell surface proteins (306). Due to the potential involvement of manganese in NeuB activity, and consequently capsule formation, the metal may play a role in protection from host complement deposition and thus in immune evasion. This mechanism of innate immune evasion is particularly useful to the pathogen during infection of the vasculature, a niche that is complement is enriched.

In the pathogenic *Neisseria*, copper plays a role in resistance to extracellular RNS (215, 319, 320). *N. gonorrhoeae* (321) and *N. meningitidis* (322) possess a surface-exposed lipid-modified azurin (294), which is a putative electron donor to peroxidases (215). This biological function is particularly relevant to macrophage infection because they are known to increase the expression of nitric oxide synthase (98) upon stimulation with LOS (323, 324). *N. gonorrhoeae* can survive in the harsh environment of macrophage phagosomes, potentially through a mechanism involving copper-bound Laz (325, 326). Interestingly, the gonococcal genome encodes a putative peptidase with a PepSy domain (Accession number WP_003702955.1). This peptidase is 34% identical and 51% similar to that produced by *P. aeruginosa* (NCBI Reference Sequence: NP_252478.1). The peptidase in *P. aeruginosa* has been shown to be copper-repressed (327). A similar putative peptidase is predicted to be produced by the meningococcus (Accession number WP_079889394.1) and is 36% identical and 50% similar to that from *P.*

aeruginosa. The role of copper in the regulation and function of this peptidase in *N. gonorrhoeae* and *N. meningitidis* is a potential focus of future investigation.

VII. The Response to Metal Overload Requires Sensing and Export of Intoxicating Metals

The host applies metal intoxication strategies to limit bacterial growth and survival. Metal intoxication is the process by which the host floods the bacterial nutrient supply with metals. The consequences of metal intoxication for bacteria include electron transport chain (ETC) inhibition, protein mismetallation, and ROS and RNS accumulation (328). To limit these consequences, bacteria utilize mechanisms that store or export excess metal and repress metal import systems (328). Metal toxicity in *N. gonorrhoeae* has also been shown in reference to Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}(243, 329). However, specific responses to overload of each metal remain poorly characterized.

While macrophages have not been shown to exert metal intoxication upon the pathogenic *Neisseria* species*,* these immune cells have demonstrated the ability to increase the concentration of zinc in the cytosol and the phagocytic vacuole via the SLC39A transporters (328, 330, 331) suggesting that host-induced zinc toxicity may be relevant to pathogenic *Neisseria* infection. Macrophages have been shown to increase phagosomal zinc concentrations to apply metal stress on invading *Mycobacterium* species (230, 332). In *Streptococcus pneumoniae*, excess zinc competes with manganese for binding to pneumococcal surface antigen A (PsaA), resulting in reduced manganese uptake, reduced oxidative stress resistance, and reduced resistance to PMN killing (333). In *E. coli*, high levels of manganese correlate with

reduced levels of Fe^{2+}, iron-containing enzymes in the ETC and TCA cycle (iron-sulfur clusters and heme-containing enzymes), and consequently, reduced levels of NADH and ATP (254). Although not empirically tested in the gonococcus, manganese intoxication has been tested and shown to be relevant to meningococcal growth. Excess manganese in *N. meningitidis* results in protein mismetalation and subsequent dysregulation of Fur-regulated genes (Table 4) (243). Under excess manganese conditions, the meningococcus expresses *mntX*, the gene encoding a manganese export protein, which is critical to survival under high manganese conditions. MntX contains predicted transmembrane domains suggesting that this protein transports manganese from the cytoplasm to the periplasm (243). The *mntX* mutant exhibited a reduced ability to survive in the blood of infected mice relative to the wild-type strain (243). Additionally, the *mntX* mutant showed reduced resistance to human serum (243). Taken together, these data suggest that the meningococcus senses high manganese during septicemic infection and responds by expressing *mntX*. It is also feasible that the meningococcus requires a manganese exporter during infection in the cerebrospinal fluid. The blood and cerebrospinal fluid are body sites that are manganese-enriched and may be a hostile environment for a pathogen lacking a Mn exporter. Metal toxicity in *N. meningitidis* has also been shown in reference to Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} (329). Conversely, *mntX* is frameshifted in 66% of sequenced *N. gonorrhoeae* strains. A *N. gonorrhoeae* strain, which was sensitive to manganese in this way, could be rescued through complementation with meningococcal *mntX* (243). Expression of *mntX* in certain gonococcal strains may enable dissemination to the blood and meninges.

Copper intoxication is used by the host to limit bacterial infection. For example, copper influx into the phagosomal compartment of macrophages results in increased killing of an *E. coli*

strain deficient in a copper efflux protein, CopA, relative to the wild type (334). The gonococcus also produces a copper efflux protein, CopA, in the cytoplasmic membrane (242). CopA likely transports copper from the cytoplasm to the periplasm. A *copA* mutant exhibited higher concentrations of internal copper and was growth impaired under high copper conditions (242). Following copper supplementation, the *copA* mutant was limited in its ability to associate with and invade primary human cervical epithelial cells; it was also less resistant to killing by nitrite and *S*-nitrosoglutathione (GSNO), a nitric oxide generator (242). This data suggests *N. gonorrhoeae* experiences copper overload within cervical epithelial cells**.** Djoko and McEwan showed that high levels of copper increase gonococcal sensitivity to the RNS generator, sodium nitrite and suggested that copper-dependent inactivation of hemoproteins involved in intracellular RNS detoxification results in RNS-dependent killing of *N. gonorrhoeae* (244). While the concept of nutritional immunity has been thoroughly studied, the concept of metal intoxication requires further exploration. Expanded application of this concept to pathogenic *Neisseriae* infection will broaden our understanding of metal homeostasis and its role in pathogenesis.

VIII. Next Steps - Comprehensive Characterization of Nutritional Immunity and Metal Intoxication Requires Insight into Integrated Metal Homeostasis

Pathogens possess mechanisms of defense against metal starvation and metal intoxication exerted by the host, as these metals play integral roles in metabolism, maintenance of cell structural integrity, and ROS and RNS resistance. Metal involvement in

these processes is often studied in isolation, meaning only one metal (i.e., zinc, manganese, or copper) is considered at a time. However, it is improbable that pathogens encounter a single type of metal depletion or stress during infection of a host whose metal allocations and concentrations are heterogeneous.

One of the most thoroughly investigated examples of metal interplay is that between manganese and zinc in *S. pneumoniae.* Cell-associated manganese in wild-type *S. pneumoniae* is substantially reduced in the presence of excess zinc, and zinc depletion of cellular manganese could be restored by the addition of excess manganese (335). Under high zinc conditions, zinc competes with and inhibits manganese binding to the manganese importer, PsaA, resulting in manganese starvation and zinc toxicity (335, 336). Zinc-induced manganese starvation leads to increased sensitivity to oxidative stress (336). Another example of metal interplay has been investigated in *S. enterica* Serovar Typhimurium. In this bacterium, *mntH* encodes a manganese importer that is iron-repressed in a Fur-dependent manner and manganese-repressed in a manganese transport repressor (MntR) -dependent manner (337). Kehres et al. hypothesized that co-regulation of *mntH* maintains an equilibrated Mn^{2+}/Fe^{2+} ratio in *Salmonella* (337). *Helicobacter pylori* expresses a metal efflux system, CznABC, which interacts with cadmium, zinc, and nickel and confers resistance to intoxicating levels of all three metals (338). *Acinetobacter baumannii,* when grown in the presence of CP that is able to simultaneously chelate zinc and manganese, exhibits reduced intracellular manganese and zinc but increased iron levels (339). In this case, CP treatment not only resulted in altered zinc, manganese, and iron homeostasis but also in reduced growth (339). In *Klebsiella pneumoniae*, the zinc efflux protein, ZntA, is responsible for exporting zinc from the cytoplasm (340). The *zntA* mutant was

shown to accumulate more manganese in addition to zinc and less iron than the wild type when subject to high zinc conditions, demonstrating integrated zinc, manganese, and iron homeostasis in wild type *K. pneumoniae* (340).

Pathogenic *Neisseriae* sense metal concentrations in the environment and respond by altering the expression of metal import or export systems and allocating these metals to metabolic and biosynthetic processes to allow for survival and virulence. *N. gonorrhoeae* and *N. meningitidis* are similar pathogens, which respond similarly to metal limitation and overload within the same host, despite causing different physiological symptoms. It is unlikely that the pathogenic *Neisseria* experience zinc, manganese, and copper starvation or intoxication in isolation from other metals. Interaction with multiple metals by neisserial proteins suggests the need for complex and integrated metal homeostasis (Table 4). This is evidenced by the ZnuCBA manganese import system in *N. gonorrhoeae*, which is manganese-regulated in a Zur-dependent manner but is also responsible for zinc import (Table 4) (204, 209, 211). More work needs to be done to characterize Zur metal sensing when zinc and manganese are present together. Gonococcal TdfJ is zinc-repressed in a Zur-dependent manner and is iron-induced (214). The zinc to iron ratio required for optimal TdfJ expression in the presence of S100A7 should be addressed in future studies. Gonococcal TbpA is both iron- (182, 299) and manganese-repressed (298) (Table 4). TbpA is an iron-repressed (182, 299) TonB-dependent transporter that pirates iron from human TFg and transports it across the outer membrane (180, 341). Considering that TbpA is also manganese-repressed and that TF can transport manganese in a manner similar to iron, it would be interesting to determine whether TbpA can pirate manganese from manganese-loaded TF. Additionally, studies regarding the application of

this potential manganese transport system to a host niche would be informative. The manganese export protein, MntX, in *N. meningitis* is required for survival under high manganese and low iron conditions, and the absence of the gene encoding this system results in mis-regulation of iron-regulated genes (Table 4) (243). It would be informative to discern the exact manganese to iron ratio required for optimal *mntX* expression and consequent serum resistance in *N. meningitidis.* In-frame MntX is only present in a subset of gonococcal strains (243).

The evolution of complex metal regulatory and import mechanisms suggests that the pathogenic *Neisseriae* possess a need for multifactorial metal homeostasis. Instances of overlap in different metal-related processes imply that the pathogenic *Neisseriae* may specifically require the integration of manganese and iron homeostasis and zinc and copper homeostasis. The exact mechanisms of integrated metal homeostasis and the host conditions under which they are relevant have not yet been fully deciphered. Further investigation into the complex metal environment sensed by the bacteria in the host could broaden our understanding of mixed metal homeostasis and the neisserial response to nutritional immunity and metal intoxication.

CHAPTER 4: STRUCTURAL AND FUNCTIONAL INSIGHTS INTO THE MECHANISM OF ZINC-DEPENDENT REGULATION BY THE ZINC UPTAKE REGULATOR (ZUR) IN *NEISSERIA GONORRHOEAE*

I. Introduction

Neisseria gonorrhoeae is the etiological agent of the sexually transmitted infection gonorrhea, an infection that impacted nearly 650,000 individuals in the Unites States alone in 2022. In 2020, the World Health Organization (WHO) estimated over 80 million new cases of gonorrhea world-wide. Not only does gonorrhea impose a significant epidemiologic burden, but it also imposes a significant financial burden. Gonorrhea is estimated to cost Americans ages 15-39 over $200 million in their lifetime (239, 342, 343). Infection by *N. gonorrhoeae* causes cervicitis in women and urethritis in men. If left untreated, the gonococcal infection can ascend to the upper reproductive tract causing massive inflammation in the fallopian tubes (salpingitis) and epididymis (epididymitis). Sequelae of disseminated infection in women is ectopic pregnancy and pelvic inflammatory disease. Further dissemination to the blood stream can lead to septic arthritis, skin rashes, and in rare cases endocarditis and meningitis. However, infections in females are often asymptomatic, leading to increased probability of sequalae. Because of the increased probability of sequelae in women, gonorrhea is expected to cost women three times as much in direct medical costs as men (342).

The current recommended treatment for gonococcal infection is a single intramuscular dose of ceftriaxone. The limited number of recommended antimicrobial treatments is the direct result of increased antimicrobial resistance in the gonococcus. Because the gonococcus so readily acquires and maintains antimicrobial resistance determinants, we are in urgent need of an effective vaccine. Unfortunately, vaccine efforts have been thwarted by high-frequency phase and antigenic variation of surface exposed vaccine targets. Surface exposed proteins that are conserved, like reduction modifiable protein (Rmp) and porin, are poor vaccine candidates because the immune response is non-productive and exacerbates infection. Thus, we are in urgent need of vaccine targets that are expressed in the multitude of gonococcal isolates, not subject to high-frequency variation, required for infection, and generate a protective immune response. The well-conserved TonB-dependent (Tdt) and ABC transporters produced by *N. gonorrhoeae* are promising targets because they meet many of these requirements. Disrupting these metal acquisition systems is a promising strategy for generating novel prophylactic treatments and vaccine-induced immunity. An immune response that could render these metal transport systems non-functional could starve and kill the pathogen. This starve and kill concept has also been demonstrated in the case of the Tdt TbpA where mutated TbpA is unable to bind the iron source TF thus killing *N. gonorrhoeae* in the absence of another iron source (344). Disruption of the function of the Tdts, TdfH and TdfJ, through mutagenesis prevents zinc uptake from the host zinc sources CP and PS respectively and kills *N. gonorrhoeae* when no other zinc source is supplied (179, 198, 201). Additionally, utilization of psorasin via TdfJ is dependent upon the high-affinity zinc transporter ZnuA. ZnuA is encoded by the *znuCBA* locus where *znuC* encodes an ATPase, *znuB* encodes the transmembrane protein and *znuA* encodes the

periplasmic binding protein. Survival at the site of infection likely requires TdfJ and ZnuA, making these proteins promising targets for novel therapeutic and vaccine developments. The genes encoding TdfJ and ZnuA are zinc-repressed by the zinc-uptake regulator, Zur (202, 214, 226).

In *N. gonorrhoeae*, ZnuABC has also been characterized as a manganese transporter that is manganese-regulated by Zur (205, 209, 211). As such, ZnuABC has been named MntCBA. These names have been used interchangeably to describe the same set of proteins encoded by the same genes. Unfortunately, when the transporter was characterized regarding manganese, the name order was reversed. Consequently, the gene with locus tag NGO0168 has been name *znuA* and *mntC*. Gene with locus tag NGO0169 has been named *znuB* and *mntB*, and gene with locus tag NGO170 was named *znuC* and *mntA.* For clarity and simplicity, hereon, the locus and encoded proteins will be referred to as *znuCBA* and ZnuABC respectively.

Many Zur proteins have been characterized in other bacteria such as *Bacillus subtilis*, *Xanthamonas campestris*, *Neisseria meningitidis*, and *Salmonella enterica*. In *B. subtilis*, Zur binding to various levels of zinc results in conformational changes in the protein that result in an active, partially active or inactive form of Zur (345). Additionally in *B. subtilis*, the ability of Zur to repress gene expression is relative to the specific DNA sequence upstream of each gene. For example, Zur dependent repression of *znuA* requires more zinc than that of *zinT*. In *X. campestris*, zinc binds up to 4 binding sites on the Zur dimer. Zinc binding at two of these sites is required for structural maintenance, while binding at the other two regulatory sites results in a change in Zur to the active conformation (346). The Zur regulon and partial mechanism have been characterized in *N. meningitidis.* In *N. meningitidis,* Zur (NmZur) regulates genes encoding

the zinc transporters such as ZnuABC and ZnuD, which is homologous to gonococcal TdfJ. NmZur was shown to regulate these genes based on specific sequences within the promoter region termed Zur boxes. Mutation of the 3' inverse repeat of the Zur box upstream of *znuD* resulted in reduced Zur binding to the promoter. Interestingly, in *S. enterica*, the *zur* and *znuC* mutants demonstrated a survival disadvantage relative to the wild type in a mouse model of infection (347), suggesting that Zur-dependent regulation and zinc acquisition may be critical to infection in *S. enterica*.

The exact mechanism of both zinc and manganese-dependent gene regulation by *N. gonorrhoeae* Zur (NgZur) has not been fully characterized in *N. gonorrhoeae*. We tested the hypothesis that genes encoding ZnuABC are Zur-repressed in a zinc- and manganese-dependent manner and that regulation requires certain structural and functional Zur features to occur. Data acquired through Zur structure modeling, phylogenetic analysis, RNA-sequencing, and RT-qPCR suggest that Zur is a zinc-specific global regulator in strain FA1090. Identifying the metal-dependent mechanism through which Zur regulates the zinc importer, ZnuA, and other Zur-regulated genes is critical to evaluating their potential as possible therapeutic targets.

II. Results

A. N. gonorrhoeae Zur Resembles Other Zinc-dependent Regulators but is Phylogenetically Distinct

To identify the amino acids involved in gonococcal Zur (NgZur) regulation, the NgZur sequence was aligned with those of Zur proteins which have been previously characterized in other bacteria (Figure 6). Fur family regulators like NgZur share a similar structure that is composed of an N-terminal DNA binding domain (DBD) and a C-terminal dimerization domain (DD) linked by a flexible hinge region containing the ligand binding domain (LBD) (Figure 6A). NgZur contains two predicted zinc coordination sites, a CXXC motif-containing site (CXXC site) and a histidine, cysteine, and glutamic acid-containing site (HCHE site) both of which are located in the ligand binding domain (LBD) (Figure 6A and 6B). These zinc binding sites have been shown to be critical to the structure and regulatory function of XcZur respectively where the CXXC site is required for Zur structural maintenance and the HCHE site is involved in differential gene regulation (346). Thus, structural maintenance and regulatory function of NgZur likely requires zinc coordination at these sites as well. Additionally, NgZur contains an arginine residue at amino acid position 24 (R24) (Figure 6A) located in the DNA-binding domain (DBD) which has been shown to be crucial for DNA binding in XcZur and is conserved across all analyzed Zur protein sequences (346). Predicted alpha helix 5 (H5) and β strand 5 (B5) contain highly-conserved, nonpolar and hydrophobic residues (i.e. V, A, L, I) (Figure 6B). These hydrophobic residues are likely involved in protein dimerization (348), and have been crystalized in *X. campestris* (346). Interestingly, NgZur and NmZur contain a unique DECH-rich

region from amino acid position 90-102 (Figure 6B). A similar HDH metal-binding motif has been identified in gonococcal ZnuA, suggesting that this region plays a role in metal-binding that is unique in the *Neisseriae* (204). Like other Fur family regulators, NgZur is predicted to form a dimer made up of 6 alpha helices and 4 β sheets per monomer (Figure 7). The predicted tertiary structure of NgZur resembles a clamp shape similar to *E. coli* Zur (EcZur) (349) and XcZur (346).

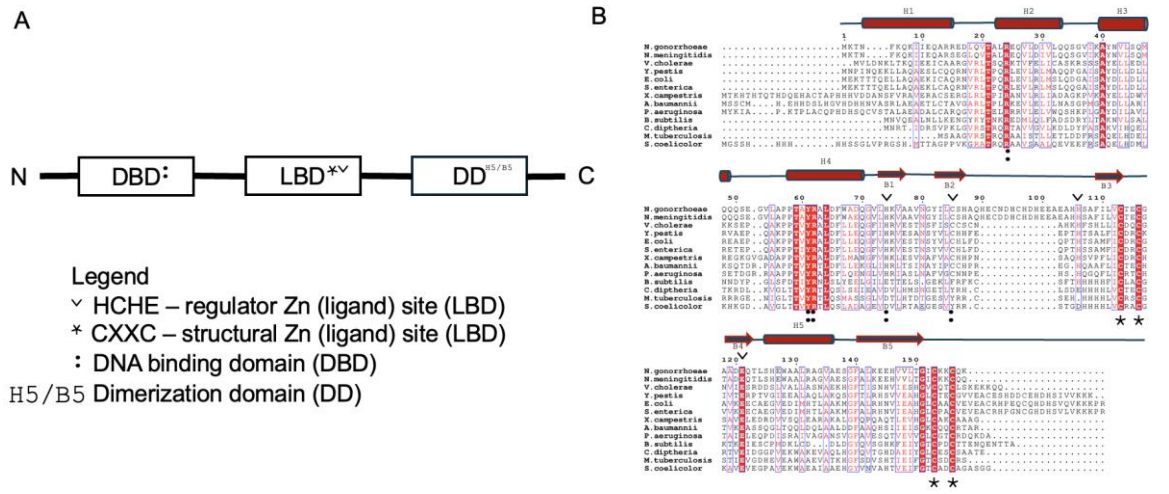

Figure 6. N. gonorrhoeae Zur Resembles Other Zinc-dependent Regulators

The general structure of Fur-family regulators is given with symbols indicating predicted functional elements of the protein (A). The multi-sequence alignment of NgZur with other characterized Zur proteins was generated using Clustal Omega and imported into ESpirit 3.0 for visualization. The NgZur sequence from strain FA1090 was imported into Jpred 4 to generate a predicted secondary structure which is overlayed the sequence alignment. :, highly conserved DNA-binding residue, upside down carrot, HCHE regulatory zinc-binding; and *, CXXC motif-containing structural zinc-binding site. The critical residues in the dimerization domain are predicted to be in alpha helix 5 (H5) and β strand 5 (B5). Red filled boxes indicates identical residues. Red text indicates similar residues, and blue open boxes indicate groups of similar residues.

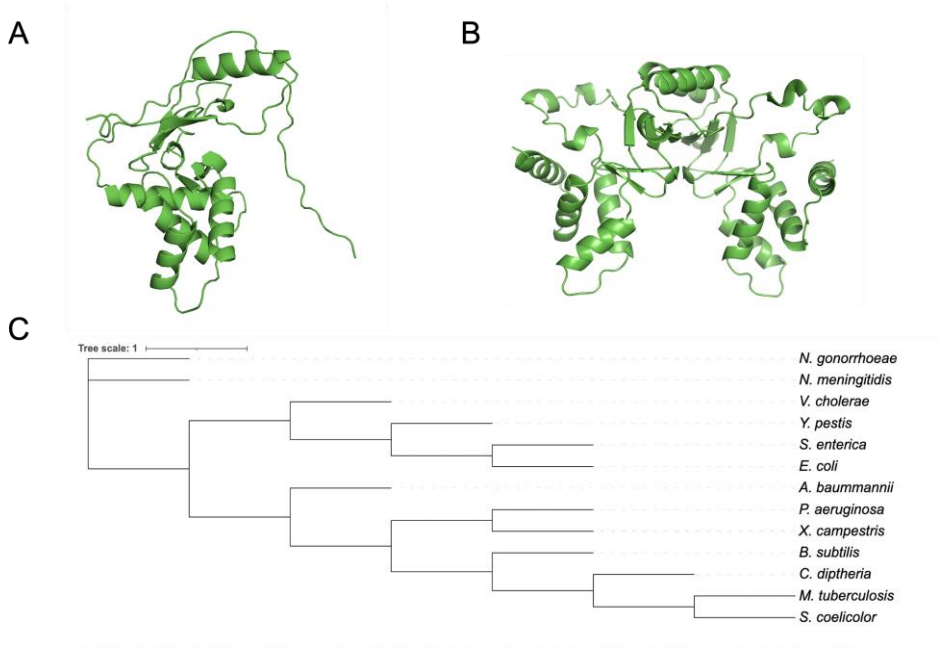

Figure 7. N. gonorrhoeae Zur is Phylogenetically Distinct from Other Characterized Zur Proteins

The homology model of gonococcal Zur from strain FA1090 was generated using the ColabFold: AlphaFold2 platform. The predicted structure of the monomer (A) and the dimer (B) is shown. The phylogenetic tree of characterized Zur proteins (C) was generated by multi-sequence alignment using Clustal Omega. The multi-sequence alignment was used to generate the phylogenetic tree with the RAxML, GAMMA WAG protein model and 1,000 bootstrap replicates.

Phylogenetic analysis revealed that NgZur and NmZur are distantly related to other characterized Zur proteins possibly due to the DECH-rich region and variation in the N- and C-termi of the proteins (Figure 7C). The phylogenetic distance based on sequence differences suggests that the role of Zur in the *Neisseriae* may be different from that in other bacteria.

B. RNA-sequencing and RT-qPCR Analyses Suggest that Zur is Not Zinc- regulated

To characterize metal-dependent regulation of *zur*, wild-type *N. gonorrhoeae* and the Zur- mutant (MCV964) were grown in the presence or absence of zinc, and RNA was isolated for RNA-sequencing and RT-qPCR. The RNA-sequencing analysis showed that the Zur regulon includes genes that are both zinc-repressed and zinc-induced (Table 5). While transcription of gonococcal *fur* is repressed by the Fur metal cofactor, iron, (207), the data presented here suggests that *zur* is regulated differently in that the gene is not zinc-repressed (Table 5). This data correlates well with the absence of a clear Zur box upstream of the *zur* gene. RNA-sequencing analysis was confirmed by RT-qPCR and showed no significant change in *zur* expression in the presence or absence of zinc (Figure 8A).

Table 5. RNA-sequencing and RT-qPCR Analysis of Zinc-dependent

Gene Identifier		L2FC (WT Zn vs. TPEN)	
Locus Tag	Gene name	RNA-seq	RT-qPCR
NGO0168	znuA	-4.96	-4.71
NGO0169	znuB	-1.96	-1.11
NGO0170	znuC	-2.04	-1.94
NGO0542	zur	ns	ns
NGO1049	ngo1049	-4.13	-4.76
NGO1442	adhP	3.42	2.08
NGO1496	tbpB	ns	ns

ns (not significant) indicates no statistical difference in expression under those conditions where significance is indicated by $P_{adj} < 0.05$ (RNA-seq) or $P < 0.05$ (RT-qPCR)

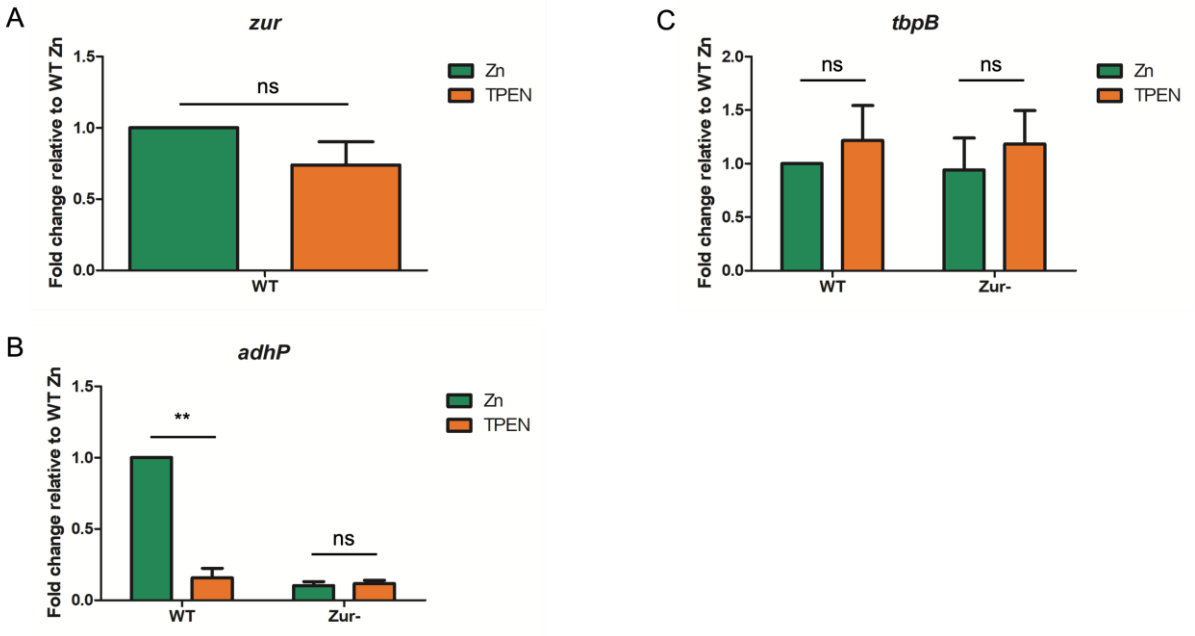

Figure 8. The Gene Encoding the Zinc Uptake Regulator (Zur) is Not Zinc Regulated.

Wild type *N. gonorrhoeae* (FA1090) and the isogenic *zur* mutant were grown in Chelex-treated defined media (CDM) in the presence of zinc (Zn) or N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN) until late log phase at which point, cells were pelleted and lysed for RNA isolation. Complementary DNA (cDNA) was made from DNAase-treated RNA and used for RT-qPCR. Expression was calculating using the $2^{\Delta CT}$ method, normalized to reduction modifiable protein (*rmpM*). Expression was calculated relative to the WT Zn condition. Depicted is expression data for *zur* (A), *adhP* (B), and *tbpB* (C). Statistical significance was calculated from the averages of three independent growth experiments using a Student's T test (ns, not significant *, $P < 0.05$, **, $P < 0.005$).

This lack of zinc-dependent regulation of *zur* may allow for constitutive expression of *zur* so that the protein can regulate genes involved in critical processes such as metabolism. Such a gene is *alcohol dehydrogenase P*, *adhP*, which was highly zinc-induced in a Zur-dependent manner (Figure 8B). As expected, *tbpB* was neither Zur- nor zinc-regulated and was used as a negative control.

C. Transcriptional Regulation of znuCBA is Both Zinc- and Zur-dependent.

After identifying the protein sequences and gene regulation patterns that result in Zur production and function, we aimed to characterize Zur-dependent regulation of the genes encoding the high affinity metal transporter, ZnuABC. ZnuABC is a zinc ABC transporter that has been shown to be Zur-repressed in a zinc-dependent manner in *N. meningitidis* (210). Thus, the genes encoding this system were expected to be similarly regulated in the closely related pathogen, *N. gonorrhoeae*. After growing the wild-type and Zur- mutant in the presence or absence of zinc, the RNA-sequencing and RT-qPCR data showed that the genes encoding this system, *znuC*, *znuB*, and *znuA* are significantly repressed in the presence of zinc in a Zur-dependent manner (Table 5, Figure 8).

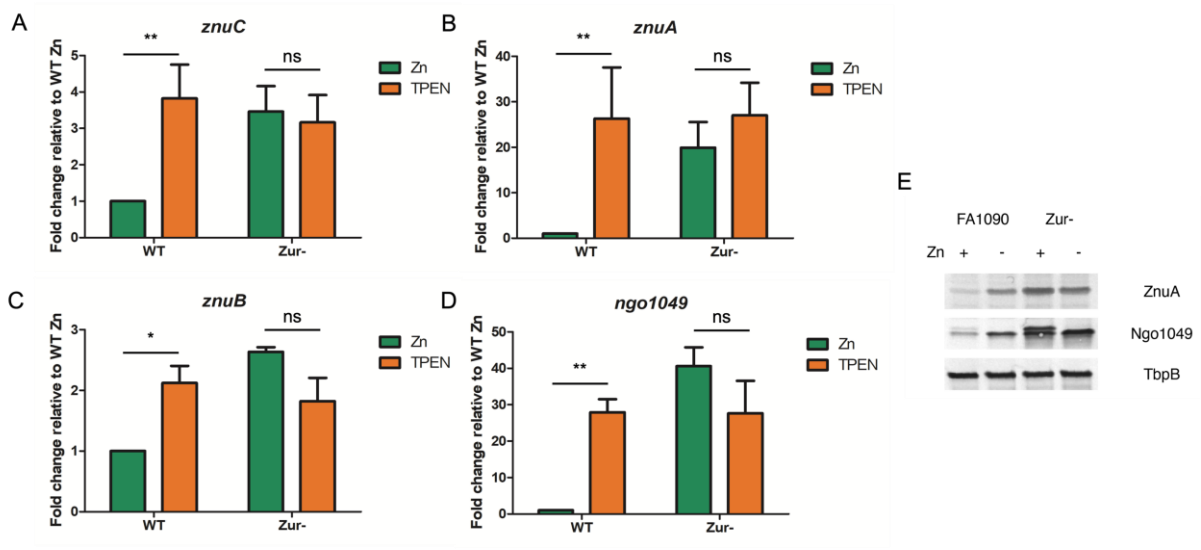

Figure 9. Transcriptional Regulation of znuCBA is Zur-dependent

Wild type *N. gonorrhoeae* strain FA1090 and the isogenic zur mutant were grown in Chelex-treated defined media (CDM) in the presence of zinc (Zn) or N,N,N′,N′-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN). Cultures were grown to late log phase then harvested for RNA isolation and western blot analysis. Complementary DNA was synthesized from DNA-treated RNA and used for RT-qPCR. Expression was calculated relative to the WT Zn condition. Depicted is expression data for *znuC* (A), *znuB* (B), *znuA* (C), and *ngo1049* (D). Statistical significance was calculated from the averages of three independent growth experiments using a Student's T test (ns, not significant *, $P < 0.05$, **, $P < 0.005$). The Western blot image shows protein expression of ZnuA (top panel) Ngo1049 (middle panel) and TbpB (bottom panel) in the presence of zinc (+) or TPEN (-) (E).

The fold-different in *znuA* regulation under differential zinc conditions (~20-40x) (Figure 9C) is much greater than that of *znuC* or *znuB* (~1.5-2.5x) (Figure 9A, 9B, and 9C), suggesting that *znuA* transcription is more sensitive to changes in zinc levels that *znuC* and *znuB*. The regulatory pattern of *znuA* is maintained at the protein level suggesting that no post-transcriptional modifications alter ZnuA protein levels (Figure 9E). Interestingly, Zur also represses a gene which encodes a protein of unknown function, *ngo1049* (Figure 9D and 9E), in a zinc-dependent manner. Zinc-dependent repression of *ngo1049* is comparable in fold-change to that of *znuA* (Figure 9B and 9D) suggesting that this protein may play a role in acclimation to differential zinc conditions.

Considering the critical role of ZnuA in zinc uptake in the presence of PS (179), it was of interest to determine the level of transcription of the gene encoding this protein relative to other genes within the *znuCBA* operon. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of each gene in the *znuCBA* operon was quantified by RNA-sequencing. FPKM data showed that in the absence of zinc, *znuC* is transcribed at much higher levels than *znuB.* However, transcript levels of *znuA* are much higher levels that of *znuC* (Figure 10A), giving the following transcriptional profile of the operon – *znuB* < *znuC* < *znuA.*

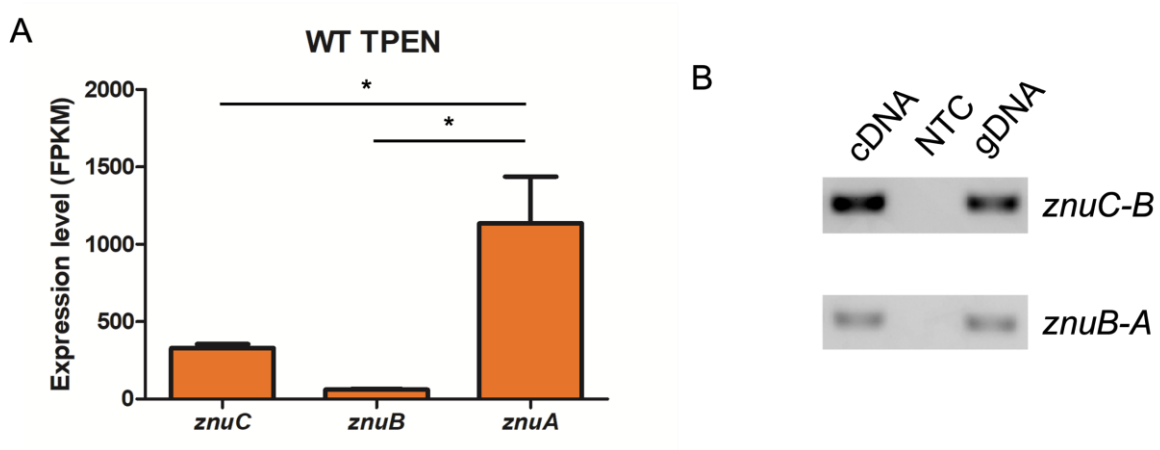

Figure 10. The znuA Gene is the Most Expressed Gene in the znuCBA Operon

N. gonorrhoeae strain FA1090 was grown in Chele**highly** x-treated Defined Media (CDM). Cultures were grown to late log phase in the presence of N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN). Cultures were pelleted and RNA was isolated fro for RNA sequencing by Novogene (A) and RT-PCR (B). Expression data shows average expression levels of *znuC*, *-B*, and *-A* as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) from 3 independent experiments. RT-PCR data shown is representative of there independent growth experiments. Statistical significance was determined by 1-way ANOVA with Bonferoni post-test.

*, $P < 0.05$.

These data suggest that a single transcript of *znuA* separate from the upstream genes is made which results in multiple ZnuA proteins service fewer ZnuB and ZnuC proteins and further highlights the importance of ZnuA under zinc limited conditions. However, RT-PCR data showing transcription of the intergenic regions between each gene in the operon demonstrates that an RNA message containing the *znuC*, *znuB*, and *znuA* co-transcript is also synthesized (Figure 10B). Together this data shows *znuA* is transcribed at high levels in response to zinc limitation being transcribed in two RNA messages, a polycistronic message and a single message. This evidence highlights the gonococcal emphasis on Zur- and zinc-dependent *znuA* transcription when faced with various zinc conditions.

D. ZnuA is Mn-repressed in 1291 but Not FA1090

To identify whether Zur regulates *znuCBA* in a manganese-dependent manner, wild-type FA1090 and the Zur- mutant were grown in the presence or absence of Mn and whole cell lysates were analyze for ZnuA protein production by western blotting. Unexpectedly, western blot data showed that ZnuA was not manganese regulated in a Zur-dependent manner (Figure 11).

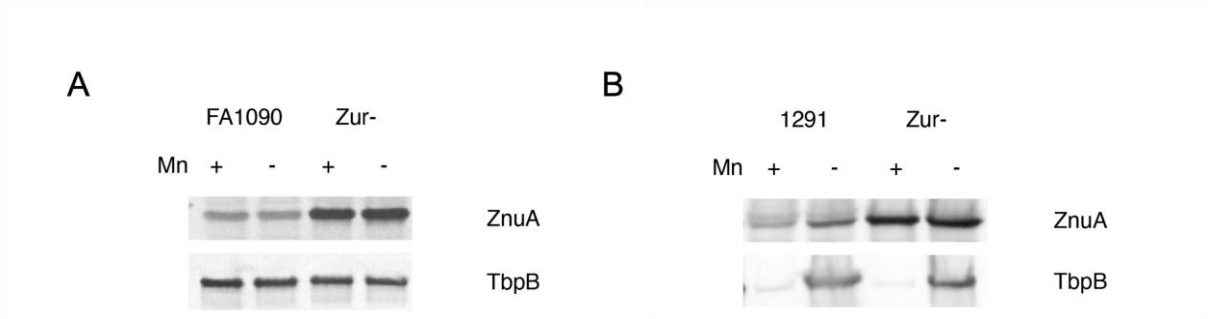

Figure 11. ZnuA Protein Levels are Manganese-repressed in FA1090 but Not 1291

Wild types *N. gonorrhoeae* (FA1090) and 1291 and the *zur* mutant of each strain were grown in Chelex-treated defined media (CDM) in the presence of $MnCl_2$ (Mn) or in CDM only to represent the manganese-deplete condition. Culture were grown until late log phase at which point, cells cultures were standardized to 100,000 KU to generate whole cell lysates for western blotting. The western blot image depictes ZnuA and TbpB (negative control) protein levels in N. gonorrhoeae grown in the presence (+) or absence (-) of Mn in strain FA1090 (A) and 1291 (B).

This data was contradictory to that in the literature demonstrating the *znuCBA* was manganese regulated in a Zur-dependent manner. However, this transcriptional pattern was demonstrated in strain 1291, leading to the hypothesis that strain differences account for differential manganese-dependent regulation of *znuCBA.* Western blot data showed that ZnuA production is manganese-dependent in strain 1291 and that this difference is no longer appreciable in the Zur- mutant, supporting the hypothesis that there are strain-dependent differences in manganese-dependent regulation by Zur. Interestingly, TbpB is manganese-repressed in strain 1291 in a Zur-independent manner, further supporting this hypothesis. Taken together this work demonstrates the Zur is a zinc- and manganese-dependent regulator. However, the metal specificity of Zur is strain dependent.

III. Discussion

The pathogen that causes gonorrhea, *Neisseria gonorrhoeae,* is both a public health and economic threat. The rapid increase of antimicrobial resistance genes in the gonococcal population has caused the number of effective treatments against the pathogen to decline rapidly. To address the very real threat of untreatable gonorrhea, novel therapeutics and effective vaccines need to be developed. A promising strategy of combating *N. gonorrhoeae* is termed “starve and kill” and aims to prevent *N. gonorrhoeae* from using host metal sequestration proteins as metal nutrient sources to kill the pathogen. Maurakis et al showed that mutagenesis of the gonococcal metal transporters TdfJ and ZnuA prevents *N. gonorrheae* from utilizing the host zinc sequestration protein, PS, a vital zinc source at the site of infection (264). The ZnuA- mutant is deficient for invasion of and survival within primary human cervical epithelial (pex) cells and forms thinner biofilms than the wild-type (205). ZnuA is also

responsible for importing manganese which directly quenches reactive oxygen species (ROS). This function is particularly vital in the context of neutrophil infection where ROS is high. Thus, ZnuA is a promising target for therapeutic development.

The genes encoding ZnuABC are regulated by the zinc uptake regulator, Zur, which derepresses the gene in the absence of zinc and represses the gene in the presence of zinc. These genes have also been shown to be manganese-regulated in a Zur-dependent manner. However the mechanism through which Zur regulates these genes in a zinc and manganese-dependent manner had not been elucidated. Therefore, we hypothesized that specific structural and functional elements of Zur are required for zinc- and manganese-dependnent regulation. Understanding the mechanism by which and the specific conditions under which Zur regulates *znuCBA* in *N. gonorrhoeae* is critical to evaluating its therapeutic potential.

This work aimed to characterize the mechanism through which Zur regulation occurs by (1) comparing NgZur to characterized Zur proteins, (2) characterizing regulation of *zur* itself, and (3) characterizing zinc- and manganese-dependent regulation of Zur-regulated genes. We used Zur sequence analysis and homology modeling, RNA-sequencing, and RT-PCR to identify the following hypothetical model of the mechanism of Zur regulation. Under zinc limited conditions, the CXXC site of the Zur monomer is occupied by zinc to maintain Zur structure, and that in the absence of zinc, the HCHE regulatory site of the Zur monomer is unoccupied, resulting in an inactive Zur conformation and de-repression of Zur-regulated genes. This condition is met at the site of infection where CP and PS sequester zinc and create a zinc-limited environment for the gonococcus. Conversely, in the presence of zinc, both the CXXC and the HCHE regulatory sites, which are within the LBD of Zur, are occupied by zinc resulting in

dimerization of monomers at the DD. The dimerized Zur protein thus forms an active conformation capable of repressing *znuCBA* and *ngo1049*, and activating *adhP.* While differential zinc conditions alter expression of Zur-regulated genes, it does not alter expression of the *zur* gene itself, resulting in a steady level of *zur* transcription potentially so that the protein which it encodes is always produced and ready to alter gene transcription when zinc levels are altered. However, this mechanism is strain-specific. This was confirmed by western blot data which showed that ZnuA protein levels change with the addition of manganese in strain 1291 but not FA1090. Similar metal-related strain differences have been previously reported. In contrast to gonococcal strain FA19, strain FA1090 does not encode a functional LF binding protein, Lbp, system which allows *N. gonorrhoeae* to utilize iron bound to LF (188, 350). TraD which is encoded by the gonococcal genetic island (GGI) in *N. gonorrhoeae,* is iron-, zinc-, and copper-regulated (351); however, the GGI itself is present in FA19 and MS11 but not FA1090 and F62 (21). The gene encoding the manganese exporter, MntX, is frame-shift mutated in ~66% of sequenced gonococcal strains (243), being found present in full-length in FA1090 but not FA19. Differences in metal-dependent regulation by Zur can be added to this list of metal-related gonococcal strain differences.

Future studies will include crystallization and mutagenesis of NgZur in complex with DNA to confirm key residues involved in zinc- and DNA binding and protein dimerization. Further studies will also identify structural and functional elements of Zur that allow for manganese-dependent regulation in strain 1291. These future directions will give insight into the manganese-dependent transcriptional response to manganese limitation *N. gonorrhoeae*.

At first glance, the ability of Zur to turn critical genes “on” or “off,” makes it seem like a promising target for a small-molecule inhibitor. It is conceivable that locking Zur in the DNA-binding conformation with a small-molecule inhibitor could turn ZnuA “off” preventing *N. gonorrheoae* from acquiring zinc from PS and thus starving and killing the pathogen. However, we show here that Zur can also activate *adhP* in the active conformation. Thus, it is also conceivable that the same small-molecule modifier could have unpredictable and undesirable off-target effects. Off-target effects of altering a global regulator have been demonstrated in MtrR, the MtrCDE efflux pump regulator. In the wild-ype, MtrR represses the MtrCDE efflux pump, preventing antimicrobials from being extruded from the cell. However, mutations in MtrR that lock the regulator in the inactive conformation cause over expression of MtrCDE, increased antimicrobial efflux, and therefore increased antimicrobial resistance (352). Mutations within the promoter regions of MtrR-repressed genes (i.e. *mtrR* and *mtrCDE)* results in similar impacts on gene expression (353). MtrR is also a global regulator, indirectly regulating genes involved in lactate utilization (*lctP*) (354) and glutamate biosynthesis (glnA)(355). Therefore, off-target effects of modifying Zur with a small-molecule inhibitor or activator need to be considered prior to pursuing Zur as a therapeutic target.

Zur may be better considered as a tool for identifying zinc-regulated virulence factors such as ZnuA that are expressed and critical to survival. A small molecule inhibitor of *Salmonella* ZnuA has been identified from a chemical library of 36 zinc-binding compounds generated by Robert Di Santo et al. (233). This di-aryl pyrrole hydroxamic acid small molecule inhibitor limited growth and intracellular survival of *Salmonella enterica* serovar Typhimurium (233), demonstrating the antimicrobial activity of a ZnuA inhibitor.

The design of such a therapeutic would be informed by the mechanism described in this work. While Zur has not been shown to have a role in survival and virulence nor is the Zur- mutant growth deficient *in vitro* (data not shown), data showing the *S. enterica* Zur mutant as less infectious in a mouse model suggests that NgZur could potentially be involved in survival and virulence in a model of gonococcal infection.

CHAPTER 5: THE MANGANESE TRANSPORTER, MNTX, MAINTAINS MANGANESE HOMEOSTASIS IN *NEISSERIA GONORRHOEAE*

I. Introduction

Pathogens and host require transition metals such as zinc, manganese, and copper to support biological functions such as modulation of gene expression, metabolism, enzymatic processing and structural maintenance of proteins. Because these metals are highly insoluble and volatile in their free state, the host goes to great lengths to stabilize these metals by keeping them bound to proteins. For example, free iron contributes to the Fenton chemistry, which generates damaging hydroxyl radicals (140). Human TF and LF bind iron, thus solubilizing and buffering the host iron pool. Other examples of metal binding proteins in the host include which binds zinc and manganese and PS which binds zinc only.

In the context of infection, the binding of host proteins to transition metals in effect removes those metals from the bacterial nutrient supply. The process through which host proteins sequester metals from the bacterial nutrient supply is termed nutritional immunity. Nutritional immunity in the context of infection by the human-restricted pathogen, *N. gonorrhoeae*, has been thoroughly investigated and reviewed (177, 178). For example, CP can create a zinc and manganese limited environment (198). However, Kammerman et al. showed that *N. gonorrhoeae* can use CP directly as a sole zinc source which allows the pathogen to grow in the face of nutritional immunity (198). The ability of CP to limit manganese and

subsequently bacterial growth has not but demonstrated in *N. gonorrhoeae* but has been demonstrated in other bacteria such as *Staphylococcus aureus* (356).

Conversely, the host can flood the bacterial nutrient supply with high amounts of metals resulting in reactive oxygen species turnover, protein mismetallation, and dysregulated gene expression in bacteria. The mechanism through which the host overloads the pathogen nutrient supply with metals is referred to as metal intoxication (210). An example of a gonococcal infection site in which the host can potentially overload the gonococcus with metal is the seminal fluid which is high in zinc (259). Additionally, the host can divert zinc to an *E. coli*-infected vacuole of a macrophage, suggesting this is a site of metal overload as well.

Manganese can be altered in the phagosome of macrophages through expression of the manganese transporter, natural resistance-associated macrophage protein 1 (NRAMP1), (357) on the phagosomal membrane (358). Unfortunately, there is controversy in the literature regarding the direction of NRAMP1 transport (359). However, it is feasible that dependent upon the direction of transport, NRAMP1 can generate a manganese intoxicating or manganese limiting environment within macrophages.

In *N. gonorrhoeae*, manganese is an important transition metal involved in oxidative stress resistance (211). Instead of utilizing a manganese-dependent superoxide dismutase (SOD) (209), *N. gonorrhoeae* uses manganese directly as a quencher of reactive oxygen species (ROS) (215). Manganese-dependent resistance to oxidative stress depends upon the manganese ABC transporter, ZnuABC. In the absence of ZnuA, *N. gonorrhoeae* is unable to import manganese and resist oxidative killing (209). The genes encoding this system are

regulated by the zinc uptake regulator, Zur, where regulation is manganese dependent (211) in a strain-specific manner (this study).

Too much manganese can have deleterious effects on the pathogen as well. *N. gonorrhoeae* lacking the putative manganese exporter, MntX, is more sensitive to high manganese concentrations than *N. meningitidis* expressing the homologous protein. However, only 34% of analyzed gonococcal strains encode a full-length MntX (243), suggesting that some strains may be more resistant to manganese overload than others.

The ability of *N. gonorrhoeae* to overcome manganese overload and maintain manganese homeostasis through expression of MntX has not been thoroughly investigated. Therefore, we hypothesized that resistance to manganese overload and maintenance of manganese homeostasis in *N. gonorrhoeae* is strain-specific and dependent upon the putative MntX. Growth assays and protein homology modeling was used to gain insight into the role of MntX in manganese homeostasis in *N. gonorrhoeae* strains FA1090 and FA19. This work demonstrates that gonococcal strains are differentially adapted to maintain different internal metal levels although they occupy the same niche and suggests that different strains have different metal requirements for survival and virulence in the human host.

II. Results

A. The Wild-type Manganese Transport Gene, mntX, is Present Select Strains of Neisseria gonorrhoeae

To elucidate the mechanism through which FA1090 is protected from manganese intoxication, we searched the FA1090 genome for a putative manganese efflux protein, MntX,

which has been characterized in the closely related pathogen, *N. meningitidis*. Sequence analysis revealed that the FA1090 gene with locus tag NGO_1768 encodes a protein that is 93% identical to meningococcal, MntX (Figure 12). The homology model of this protein shows that it is made up of 6 transmembrane alpha helices (Figure 12B) suggesting localization in the membrane. The gene which encodes this protein is frame-shift mutated in strains FA19 and 1291 (Figure 12A), suggesting that MntX may play a role in resistance to manganese intoxication in FA1090 but not in FA19 and 1291. Sequence analysis of 159 gonococcal strains isolated between 2003 and 2022 demonstrated that 80% of these contemporarily isolated strains do not encode a full-length MntX, suggesting that many strains are sensitive to manganese overload.

B. Sensitivity of N. gonorrhoeae to Manganese Overload is Strain-specific

To identify whether manganese accumulation differed between gonococcal strains, we grew strains FA1090, the genome of which encodes full-length MntX, and FA19, the genome of which encodes a truncated MntX, in the chelex-treated media (CDM) in presence or absence of manganese. ICP analysis showed that strain FA19 accumulates more internal manganese than FA1090 and that this difference is independent of other metals such as iron and zinc, demonstrating specificity (Figure 13).

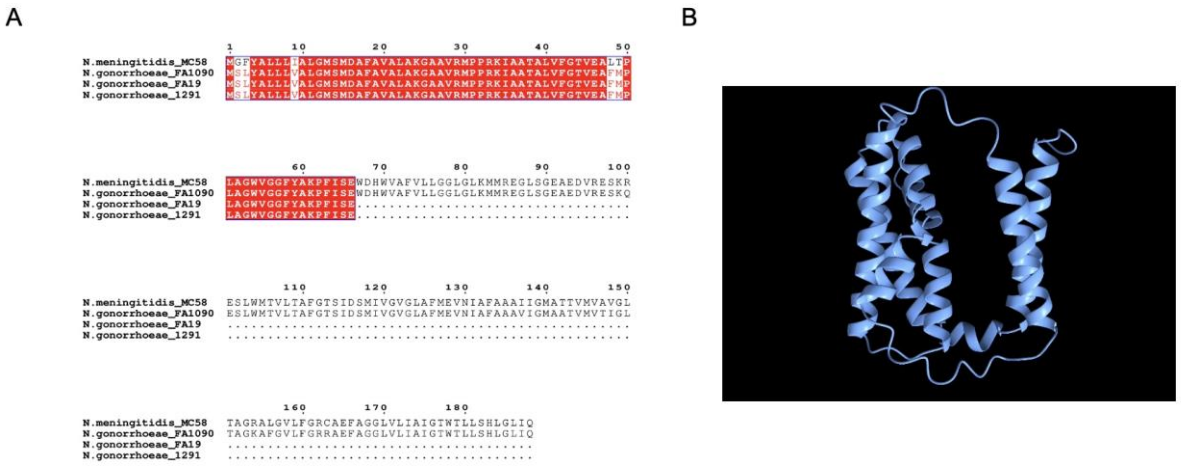

Figure 12. The N. gonorrhoeae Strain FA1090 Genome Encodes and MntX Protein Similar to that in N. meningitidis

The multi-sequence alignment of NgMntX with NmMntX was generated using Clustal Omega and imported into ESpirit 3.0 for visualization (A). Red filled boxes indicates identical residues. Red text indicates similar residues, and blue open boxes indicate groups of similar residues. The homology model of MntX from *N. gonorrhoeae* strain FA1090 was generated in AlphaFold (B).

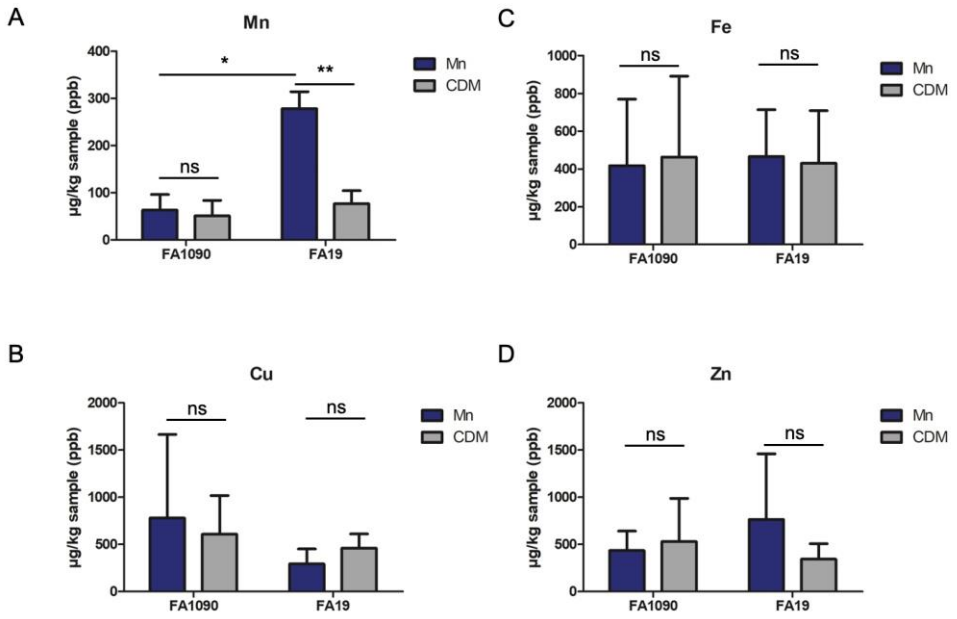

Figure 13. Manganese Levels Fluctuate in FA1090 but Not FA19

N. gonorrhoeae strains FA1090 (MntX+) and FA19 (MntX-) were passaged on GCB plates containing 7 µM EDTA grown in Chelex-treated defined media (CDM). Once cultures reached early log phase, cultures were diluted by half, and manganese was added to the manganese-replete condition (Mn). Manganese was excluded from CDM for the manganese-deplete condition (CDM). Cultures were washed with Chelex-treated HEPES and pelleted prior to inductively coupled plasma mass spectrometry (ICP-MS). Data shows µg manganese (A), copper (B), iron (C), and zinc (D) per kg pellet weight.

To identify whether manganese accumulation correlated with manganese sensitivity, FA1090 and FA19 were grown in CDM at increasing manganese concentration. As manganese concentrations increase, FA19 and the isogenic Zur- mutant experiences a growth deficiency while growth of FA1090 and the isogenic Zur- mutant remains unaffected (Figure 14A-D). This data demonstrates that FA19 experiences manganese overload and suggests that different gonococcal strains maintain different internal metal pools.

C. ZnuA and Zur are Involved in Maintaining Internal Manganese Levels

In *N. gonorrhoeae*, the Zur-repressed ABC transporter, ZnuABC, is responsible for the high affinity transport of manganese (205). Therefore, I hypothesized that the ZnuA- mutant would be less sensitive to manganese overload because this mutant lacks the ability to transport toxic levels of manganese. Growth of the ZnuA- mutant in the presence of high manganese concentrations is statistically significantly different from growth in the absence of manganese (Figure 14E and F), suggesting that manganese sensitivity is ZnuA-dependent. Because Zur represses *znuA* in a manganese-dependent manner (211), I hypothesized that, the Zur- mutant would be more sensitive to manganese intoxication than the wild type. When grown in the presence of intoxicating manganese concentrations, the Zur- mutant was more manganese-sensitive than the wild type (Figure 14D and F), suggesting a role for Zur-dependent regulation of internal manganese homeostasis.

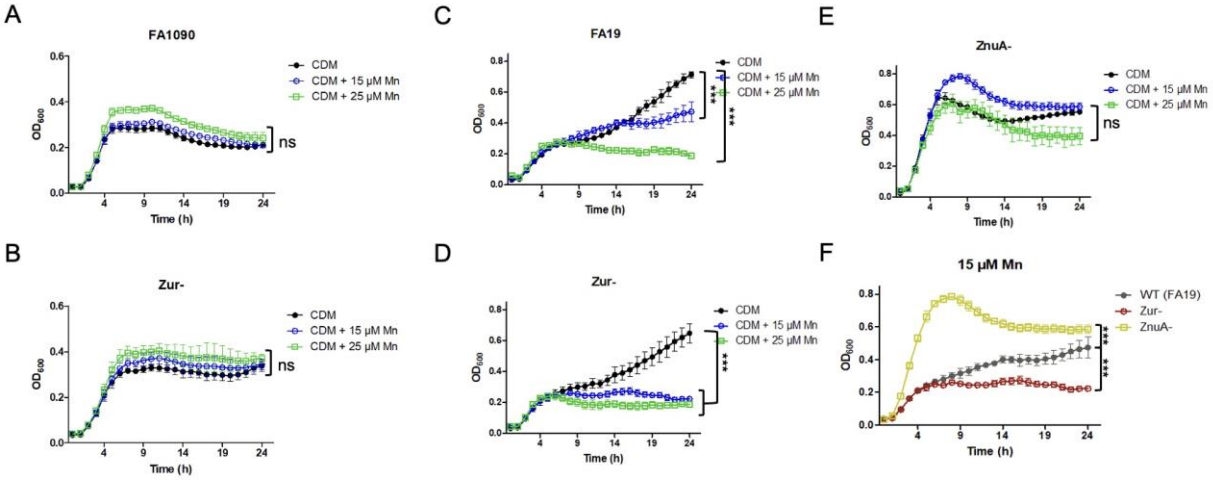

Figure 14. Manganese Overload is Strain-specific and ZnuA- and Zur-dependent

N. gonorrhoeae strains FA1090 (A), the FA1090 Zur- mutant (MCV964) (B), FA19 (C), the FA19 Zur- mutant (MCV963) (D), and the FA19 ZnuA- mutant (E) were passaged onto GCB containing 25µM $ZnSO_4$ then grown in Chelex-treated CDM with the following supplements, 5 µM $ZnSO_4$, 5 mM D-mannitol, 7.5 µM 30% saturate human transferrin, and Kelloggs's supplement 1 with or without the addition of 15 µM or 25 µM $MnCl_2$. Cultures were incubated for 24 hours under standard conditions. Growth of FA19 and the Zur- and ZnuA- mutants grown under the above conditions and 15 µM $MnCl_2$ is replotted in F. Statistical significance was calculated by two-way ANOVA with Bonferoni post-test.

***, $P < 0.001$.

III. Discussion

N. gonorrhoeae utilizes transition metals in various processes such as macromolecule biosynthesis (310, 312), regulation (211), and oxidative and nitrosative stress (209, 319). The metal nutrients required for these processes must be acquired from a sole source the human host, as *N. gonorrhoeae* is a human-restricted pathogen. Host innate immunity proteins bind transition metals, effectively sequestering them away from the bacteria in a process termed nutritional immunity. This metal sequestration by host nutritional immunity proteins can inhibit growth, survival, and pathogenesis of *N. gonorrhoeae* (179, 187, 198, 344). Conversely, the host can overload the pathogen with metals through a much less appreciated mechanism termed metal intoxication. Although little evidence demonstrates metal intoxication in *N. gonorrhoeae*, evidence for this innate immune mechanism has been demonstrated in the context of macrophage infection by *Escherichia coli.* Macrophages infected with *Escherichia coli*, upregulated copper transporter 1 (CTR1) and re-localize the transporter to the phagosomal membrane to mediate copper-induced killing of the bacteria (334). *N. gonorrhoeae* expressed a functional copper export protein, CopA (242). However, the conditions under which copper intoxication occurs in the context of gonococcal infection has not been investigated. In addition to copper-mediated killing mechanisms, macrophages utilize zinc-mediated killing mechanisms. Specifically, infected microphages divert zinc to *E. coli*-containing intracellular vesicles (360). In a mouse model of septicemia, *N. meningitidis* lacking the manganese efflux protein, MntX, exhibited reduced survival relative to the MntX-expressing strain, suggesting that the host overloads the pathogen with manganese in the blood (243).

Thus, the aim of this work was to identify the role of the putative manganese transporter, MntX, in *N. gonorrhoeae*. However, the genomes of only a subset of gonococcal strains encode the full-length MntX protein. Therefore, I hypothesized that resistance to manganese intoxication requires the manganese exporter, MntX, and that this resistance is strain-specific. Growth curve analysis suggests that in strain FA1090, the full-length MntX protein helps to maintain steady internal manganese levels, and this manganese does not impact growth. Conversely, in strain FA19, MntX is not produced, and manganese accumulates in the cytoplasm at higher levels and leads to manganese intoxication. In *N. meningitidis*, the deleterious effect of excess manganese can be attributed in part to the overlap in the iron and manganese regulons. The ferric uptake regulator, Fur, can bind manganese in addition to iron and can repress iron import genes in the presence of manganese (213). Consequently, under iron-limited conditions, mis-metallation of Fur by manganese can result in repression of iron import genes, leading to iron starvation when manganese is high. In the context of this study, the genome of both strains FA1090 and FA19 encode the iron importer, TbpAB. However, *tbpAB* is manganese repressed only in strain FA19, while the operon is not manganese regulated in strain FA1090. Therefore, I submit the following hypothetical model of manganese homeostasis in *N. gonorrhoeae*. During iron limitation, manganese-resistant strains (i.e. FA1090), resist Fur mis-metalation by removing manganese from the cytoplasm via MntX. This prevents repression of iron import genes and subsequent iron starvation. Conversely, in strains lacking MntX (i.e. FA19), manganese is able to accumulate in the cytoplasm allowing it to mis-metallate Fur and repress iron import genes. This results in iron starvation under manganese intoxicating conditions. Future directions will test this model by growing FA19 in the presence

or absence of free iron or human TF and intoxicating manganese. Hypothetically, the supplementation of FA19 with free iron would properly metalate iron-binding proteins so that when manganese is added, the intoxicating effects of protein mis-metalation are mitigated and MntX is no longer essential.

This study provides evidence for the existence of a novel manganese exporter, MntX, in *N. gonorrhoeae* and broadens our current understanding of gonococcal pathogenesis and the response to metal alterations in the host. This study also supports the exploitation of manganese overload as a novel therapeutic strategy.

CHAPTER 6: ZINC-DEPENDENT REGULATION AND CARBON UTILIZATION OVERLAP IN *N. GONORRHOEAE*

I. Introduction

Neisseria gonorrhoeae causes the sexually transmitted infection, gonorrhea. Gonorrhea manifests as two major clinical presentations, those being urogenital infection and disseminated infection. Urogenital infection is the result of gonococcal invasion of mucosal epithelial cells and PMNs while disseminated infection is the result of infection ascension to the upper reproductive tract and to the blood stream where it is carried to disseminated niches such as the synovial joints, and in cases of severe infection, endocardial cells and the meninges.

As a fastidious, obligate human pathogen, *N. gonorrhoeae* must acquire micronutrients for survival and virulence from its human host. Such nutrients include 5% CO_2 (5, 6), oxaloacetate, hypoxanthine, uracil, B-vitamins, metals like magnesium, iron, zinc, and amino acids among others (179, 187, 198, 201, 214, 222, 344, 361). This bacterium is also a metabolic specialist, growing only in the presence of glucose, lactate, and pyruvate (7-9). Unless cultured in the laboratory under defined conditions, *N. gonorrhoeae* must acquire these micronutrients from the sole source, the human host. The pathogen is further restricted to specific host-cell niches that provide these micronutrients such as that within PMNs, mucosal epithelial cells, and disseminated niches. Each niche must supply the gonococcus with a highly specific set of nutrients. The epithelial surface which *N. gonorrhoeae* infects can be quite dynamic in nutrient availability. Epithelial cells are rich in PS (264) which sequesters zinc from the pathogen's

nutrient supply. Additionally, the influx of PMNs to the site of infection contain CP which sequesters both zinc and manganese. Not only does the presence of PMNs impact zinc and manganese availability, but it also impacts sugar availability. In the case of gonococcal infection of PMNs, Potter et al. showed that a subset of genes involved in glycolysis were predicted to be non-essential when *N. gonorrhoeae* was grown in the presence of PMNs. This suggests that PMNs provide a carbon source for *N. gonorrhoeae* other than glucose that allows the pathogen to bypass glycolysis. Instead, *N. gonorrhoeae* ferments lactate produced by PMNs instead of relying on the glycolytic pathway (362). Interestingly, Pawlik et al showed that genes related to metabolism and protein synthesis such as alcohol dehydrogenase P (*adhP*), NADPH-dependent 7-cyano-7-deazaguanine reductase (*queF*), and 7-cyano-7-deazaguanine reductase (*queC*) are zinc-regulated in the closely-related pathogen *Neisseria meningitidis*. Data from Potter et al. and Pawlik et al. together suggest that the zinc availability may impact metabolism. While *N. gonorrhoeae* is sure to face a complex milieu of nutrients like sugars and metals in the host, only few have investigated the interplay between micronutrients. Further, none have investigated the impact of metal availability on carbon utilization. Thus, I hypothesized that carbon metabolism genes are differentially regulated by zinc in *Neisseria gonorrhoeae*. RNA-sequencing data showed that genes involved in glycolysis and pyruvate metabolism are significantly differentially regulated by zinc, suggesting that the zinc and carbon metabolism transcriptomes overlap in *Neisseria gonorrhoeae.* Understanding the link between metal homeostasis and essential carbon metabolism pathways within different host cell niches could give new insights into gonococcal pathogenesis.

II. Results

A. Over 200 Genes are Differentially Regulated by Zinc in N. gonorrhoeae

To identify zinc dependent regulation of carbon metabolism genes, *N. gonorrhoeae* was grown in chelex-treated defined media in the presence of zinc or the zinc chelator, N,N,N,',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN). RNA-sequencing analysis showed that zinc alters the expression of over 200 genes in *N. gonorrhoeae*. Of the over 200 significantly differentially regulated genes, 89 genes were zinc-induced and 131 genes were zinc-repressed (Figure 15).

B. Differentially Expressed Genes are Involved in Pyruvate Metabolism

The Kyoto Encylcopedia of Genes and Genome (KEGG) analysis showed that 7 of those significantly zinc-regulated genes are involved in pyruvate metabolism (Figure 16). The gene encoding the L-lactate dehydrogenase, *lldD*, is significantly zinc-repressed (Table 6). LldD was shown to be required for growth in PMNs and in primary cervical epithelial cells under microaerobic conditions (363). The gene encoding the D-lactate dehydrogenase, *ldhA*, was also significantly zinc-repressed (Table 6). LdhA was shown to be required for growth in polymorphonuclear leukocytes but not in primary cervical epithelial cells under either aerobic or microaerobic conditions. Interestingly, phosphate acetyltransferase (*pta*) and dihydrolipoamide dehydrogenase (*dldH*) were both zinc-repressed (Table 6).

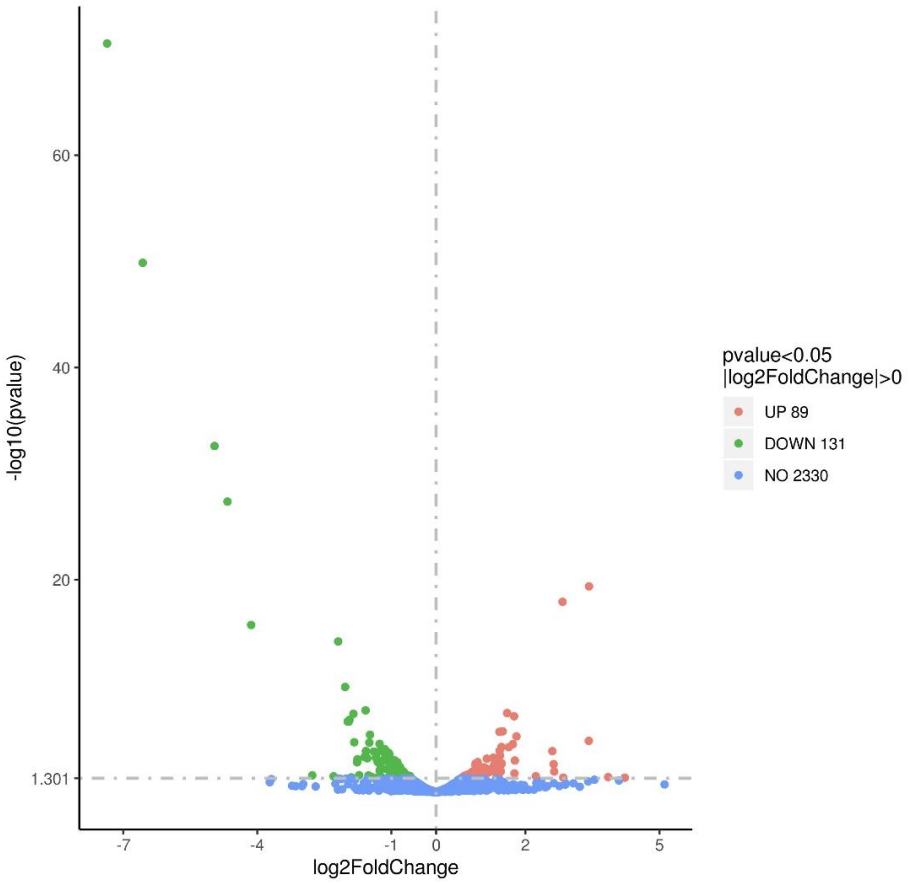

Figure 15. Over 200 genes are Differentially Zinc-regulated

N. gonorrhoeae strain FA1090 was grown in chelex-treated defined media (CDM) in the presence of 20 µM $ZnSO_4$ or 7µM N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN) to late log phase at which point cells were pelleted and RNA was isolated for RNA-sequencing by Novogene. Plotted is the Log2FoldChange of each significantly zinc-regulated gene by the –log10(pvalue) under these conditions. Gene dots indicate downregulated (zinc-pressed) genes and red dots indicate upregulated (zinc-induced) genes. Blue dots indicate non-differentially regulated genes that fall below the significance threshold (horizontal dashed line). The foldchange threshold is indicated by a vertical dashed line.

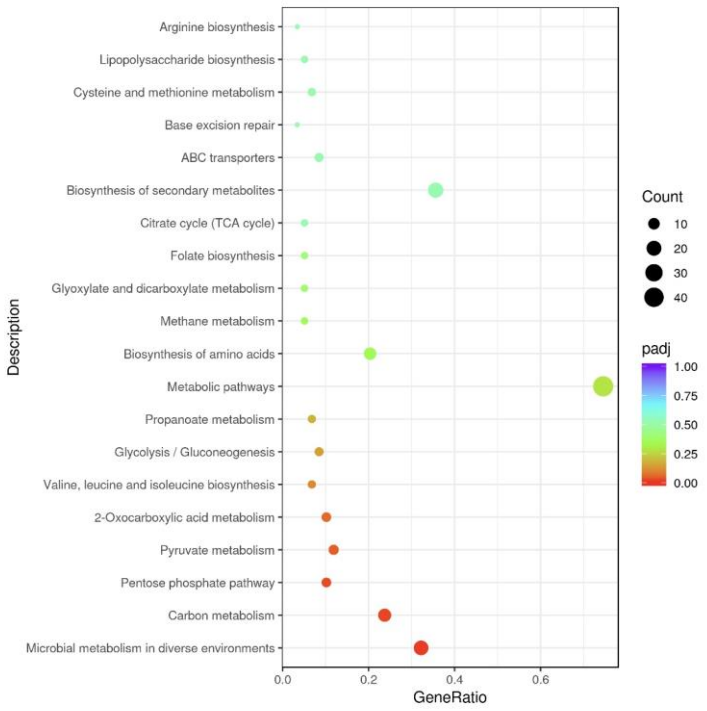

Figure 16. Significantly Zinc-regulated Genes are Involved in Pyruvate Metabolism.

Wild-type strain FA1090 and the Zur- isogenic mutant of *N. gonorrhoeae* were grown in chelex-treated media (CDM) in the presence (20 µM $ZnSO_4$) or absence (7 µM TPEN) of zinc and pelleted for RNA isolation. RNA was submitted to Novogene for RNA-sequencing and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. “Gene ratio” is the percentage of significantly differentially expressed genes in the given Gene Ontology (GO) term. The size of the dots indicate the number of genes that were enriched in that biological process. The color indicates heat mapped significance values.

Table 6. Differential Expression of Pyruvate Metabolism Genes is Zinc-dependent Regulation

Locus Tag	Gene name	L2FC (WT Zn vs. TPEN)	Gene description
NGO0916	lpdA	1.41	dihydrolipoamide succinyltransferase
NGO0639	lldD	-0.8	lactate dehydrogenase
NGO0848	lueA	-0.76	2-isopropylmalate synthase
NGO1336	ldhD	-0.74	lactate dehydrogenase
NGO0214	pta	-0.72	phosphate acetyltransferase
NGO1521	ackA	1.35	acetate kinase
NGO0925	dldH	-0.55	dihydrolipoamide dehydrogenase

ns (not significant) indicates no statistical difference in expression under those conditions

where significance is indicated by $P < 0.05$ determined by RNA-sequencing

C. Genes Involved in Pyruvate Metabolism are Zinc Regulated in a Zur-independent Manner

Transcript quantification data expressed as fragments per kilobase of transcript per million mapped reads (FPKM) demonstrated that zinc-dependent regulation of these genes was independent of the zinc uptake regulator, Zur, (Figure 17). This was shown by zinc-dependent regulation of each gene in the Zur- mutant. Zur-independent regulation of pyruvate metabolism suggests that zinc-dependent regulation can occur through multiple mechanisms in *N. gonorrhoeae*.

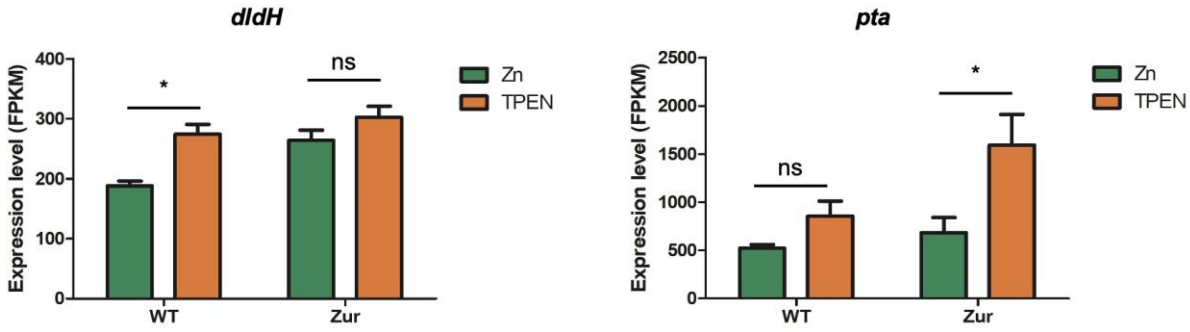

Figure 17. Pyruvate Metabolism Genes are Zn and Zur Regulated

N. gonorhoeae strain FA1090 and the Zur- isogenic mutant were grown in chelex-treated defined media (CDM) in the presence or absence of 20 µM $ZnSO_4$ or 7µM N,N,N′,N′-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN). At late log phase, cultures were pelleted for RNA isolation and RNA-sequencing. Expression of *dldH* (A) and *pta* (B) is shown as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) in the presence (green) and absence (orange) of Zn. Statistical significance was calculated from the averages of three independent growth experiments using a Student's T test (ns, not significant *, $P < 0.05$).

III. Discussion

The human-restricted pathogen, *N. gonorrhoeae*, infects mucosal surfaces, polymorphonuclear leukocytes, and disseminated niches such as the blood and synovial joints. Each location must supply micronutrients including carbon sources and metals. PMNs are a host-cell niche of particular interest because *N. gonorrhoeae* survives the hostile phagosomes within these cells (364-367). These cells are hostile because they exhibit oxidative and non-oxidative antimicrobial activity and contain high levels of the nutritional immunity protein, CP, which limits the zinc and manganese nutrient supply (261). However, *N. gonorrhoeae* can utilize PMNs as a lactate source and the CP released by these cells as a zinc source (198, 362). However, the transcriptional response to this dynamic nutrient environment has not been characterize. Thus, I hypothesized that the carbon- and zinc-regulated transcriptomes overlap in *N. gonorrhoeae*. To test this hypothesis, RNA-sequencing was conducted using samples collected from *N. gonorrhoeae* grown in the presence and absence of zinc.

RNA-sequencing analysis demonstrated that over 200 genes are differentially regulated by zinc and that 7 of those genes are involved in pyruvate metabolism. Phosphate acetyltransferase, *pta*, and dihydrolipoamide dehydrogenase, *dldH*, were significantly zinc repressed. DldH is a part of the pyruvate dehydrogenase complex which oxidizes pyruvate to give acetyl-CoA. Acetyl-CoA is then converted into acetyl phosphate by Pta leading to acetate production. Interestingly, these two genes were predicted to be non-essential in *N. gonorrhoeae* when grown in the presence of PMNs compared with *N. gonorrhoeae* grown in the absence of PMNs (362). Other predicted non-essential genes under PMN+ conditions include pyruvate kinase (*pyk*), citrate synthase (*glta*), aconitase B (*acnB*), and isocitrate

dehydrogenase (*idh*). Additionally, flux through the pyruvate synthesis pathway and the Tricarboxylic Acid (TCA) Cycle were reduced when *N. gonorrhoea*e was grown in the presence of PMNs, which provide alternative carbon sources including lactate and pyruvate. In short, the TCA cycle and therefore zinc-dependent regulation of genes involved in the TCA cycle can be bypassed in *N. gonorrhoeae* if grown in the presence of PMNs producing lactate. One might speculate then that zinc-dependent regulation of TCA cycle genes helps *N. gonorrhoeae* adapt to changes in carbon conditions that correspond to different host-cell niches. For example, in the infected male urethra where the levels of zinc are high, *pta*, *dldH,* and other pyruvate metabolism genes are zinc repressed. At this infection site, *N. gonorrhoeae* relies on lactate and pyruvate as alternative carbon sources that are produced by PMNs which are often enrich in the infected male urethra. However, following transmission to female reproductive tract, *N. gonorrhoeae* experiences a zinc-depleted environment, which induces pyruvate metabolism genes (i.e. *dldH* and *pta*) allowing *N. gonorrhoeae* to utilize glucose as a primary carbon source. The female infection site which is commonly devoid of PMNs during asymptomatic infection. Therefore, further investigation of the role of zinc-dependent regulation in carbon utilization could shed light on gonococcal metabolism and pathogenesis in different host-cell niches including in localized infection niches (i.e. mucosal epithelial cells and polymorphonuclear leukocytes) and disseminated infection niches (i.e. blood stream and synovial joints).

CHAPTER 7: SUMMARY AND PERSPECTIVES

I. Gonorrhea

The Gram-negative bacterium, *Neisseria gonorrhoeae*, causes the sexually transmitted infection, gonorrhea. Each year, over 600,000 cases of gonococcal infection are reported to the CDC (49), and the World Health Organization (WHO) estimated over 80 million new cases of gonorrhea in 2020 (368). Symptoms of urogenital infection include cervicitis in women and urethritis in men. Disease sequelae include pelvic inflammatory disease, ectopic pregnancy, and infertility. Disseminated gonococcal disease is characterized by synovial joint infection, and in severe but less frequent cases, septicemia and endocarditis. The gonococcus causes disease by first attaching to the mucosal epithelial surface via a surface-localized pilus and invading the cell through Opa (94). *N. gonorrhoeae* expressed multiple different variants of Opa, and variable expression determines host cell tropism. For example, *N. gonorrhoeae* expressing OpaI interact with CEACAM1 and CEACAM3 on neutrophils(100), and Opa52-expressing gonococci interact with epithelial cells (369). The immune response to the gonococcus is skewed away from a T_H1/T_H2 response and toward a T_H17 response (84) resulting in an influx of polymorphonuclear leukocytes or neutrophils. This excess of neutrophils is the purulent exudate commonly observed in infected male patients.

II. Gonorrhea Treatment and Prevention

Previously, gonococcal infections could be treated effectively with multiple classes of resistance-free antimicrobials such as the sulfonamides and penicillins in the 1930s and 1940s. However, resistance to these antimicrobials arose soon after in the 1950s and 1960s.

Tetracyclines were then the first line of resistance-free antimicrobial defense against the pathogen until high-level resistance prevented its use in the 1990s. Similarly, the cephalosporins were recommended until high-level resistance limited their utility in the early 2000s (370). The ability of the gonococcus to acquire and maintain antimicrobial resistance determinants, has created a bottleneck of effective antimicrobial treatments over time. Consequently, the last CDC-recommended line of defense against *N. gonorrhoeae* is a singular intramuscular dose of ceftriaxone (72). Thus, there is an urgent need for novel therapeutics and an effective vaccine.

Vaccine efforts have been thwarted by the ability of the gonococcus to suppress the immune system, mimic host cell factors, and alter surface exposed structures through antigenic and phase variation. A great example of this is gonococcal porin which is antigenically variable, inhibits dendritic cell-activated T-cell proliferation (83), and is masked by LOS sialylation (112). Additionally, the antibody response against porin is blocked by the antibody response against RmpM (124, 125). The vast majority of other surface-exposed gonococcal proteins such as Opa, Pilin, LOS, Rmp, and IgA protease similarly contribute to immune evasion and complicate vaccine development. To date, the correlates of a protective and lasting immune response against *N. gonorrhoeae* are unknown. However, vaccines against the closely related pathogen, *N. meningitidis,* that are cross-protective against *N. gonorrhoeae* have shown promise. In 2008, a mass vaccination event occurred in New Zealand in which individuals ages 15-30 were vaccinated against *N. meningitidis* using the MenZB vaccine. This vaccine contained meningococcal outer membrane vesicles (OMVs). Petousis-Harris et al. hypothesized that the immunological response to meningococcal OMVs would be cross-protective against *N.*

gonorrhoeae due to the ~90% genetic similarity between *N. meningitidis* and *N. gonorrhoeae* (371). Following this vaccination event, gonorrhea cases decreased substantially, and meningococcal vaccine effectiveness against gonococcal infection was an estimated 31% (371). The meningococcus serogroup B vaccine, 4CMenB (Bexsero), contains the MenZB OMV antigens in addition to three recombinant proteins; Neisserial Adhesin A (NadA), factor H binding protein (fHbp) fused to genome-derived neisserial antigen 2091 (GNA2091), and Neisserial heparin binding protein (NHBP) fused to GNA1030 (372). The fusion GNAs, when used to passively immunize rats against *N. meningitidis*, elicit a protective response (373). 4CMenB generates antibodies against *N. meningitidis* that are cross-reactive with *N. gonorrhoeae* due the high sequence identity of the homologous proteins between the species (77). Consequently, gonococcal OMV vaccines containing immunogenic proteins as well as passive immunization with antibodies raised against these proteins are currently being investigated as promising vaccine and therapeutic candidates respectively. Interestingly, the OMV protein antigens in 4CMenB include the high-affinity metal transporters known as TonB-dependent transporters (Tdts) (77). The Tdts have been previously described as promising vaccine candidates because they are surface-exposed, highly conserved across strains, required for survival and virulence, and are not subject to high-frequency variation (177, 187). Another protein of interest included in 4CMenB are the high-affinity periplasmic metal transporter, ferric iron binding protein A (FbpA), which is required for iron transport following import via the Tdt, transferrin binding protein A (TbpA) (208). Novel vaccine an therapeutic efforts will target these metal transporters which are so crucial to gonococcal infection.

III. Research Objectives

The pathogenic Neisseria require transition metals for various biological processes such as sugar (312) and lipid biosynthesis (310), oxidative and nitrosative stress resistance (209, 211, 319), gene regulation, and metabolism (210). As a human-restricted pathogen, *N. gonorrhoeae* must obtain its critical metal nutrients from the host. However, host microenvironments effectively control the bacterial metal nutrient supply by either limiting metal availability or overloading the microenvironment with these metal nutrients. The mechanism through which host innate immunity proteins sequester and limit transition metals is termed nutritional immunity. Conversely, the mechanism through which the host overloads the bacterial nutrient supply with metals is termed metal intoxication. Thus, *N. gonorrhoeae* must maintain internal metal homeostasis. This can be achieved by altering metal import, export, and storage. Alterations in these processes maintain a homeostatic level of metal nutrients within the bacterial cell. Metal homeostasis and subsequent alterations in metal transport and storage is critical for expression of potential vaccine targets such as the Tdts and periplasmic metal transporters in OMVs. Therefore, the objective of this work was to characterize zinc, manganese and to a lesser extent copper homeostasis in *N. gonorrhoeae.* To accomplish this, a comprehensive literature review of Neisserial zinc, manganese, and copper homeostasis in the context of nutritional immunity and metal intoxication was conducted. The mechanism through which *N. gonorrhoeae* senses zinc and manganese specifically through the zinc uptake regulator Zur which alters global gene regulation was characterized, and the mechanism through which *N. gonorrhoeae* maintains manganese homeostasis via the putative manganese exporter, MntX, was identified.

IV. Project Summary

During infection by *N. gonorrhoeae*, the host deploys nutritional immunity proteins, namely, PS and CP, to limit the bacterial nutrient supply of zinc and manganese. PS sequesters zinc while CP sequesters both zinc and manganese. CP is maintained at high levels within a neutrophil which is the primary immune cell type that responds to gonococcal infection (84, 261). PS is enriched in epithelial cells of the lower genital (264). Therefore, *N. gonorrhoeae* experiences a zinc- and manganese-depleted environment during infection. In response, the Tdts, TdfH and TdfJ, bind to CP and PS respectively and utilize them directly as zinc sources (179, 198, 201). Utilization of PS as a sole zinc source requires the high-affinity zinc transporter, ZnuABC (179). ZnuA is the periplasmic zinc binding protein, ZnuB is the associated transmembrane protein, and ZnuC is the ATPase that provides energy for zinc transport. The genes encoding this system, *znuCBA*, are regulated by the zinc uptake regulator, Zur. In the presence of zinc, Zur represses *znuCBA* in a zinc-dependent manner, while in the absence of zinc, Zur de-repressed the *znuCBA* locus allowing for transcription of the operon. Confusingly, ZnuABC was named MntABC when it was implicated in manganese transport (205, 209), and Zur was named PerR when it was implicated in manganese-dependent response to peroxide stress (211). To clarify, both sets of nomenclature refer to a single genetic sequence identified by the locus tags ngo0168, ngo0169, ngo0170 (*znuCBA*/*mntABC*), and ngo0542 (*zur*/*perR*). For simplicity the operon and its gene products will be referred to using the znu nomenclature hereon. Unfortunately, the data implicating *znuCBA* as encoding a transporter that binds both zinc and manganese has been retrieved from multiple gonococcal strains under various metal conditions leading to confusion regarding metal specificity of the transporter itself and of the

protein that regulates it, Zur. This confusion begged the overarching research question of whether *znuCBA* is both zinc and manganese regulated in a single strain and more broadly, whether Zur is involved in both zinc and manganese homeostasis. To answer these questions and to clarify the nomenclature in the literature, the mechanism of Zur and zinc-dependent regulation of *znuCBA* was analyzed via Zur protein sequence analysis, reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR), and RNA-sequencing. The Zur protein sequence analysis showed that the *Neisseria* produce a Zur protein which is much different from other characterized Zur proteins, containing a DECH-rich region of unknown function. Additionally, gonococcal Zur contains two putative zinc binding sites per monomer. Based on sequence identity with functionally characterized Zur proteins, it is feasible that zinc binding at the CXXC site of gonococcal Zur is required for structural integrity, while zinc binding at the HCHE site is required for regulatory function. The zinc binding domain which includes the CXXC site and the HCHE site is positioned between the dimerization domain and the ligand binding domain. Arginine at position 24 (R24) within the DNA-binding domain is likely required for the Zur-DNA interaction. The RT-qPCR data showed that *znuCBA* is Zur-regulated in a zinc-dependent manner. The RNA-sequencing data showed that *znuA* is the most highly expressed gene within the operon under zinc limited conditions. Considering that *znuA* is the last of the three genes in the operon, this data also suggests that multiple messages are transcribed from the *znuCBA* locus. Multiple *znuA* transcripts with fewer *znuC* and *znuB* transcripts suggest that multiple ZnuA proteins service fewer ZnuB and ZnuC proteins, and emphasizes the critical role of ZnuA in zinc transport. The RNA-sequencing data also demonstrated that Zur is a global zinc-dependent regulator, regulating genes involved in metabolism such as alcohol dehydrogenase P

(*adhP*) and genes of unknown function such as *ngo1049* in addition to zinc transport genes such as *znuCBA*. Therefore, the following model of the mechanism of Zur-dependent regulation is proposed. To maintain structural integrity, the CXXC site is always bound to zinc. However, in the presence of excess zinc, the HCHE site is occupied by zinc, allowing dimerization of Zur at the dimerization domain. Zinc binding allows Zur to form a conformation that is capable of binding DNA and allows R24 to bind DNA. Zinc-bound Zur binds to promoters upstream of *znuC*, *znuA*, *ngo1049*, and *adhP* resulting in altered expression of these genes. Moving forward, the role of the DECH-rich region of Zur should be further investigated. Additionally, reporters using Zur-regulated promoters such as those upstream of *znuA*, *tdfH* and *tdfJ* should be generated to identify expression of these virulence factors in a cell model of infection. Identifying to what extent *znuA*, *tdfH* and *tdfJ* are expressed in a cell model of infection is critical to evaluating the effectiveness of a vaccine that aims to include these proteins.

The zinc regulated genes, *znuA*, *adhP*, and *ngo1049* were among a total of 200 genes that were differentially expressed in the presence and absence of zinc. Seven of those significantly differentially expressed genes are involved in pyruvate metabolism. Five genes were repressed in the presence of zinc and two genes were induced in the presence of zinc. Interestingly, two of the zinc-repressed genes, *ngo0214* which encodes a phosphate acetyltransferase and *ngo0925* which encodes a dihydrolipoamide dehydrogenase (DldH), have been previously predicted to be essential only in *N. gonorrhoeae* grown in the absence of neutrophils (362). Pta and DldH are involved in glucose metabolism and provide precursors that enter the tricarboxylic acid (TCA) cycle. They were also predicted to be non-essential when *N. gonorrhoeae* was grown in the presence of PMNs. Potter et al. showed that PMNs provide a

carbon source that allows *N. gonorrhoeae* to bypass the tricarboxylic acid (TCA) cycle (362). Specifically, PMNs provide lactate for gonococcal fermentation. Take together, this data suggests that when glucose is the predominant carbon source, zinc-dependent expression of *dldH* and *pta* supports metabolism of *N. gonorrhoeae*. However, when lactate is the predominant carbon source, zinc-dependent expression of *dldH* and *pta* is non-essential because the TCA cycle which involves these genes is itself bypassed, and the nutritional need for carbon breakdown is met by lactate fermentation.

The zinc-dependent regulation of *dldH* and *pta* may be linked to niche adaptation. Male seminal fluid is high in zinc (259) and neutrophil-rich during gonococcal infection (87) while the female genital tract is relatively limited in zinc (264) and PMNs during asymptomatic infection. In male seminal fluid, zinc-dependent repression of *dldH* and pta support more efficient growth in the presence of lactate secreted by PMNs. Conversely, the de-repression of *dldH* and *pta* in the female genital tract may support glycolysis where neutrophils are often not present to secrete high levels of lactate and where zinc is maintained at low levels due to the presence of the nutritional immunity protein PS. Future work will investigate the impact of zinc-dependent regulation of carbon utilization genes on gonococcal survival when grown in the presence of glucose, lactate and pyruvate, the only three carbon sources utilized by *N. gonorrhoeae*. It would be interesting to then identify the impact of zinc-dependent regulation of carbon utilization genes on gonococcal survival in different host-cell niches.

To this point, characterization of zinc-dependent regulation and homeostasis in *N. gonorrhoeae* has been described. However, recall that an objective of this work was to

characterize the mechanism through which *N. gonorrhoeae* senses both zinc and manganese specifically through the zinc uptake regulator Zur in a single strain.

To continue to address this aim, RT-qPCR was completed to identify whether *znuCBA* was Zur regulated in a manganese-dependent manner. Interestingly, *znuCBA* was not manganese regulated in strain FA1090. This is contradictory to the literature and begged the question as to whether the observed differences were due to the strain differences or suboptimal experimental conditions. To answer this question, strain 1291, which was shown in the literature to exhibit manganese-dependent regulation of *znuCBA* (211), was grown in the same metal limitation conditions under which strain FA1090 was grown. Under these conditions, in strain 1291, *znuCBA* was not regulated in a manganese-dependent manner. This confirmed that suboptimal experimental conditions were utilized. Experimental conditions were then optimized to further limit manganese so that when manganese was added in excess, a difference in regulation would be visible if it occurred. Following metal condition optimization, western blot data showed that ZnuA protein levels were reduced in the presence of manganese in strain 1291 in a Zur-dependent manner. The optimized experimental conditions were then used to characterize manganese-dependent production of ZnuA in strain FA1090. Interestingly, ZnuA protein levels were not altered by the presence or absence of manganese, suggesting that manganese homeostasis in *N. gonorrhoeae* is different between strains. To identify the factors that could explain these differences, the sequences of manganese related genes were compared between strains FA1090, 1291, and FA19. Sequence analysis showed that the genome of strain FA1090 encodes a full-length putative manganese exporter, MntX. In strains 1291 and FA19, a nonsense mutation at position 67 results in a

truncated MntX protein. The sequence between 1291 and FA19 was identical. FA1090 was used to represent an MntX positive strain, and FA19 was used to represent an MntX negative strain therefore strain 1291 was omitted from further analysis. Considering that the host utilizes metal intoxication in addition to nutritional immunity, it was of interest to characterize manganese internalization and growth of these strains under intoxicating levels of manganese. ICP-MS data showed that the addition of manganese does not alter internal manganese pools in FA1090 while the excess manganese accumulates in FA19. Growth assay data showed that strain FA1090, the genome of which encodes a full-length MntX, is not sensitive to high manganese levels. However, strain FA19, the genome of which encodes a truncated MntX, is sensitive to high levels of manganese. The isogenic Zur- mutant was more sensitive to manganese intoxication than FA19, suggesting that in the absence of Zur, *znuA* is fully derepressed allowing high-affinity transport of excess manganese into the cell. This was supported by data showing that the ZnuA- mutant was not sensitive to manganese intoxication and suggests that the absence of ZnuA prevents high-affinity transport of intoxicating manganese. Together, this data suggests that manganese levels in *N. gonorrhoeae* differs between strains, and that manganese homeostasis may involve the manganese transporter, MntX. In *N. meningitidis*, the absence of MntX results in accumulation of manganese in the cytoplasm which leads to protein mismetallation and gene dysregulation (243). Specifically iron-regulated genes become dysregulated when MntX is absent. Additionally, the MntX- mutant is more sensitive to iron limitation (243), suggesting that at intoxicating manganese levels, iron is replaced by manganese in iron-containing proteins causing protein dysfunction. In *N. gonorrhoeae*, the ferric uptake regulator, Fur, can bind manganese (213), which would repress

expression of iron import genes. Therefore, it is feasible that under iron-deplete but manganese-replete conditions, gonococcal strains lacking a full-length MntX (i.e. FA19) experience Fur mis-metalation with manganese which represses iron import under iron limited conditions resulting in iron starvation. Conversely, in strains expressing a functional MntX (FA1090), manganese is exported from the cytoplasm preventing Fur mis-metalation and subsequent repression of iron importers. This allows for growth under iron-limited and manganese-intoxicating conditions. This data generated from this study suggests a novel mechanism of defense against host-mediated metal intoxication.

The data presented in this work demonstrates that *N. gonorrhoeae* maintains internal zinc homeostasis via transcriptional regulation that alters zinc transport and that homeostatic metal conditions may contribute to gonococcal carbon metabolism. Additionally, this works provides evidence for a novel mechanism through which manganese homeostasis in *N. gonorrhoeae* contributes to resistance of host-mediated metal intoxication. Take together, this work gives new insights into gonococcal adaptation to changes is micronutrients within the host.

V. Future Perspectives

As metal transports are often required for growth, highly conserved across gonococcal strains, and are not subject to high-frequency variation, they represent promising targets for the treatment and prevention of gonorrhea. However, understanding the mechanisms underlying their expression and when these targets are turned on or off is crucial to predicting therapeutic effectiveness. Specifically, the data presented in this work further uncovers the mechanism of Zur- and zinc-dependent regulation of metal transporters such as ZnuA, TdfH, and TdfJ. TdfH

and TdfJ are currently being investigated as promising vaccine targets to be included in OMV-based vaccine strategies. However, our understanding of the extent to which these transporters are expressed in different host cell niches is limited. Therefore, the opportunity remains to assess expression of these targets in cell culture via reporter assays. Additionally, our understanding of metal-dependent regulation of these genes is one-sided, only considering the concentrations of one metal at a time. It is more likely that the niches occupied by *N. gonorrhoeae* during infection contain various levels and ratios of multiple metals such as iron, copper, and cobalt in addition to zinc and manganese. This idea of metal interplay impacting gonococcal metal homeostasis and subsequent growth and survival has been reviewed more extensively by Branch et al. (374) (see also chapter 3).

Although metal transporters are promising vaccine and therapeutic targets, *N. gonorrhoeae* retains the ability to acquire and maintain mutations that confer resistance to antimicrobials and assist in immune evasion. It is feasible that mutations that arise in these transporters could make them less susceptible to therapeutics or help *N. gonorrhoeae* evade the immune system. To make matters worse, antimicrobial treatment failures continue to occur (73, 375-377), and accurate and timely diagnosis of both symptomatic and asymptomatic patients is challenging. The most sensitive and widely used diagnostic test is nucleic acid amplification technologies (NAATs). However, NAATs do not provide information about antimicrobial susceptibility. Information about antimicrobial susceptibility requires viable culture testing which is time-consuming and quite difficult due to the fastidious nature of *N. gonorrhoeae* (378). Before 2020, presumptive treatment of *N. gonorrhoeae* included both oral azithromycin and intramuscular ceftriaxone. This inclusion of azithromycin was to treat

potential co-infections with *Chlamydia trachomatis* (379); however, the presumptive use of this dual therapy has led to increased azithromycin resistance in *N. gonorrhoeae* isolates (380). Presumptive treatment with the current CDC-recommended antimicrobial, ceftriaxone, can be effective if the strain with which the patient is infected is susceptible to ceftriaxone. Otherwise, treatment with ceftriaxone can lead to increased resistance in the gonococcal population (379, 381).

The ongoing genetic change of *N. gonorrhoeae* and subsequently dwindling number of effective treatment strategies calls for a novel approach of combating gonococcal infection. The evidence presented in this work that demonstrates the antimicrobial effect of manganese reveals an opportunity for the development of manganese intoxicating drugs for the treatment of gonorrhea. One possible treatment strategy includes the development of a prophylactic manganese-embedded intravaginal ring (IVR). An IVR is a ring-shaped medical device made of biocompatible, flexible, and permeable material that is inserted into the vagina for slow drug delivery over time. IVRs have been shown to be a safe (382) and effective (383) means of prophylaxis against human immunodeficiency virus (HIV). While safe and effective, women using these medical devices also tend to adhere to their use continuously over time and report the comfortability of the device (384).

An IVR has been developed to limit growth of *Candida albicans* (385), and a separate nonspecific IVR has been developed to limit growth of bacterial vaginosis causing bacteria, *Chlamydia trachomatis*, *N. gonorrhoeae*, HIV and HSV-2 (386). The active ingredients in this IVR are zinc, copper, and lactide. The intended effect of copper and zinc in this IVR are not explicitly disclosed. However, copper and zinc can have intoxicating effect on bacteria such as *E. coli*

(334) and S Typhimurium (360); therefore, zinc and copper intoxication may be a likely intended effect of the active ingredients in the IVR. Evidence of copper intoxication has been demonstrated in *N. gonorrhoeae* (242), while evidence of zinc intoxication has not. Unfortunately, the gonococcal genome encodes a copper exporter, CopA (242), that can confer resistance to copper in the IVR. Inclusion of the antimicrobial manganese in this IVR could help avoid treatment failure due to production CopA, in *N. gonorrhoeae*. A gonococcal strain would need to express functional MntX, CopA, and potentially a zinc export protein to resist killing by this IVR. Fortunately, it is unlikely that a zinc exporter would be expressed in the vaginal tract considering that genes encoding zinc exporters are often zinc-induced (387, 388) and that the vaginal tract is a zinc-limited niche. Therefore, an IVR embedded with intoxicating manganese and copper is a multi-layered approach to prevent gonococcal infections, limit prevention failure, and potentially slow the development of antimicrobial drug resistance.

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VITAE

Alexis Hope Branch was born in the flesh on October 19th, 1996, to Janet M. Branch and Paul M. Branch Jr. in Richmond VA. She attended Manchester High School in Midlothian, VA where she prepared for her undergraduate degree by taking courses such as Anatomy and Physiology, Physics, and Calculus. In 2018, Alexis began a fellowship as an NIH-funded Initiative for Maximizing Student Development (IMSD) undergraduate scholar under the direction of Dr. Sarah E. Golding at Virginia Commonwealth University in Richmond, VA. As an IMSD scholar she conducted research on the antimicrobial resistant pathogen, *Neisseria gonorrhoeae*, under the direction of Dr. Cynthia N. Cornelissen. In 2019, she earned her B.S. in Biology with a minor in Chemistry and honors in research from Virginia Commonwealth University in Richmond, VA. She then matriculated into the doctoral program at the Institute for Biomedical Sciences (IBMS) at Georgia State University in Atlanta, GA. She was born again of the spirit on January 29th, 2023, when she accepted Christ as her personal Savior at First Baptist Church of Atlanta in Atlanta, GA. Alexis finished her PhD in the fall of 2024 and will begin a post-doctoral fellowship as an NIH-funded Seeding Postdoctoral Innovators in Research & Education (SPIRE) scholar at the University of North Caroline at Chapel Hill under the direction of Dr. Robert Nicholas.