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# Structure-Function Relationships in HpuAB, the Gonococcal Bipartite TonB-dependent Transport System

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by

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ABSTRACT

Gonorrhea, a prevalent sexually transmitted infection affecting millions annually worldwide, is caused by *Neisseria gonorrhoeae* (*Ngo*), a "superbug" resistant to all antibiotic classes. Compounding the challenge, the absence of protective immunity upon infection allows for reinfection, and a viable vaccine against gonococcal infection remains elusive.

In response to infection, the human host deploys nutritional immunity, sequestering essential metals like iron from invading bacteria, using metal binding proteins. To overcome this, *Ngo* employs outer-membrane TonB-dependent transporters (TdTs), like HpuAB, to acquire iron from host metal binding proteins, such as hemoglobin (Hb).

Part of our study focused on HpuA, the lipoprotein component of the HpuAB system. Mutations targeting hydrophobic residues crucial for Hb interaction were studied. Results demonstrated that without HpuB, strains failed to grow, emphasizing HpuB's role in iron internalization. Notably, when HpuB is produced, deletion and insertion mutations in loop 2 of HpuA affected growth and binding to Hb.

Further investigations into HpuB, the transmembrane protein of the HpuAB system, uncovered essential loop regions for binding and growth on Hb as a sole iron source. Deletion mutations in loops 2, 3, and 4 facilitated binding and growth independently of HpuA production. Intriguingly, mutations in loop 7 abrogated binding and impaired growth in the absence of HpuA, but partial growth and full binding recovery occurred when HpuA was present. This highlighted the importance of loop 7 in iron acquisition and suggested a potential role for both HpuA and HpuB in the binding Hb.

As a combination of non-binding TdT mutants is hypothesized to have the potential to improve vaccine efficacy and provide protection, identifying non-binding HpuB mutants could be important. In summary, this research sheds light on the intricacies of the HpuAB system, contributing valuable insights that could inform the development of an effective gonorrhea vaccine.

INDEX WORDS: *Neisseria gonorrhoeae*, TonB, heme transport, sexually transmitted infections

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**DEDICATION**

I would like to dedicate this work to my family close and extended and to my friends! To my parents Alfred and Emilia, for instilling in me the drive to never give up, for showing me the importance of hard work and discipline, and for encouraging me to see the sky as the only limit. By Immigrating to America, you sacrificed your lives for my sisters and I, just for our future and I know this was not easy, and for that I will eternally be grateful. To my husband Pierre, thank you for your love and for always pushing me to be the better version of myself, thank you for never letting me give up, you truly complete me. To my sister Bida, for taking up the load and role of being the first born, thank you for always leading by example and making sure your sisters were taken care of. Thank you for the love and support Ibrahim and the kids, Samira, Ismael and Mimi have given me throughout the years. To my younger sister Vanessa, your drive and determination to get what you put your heart into is truly inspiring, thank you. I pray that the PhD in Aerospace engineering you're pursuing take you to the greatest places, fly high as the sky is the limit! Finally, to my baby sister Elodie, for your empathy, your love of family and your passion, thank you for the joy you bring to those around you. Hope this master's in public health you're pursing place you in rooms that will change the world for the better. This journey could not have been possible without the love of these family members that I will always love and cherish. Vous êtes ma vie entire, que le bon Dieu vous rende tout votre soutien et votre amour au centuple. To my friends which have been instrumental for my success with their help, their wellness checks and for always encouraging me, thank you. A special thanks to Sandhya Padmanabhan, Diariétou Badji, Jeanne Diop, Theodora Pereira, Pooneh Tavakoley, Elisa Diatta, and Fatou Lindor Seck.

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**TABLE OF CONTENTS**

## LIST OF TABLES

**LIST OF FIGURES**

## List of Abbreviations

| | |
|---|---|
| > | greater than |
| < | less than |
| % | percent |
| ~ | approximately |
| °C | degrees Celsius |
| α | alpha |
| β | beta |
| Δ | deletion |
| ΔH548 | histidine 548 deletion |
| µg | microgram |
| µL | microliter |
| µM | micromolar |
| 2D | two-dimensional |
| $A_{450}$ | absorbance at 450 nanometers |
| ABC | ATP-binding cassette |
| AP | alkaline phosphatase |
| apo- | empty/no metal bound |
| ATP | adenosine triphosphate |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| BSA | bovine serum albumin |
| C- | carboxy |

| | |
|---|---|
| CDC | Centers for Disease Control and Prevention |
| CDM | Chelex-treated chemically-defined medium |
| CEACAM | carcinoembryonic antigen-related cell adhesion molecules |
| CFU | colony forming unit(s) |
| $CO_2$ | carbon dioxide |
| DAB | 3,3'Diaminobenzidine |
| $dH_2O$ | deionized water |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| Fe | iron |
| $Fe^{2+}$ | ferrous iron |
| $Fe^{3+}$ | ferric iron |
| fur | ferric uptake regulator |
| g | gram |
| GCB | gonococcal broth |
| h | hours |
| $H_2SO_4$ | sulfuric acid |
| Hb | hemoglobin |
| HCl | hydrochloric acid |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HIV | human immunodeficiency virus |

| | |
|---|---|
| Hp | haptoglobin |
| HRP | horseradish peroxidase |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IL | interleukin |
| IPTG | isopropyl ß-D-1-thiogalactopyranoside |
| kDa | kilodalton |
| LB | Luria-Bertani |
| LOS | lipooligosaccharide |
| LPS | lipopolysaccharide |
| M | molar |
| mg | milligram |
| min | minute |
| mL | milliliter |
| mM | millimolar |
| MSM | men who have sex with men |
| MW | molecular weight |
| N- | amino |
| NaCl | sodium chloride |
| $NaHCO_3$ | sodium bicarbonate |
| NBT | nitro blue tetrazolium |
| nm | nanometer |

| | |
|---|---|
| nM | nanomolar |
| $OD_{600}$ | optical density read at 600 nanometers |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDB | protein data bank |
| poly-G | poly-guanine |
| RNA | ribonucleic acid |
| RPM | revolutions per minute |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| STI | sexually transmitted infection |
| TBS | tris buffered saline |
| TBST | tris buffered saline + Tween 20 |
| Tdf | TonB-dependent function |
| TdT | TonB-dependent transporter |
| TGF-β | transforming growth factor beta |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TPEN | N,N,N’,N’-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine |
| WCL | whole-cell lysate |
| WHO | World Health Organization |
| WT | wild type |

## Preface

Before you lies the dissertation named “Structure-Function Relationships in HpuAB, the Gonococcal Bipartite TonB-dependent Transport System”, most of which sums up my research efforts at Georgia State University, during my PhD training in the laboratory of Dr. Cynthia Nau Cornelissen. I conducted the research described herein between September 2017 and July 2023, after a postbaccalaureate year on the same topic between July 2016 and June 2017. After being fortunate enough to have substantial experience in laboratory settings through internships and I decided to pursue a PhD with the expectation that I will use my degree in a research position in government agencies or industry. I was lucky to find a laboratory with a welcoming mentor that I chose to follow from Virginia Commonwealth University to Georgia State University, a third of the way through my PhD. As I am writing, I am experiencing a combination of bittersweet emotions as I bid farewell to this PhD journey, that has deeply influenced the last six and a half years of my life. While I feel sad, leaving it behind, I am also filled with excitement for the opportunities that lie ahead.

This document was written in October 2023.

I hope you find pleasure in your reading.

OAA

# CHAPTER 1. INTRODUCTION

## I. Neisseriaceae Family

The *Neisseriaceae* family has been classified since 1933 and was composed of four genera: *Neisseria*, *Acinetobacter*, *Kingella*, and *Moraxella*. These non-spore-forming Gram-negative bacteria constituted of aerobes or facultatively anaerobes, rod-shaped or coccoid-shaped, and oxidase-positive or catalase-positive organisms. The *Neisseriaceae* family has been updated multiple times over the years and now, based on ribosomal nucleic acid (RNA) analyses, the genera Moraxella and Acinetobacter are part of the *Moraxellaceae* family (1, 2). Moreover, the genera *Eikenella*, *Simonsiella*, and *Alysiella* (3) were added to the family *Neisseriaceae*, which now constitutes a major branch of the β-Proteobacteria.

The *Kingella* and *Eikenella* genera are mostly composed of human commensals in the bowel and oral cavity, which occasionally cause opportunistic infections (4). The genus discovered in 1879, *Neisseria*, however, is composed of both numerous commensals and two pathogenic species, *N. meningitidis* and *N. gonorrhoeae* (5). They are non-motile diplococci with flattened sides, that grow optimally in the presence of enough $CO_2$ (6) and in the 35-37°C temperature range. Human-associated commensal and pathogenic *Neisseria* have a particularity, they can produce acid from carbohydrates and sugars such as glucose or maltose, and polysaccharide from sucrose (7). Another particularity is their ability to reduce nitrite to nitric oxide (8). The two pathogenetic species have considerable similarity at the genome level, however, the disease manifestation is very different.

## II. Pathogenic Neisseria

Most of the commensal *Neisseria* species rarely have clinical presentations and colonize the oronasopharynx without causing disease, however, sometimes they can lead to opportunistic infections in individuals with weakened immune systems (5, 9). These species have been associated with cases of endocarditis, meningitis, septicemia, otitis, bronchopneumonia, and some genital tract diseases (5). The pathogenic *Neisseria* species, *N. gonorrhoeae* and *N. meningitidis* are both human specific. *N. meningitidis* can be carried asymptomatically and frequently colonizes the nasopharynx of healthy individuals (10). A very small proportion of infections lead to dissemination to the brain and the meninges, causing disease (11). Contrary to the meningococcus, which can be considered part of the normal flora, the gonococcus is always considered pathogenic. *N. gonorrhoeae* primarily infects the mucosal surfaces of the urogenital system, the oronasopharynx, and the rectum. Finally, these two pathogenic species are similar at the genomic level, yet different in disease outcomes; the particularity of the meningococcus is the presence of an antiphagocytic polysaccharide-based capsule that distinguishes it from the gonococcus.

## III. Meningococcal Disease

### *a) Epidemiology and Incidence*

In approximately 10% of humans, *N. meningitidis* colonizes the nasopharynx, where it predominately exists as a member of the normal flora. Twenty-five percent of these carriers have a meningococcal colonization that lasts months, a third have an intermittent colonization, and about forty percent are transiently colonized (12-14). Meningococcal transmission can occur person to person by direct contact with nasal or oral secretions or through inhalation of

large droplet nuclei (15). Environmental factors such as tobacco smoke exposure seem to be correlated with meningococcal carriage increase and lead to rising risks of infection (16). Moreover, individuals at greater risk of periodic and more severe meningococcal infections seem to have defects in the complement pathway or regulation (17, 18). During meningococcal outbreaks, 90-100% carriage rates have been recorded, especially in closed settings such as college dormitories and military camps (12, 19-21). Meningococcal disease incidence is rare, despite high prevalence of meningococcal carriage, with rates ranging from 1 to 1000 cases per 100,000 people per year, depending on the location (19). Geographic location and age constitute a big determinant of meningococcal carriage. For populations living in North America and Europe, carriage rates are very low early in life, before considerably increasing during adolescence, peaking in 20-24 years bracket, and finally declining later (22). Meningococcal infection may also be influenced by the season as when epidemics arise at the end of the dry season in “the meningitis belt” of Africa, from West Africa (Sénégal) to East Africa (Ethiopia) (23, 24). As indicated in Woringer *et al.*, “The current understanding of the phenomenon includes a combination of seasonal meningococcal and pneumococcal hyperendemicity related to climatic and environmental factors” (25). Finally, meningococcal infection can vary based on infection with different serogroups. The meningococcal strains are separated into 13 serogroups based on the chemical structure of the polysaccharide capsule. Of the 13, serogroups A, B, C, W, X and Y are responsible for more than 90% of disease worldwide (19). For instance, in the meningitis belt of Africa and in Asia, Serogroup A is the primary cause of pandemic outbreaks, that can reach up to 1% of the population, every 5-10 years since 1905 (26). In the Americas, Europe and Australia however, serogroup B and to a lesser extent

serogroup C, are responsible for most infections, with usually less than 10 cases per 100,000 per year (26).

***b) Disease***

During colonization, *N. meningitidis* use a multifactorial process involving pili, twitching motility, LOS, opacity proteins, and other surface proteins to facilitate binding to the mucosal epithelial cells in the nasopharynx or oropharynx (27). The meningococcal adhesins stimulate epithelial cells to uptake meningococci into phagocytic vacuoles. These vacuoles may cross the mucosal epithelium, allowing the bacteria to enter subepithelial tissues (28). In a small percentage of individuals, *N. meningitidis* can, at this stage, penetrate the mucosa and disseminate into the bloodstream, leading to one of the two major types of meningococcal disease (27, 29). This resulting meningococcemia can lead to colonization of the meninges, the pericardium, and the large joints (30). Interestingly, *N. meningitidis* can be found in the bloodstream of 75% of patients with meningococcal disease, but meningococcemia, occurs in only 5 to 20% of those patients (31). Signs and symptoms of meningococcemia include an abrupt onset of fever, chills, feeling unusually weak and tired, and a petechial or purpuric rash may be present, often on the hands and feet (32). It is also often associated with the rapid onset of hypotension, acute adrenal hemorrhage, and multiorgan failure (31). However, when the vacuole crosses the mucosal epithelium and the bacteria enter the subepithelial tissues, infection of the meninges, which surround the brain and spinal cord leads to the second major type of meningococcal disease, meningococcal meningitis. Signs and symptoms of meningitis include sudden onset of headache, high fever, stiff neck, nausea, vomiting, sensitivity to light and/or mental confusion, changes in behavior such as confusion, sleepiness, and being hard to

wake up (24). Meningitis patients need immediate attention to minimize life-threatening disease progression. Finally, in 5 to 15% of patients, invasive meningococcal infection can result in a hard to detect pneumonia because the isolation of *N. meningitidis* from sputum cannot distinguish carriage from active infection (31).

***c) Prevention***

Developing vaccines for meningococcal infection worldwide has been the approach towards prevention of meningococcal disease. Since 1981, a vaccine, MPSV4, that is 85% effective in adults has been available. This vaccine was a polysaccharide capsule vaccine against *N. meningitidis* serogroups A, C, Y, and W. Unfortunately, this polysaccharide only vaccine had a limited effectiveness as it does not lead to immunologic memory due to stimulation of T-cells and not B cells, having therefore no effect on meningococcal carriage (33-35). An upgrade of the polysaccharide vaccine was done, to address the aforementioned issues, with the addition of a protein conjugate. This led to the introduction, in the 2000s, of quadrivalent vaccines MenACWY, Nimenrix, Menactra and Menveo (33, 36, 37). A combined conjugate meningococcal vaccine, Menhibrix, containing *N. meningitidis* serogroup C and Y capsular polysaccharides, as well as *Haemophilus influenza* type b capsular polysaccharide was also introduced (38). Monovalent conjugate vaccines against serogroups A (1 vaccine) and C (3 vaccines) also exist (39). These vaccines have been successful against the strains they cover; however, a vaccine was yet to be developed against serogroup B, which causes 50% of invasive meningococcal disease globally and 32% in the United States (40). The reason for the delay in vaccine development for serogroup B has to do with the polysaccharide capsule target. In the other serogroup, except serogroup A, this target is composed of sialic acid whereas in

serogroup B the capsule has sialic acids with specific α2-8 linkage structures found in many human tissues, especially in the central nervous systems of fetuses and children (41-43). This capsule was, therefore, avoided because the molecular mimicry was likely to decrease immunogenicity due to tolerance in humans and/or to induce autoimmunity against self-antigens (44). To find alternatives for serogroup B, a reverse vaccinology process during which a genomic search for potential antigens and the use of recombinant DNA technology to produce and test these antigens for suitability, was used (45). Outer-membrane structures such as factor H binding protein (fHbp), *Neisseria* adhesion A (NadA), and *Neisseria* heparin-binding antigen (NHBA) were, therefore, identified as vaccine candidates for serogroup B. These three proteins with detoxified outer membrane vesicles from the New Zealand outbreak strain NZ98/254 lead to the formulation of a four-component vaccine, 4CmenB/Bexsero (46). Based on the antigenic presentation of the target strain and the subtype of PorA, 4CMenB is between 80-96% effective. For instance the target strain is sensitive to 4CMenB if it expresses the P1.4 subtype of PorA or if one of the three antigens (fHbp, NadA and NHBA) is highly expressed (47, 48). When 1 of the 3 vaccine antigens is highly expressed, the probability of being killed by the host antibodies is 80%, and the probability climbs to 96% if 2 out of 3 proteins are highly expressed (48). Trumemba, another serogroup B vaccine, contains two distinct fHbp variants (49). In the United States, the quadrivalent meningococcal conjugate (MenACWY) vaccine is recommended for all adolescents aged 11 to 12 years with a booster dose for adolescents aged 16, whereas vaccination against serogroup B meningococcal disease is recommended at the age of 16 (50).

### *d) Detection and Treatment*

Meningococcal disease can be difficult to diagnose since its signs and symptoms can be like other diseases. Blood sample or cerebrospinal fluid (fluid near the spinal cord) sample can be collected for detection. When samples are positive for *N. meningitidis*, a culture allows the identification of the specific subtype that is causing the infection to better inform antibiotic choices (51, 52). These methods for meningococcal diagnosis are sometimes too slow and frequently compromised by prior antibiotic treatment. Therefore, the introduction of sensitive quantitative Polymerase Chain Reaction (qPCR) assays significantly improved laboratory detection rates and time required to confirm invasive meningococcal disease (53). This allows medical staff to choose the most effective antibiotic, for the subtype, in a timely manner.

In the 1930s, sulfonamides were first introduced to treat meningitis. Due to an early resistance to sulfonamides, penicillin was introduced and still remains the treatment used today (54). Up to a 100 years ago meningococcal infection carried a case fatality rate of approximately 70%. With the introduction of antimicrobial therapies, there was a significant drop in the mortality rate, down to a stable 9-12% rate, over the last 20 years (55). However, the mortality rates only dropped to 40% in cases of meningococcemia (31). Based on how serious the infection is, breathing support, medications to treat low blood pressure, surgery to remove dead tissue and wound care for parts of the body with damaged skin might be required (51). Interestingly, with the now low mortality rates, still 11-19% of convalescent patients will have serious sequalae with complications such as neurological disability, hearing loss, and loss of limb(s) (30, 56). With the severity of meningococcal diseases, people in close contact with infected individuals are at risk of developing meningococcal disease within 5-10 days after

contact (15). Therefore, to limit the spread, antimicrobial chemoprophylaxis treatments, that eliminate meningococcal nasopharyngeal carriage, are also prescribed for caretakers and frontline medical staff. These approved systemic antibiotics are rifampin, ciprofloxacin, and ceftriaxone. Resistance to antimicrobial drugs is rare in *N. meningitidis*; however, early on resistance to sulfonamides and now resistance to other drugs, like penicillin, are being recorded (54, 57, 58).

### *e) Animal Models of Infection*

To investigate meningococcal infection, many different animal models have been developed. In mice and infant rats, intraperitoneal models of infection, used to characterize virulence and protection profiles from immunization, have been developed (59). The intranasal mouse model however, helps to characterize the mechanisms of pathogenesis and disease progression (60, 61). With *N. meningitidis* being a human-specific pathogen, both animal models of meningococcal disease investigation are limited. Consequently, humanized transgenic mice, that express human proteins, and closely mimic human infection are being developed (62-65). These transgenic mouse models will allow for a better, more detailed characterization of the meningococcal-host interactions that lead to infection.

## IV. Gonococcal Disease

### *a) Epidemiology and Incidence*

*Neisseria gonorrhoeae* is the etiological agent of the sexually transmitted infection (STI), gonorrhea. The disease was discovered over a century ago and as currently the second most common, reportable infectious disease in the United States; it remains a medical issue till this day. Gonococcal cases yearly go as high as 82.4 million in the world, according to the 2020

World Health Organization's (WHO) estimates, with over 710,151 of them in the United States alone, according to the Centers for Disease Control's (CDC) 2021 estimates (66, 67). In 2018, treatment costs for gonococcal infections in the United States was $271 million (68). A gonococcal infection does not lead to protective immunity, contributing therefore to the high number of cases (69). The gonococcus mostly spreads through direct sexual contact, this contributes to the correlation between age and infection. Adolescents and young adults between ages 15-29 represent the majority of the new gonococcal cases recorded each year. However, other factors such as socioeconomic status, sexual orientation, number of sexual partners, and race also contribute to the high number of cases (67, 68). For instance, ethnic minority groups like Latinos, those of African descent, and Native Americans represent an extremely large proportion of overall gonococcal infections (69). Similarly, infections among men who have sex with men (MSM) increase yearly and are more prevalent in HIV positive individuals, as gonococcal infection seems to increase HIV viral replication and gene expression (70-72). Interestingly, among individuals carrying gonococcal infections, co-infection with *Chlamydia trachomatis* is also very common (73). Among individuals with gonorrhea, women are often reservoirs of *N. gonorrhoeae* as they are frequently asymptomatic, about 50% of them, which means more cases are reported in men than in women (74, 75). This consequently leads to an increased spread and more severe consequences post infection (75).

***b) Disease***

The most commonly known form of gonococcal infection is gonorrhea, a genital tract disease. The gonococcus, following transmission, comes in close contact and adhere to the epithelium, with the help of surface structures, before replicating and colonizing the area (76).

After colonization, a small percentage of adherent gonococci are capable of invasion and transcytosis into the epithelial cell layer, subsequently leading to local infections or dissemination (77). *N. gonorrhoeae* primarily infects mucosal and glandular surfaces of the genito-urinary system, but can also infect the rectal, pharyngeal, and conjunctival tissues (78). The infection results in local inflammation with recruitment of neutrophils and macrophages to the site. The usual presentation of gonococcal infection in men is urethritis, which can ascend, in the rare absence of treatment, to cause more severe outcomes such as prostatitis and epididymitis (79, 80). Symptoms begin about 2 to 5 days post exposure in men which are symptomatic 90 % of the time (80). Typical infections in women however, initially begins 5 to 10 days post exposure, with cervicitis before ascending, in asymptomatic cases. These ascending, untreated infections can lead to severe secondary sequalae, including pelvic inflammatory disease, salpingitis, and fallopian tubes damage with infertility consequences (74, 81). For both men and women, asymptomatic infections (75) can cause dissemination, which can consequently lead to septic arthritis, endocarditis, and meningitis (82-85). Finally, the least common manifestation of gonococcal infections which arise in the mouth, throat, rectum, and eyes (in neonates during birth through passage of the birth canal), are often difficult to diagnose and treat (86, 87).

### *c) Immune Responses to Gonorrhea*

A gonococcal infection induces local production of transforming growth factor β (TGF-β) and interleukin 10 (IL-10) that skews the immune response away from the adaptive Th1 and Th2 responses to an innate Th17 response (88). In the presence of the gonococcus, the Th17 response enhances a non-protective neutrophilic inflammatory response by producing IL-17

and IL-22, which recruits neutrophils in both the human and mouse reproductive tracts (89). Interestingly, female mice clear gonococcal infections faster with protection from reinfection, when immunized with gonococcal antigens plus IL-12, a Th1 activating cytokine (90). Similarly, anti-TGF-β antibody treatment redirected the immune response from Th17 to Th1 and Th2 leading to protective immunity, protective antibodies, and reinfection resistance (91).

***d) Prevention Challenges***

Although vaccine strategies have proven to be successful for meningococcal disease, the same is not true for gonococcal disease. Due to the ability of *N. gonorrhoeae* to undergo high-frequency antigenic and phase variation of its surface antigens, the development of vaccines has been very challenging. Because of these variations, the pathogen can express a large array of proteins on its surface forcing the host to produce antibodies to these constantly evolving surface antigens (92). Moreover, natural infection does not elicit a protective immune response (69). Unfortunately, there is no effective vaccine against gonorrhea, available yet. Interestingly, increasing evidence shows that the Bexsero vaccine, for meningococcus, may elicit some protection against gonococcal infection, as vaccination elicited the production of cross protective antibodies (93). The recommendation for prevention today is to use safe sex practices, regularly have effective screening when exposed and contact tracing to limit outbreaks.

***e) Detection and Treatment***

Several methods can be used to detect *N. gonorrhoeae*. First, samples are collected from the first urine of the day or from swab specimens from the mucosa of the urogenital tract, the rectum, the pharynges, and the conjunctiva (94). Then, the bacteria from the samples are

cultured to perform the detection tests. One way to detect the gonococcus is by performing light microscopy of stained smears, a technique that is sensitive and specific, but only allows the visualization of gonococci in urethral or cervical mucus (95). Performing nucleic acid amplification tests (NAATs) is another was to detect gonococci as this technique is 95% sensitive and specific for cervical swabs, urethral swabs, and first-catch urine in men (96).

Contrary to *N. meningitidis*, *N. gonorrhoeae* is highly capable of acquiring resistance to antibiotics and even more intriguing, the gonococcus maintains these resistances over time. Because it has become increasingly drug resistant, evidence shows that current pharmacotherapies will soon be ineffective (97-102). Sulfonamides were first used to treat gonococcal infections before resistance started arising less than 10 years later, then as in the meningococcal infections a switch to penicillin was made. Since the introduction of penicillin, resistance to every class of antimicrobial drug utilized for treating *N. gonorrhoeae* has emerged (95, 103, 104). With WHO and CDC recommend that the treatment options for *N. gonorrhoeae* should be easily accessible, cost effective, and have more than a 95% cure rate as a single dose (105, 106); antimicrobial resistance led, since the 1930's, to many updates of the treatment guidelines, with the most recent one in 2020 (107). Now, for all uncomplicated gonococcal infections, a 250 mg intramuscular dose of ceftriaxone is the recommended treatment. As recently as 2015, the recommended treatment was a one-time dual therapy of 250 mg intramuscular ceftriaxone plus 1 g oral azithromycin (100). This 2015 treatment would also treat Chlamydia co-infection. For individuals with β- lactam sensitivity, alternative drug combinations, with promising results in clinical trials, such as 2 g azithromycin and either 240 mg gentamycin or 320 mg Gemifloxacin, have also been identified (108, 109). As the last

methods of treatment are dwindling down, there is a dire need for vaccine and therapeutic strategies to avoid an era of untreatable gonococcal infection.

### *f) Animal Models of Infection*

Animal models of gonococcal infection have been difficult to establish due to the human specific nature of the pathogen. The first genital tract infection model successes were in chimpanzees; however, because their use is too costly, using chimpanzees as models for gonococcal infection is not practical (110, 111). An alternative mouse model of infection was developed after finding that gonococcal colonization is affected by the murine estrous cycle (112, 113). How estrogen allows for gonococcal colonization has yet to be understood; however, different hypotheses have been proposed. One suggests rearrangements of genital tissue (114), another suggests that estrogen might temporarily suppress the mouse immune system (115, 116), and a third suggests that the estrous cycle causes variation of innate immunity mediators (117). This aforementioned model of infection is the 17 β-estradiol treated germ-free BALB/c mouse that allows long-term colonization (112, 116). This model is particularly useful because the site of infection is like that of humans, with similar carbon availability, mucus properties, and commensal burden (118, 119). Moreover, this mouse model does not develop a humoral memory response to gonococcal infection, like humans. Depending on the method of estrogen administration, murine colonization can last up to 40 days with a typical window of 12-14 days, which affords enough time for *in vivo* characterization of gonococcal infection. Some of these experiments have proven to be useful in the initial characterization of vaccine candidates (116).

Nonetheless, this 17 β-estradiol mouse model has its limitations. For instance, the mouse vaginal pH is considerably higher than the human one, and mice do not undergo a period of menstrual bleeding (116). With these examples alone, the differences in the physiology of the genital tract can have an impact on the survival of the gonococcus, especially as these parameters are not testable in this model. Finally, as a human specific pathogen, there are numerous host-pathogen interactions that do not take place in this model, because the interaction requires human proteins such as cell surface receptors and complement regulatory proteins. Additionally, to grow and survive, *N. gonorrhoeae* only uses the human specific version of the following iron sources: transferrin and lactoferrin (120). Therefore, to overcome these limitations, efforts to humanize mice are constantly ongoing, to reinforce their effectiveness as an infection model. Many transgenic mice have been developed so far, with some that can express human CEACAM1 (62), human factor H (121), and human transferrin (63). These constitute single transgene mice; however, many more are currently being developed, with the goal being to develop mice that can express multiple human proteins for a closer model of human infection.

## V. Virulence Factors of Neisseria Gonorrhoeae

With years and years of co-evolution with the human host, gonococci possess an array of surface-associated virulence factors important for host pathogen interactions and infection. These proteins as seen in figure 1, play a role in adhesion, invasion, nutrient acquisition, dissemination, immune suppression, and immune evasion (122). Because many of the genes, encoding these virulence factors, are subject to phase and/or antigenic variation at high frequency, they contribute even more to gonococcal immune evasion. During phase variation,

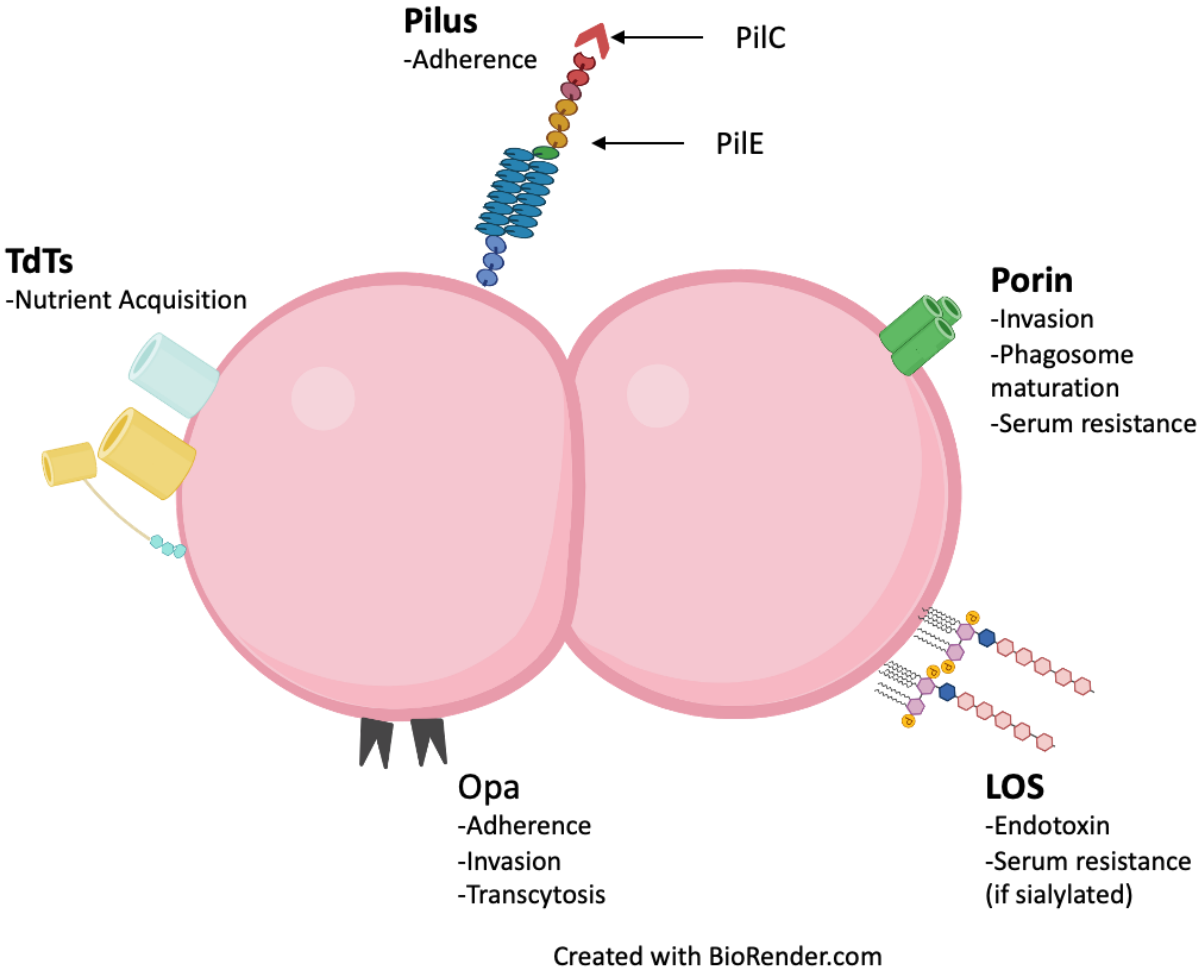



**Figure 1: Overview of Surface-Associated Virulence Factors in Neisseria Gonorrhoeae.**
This cartoon image shows the surface of gonococcal cells, with its different virulence factors.

the expression of a gene can be reversible and be turned on or off whereas during antigenic variation, an antigen, expressed on the cell surface, can have diverse alternative forms.

***a) Type IV Pilus***

Many Gram-negative bacteria like, *N. gonorrhoeae*, produce a type IV pilus comprised of a filamentous polymer of pilin proteins. The polymer is several micrometers (μm) long, has a 6 nanometers (nm) diameter and protrudes from the surface of the gonococcal cell (123). During synthesis, 23 genes ensure that the pilus undergo assembly, functional maturation, counter retraction, and emergence on the cell surface (124, 125). The 15 proteins, identified by systemic genetic analyses, making up the pilus are also involved in its biogenesis, assembly, and disassembly (125). Many subunits of PilE, the major structural pilin, assemble into a helix to form the general filament. Then some minor pilin proteins, PilC, PilV, and PilX, are incorporated into the filament to control its function (126, 127). An ATPase PilT and PilC pilin coordinate the rapid assembly and disassembly of the pilus leading therefore, to "twitching motility", a quasi-motility of the gonococcus (128, 129). The gonococcal type IV pilus performs numerous roles, including serving as a major adhesin for attachment to the epithelium (123, 130), and playing a role in self agglutination or microcolony formation (126). The pilus also plays an important role in naked foreign DNA uptake, from the extracellular milieu, leading to lateral gene transfer that contributes to genetic diversity and the rise of antimicrobial resistance (131, 132). This feature also increases the frequency of transformation of the gonococcus, making it naturally competent. The gonococcal pilus is one the virulence factors that undergoes both high frequency phase and antigenic variation. During phase variation, the *pilC* gene that contains a poly-G tract within its coding sequence, can slip in and out of frame during DNA replication.

These frameshift mutations, caused by changes in the length of the poly-G tract that create a premature stop codon, consequently, turn the *pilC* gene on or off (133). During antigenic variation however, a non-reciprocal, RecA-dependent genetic recombination between the *pilE* expression locus and one of the many genes in the silent *pilS* locus occur; this recombination results in antigenically distinct genes (134, 135).

***b) Opacity Proteins***

The opacity proteins, also known as Opas, originally were named based on how they affected the opacity of gonococcal colonies viewed on solid media (136). They are a family of transmembrane proteins that are closely related and are found in the outer membrane of *N. gonorrhoeae*. Opas are composed of an eight-stranded β-barrel with four surface-exposed loops (123). Generally, the gonococcal chromosome has up to 11 *opa* loci and the meningococcal chromosome has 4 to 5 *opa* loci (137). Opas are considered invasins that allow invasion of epithelial cells and leukocytes such as neutrophils (137-141) and their expression in natural infections (142) and experimental infections (143) shows their importance in gonococcal infection. The Opas are also known to have a role in modulating the host immune response.

Based on their human receptor tropisms, Opas are classified as binding two sets of host receptors, the carcinoembryonic antigen-related cell adhesin molecules (CEACAMs) (144, 145), and the heparin sulfate proteoglycans (146, 147). For example, long-term colonization in the lower genital tract is associated with Opas binding CEACAM5 and enhances penetration of the epithelial tissues that may lead to dissemination is associated with Opas binding CEACAM1 (148). While performing their immune-modulatory capabilities, Opas can arrest activation and

proliferation of CD4+ T-cells (149), by binding the CEACAM1 receptors on the T cells. Similarly, by binding the CEACAM1 receptors on the B cells, Opas cause death of B cells, therefore, lowering antibody production (150). Moreover, Opas were shown to have an impact on skewing the host immune response from the Th1/Th2 responses to the Th17 response mentioned above (88, 151).

Finally, the Opas are also subject to both high frequency antigenic and phase variation. Every *opa* gene has tandem repeats, $[CTCTT]_n$ that encode a leader peptide, in the 5' region of the *opa* genes. Slipped-strand mispairing within this repeat leads to phase variable expression of Opas (152, 153). This results in the gonococcus expressing none, one, or multiple Opa proteins at once (152-154). Antigenic variation is also seen during lateral gene transfer, when there is recombination between *opa* loci leading to hybrids (155).

***c) Porin***

Porin is the most abundant protein found on the outer membrane of *Neisseria spp* and make about 60% of these outer-membrane proteins (156, 157). Porins form a trimer and each of the monomer forms a β-barrel (158, 159). Porins function as nutrient transport channels that are crucial for the survival of the pathogenic *Neisseria* (123, 160). Another role played by porins is to contribute to the pathogenesis of *N. gonorrhoeae*. For instance, porins can translocate into the host cell membranes and disrupt host cell signaling that lead to phagosome maturation and degranulation (141, 161). This consequently allows the gonococcus to survive in phagosomes (161-163).

Two classes of porins produced by *N. meningitidis* are the large phase-variable class, PorA and the smaller class, PorB (164, 165). Interestingly, PorA is not expressed in *N.*

*gonorrhoeae* because it is a pseudogene; the gonococcus therefore, only expressed PorB (166, 167). In turn, PorB is subclassified as PorB1A, which is strongly associated with disseminated phenotypes or PorB1B, which is primarily found in local infections (167, 168). PorB1A binds factor H and with PorB1B they both bind the C4b binding protein of the complement pathways, mediating serum resistance among gonococcal isolates (169-171). Taken together, these reiterate the role of porins in the pathogenesis of *N. gonorrhoeae*.

***d) Lipooligosaccharide (LOS)***

The major glycolipid expressed on the outer membrane of Gram-negative bacteria is the lipopolysaccharide, LPS. Three structural regions make up the LPS: a membrane anchor domain, Lipid A, a short hydrophilic oligosaccharide core, and a variable length polysaccharide made of repeating O-antigen (172). In most species, LPS is a highly immunogenic virulence factor. In pathogenic *Neisseria*, because the LPS lacks the O-antigen, it is referred to as lipooligosaccharide (LOS) (173). LOS, like the pilus and Opas might also have an immune-modulatory role as it was shown to be involved in the manipulation of host dendritic cells, skewing the immune response to favor bacterial survival (174). Although LOS usually serves as an endotoxin, it is crucial for the virulence of the gonococcus. For instance, LOS mimics the human glycosphingolipids (175) and bind the human asialoglyco-protein receptor (ASGP-R) found on human sperm (176), increasing therefore, invasion of urethral epithelial cells (176, 177). In another example, sialylation of some gonococcal LOS confers serum resistance (178). *N. gonorrhoeae* cannot make sialic acid, but rather produce sialyltransferases (179-181) that permits the usage of host-derived cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA) as a sialyl donor (182, 183).

As a virulence factor, LOS is subject to phase variation. A variety of genes encoding glycosyltransferases, responsible for the extension of the carbohydrate polymer (184) and the modification of the terminal sugar of the LOS, undergo phase variation and therefore contribute to the phase variability of LOS. These genes: *lgtA*, *lgtC*, and *lgtD* contain poly-G tracts within their coding regions that can slip, and the resulting frameshift mutations lead to variations in the terminal sugars of LOS, thereby also contributing to an antigenic diversity of the LOS (185). Interestingly, LOS expression can impair Opa-mediated invasion of some cell lines (186), suggesting that LOS undergoes antigenic phase variation that enables the gonococcus to alternate between an invasive and serum-resistant phenotype for better survival.

***e) IgA1 Protease***

The human mucosa is the site where most neisserial infections occur. It contains many antibodies including the predominant subclass IgA, which has neutralization activity against pathogens (187). To overcome this neutralizing activity, *Neisseria spp.* produce a proteolytic enzyme, IgA protease, that cleaves IgA and counteracts its effect (188, 189). Immature IgA protease is secreted, followed by endoproteolytic cleavage steps that result in the mature form of the protease (190, 191). Immunoglobulin IgA1's heavy chain contains a proline-rich hinge region that is the target of the mature IgA protease (188, 190). However, in this proline-rich region, two cleavage sites are identified and the usage of one over the other depends on which of the two classes of IgA protease *Neisseria* expresses. When class 1 is expressed, the enzyme cleaves a proline-serine bond at residue 237, whereas expression of class 2 leads to the cleavage of a proline-threonine bond at residue 235 (188, 192, 193). Another role for IgA

protease is to cleave lysosome-associated membrane protein 1 (LAMP1) found in lysosomes and late endosomes (193), therefore allowing the survival of intracellular *Neisseria* in lysosomes and endosomes (193, 194). Experimental human infection studies showed that IgA protease is not essential for gonococcal colonization; however, it was also shown that the protease play an important role in transcytosis, therefore there is conflicting evidence about whether IgA1 protease is necessary for pathogenicity (187, 194).

Finally, in *N. gonorrhoeae*, IgA protease might be subject to antigenic variation. There is evidence that horizontal gene transfer contributes to recombinational events, indicated by a mosaic-like organization among protease genes (195, 196).

## VI. Host Iron Homeostasis

Bacteria need nutrients to grow and cause disease. A nutrient that is crucial for their metabolism is iron, therefore, bacteria absolutely need to develop ways to acquire this important metal to survive and cause disease (197-199). For that reason and more, the human host has evolved mechanisms to keep free iron at levels that are too low to allow invading pathogens to use it. This, consequently, deprives bacteria of iron and reduces their capacity to infect. This tactic used by the host is called nutritional immunity and it can be effective against many infectious microbes (200-202).

### *a) Iron*

Iron is a key catalytic element in enzymatic proteins as it plays a key role is electron tranfer. It plays an important role in physiological processed in the human body such as respiration, nucleoside biosynthesis, erythropoiesis, immune function, and host defense. It also has a crucial role in cellular activities such as DNA replication and repair, and mitochondrial

function. Finally, iron is a critical component of enzymatic reactions, where is serves as a cofactor and structural complexes like iron-sulfur clusters (203, 204). Elemental iron exists in two interchangeable redox states: ferrous (Fe2+) and ferric (Fe3+). Curiously, this essential iron can be damaging to humans through the Fenton reaction. During this reaction, hydroxyl radicals and higher oxidation states of the iron are generated, and in turn these oxidize a wide range of substrates, causing biological damage (205-207). Therefore, there is a need for a tight regulation of iron in the aerobic environment of the mammalian body (208).

### *b) Iron Regulation in Humans*

To prevent damage and iron toxicity, the level of iron in the body is maintained at low levels (~$10^{-18}$ M in plasma) using an iron sequestration tactic (209). Iron-scavenging extracellular transport proteins such as hemoglobin, transferrin, lactoferrin, and lipocalin, and intracellular ferritin are the main agents for iron sequestration that render free iron extremely scarce (209, 210). Approximately 70% of all the iron in the body is stored by hemoglobin in erythrocytes, whereas ferritin stores about 25% of iron in organs like the liver (211, 212). Hepcidin, a peptide hormone produced in the liver, and its receptor ferroportin play a crucial role in iron homeostasis (213). Ferroportin is a receptor on hepatocytes and macrophages that exports iron into plasma. When iron levels are too high or during infection and inflammation, increased hepcidin binds ferroportin resulting in the endocytosis of the ferroportin receptor to prevent cell iron export into the plasma (211, 214, 215). Therefore, this tight iron regulation in the host not only prevents harmful biological outcomes, but it also leads to restriction of iron from invading pathogens (201). Paradoxically, most of these iron-scavenging extracellular transport

proteins that allow iron sequestration are used by pathogenic *Neisseria* as actual sources of iron.

## VII. Host Iron Sources

The iron sources discussed here mediate iron sequestration and can be utilized by host cells with a metabolic requirement for iron. They can also be utilized by pathogenic bacteria to overcome iron deplete conditions imposed by the host during nutritional immunity.

### *a) Transferrin*

Transferrin (Tf), an 80 kDa glycoprotein, is one of the most abundant iron transporters found in human serum (216). Tf has a bi-lobed structure with a N-terminal and a C- terminal lobe, each of which has a high-affinity binding site for a single Fe3+ atom (217, 218). As the N- and C lobes are ~40% identical, they probably arose from gene duplication followed by fusion (217). In human serum, Tf concentration is about ~25 to 50 μM with only 30% of the iron binding sites loaded. Interestingly, iron is not evenly distributed between the two lobes but rather, there is a slight preference for N-lobe iron binding (216, 219, 220). A Tf receptor is present on all iron requiring cells and there is a slight bias towards ferrated Tf (221). Upon binding to this receptor, the ferrated-Tf-receptor complex undergoes endocytosis (222, 223) and the acidity of the endosome leads to the release of the iron before the apo-Tf-receptor complex is recycled back to the cell surface (216). Once on the surface, the apo-Tf is displaced by a new ferrated transferrin molecule, which binds the receptor with higher affinity than the apo form at neutral pH; therefore the apo-Tf is released back into the serum (222, 223).

***b) Lactoferrin***

Lactoferrin (Lf) is also an an 80 kDa glycoprotein of the transferrin family. Like Tf, Lf is also bi-lobed with both lobes being 37% identical and each having a high-affinity Fe3+ binding site (224, 225). It is one of the most abundant iron binding proteins found in human secretions such as tears, nasal and bronchial secretions, saliva, bile, gastrointestinal fluids, semen, vaginal fluids, and urine (226, 227). Lf is the second most abundant protein found in colostrum and breastmilk, and can also be found in amniotic fluids, blood plasma, and some leukocytes (222, 228-230). Lf is an important component of iron homeostasis because it is found in such a wide array of anatomical sites and it is also able to retain iron binding over a wide range of pHs, making it therefore very efficient at iron restriction and bacterial growth suppression (225). Besides the tactic of iron sequestration, Lf can use an autoproteolysis approach to have antimicrobial activity. During autoproteolysis, the N-terminus of Lf is cleaved resulting in the production of lactoferricins, which are small antimicrobial compounds that use charge disruption to interact with bacterial surface components causing therefore, membrane destabilization and cell lysis (231-234).

***c) Heme/Hemoproteins***

Heme is a small porphyrin ring that contains approximately 70% of iron in the human body, each heme compound coordinates a single ferrous iron atom (235). Heme plays an important role in oxygenation reactions, oxidative stress responses, electron transport, oxygen transport, oxygen sensing, and oxygen storage (236). Just like with iron, excess of this vital biomolecule is toxic to cells. This toxicity is due to its lipophilic nature and its high catalytic ability to produce reactive oxygen species (236). To avoid heme toxicity, 95% of heme is

sequestered in hemoproteins. When hemolysis occurs, Hb is broken down into αβ dimers with 2 heme groups bound (237). At this stage, toxicity prevention efforts are taken over by haptoglobin, which rapidly binds αβ dimers of Hb and shuttles them to the liver for clearance (238-240). Albumin and hemopexin are also proteins that can sequester excess heme, or heme released from damaged hemoproteins, to avoid heme toxicity (238).

***d) Ferritin***

Ferritins are heteropolymers composed of 24 subunits of two types: the heavy (H) chain and the light (L) chain, which are both required for the function of ferritin. At about 450 kDa, the subunits of the mature protein are arranged in a nearly spherical structure that can impressively accommodate up to 4500 iron atoms (241-243). Contrary to the iron sources mentioned above, ferritin binds excess intracellular iron. Inflammation and infection cause a drop in serum iron levels and an increase in cell-associated iron, leading to upregulation of ferritin in macrophages and more iron storage per ferritin molecule (244, 245). In iron deplete conditions, iron from ferritin is released by proteasomal degradation (246, 247). It was previously shown that ferritin knockout mice are nonviable; this emphasizes the crucial role of ferritins in mammalian survival (248).

## VIII. Iron Acquisition by Pathogenic Neisseria

As the pathogenic *Neisseria spp.* evolved to be human-specific, they found ways to adapt to the host environment. One way to ensure their survival is to have access to the critical metal nutrient, iron. To survive within the host, *N. meningitidis* and *N. gonorrhoeae* evolved an assortment of iron transporters, or acquisition systems, that directly hijack iron from host iron and heme sequestering proteins. To function, these high-affinity TonB-dependent outer-

membrane transporters (TdTs), require the presence of an outer-membrane receptor, the Ton motor complex, and a dedicated ABC transporter. There are eight TdTs found in *N. gonorrhoeae* with their corresponding ABC transporters and the Ton motor complex (249). Demonstrating the same overall structure, the 8 gonococcal TdTs, seen in figure 2, encompass a 22-stranded β-barrel that spans the outer membrane, 11 flexible extracellular loops, and a N-terminal folded plug domain that blocks the pore of the β-barrel (250-252).

***a) Two-Component TonB-Dependent Transporter***

In *N. gonorrhoeae*, there are 3 distinct two-component systems for iron uptake. These two-component systems are composed of 2 proteins: an integral outer-membrane TonB-dependent transporter and a lipid-modified accessory protein, called lipoprotein, tethered to the outer membrane. The first and best studied system of this kind is the transferrin-iron uptake system which has the transferrin binding protein A (TbpA) as transporter and the transferrin binding protein B (TbpB), as lipoprotein. TbpAB binds the C-lobe of both apo- and Fe-transferrin (Tf) but strips Fe-Tf of its iron to facilitate iron passage across the outer membrane (253, 254). The lipoprotein TbpB is bi-lobed with homologous C-lobe and N-lobes. The N-lobe exclusively binds the C-lobe of ferrated-Tf (Fe-Tf) (255, 256). Despite not being necessary for Fe-Tf internalization, TbpB was shown to greatly increase the efficiency of iron import, by TbpA, due to its exclusive recruitment of iron loaded Tf (254, 255, 257-260). The TbpAB system is not subject to phase variation, and it is expressed in all gonococcal and meningococcal strains (261). The genes encoding the TbpAB system are organized in an iron-repressed bicistronic operon, with *tbpB* upstream of *tbpA* (261, 262). Interestingly, TbpB is antigenically variable unlike TbpA which is very well conserved with >95% sequency identity

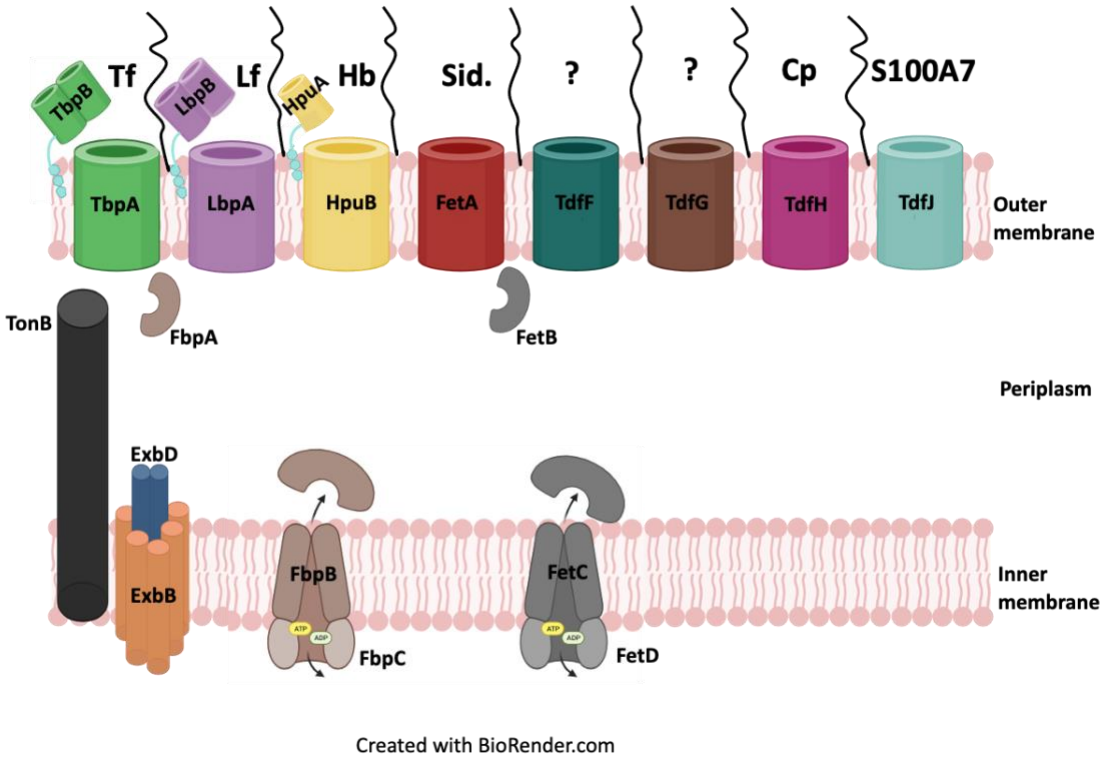



**Figure 2: Overview of the 8 Gonococcal TonB-Dependent Transporters (TdTs): Single and Two-Components TdTs and ABC Transporters.**

Cartoon schematics showing the eight TdTs produced by the gonococcus, represented by barrels. The TdTs are in the outer membrane, and their respective ligands are written. The lipoprotein components associated with TbpA, LbpA, and HpuB, are also showed to be anchored to the outer membrane. TonB, ExbB, and ExbD machinery, which energizes the transport of metals through the TdTs, is shown in the inner membrane. The ABC transport systems, which allow the internalization of metals across the inner membrane, are also shown in the inner membrane and the periplasmic space. Ligands: Tf = Transferrin; Lf = Lactoferrin; Hb = Hemoglobin; Sid = Xenosiderophore; Cp = Calprotectin.

across all gonococcal strains (263, 264). Experimental urethral infection studies showed that a gonococcal strain incapable of utilizing Tf could not to establish infection (265) and human infection trials showed that the absence of TbpAB resulted in attenuated virulence (265). Taken together, the studies confirm the importance of the Tf receptor, TbpAB, for gonococcal pathogenesis.

The second best studied two-component system strips the iron from human lactoferrin. The lactoferrin-iron acquisition system, LbpAB, is comprised of the lactoferrin binding protein A (LbpA) as the transporter and the lactoferrin binding protein B (LbpB) as the lipoprotein. With a high similarity to TbpA, LbpA structure has yet to be solved, but prediction models suggest a function similar to that of TbpA (266). Nevertheless, the solved structure of bi-lobed LbpB, complexed to Lf, confirmed that, just like with TbpB, LbpB's N-lobe exclusively binds the C-lobe of ferrated Lf (Fe-Lf) (267). This, consequently, suggests the role of LbpB in increasing the efficiency of iron uptake by LbpA, like with TbpB. Additionally, though, LbpB was found to protect *Neisseria spp.* from the lactoferrin-derived antimicrobial peptide, lactoferricin (268). LbpAB is very similar to TbpAB, with their genes also arranged in an iron-regulated bicistron, with *lbpB* upstream of *lbpA* (269, 270). Although 100% of meningococcal isolates express LbpAB, only ~50% of gonococcal strains express the Lf system (271), and contrary to TbpB, LbpB is phase variable due to a polyC-tract in the coding region (270, 271). Although not required for human infection, LbpAB allows iron acquisition in the absence of the TbpAB as shown by recovery of the lost infection phenotype seen in the *tbp* mutant (265, 271).

The third two-component system, the hemoglobin-haptoglobin uptake system, HpuAB, allows iron acquisition from hemoglobin (Hb) and hemoglobin-haptoglobin (Hb-Hp) complexes

(272-275). HpuAB is found in both pathogenic *Neisseria spp*, however a single component form of this Hb transporter, HmbR, is expressed only in *N. meningitidis* as its gonococcal ortholog is a pseudogene (272). HpuAB is composed of HpuB the transporter and HpuA the lipoprotein, and both proteins are required for heme internalization, unlike TbpB and LbpB which are not necessary for transport (273, 274, 276). Interestingly, the Hpus extract heme from Hb, for transport, instead of iron like with the Tf and Lf systems (277). HpuAB is less characterized but attempts at a crystal structure for the gonococcal HpuA, that is about half the size of the other lipoproteins were made. Based on the known crystal structure of *Kingella denitrificans*'s HpuA, a one-lobed lipoprotein structure, that is structurally similar to the C-lobe of TbpB and LbpB was obtained (278). HpuAB is found in less than 1% of gonococcal strains yet phase-on isolates are found in women infected during the first half of their menstrual cycle (279). Like the Lf system, HpuAB is phase variable due to a polyG-tract in the coding region of *hpuA* (280, 281). Heme acquired through this receptor is sufficient as a sole iron source to support gonococcal growth (273). Surprisingly, meningococcal HpuAB is unique in that it can bind to an array of other species' hemoglobin unlike with the Tf and Lf systems that exclusively bind a human ligand (282). Taken together, these studies have yet to establish the role of HpuAB in gonococcal pathogenesis.

***b) Single-Component TonB-Dependent Transporter***

In *N. gonorrhoeae*, there are 5 distinct single-component transport systems, for iron or zinc uptake. These single-component TdTs only have a transmembrane protein and do not possess a lipoprotein.

The first out of the five, FetA, is particular as its ligand is not a human protein but rather a bacterial siderophore. FetA allows *Neisseria* to uptake siderophores, which are small iron chelating compounds, produced by other bacterial species as part of their iron scavenging activity (283). As *Neisseria* themselves do not produce siderophores, they hijack the siderophores made by other bacteria (284, 285). The solved crystal structure of *Neisseria* FetA revealed a trimer, with the size of the monomers at 76 kDa each. FetA was also found to interact with free iron atoms, suggesting some capacity for non-siderophore-associated iron uptake (286). FetA undergoes phase variation due to a poly-C tract between the promoter's -10 and -35 region resulting in changes in the strength of the promoter, affecting therefore, gene expression (287).

The remaining four TdTs are together called TonB-dependent function (Tdf) F, G, H, and J (288). Little is known about TdfF and TdfG, their human ligands have yet to be identified but their genes are repressed in the presence of iron, suggesting a role in iron uptake (261, 289, 290). TdfF is known to be required for gonococcal survival in human cervical epithelial cell culture (291). TdfF is expressed by both pathogenic *Neisseria* whereas TdfG is only expressed in the gonococcus. TdFH, called calprotectin binding protein A (CbpA) in *N. meningitidis*, allows the uptake of zinc from human calprotectin found in abondance in neutrophils (292-294). TdfH is a 100 kDa protein that targets calprotectin's Site I, a non-canonical Zn2+/Mn2+ binding pocket (295). TdfH is not known to exhibit any phase or high-frequency antigenic variation. The last TdT, TdfJ or zinc uptake D (ZnuD) in *N. meningitidis*, is an 85 kDa protein that also allows uptake of zinc, from human S100A7 (249, 292, 296, 297). TdfJ can directly bind to free zinc, which is then internalized and can also be found in commensal *Neisseria* species (298). It was

previously found that a mutant unable to produce ZnuD was defective for systemic infection by *N. meningitidis* in mice; however, this mutant could still locally infect the nasopharynx, therefore, suggesting a potential role for TdfJ in the bloodstream (298). Interestingly, *tdfJ* was found to be both transcriptionally induced by iron and repressed by zinc yet the mechanism of iron regulation of *tdfJ* is unresolved (289). Curiously, ZnuD contained a consensus-like hemin-binding motif, and even co-sediments with hemin when overexpressed in *E. coli*, however, is not known to contribute to heme uptake by *Neisseria* (299). Finally, TdfJ is also not known to exhibit any phase or high-frequency antigenic variation.

### *c) The Ton Motor Complex*

In these 8 Ton-B dependent transporters described above, following ligand binding to the receptor, the transmembrane transport of iron, siderophores, heme and zinc is dependent on the Ton motor complex for metabolic energy (300, 301). The Ton complex is composed of 3 proteins, TonB, ExbB, and ExbD, all localized in the cytoplasmic membrane and their respective genes are encoded in an iron repressed operon (289). TonB has 3 functional domains: an N-terminal transmembrane domain, a proline-rich spacer in the periplasm, and a C- terminal domain that interacts with TdTs (302). Although not completely characterized, the general function of the Ton motor complex was established. Upon ligand binding to its corresponding TdTs, a conformational change within the transporter exposes the TonB-box, a conserved region within the plug domain of TdTs, to the periplasm. In the periplasmic space, the C-terminal domain of TonB forms a stable complex with the TonB box (303). The next exact step of this mechanism is unclear; however, it has been demonstrated that the energy that drives the Ton system is harnessed from the proton motive force (250, 304, 305). One thought is that

TonB, energized by the proton motive force, interacts with the TonB box to displace or alter the conformation of the TdT plug domain, consequently facilitating transfer of the metal substrate across the membrane and into the periplasm. More studies are needed to completely establish the steps required for TonB-dependent substrate acquisition.

### *d) ABC Transporters*

Once in the periplasmic space, the transferred substrates discussed above are shuttled through the cytoplasmic or inner membrane into the cytoplasm. This transport is facilitated by ATP-binding cassette (ABC) transporters. ABC transporters are composed of three parts: a periplasmic binding protein that binds metal ions in the periplasm and takes it to the second part, a cytoplasmic permease that forms a pore in the cell membrane for entry into the cell, and an ATP-ase that hydrolyzes ATP to energize the system (306, 307). So far, three ABC transporters have been found to service the TdTs: FbpABC shuttles the iron transported by TbpAB and LbpAB (308-310), FetBCD shuttles the iron transported by FetA (284), and ZnuABC shuttles the zinc transported by TdfH and TdfJ (249, 311, 312). FbpABC and FetBCD, are iron repressed (287, 289). In different studies, MntABC, also called ZnuABC, has been found to shuttle both manganese and zinc, suggesting a dual function for ZnuABC (313). An analogous ABC transporter is hypothesized to exist for heme transport, but to date none is known to exist in the *Neisseria*. Similarly, interactions between any ABC transporters and TdfF or TdfG have not yet been discovered, but it is conceivable that they could exist.

### *e) Iron Regulation in Neisseria*

The *Neisseria* must maintain adequate iron uptake and homeostasis, just like the mammalian hosts that were previously discussed. TDTs which are iron uptake systems are tighly

repressed by iron, a process mediated by the ferric uptake regulator (Fur) protein (314). When available iron is high, Fur monomers complex with Fe3+ ions before dimerizing, then dimerized Fur binds the "Fur box," a specific DNA sequence that overlaps with the promoters of iron-regulated genes (314, 315). This Fur-Fur box binding interaction physically inhibits access to the promoter by RNA polymerase, thereby, repressing these iron-regulated genes (285, 316). As human specific pathogens, *Neisseria* needs to adjust quickly to the iron limited environment imposed by the host. As iron stores are depleted, the insufficient presence of Fe3+ keeps Fur in its monomeric form leading to transcription of the iron-regulated genes by RNA polymerase (315, 317, 318). Therefore, in iron deplete conditions, *Neisseria* can upregulate their TdTs to ensure their survival.

## IX. Research Objectives

The goal of the research described in this dissertation is to better understand the not so well-characterized HpuAB hemoglobin transport system. This work was initiated to better define the structure-function relationships in these proteins. Throughout this thesis we pursued three primary objectives. 1) We tried to establish the importance of several residues, in the lipoprotein HpuA, for the growth and binding of *N. gonorrhoeae* with Hb as a sole iron source, in the presence or absence of the transporter HpuB. This work was also proposed by Dr. Stephen Hare as a continuation of the study by Wong *et al.*, where these same residues were previously shown to be important for gonococcal binding to Hb, in vitro and in the absence of HpuB. 2) We investigated the role of extracellular loops 2, 3, 4 of HpuB and particularly extracellular loop 7 and its heme binding motifs with respect to human Hb binding and heme/iron uptake. 3) As previously seen in in the meningococcus, we investigated the ability of

*N. gonorrhoeae* to bind an array of hemoglobin from different species. These studies have helped better characterize the HpuAB utilization system and have provided more insight into the use of these TdTs as vaccine targets.

# CHAPTER 2: BACTERIAL APPROACHES TO HEME UPTAKE, FROM MULTIPLE SOURCES

## I. The Different Sources of Heme

Heme is an iron protoporphyrin IX involved in many essential functions both in prokaryotes and eukaryotes. It is a prosthetic group found in hemoglobin and cytochromes. Heme works as a tetrapyrrole-based cofactor for many enzymes and is thought to be required for many processes including oxygen metabolism, and respiration (319, 320).

Heme is a readily available source of iron for pathogenic organisms. In the human host, 95% of iron is stored in the form of heme or heme-binding proteins, which can in turn be useful sources of iron for nutrition of pathogens (321, 322). During infection, bacteria attempt to obtain iron from host heme sources such as hemoglobin, haptoglobin-hemoglobin, and hemopexin-heme. Moreover, some pathogenic bacteria can either secrete hemolysins to release hemoglobin or produce proteases to degrade hemoglobin to access heme and consequently obtain iron (323). These heme-binding proteins keep free heme away from invading pathogens through a process called nutritional immunity. However, bacteria have evolved sophisticated ways to overcome this depletion by hijacking the following heme-binding proteins and using them as heme sources to cause pathogenesis (235).

### *a) Hemoglobin and Haptoglobin-Hemoglobin*

Hemoglobin is found in red blood cells and functions as an oxygen transporter. It forms a tetramer composed of two α- and two β-chains (324). Each of these subunits bind a single heme molecule, thus one hemoglobin contains four heme groups. Hemoglobin is a hemoprotein, that can be utilized by many bacterial pathogens as a source of heme.

After hemolysis, free hemoglobin dimers are bound by haptoglobin in the body with high affinity, to form haptoglobin-hemoglobin complexes. Similarly, these complexes represent another source of iron (277, 325). However, only some bacteria can utilize haptoglobin-hemoglobin complexes as a heme source (326-328).

***b) Heme-Hemopexin***

Hemopexin is a major carrier of free heme in plasma. This hemoprotein functions to prevent oxidative stress incurred by the presence of free heme and has an affinity for heme that is higher than that of the other heme-bound proteins (329, 330). An example of a pathogen capable of direct hemopexin utilization has yet to be shown, but multiple pathogens have been reported to bind and/or indirectly use hemopexin (331).

***c) Heme-Albumin***

Heme-albumin is the most abundant protein found in plasma and is capable of binding heme. Although no albumin-specific receptors have been described yet, human serum albumin can be a good heme source for pathogens in laboratory conditions. Albumin not only shields the heme from chemical reactivity by binding it, but it also allows for hemophore-mediated heme uptake which in turn mitigates hemopexin-mediated inhibition of *Candida albicans* (332).

***d) Hemophores***

Hemophores are functionally analogous to siderophores; hemophores are bacterial secreted proteins that target heme instead of iron (333). They are thought to be indirect sources of heme because they are extracellular proteins that bring heme, previously acquired from other heme sources, to specific outer membrane receptors (334).

These aforementioned heme-containing proteins constitute a gold mine for infectious bacteria as heme-containing proteins represent numerous sources of the essential nutrient, iron. Some bacterial species, such as *Haemophilus influenzae*, can have multiple heme receptors that are either expressed in different environments or bind different kinds of hemoproteins (325, 331). However, other bacteria, like *Yersinia enterocolitica*, only have one hemoprotein receptor, HemR, that binds multiple hemoproteins by only recognizing their bound heme molecule (335).

## II. Gram-Negative Bacterial Uptake of Exogenous Heme

Over thirty different outer membrane heme receptors in Gram-negative bacteria have been well characterized (336). The structure of these receptors includes a beta-barrel with extracellular loops that bind to free heme, hemoproteins, or hemophores. Heme-binding proteins seem to contain two conserved histidine residues located between two conserved motifs, FRAP and NPNL, although not all possess the histidine residues (335, 337). During transport through the outer membrane, the TonB/ExbB/ExbD system transduces energy from the proton motive focrce to the outer membrane to allow the uptake of heme (338). Once through the outer membrane receptor, a periplasmic heme transport protein (part of an ABC transport system) takes the heme through the periplasm. Next, via active transport, the transmembrane transport proteins of the ABC transport system shuttle the heme across the inner membrane, into the cytoplasm. The best characterized periplasmic heme transport proteins are ShuT in *Shigella dysenteriae* and PhuT in *Pseudomonas aeruginosa* (339, 340).

The heme uptake systems are divided into three classes: direct uptake, bipartite heme receptors and hemophore-mediated heme uptake systems (337). The differences between these three classes are discussed below.

***a) Direct Uptake***

During direct uptake, a single specific outer membrane receptor sequesters heme from free heme or host hemoproteins and internalize it into the periplasm in a TonB-dependent manner. As described above the heme is then shuttled into the cytoplasm with the help of an ABC transporter. The most well-characterized direct heme uptake systems are HemRSTUV in *Yersinia enterocolitica*, and PhuSTUVW in *Pseudomonas aeruginosa* where HemR, and PhuR are the respective receptors (341, 342).

***b) Bipartite Heme Receptors***

A bipartite receptor utilizes two proteins for heme uptake has been described in bacteria such as *N. meningitidis*, *N. gonorrhoeae* and *Porphyromonas gingivalis* (275, 343). The TonB-dependent heme receptors that mediate heme transport are HpuB and HmuR in the *Neisseria* species and in *P. gingivalis,* respectively. Another component of the bipartite receptor is a lipid anchored lipoprotein such as HpuA (*Neisseria*) and HmuY (*P. gingivalis*) which also help in the binding of heme-containing proteins. It was previously found that the HpuAB bipartite heme receptor was required for the utilization of either hemoglobin or haptoglobin-hemoglobin complexes (277).

***c) Hemophore-Mediated Heme Uptake***

There are two types of hemophore-mediated heme uptake systems in Gram-negative bacteria, HasA and HxuA.

The Heme Acquisition System (Has) or HasA is a hemophore-mediated heme uptake system that has been identified in many bacteria such as *Serratia marcescens* ($HasA_{SM}$), *Pseudomonas aeruginosa* ($HasA_{PA}$), and *Yersinia pestis* ($HasA_{YP}$) (344-346). HasA are the first class of hemophores identified. HasA are also a family of highly conserved proteins secreted by ABC transporters which in turn provide the energy required to export the hemophore in the external medium (347). There is passive transfer of heme from the heme-carrier proteins to the hemophore which have a higher affinity (348). Hemophores capture free heme, or heme from heme-carrier proteins, and present it to a TonB-dependent, hemophore-specific, outer membrane receptor, HasR.

Hxu is another identified hemophore-mediated heme uptake system, it is synthesized from the *hxuCBA* gene cluster. Hxu allow *Haemophilus influenza* type B to acquire exogenous heme from heme-hemopexin for aerobic growth and it interacts with hemopexin, by preventing it from sequestering heme (331). However, contrary to HasA above, HxuA does not bind to heme alone (349). The process by which heme is handed to HxuA remains to be determined, but the heme-hemopexin-HxuA complex is found in the external medium and is also presented to a TonB-dependent outer membrane receptor, HxuC for transport across the membrane (350).

**III. Gram-Positive Exogenous Heme Uptake Systems**

Heme uptake in Gram-positive bacteria is not as understood as in Gram negative bacteria, but they both seem to require ABC transporters (337). The ABC transporters allow the internalization of heme via active transport. The different heme transport systems are discussed below.

### *a) Near Transporter (NEAT)-Mediated Heme Uptake Systems*

The NEAT domain is a poorly conserved 120-125 amino acid regions that is encoded in variable numbers near ABC iron transporter genes; NEAT stands for near transporter (351). NEAT domains can bind heme, hemoglobin, or hemoglobin-haptoglobin. A few NEAT-type heme uptake systems are discussed below.

In *Staphylococcus aureus*, Iron-regulated Surface Determinant (Isd) genes, *isdA-F*, encode a NEAT-type heme acquisition system. Hemoglobin is bound by the cell surface protein, IsdB, which contains $NEAT1_{IsdB}$ and $NEAT2_{IsdB}$ domains. The $NEAT1_{IsdB}$ domain is required and sufficient for hemoglobin binding and the $NEAT2_{IsdB}$ domain is required and sufficient for heme binding (352). Heme from hemoglobin is removed by IsdB and passed to another surface protein containing one NEAT-domain, IsdA, which in turn transfers it to IsdC, a cell wall protein (353). IsdC shuttles the heme through the cell wall and hands it off to the membrane transporter IsdDEF for transport in the cytoplasm (354). IsdH, in *S. aureus*, is another NEAT-domain containing cell surface protein, it can bind heme ($NEAT3_{IsdH}$), and hemoglobin or haptoglobin-hemoglobin complexes ($NEAT1_{IsdH}$ and $NEAT2_{IsdH}$) (355). As described above, IsdH also shuttles heme to IsdA then IsdC.

Another example, the heme uptake system Shp-Shr-HtsABC, was identified in *Streptococcus pyogenes*. The cell surface protein, Shr, binds hemoglobin and the hemoglobin/haptoglobin complex then hands off heme to Shp, another cell surface protein containing NEAT domains (356-358). Heme is finally passed to the ABC transporter, HtsABC (or SiaABC), to be transported across the membrane (359).

Finally, the only hemophore-mediated uptake described in Gram-positive bacteria was found in *Bacillus anthracis* and is composed of three proteins: IsdX1 containing one NEAT domain, IsdX2 containing five NEAT domains, and IsdC (360). IsdX1 and IsdX2 are the secreted hemophores that take heme from hemoglobin and hand it to IsdC, which is cell wall-anchored (361). Moreover, Hal and BslK were proteins identified in *B. anthracis* and found to have a NEAT domain that bind and acquire heme from hemoglobin (362, 363). As in *S. aureus*, these proteins bind and transfer heme to the Isd system via the cell wall protein IsdC, followed by IsdDEF transport across the cell membrane.

***b) Non-NEAT Mediated Heme Uptake Systems***

The direct uptake of heme is facilitated by cell surface heme receptors, cell wall chaperone proteins and ABC transporters. The cell surface heme receptors initially bind heme then a heme transfer cascade takes heme to cell wall proteins which then hand it off to ABC transporters that in turn translocate the heme across the cell membrane (236).

In *Corynebacterium diphtheriae*, two heme uptake systems were identified, HtaAB-HmuTUV which binds hemoglobin and ChtABC-CirA which binds both heme and hemoglobin (364, 365). In the HtaAB-HmuTUV system, cell surface exposed protein HtaA binds hemoglobin then passes heme to HtaB, another cell surface protein that will then hands it to HmuT. Eventually, HmuT, which is cell wall-associated, and the ATP transporter HmuUV enable the transport of heme into the cytoplasm (364, 366, 367). The exact transport order of heme between HtaAB and HmuTUV has yet to be defined.

Similarly, ChtAB and CirAChtC are exposed to the surface, bind heme and hemoglobin and function just like HtaAB. It is also thought that ChtAB and ChtC are a result of gene duplication of HtaAB (365).

**IV. Mycobacterium Tuberculosis Utilization of heme**

*Mycobacterium tuberculosis* utilizes heme as an iron source. This heme uptake system has been more recently discovered and is sufficient to rescue the growth of a heme auxotroph (368). A secreted hemophore Rv0203 binds heme and transfers it to the transmembrane proteins MmpL11 and MmpL13 (369, 370). Rv0203 and HasA have a similar heme binding motif but no structure or sequence similarities (371).

**V. Heme Processing After Transport in the Bacterial Cytoplasm**

Not much is known about the fate of heme in the cytoplasm of bacterial cells. One hypothesis, “heme hijacking”, is that bacteria directly use exogenous heme from the host. This would be more energetically advantageous, since heme biosynthesis require multiple enzymatic steps (372). Furthermore, heme biosynthetic mutants were found to also require exogenous heme as an iron source (373-375). This solidifies a “heme hijacking” hypothesis, during which the host heme can be used in some bacterial heme proteins (319).

In contrast, in many bacteria cleavage of heme is the way to have access to iron, especially during iron restriction, suggesting another fate for the cytoplasmic heme (376-378). The cleavage of heme is done by heme oxygenases, which degrade heme into iron and heme degradation products (379). Many bacteria have canonical heme oxygenases, homologous to the mammalian oxygenase HO-1, that degrade heme into ferrous iron, biliverdin, and carbon monoxide (377). However, new classes of non-canonical heme oxygenases, not homologous to

the human one, were found in *S. aureus* and *M. tuberculosis* (378, 380, 381). The heme degrading enzymes in *S. aureus*, IsdI and IsdG, produce iron, staphylobilins and formaldehyde as heme degradation products (382, 383). MhuD, in *M. tuberculosis* is another non-canonical oxygenases that degrade heme to liberate iron and mycobilin (381). Additionally, a new structural class, the "split-barrel fold" class, of heme oxygenases has been identified and includes HugZ in *Helicobacter pylori* and ChuZ in *Campylobacter jejuni* (384, 385).2

## VI. Heme: Biosynthesis, Regulation and Toxicity

Heme biosynthesis is a process during which heme is synthesized following three conserved steps. During the process, the amino levulinic acid (ALA) heme precursor is converted to a coproporphyrinogen III which can be utilized by different pathways for the synthesis of various tetrapyrrole-based cofactors such as heme. As reviewed in Choby *et al.*, the role of heme synthesis in pathogenesis of bacteria is not well understood, but in *S. aureus*, heme biosynthesis is vital to cause disease in murine models of infection (386). It was also discovered that prokaryotes have three distinct heme biosynthetic pathways (387, 388). Some bacteria, although not all, can also synthesize heme endogenously through the coordinated effort of enzymes as with *S. aureus* (389). However, some bacteria can both synthesize endogenous and acquire exogenous heme. The existence of heme storage proteins has also been hypothesized in bacteria that acquire exogenous heme, as heme biosynthesis is strictly regulated. As suggested by Anzaldi and Skaar, a way to understand the duality of heme acquisition and biosynthesis probably depends on the energetic costs of heme biosynthesis compared to those of heme acquisition or uptake (236).

Bacteria have evolved to sense iron depletion, due to the presence of Fur (ferric uptake regulator), the iron-dependent repressor (390). Presumably, heme levels can also be sensed by bacterial pathogens with the help of heme-sensing systems that signal contact with heme-rich tissues (390). Heme uptake regulation during infection is often negatively controlled by Fur, with the expression of the genes involved in heme catabolism often repressed by iron levels and de-repressed by heme levels in the environment. Many of the genes involved in heme acquisition are regulated by the Fur repressor or by the functionally similar Diphtheria Toxin Repressor (DtxR) (391). Many oxygeneases are also Fur-regulated such as IsdG and IsdI in *S. aureus*, PigA/HemO in *P. aeruginosa* and ChuZ in *Campylobacter jejuni* (392-394). Another regulatory mechanism involves specific extracytoplasmic function (ECF) sigma and anti-sigma factors. For instance, in *Bordetella pertussis*, the ECF sigma factor HurI and its anti-sigma factor HurR control the expression of the *bhuRSTUV* heme uptake operon. The iron starvation induces the heme uptake regulator genes, *hurIR*. However, only the presence of heme, sensed by BhuR the outer membrane receptor protein, induces HurI-mediated transcriptional activation of the *bhuRSTUV* promoter (395, 396).

Conversely, bacteria sense excess intracellular heme, which is toxic to them, therefore forcing these pathogens to require heme detoxification tactics (386). The toxicity of heme is due to its ability to catalyze the formation of reactive oxygen species. Although there is no direct correlation between DNA damage and heme toxicity, it was suggested that the two can explain why heme can be toxic to bacteria as iron-mediated production of reactive oxygen species can damage DNA, lipids, and protein (397-400). Little is known about the mechanism of heme toxicity and much about its process have yet to be discovered. Gram-negative bacteria

tend to be less sensitive to heme toxicity than their Gram-positive counterparts (401). Bacteria detect the presence of toxic levels of heme with the help of a heme sensor system and consequently use specific methods to prevent heme toxicity (402-404). Pathogens have evolved many strategies to overcome heme toxicity such as repression of heme internalization and heme biosynthesis systems. The other strategies employed by pathogens to overcome heme toxicity are the activation of export (efflux pumps removing heme or toxic metabolites of heme accumulation), sequestration (sequester heme into nontoxic molecules), and degradation (heme oxygenase-mediated degradation) systems (403, 405, 406). For example, in *S. aureus*, the heme sensing two-component system, HssRS, regulates the expression of the heme-regulated ABC transporter HrtAB, which exports molecules (heme or other) that accumulates in the cytoplasm upon heme exposure, thereby mitigating heme toxicity (404).

Iron and heme sensing mechanisms are important processes that lead to the tight regulation of heme biosynthesis, uptake, degradation, and efflux. Consequently, this tight regulation, has a profound impact on the survival of pathogens and their ability to cause disease.

## VII. Perspectives

With the lack of vaccine against sexually transmitted infections (STI), new strategies to combat the global STIs are being investigated. As many microbial pathogens are highly susceptible to porphyrin-based compounds, one such strategy is to look at porphyrins and metalloporphyrins as topical microbicides to block bacterial STI. It was previously found that heme-analogous compounds composed of a porphyrin moiety and a non-iron metal ion, different from iron, inhibited many bacterial pathogens, by replacing heme as a cofactor and

preventing many enzymes from functioning (407-409). Therefore, understanding the mechanism of heme uptake in pathogenic bacteria as well as its role in pathogenesis can lead to the development of novel therapeutic strategies against bacteria capable of utilizing exogenous heme. Therefore, understanding the metabolism of heme in pathogenic bacteria as well as its role in pathogenesis can lead to the development of novel therapeutic strategies against bacteria capable of utilizing exogenous heme.

# CHAPTER 3: MATERIALS AND METHODS

Bacterial strains used in this work can be found in Table 1 with their genotypes. A list of the plasmids and their descriptions is in Table 2. Finally, the oligonucleotides used in this work are listed in Table 3.

## I. Bacterial Strains and Growth Conditions

Luria-Bertani (LB) medium used to culture *E. coli* strains was supplemented with antibiotics at the following concentrations: 100 μg/mL for carbenicillin, 34 μg/mL for chloramphenicol, 50 μg/mL Kanamycin, 50 μg/mL streptomycin and 50 μg/mL for spectinomycin. GC medium base (Difco) agar with Kellogg's supplement I (410) and 12 μM $Fe(NO_3)_3$ (constitute a GCB plates) was used to grow and maintain strains of *N. gonorrhoeae* at 36$^{o}$C with 5% CO2. GCB plates were sometimes supplemented with 1 mM Isopropyl-β-D-thiogalactoside (IPTG) and 12.5 μM deferoxamine mesylate (Desferal/DFO), lacking $Fe(NO_3)_3$ (GCB/DFO/IPTG plates) to induce relevant protein production. The addition of DFO deprived gonococcal strains of iron, and IPTG induced the promoter behind which the mutations are inserted. When *N. gonorrhoeae* was grown in liquid culture, colonies from GCB/DFO/IPTG plates collected with a cotton swab and resuspended in chemically defined medium (CDM), that had previously been treated with Chelex-100 resin (Bio-Rad). For whole-cell ELISA, cultures were grown until reaching exponential phase before back dilution with CDM and treatment with 1 mM IPTG. One hour post back dilution, IPTG was added and the growth was allowed to proceed for 4 hours, after which a standardized density of whole cells was added to a 96-well MaxiSorp microtiter dish (Nunc) plate for ELISA.

## II. Gonococcal Mutant Construction

Ectopic *hpuA* mutants with or without native *hpuB* were constructed using wild-type (WT) or mutated *hpuA* gene sequences (strain FA19) obtained from Dr. Stephen Hare (Table 1). Similarly, ectopic *hpuB* mutants with or without native *hpuA* were constructed using wild-type (WT) or mutated *hpuB* gene sequences (strain FA19) submitted to Genewiz Inc. for *de novo* synthesis (Table 1). These genes were subsequently subcloned into pVCU234 SmaI site (Table 2), using restriction endonucleases from New England BioLabs (NEB). Eight nucleotides (3 nucleotide from the SmaI site and 5 more nucleotides) space separate the plasmid ribosome-binding site from the gene start codon. For gonococcal transformations, these plasmids (Table 2) were linearized with PciI before being used to transform piliated strains. Strains RSC150 (*hpuA*- *hpuB*-) and RSC125 (ectopic *hpuA+, hpuB+*) were used to make the *hpuA* mutants and strains RSC150 (*hpuA*- *hpuB*-) and RSC275 (*hpuA*+, ectopic *hpuB+*) were used to make the *hpuB* mutants. GCB plates supplemented with chloramphenicol at 1 μg/ mL, were used to select for transformants and PCR (using primers in Table 3), followed by sequencing, allowed the verification of the *hpuA* and the *hpuB* genes.

**Table 1. Strains Used in This Study**

| Strain | Genotype | Reference |
|---|---|---|
| ***E. coli*** | | |
| TOP 10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ- | Invitrogen |
| DH5α | F + endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYAargF)U169, hsdR17(rK–mK+), λ– | NEB |
| ***N. gonorrhoeae*** | | |
| FA19 | Wildtype | (411) |
| HpuA project | | |
| RSC150 | FA19 *hpuA::Ω (hpuA- hpuB-)* | This Study |
| RSC151 | FA19 *hpuA::kan (hpuA-hpuB+)* | This Study |
| RSC152 | RSC150 + ectopic WT *hpuA* | This Study |
| RSC153 | RSC150 + ectopic Y78A mutant *hpuA* | This Study |
| RSC154 | RSC150 + ectopic Y81A mutant | This Study |
| RSC155 | RSC150 + ectopic Δ77-82 mutant *hpuA* | This Study |
| RSC156 | RSC150 + ectopic Δ111-116 mutant *hpuA* | This Study |
| RSC157 | RSC150 + ectopic Δ225-231 mutant *hpuA* | This Study |
| RSC186 | RSC150 + ectopic F79A mutant *hpuA* | This Study |
| RSC187 | RSC150 + ectopic Y111A mutant *hpuA* | This Study |
| RSC188 | RSC150 + ectopic D115A mutant *hpuA* | This Study |
| RSC189 | RSC150 + ectopic F116A mutant *hpuA* | This Study |
| RSC190 | RSC150 + ectopic S140R mutant *hpuA* | This Study |
| RSC191 | RSC150 + ectopic G141R mutant *hpuA* | This Study |
| RSC192 | RSC150 + ectopic Y226A mutant *hpuA* | This Study |
| RSC193 | RSC150 + ectopic Y227A mutant *hpuA* | This Study |
| RSC194 | RSC150 + ectopic F295A mutant *hpuA* | This Study |
| RSC195 | RSC150 + ectopic R299A mutant *hpuA* | This Study |
| RSC196 | RSC150 + ectopic CC136/137SS mutant *hpuA* | This Study |
| RSC117 | RSC150 + ectopic S112A mutant *hpuA* | This Study |
| RSC118 | RSC150 + ectopic S113A mutant *hpuA* | This Study |
| RSC119 | RSC150 + ectopic P114A mutant *hpuA* | This Study |
| RSC125 | RSC151 + ectopic WT *hpuA* | This Study |

| | | |
|---|---|---|
| RSC126 | RSC151 + ectopic Y78A mutant *hpuA* | This Study |
| RSC127 | RSC151 + ectopic Y81A mutant | This Study |
| RSC128 | RSC151 + ectopic Δ77-82 mutant *hpuA* | This Study |
| RSC129 | RSC151 + ectopic Δ111-116 mutant *hpuA* | This Study |
| RSC130 | RSC151 + ectopic Δ225-231 mutant *hpuA* | This Study |
| RSC131 | RSC151 + ectopic F79A mutant *hpuA* | This Study |
| RSC132 | RSC151 + ectopic Y111A mutant *hpuA* | This Study |
| RSC133 | RSC151 + ectopic D115A mutant *hpuA* | This Study |
| RSC134 | RSC151 + ectopic F116A mutant *hpuA* | This Study |
| RSC135 | RSC151 + ectopic S140R mutant *hpuA* | This Study |
| RSC136 | RSC151 + ectopic G141R mutant *hpuA* | This Study |
| RSC137 | RSC151 + ectopic Y226A mutant *hpuA* | This Study |
| RSC138 | RSC151 + ectopic Y227A mutant *hpuA* | This Study |
| RSC139 | RSC151 + ectopic F295A mutant *hpuA* | This Study |
| RSC140 | RSC151 + ectopic R299A mutant *hpuA* | This Study |
| RSC141 | RSC151 + ectopic CC136/137SS mutant *hpuA* | This Study |
| RSC121 | RSC151 + ectopic S112A mutant *hpuA* | This Study |
| RSC122 | RSC151 + ectopic S113A mutant *hpuA* | This Study |
| RSC123 | RSC151 + ectopic P114A mutant *hpuA* | This Study |
| | | |
| <u>HpuB project</u> | | |
| | | |
| RSC275 | RSC151 + pGSU451 *hpuB::Ω* (*hpuA+ hpuB-*) | This Study |
| RSC276 | RSC150 + ectopic WT *hpuB (hpuA- hpuB+)* | This Study |
| RSC277 | RSC150 + ectopic Δ236-246 mutant *hpuB* | This Study |
| RSC278 | RSC150 + ectopic Δ306-311 mutant *hpuB* | This Study |
| RSC279 | RSC150 + ectopic Δ366-370 mutant *hpuB* | This Study |
| RSC281 | RSC150 + ectopic Δ538-544 mutant *hpuB* | This Study |
| RSC282 | RSC150 + ectopic Δ555-559 mutant *hpuB* | This Study |
| RSC283 | RSC150 + ectopic ΔH548 mutant *hpuB* | This Study |
| RSC284 | RSC275 + ectopic WT *hpuB (hpuA+ hpuB+)* | This Study |
| RSC285 | RSC275 + ectopic Δ236-246 mutant *hpuB* | This Study |
| RSC286 | RSC275 + ectopic Δ306-311 mutant *hpuB* | This Study |
| RSC287 | RSC275 + ectopic Δ366-370 mutant *hpuB* | This Study |
| RSC289 | RSC275 + ectopic Δ538-544 mutant *hpuB* | This Study |
| RSC290 | RSC275 + ectopic Δ555-559 mutant *hpuB* | This Study |
| RSC291 | RSC275 + ectopic ΔH548 mutant *hpuB* | This Study |

**Table 2. Plasmids Used in This Study**

| Plasmid | Description | Reference |
|---|---|---|
| pKH37 | Gonococcal complementation vector | (412) |
| pVCU234 | pKH37 + Ribosome Binding Site | (413) |
| | | |
| HpuA project | | |
| pVCU235 | pVCU234 + WT *hpuA* | (413) |
| pVCU236 | pVCU234 + Y78A | (413) |
| pVCU238 | pVCU234 + Y81A | (413) |
| pVCU249 | pVCU234 + Δ77-82 | (413) |
| pVCU250 | pVCU234 + Δ111-116 | (413) |
| pVCU251 | pVCU234 + Δ225-231 | (413) |
| pVCU252 | pVCU403 + *hpuA* locked phase on R299A at SmaI site | (413) |
| pGSU421 | pVCU252 + *Ω* cassette at PpuMI site | This Study |
| pGSU422 | pVCU252 + *kan2* cassette transposon kit | This Study |
| pGSU423 | pVCU234 + F79A | This Study |
| pGSU424 | pVCU234 + Y111A | This Study |
| pGSU425 | pVCU234 + D115A | This Study |
| pGSU426 | pVCU234 + 116A | This Study |
| pGSU427 | pVCU234 + S140R | This Study |
| pGSU428 | pVCU234 + G141R | This Study |
| pGSU429 | pVCU234 + Y226A | This Study |
| pGSU430 | pVCU234 + Y227A | This Study |
| pGSU431 | pVCU234 + F295A | This Study |
| pGSU432 | pVCU234 + R299A | This Study |
| pGSU433 | pVCU234 + CC136/137SS | This Study |
| pGSU456 | pVCU234 + S112A | This Study |
| pGSU457 | pVCU234 + S113A | This Study |
| pGSU458 | pVCU234 + P114A | This Study |
| HpuB project | | |
| pGSU442 | pVCU234 + WT *hpuB* | This Study |
| pGSU443 | pVCU234 + Δ236-246 (immature site) | This Study |
| pGSU444 | pVCU234 + Δ306-311 | This Study |
| pGSU445 | pVCU234 + Δ366-370 | This Study |
| pGSU446 | pVCU234 + Δ424-429 | This Study |
| pGSU447 | pVCU234 + Δ538-544 | This Study |
| pGSU448 | pVCU234 + Δ555-559 | This Study |
| pGSU449 | pVCU234 + ΔH548 | This Study |
| pGSU450 | pVCU403 + *hpuA* (downstream of polyG tract) + half of *hpuB* | This Study |
| pGSU451 | pGSU450 + Ω cassette in *hpuB* | This study |

**Table 3. Primers Used in This Study**

| Primer | Sequence | Purpose/Target |
|---|---|---|
| oVCU816 | CTAGGCACCCCAGGCTTTACAC | pKH37 sequencing |
| oGSU053 | CAACGCCAACCGCAACAC | *hpuB* sequencing - F |
| oGSU054 | CCAACGAGCTGTCTAATTTGTGGACGG | *hpuB* sequencing – F |
| oGSU055 | GCATACGCTACGACAAAAACAGC | *hpuB* sequencing - F |
| oGSU086 | CGC CCA GCA GAT ACC TTA CC | *hpuB* sequencing - F |
| oGSU087 | CCT GGG TGA AAG GCA TAG AG | *hpuB* sequencing - F |
| oGSU152 | CGACCGCCACGCAGGGCAGC | *hpuA* sequencing - F |
| oGSU194 | CGTTCTGCTCTATACCCACGT | pVCU234 sequencing - R |
| oGSU224 | TTAAAAGGAGCCCGGGATGAAATACAAAGCCCTGCCC | In-fusion *hpuA* into pVCU234 SmaI site- F |
| oGSU225 | CGGGCCCCCCCTCGAGTTATTGTTTAGTAAGGTGGTTGGT | In-fusion *hpuA* into pVCU234 SmaI site- R |
| oGSU226 | GCTGCCCTGCGTGGCGGTCG | *hpuA* sequencing - R |
| oGSU270 | GGGCGTGGATAGGTCGGGGATCCGGTGATTGATTGAG | In-fusion Ω cassette in pVCU252 PpuMI site =pGSU421- F |
| oGSU271 | TTTCGCCGTTGGGACGGGGATCCGGTGATTGATTGAG | In-fusion Ω cassette in pVCU252 PpuMI site =pGSU421- R |
| oGSU275 | GTCATCCACCGGATCAATTCCCCTG | Ω sequencing primer- F |
| oGSU276 | GCATCCAAGCAGCAAGCGCGTTAC | Ω sequencing primer- F |
| oGSU277 | TAACGCTATGGAACTCGCCG | Ω sequencing primer- F |
| oGSU280 | GTTCGCCCAGCTTCTGTATG | Ω sequencing primer- R |
| oGSU284 | GTAATCCGAGTTGGCATTGTCC | *hpuB* sequencing – R |
| oGSU295 | AACGGGAAACGTCTTGCTCGAG | Tn5 <Kan2> sequencing primer- F |
| oGSU296 | TCGCTCAGGCGCAATCACGAAT | Tn5 <Kan2> sequencing primer- F |
| oGSU297 | CTCGAGCAAGACGTTTCCCGTT | Tn5 <Kan2> sequencing primer- R |
| oGSU298 | AGGTTTCGTCCGAAGTCGGT | *hpuB* sequencing – R |
| oGSU372 | CTCTAGAGGATCCCCGGGGGCAATATCGAAAACATCAACACCG | In-Fusion *hpuA* (downstream of polyG tract) + half of *hpuB* into pVCU403 SmaI- F |
| oGSU376 | ACACACTGGCAGGCAAAATCCT | *hpuA* sequencing - F |
| oGSU377 | GGTTTGGTAATTGACTGCGC | *hpuB* sequencing - R |
| oGSU378 | TCGAGCTCGGTACCCGGGTACAGTTTTGCGAAATAACT | In-Fusion *hpuA* (downstream of polyG |

| | | |
|---|---|---|
| | | tract) + half of *hpuB* into pVCU403 SmaI- R |
| oGSU379 | AATTAAAAGGAGCCCAATTCATGCCCATTCCCTTCAAACC | In-Fusion *hpuB* into pVCU234- F |
| oGSU380 | GAATTCCTGCAGCCCTTAGAACTTCGCTTCGATGGTG | In-Fusion *hpuB* into pVCU234- R |
| oGSU381 | TTGTTCGGCGCGTACATCCGGTGATTGATTGAG | In-fusion Ω cassette into pGSU450- F |
| oGSU382 | TTGAAGTTGCCGTACATCCGGTGATTGATTGAG | In-fusion Ω cassette into pGSU450- R |

## III. Protein Purification, Crystallization, Data Collection and Structure Determination

The meningococcal and gonococcal HpuA's were produced by a neisserial SLAM-mediated secretion system, adapted into a protein production C43 DE3 strain of *Escherichia coli* (Sigma Aldrich), that was developed in the laboratory of Dr. Trevor Moraes (University of Toronto). The secreted protein was affinity purified and enriched by nickel-affinity EXCEL column chromatography (Cytiva) from the culture supernatant. Purified protein was dialyzed into PBS, assessed for purity and concentration by SDS-PAGE analysis before screening in multiple crystallization screens, with or without the C-terminal histidine tag. Five crystallization screens were used: Index HT crystallization screen (Hampton Research) and the entire MCSG crystallization suite (Anatrace), consisting of MCSG 1 through 4. Protein was screened at multiple concentrations ranging between 13 to 35 mg/mL and an Arts Robbin Gryphon drop setting (Arts Robbin Instruments) was used to place 0.5 uL of protein solution with 0.5 uL of crystallization buffer in sitting-drop vapor diffusion 96-well plates that were stored at ambient temperature and monitored in a RockImager182 imaging system (Formulatrix).

The meningococcal HpuA crystallized readily in over 100 different conditions, and a select number of these crystals were soaked in cryoprotectant made with mother liquor supplemented with 20% (v/v) glycerol before flash frozen in liquid nitrogen and sent to the synchrotron facility for screening and data collection. The gonococcal HpuA did not crystallize as readily, and our initial attempts resulted with protein crystals containing a partially degraded HpuA. Several loop deletion mutants were engineered to increase stability; one loop deletion construct resulted in diffraction quality crystals containing the intact protein from which the final structural dataset was collected from.

Diffraction data was collected at the AMX (414) and FMX (415)beamlines (17-ID-1 and 17-ID-2) of the National Synchrotron Light Source II at Brookhaven National Laboratory. Datasets were processed, scaled, and merged using the autoPROC (416); molecular replacement solutions were solved by Phaser MR (417)using input models generated from the Alphafold2-based ColabFold server. Structural refinement was done through Phenix Refine (418), Refmac (419), and manual adjustment and building in COOT (420)to Rwork/Rfree values below 0.20/0.25. The structural images were prepared using Pymol and further refinement is needed for the structures to be deposited into the Protein Data Bank.

**IV. HpuB Peptide-Specific, Guinea Pig, Polyclonal Antiserum**

The amino acid sequence of HpuB was analyzed to identify potential regions to target for antibody generation. Peptide $^{420}$NSDYSYFAKLYDPK$^{433}$ was chosen based on its surface accessibility (in extracellular loop 5) and immunogenic profile using the Hopp-Woods algorithm. The HpuB peptide was synthesized using standard Fmoc solid-state peptide chemistry and conjugated to KLH, then emulsified 1:1 (volume) with Freund's adjuvants for immunization. Two guinea pigs were immunized subcutaneously over a period of ~10 weeks, with terminal bleeds taken at the end. Peptide and polyclonal antiserum were generated by Biosynth.

**V. Western Blotting**

Gonococcal suspensions, in phosphate buffer saline (PBS), were standardized to an optical density of 100,000 Klett units (KU) x µL before being centrifuged; pellets were resuspended in 2X Laemmli solubilizing buffer (BioRad) and stored at -20°C. Subsequently, whole-cell lysates (WCL) were thawed, combined with 5% β -mercaptoethanol, and boiled for 5 min. Precast 4 to 20% gradient polyacrylamide gels (BioRad) were used to separate the protein

samples. For the *hpuA* mutants, a transfer onto a nitrocellulose membrane was done first, before a ponceau stain was applied to verify equal protein loading. However, for the *hpuB* mutants, a stain free image was taken to show equal protein loading, followed by transfer onto a nitrocellulose membrane. Next, the blots were blocked in 5% (wt/vol) bovine serum albumin (BSA) dissolved in Tris Buffered Saline without (for *hpuA* mutants) or with (for *hpuB* mutants) 0.05% Tween 20 (TBS or TBST with Tween20). The blots were then probed with either HpuA C-terminal peptide-specific rabbit polyclonal antiserum (280) (1:2000 in blocker) or HpuB peptide-specific guinea pig polyclonal antiserum (1:7000 in blocker) described above, for 1 h at room temperature (RT). Next, the blots were washed three times with TBS or TBST and then probed with AP-conjugated anti-rabbit IgG secondary antibodies (1:5,000 in blocker) or AP-conjugated anti-guinea pig IgG secondary antibodies (1:10,000 in blocker) respectively, for 1 h. Finally, the blots were washed again using TBS or TBST and developed using Nitrobluetetrazolium (NBT) 5-bromo-4-chloro-3-indolylphosphate (BCIP) tablets (Sigma). A final image of the blots was taken on a Bio-Rad ChemiDoc gel imaging system using colorimetric detection.

**VI. Iron-Restricted Growth Assays with Human Hemoglobin (hHb)**

Adapted from previous methods (421), strains were streaked onto GCB/DFO/IPTG and incubated at 36°C with 5% $CO_2$ for 16-19 hours. Iron-starved strains were resuspended into PBS at a starting optical density ($OD_{600}$) of 0.15. To test hemoglobin (Hb) utilization, cells were diluted to $OD_{600}$ of 0.002 in 1X CDM and were added to a Falcon™ 96-Well, Non-Treated, Flat-Bottom Microplate. To the plate, the following additions were made: 5 µM DFO, 1 mM IPTG, 1 mM mannitol, 6 mM $NaCO_3$, and 0 µM (negative control) or 1 µM human Hb. This assay was also used to test the ability of *N. gonorrhoeae* to utilize and growth with Hb produced by

different species, using 1 µM of each species of Hb. BioTek Cytation5 and SynergyH1 plate readers were used to incubate the cultures grown at 36°C, shaken at 225 RPM with 5% $CO_2$ and to measure the $OD_{600}$ every 30 min for up to 24 hours, to track the growth of the strains.

**VII. Whole-Cell ELISA**

For the HpuA project: Gonococci were iron stressed by overnight growth on GC/DFO/IPTG medium agar plates. Colonies from the plates were inoculated into CDM and cultures were grown as described above until exponential phase before back dilution with CDM and treatment with 1 mM IPTG; the growth was allowed to proceed for 4 hours post back dilution.

For the HpuB project: Gonococci were iron stressed by overnight growth on GC/DFO/IPTG. Whole gonococcal cells were collected from these plates, resuspended in PBS, to an $OD_{600}$ of 1.

The resulting cultures or cell suspensions respectively, were standardized to an optical density of 10,000 KU x 1 µL before being added in triplicate to a 96-well MaxiSorp microtiter dish (Nunc) plate. The dried microtiter plate was then blocked with 200 µL of 5% (wt/vol) non-fat dry milk in TBS, added for 1 h of incubation. After the blocker was removed, 150 nM (HpuA project) or 4 nM (HpuB project) HRP-conjugated hHb in blocker was added for 1 h of incubation at RT. Next, the cells were washed five times with TBS. Later, 50 µL (HpuA project) or 43 µL (Hpub project) of 3,3’,5,5’-Tetramethylbenzidine (TMB) ELISA substrate solution (Thermo) was added to detect the amount of HRP-hHb bound to the cells in each well. After colorization, corresponding volumes of 0.18 M sulfuric acid ($H_2SO_4$) was added to each well to stop the

reaction. Coloration was quantified by reading the absorbance at 450 nm using a Cytation5 plate reader (BioTek).

**VIII. Protein Complex Prediction by Alphafold2 and ColabFold**

For all protein complex predictions, the Google Colaboratory version of the AlphaFold2 (422) software, ColabFold (423), was used with default setting (model type=alphafold2_multimer_v3, num_recycles=3, num_relax=0). Models were generated using the multimer mode (424) for the ternary complex of HpuAB and the tetrameric hemoglobin, the binary complex of HpuB and hemoglobin, and the different HpuB mutant variants generated in this study. Since ColabFold currently cannot incorporate ligands and cofactors into their structural predictions, the known crystal structure of the oxygenated hemoglobin (PDB:1HHO) was superimposed onto the model to correctly position the heme group within the hemoglobin. Visualization and images were prepared using Pymol (Schrödinger LLC).

**IX. Surface Exposure Testing Using Trypsin Digestion**

Protease accessibility assays, following a protocol previously described (257), were done using trypsin. Iron-stressed gonococcal inocula with an optical density $OD_{600}$ of ~0.4, were treated with 5 µg reconstituted trypsin (Sigma) per mL of culture. Then at various timepoints after trypsin addition, 0.6 trypsin-inhibiting units of aprotinin (Sigma) were used to stop the reaction (25 µL trypsin /5 mL of culture with 150 µL aprotinin). One milliliter of culture was collected before the addition of trypsin, as no treatment control, then the 5 mL culture treated with trypsin was kept at 36°C with 5% CO2. Another milliliter of culture was collected at the following time points 0, 10, 20, 30, and 40 minutes. The resulting trypsin-treated cells were

pelleted and resuspended in 2X Laemmli solubilizing buffer. As described above, western blotting was used to detect HpuB in these whole-cell lysates.

**X. ELISA Competition Assay**

RSC284 (HpuB project positive control A+B+) was iron stressed by overnight plate growth and resuspended as described above. Whole cells were standardized to an optical density of 0.4 ($OD_{600}$). In the MaxiSorp microtiter plate, 100 µL of the cell suspension were applied in quadruplicate for each condition and allowed to dry. The dried cells were blocked with 200 µL of 5% (wt/vol) non-fat dry milk in TBS, for 1 hour. One hundred microliters of each of the following conditions, incubated for 1 hr at RT, were added: blocker only as a negative control, 5 nM HRP-hHb as a positive control and 5 nM HRP-hHb with 20 X excess competitor. Unlabeled human, mouse, porcine and rat Hb were used as competitors. The wells were then washed and developed as described above with TMB and $H_2SO_4$. This time, the blocking of Hb binding (due to the presence of excess unlabeled Hbs) was quantified by reading the absorbance at 450 nm using a Cytation5 plate reader (BioTek).

**XI. Statistics**

Using a Student's *t* test, after analysis of variance, allowed for a comparison of results between the positive control or the negative control (when mentioned) and mutant strains. Pairwise comparisons with a *P*-value < 0.05 were considered statistically significant. All the experiments shown (unless otherwise mentioned) are the means of three or four biological replicates (± SEM).

# CHAPTER 4: THE IMPACT OF MUTATING HPUA, ON GONOCOCCAL GROWTH AND BINDING

## I. Introduction

Gonorrhea, the sexually transmitted infection caused by *Neisseria gonorrhoeae*, affected in 2021, 82.4 million people around the world based on the WHO's estimates, and about 710,151 of those cases were reported in the United States alone that same year (66, 67). One factor contributing to the rise in the number of gonococcal cases is the absence of protective immunity following an infection (69, 151). Moreover, another contributor to the rising number of cases is that 80% of the gonococcal cases in women are asymptomatic, leading to a wider spread and patient seeking treatment late (75). Urethritis in men and cervicitis in women are the first manifestations of a gonococcal infection, however the infection can progress to severe clinical sequels, such as infertility and dissemination, when left untreated (74, 79, 84). To make matters worse, *N. gonorrhoeae* is becoming impossible to treat, as it has developed a resistance to all classes of antibiotics used to treat it to date (66, 95). With multidrug resistant isolates on the rise, the last recommended treatment option in 2010, a dual therapy with ceftriaxone and azithromycin, already rendered obsolete has just been updated to ceftriaxone only, in December of 2020 (107, 425). Even though, there has already been reports of ceftriaxone resistance (97-99, 426), it has continued to be rare, however, azithromycin resistance increased, prompting a reevaluation of the recommended treatment. There is currently no effective vaccine available against *N. gonorrhoeae*, tallying this to the soon to be non-existent list of therapeutic options, make gonorrhea a public health threat that urgently needs be addressed.

Finding the best targets for treatment and prevention of gonorrhea is crucial. As some are considering redeploying older antibiotics, often used for other diseases, to treat ceftriaxone-resistant gonococcal infections (427), one strategy to develop therapeutics is to focus research efforts on how *N. gonorrhoeae* acquires essential metals to cause pathogenesis. During infection, the human host uses metal binding proteins, like transferrin, lactoferrin, and hemoglobin, to sequester iron away from *N. gonorrhoeae* in an effort to keep it from causing disease, a process called nutritional immunity (200). To overcome this challenge of metal limitation, *N. gonorrhoeae* expressed highly conserved TonB-dependent Transporters (TdTs) to acquire metals directly from the host metal-binding proteins (316, 428, 429). Interestingly, most of the TdTs do not undergo high-frequency antigenic and phase variation and they are highly conserved among all pathogenic Neisseria strains (316). Therefore, the importance of these TdTs warrant a thorough dive into their potential as vaccine targets (430).

The hemoglobin transport system (HpuAB) allows *N. gonorrhoeae* to acquire iron from hemoglobin (Hb). This TdT is a bipartite receptor composed of a lipoprotein HpuA and a transmembrane protein HpuB which are expressed only in isolates from women in the first half of the menstrual cycle because of phase variation (279). Both HpuA and HpuB are thought to be necessary for effective binding and iron uptake from Hb.

In a study by Wong *et al.*, the residues of HpuA in *Kingella denitrificans* (KdHpuA) were used for molecular replacement to solve the C-terminal beta barrel of gonococcal HpuA and It was also found that mutating hydrophobic HpuA residues interacting with Hb caused a binding defect (278). The results of this study were encouraging but the binding interaction was tested in a pull-down assay which was in vitro. Most of the interaction was also studied between

KdHpuA and Hb only. Therefore, a further investigation was done here to evaluate the extent to which these mutated hydrophobic residues are defective in *N. gonorrhoeae* for binding and growth on human hemoglobin. We built on the study by Wong *et al.*, and ectopically inserted the mutated versions of *hpuA,* on the gonococcal chromosome behind an IPTG-inducible promoter, to better understand the structure-function relationships of this lipoprotein. Growth on Hb as a sole iron source and Hb binding assays were performed.

## II. Results

### *a) Ngo HpuA Structure*

The crystal structure of the gonococcal HpuA C-terminal beta-barrel was previously determined by Wong *et al.,* (PDB:5EE2), using a molecular replacement strategy with the crystal structure of the *Kingella denitrificans* HpuA (PDB:5EC6) (278). Our collaborators in the Moraes lab attempted to resolve the full-length structure of the gonococcal HpuA and interestingly also ended up crystallizing only the barrel-domain of the protein, suggesting that here might be an inherent instability in the full-length protein during the crystallization process. Using sequence and structural alignments against the meningococcal HpuA structure and an Alphafold generated model of the gonococcal HpuA, an extended loop in the handle domain that was unique to the gonococcal HpuA was identified. It was hypothesized that this loop might contribute to the instability of the full-length protein, therefore a loop-deletion mutant was engineered by removing the loop residues GAGSASDAPSRSRRSLDAAP which was replaced with the DTGS residues from the meningococcal HpuA. The full-length HpuA structure was obtained with a loop deletion in the handle domain and can be seen in Fig. 3.

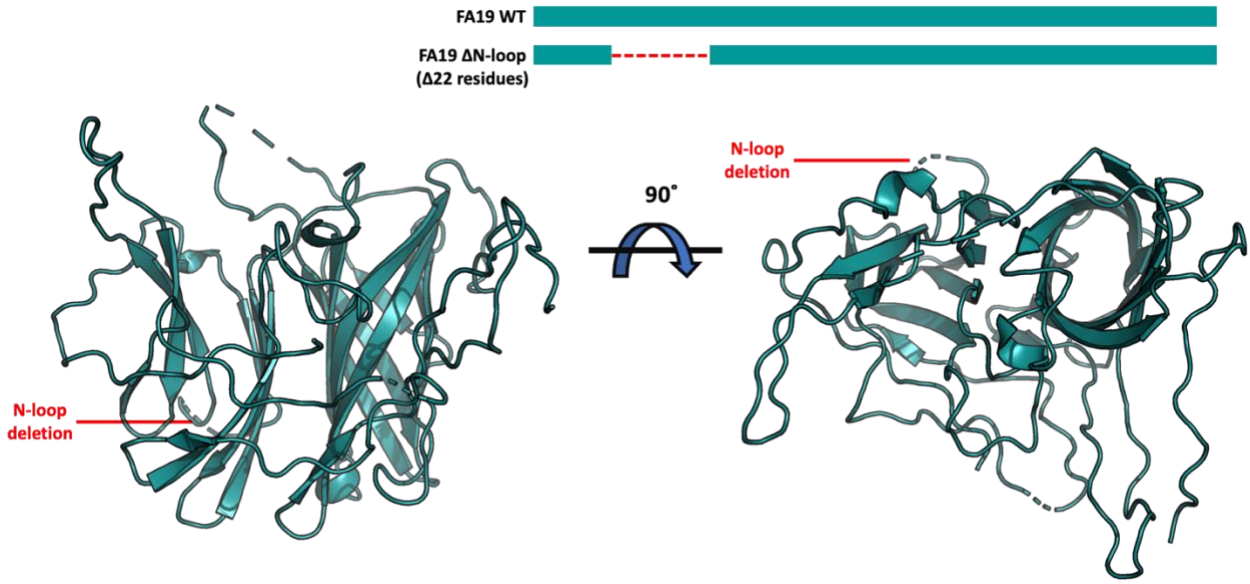



**Figure 3: Crystal Structure of HpuA.**

The crystal structure of full length HpuA was solved. The extended loop region in the handle domain was removed to make the protein stable in crystallization. The gonococcal HpuA residues GAGSASDAPSRSRRSLDAAP, found in the handle domain, were replaced with residues DTGS from the meningococcal sequence. A full length HpuA structure was obtained with a shortened handle domain. The crystal structure was solved by Dr. Dixon Ng, from the Moraes lab, at the University of Toronto.

### *b) HpuA Genes Integrated in the Ectopic Site are Induced by IPTG*

HpuA and HpuB are only expressed in iron deplete conditions and are phase variable. To get the maximum level of expression of *hpuA* and *hpuB* in the native site, the appropriate degree of iron limitation is necessary, possibly making it difficult to express comparable levels of mutated *hpuA* genes in the native site. To overcome this challenge, we used two strains mutated at the native locus. With *hpuA* preceding *hpuB* in the same operon, one strain contained an omega cassette in *hpuA* (RSC150), so it lacked both *hpuA* and *hpuB;* the other strain contained a non-polar kanamycin cassette (RSC151) which inactivates the *hpuA* gene but leaves hpuB expressed natively (Table 4). A complementation vector, pVCU234, was used to ectopically add back wild type or mutated versions of *hpuA* in RSC150 (*hpuA*- *hpuB*-) or RSC151 (*hpuA*- *hpuB+*). This vector contains an Isopropyl β-d-1-thiogalactopyranoside (IPTG) inducible promoter followed by a strong ribosome binding site (Fig. 4). Using this approach and adding a 1 mM of the inducer, we can more precisely control protein synthesis and, consequently, compare more reliably protein properties.

**Table 4. HpuA Loop Deletion Mutations**

| Position | Wild type | Mutations |
|---|---|---|
| 77-82 | $^{77}$DYFGYK$^{82}$ | Deletion |
| 78 | Tyrosine | Alanine |
| 79 | Phenylalanine | Alanine |
| 81 | Tyrosine | Alanine |
| 111-116 | $^{111}$YSSPDF$^{116}$ | Deletion |
| 111 | Tyrosine | Alanine |
| 112 | Serine | Alanine |
| 113 | Serine | Alanine |
| 114 | Proline | Alanine |
| 115 | Aspartate | Alanine |
| 116 | Phenylalanine | Alanine |
| 136-137 | Cysteine/Cysteine | Serine/Serine |
| 140 | Serine | Arginine |
| 141 | Glycine | Arginine |
| 225-231 | $^{225}$SYYGTLA$^{231}$ | Deletion |
| 226 | Tyrosine | Alanine |
| 227 | Tyrosine | Alanine |
| 295 | Phenylalanine | Alanine |
| 299 | Arginine | Alanine |

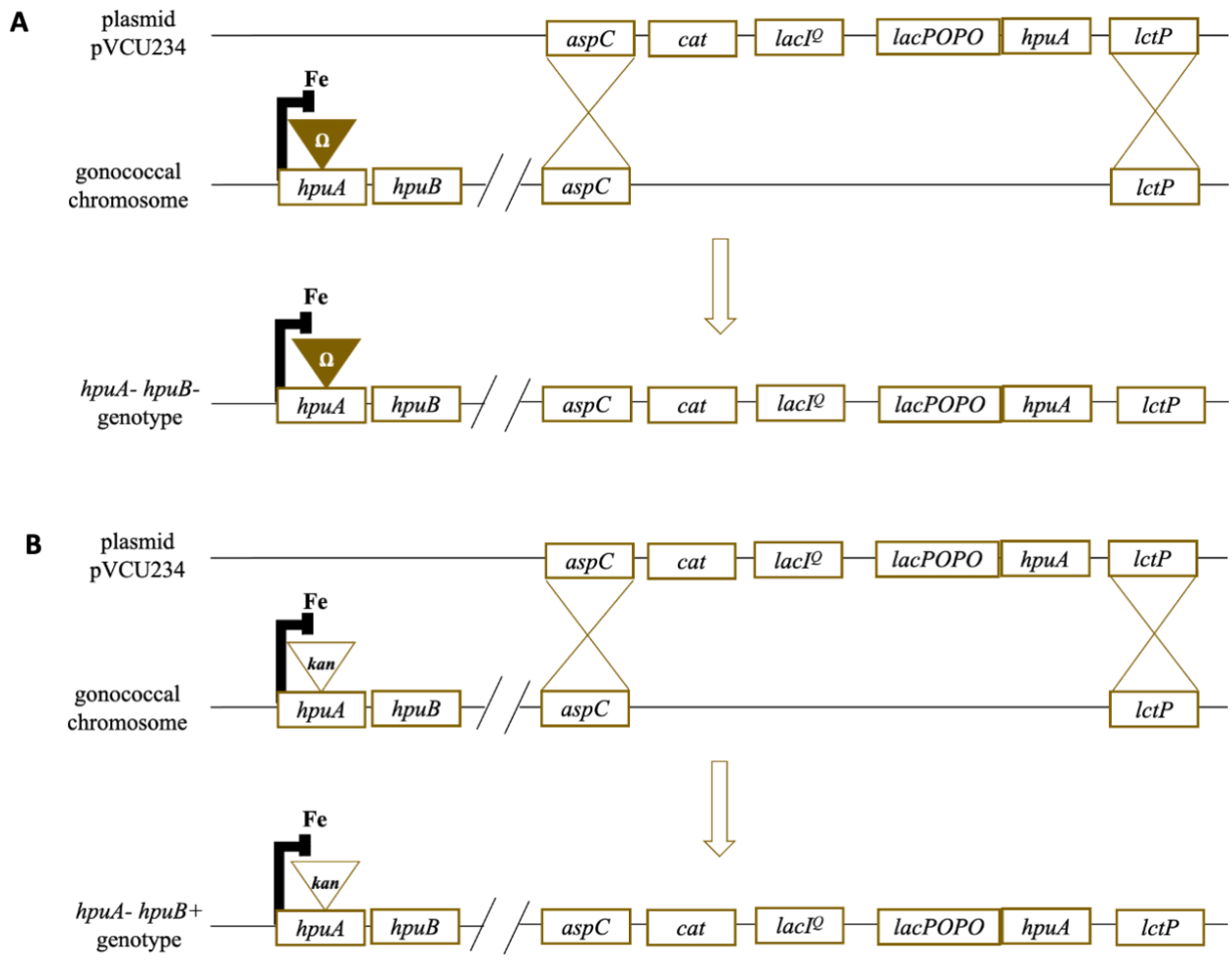



**Figure 4: Diagram Showing the H*puA* Mutations Ectopically Inserted into the Gonococcal Chromosome Generating Gonococcal Strains that Were Either H*puB*- (Ω Cassette) or H*puB*+ (K*an* Cassette).**

Wild type and mutated versions of *hpuA* were cloned into a complementation vector, pVCU234, behind an IPTG inducible promoter. Different *hpuA* mutants were inserted between the *aspC* and *lctC* loci following transformation of Ngo. The chromosome of the recipient strain is either *hpuA*$^{-}$ *hpuB*$^{-}$ (Ω cassette) or *hpuA*$^{-}$ *hpuB*$^{+}$ (*kan* cassette). *lacPOPO* promoter is induced by IPTG.

The mutations were developed to be inducible, as shown by the final genotype's diagram, but we needed to verify that they made the right gene under IPTG. The *hpuA* mutants were grown in the presence or absence of IPTG, then, to detect the correct gene product, a western blot was done. This western blot allowed the confirmation of HpuA expression in the presence of the inducer, IPTG (Fig. 5). As controls, we used RSC150 (A-B-) representing the negative control and RSC125 (A+B+), containing native *hpuB*, representing the positive control. As a means to confirm equal protein loading, ponceau staining, following the transfer step, established that the lanes were loaded equally (data not shown). HpuA was found to be expressed only in the presence of the inducer, IPTG, whereas the absence of IPTG did not lead to any expression of HpuA. We next proceeded to further characterize these mutated strains in the rest of the study.

***c) Very Few Mutants Exhibit Growth Impairment on Hb as a Sole Iron Source***

As this study looks at how mutating *hpuA* of the HpuAB transport system affect its function, we wanted to establish whether these mutations would affect *N. gonorrhea's* ability to grow on Hb as a sole iron source. To do so, the strains in both background (RSC150 and RSC125) with or without the transmembrane protein, HpuB, were grown in iron deplete conditions, a growth assay was performed. An iron chelator, Desferal (DFO) was added, and the strains were also supplemented with IPTG plus 0 or 1 µM Hb as the sole source of iron. Using GraphPad Prism, the resulting growth curves were used to calculate the area under the curve that is shown in Fig. 6. When 0 µM Hb was supplemented, no growth was observed for any of strains regardless of the expression of native *hpuB* (10A and 4B). When the strains were supplemented with 1 µM Hb, the *hpuA* mutants showed diverse levels of growth.

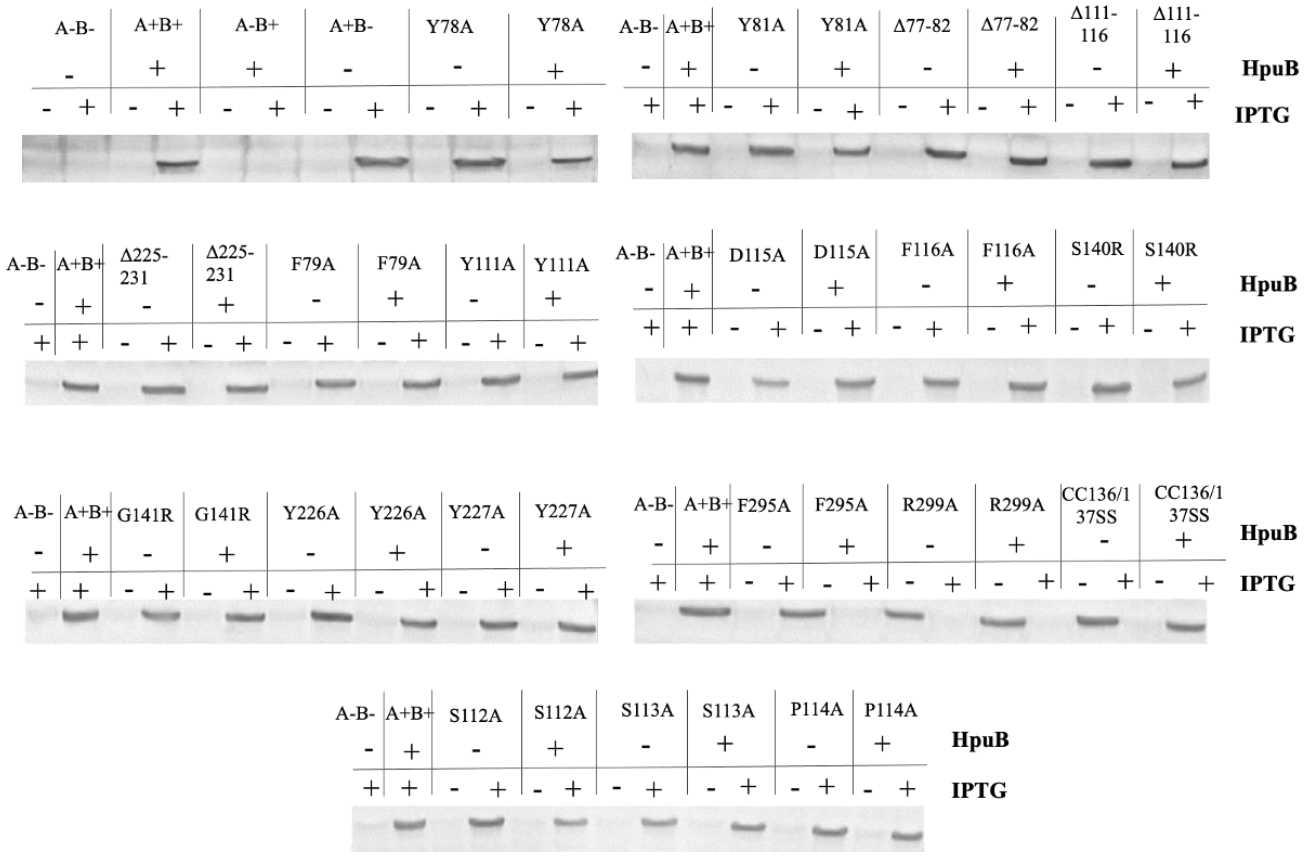



**Figure 5: Only IPTG Induces the Strains Expressing Mutated *HpuA* Genes.**

*hpuA* mutant strains were grown in Fe limiting conditions with (+) or without (-) 1 mM IPTG. Cells were collected from plates and resuspended into PBS. From these suspensions, whole-cell lysates were prepared by standardizing the cell suspensions to an $OD_{600}$ of 1, pelleting cells and resuspending them in lysis buffer. Western blots were performed to characterize the production of HpuA in the presence or absence of IPTG using anti-HpuA antibody in rabbit. The strain with WT *hpuA* gene in the ectopic site and native *hpuB* gene (RSC125) was used as a positive control (A+B+) whereas the strain lacking both native *hpuA* and *hpuB* genes RSC150 (A-B-) was used as a negative control. Equal loading of each lane is confirmed by ponceau staining following transfer. The data shown represents 3 biological replicates.

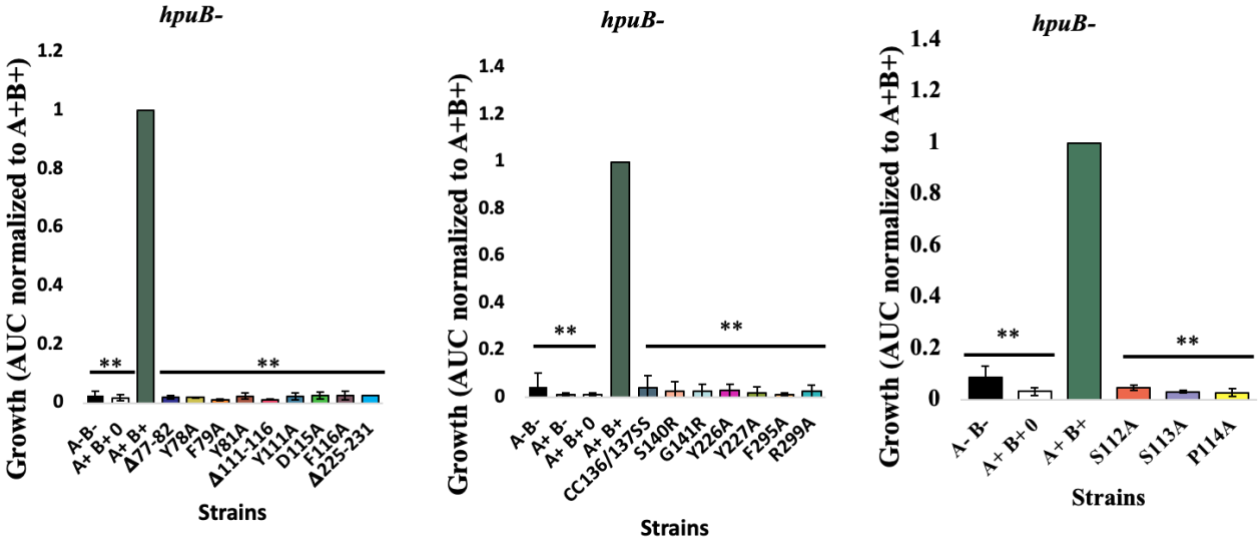
As anticipated, the negative control, RSC150 (A-B-), exhibited the minimum amount of growth and the positive control, RSC284 (A+B+), showed the highest amount growth. We also assessed the growths of strains RSC151 (*hpuA- hpuB+*) and RSC152 (*hpuA+ hpuB-*) as possible negative controls as HpuA and HpuB are thought to be both required for Hb utilization. As suggested before, we saw that either strains, containing HpuA or HpuB alone, is not able to grow on Hb as a sole iron source, suggesting that both might be required to allow iron uptake from Hb (Fig. 6C, D). In the absence of the transmembrane protein hpuB, none of the strains were able to grow and all showed significantly lower growth than A+B+, at A-B- levels (Fig. 6C). This might strengthen the thought that both HpuA and HpuB are required for Hb utilization.

In contrast, when HpuB was natively expressed, only strains $\Delta$111-116 and P114A exhibited significantly lower growth than A+B+ and the rest of the strains were able to grow at A+B+ levels (Fig. 6D). Both strains with impaired growth have mutations located in loop 2 of HpuA. With this observation, we decided to focus our next assay on the mutations located in loop 2 and a few more mutations assess the importance of loop 2 in binding.

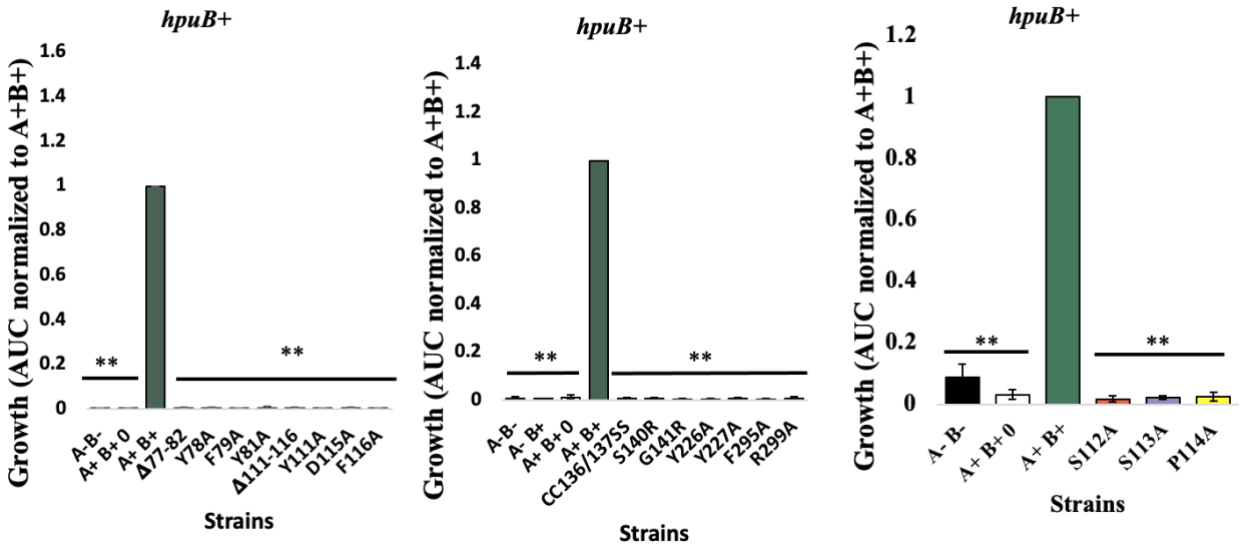
***d) Some of the Loop 2 Mutants Demonstrate Impaired Binding to Hb***

Using whole-cell ELISA, we determined the binding ability of the *hpuA* mutants, in the presence or absence of HpuB. After inoculating the strains from GCB/DFO/IPTG plates into CDM, cultures were grown as described above until exponential phase, with treatment with 1 mM IPTG. After standardization the cell cultures were added to a 96-well ELISA plate and left to dry. Next, the cells were probed with horseradish peroxidase (HRP)-labeled hemoglobin (Hb-HRP) to determine the binding levels of each strain (Fig. 7). Different levels of Hb binding were observed across the strains. As expected, we observed the least detectable level of binding

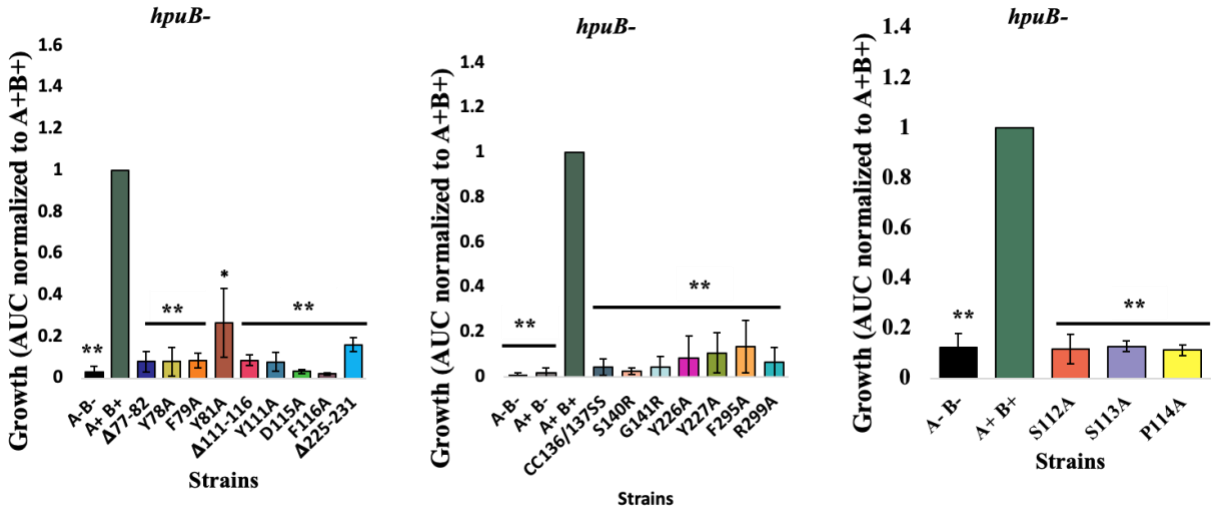
**A**


hpuB-
Growth (AUC normalized to A+B+)
Strains
A-B-
A+ B+0
A+ B+
Δ77-82
Y78A
F79A
Y81A
Δ111-116
Y111A
D115A
F116A
Δ225-231
hpuB-
Growth (AUC normalized to A+B+)
Strains
A- B-
A+ B+0
A+ B+
CC136/137SS
S140R
G141R
Y226A
Y227A
F295A
R299A
hpuB-
Growth (AUC normalized to A+B+)
Strains
A- B-
A+ B+0
A+ B+
S112A
S113A
P114A

**B**


hpuB+
Growth (AUC normalized to A+B+)
Strains
A-B-
A+ B+0
A+ B+
Δ77-82
Y78A
F79A
Y81A
Δ111-116
Y111A
D115A
F116A
hpuB+
Growth (AUC normalized to A+B+)
Strains
A- B-
A+ B+0
A+ B+
CC136/137SS
S140R
G141R
Y226A
Y227A
F295A
R299A
hpuB+
Growth (AUC normalized to A+B+)
Strains
A- B-
A+ B+0
A+ B+
S112A
S113A
P114A

**C**




**D**

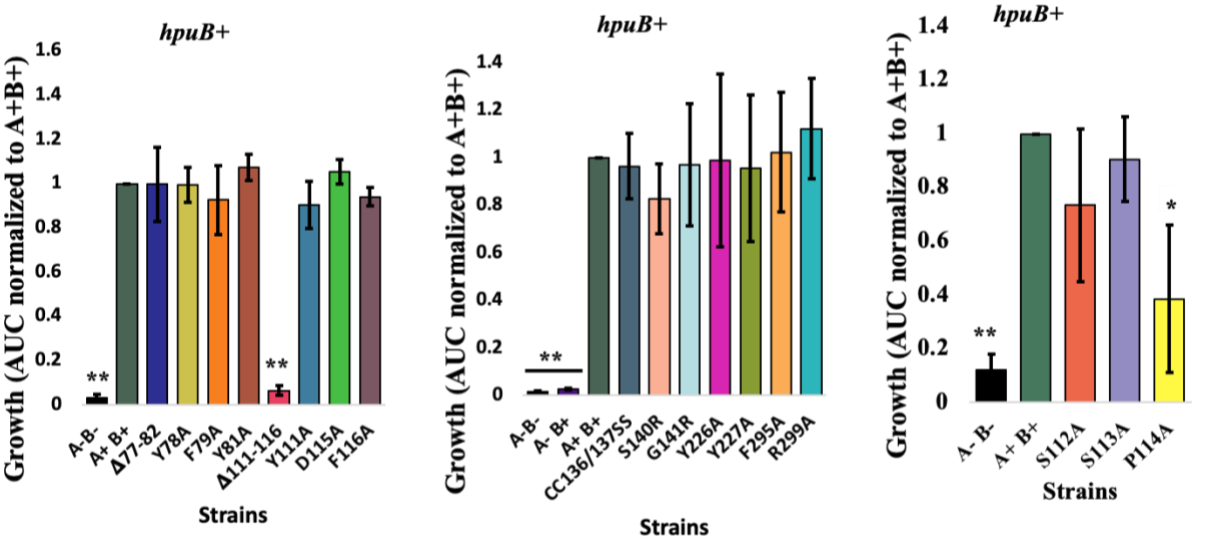



**Figure 6: Δ111-116 and P114A are Impaired for Growth on Hemoglobin as a Sole Iron Source, When HpuB is Expressed.**

Gonococci were grown on GCB/DFO/IPTG plates before being resuspended in CDM. The cell suspensions were standardized to an $OD_{600}$ of 0.002 before being added to a 96-well plate containing DFO, IPTG and 0 or 1 µM hHb. Cells were grown for 21 hours, during which the $OD_{600}$ was recorded in 30-minute intervals to assess the growth of strains in the *hpuA*- background (A) and the *hpuB+* background (B). A-B- is used as a negative control and A+B+ as a positive control. A+B- as well as A-B+ are also controls as both proteins are thought to be required for growth. From the growth curves, the area under the curve (AUC) was calculated using GraphPad Prism then normalized to growth by the A+ B+ strain. The data from three biological replicates were analyzed to generate the means and standard deviations shown. Statistically significant differences were assessed by the Student's *t*-test relative to the A+B+ strain (*, $p < 0.005$; **, $p < 0.0005$).

with the negative control, RSC150 (A-B-), and the most level of binding with the positive control, RSC125 (A+B+).

For the strains lacking native HpuB, Y78A, Y81A, Δ111-116 and Y111A mutations were binding Hb at A+B+ level, while S112A, S113A, P114A, D115A and F116A had significantly reduced Hb binding when compared to A+B+ (Fig. 7A). Interestingly, RSC152 (A+B-) also bound to Hb at A+B+ level suggesting that HpuA, expressed in the ectopic site, alone can allow binding to Hb.

When HpuB is present in the native site, all mutations, except S112A, S113A, P114A, and F116A bound Hb at levels comparable to that of A+B+. S112A, S113A, P114A and F116A showed a significant decrease in binding when compared to A+B+ (Fig. 7B). RSC151 (A-B+) showed a significant decrease in Hb binding, indicating that HpuB alone, expressed natively, does not allow binding to Hb. Taken together, these data suggest that positions 112 through 114 and 116 of loop 2 might have an important role in binding to Hb.

## III. Discussion

The human host sequesters, away from invading bacteria, the key nutrients needed to grow and cause disease, using nutritional immunity factors, this process is referred to as nutritional immunity (201). Some of these essential nutrients are transition metals such as iron, zinc, manganese, and cobalt. To overcome these defensive mechanisms imposed by the host, many bacteria started to secrete siderophores, which are small organic molecules, that scavenge free metals that they encounter (431, 432). Once loaded with metals, the siderophores become a source of said metal when the bacteria encounter them.

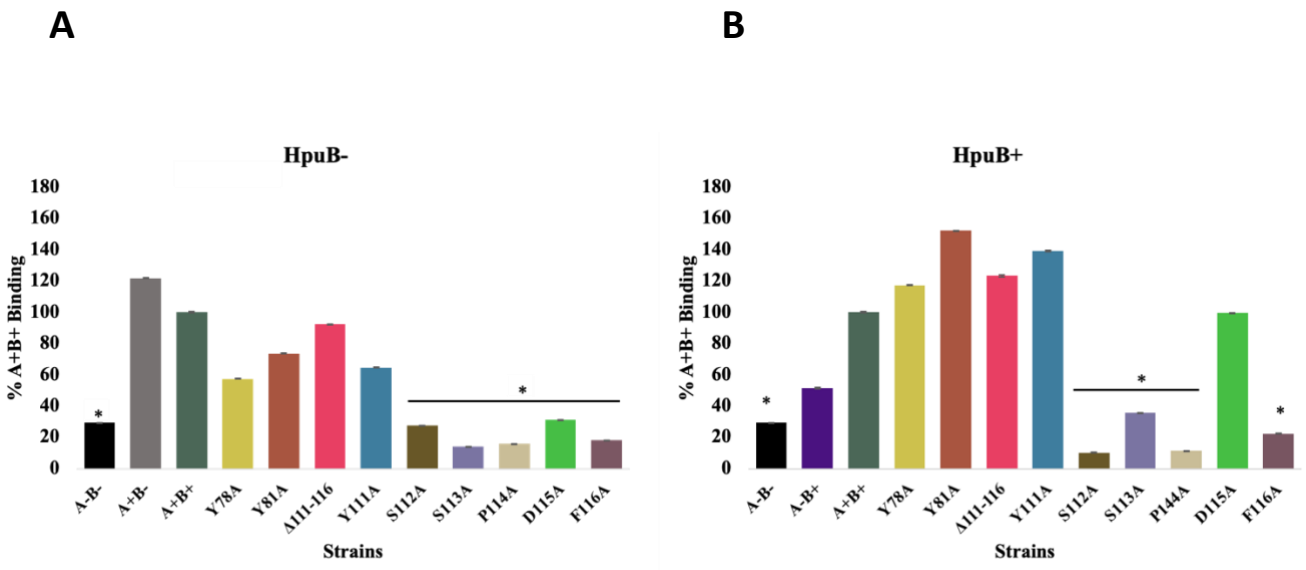



**Figure 7: S112A, S113A, P114A, and F116A *HpuA* Mutants Have a Binding Defect Regardless of the Expression of HpuB.**

Gonococcal hpuA mutants were grown before being standardized to 10,000 KU x 1 µL, the cell suspensions were then added to a 96-well ELISA plate and allowed to dry prior to blocking. The ELISA plate was then probed with HRP-conjugated hHb before being washed and developed with a TMB substrate. The binding ability of strains without (A) and with (B) native hpuB was assessed. The absorbance at 450 nm was read to quantify the signal. A-B- is used as a negative control and A+B+ as a positive control. A+B- as well and A-B+ are also used as controls since both HpuA and HpuB are thought to be required for binding. All the strains were normalized to A+B+ and showed as a percentage of A+B+. These data are representative of one of three biological replicates. A Student's t-test was used to assess the statistically significance differences relative to A+B+ (*, $p < 0.05$).

*N. gonorrhoeae* not only does not secrete its own siderophores, but it can utilize metal-loaded xenosiderophores secreted by other bacteria in the environment (433). Nevertheless, to overcome these metal restricted conditions, *N. gonorrhoeae* also started expressing special TonB-dependent outer membrane transporters (TdTs) capable of binding and directly hijacking the host 74utriational immunity proteins of their metal cargo. Many TdTs have been identified such as TdfH and TdfJ which respectively pull zinc from calprotectin and S100A7, as well as TdfF and TdfG that acquire iron from unknown ligands and FetA, which hijacks the xenosiderophores. The rest of the TdTs are bipartite receptors composed of two proteins, a lipoprotein and a transmembrane transporter, that pull iron from their ligands (434). These bipartite TdTs are TbpAB which binds transferrin, LbpAB that bind lactoferrin and HpuAB that binds hemoglobin and is the focus of this investigation.

It was previously found that the lipoprotein TbpB, of TbpAB, facilitates binding of human transferring but is not essential for the uptake of iron from transferrin (258, 435). Similarly, the lipoprotein LbpB, of LbpAB, is suggested to facilitates binding but is not essential for the uptake of iron from Lactoferrin (267, 270, 436). Since the bipartite transporters TbpAB and LbpAB, have lipoproteins that are not necessary for utilizing their ligands, the role of liproprotein, HpuA, in the utilization of Hb was looked at with these mutants. Moreover, the first direct interaction between HpuA and Hb was recorded in Wong *et al.*, where the mutations in the gonococcal HpuA's residues, thought to be interacting with Hb, caused a binding defect in pulldowns assays (278). Therefore, in our study, we also tried to investigate the effect of these same mutations on the ability of live *N. gonorrhoeae* to bind Hb and grow.

A crystal structure of the full *N. gonorrhoeae* HpuA was obtained and the HpuA mutations generated previously (278) were placed in an ectopic site, behind a *lac* promoter on the gonococcal chromosome. The expression of the mutants was verified via western blot only in the presence of IPTG which de-represses the promoter. After growth with and without Hb as a sole iron source, the mutants *hpuA* only grew in the presence of the transmembrane protein HpuB and the presence of Hb. This suggests that the lipoprotein alone is not capable of utilizing Hb and that HpuB is required for acquisition. This is biologically logical as the HpuB is the barrel through which the heme is internalized across the membrane. Interestingly, when strains RSC151 and 152, which respectively contained HpuB only or HpuA only, were grown on Hb as a sole iron none of them could grow. This confirms the literature about the requirement of both proteins for heme internalization (437). In the presence of HpuB all the mutants that were tested for growth were able to grow except $\Delta$111-116 and P114A which had a significant growth defect. This suggested that the mutations did not have an effect on growth and residues 111 through 116 of loop 2 might have an important role for heme uptake. Taken together, the binding ability of all the mutants, generated within the 111-116 region, was investigated without the presence of HpuB to see HpuA's contribution to binding and with the of HpuB to see how much of the HpuAB binding is contributed by HpuB.

For the strains lacking native HpuB, mutations S112A, S113A, P114A, D115A and F116A exhibited a binding defect contrary to $\Delta$111-116 and Y111A mutations which did not exhibit a binding defect. Additionally, RSC152, which only expresses HpuA, was also able to bind to HRP-Hb. This suggests a possible direct interaction between HpuA and Hb.

However, when HpuB was expressed, only mutations S112A, S113A, P114A, and F116A had a binding defect whereas Δ111-116, Y111A and D115A were binding at A+B+ levels. Unexpectedly, RSC151 which only expressed HpuB also had a binding defect. This suggests that residues 111-116 might be important for uptake but not binding. Moreover, the binding defect of P114A, regardless of the presence of HpuB, and its decreased growth highlights the possible importance of the proline residue, in both binding and uptake. Surprisingly, the binding defect observed in S112A, S113A, P114A, and F116A did not translate into a growth defect. A possible explanation for the binding defect of RSC151 and deletions S112A, S113A, P114A, and F116A was that the *kanamycin* gene placed in *hpuA* might have had an unwanted polar effect after all, causing a variation in the amount of HpuB natively expressed under iron restriction. Nevertheless, the construct used still allows the investigation of the growth phenotypes of these mutants. Because during a growth assay, the restriction of iron is more stringent, so HpuB is likely expressed at higher levels and the strains are allowed to grow longer, giving the cells enough time to make HpuB that utilize the sole source of iron, Hb.

Overall, there might be a direct interaction between HpuA and Hb, but this possible interaction will need to be investigated further as HpuA expression can be controlled at the ectopic site where it is almost certainly made at high levels. A better control of the expression of HpuB will also be needed to clearly differentiate the contribution of either of these proteins to binding and/or growth on Hb. This study and the suggested implementations will elucidate whether both HpuA and HpuB are required for binding and growth on Hb and whether HpuA is only necessary for uptake like the other lipoproteins, TbpB and LbpB. Finally, future works will

not only help better understand the interaction between HpuAB and Hb, but they will also help determine the potential of each of these proteins, HpuA and HpuB, to be a vaccine target.

# CHAPTER 5: LOOP 7 OF *NEISSERIA GONORRHOEAE* HPUB RECEPTOR PLAYS AN IMPORTANT ROLE IN HEMOGLOBIN BINDING, EXTRACTION, AND UPTAKE

## I. Introduction

In the previous chapter, we specifically mutated the lipoprotein HpuA to see how it impacts *N. gonorrhoeae*'s ability to bind and grow on human hemoglobin (hHb). We also evaluated the impact of the presence of HpuB on these mutations. In this chapter, we are investigating mutations in HpuB, which is the actual transmembrane protein through which the heme moiety enters the bacterium. Both HpuA and HpuB are thought to be required to bind and utilize hHb. Very little is known about the importance/role of the extracellular loops of HpuB during the binding uptake process. Since there is a selective advantage to expressing HpuAB during menses, HpuB mutants unable to bind and grow on hHb, as in the protection studies mentioned before, could be considered as part of a vaccine cocktail against *N. gonorrhoeae*. To identify necessary domains of the HpuB loops, we targeted loops 2, 3, 4, and 7. We targeted the conserved NPEL domain and the conserved histidine of loop 7, as well as hypothetical α-helix at position 538-544. Therefore, we hypothesized that deletion of HpuB extracellular loops will reduce or abrogate Hb binding and consequently cause defect in growth with Hb as a sole iron source. We also hypothesized that residues in HpuB's loop 7 may play a crucial role in hemoglobin binding and heme transport.

This current study aims to elucidate the interaction between gonococcal HpuB and hHb and to contribute to a detailed structure-function analysis of the gonococcal HpuAB system. HpuB extracellular loop deletion mutants were analyzed for their binding ability to hHb and growth on hHb as a sole iron source, to better understand the binding and heme internalization

mechanisms. In this current study, we found that mutations targeting loop 7, which contains conserved, putative heme-binding motifs, demonstrated impaired binding and growth, highlighting the importance of this loop in HpuB functions. We also found that the lipoprotein, HpuA, is crucial to restoring Hb binding and iron internalization for the loop 7 mutants that lack these conserved motifs.

## II. Results

### *a) Hypothetical Membrane Topology of HpuB as a Foundation for Mutagenesis*

Geneious was used to create a sequence alignment between meningococcal TbpA (for which the crystal structure is known [PDB 3V89]), gonococcal TbpA (WP_003693614) and HpuB (WP_003687084). Next, using the HpuB sequence in NetSurfP 2.0 and information about structural features of TbpA, the beta strands of HpuB were predicted. Finally, TOPO2 was used to create a transmembrane protein 2D topology and PHYRE2 was used to predict folded structure of HpuB. A representation of the hypothetical HpuB topology model with extracellular loops, helices and plug domain is shown in Fig. 8. The arrows indicate the location of the mutations created: Δ236-246 (loop 2), Δ306-311 (loop 3), Δ366-370 (loop 4), Δ538-544 (hypothetical α-helix in loop 7), ΔH548 and Δ555-559 (predicted conserved motifs in loop 7). While comparing the sequence of the gonococcal HpuB to that of other bacterial heme uptake systems, we identified 3 conserved motifs (Fig. 9), a histidine located between a FRAP motif and a NXXL (NPEL in *N. gonorrhoeae* FA19) motif. Mutations were made by deleting either the histidine or the NPEL motifs but not the FRAP motif as it is predicted to be part of the barrel (438).

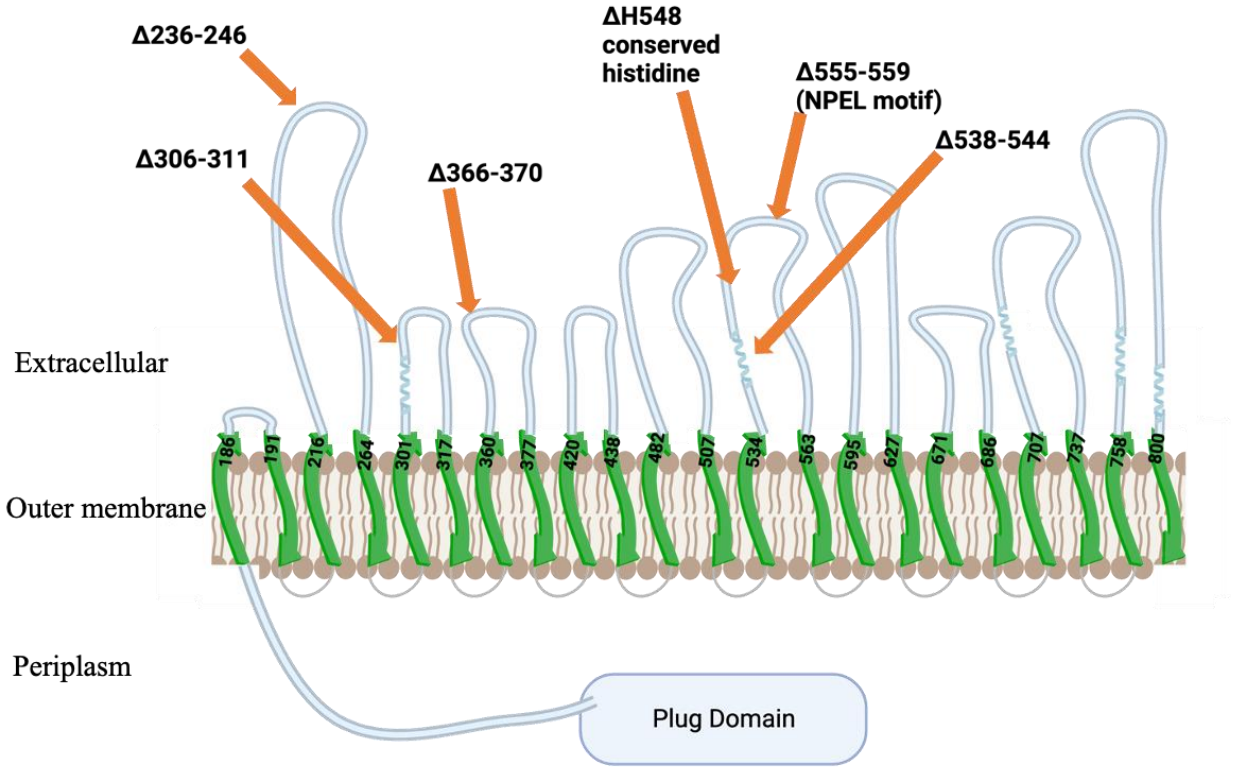



**Figure 8 : Hypothetical Topology Map of HpuB as a Foundation for Mutagenesis.**

The putative loops are extracellularly located with loop helices represented by coil-like regions. Beta strands are shown in green within the outer membrane. The orange arrows indicate the locations of the deletion mutations generated in this study. Image created with BioRender.com.

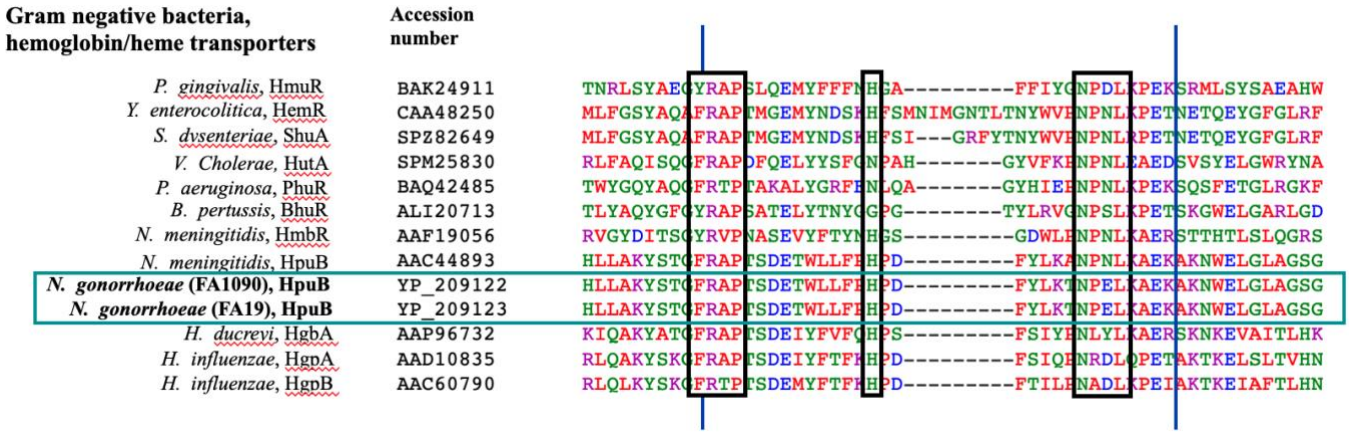



**Figure 9: Muti-Sequence Alignment of Gram-Negative Hemoglobin/Heme Transporters.**

Only loop 7 is shown and it is delineated by the blue lines. The different bacteria used are shown with their corresponding accession number. The conserved FRAP (YRAP and FRTP variation seen), Histidine (sometimes absent) and NXXL motifs are shown in the black rectangles. The amino acid sequence of *N. gonorrhoeae*, with the FRAP, histidine, and NPEL motifs, is shown in the cyan rectangle.

### *b) Mutated HpuB Genes Were Created and Confirmed to be Expressed from an Inducible Promoter in an Ectopic Site in the Gonococcal Chromosome*

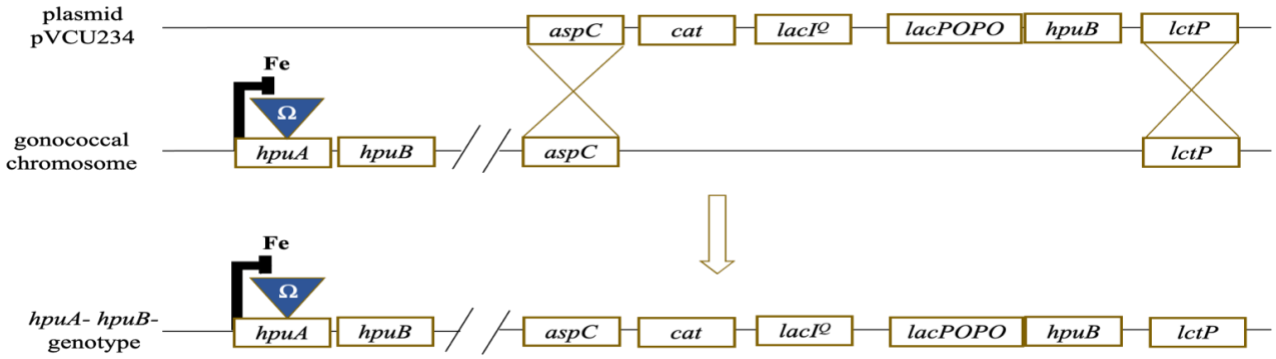
The *hpuA* and *hpuB* genes are natively expressed only under iron-restricted growth conditions (273, 280). To avoid fluctuations in protein expression levels under iron restriction, we constructed two strains mutated in the native locus; one was unable to produce either HpuA or HpuB (RSC150). The other mutant strain had a locked, phase-on *hpuA* gene and an inactivated *hpuB* gene in the native chromosomal site (RSC275) (Table 5). Plasmid pVCU234, a complementation vector with an isopropyl β-d-1-thiogalactopyranoside (IPTG) inducible promoter followed by a strong ribosome binding site, was used to ectopically insert wild-type (WT) or mutated versions of *hpuB* into the RSC150 (*hpuA- hpuB-*) or RSC275 (*hpuA+ hpuB-*) backgrounds (see Fig. 10A and B for schematic). Using this approach allowed for better control over the production of HpuB*,* with the addition of 1 mM IPTG, rather than iron restricted growth conditions.

After growing the *hpuB* mutants with or without IPTG, a western blot was performed to confirm the production of HpuB in the presence of IPTG (Fig. 11). Strain RSC150 (A-B-) was used as a negative control whereas RSC284 (A+B+) with a native and locked phase-on *hpuA* gene was used as a positive control. A stain free gel confirmed that the lanes were loaded consistently (data not shown). Immunoblot analysis using a HpuB peptide-specific polyclonal antiserum (ordered from Biosynth), confirmed that HpuB production was regulated by IPTG addition to all the mutant strains. Following this confirmation, we progressed to characterization of the binding and growth phenotypes of the mutants.

**Table 5. HpuB Loop Deletion Mutations**

| Position | Wild type | Mutations |
|---|---|---|
| 236-246 | $^{236}$YVFDPANPSPS$^{246}$ | Deletion |
| 306-311 | $^{306}$TGTTTS$^{311}$ | Deletion |
| 366-370 | $^{366}$NYDTN$^{370}$ | Deletion |
| 538-544 | $^{538}$TSDETWL$^{544}$ | Deletion |
| 555-559 | $^{555}$TNPEL$^{559}$ (heme motif) | Deletion |
| 548 | Histidine (heme motif) | Deletion |

**A**




**B**

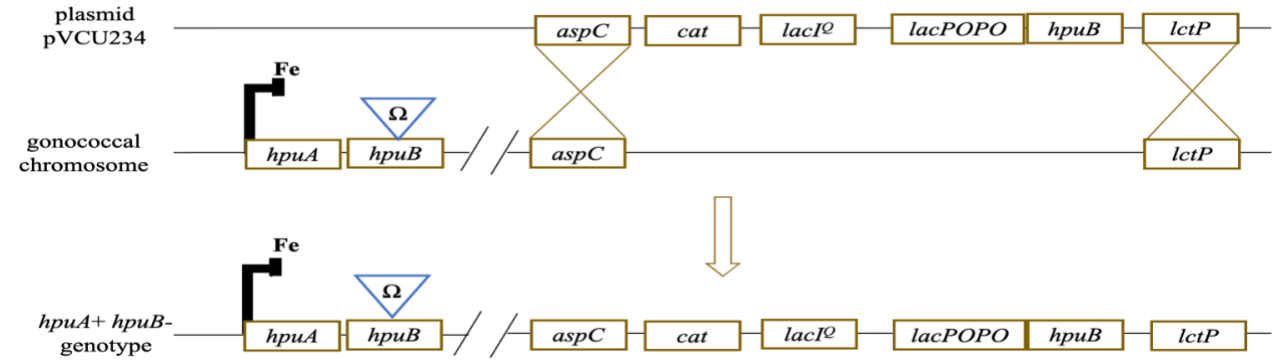



**Figure 10: Diagram Demonstrating How HpuB Mutations Were Moved into the Gonococcal Chromosome Generating Gonococcal Strains that Were Either *HpuA*- (Panel A) or *HpuA+* (Panel B).**

Wild type and mutated versions of *hpuB* were cloned into a complementation vector, pVCU234, behind an IPTG inducible promoter and ribosome binding site. Transformation of *N. gonorrheae* results in wild-type or mutated versions of *hpuB* being inserted into an ectopic site between the *aspC* and *lctC* loci. The chromosome of the recipient strain is either *hpuA- hpuB-*, i.e RSC150 (panel A) or *hpuA+ hpuB-*, i.e RSC275 (panel B). *lacPOPO* represents the IPTG inducible promoter and Fe represents the iron repressed promoter.

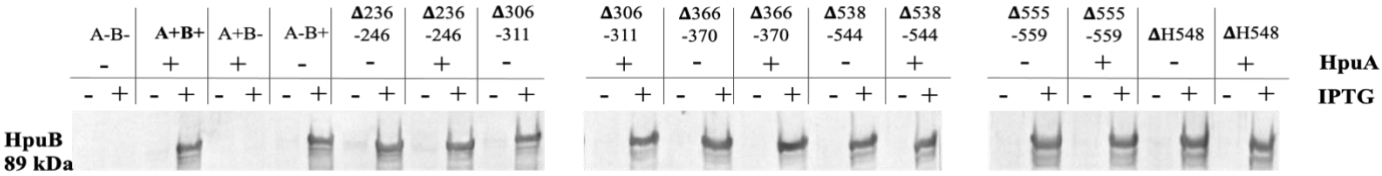



**Figure 11: Mutated *HpuB* Genes Were Created and Confirmed to be Expressed from an Inducible Promoter in an Ectopic Site in the Gonococcal Chromosome.**

HpuB mutant strains were grown on GCB plates containing DFO (Fe limiting), with (+) or without (-) 1 mM IPTG. Cells were collected from plates and resuspended into PBS. From these suspensions, whole-cell lysates were prepared by standardizing the cell suspensions to an $OD_{600}$ of 1, pelleting cells and resuspending pellets in lysis buffer. Western blots were performed to characterize the production of HpuB in the presence or absence of IPTG using anti-HpuB antibody. The strain with WT *hpuB* gene in the ectopic site and native *hpuA* gene was used as a positive control (A+B+). The strain lacking both native *hpuA* and *hpuB* genes (A-B-) was used as a negative control. Equal loading of each lane is confirmed by examination of stain-free gels before electro-transfer for western blot (not shown). Data shown is representative of 3 biological replicates.

### *c) Loop 7 HpuB Mutants Were Impaired for Growth on Hemoglobin as a Sole Iron Source*

We evaluated whether these mutations impact the ability of *N. gonorrhoeae* to utilize hemoglobin and whether the presence or absence of the lipoprotein, HpuA, also impacted utilization. We performed growth assays in metal-restricted conditions, with Desferal (DFO, an iron chelator) and IPTG to induce HpuB expression before adding 0 or 1 µM human hemoglobin (hHb) as the sole source of iron. The growth of all the mutant strains, under iron restriction and with the presence of IPTG, was compared to that of the positive control A+B+ (Fig. 12). In the absence of any iron source (0 µM hHb), no growth was observed for any of strains regardless of the expression of native *hpuA* (Fig. 12A and B). However, when 1 µM hHb was added, the *hpuB* mutants demonstrated different levels of growth. As expected, the negative control, RSC150 (A-B-), exhibited no detectable growth and the positive control, RSC284 (A+B+), demonstrated the most robust growth.

When HpuA is not expressed, the Δ236-246 and Δ306-311 mutations resulted in strains that demonstrated growth levels similar to the A+B+ strain; Δ366-370 was both significantly lower than A+B+ and higher than A-B-. The loop 7 mutants Δ538-544, ΔH548, Δ555-559 all demonstrated a significant growth defect, comparable to the A-B- strain (Fig. 13A). HpuA and HpuB are both thought to be required for hHb utilization (280). To assess the requirement for HpuA in the mutant strains generated in this study, we measured the growth of RSC275 (A+B-) and RSC276 (A-B+), both strains grew to the extent of the A-B- strain (Fig. 13A). This observation supports the importance of producing both HpuA and HpuB for utilization of hHb.

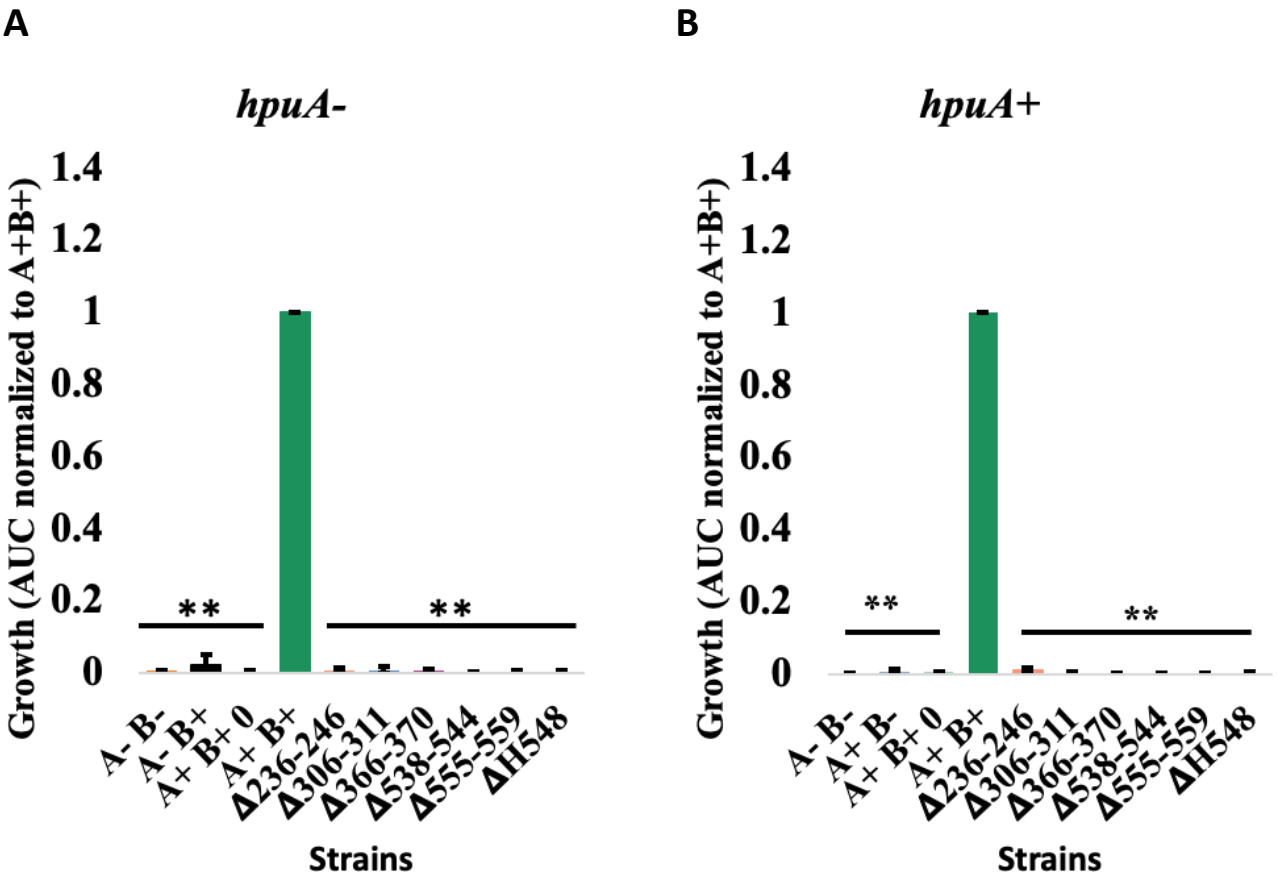



**Figure 12: *HpuB* Mutants are Impaired for Growth in the Absence of hHb.**

Gonococci were grown on GCB/DFO/ IPTG plates before being resuspended into CDM. The cell suspensions were standardized to an $OD_{600}$ of 0.002 before being added to a 96-well plate containing DFO, IPTG and 0 µM hHb. Cells were grown for 21 hours while the $OD_{600}$ was recorded in 30 minutes intervals to assess the growth of strains without native *hpuA* (A) and strains with native *hpuA* (B). A-B- is used as a negative control and A+B+ as a positive control with 1 µM Hb. A+B+ 0 µM Hb, A+B- as well as A-B+ also represent controls. The area under the curve (AUC) was calculated using GraphPad Prism then normalized to A+B+. Three biological replicates are represented with their means and standard deviation shown. A Student's *t*-test was used to calculate the statistical significance relative to A+B+ (**, $p < 0.005$).

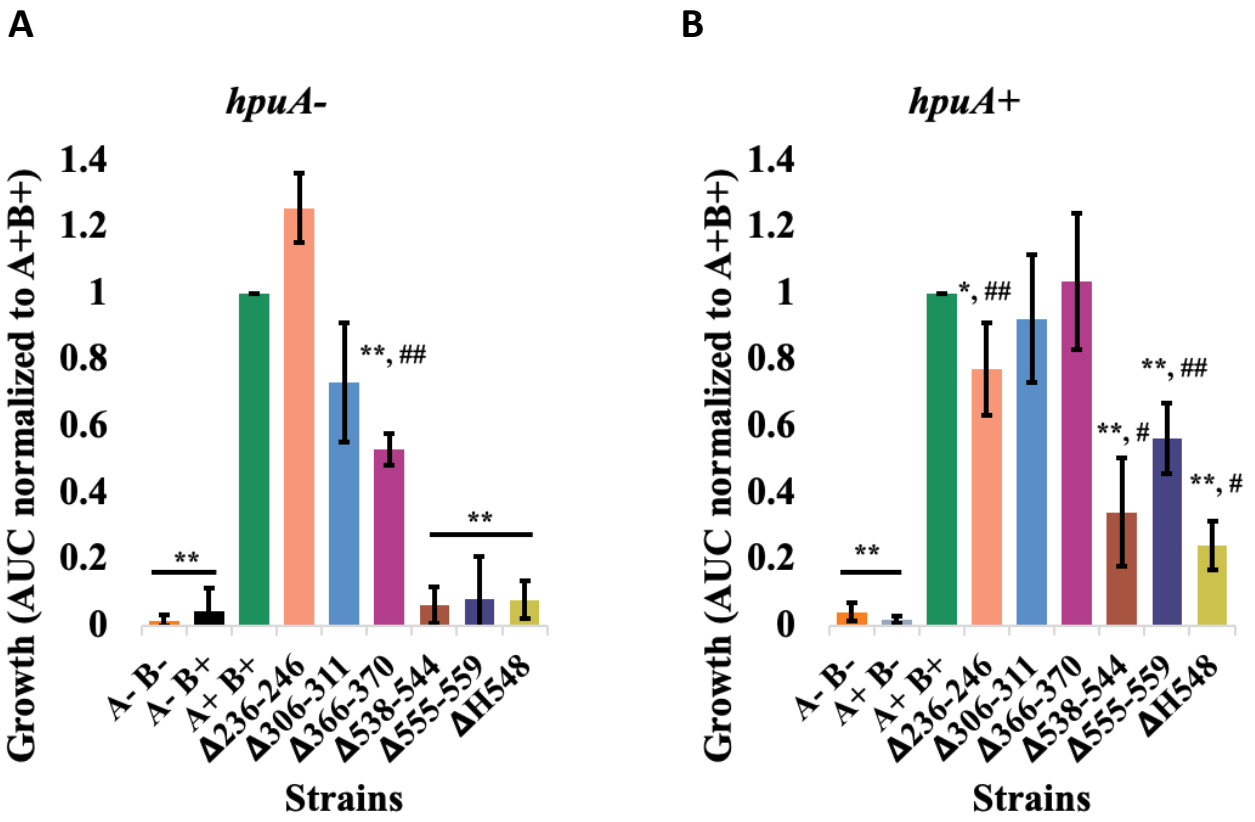



**Figure 13: Loop 7 *HpuB* Mutants are Impaired for Growth on Hemoglobin as a Sole Iron Source.**

Gonococci were grown on GCB/DFO/IPTG plates before being resuspended in CDM. The cell suspensions were standardized to an $OD_{600}$ of 0.002 before being added to a 96-well plate containing DFO, IPTG and 1 µM hHb. Cells were grown for 21 hours, during which the $OD_{600}$ was recorded in 30-minute intervals to assess the growth of strains in the mutant *hpuA* background (A) and in the wild-type *hpuA* background (B). A-B- is used as a negative control and A+B+ as a positive control. A+B- as well as A-B+ also represent controls as both proteins are thought to be required for growth. From the growth curves, the area under the curve (AUC) was calculated using GraphPad Prism then normalized to growth by the A+ B+ strain. The data from three biological replicates were analyzed to generate the means and standard deviations shown. Statistically significant differences were identified by using the Student's *t*-test relative to the A+B+ strain (*, $p < 0.05$; **, $p < 0.005$) and to A-B- strain (#, $p < 0.05$; ##, $p < 0.005$).

In strains where HpuA was expressed, the Δ306-311 and Δ366-370 mutations resulted in growth levels similar to that of the A+B+ strain. In contrast the Δ236-246 strain grew significantly below that of the A+B+ strain but still higher than A-B-. The loop 7 mutants, Δ538-544, Δ555-559, ΔH548, demonstrated a significant decrease in growth although they recovered some growth compared to when HpuA is not expressed (Fig. 13B).

***d) Loop 7 HpuB Mutants Recover hHb Binding Ability When HpuA is Produced***

To determine the binding phenotypes of these *hpuB* mutants, we performed whole-cell ELISA. As shown in Fig. 14, various levels of hHb binding were observed for the mutant strains. As expected, the negative control, RSC150 (A-B-), showed the least detectable binding and the positive control, RSC284 (A+B+), showed the highest level of binding. When HpuA was absent, Δ236-246 and Δ306-311 mutants bound hHb to the level of the A+B+ strain, while Δ366-370 demonstrated significantly reduced hHb binding. The loop 7 mutants Δ538-544, Δ555-559, ΔH548 exhibited profound hHb binding defects, comparable to that detected with the A-B- strain (Fig. 14A). RSC276 (A-B+) also bound hHb at the level demonstrated by the A+B+ strain, suggesting that HpuB alone can accomplish binding of hHb in this system.

When the lipoprotein HpuA was expressed in the native site, all mutants, except for Δ366-370, demonstrated hHb binding levels similar to that of the A+B+ strain. The Δ366-370 mutation resulted in a strain demonstrating a significant decrease in binding when compared to the A+B+ strain (Fig. 14B). RSC275 (A+B-) showed no detectable hHb binding, indicating that production of HpuA alone does not allow hHb binding.

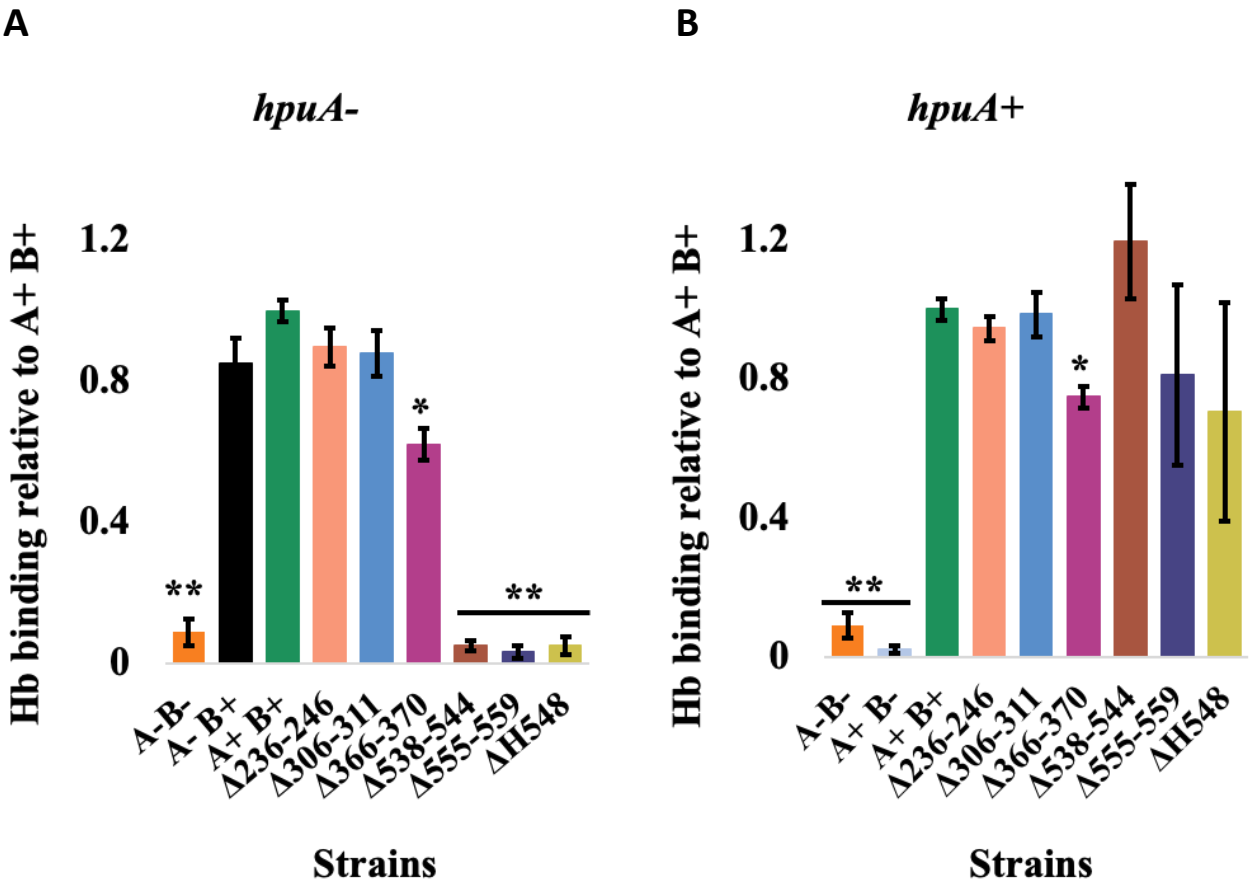



**Figure 14: Loop 7 H*puB* Mutants Recover hHb Binding Ability When HpuA is Produced.**
Gonococcal *hpuB* mutants were grown on GCB/DFO/IPTG plates before being resuspended in PBS to an $OD_{600}$ of 1. Cell suspensions were added to a 96-well ELISA plate and allowed to dry prior to blocking. The ELISA plate was then probed with HRP-conjugated hHb before being washed and developed with a TMB substrate. The binding ability of strains without (A) and with (B) native *hpuA* was assessed. The absorbance at 450 nm was read to quantify the signal. A-B- is used as a negative control and A+B+ as a positive control. A+B- as well and A-B+ are also used as controls since both HpuA and HpuB are thought to be required for binding. All the strains were normalized to A+B+ and showed as a percentage of A+B+. Three biological replicates are represented with their means and standard deviation shown. A Student's *t*-test was used to assess the statistically significance differences relative to A+B+ (*, $p < 0.005$; **, $p < 0.0005$).

### *e) Mutated HpuB Variants are Expressed on the Gonococcal Cell Surface and Susceptible to Trypsin Digestion*

To ensure that the mutations generated did not affect the surface exposure and the overall conformation of the resulting HpuB proteins, we conducted a cell-surface protease digestion. The mutated strains were grown on plates supplemented with both DFO and IPTG. Following overnight growth, colonies were recovered from plates and resuspended in PBS; standardized cell suspensions were subjected to a time course of trypsin digestion (257) before whole-cell lysates were prepared. The lysates were then subjected to SDS-PAGE and transferred to nitrocellulose for subsequent detection of HpuB and proteolysis products using the HpuB peptide-specific polyclonal antiserum (Fig. 15). It is important to note that only the proteolytic products containing the peptide epitope, used to generate this antiserum, were detected. A proper extracellular localization is confirmed by having a digestion pattern similar to that of the A+B+ wild-type strain. Over the time course utilized, the full length HpuB (~89 kDa), was digested into ~ 65kDa, ~50 kDa, and ~25 kDa fragments as seen in the A+B+ control strain. Mutations at locations $\Delta$236-246, $\Delta$306-311 and $\Delta$366-370 resulted in HpuB-producing strains that display digestion patterns identical to that of the A+B+ control, indicating proper folding and surface exposure. The loop 7 mutants, however, demonstrated more proteolytic products than the A+B+ strain, with additional fragments at ~55 kDa, ~44 kDa, and ~30 kDa, consistent with surface presentation but also additional exposure of trypsin sensitive digestion sites (Fig. 15). Taken together, these data indicate that all the mutants generated in this study presented HpuB on the gonococcal cell surface.

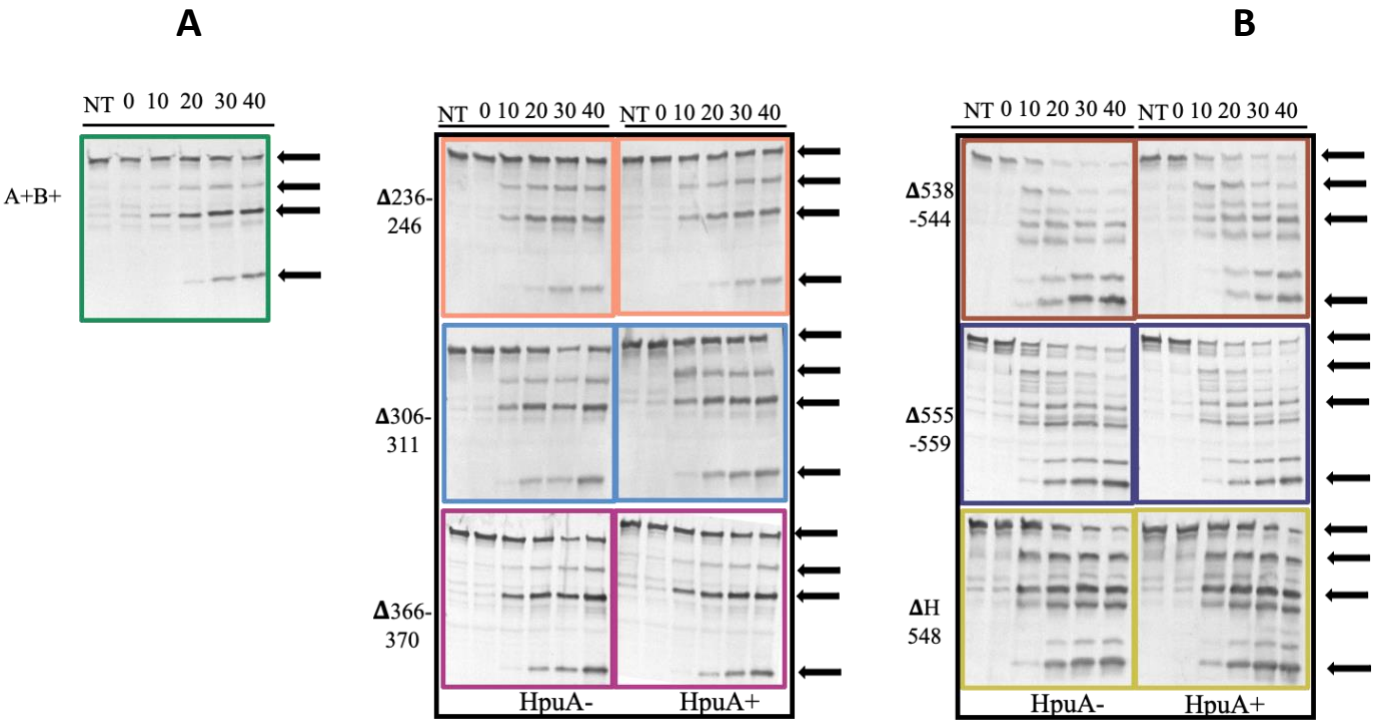



**Figure 15: Mutated HpuB Variants are Expressed on the Gonococcal Cell Surface and Susceptible to Trypsin Digestion.**

Strains were grown on GCB/DFO/IPTG plates and resuspended in PBS to an OD600 of 0.4. Iron-starved whole cells were then treated with trypsin for 0, 10, 20, 30 and 40 min before the reaction was stopped with aprotinin. NT represents no treatment. Next, the cells were pelleted, and the lysates were subjected to SDS-PAGE and western blot. The blots were probed with anti-HpuB antibody followed by an AP-conjugated IgG secondary antibody. NBT/BCIP was used to develop the blots. A+B+ shows the positive control trypsin digestion pattern (A). The digestion pattern of the mutants is shown (B). The black arrows indicate the proteolytic products seen in the positive control.

### *f) The Deletions in Loop 7 Move Histidine 548 Further Away from the Closest Heme Group*

After obtaining the results described above, we sought to better understand the role of loop 7 and particularly the role of the conserved histidine and the NPEL motif, which are conserved among heme transporters of many Gram-negative bacteria. To analyze the interfaces in a HpuA-HpuB-Hb ternary complex, the Alphafold2-based Colabfold running in Alphafold-multimer (422-424) mode was used to generate predictive models of the three proteins in complex. A known Hb-heme crystal structure (PDB:1hho) was super-imposed onto the complex to identify the locations of the heme groups. Our predicted ternary structure shows HpuB in green, HpuA in blue, Hb in red, and the corresponding heme group in white with its $Fe^{2+}$ as a red ball (Fig. 16A). A zoomed-in image of the interaction shows HpuB in green with residue H548 highlighted in red and the distance between the closest heme group and H548 is measured in angstroms (Å); the distance between the closest heme group and H548 is 4.6 Å (Fig. 16B). However, this distance increases to 18.1 Å when the hypothetical α-helix Δ538-544 is deleted (Fig. 16C) and to 22.3 Å when the NPEL motif Δ555-559 is deleted (Fig. 16D). Interestingly, in the predicted binary complex of HpuB and Hb only, the distance between residue H548 and the heme group is 5.7 Å, in the mutants this distance is 14.2 Å when Δ538-544 is deleted and 10.5 Å when Δ555-559 is deleted (Fig. 17). The relative position of the histidine, thought to be essential for receptor function (335), changes with the Δ538-544 and Δ555-559 deletions pushing the histidine further away from the surface and from interacting with the heme group in the hHb.

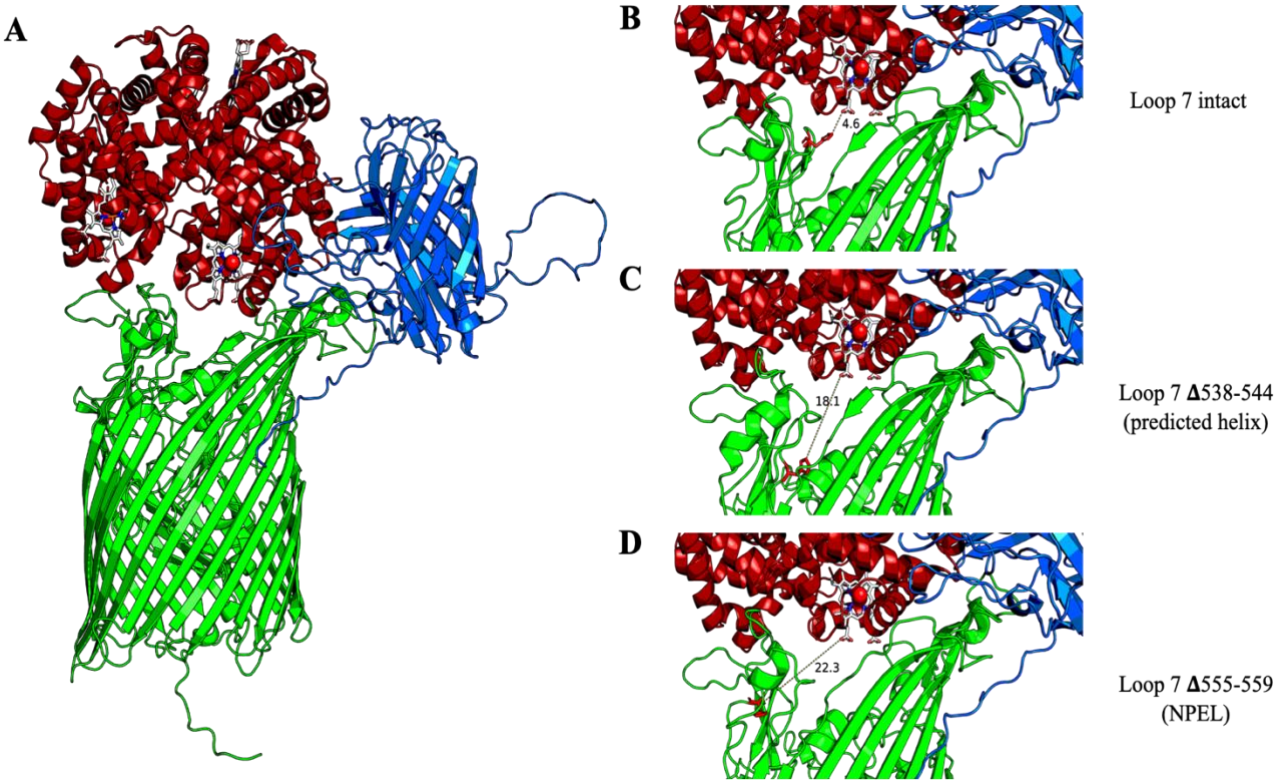



**Figure 16: The Deletions in Loop 7 Move Histidine 548 Further Away from the Closest Heme Group. HpuA-HpuB-Hb Structure.**

AlphaFold2 was used to get a prediction model of the structures. A known Hb-heme crystal structure (PDB:1hho) was super imposed to show the heme groups. HpuB is in green with H548 highlighted in red, HpuA in blue, Hb in red, heme group in white with is $Fe^{2+}$ as a red dot. The distance in angstroms (Å) between the heme group and histidine at position 548 is measured. The model was made by Dr. Dixon Ng, from the Moraes lab, at the University of Toronto.

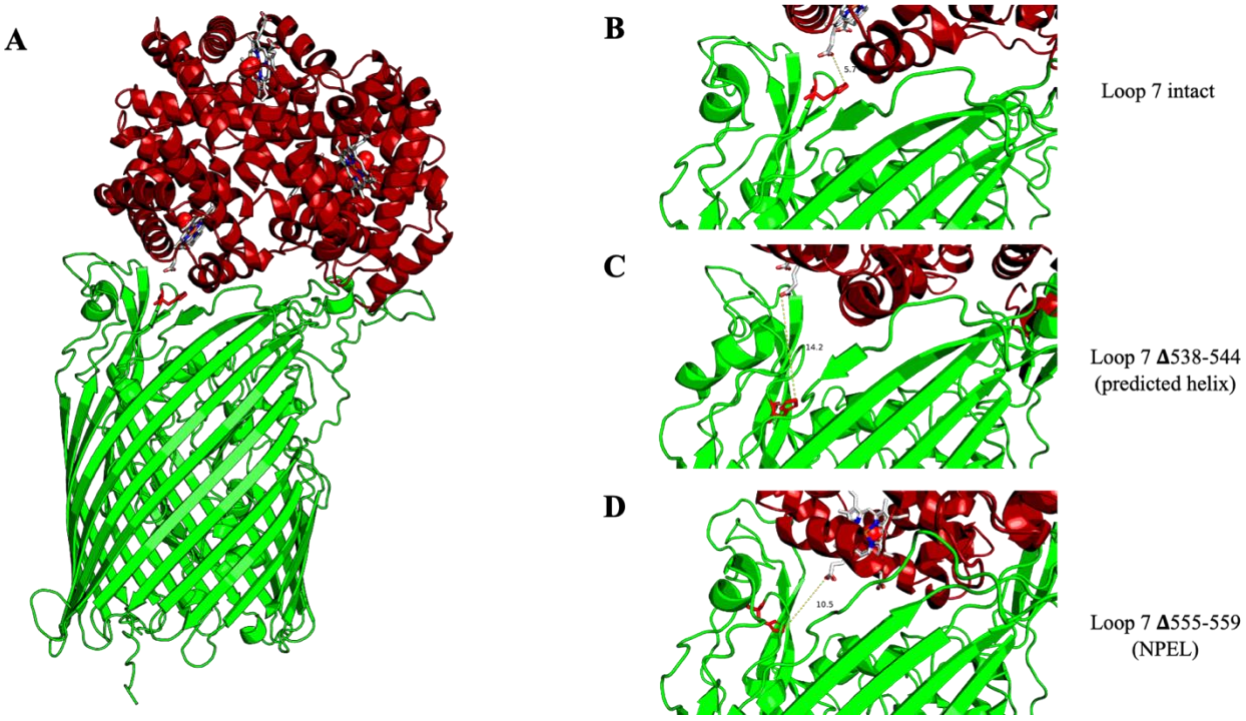



**Figure 17: The Deletions in Loop 7 Move Histidine 548 Further Away from the Closest Heme Group. HpuB-Hb Structure.**

AlphaFold2 was used to get a prediction model of the structures. A known Hb-heme crystal structure (PDB:1hho) was super imposed to show the heme groups. HpuB is in green with H548 highlighted in red, Hb in red, heme group in white with is $Fe^{2+}$ as a red dot. The distance in angstroms (Å) between the heme group and histidine at position 548 is measured. The model was made by Dr. Dixon Ng, from the Moraes lab, at the University of Toronto.

### *g) N. Gonorrhoeae Can Bind and Utilize Hemoglobin Produced by Animals Other Than Humans, as a Sole Iron Source*

To date, *N. gonorrhoeae* has been demonstrated to bind and utilize TdTs ligands primarily of human origin (295, 439) To determine if this concept applies to the HpuAB system and whether *N. gonorrhoeae* only binds and utilizes hemoglobin of human origin, we performed a competition assay and a growth assay. A wavelength scan (Fig. 18) was also conducted to ensure that the purchased Hb from all species of origin, were in the reduced (ferrous iron) form.

A competition assay was conducted to establish whether *N. gonorrhoeae* can bind to Hb from mouse (mHb), pig (pHb) and rat (rHb). RSC284 (A+B+) was grown as described above and tested by whole-cell ELISA for hHb binding in the presence of other species of Hb as competitors. As expected, the lowest detectable level of binding was seen in the negative control (blocker only) and the highest level of binding was detected when hHb-HRP was allowed to bind without competitor (Fig. 19). Unlabeled human, mouse, pig, and rat hemoglobin all competed with HRP-conjugated hHb, as seen by a significant decrease in labeled hHb binding. *N. gonorrhoeae* can, therefore, bind mouse, pig, and rat Hb in addition to hHb.

Given this result, we next assessed the ability of the gonococcus to utilize Hb from different species by conducting a growth assay as described above, but this time, either hHb, mHb, pHb or rHb was provided as the sole source of iron. The growth of RSC284 (A+B+) with Hb from different species was compared to the growth of A+B+ in the presence of hHb (Fig. 20). An AUC calculation was used to represent the total amount of growth over the entire assay. The condition to which no iron source was added, as expected, demonstrated the least amount of

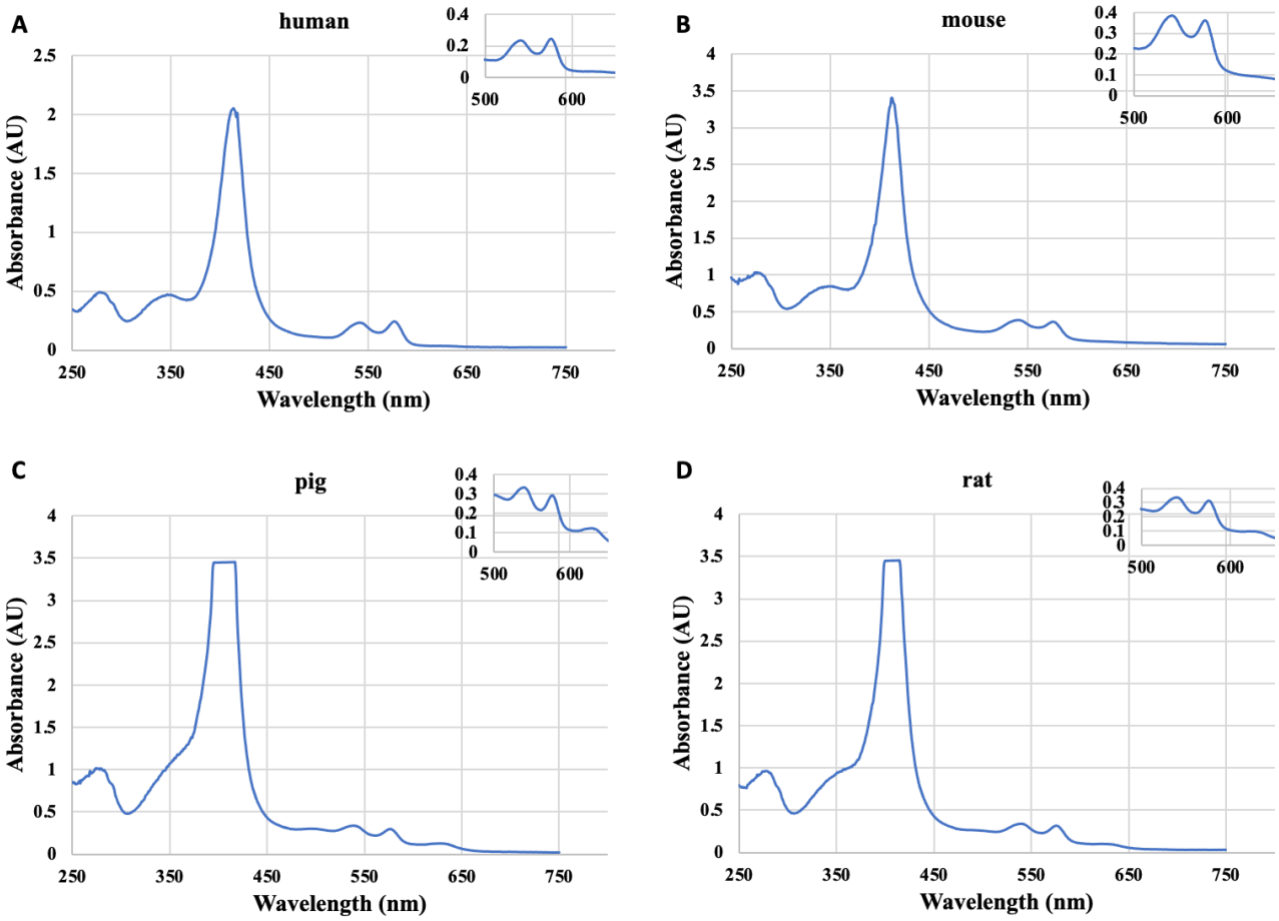



**Figure 18: The Hemoglobin of Different Species is in the Reduced State, Oxyhemoglobin.**
Soret bands are showing peaks for each Hb tested. Each of the different Hb species human (A), mouse (B), pig (C) and rat (D) were diluted to ~10 µm in HEPES. A spectrophotometer was used to do a wavelength scanning which generates peaks for reduced Hb at ~410 nm and peaks for oxidized Hb at ~405 nm. Strong alpha and beta peaks, at ~550-600 nm (shown in top right corner of each graph), are also indicative of ferrous Hb (reduced Hb). Although the pig and rat Hbs are showing a wide peak between 405-410 nm, the alpha and beta peaks at ~550-600 nm confirm the ferrous state of the Hb, suggesting that the stock used were too concentrated.

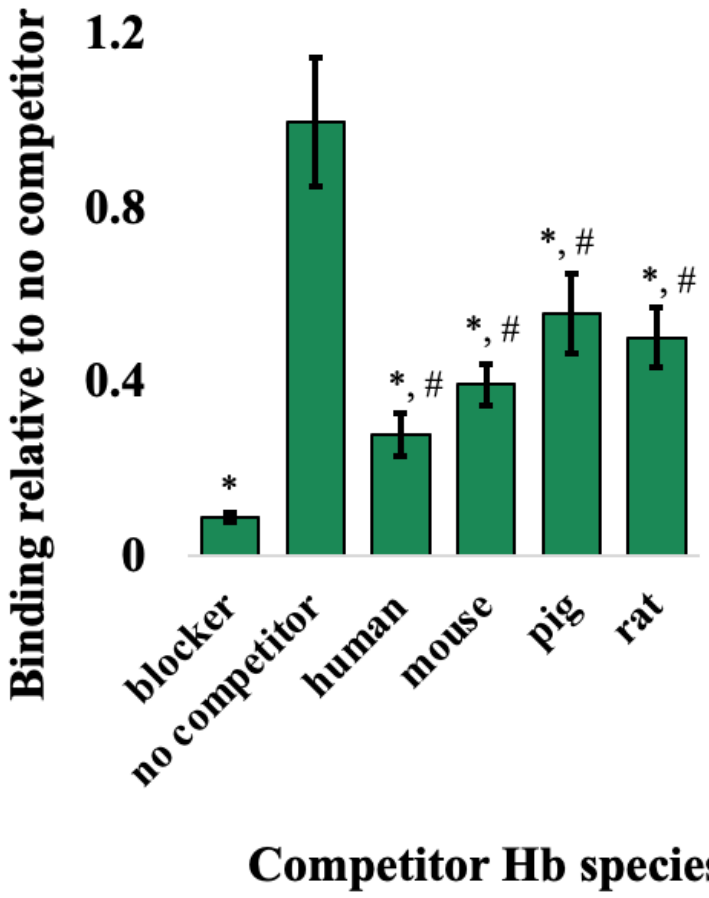



**Figure 19: *N. Gonorrhoeae* Can Bind Hemoglobin Produced by Animals Other than Humans.** A competition assay was used to establish whether *N. gonorrhoeae* can bind to Hb from mouse (mHb), pig (pHb) and rat (rHb). A+B+ was grown on GCB/DFO/IPTG plates before being resuspended in PBS to an $OD_{600}$ of 1. The cell suspension was added to a 96-well ELISA plate and allowed to dry prior to blocking. The ELISA plate was then probed with either no Hb (i.e blocker), 5 nm hHb-HRP without competitor or 5 nm hHb-HRP + 20X (100 nm) excess competitor. The competitors used were unlabeled hHb, mHb, pHb or rHb. Next, step was to develop with TMB. 5 nm hHb-HRP + no competitor represents the positive control, and the blocker condition represents the negative control. Statistically significant differences were assessed by the Student's *t*-test relative to the no competitor condition (*, $p < 0.0005$) and the blocker condition (#, $p < 0.0005$). The means and standard deviation of 4 biological replicates is shown.

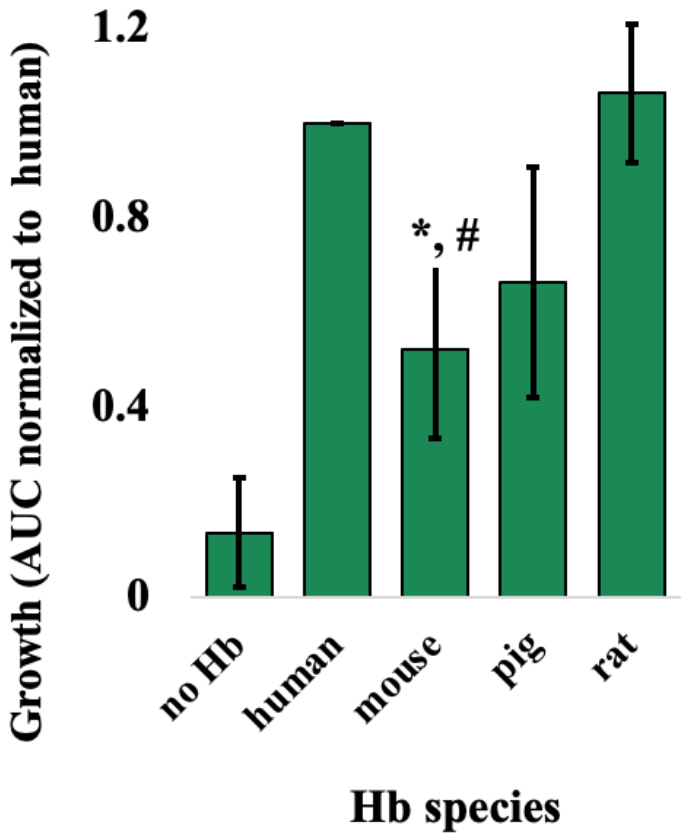



**Figure 20: *N.Gonorrhoeae* Can Grow on Hemoglobin, Produced by Species Other than Human, as a Sole Iron Source.**

A growth assay was used to establish whether *N. gonorrhoeae* could grow on different Hb species, as their sole iron source. A+B+ was grown on GCB/DFO/ IPTG plates before being resuspended into CDM. The cell suspension was standardized to an $OD_{600}$ of 0.002 before being added to a 96-well plate containing DFO and IPTG with no Hb or 1 µM human, mouse, pig, or rat Hb. Cells were grown for 21 hours while the $OD_{600}$ was recorded in 30 minutes intervals to assess the growth of A+B+ with hemoglobin of different species. The human Hb condition represents the positive control and the no Hb condition represents the negative control. From the growth curves, the area under the curve (AUC) was calculated using GraphPad Prism then normalized to growth on human Hb. Three biological replicates are represented with their means and standard deviation shown. Statistically significant differences were assessed by the Student's *t*-test relative to the human Hb condition (*, $p < 0.05$) and no Hb (#, $p < 0.05$).

growth while the highest amount of growth was detected when hHb was provided. The A+B+ strain was similarly able to utilize hHb, pHb and rHb for growth in this assay. However, mHb supported growth of A+B+ to some extent, with levels significantly higher than the no iron control but growth was significantly lower than detected in the hHb condition. In contrast to the published observations with other gonococcal nutrient transport systems, these results indicate that *N. gonorrhoeae* can bind to and utilize hemoglobin from different species, in addition to human.

## III. Discussion

The fight against gonorrhea, a serious public health concern, persists. There is no protective immunity elicited following infection; moreover, the number of gonococcal isolates resistant to all available antibiotics is dramatically increasing. The lack of an efficacious vaccine against *N. gonorrhoeae* does not help our odds at beating this pathogen; however, the development of an effective vaccine could significantly decrease disease incidence. Like other Gram-negative bacteria, *N. gonorrhoeae* requires metal inside the human host but is confronted with nutritional immunity imposed by the host. The human host restricts essential nutrients, like iron, in an effort to prevent bacteria from colonizing and causing disease. To overcome these metal-limited conditions imposed by the host, *N. gonorrhoeae* expresses eight outer membrane TdTs that bind to host metal-sequestering proteins to bypass nutritional immunity and access their metal load (429). Of these TdTs, three are bipartite receptors consisting of a lipoprotein and a transmembrane protein: TbpAB, and LbpAB which respectively

enable iron uptake from transferrin and lactoferrin (257, 269, 270, 440, 441). HpuAB, the subject of the current investigation facilitates iron acquisition from hemoglobin (273, 280).

In our study, we hypothesized that deletion of selected, putative HpuB loops would reduce or abrogate Hb binding and consequently cause defects in growth with Hb as a sole iron source. We expected that these deletion mutations would help identify domains of the gonococcal HpuB transporter that are essential for ligand binding and iron uptake functions. Loops 2, 3, 4 of HpuB were targeted in the current study; loop 7, which contained conserved motifs found in many heme transporters was also mutagenized. These loops were selected due to homology with similar ligand binding domains in TbpA (282, 442-445) and in the meningococcal Hb receptor, HmbR (274, 446). TbpA and HmbR are approximately 25% and 28% identical to HpuB, respectively.

Both HpuA and HpuB were thought to be required for Hb utilization (280, 281, 300). In our study, we generated HpuB loop deletion mutants, near the N-terminus between residues 236 and 559. Surprisingly, we observed that RSC275 (A+B-), that only expresses the lipoprotein HpuA, could neither bind Hb nor utilize it for growth whereas RSC276 (A-B+), that only expresses the transmembrane protein HpuB, could bind hHb but not utilize it as a sole iron source to grow. This suggests a potential role for HpuA in increasing the efficiency of iron uptake by HpuB, as seen with the lipoprotein of the TbpAB system (258).

In the absence of the lipoprotein HpuA, mutant strains with deletions of $\Delta$236-246 and $\Delta$306-311 were able to bind to and utilize hHb at A+B+ control levels while $\Delta$366-370 bound to and utilized Hb at levels significantly lower than that of the A+B+ positive control. This is interesting as RSC276, which also does not express HpuA, cannot utilize hHb when producing

the WT version of HpuB, suggesting that these mutations allowed for the need of HpuA to be bypassed during internalization.

In a study by Chen *et al.,* (276) a strain lacking HpuA and producing WT HpuB was unable to grow on hHb (Hb-). However, after growth on Hb plates, discovered Hb+ revertants, that resulted from single point mutations in HpuB (clustered towards the C-terminus) restored growth on Hb as a sole iron source, in the absence of HpuA. They attributed this restored growth to the presence of free heme, released from Hb, externally that was then internalized. In their work, they also suggested that the requirement for HpuA, during iron acquisition, could be bypassed by the point mutations in HpuB. Given the likeness in growth phenotypes, when HpuA is absent, between these strains with spontaneous mutations and the generated strains of our study, it could be biologically relevant for the gonococcus to have ways of overcoming the need for HpuA.

Our study also showed that, when HpuA was expressed, Δ236-246, Δ306-311 and Δ366-370 were all able to bind and grow on hHb, with Δ236-246 growing significantly lower and Δ366-370 binding significantly lower than A+B+. Additionally, when looking closely at the AlphaFold2 models, the locations of the deletions in loop 2, 3, and 4, all seemed to be near the hHb and/or HpuA interface. Therefore, a possible explanation for the ability of the strains, lacking HpuA, to grow on hHb could be that the mutations in HpuB circumvent the requirement for HpuA, by making the barrel more accessible and allowing more efficient heme internalization. We speculate that a potential mechanism to explain our results is that during hHb binding and metal internalization, HpuA acts by pulling loops away from the orifice of the barrel to facilitate heme uptake, as seen by the enhanced growth phenotypes when HpuA was

present. The HpuB deletions could, therefore, possibly be enough to move domains of these loops out of way and to enable heme uptake, even when HpuA is absent.

In contrast, the HpuB loop 7 deletion mutants demonstrated abrogated binding and growth on hHb when HpuA was not expressed. Interestingly, when HpuA was expressed, these loop 7 mutants completely recovered their hHb binding abilities; we observed partially recovered growth on hHb although this was still significantly reduced compared to the control A+B+ strain. This points to the importance of loop 7 residues, 538-544 (hypothetical α-helix), H548 (histidine motif) and 555-559 (NPEL motif), for growth on hHb, but also the importance of HpuA in facilitating the binding of hHb with HpuB in these loop 7 mutants.

Many heme uptake systems in Gram-negative bacteria contain heme-binding motifs, including two conserved histidine residues located between a FRAP and a NXXL motif (335). The histidine residues are not found in all Gram-negatives and looking at the sequence from *N. gonorrhoeae* strain FA19, we identified one histidine residue between a FRAP and a NPEL motif. AlphaFold 2 prediction models of Hb-HpuB with and without HpuA, with a focus on loop 7, were also generated in this study. The distance between the identified histidine at position 548 (H548) and the heme group of the closest Hb subunit, thought to be the heme source, was measured. In the presence of HpuA, the distance between H548 and the heme group is shorter than when HpuA is absent. This could explain a possible role for HpuA, which is to help stabilize HpuB in a conformation that favors a better binding affinity and a shorter required distance between H548 and the heme to be transported across the barrel. This would be different from the other bipartite systems (TbpAB and LbpAB) where the lipoprotein is directly capturing its cognate substrate and facilitating transfer in complex with the barrel protein. Interestingly,

regardless of HpuA expression, when comparing this measured distance in intact HpuB to that of HpuB Δ538-544 (hypothetical α-helix), or HpuB Δ555-559 (NPEL motif), the distance is longer in the deletion mutants where H548 seem to be pointing away from the surface and into the barrel. This could explain the growth defect seen in the loop 7 mutants as H548 would be too far away to interact with hHb, possibly preventing uptake. Finally, as the presence of HpuA restored the binding capability of the loop 7 mutants, another possible role for HpuA is to aid in ligand binding. Taken together, a possible mechanism of function could be that HpuA helps better stabilize the binding between HpuB and hHb bringing, therefore, the heme group close enough to the putative heme binding motif, H548, that possibly helps to initiate heme uptake.

These observations mentioned above can also account for the slight differences seen in the protease accessibility assay of the loop 7 mutants. These mutations may not have disturbed the conformational structure of HpuB, but rather the H548 pointing towards the barrel could have exposed other residues, not usually extracellular, to trypsin causing more proteolytic products. Additionally, given that HpuB represents the only portal of iron from Hb through the outer membrane, the fact that HpuA rescues the loop 7 mutations, binding defect, indicates that this mutated protein must be conformationally correct and functional.

In a previous study, it was noted that although heme did not compete with $^{125}$I-labelled hHb for binding the meningococcal HpuAB, this system did not discriminate between Hb of different species, possibly due to HpuAB specifically recognizing the heme moiety of Hb (282). A study of meningococcal HmbR also showed a significant difference in the abilities of some animal Hbs to serve as iron sources or to enable the growth stimulation accomplished by hHb (326). In our gonococcal study, the wild-type A+B+ strain was able to bind HRP-conjugated hHb

and the binding was also inhibited by unlabeled human, mouse, pig, or rat Hb species, indicating that the gonococcal receptor system, similar to that described for *N. meningitidis* recognized other species of Hb other than that from humans. Subsequently, we determined that Hb from these different species also allowed growth of the wild-type strain at levels lower than that generated with hHb, but the growth with the alternative forms of Hb still resulted in growth significantly above background. These results are interesting as precedent suggested that *N. gonorrhoeae* exclusively bound to the human forms of the nutritional immunity proteins, including human transferrin, calprotectin and S100A7 (249, 295, 439). However, the sequence conservation of Hbs from the different species is very high, consistent with the ability of the HpuAB system to recognize multiple forms of Hb from distinct animal species. While transgenic mouse models of disease are being developed (63, 65), to assess the contributions of TbpAB and the zinc transport systems to virulence, this may not be necessary for the HpuAB system as the mouse Hb can presumably be employed as an iron and heme source if it is available during experimental infections.

Although most of the gonococci isolated from humans do not express the hemoglobin receptor, the genes encoding the HpuAB system are maintained (273, 280) and could have a role in pathogenesis. One study concluded that the hemoglobin receptors facilitate invasion and dissemination of *N. meningitidis* in the vascular system (272). Moreover, during infection in the early phases of a woman's menstrual cycle, expression of the gonococcal Hb receptor appears to have a selective advantage. (279). Therefore, elucidating the advantages of expressing these hemoglobin receptors would be crucial in determining their potential as vaccine components.

In summary, this study characterized structure-function relationships in the gonococcal TonB-dependent transporter, HpuB. We showed that some mutations in HpuB bypassed the need for the lipoprotein, HpuA, for binding and uptake. We also demonstrated the particular importance of the putative heme motifs, in loop 7, for the internalization of heme. Our findings also highlighted the importance of HpuA, for binding hHb, as the lipoprotein completely rescued the binding defect of the loop 7 mutants. We saw the importance of residues 538-544 which constitute a hypothetical α-helical region in loop 7. This region could have a significant function in the interaction of HpuB with hHb, and in ensuring heme extraction from Hb as seen previously with loop 3 helices of both TbpA and TdfJ (296, 439, 447). Therefore, acquiring a high-resolution structure of HpuB, either by protein x-ray crystallography or cryo-electron microcopy, will help better inform the choice of future mutations and even help decide whether to target helices found on the extracellular loops as in other studies (296, 439, 447). As previously published, Transferrin-Binding Protein B (TbpB) and factor H binding protein (fHbp) mutants unable to bind host ligands demonstrated better vaccine potential (121, 448, 449). Although a similarly enhanced immunogenicity has not yet been described for the TdTs following mutagenesis, it is hypothesized that, a cocktail of non-binding mutants from the TdTs, could enhance vaccine potential and offer protection. Therefore, further characterizing HpuB and other TdTs for vulnerable surface-exposed, ligand-binding motifs is an important step towards potentially developing a protective anti-gonococcal vaccine.

# CHAPTER 6: SUMMARY AND PERSPECTIVES

The Centers for Disease Control and Prevention listed *Neisseria gonorrhoeae* as an urgent threat pathogen for increased antimicrobial resistance. As of 2019, it is one of only five pathogens in this highest-level category of threat (450). *N. gonorrhoeae* is a serious public health concern with a yearly estimate of 710,151 new infections in 2021 and a financial burden as high as $271 million in 2018, in the United States alone (68). The lack of protective immunity from previous infections contributes to this public health issue, as high-risk individuals can be reinfected repeatedly (69). Moreover, worldwide reports of increasingly antibiotic resistant clinical isolates combined with no vaccines available for prevention intensified the gravity of this public health problem (99, 107). Taken together these issues raise real concerns that we may be reaching a stage of untreatable gonorrhea, and there is, therefore, an urgent need for therapeutic and preventive strategies. One approach to the development of vaccines is to study the outer membrane TonB-dependent transporters (TdTs) as vaccine targets (428, 451).

In our studies, we focused our work on the gonococcal bipartite TdT HpuAB which is expressed in many *Neisseria* species but is subjected to recombination and immune selection (272, 452, 453). The HpuAB system undergoes phase variation due to slip strand mispairing, but is found to be expressed in women in the first two weeks of their menstrual cycle (279). HpuAB functions by hijacking heme or iron from hemoglobin (Hb) and hemoglobin bound haptoglobin (Hb-Hp). Moreover, HpuAB is thought to be important for meningococcal invasion and dissemination in the vascular system (272). This suggests a possible role in for HpuAB in gonococcal dissemination.

To investigate the structure-function relationships of the HpuAB transport system, our work began with attempts to assess the binding and growth ability of gonococcal strains with mutated hydrophobic residues in HpuA, from the study by Wong *et al.,* (278), that seemed to directly interact with Hb. In Wong *et al.,* (278), the crystal structure of HpuA was solved and the first binding interaction between HpuA and Hb was shown in a pulldown assay, in the absence of the transmembrane protein, HpuB. In our study, and in the presence of HpuB, mutants containing HpuA Δ111-116 and HpuA P114A were incapable of growth on Hb as sole iron source, suggesting the importance of the deleted residues Δ111-116 and the mutated residue P114A in the growth on Hb. Next, our binding assay showed that, regardless of the production of HpuB, S112A, S113A, P114A, and F116A mutants, within *hpuA* loop 2, were unable to bind Hb. Additionally, D115A was defective, for binding in the absence of HpuB and recovered its binding ability in the presence of HpuB. Our results, show a direct interaction between HpuA and human Hb, similar to that seen in Wong *et al.,* (278). However, some strains, with *hpuA* mutated in the loop 2 region, were found to have no growth defect but seemed to be impaired for binding, which was surprising as we expected that a binding defect would lead to a growth defect. A possible explanation for this is that the level of HpuB produced in the native site may have been reduced because of the non-polar *kan* cassette placed in *hpuA*. Another surprising observation is that a strain expressing *hpuA* only and not *hpuB* was able to bind at wild type levels. This could be due to an overexpression of *hpuA* from the ectopic site. Thus more characterization and using a different approach such as moving both genes in the ectopic site will be required to, more completely, assess the role of the HpuA loop regions on Hb utilization.

We then aimed to investigate the structure function relationship of HpuB with Hb to identify key residues important for binding and growth on Hb as a sole iron source. Based on their importance in the binding and/or growth of gonococcal TbpA on transferrin or that of the meningococcal HmbR on Hb, loops 2, 3 and 4 were targeted. In previous works, it was also found that mutagenesis of the helix in loop 3 of TbpA or TdfJ was sufficient to cause a binding defect or abrogated growth on transferrin or S100A7, respectively (296, 439, 447). With these previous studies, the presence of a hypothetical α-helix and conserved motifs, found in a wide range of heme transporters, made the gonococcal HpuB loop 7 an interesting target. We hypothesized that HpuB's loop 7 was important for iron piracy from Hb.

Deleted residues in loops 2, 3 and 4 did not exhibit a binding or growth defect regardless of the presence of the lipoprotein, this suggests that the requirement for HpuA was bypassed maybe because the deletions allowed the HpuB orifice to be more accessible. However, loop 7 mutants with deleted histidine or NXXL conserved motifs found in heme transporters, or with the deleted hypothetical α-helix were found to be defective for growth on Hb, this highlights the importance of loop 7 in the acquisition of iron from Hb. Interestingly, as seen with TbpA and TdfJ, in our work a deletion of a hypothetical α-helix in loop 7 of HpuB also abrogated growth on Hb. Taken together, these results suggest that α-helices in the extracellular loops of TdTs play an important role in metal uptake and that the helical shape might be more important for metal piracy than its amino acid sequence. Therefore, future studies should particularly explore the individual residues that constitute the hypothetical α-helix of HpuB loop 7 to better characterize its role in iron or heme piracy.

Moreover, when comparing the prediction model of Hb-HpuB to that of Hb-HpuB-HpuA ternary complex, specifically loop 7 and its conserved motifs found in heme transporters, the distance between the histidine at position 548 and the closest heme group was found to be shorter in the absence of HpuA, suggesting a potential role for HpuA in helping stabilize HpuB in a conformation that favors a better binding affinity. Additionally, when comparing this same distance in the ternary complex, it was found to be shorter in wild type HpuB than in HpuB loop 7 mutants ($\Delta$538-544 hypothetical helix, or $\Delta$555-559 NPEL motif). Taken together, a possible mechanism of function could be that HpuA helps better stabilize the binding between HpuB and hHb which places the heme group close to the histidine motif H548, that possibly helps initiate heme uptake.

In our study we also found that *N. gonorrhoeae* could bind and utilize mouse, pig, and rat Hb as sole sources of iron. This was interesting because contrary to our data, it was previously found that *N. gonorrhoeae* exclusively bound to the human forms of the nutritional immunity proteins, including human transferrin, calprotectin and S100A7 (249, 295, 439). However, this can be due to the high sequence conservation of Hbs from the different species, or to the fact that the HpuAB might only recognize the heme moiety of Hb and can therefore, recognize multiple forms of Hb from distinct animal species.

Pathogenic *Neisseria* species employ TonB-dependent transporters (TdTs) for metal acquisition. These TdTs possess characteristics that make them strong candidates for vaccines, such as widespread presence among pathogenic strains, surface exposure, limited antigenic variation, increased expression during infection, and the ability to trigger a protective immune response (454). Multiple studies have found that when the TdTs are used as immunogens in

animals, they elicit bactericidal and cross protective immune responses that are more robust than during natural infection (454-456). Moreover, in previous studies, Transferrin-Binding Protein B (TbpB) and factor H binding protein (fHbp) mutants incapable of binding to host ligands, were found to elicit a more effective immune response (121, 448, 457, 458). A similarly enhanced immunogenicity with non-binding TdTs has yet to be described, however, the hypothesis is that a cocktail of non-binding TdTs mutants could enhance vaccine potential and offer protection. A suggested mechanism is that, compared to the host ligand-bound bacterial protein, the protein incapable of binding human ligand has more exposed epitopes that can be recognized by the immune system, therefore increasing the production of blocking antibodies. Consequently, there needs to be more structure-function characterization studies of the TdTs, as with the HpuAB system presented above, to identify non-binding mutants with the least altered overall protein structure as well as studies that evaluate the immunogenicity of these non-binding mutants in transgenic mouse models.

The TdTs are subjected to strong selective pressure that can drive variation (459). Moreover, because more than one TdT can import the same cargo (iron, zinc, siderophores, etc.) in the same strain, there is a possibility for redundancy between these systems, potentially making it necessary to target multiple TdTs to establish long-term protection rather than just a single TdT. While HpuAB may not be considered an efficacious vaccine candidate due to its phase variation and expression only in a small subset of gonococcal isolates, it may still be worth including in a vaccine cocktail. It is possible that when the iron-bound ligands, other than Hb, are absent or the other iron acquisition mechanisms are successfully compromised by

blocking antibodies, utilization of the HpuAB system may increase as a compensatory mechanism.

Additionally, for a gonococcal vaccine to work, considering the route of administration, population of people to target, and when to administer, is needed. Work in transgenic mice models will help narrow down the vaccination routes and choice of adjuvant, however, only clinical trials will be able to clearly determine which antigen combination is best suited (460-462). Moreover, the group of people that will first receive the gonococcal vaccine needs to be considered as this can impact the rate at which the cases will decrease. For instance, a study using an individual-based site-specific mathematical model simulation, predicted that administering the vaccine to 30% of a population of 10,000 men who have sex with men (MSM) could, within 2 years, reduce disease incidence by 62 % with a vaccine that has 50% efficacy or 94% with a 100% protective vaccine (463). This same study also predicted that with access to vaccine boosters, gonorrhea would be eradicated in eight years with efficacies of 50%, or more, with protection lasting two years (463). According to this study, a vaccine with relatively modest efficacy could, when combined with a sensible immunization plan, significantly and quickly reduce the frequency of gonorrhea. Additionally, determining when to administer the vaccine is crucial for the hope to eradicate this disease. As a sexually transmitted infection that highly affects individuals within the 15-24 years age range (68), pre-teens are also likely the targets for the introduction of the gonococcal vaccine. This could prove challenging depending on the parents' approval of the vaccine for their children and the rise of the anti-vaccine movement. Therefore, assessment of vaccine acceptance using tools such as the WHO Vaccine

Hesitancy Scale (464, 465), will also help with more realistic projections to better prepare for the arrival of an effective gonococcal vaccine.

The HpuAB system is not well characterized, and much is needed to better understand how the gonococcus utilizes Hb as an iron source via this system. The work on HpuA, in collaboration with Dr. Dixon Ng from the Moraes lab at the University of Toronto, allowed for the complete structure of the gonococcal HpuA to be solved, unlike in Wong *et al.,* (278), where only the barrel of HpuA was solved. With this now complete structure, future directions will include testing more residues to identify non-ligand binding HpuA. Similarly, future directions will focus on solving the crystal structure of HpuB to better inform the approach to developing non-binding HpuB mutants, although the loop 7 of HpuB seem a good start for identifying non-binding versions of the protein. Studying the HpuAB system in *N. gonorrhoeae* will also contribute important overlapping information that can serve the characterization of this same transport system expressed in other bacteria including *Neisseria meningitidis*, *Kingella* and *Eikenella*.

In conclusion, the work presented here has contributed new information on the structure-function relationships of each of the components of the HpuAB bipartite receptor. The findings have helped identify the domains that are essential for protein function and the regions that need closer investigation to fine-tune a non-binding mutant with the least altered overall protein structure. This work may help in the development of an efficacious TdT based vaccine or the identification of novel therapeutic targets to combat this worrisome public health threat, gonorrhea.

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## VITAE

Olivia Awate was born on September 21, 1992, in Dakar, Senegal. She attended Sacred Heart High School in Dakar, where she was very active in the school's handball team. In 2016, she received her B.S. in Biology from The College of William and Mary in Williamsburg, VA. After graduation, she did a one-year postbacclaureate program before enrolling in the BSDP PhD program at Virginia Commonwealth University in Richmond, VA. She then moved with her advisor and continued her PhD by matriculating into the PhD program at the Institute for Biomedical Sciences at Georgia State University in Atlanta, GA, both under the direction of Dr. Cynthia Nau Cornelissen. She finished his PhD in Fall 2023 and is in her interview process for a potential future position.

Her publications and presentations are listed below.

### Publications

**Awate O.A.**, Ng D., Stoudenmire J.L., Moraes T.F., Cornelissen C.N. BioRxiv 2023. Investigating the importance of surface exposed loops in the gonococcal HpuB transporter for hemoglobin binding and utilization. bioRxiv:2023.10.30.564842

### Presentations

**"**The Gonococcal Hemoglobin Transport System HpuAB"; Talk delivered at the TonB-Dependent Transporter grant meeting, in person, August 2023

**“**Structure-function analysis of the gonococcal TonB-dependent transporter, HpuB”; Short talk and poster delivered at the International Pathogenic Neisseria Conference, in person, September 2023

“Structure-Function Relations of the Hemoglobin TonB-dependent Transporter, HpuB”; Poster delivered at the Mid-Atlantic Microbial Pathogenesis Meeting, in person, February 2023

**“**The Gonococcal HpuAB System: Functional Analysis of HpuA”; Talk delivered at the TonB-Dependent Transporter grant meeting, in person, August 2022

**“**The Gonococcal HpuAB System: Functional Analysis of HpuA”; Short talk delivered at the NIH Sexually Transmitted Infections Cooperative Research Centers U19 meeting, virtual, July 2022

**“**Structure Function Relationships of the Gonococcal HpuAB System”; Poster delivered at the American Society for Microbiology, in person, June 2022

**“**A Tale of Resistant Gonorrhea”; Short talk delivered at GSU 3-minute thesis competition, preliminary round, in person, March 2022

**“**The Gonococcal Hemoglobin Transport System HpuAB: Structure-Function Analysis”; Talk delivered at the *Neisseria gonorrhoeae* Research Society (NgoRS) Conference, virtual, January 2022

**“**How Close Are We to Untreatable Gonorrhea”; Poster tweets delivered at the Under the Peachtree Twitter Conference (The Atlanta 500 Women Scientists), virtual, October 2021

**“**Functional Analysis of the Gonococcal Hemoglobin Receptor, HpuA”; Poster delivered at the International Pathogenic Neisseria Conference, in person, September 2018

**“**Functional Analysis of the Hemoglobin Receptor, HpuA, Produced by *Neisseria Gonorrhoeae*”; Poster delivered at the American Society for Microbiology, in person, June 2018

**“**Functional Analysis of HpuA, the Hemoglobin Receptor of *Neisseria Gonorrhoeae*”; Poster delivered at the Mid-Atlantic Prep and IMSD Research Symposium (MAPRS), in person, May 2017

**“**Functional Analysis of HpuA Hemoglobin Binding in *Neisseria Gonorrhoeae*”; Poster delivered at the Annual Biomedical Research Conference for Minority Students, in person, November 2016