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CHARACTERIZATION OF THE EFFECTS OF IRON ON *NEISSERIA*
GONORRHOEAE SURFACE PROTEIN MODULATION AND HOST CELL
INTERACTIONS

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

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Dedicated to my family

Acknowledgements

First, I would like to express my gratitude to my advisor Dr. Cynthia Nau Cornelissen for her guidance, support, and understanding over the years. She has been a great mentor and teacher. Thank you for giving me this opportunity.

I would sincerely like to thank my graduate committee members: Dr. Barbour, Dr. Carlyon, Dr. Lloyd, and Dr. Jefferson whose valuable suggestions and discussions have helped me think about my research from different viewpoints. They all have been a source of encouragement on my committee. Special thanks to Dr. Francine Cabral whose kind words of encouragement helped probably more than she realizes.

To the current and past members of the Cornelissen lab, who have been there through the good times and hard times: Devin Cash, Angie Claxton, Jeremy Cornelissen, Mary Katherine Dickinson, Sophonie Jean, Dr. Shreni Mistry, Dr. Aimee Hollander, Dr. Rosuany Velez, Heather Strange, and Tyler Thomas. You all have been great friends and colleagues over the years. Life in the lab would certainly not have been the same without you. Thanks for the constant humor and encouragement when it was clearly needed. I look forward to continued friendships.

To my wonderful friends who have supported me through these past years: Dara, Chioma, Soso, Rita, Sena and the rest of “us”. They have been my personal cheerleaders who believed in me even in the times I may not have believed in myself. You’ve kept me going when I didn’t want to. To Damola, thank you for being a big brother.

Most of all, I am grateful for my family whose love and support has brought me this far. Thanks to my parents, Lateef and Laide Oki, who are always there for me no matter the circumstance. They have done their best to give my siblings and me the best lives they can afford and taught us to be the best we can be. Oyinda, Kenny, and Rahman, I wouldn’t trade you guys for the world! To my Aunt Mariam and Uncle Lati, Thank you for being second parents. To Dr. Ejire, you have been a great guardian and mentor and I would not be here today without you. Thank you for being there. Last but not least, to the rest of my family in Nigeria, thank you for your support and encouragement.

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List of Abbreviations

α	alpha, anti
β	beta
Δ	delta, deletion
Ω	omega
$^{\circ}\text{C}$	degrees Celsius
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
2D-DIGE	two-dimensional differential in gel electrophoresis
ABC	ATP binding cassette
ASGP-R	asialoglycoprotein receptor
AP	alkaline phosphatase
Amp^{r}	ampicillin resistance
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin

BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bfr	bacterioferritin
bp	base pair
CD46	cluster of differentiation 46
CDC	Centers for Disease Control and Prevention
CDM	chemically defined media (Chelexed)
cDNA	complementary DNA
CEACAM	carcinoembryogenic antigen-related cell associated molecule
CFU	colony forming units
CO ₂	carbon dioxide
CR3	complement receptor 3
DGI	disseminated gonococcal infection
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECL	enhanced chemoluminescence
EDTA	ethylene diamine tetraacetic acid
Erm ^r	erythromycin resistance
Fbp	ferric binding protein
FBS	fetal bovine serum
Fe	iron

Fe(NO ₃) ₃	ferric nitrate
Fur	ferric iron uptake regulator
<i>g</i>	gravity
GCB	gonococcal growth media
GCU	gonococcal uptake sequence
GGI	gonococcal genetic island
H ₂ O ₂	hydrogen peroxide
HEPES	4-(2-hydroxyethyle)-1 piper azine-ethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HSPG	heparin sulfate proteoglycan
HS TBS	high salt Tris-buffered saline
IgA	immunoglobulin A
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
iTRAQ	isobaric tags for relative and absolute quantification
Kan ^r	kanamycin resistance
kDa	kiloDalton
KU	klett unit
L	liter
LB	Luria Bertani

LC-MS	liquid chromatography-mass spectrometry
LNnT	lacto-N-neotetraose
Lf	lactoferrin
LHr	lutropin receptor
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LS TBS	low salt Tris-buffered saline
M	molar
mAMP	milliampere
mg	milligram
MgCl ₂	magnesium chloride
min	minutes
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
Na ₂ CO ₃	sodium carbonate
Na ₂ HCO ₃	sodium bicarbonate
NaNO ₂	sodium nitrite
NBT	nitro blue tetrazolium

NICS	neisseria intergenic complementation site
NspA	Neisseria Surface Protein A
nM	nanometer
OD	optical density
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	opacity protein
ORF	open reading frame
PBS	phosphate saline buffer
PCR	polymerase chain reaction
pI	isoelectric point
PID	pelvic inflammatory disease
Pil	pilin
PTM	post-translational modification
RBS	ribosomal binding site
RMP	reduction-modifiable protein
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spec ^r	spectinomycin resistance
SREC	scavenger receptor
Strp ^r	Streptomycin resistance
Tbp	transferrin binding protein
Tdf	tonB-dependent transporter
TE	Tris-EDTA
Tf	transferrin
Tn	transposon
Tween 20	polyoxyethylene sorbitan monolaurate
WB	western blot
WHO	world health organization
WT	wild-type
Wt	weight

ABSTRACT

CHARACTERIZATION OF THE EFFECTS OF IRON ON *NEISSERIA GONORRHOEAE* SURFACE PROTEIN MODULATION AND HOST CELL INTERACTIONS

By Aminat Taiwo Oki

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

Virginia Commonwealth University, 2014

Major Director: Cynthia Nau Cornelissen, Ph.D.

Professor, Department of Microbiology and Immunology

Iron is an essential nutrient that is sequestered by iron-binding proteins in the human host resulting in a hostile environment for microbes. *Neisseria gonorrhoeae*, however, can utilize numerous iron-binding proteins such as transferrin and lactoferrin to acquire this nutrient. During initial infection, gonococci have access to transferrin and lactoferrin present in semen and

vaginal fluids, as well as to hemoglobin present in blood during menses or disseminated infections. Consequently, the gonococcus likely encounters conditions of high iron at some stages in the course of natural infection. Potential contributions of iron to gonococcal invasion have however been largely overlooked in the field as most studies investigating invasion represent iron depleted environments. Considering the link between menses in women and ascending gonococcal infections, we hypothesized that high iron concentrations present at this time triggers the induction of membrane proteins that enhance gonococcal pathogenesis. Here, we report the gonococcal iron-induced surface proteome and show evidence of post-translational modification of many of these proteins. We also present evidence of an iron enhanced, Opa-independent invasion mechanism. Finally, we investigated the role of NspA, TdfJ and NGO1063 on Opa-independent iron induced invasion. Our studies underscore the importance of investigating the effect of iron on gonococcal host cell interactions. Given the potential clinical relevancy of this phenomenon, data from our studies represent a solid framework for further investigation of gonococcal pathogenesis.

CHAPTER 1: INTRODUCTION

I. Pathogenic Neisseria

Most *Neisseria* species, such as *N. lactamica* and *N. mucosa*, are commensals and are considered normal flora of the human oro- and nasopharynx. Occasionally, these commensals may act as opportunistic pathogens (96). The two pathogenic species of the genus are *N. gonorrhoeae* and *N. meningitidis*. The two organisms are closely related, having 80-90% sequence identity (171), but they both have different niche preferences and cause very different diseases. *N. gonorrhoeae* is the etiological agent of the sexually transmitted infection gonorrhea while *N. meningitidis* can cause meningococcal septicemia and meningitis. They are both obligate human pathogens with no other known reservoirs (127). One major difference between the two organisms is the expression of a polysaccharide capsule by *N. meningitidis*; the serological difference of this capsule between strains forms the basis of serogroup classification.

II. *Neisseria meningitidis*

N. meningitidis, the meningococcus, is a Gram-negative diplococcus that colonizes the nasopharynx of humans mainly as a commensal and is carried by 10-35% of the healthy adults in non-endemic geographical areas (30, 158). In some cases, meningococci disseminate from the nasopharynx into the bloodstream causing septicemia or even transverse the blood-brain barrier and result in meningitis. Mechanisms that trigger the change from colonization to disease state of

the organism are not well understood, but environmental factors such as dry air and smoke (60), host susceptibility factors like age and recent upper respiratory tract infection (173), and bacterial genetics (127) play a role.

III. *Neisseria gonorrhoeae*

A. Epidemiology

Neisseria gonorrhoeae is a Gram-negative diplococcus and the etiological agent of the sexually transmitted infection, gonorrhea. Gonorrhea is a significant world health concern with an estimated 106 million new cases of infection each year (191). In the United States, gonorrhea is the 2nd most common reportable infectious disease with CDC estimates of over 820,000 new infections annually (31, 33). While *N. gonorrhoeae* typically colonizes the mucosal epithelia of the male urethra and female uterine cervix, infections can also occur on the rectum, the throat and the conjunctiva. Transmission generally occurs through close sexual contact but it can also be transmitted from mother to baby (conjunctiva of the eye) if the mother is infected at the time of birth. As a prophylaxis, newborns are treated with antimicrobial agents (erythromycin) to avoid neonatal infection (143).

B. Infection

The most common genital tract manifestation of the disease is acute urethritis in men and cervicitis in women accompanied by purulent genital discharge containing neutrophils filled with gonococci. Asymptomatic infections occur in about 15% of men and up to 50% of women (184). Untreated infections can lead to ascending gonococcal infection in up to 45% of infected women (20, 155), and often results in serious complications such as pelvic inflammatory disease (PID), and subsequent infertility and ectopic pregnancies due to fallopian tube scarring and blockage (86). The highly asymptomatic nature of the infection results in greater morbidity in women, making gonococcal infection a major concern in women's health. Untreated infections in men can lead to epididymitis, prostatitis, and infertility. Although uncommon, disseminated disease can cause arthritis-dermatitis syndrome, endocarditis and/or meningitis (53). In addition to sequelae, gonococcal infection has also been correlated with increased HIV transmission rates (37, 119).

C. Treatment

Antibiotic therapy is effective in treating gonococcal infections; however, over the years, *Neisseria gonorrhoeae* has steadily become increasingly resistant to a broad spectrum of antibiotics. Sulfonamides were introduced as antibiotic therapies for the treatment of gonorrhoeae in 1936 (99) and since then, *N. gonorrhoeae* has acquired resistance to most antibiotics used for treatment. Penicillin, tetracycline, and fluoroquinolone antimicrobials are no longer recommended as appropriate treatment options for gonorrhea in the United States with ceftriaxone (an extended spectrum cephalosporin) being the only current recommendation (32).

This limits the recommended treatment to a single class of antibiotics, the cephalosporins. Resistance to this class of drugs has emerged in Japan and China with few sporadic isolates in the United States (11, 133) and untreatable disease is anticipated in the near future (174). Importantly, the infection elicits no protective immune response and there is currently no vaccine for preventive measures.

IV. Gonococcal adherence to and invasion of epithelial cells

To initiate infection, bacteria first attach to the host cells. Attachment is followed by either extracellular colonization or by invasion of cells to establish intracellular accommodation and evasion of host immunity. Bacterial pathogens take advantage of host cell surface structures to promote adherence and invasion.

N. gonorrhoeae expresses several factors that help ensure its successful colonization of the human genital tract. Gonococci first attach to host cells via type IV pili (165). At later times after initial infection, the pili retract bringing the gonococcus into close proximity to epithelial cells and allows for tight association with the host cell plasma membrane (127). Close contact eventually results in engulfment of the organisms (69). An overview of gonococcal and host factors that may be involved in adherence and invasion are shown in schematic form in Figure 1. Gonococci may survive intracellularly, in or transcytose through the host cell (126, 138). See Merz and So for review (127). Discussed below are gonococcal structures that have been demonstrated to contribute to the process of adhesion and invasion. It is important to note that many of these structures are subject to high frequency phase and/or antigenic variation which

contribute immensely to the bacterium's immune evasion abilities and has complicated vaccine development efforts by the scientific community.

Figure 1. Schematic overview of gonococcal interactions with epithelial cells. To initiate infection, gonococci must first attach to the host cells to avoid clearance by innate immune mechanisms. Pili mediate primary interaction with host epithelial cells. Pili are eventually retracted, bringing the gonococcus into close proximity with epithelial cells. Interaction of outer membrane proteins and their cognate host receptors result in internalization of the gonococcus. For example, Opa proteins may bind HSPGs and CEACAMs. Opas have also been shown to bind integrin receptors via fibronectin and vitronectin. Interactions between other surface structures such as Porin and LOS have also been shown to result in internalization.

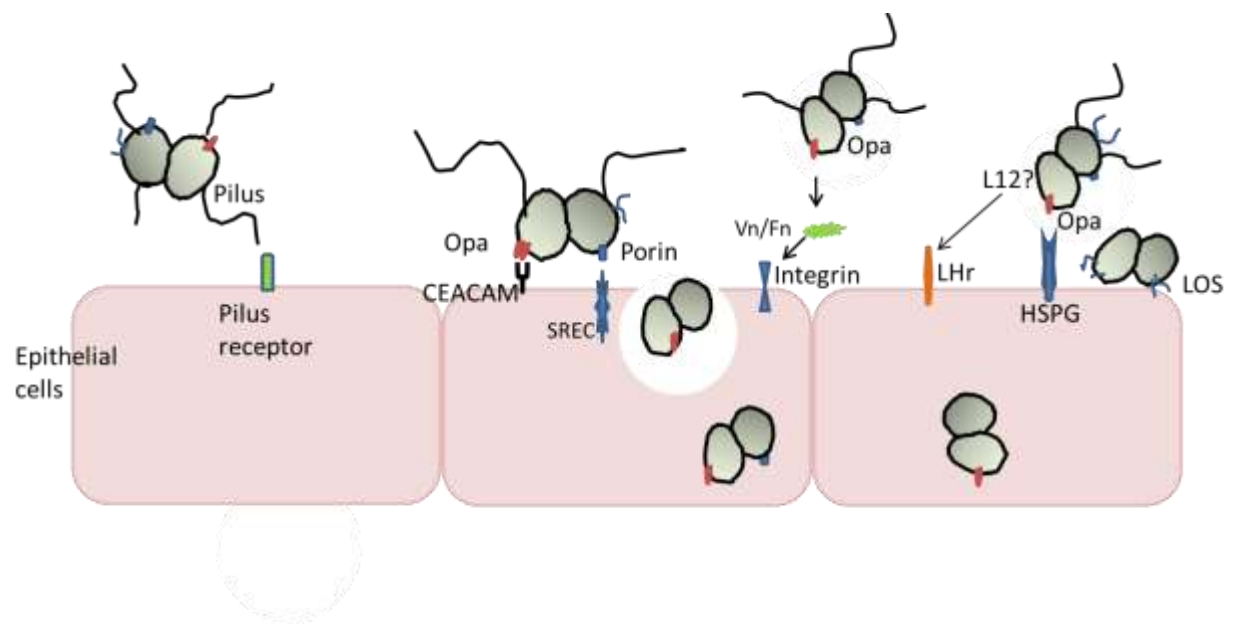


Figure 1. Schematic overview of gonococcal interactions with epithelial cells

A. Pilus

Neisseria gonorrhoeae produce type IV pili, filamentous structures that protrude from the surface of the organism. These structures, which undergo high frequency variation, mediate bacterial aggregation (166) and adhesion to host cells (165) likely by helping overcome electrostatic repulsion between host cells and the surface of the gonococci (81). Pili are also required for natural DNA competence of the gonococcus (62) and impart twitching motility by rapid extension and retraction (125). Non-piliated gonococci are less virulent in organ cultures of human fallopian tubes (121) and unable to initiate infection in a human male challenge model (101) demonstrating the critical role of this structure in natural infection.

The gonococcal pilus is composed of two main subunits; *pilE* (or pilin) and pilC. PilE is the major structural subunit; the shaft is composed of pilE subunits arranged in a helical array (42). PilC is the tip associated adhesin (144). Pili are able to agglutinate erythrocytes in a PilC-independent manner (146) and different pilE variants display different degrees of adhesion to epithelial cells (131), indicating that PilE also has adhesive properties. Pilin antigenic variation is the result of high frequency intergenic gene conversion between the expressed *pilE* gene and a portion of one of several truncated silent pilin genes (*pilS*) located on multiple loci in the genome via a RecA-dependent pathway (71, 148). Gonococcal strain FA1090 for example, contains 19 unique *pilS* copies located in 6 different loci (76). This variation gives rise to new pilin variants that differ in their ability to be expressed or assembled into pili. Thus, antigenic variation can result in a *pilE* variant defective in fiber formation; leading to a non-piliated phenotype. All *pil* genes contain variable *pil* gene segments (cassettes) interspersed with short conserved regions (84). The *pilS* copies differ from *pilE* in that *pilS* genes lack a promoter, ribosomal binding site, and a portion of 5' region found at *pilE* (70, 72, 149). These copies are

comprised of a 5' semivariable region, corresponding to the central ~250 bp of *pilE*, and a 3' hypervariable loop and tail. Within these variable regions are short regions of sequence conserved among all *pilS* copies and *pilE* that are likely the sites of homology during recombination. A guanine quartet/quadruplex (G4) forming sequence that is located only upstream of *pilE* is necessary for antigenic variation as mutations that disrupt the formation of the structure also disrupt antigenic variation (27). It has been proposed that the initiation of pilin antigenic variation begins with transcription of the *pilE* G4-associated sRNA which forms an RNA:DNA hybrid on the C-rich strand of the *pilE* G4 sequence thus facilitating the formation of a G4 structure by the unpaired G-rich strand. It is yet to be determined whether proteins necessary for antigenic variation bind the G4 structure to initiate the event. Phase variation is RecA-independent and occurs when a poly C tract located at the beginning of the *pilE* gene causes a slipped strand mispairing event that results in a frame-shift (106). This event occurs at a frequency of 10^{-4} per cell per generation and results in reversible on and off switching of pilin (106, 198). Additionally, pilin undergoes post-translational modifications such as glycosylation and phosphorylation (82, 160).

Most strains carry two *pilC* loci, both of which undergo phase variation. PilC expression is required for pilus assembly and pilus mediated adherence (97, 144, 146). In the absence of PilC, Pile is processed by removal of approximately 39 N-terminal amino acids and secreted as a soluble pilin molecule called S pilin (73). The ATPase motor protein, PilT, mediates pilus retraction and is required for twitching motility and competence for natural transformation (193). Other minor pilus associated proteins are important for pilus biogenesis and function (24, 28).

The host cell receptors that engage gonococcal pili are not quite clear. Early studies identified CD46 as the putative receptor on human epithelial cells (98). However, more recent

studies demonstrate that pilus-mediated gonococcal infection of epithelial cells can occur in a CD46-independent manner (103, 172). Edwards *et al.* showed that gonococcal pili bind to $\alpha 1$ and $\alpha 2$ integrins on urethral epithelial cells (49). Therefore, it is possible that host factors other than CD46 are involved in mediating this interaction.

B. Opacity-associated proteins (Opa)

Opa proteins are a family of 27-31 kDa proteins that mediate adherence to and trigger invasion of epithelial cells (164) and leukocytes (110). They are integral outer membrane proteins that impart an opaque appearance to colonies expressing them (164). Opa proteins can be categorized into two groups based on the host cell molecules they bind. One group can bind directly via host cell surface-associated heparin sulfate proteoglycans (HSPGs), or indirectly via extracellular matrix proteins like vitronectin and fibronectin (64, 176, 179). The other group of Opa proteins binds to carcinoembryonic antigen (CEA) related cell adhesion molecule (CEACAM) receptors (67, 181).

Gonococcal strains possess up to 11 *opa* loci in the genome, all of which can be expressed independently of each other (13). As a result, a given bacterium can express none, one or multiple different Opa proteins; individual organisms have been shown to simultaneously express up to 4 different Opas (18). This high variability results in a constant heterogeneous population of bacteria. Although the crystal structure of Opa proteins has not been solved, the Opas are structurally similar to the conserved Neisseria Surface protein A (NspA) whose crystal structure has been reported (180). Structurally, the Opas are made up of eight transmembrane domains that form a β -barrel with four surface exposed loops. The Opa proteins undergo

frequent phase variation ($\sim 10^{-3}$ per cell per generation) due to slipped-strand mispairing of tandem pentameric (CTCTT) repeats present within the signal peptide coding region of the *opa* gene (80, 159). Deletion or insertion of a repeat causes a shift in the reading frame. Additionally, antigenic variation occurs as a result of recombination between *opa* genes (85).

Unlike pili, Opa proteins are not necessary for initial colonization of the host as experimental infections carried out in human males with Opa⁻ organisms resulted in successful colonization (150). However, there seems to be a strong selection for Opa expression *in vivo* as clinical isolates are Opa⁺ (93). Similarly, gonococci recovered from studies with male volunteers infected with transparent Opa⁻ bacteria (150) were Opa⁺ suggesting there is a strong selection for Opa expression *in vivo*. These studies also revealed that there was no specific selection for any one Opa. Conversely, Opa⁻ bacteria predominate during menses in women (92). Opa⁻ variants were also preferentially isolated from fallopian tubes of women with acute salpingitis (46) suggesting that Opas are not selected for during infection of the upper female genital tract. Together, these data suggest that in addition to Opas, other virulence factors are likely to contribute to invasion of host cells.

C. Lipooligosaccharide (LOS)

Most Gram-negative bacteria have lipopolysaccharide (LPS) on their outer membrane. LPS is composed of three polysaccharide chains attached to a lipid A moiety, which anchors the molecule in the outer membrane. *N. gonorrhoeae* produces a short type of LPS known as lipooligosaccharide (LOS) because the core oligosaccharide (sugar) structure is highly branched and it lacks repetitive O-antigen side chains. The sugars are added by glycosyltransferases

encoded by *lgt* genes (95). While the core structure is highly conserved, the *lgt* genes undergo rapid phase variation, resulting in the expression of several terminal oligosaccharide structures on the outer membrane of *N. gonorrhoeae* at any given time (25). The *lgt* genes are phase variable due to polynucleotide tracts within their coding sequence that can cause slipped strand mispairing. The resulting heterogeneous combination of these enzymes results in the antigenic variation of the LOS structure.

LOS also contributes to host cell invasion. The lacto-*N*-neotetraose (LNnT) moiety present on certain LOS structures mimic human paragloboside which is present on many human cells. LNnT interacts with the asialoglycoprotein receptor (ASGP-R) (77); and facilitates the adherence of gonococci to human sperm (77, 78) and the invasion of human urethral epithelial cells (77). This interaction results in pedestal formation in host cells and subsequent internalization of the bacteria. Studies have shown that the LNnT epitope is selected for in men during human volunteer studies and also in clinical isolates from naturally occurring gonococcal urethritis (147) signifying its importance. LOS containing LNnT was also found to promote invasion of cultured human cervical epithelial (ME180) cells in an Opa-independent but pili-dependent manner (154), emphasizing the relevance of pilus expression in gonococcal infections.

LOS side chains also terminate in epitopes that mimic sugar moieties of mammalian glycosphingolipids (79, 117, 141). This helps in immune evasion and also allows the gonococcus to utilize host molecules that normally associate with the mimicked structure. Additionally, LOS can be modified by the addition of terminal sialic acid moieties. Gonococci are unable to synthesize sialic acid; instead they hijack host derived cytidinemonophosphate-*N*-acetylneuraminic acid (CMP-NANA) for use as the sialyl donor (9, 153). This modification

significantly inhibits LOS- and Opa -mediated invasion and adhesion(77, 177) but also confers protection from complement and ingestion by professional phagocytes (182). These data suggest that LOS antigenic and phase variation may allow *N. gonorrhoeae* to fluctuate between invasive and serum resistant phenotypes to promote bacterial survival (177).

D. Porin

Porins are the most abundant proteins in the gonococcal outer membrane. These proteins form anion selective ion channels that are essential for viability (65). The channels allow the passage of small nutrients and waste products across the outer membrane. *N. gonorrhoeae* expresses two different *porin* genes, PorBIA and PorBIB, which form the basis of the serotyping system (105).

Porins play a multifaceted role in gonococcal pathogenesis. These proteins are capable of translocating into eukaryotic cell membranes where they form voltage-gated channels that result in the modulation of host cell signaling events (116, 145, 186). Porins can also translocate into the mitochondria of host cells, triggering cytochrome C release and apoptosis (130), potentially causing cytotoxicity observed in fallopian tube organ cultures and shedding of epithelial cells during mucosal infection (43). However, the role of porins in apoptosis is controversial as gonococcal infection inhibits apoptosis of primary human male urethral epithelial cells (14).

Porins also play a role in immune evasion by impairing phagocyte function. Studies have shown that porin selectively interferes with the signaling machinery of polymorphonuclear leucocytes (PMNs) resulting in inhibited degranulation of PMNs, and a down-regulation of actin polymerization, opsonin receptor expression and phagocytosis of gonococci (17, 75).

Additionally, porin can also inhibit phagosome maturation (129) suggesting that porins play an important role in intracellular survival of *N. gonorrhoeae*.

Gonococcal PorBIA is associated with increased bacterial invasiveness; strains harboring this allele are more resistant to killing by normal human serum and invade cells to a greater extent than gonococci bearing the PorB1B protein *in vitro* (178). This porin-dependent adherence to and invasion of host cells only occurs under low phosphate conditions that mimic systemic bloodstream infections and is thought to be especially relevant during disseminated gonococcal infection (108). Scavenger receptor, SREC, was reported to be the host receptor that engages PorBIA (139). All together, these reports indicate the essential role that porins play in gonococcal pathogenesis.

E. Ribosomal protein L12

The lutropin receptor (LHr), which is expressed by endometrial and fallopian tube epithelia, has been implicated in an enhanced invasion phenotype of *N. gonorrhoeae*. Specifically, LHr plays a dual role as both a tissue-specific environmental signal that induces an enhanced-gonococcal invasion phenotype as well as being the specific host cell receptor engaged during internalization (156, 157). This interaction was found to be competitively inhibited by human chorionic gonadotropin (hCG), the natural ligand for LHr (156). Subsequent studies revealed that the ribosomal protein, L12, was a membrane associated and surface exposed “hCG-like” protein that likely serves as a gonococcal ligand that mediates this interaction (157).

V. Iron acquisition and available host iron sources

Iron is an essential element for most living organisms and plays a crucial role in a variety of cellular functions in metabolism, growth and differentiation as well as oxygen transport, DNA synthesis and energy production (23). However, excess free iron is toxic. Under most physiological conditions, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) which can activate the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}\cdot$), leading to the partial reduction of oxygen into free hydroxyl radicals that can cause oxidative stress-induced damage of DNA, lipids and proteins leading to cell damage and death (4, 120, 185). To protect against this effect, iron is sequestered by human iron binding proteins that tightly regulate iron concentrations. Iron is transported in blood mainly by transferrin and hemoglobin. Thus, the amount of free iron available to invading bacteria is much less than what is required to replicate and cause disease (152).

In humans, the major iron transport proteins are transferrin (Tf) in the blood, and lactoferrin (Lf) in lymph and mucosal secretions like semen and vaginal fluids. Lactoferrin also has antimicrobial activities. The bactericidal activity of LF was originally ascribed to its iron chelating activity but was later demonstrated to stem from its proteolysis into lactoferricin (see Farnaud and Evans for review (59)). Iron is also bound by heme, the heme binding proteins, hemoglobin and hemopexin, and haptoglobin-hemoglobin. Hemoglobin functions primarily as an oxygen transporter and is found in erythrocytes but is released into the blood upon lysis of these cells. Ferritin is the major iron storage protein found intracellularly but can also be found in low concentrations in serum (1).

N. gonorrhoeae is a very well-adapted human pathogen and has evolved a repertoire of high affinity iron uptake systems that facilitate iron acquisition from human iron-binding

proteins. It is able to remove iron from transferrin, lactoferrin, and hemoglobin in a TonB-dependent manner. The TonB-ExbB-ExbD complex transduces energy generated by proton motive force in the cytoplasmic membrane to TonB-dependent transporter (Tdt) proteins in the outer membrane (104, 134). Iron stripping and internalization is an energy dependent process, thus inactivation of the TonB system abrogates the function of these transporters.

Additionally, it is important to note that while *N. gonorrhoeae* does not secrete any known siderophores (iron scavenging molecules) (189), it is able to use siderophores produced by some other organisms such as *Salmonella* and *E. coli* as a source of iron (29, 162). These all indicate that there are many sources of iron accessible to the gonococcus *in vivo*.

Studies have shown that gonococci require iron for intracellular survival *in vitro* (201); the expression of either the transferrin receptor or lactoferrin receptor is necessary to establish infection in human males (5, 39) and the hemoglobin receptor is selected for in gonococci isolated from women during menses (7). These studies emphasize the connection between iron acquisition and the ability of *N. gonorrhoeae* to initiate infection *in vivo*. Thus iron plays an important role during natural infection and it is important to study the contribution of iron to the invasion process.

Many cell culture systems used to characterize gonococcal invasion represent iron-restricted conditions as cell culture media generally has a low iron content. Also, the media is often supplemented with fetal bovine serum (FBS), which contains growth factors, to help ensure the health of eukaryotic cells. However, FBS also contains bovine transferrin. Since *N. gonorrhoeae* can only use human transferrin, FBS effectively chelates free iron in the media in a form that is inaccessible to the gonococcus. It is important to note that Shaw and Falkow (151), who developed a model for gonococcal infection in HecIB cells, observed that gonococcal

invasion was sensitive to fetal calf serum and supplementation with iron enabled gonococcal invasion. This finding was confirmed by Chen *et al.* (34) and Heine *et al.* (83). However, subsequent studies characterizing *N. gonorrhoeae* invasion do not mention iron supplementation. Thus, the contribution of iron to the process of invasion has been largely overlooked.

VI. Iron regulation and iron regulated proteins

The best characterized mechanism of iron regulation is via the ferric iron uptake regulator (Fur) protein. In the presence of ferrous iron, an iron-Fur complex is formed that binds target DNA regulatory sequences (known as Fur box) and typically results in the repression of transcription of many iron-repressible genes (58). In the absence of iron, this process is reversed and the genes are transcribed. Many bacteria, including the *Neisseriae*, have both iron repressible and iron induced genes but the mechanism of iron induced gene regulation is not clearly understood. However, Fur-mediated activation of genes has been reported in a variety of bacteria (26, 57, 63) including the *Neisseria* (68, 197). By directly repressing the antisense small RNA *nrrF*, Fur indirectly acts as a positive regulator for the succinate dehydrogenase genes, *sdhA* and *sdhC*, in *N. meningitidis* (124). Fur has also been reported to function directly as an activator by binding upstream of the promoter of certain genes (44) where it presumably blocks the binding of a repressor or aids transcription by recruiting RNA polymerase (197).

Considering the link between iron and gonococcal infection, and the ability of the gonococcus to use multiple host iron binding proteins, it is expected that the gonococcus experiences certain periods of high internal iron content during natural infection. At this time, iron induced proteins are expected to be expressed in the host environment. While the gonococcal iron-restricted surface proteome has been studied at length, little is known about the

iron induced proteome. The gonococcal iron-induced transcriptome has been reported (47, 90), but due to post-transcriptional events such as modifications, mRNA half-lives, and other protein processing events, mRNA expression may not correlate with protein expression. We expect that iron regulated surface proteins could play crucial roles in gonococcal pathogenesis. Furthermore, the identification of these surface proteins will provide additional potential targets for the development of therapeutics or vaccines against this evasive pathogen.

VII. Gonococcal vaccine development

Gonococcal vaccine development has proven a challenge thus far due to the wide range of immune evasion strategies employed by *N. gonorrhoeae*. Major immune evasion strategies include its ability to survive intracellularly in host cells, phase and antigenic variation of many of the surface proteins, and mechanisms of molecular mimicry as discussed earlier in this chapter.

The success of early vaccine attempts were compromised by these immune evasion strategies. For instance, Pilus was evaluated as a vaccine antigen but a pilus-based vaccine failed to protect male volunteers against gonococcal urethritis (22), presumably due to high frequency antigenic variation of this appendage. Subsequently, porin was evaluated as a vaccine candidate because it is not subject to phase or antigenic variation (190). However, LOS sialylation and antibodies directed against another OM protein (Rmp), which associates with porin, are capable of blocking porin antibodies, thus inhibiting their bactericidal activity (140).

Reports have shown that the gonococcus also interferes with the immune response in many ways. *N. gonorrhoeae* suppresses the adaptive immune response (89, 115), induces apoptosis of antigen presenting cells (48), and inhibits T cell proliferation (199). These numerous

strategies are likely the reason gonococcal infections do not elicit protective immunity. Since great strides are being made to elucidate gonococcal pathogenesis, the scientific community is now in a better position to develop potentially effective vaccines.

With anticipated untreatable gonococcal infections in the near future, there is an urgent need for a gonococcal vaccine. An attractive candidate is the transferrin iron acquisition system since the proteins involved are surface exposed, not subject to phase and antigenic variation, and expressed in most strains. It has also been shown that recombinant Tbp proteins conjugated to the cholera toxin B subunit are capable of inducing antibody responses in the serum and female genital tract of mice (137), suggesting that these antigens could be components of an efficacious vaccine. Other non-variable antigens that are attractive candidates in the development of an effective gonococcal vaccine include the TonB dependent receptor, TdfJ, and the outer membrane channel for pilus extrusion, PilQ. To increase the likelihood of achieving successful and broad protective immunity, a multivalent vaccine would be preferable. By deducing the components of the gonococcal iron induced outer membrane proteome, we will be able to identify more targets that have a high potential of being expressed and relevant *in vivo*.

VIII. Objectives

- 1). Identify the gonococcal iron induced outer membrane proteome
- 2). Characterize the contribution of iron to the gonococcal epithelial cell invasion process
- 3). Describe the contribution of selected iron-induced proteins in invasion of epithelial cells by *N. gonorrhoeae*.

CHAPTER 2: MATERIALS AND METHODS

I. Bacterial growth conditions

Gonococcal strains were routinely grown on GC medium base (GCB; Difco) containing supplement I (101) and 12 μ M ferric nitrate at 37°C in an atmosphere enriched with 5% CO₂. Antibiotics were used at the following concentrations when indicated: 50 μ M kanamycin, 50 μ M spectinomycin, or 1 μ M erythromycin for selection of gonococcal mutant strains. For induction of gene expression, the complemented mutant strain was grown in the presence of IPTG at indicated concentrations. For iron-depleted growth conditions, gonococci were grown in a defined medium that was treated with Chelex 100 (BioRad) to render it iron free (CDM) (189). For iron-replete conditions, gonococcal strains were grown in CDM for one mass doubling before the addition 24 μ M ferric nitrate. All glassware was washed with nitric acid and rinsed extensively with deionized water to remove residual iron. When indicated, strains were grown in GCB broth containing supplement I and 24 μ M ferric nitrate. All liquid cultures were grown at 37°C with 5% CO₂. *E. coli* strain, TOP10 (Invitrogen) was used for cloning and propagation of plasmids. *E. coli* was cultured in Luria-Bertani (LB) broth or on LB agar containing 100 μ M ampicillin, 50 μ M kanamycin, or 50 μ M spectinomycin as needed at 37°C shaking at 225 rpm. *E.coli* strains were transformed by heat shock following manufacturer's protocol (Invitrogen).

II. Cell culture methods

The ME180 human cervical epithelial cell line (HTB 33; American Type Culture Collection) was cultured in McCoy's 5A medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BenchMark). Cells were maintained at 37°C, 5% CO₂ containing atmosphere.

A. Passing cells

When cells were close to confluent, the medium was aspirated, first washed with 3mL Trypsin-EDTA, and then incubated with 2 ml Trypsin/EDTA at 37°C for 3 minutes. Monolayers were gently scrapped with a cell scrapper. After cell detachment, 8 ml of fresh medium was added and cells were centrifuged at 800rpm for 5 minutes. The supernatant was aspirated and pellet re-suspended in fresh media. A fraction was transferred to a new flask which volume depended on the desired dilution. Medium was added to reach a final volume of 10 ml and the cells were grown again under the aforementioned conditions. Twenty-four hours prior to infection, ME180 cells were seeded in 12-well plates at 2.5×10^5 cells/well and incubated at 37°C in 5% CO₂.

B. Infection of cells with *Neisseria gonorrhoeae*

Two days prior to infection, gonococcal strains were propagated on GC agar plates in 5% CO₂-containing atmosphere at 37°C. The following day, colonies were checked under a binocular microscope for piliation and Opa expression status by colony morphology (164). Piliated, transparent colonies were picked and streaked onto a new plate. On the day of infection,

bacteria were harvested from GC agar plates using a cotton swab and then re-suspend in 5 ml of CDM. Bacterial density was assessed using a Klett meter. Infection inoculum was prepared by diluting each strain in medium supplemented with 10% FBS at a calculated amount to generate a multiplicity of infection (MOI) of 10. Indicated amounts of ferric nitrate or human transferrin were added to generate iron replete conditions.

C. Gentamicin protection assay

ME180 cells were infected for 4 hours followed by 3 phosphate buffered saline (PBS) washes to remove extracellular, non-adherent bacteria. Monolayers were then treated with cell culture medium containing 100 μ g/mL gentamicin for 1 hour to kill extracellular gonococci. Gentamicin was removed and cells were washed three times with PBS. To collect and enumerate internalized gonococci, monolayers were treated for 10 minutes with a saponin-EDTA mix (PBS, 2mM EDTA, 0.5% saponin) and cells were lysed by vigorous pipetting. 20 μ l of serially diluted samples were plated on GCB plates and incubated at the described growth conditions. Viable cells were enumerated after 24 hours and percent invasion (internalized bacteria divided by total bacteria) was calculated. For each assay, values were derived from the mean of two independently infected cell monolayers. Each assay was independently conducted at least three times and data presented are a mean and standard deviation of these experiments.

D. Adherence assay

For adherence assays, 2 hours of infection was allowed then monolayers were washed five times with PBS to remove extracellular, non-adherent bacteria and then cells were treated with saponin-EDTA mix as described above to collect the adherent and internalized populations. For each assay, values were derived from the mean of two independently infected cell monolayers. Each assay was independently conducted at least three times and data presented are a mean of these experiments.

III. Nucleic acid methods

A. Chromosomal DNA isolation

Gonococcal strains were streaked and grown overnight on GC agar plates. To isolate DNA, colonies were removed using cotton swabs and re-suspended in TE buffer. Cells were lysed by treatment with lysozyme (Sigma -aldrich), and RNA and proteins were removed by treatment with RNaseA and proteinase K, respectively. Cell debris was removed and the supernatant was carefully transferred to a new tube. DNA was precipitated by the addition of isopropanol and DNA was spooled out using a sterile, disposable glass pipet. DNA was dissolved in TE overnight then placed at -20°C for long -term storage.

B. Plasmid DNA isolation

The *E. coli* strains carrying plasmids of interest were grown as described. Plasmids were purified from the overnight cultures in LB broth using a QIAprep Spin Miniprep kit (Qiagen) according to manufacturers' recommendations.

C. RNA isolation and purification

Gonococcal strains were grown in CDM as described above. After one mass doubling, 24 μ M ferric nitrate was added to iron replete conditions and the cultures were grown for an additional 2 hours. RNA was stabilized using RNAProtect (Qiagen) and total RNA was isolated from cultures using the RNeasy minikit (Qiagen) following manufacturer's recommendations. Samples were treated twice with DNase (Qiagen) to remove any contaminating DNA, and SUPERase-In (Ambion) was added prior to storage at -20°C.

D. Qualitative reverse transcriptase PCR (RT-PCR)

cDNAs to portions of transcripts of interest were generated using the Themoscript reverse transcriptase PCR system (Invitrogen). Synthesized cDNA was used as PCR template for PCR with MyTaq polymerase (Bioline). Primers used are listed in Table 2. To detect potential DNA contamination, parallel no reverse transcriptase control reactions were conducted as well as no-template PCR reactions. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

IV. Bacterial strains and mutant construction.

All strains and plasmids used in this study are listed in Table 1. Strains FA19 and FA1090 have been previously described (18, 128). The FA1090 genome was sequenced at the University of Oklahoma and annotated by Los Alamos National Laboratory (available at: <http://www.stdgen.lanl.gov>). The partial Opa deletion strain FA1090 Δ *opaKEB*, in which three of the translucent *opas* genes have been deleted (Criss, unpublished), and the complete Opa deletion strain (FA1090 Opaless) (10), were generated by sequential deletion of individual *opa* genes. These strains were created in an FA1090 background expressing the 1-81-S2 pilin sequence with a point mutation in the G-quadruplex region upstream of *pilE* (27) that blocks pilin antigenic variation (FA1090 1-81-S2 P_{nv}⁺) to ensure all bacteria express the same pilin (10).

For ease of transformation, pVCU403 was constructed in a manner similar to the pUP1 plasmid (56) by annealing oligos oVCU568 and oVCU569 (See Table 2 for primers), which encode the 10-bp gonococcal uptake sequence in inverted repeat form leaving a 5' *Hind*III and 3' *Pst*I site. The annealed linker was then cloned into the multiple cloning site of pUC18 (Invitrogen) by ligation into the *Hind*III and *Pst*I sites.

To generate the *nspA* mutant, the *nspA* ORF was amplified from *N. gonorrhoeae* strain FA19 using primers oVCU478 and oVCU479 with 5' *Pac*I and *Sca*I sites respectively, and cloned into pCR2.1-TOPO (Invitrogen) resulting in pVCU401. The fragment was subsequently removed from pVCU401 and ligated into pUC18 (generating pVCU402). In vitro mutagenesis was carried out using the EZ-Tn5TM <KAN-2> insertion kit (EPICENTRE) to disrupt *nspA* with the Tn5 cassette encoding kanamycin resistance (pVCU404). The fragment containing *nspA*::Tn5 was sub-cloned into pVCU403 to generate pVCU405. This plasmid was then linearized using *Sca*I and was used to

transform FA1090 Δ *opaKEB*. For complementation, primers oVCU735 and oVCU479, with flanking *PacI* and *ScaI* sites respectively, were used to amplify *nspA* with its native ribosome binding site from strain FA1090. The amplified DNA was digested with *PacI* and *ScaI* and cloned into the similarly cut expression plasmid pGCC4 (123). The resulting construct, pVCU430, was confirmed by DNA sequencing. This plasmid was used to transform MCV406 to generate the complemented mutant strain MCV411. This strain was confirmed to exhibit wild-type expression of NspA in the presence of 2mM IPTG by western blot analysis of gonococcal whole cell lysates.

To generate the *tdfJ* mutant, the Ω cassette (135) was ligated into the unique *HincII* site of pVCU703 (74) which contained the sequence encoding *tdfJ* ORF on pCR 2.1 TOPO (generating pVCU425). TOP10 cells were transformed with the plasmid and transformants were selected on LB-agar plates containing 50 μ g spectinomycin. The resulting plasmid was linearized and used to transform FA1090 Δ *opaKEB* to generate MCV407. Transformants were selected for by plating on GC agar containing 50 μ g/ml spectinomycin. The disruption of *tdfJ* by the Ω cassette was confirmed by PCR analysis. For complementation, primers oVCU749 and oVCU742, with flanking *PacI* and *FseI* sites respectively, were used to amplify *tdfJ* plus 30bp of upstream sequence. The amplified DNA was digested with *PacI* and *FseI* and cloned into the similarly cut expression plasmid pGCC4. The resulting construct, pVCU432, was confirmed by DNA sequencing. This plasmid was used to transform MCV407 to generate the complemented mutant strain MCV413. TdfJ expression was confirmed after induction with 0.25mM IPTG by western blot analysis of gonococcal whole cell lysates. To generate the NspA⁻/TdfJ⁻ mutant (MCV408), MCV406 was transformed with pVCU425.

To construct the *NGO1063* mutant, a region containing the *NGO1063* ORF plus 349bps of upstream region and 298bp of downstream sequence was amplified from strain FA1090 using

primers oVCU695 and oVCU696 with 5' XbaI and SacI sites respectively, and cloned into pCR2.1TOPO (pVCU420). The fragment was subsequently cloned into pVCU403 resulting in pVCU308. In vitro mutagenesis was carried out on plasmid pVCU308 using the EZ-Tn5TM <KAN-2> insertion kit (EPICENTRE) to disrupt *NGO1063* with the Tn5 cassette encoding kanamycin resistance (resulting in pVCU309). This plasmid was then linearized and used to transform wildtype FA1090 (generating MCV309). Mutants were selected for by plating on GC medium with kanamycin.

Table1. Strains and plasmids used in this study

Strain or plasmid	Description	Ref. or Source
<u><i>N. gonorrhoeae</i> strains</u>		
FA1090	Wildtype	(18)
FA1090 Δ <i>opaKEB</i>	FA1090 Δ <i>opaKEB</i>	Criss, unpub.
FA1090 opaless	FA1090 Δ <i>opaA-K</i>	(10)
FA19	Wildtype	(128)
MCV656	FA1090 <i>tonB</i> Ω (Str ^r ,Spc ^r)	(74)
MCV309	FA1090 <i>NGO1063::Kan</i> (Str ^r ,Spc ^r , Kan ^r)	This study
MCV406	FA1090 Δ <i>opaKEB nspA::Tn5</i> (Str ^r ,Spc ^r , Kan ^r)	This study
MCV407	FA1090 Δ <i>opaKEB tdfJ::\Omega</i> (Str ^r ,Spc ^r)	This study
MCV408	FA1090 Δ <i>opaKEB nspA::Tn5 tdfJ::\Omega</i> (Str ^r ,Spc ^r , Kan ^r)	This study
MCV411	FA1090 Δ <i>opaKEB nspA^c</i> (Str ^r ,Spc ^r , Kan ^r , Erm ^r)	This study
MCV413	FA1090 Δ <i>opaKEB tdfJ^c</i> (+30bp upstream sequence) (Str ^r ,Spc ^r , Erm ^r)	This study
<u><i>E.coli</i> Strains</u>		
TOP10	F ⁺ [lacIq, Tn10(TetR)] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80lacZ Δ M15 Δ lacX74 <i>recA1 araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
<u>Plasmids</u>		
pCR2.1 TOPO	Cloning vector; Kan ^r Amp ^r	Invitrogen
pUC18	Cloning vector; Amp ^r	Invitrogen
pGCC4	Complementation vector; Erm ^r	(123)
pVCU403	pUC18 vector containing gonococcal uptake sequence (Amp ^r)	This study
pVCU401	pCR2.1 TOPO containing <i>nspA</i>	This study
pVCU402	pUC18 containing <i>nspA</i>	This study

pVCU404a	pUC18 <i>nspA</i> ::Tn5	This study
pVCU405	pVCU403 containing <i>nspA</i> ::Tn5	This study
pVCU430	pGCC4 containing FA1090 <i>nspA</i> with native rbs	This study
pVCU703	pCR2.1 NGO1205	(74)
pVCU418	pCR2.1 <i>NGO1205</i> :: Ω	This study
pVCU425	pVCU403 containing <i>tdfJ</i> :: Ω	This study
PVCU432	pGCC4 containing <i>tdfJ</i> with 30bp upstream sequence	This study
PVCU420	pCR2.1 NGO1063 (+349bps of upstream and 298bp of downstream sequence	This study
pVCU308	pVCU403 containing NGO1063 (+349bps of upstream and 298bp of downstream sequence	This study
pVCU309	pVCU403 containing NGO1063::Tn5 (+349bps of upstream and 298bp of downstream sequence	This study
pUNCH606	pBS-sk-fur Ω	(135)

Table 2. Primers used in this study

Primer	Sequence	Target
oVCU110	5'-TATCGGAACGTACCGGGTAGC-3'	<i>16S rRNA</i> RT PCR
oVCU111	5'-GTATTACCGCGGCTGCTGGCA-3'	<i>16S rRNA</i> RT PCR
oVCU478	GCGGGCGGCCTTAATTAAATGAAAAAAGCACTTGCC-3'	<i>nspA</i>
oVCU479	5'-AGTACTTCAGAATTTGACGCGCACGCCG-3'	<i>nspA</i>
oVCU568	5'-AGCTTGCCGTCTGAAATGGTTTCAGACGGCATGCACTGCA-3'	GCU seq
oVCU569	5'-GTGCATGCCGTCTGAAACCATTTTCAGACGGCA-3'	GCU seq
oVCU578	5'-CACGCTCCCGTTAGTCACGC-3'	<i>nspA</i> RT-PCR
oVCU584	5'-AGCCTCAAGCTCTTTAGGTTCTGCCAAAGG-3'	<i>nspA</i> RT-PCR
oVCU585	5'-TTGACTTTGCCGACGTAGTTGTAGCGGT-3'	<i>nspA</i> RT-PCR
oVCU634	5'-GCTATGCCGTTACACCCAAT-3'	<u><i>nspA</i></u> RT-PCR
oVCU685	5'-CCGGAACGGACGACCCCG-3'	<i>pile</i>
oVCU686	5'-CCCTACTTCCGCAAACCACACC-3'	<i>pile</i>
oVCU687	5'-GTGGAATGACGGGATTTA-3'	<i>pile</i>
oVCU688	5'-TGTTGAAGGGCGGATTAT-3'	<i>pile</i>
oVCU695	5'-CGCACAAACGTAAACGCCCGAAT-3'	<i>ngo1063</i>
oVCU696	5'-GGATATTTGGCTGCACAACGGCT-3'	<i>ngo1063</i>
oVCU735	5'- GCGGGCGGCCTTAATTAACCATAAAGGAACCAAAATATGAAAAAAGCA TTGCC-3'	<i>nspA</i>
oVCU742	5'-TACAAAGGCCGGCCTCAAACTTCACGTTTACGC-3'	<i>tdfJ</i>
oVCU749	5'-GCGGGCGGCCTTAATTAATGCAATGTTATATAATAACAAAATTTTGAAA TGCGACGAG-3'	<i>tdfJ</i>
oVCU766	5'-CCGTCATCCATACCGACAAA-3'	<i>ngo0232</i> RT-PCR
oVCU767	5'-GGAACGGCTCGGTGATAAA-3'	<i>ngo0232</i> RT-PCR
oVCU768	5'-AAGGCTGTTTCGACGAACT-3'	<i>apaH</i> RT-PCR

oVCU769	5'-GTTTCGGCTTCGGCTTCT-3'	<i>apaH</i> RT-PCR
oVCU770	5'-CTCTCCCGCCTGGGTAT-3'	<i>trkH</i> RT-PCR
oVCU771	5'-CAGTCCCGACATCGATTCAA-3'	<i>trkH</i> RT-PCR
oVCU772	5'-TTGCCGTCTTCAACGAAGT-3'	<i>ngo0232</i> RT-PCR
oVCU773	5'-CAACGTCATCTCGCTGGAC-3'	<i>apaH</i> RT-PCR
oVCU774	5'-CGGCGATTATTTGGGCATAG-3'	<i>hemN</i> RT-PCR
oVCU775	5'-GCCTTGCCGTTTCGATTT-3'	<i>hemN</i> RT-PCR

V. Bacterial outer membrane and whole cell lysate preparations.

Gonococcal strains FA19 and FA1090 grown overnight on GCB agar were re-suspended and grown in chelex-treated chemically defined medium (CDM) at 37°C in 5% CO₂ atmosphere with vigorous shaking through one mass doubling. The cultures were then back diluted with fresh CDM; 24µM ferric nitrate was added to the iron-replete condition and cultures were then grown for an additional 4 h. Cells were harvested by centrifugation.

A. Isolation of outer membrane proteins (OMP)

For Sarkosyl insoluble outer membrane preparations, gonococcal cells were re-suspended in 10mM HEPES (N-2-hydroxyethylpiperazineN9-2-ethanesulfonic acid) (pH 7.4) at 1/40 of the original culture volume, and frozen at -20°C. Frozen cells were thawed and passed through a French pressure cell at 16 000 *psi*, intact cells were removed by centrifugation at 8 500 x *g* for 20 minutes. The supernatant was collected and total membranes were isolated by centrifugation at 140 000 x *g* for 1 hour at 4 °C. To isolate outer membrane proteins, the membrane fractions were solubilized in 1% Sarkosyl (International Biotechnologies Inc.) and incubated on a nutator at 37°C for 30 minutes. The insoluble outer membranes were isolated by centrifugation at 140 000 x *g* for 1 hour and the supernatant discarded. The membrane preparation was washed once with 1% Sarkosyl and then twice with 10mM HEPES. The final outer membrane fraction was suspended in 10mM HEPES and stored at -80°C.

B. Isolation of Outer membrane vesicles/blebs

For isolation of outer membrane blebs, gonococcal cells were re-suspended in 1X lithium acetate buffer and incubated for 3 hours under growth conditions described above. In initial preparations, cells were passed through a 25-gauge syringe. Subsequent bleb preparations eliminated this step. Whole cells were removed by centrifugation at 37 000 x *g* for 20 minutes. The supernatant was then collected and subjected to high speed centrifugation at 140 000 x *g* for 2 hours to isolate blebs. The blebs were washed twice with 10mM HEPES as described above, suspended in 10mM HEPES and stored at -80°C. Protein concentrations were determined by bicinchoninic acid assay (Pierce).

C. Preparation of whole cell lysates

After growth, liquid bacterial cultures were standardized to a constant cell number then centrifuged for 10 minutes at 13 000 rpm. Cell pellets consisting of approximately 1×10^{12} CFU were re-suspended in 100µl of 2X Laemmli solubilizing buffer (111) and stored at -20°C.

VI. Protein analysis methods

A. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Outer membrane proteins re-suspended in 2X Laemmli solubilizing buffer and whole cell lysates were treated with 5% β-mercaptoethanol and boiled for 5 minutes. Whole cell lysates were

drawn through a 28-gauge syringe to reduce viscosity. Proteins were then separated by electrophoresis on 10 or 15% polyacrylamide gels.

B. Coomassie® stain of protein gels and mass spectrometry analysis

SDS-PAGE gels were stained with Coomassie blue (0.25% Coomassie R-250, 50% methanol, 10% glacial acetic acid) following electrophoresis. Gels were incubated in Coomassie blue stain overnight at room temperature and then destained in 20% methanol and 5% glacial acetic acid at room temperature until background staining was minimized. Bands of interest were excised from the stained gel and submitted to the VCU Mass Spectrometry Resource Center for identification. The sample was digested overnight with trypsin and the resulting peptides were analyzed on a LC-MS system that consisted of a Thermo Electron Deca XP Plus mass spectrometer with a nanospray ion source interfaced with a reversed-phase capillary column.

C. Western blot

Proteins separated on SDS-PAGE gels were transferred to nitrocellulose membrane (Schleicher and Schull) in 20mM Tris base, 150mM glycine and 20% methanol in a submerged transfer apparatus (Biorad) at constant current of 28mA for 16 hours. Before western blotting, to confirm equal protein loading in each lane, the nitrocellulose membranes were stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) for 10-12 minutes and excess stain rinsed with distilled water.

i. TbpA detection

To detect TbpA, nitrocellulose membranes were blocked with 5% BSA in high salt Tris-buffered saline (HS-TBS: 20mM Tris base, 500mM NaCl). The blocked membrane was probed with 1:1000 dilution of primary anti-TbpA polyclonal antibodies for 1 hour as described (41). After washing with HS-TBS, membranes were probed with a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Biorad) at a dilution of 1:5000 for 1 hour. The membrane was washed in HS-TBS with 0.05% Tween 20 (Sigma). Blots were developed using nitroblue tetrazolium and 5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Sigma) in buffer containing 100mM Tris (pH 9.5), 50mM MgCl₂, and 100mM NaCl.

ii. TbpB detection

To detect TbpB, the nitrocellulose membranes were blocked with low-salt Tris buffered saline (LS-TBS) containing 5% non-fat dry skim milk (Sigma) for an hour. Membranes were probed with 1:1000 diluted primary anti-TbpB polyclonal antibodies (136) then washed in low salt-TBS and 0.05% Tween-20. Next, the membranes were probed with goat anti-rabbit alkaline phosphatase conjugated (Biorad) secondary antibody at a 1:5000 dilution. The membranes were developed with NBT/BCIP developing system.

iii. NspA detection

To detect NspA, nitrocellulose membranes were blocked with low-salt Tris buffered saline (LS-TBS) containing 5% non-fat dry skim milk (Sigma) for an hour. Membranes were probed with 1:1000 diluted primary anti-NspA polyclonal antibodies (Gift from Dr. Fred Sparling), washed in

low salt-TBS and 0.05% Tween-20, and probed with goat anti-rabbit alkaline phosphatase conjugated (Biorad) secondary antibody at a 1:5000 dilution. The membranes were developed with NBT/BCIP developing system.

iv. TdfJ detection

For TdfJ detection, nitrocellulose membranes were blocked with low-salt Tris buffered saline (LS-TBS) containing 5% non-fat dry skim milk (Sigma) for an hour. Membranes were probed with 1:100 diluted primary anti-TdfJ anti-peptide antibodies (guinea pig 117), washed in low salt-TBS and 0.05% Tween-20, then probed with goat anti-guinea pig horseradish peroxidase conjugated (Biorad) secondary antibody at a 1:3000 dilution. The membranes were developed with an ECL kit (Pierce).

D. 2 Dimensional Differential In Gel Electrophoresis (2D DIGE) performed at UNC Systems Proteomics Center

Gonococcal Sarkosyl insoluble Protein samples were cleaned using Methanol Chloroform and rehydrated in standard Lysis Buffer (8M urea, 4% Chaps, 30mM tris pH8.5). The protein concentration of each sample was determined using the GE Healthcare 2D Quant kit, following the manufacturer's instructions. Proteins were labeled with Cy dyes and then separated by isoelectric focusing on a 24cm Immobiline Strip (pH 3-10) and then subsequently subjected to SDS-PAGE. Each gel was scanned on a Typhoon Trio+ imager & then analyzed using DeCyder 2D software (version 7.0). Three biological samples were analyzed and student's *t*-test ($P \leq 0.05$) used for statistical comparison.

E. iTRAQ analysis performed at the UNC Proteomics Facility

Briefly, proteins were reduced, alkylated, and digested using a modification of the FASP protocol (192) with trypsin as the protease. The peptides were iTRAQ labeled and pooled. For a 4-plex run, the following iTRAQ tags were used to label peptides: 113 for –Fe sample 1, 114 for +Fe sample 1, 115 for –Fe sample 2, 116 for +Fe sample 2. In a second 4-plex run, biological replicate 3 was labelled as follows: 113 for –Fe 3, 114 for +Fe 3, 115 for –Fe 3, 116 for +Fe 3. The pooled samples were lyophilized and re-suspended in 2% acetonitrile/0.1% formic acid. Analysis by LC-ESI-MS/MS using a 120 min. gradient was performed on an LTQ Orbitrap Velos mass spectrometer. MS spectra were searched using the mASCOT algorithm. Searches were conducted against the Uniport *Neisseria gonorrhoeae* reference database (ATCC_700825 downloaded on 11-29-12). Two runs were performed for each sample and the results were combined.

VII. Sequence Analysis

To determine the sequence of *pilE*, colony boilates from each strain was used in a PCR amplification reaction in which the following primers were used to amplify the full length gene, oVCU687 and oVCU688. The resultant PCR product was purified using Qiagen's PCR purification kit and the samples sequenced by the VCU Nucleic Acid Research Facilities using nested primers oVCU685 and oVCU686. These primers were gifts from Dr. P. Fred Sparling.

The sequences of *nspA*, *tdfJ*, and *NGO1063* were also confirmed via sequencing using commercially available TOPO specific primers (M13F and M13R) as well as 5' and 3' gene specific primers (Table 2) by the VCU Nucleic Acid Research Facilities.

VIII. Statistical Analysis

Statistical significance of adherence, invasion, and intracellular survival assays were assessed by a Student's *t*-test in which a *P* value of ≤ 0.05 was considered significant.

IX. Bioinformatics Analysis

Subcellular localization of identified proteins was predicted using CELLO 2.5 (195, 196) and SOSUI_{GramN} (88). In cases where the programs predicted different subcellular localizations for a protein, the protein localization was classified as “unknown”.

CHAPTER 3: DIFFERENTIAL PROTEOMIC ANALYSIS OF *NEISSERIA GONORRHOEAE* OUTER MEMBRANE PROTEINS IN RESPONSE TO HIGH IRON GROWTH CONDITIONS

I. Background

With the threat of untreatable gonorrhoeae on the horizon, the need to identify new treatment and preventive options is more important now than ever. Many of the key virulence factors expressed on the surface of *N. gonorrhoeae*, such as Pili, Porin and Opas, are subject to high frequency antigenic and phase variation. Even in the absence of variation, gonococci succeed in evading the immune system by mimicking host molecules and dampening the adaptive immune response. The complex and variable virulence mechanisms employed by the gonococcus have contributed to failed vaccine attempts to protect against gonococcal disease. Equipped with a better understanding of gonococcal pathogenesis, efforts in vaccine development are being renewed. Consequently, the search for novel surface exposed proteins that are expressed during natural infection is critical.

Iron acquisition is an important virulence determinant and has been correlated with pathogenicity in many microorganisms. *N. gonorrhoeae* is able to acquire iron from a myriad of host iron sources, thus likely experiencing stages of high internal iron pools during natural infection. We hypothesized that iron increases the expression of membrane proteins involved in virulence of *N. gonorrhoeae* and that this phenotype may be more relevant during natural infection than previously assumed. Identifying these iron induced proteins that potentially

contribute to host cell interactions will expand our knowledge of gonococcal pathology and help aid the development of therapeutics.

N. gonorrhoeae expresses a number of iron regulated proteins. However, most of the well-studied iron-regulated proteins are iron-repressed and are usually involved in iron acquisition such as the transferrin binding proteins (8, 40), the lactoferrin binding proteins (16), hemoglobin binding proteins (35), and the ferric binding proteins (35, 189). Although earlier studies were focused on identifying iron repressed proteins, iron induced proteins can be observed in 1D analysis of membrane proteins isolated from specifically iron starved cultures compared to culture grown in plain supportive media (132, 189). A traditional 2D proteomic analysis was previously carried out with gonococcal strain F62 comparing whole cell lysates and membrane proteins of cultures grown under iron depleted conditions to that grown under iron replete condition (170). In that analysis, a large number of iron induced spots were observed (45 of 60 confirmed differentially expressed spots); however, protein identities were not determined. We expect these iron induced surface proteins may be crucial in the pathogenesis of invasive gonococcal infection and be useful therapeutic targets.

In the current study, we conducted a comparative and quantitative proteomic profiling of the outer membrane proteome of *N. gonorrhoeae*. Membranes were isolated from parallel cultures grown under iron-restricted and high iron conditions and analyzed using a variety of techniques to increase the breadth and depth of coverage. Conventional 1D-SDS PAGE analysis was first carried out to gauge the size of the iron induced proteome. This analysis was followed up with 2D-DIGE analysis, a variation of traditional 2D gel electrophoresis in which proteins are labeled with fluorescent dyes prior to electrophoresis and then co-separated in the same gel. Finally, we conducted a third analysis using a gel free approach in which isotope tagging for

relative and absolute quantification (iTRAQ) combined with high performance liquid chromatography – electrospray tandem mass spectrometry (LC-ESI-MS/MS) was used to determine and quantify all proteins present in the samples. In this technique, amine specific isobaric tags are used to label peptides at the N-terminus and lysine side chains. These tags are also fragmented during peptide fragmentation releasing reporter ions. The intensity of these ions is then used to determine relative peptide abundance measurements between samples (3).

II. Results

To analyze the outer membrane (OM) proteins preferentially expressed under high iron growth conditions, gonococcal strains were cultured under iron-restricted (-Fe) and iron replete (+Fe) conditions. Free iron was removed from chemically defined media (CDM) by treating with chelex and ferric nitrate was reintroduced into the high iron culture conditions. Sarkosyl extraction was used to isolate outer membrane proteins from total membrane preparations. This treatment can be harsh and the potential loss of lipoproteins anchored to the membranes was a concern. To ensure a more comprehensive representation of the gonococcal OM proteins, we also purified outer membrane vesicles (OMVs/membrane blebs) from the cultures. Membrane blebs contain mostly outer membrane proteins with some periplasmic material and are a commonly used source for outer membrane proteins (38). For comparison, two gonococcal strains, FA19 and FA1090 were analyzed.

A. Preliminary SDS-PAGE analysis of gonococcal outer membrane fractions reveals iron induced proteins.

After preparation and standardization of samples, proteins were separated on SDS-PAGE gels and stained with Coomassie Blue for visualization to obtain an initial overview of differential protein expression between +Fe and -Fe populations. As internal controls, we verified that cultures were properly iron stressed by probing for the expression of known iron regulated proteins, transferrin binding protein A (TbpA) and transferrin binding protein B (TbpB). TbpB is a lipoprotein thus its presence can be used to determine whether lipoproteins are represented in our samples. As expected, both TbpA and TbpB were repressed under +Fe culture conditions in both sample preparations verifying sufficient iron stress. TbpB was also detected in both preparations indicating that lipoproteins were represented (Figure 2).

Iron-induced proteins were detected in protein profiles of both FA19 and FA1090 samples (Figure 3). Iron induced bands were carefully excised from the gels and identified by mass spectrometry. A summary of identified proteins is listed in Table 3. Many ribosomal proteins were identified in the samples. While ribosomal gene transcripts have been reported to be up-regulated under iron replete conditions (47), we suspected that some of the ribosomal proteins identified could be artifacts of the procedure used in membrane vesicle isolation. For this reason, we opted out of passing the cultures through a syringe for the subsequently prepared FA1090 samples to reduce the chances of shearing whole cells.

Figure 2. TbpA and TbpB detection in OMVs and Sarkosyl insoluble outer membrane preps. Western blot analysis of TbpA and TbpB expression in OMV and Sarkosyl insoluble outer membrane (Sark) preps from FA19 cultures iron stressed for 4 hours.

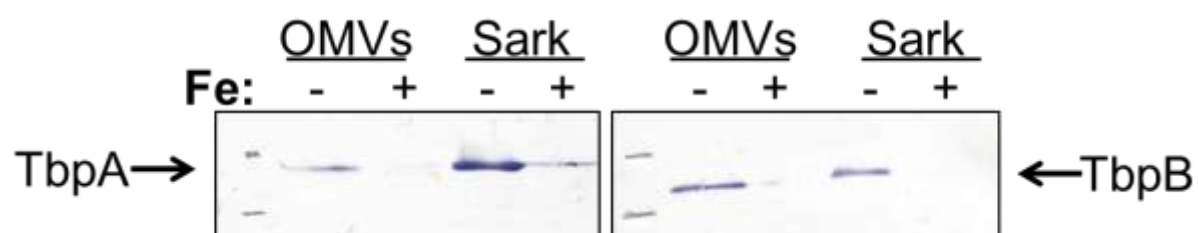
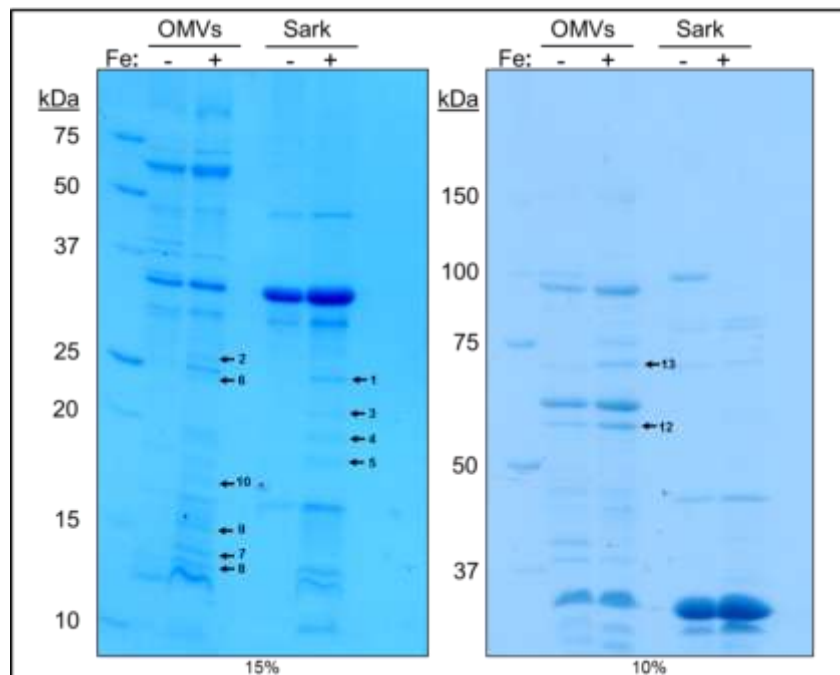


Figure 2. TbpA and TbpB detection in OMVs and Sarkosyl insoluble outer membrane preps

Figure 3. SDS-PAGE analysis of membrane preparations. 10% and 15% Coomassie Blue stained SDS-PAGE gels showing protein profiles of OMVs and Sarkosyl insoluble outer membrane (Sark) preps from strain FA19 (**A**) and 15% SDS-PAGE gel showing protein profiles from strain FA1090 (**B**). Cultures were iron stressed for 4 hours. Bands of interest, indicated by arrows, were excised and identified via mass spectrometry.

A



B

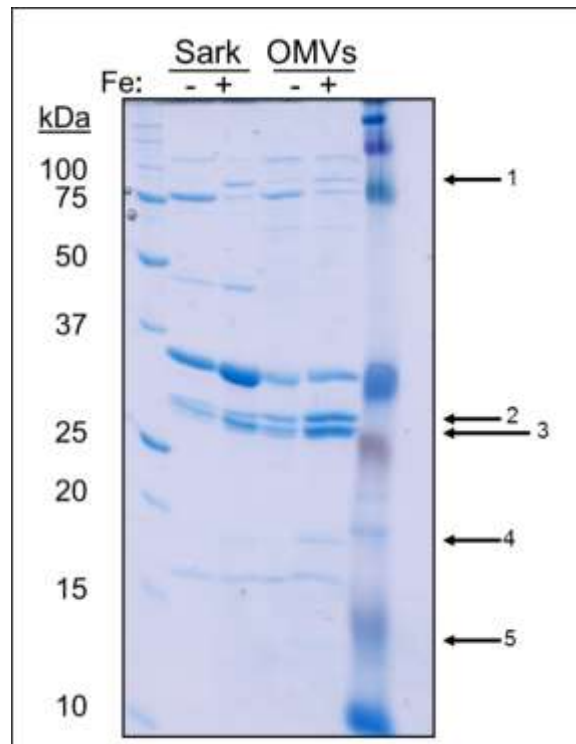


Figure 3. SDS-PAGE analysis of membrane preparations

Iron induced proteins identified in both strains FA19 and FA1090 include Omp85, the TonB-dependent transporter TdfJ, NspA, and the hypothetical protein NGO1063. Multiple proteins were identified for several of the excised bands indicating that the proteins were not completely resolved. This was anticipated as relatively low resolution is a common limitation of this technique. Since FA1090 is a gonococcal type strain, and its complete genome sequence is available, all further characterizing of the iron induced proteome was focused on this strain.

Table 3. Iron induced proteins identified from 1D SDS-PAGE analysis of FA19 and FA1090 Sarkosyl insoluble outer membrane proteins and OMVs

Band no.	ORF	Protein	Pred. Mol wt. (kDa)	Prep
FA19				
1	NGO1837	50S Ribosomal Protein L4, RplD	23.3	Sarkosyl
2	NGO0436	3-methyl-2-oxobutanoate hydroxymethyltransferase, PanB	27.7	OMVs
3	NGO0442	50S Ribosomal Protein L25, RplY	21	Sarkosyl
4	NGO1827	50S Ribosomal Protein L5, RplE	20.4	Sarkosyl
	NGO1825	50S Ribosomal Protein L6, RplF	18.9	
5	NGO0233	Outer membrane protein, NspA	18.3	Sarkosyl
	NGO0794	Bacterioferritin B, BfrB	18	
6	NGO1819	30S Ribosomal Protein S4, RpsD	23.2	OMVs
7	NGO1826	30S Ribosomal Protein S8, RpsH	14.1	OMVs
	NGO0584	50S Ribosomal Protein L9, RplI		
8	NGO1824.1c	50S Ribosomal Protein L18	12.8	OMVs
	NGO1063	Putative Lipoprotein	13.5	
	NGO1829	50S Ribosomal Protein L14, RplN	13.4	
	NGO1833	50S Ribosomal Protein L22, RplV	11.9	
9	NGO0282	Putative lipoprotein	17.5	OMVs
	NGO1855	50S Ribosomal Protein L11, RplK	15	
10	NGO1844	30S Ribosomal Protein S7, RpsG	17.6	OMVs
12	NGO1375	Hypothetical protein	66.1	OMVs
13	NGO1028	Transketolase, Tkt	71.6	OMVs
FA1090				
1	NGO1801	Outer membrane protein, Omp85	87.9	Sark
	NGO1205	TonB-dependent receptor, TdfJ	85.7	
2	NGO1861c	Opacity protein E, OpaE	27.1	OMVs
3	NGO1656	Hypothetical protein	31.5	OMVs
	NGO0070	Opacity protein B, OpaB	29.6	
	NGO1513	Opacity protein D, OpaD	31	
	NGO1577	Outer membrane protein III, Rmp	25.5	
4	NGO0233	Outer membrane protein, NspA	18.3	OMVs
5	NGO1063	Putative lipoprotein	13.5	OMVs
	NGO0581	30S ribosomal protein, S6	13.8	

B. 2D DIGE analysis aids in the identification of more iron induced proteins

Due to the incomplete resolution of proteins confounding 1D SDS-PAGE analysis, we attempted to further resolve the proteins using two-dimensional differential in gel electrophoresis (DIGE) so as to identify the true iron induced surface proteins. In this method, Sarkosyl insoluble OM preparations were differentially stained with fluorescent dyes, mixed at an equal ratio, and then co-subjected to 2-D gel electrophoresis. Images were captured at the wavelengths of respective dyes used and then merged. The difference between spots was determined using image analysis software. A summary of workflow for sample preparation and analysis via this technique is depicted in schematic form in Figure 4. This approach was preferable to traditional 2D analysis because running both samples in the same gel eliminates gel-to-gel variation. Three biological replicates were processed to enable statistical analysis and the DIGE staining pattern was alternated to avoid potential bias due to fluorescence capacity of any particular dye. 546 statistically significant ($P \leq 0.05$) spots were detected, 149 of these were iron up-regulated and 177 down-regulated by at least 1.8 fold. A representative fluorescent 2D gel image of the analysis is shown in Figure 5A.

49 iron induced (by at least 1.8 fold) true spots were selected for identification by mass spectrometry. Results were matched with tryptic peptides predicted from a protein sequence database derived from gonococcal genome data. This technique allowed us to identify more iron induced proteins (with a threshold of ≥ 1.8 fold increase), including some that had been previously identified in the preliminary 1D SDS-PAGE analysis (NGO1063, TdfJ, Omp85, and OpaD). However, multiple spots were identified to be the same proteins indicating the presence of multiple electrophoretic species. These proteins include: TdfJ, for which 7 species were identified, Omp85 (4 species), BfrA (8 species), and NGO1873 (8 species). Figure 5B indicates

the location of some of these proteins on the pick gel. Highly up-regulated proteins identified were Bacterioferritin (BfrA), OmpH, NGO1701, NGO1063 and NGO1729. Other iron induced proteins include the 50S ribosomal binding protein L25, TdfJ, and Omp85. Interestingly, Omp85 and TdfJ often resolved together both in 1D and 2D analysis. These proteins have identical pIs and an almost identical predicted mass, but the observed association could be due to redundancy of an abundant peptide. A summary of identified iron induced proteins with the range of their observed fold change (in the case of multiple species) is reported in Table 4. We were unable to resolve a fair amount of proteins as can be seen by the smear in Figure 5.

Figure 4. Work flow of 2D-DIGE analysis. Sarkosyl insoluble outer membranes from cultures grown under iron replete and iron deplete conditions were differentially labeled using fluorescent dyes Cy5 and Cy3 respectively. Samples were mixed at a 1:1 ratio and separated by isoelectric points via isoelectric focusing. Gel strip was subsequently overlaid on a polyacrylamide gel and subjected to SDS-PAGE.

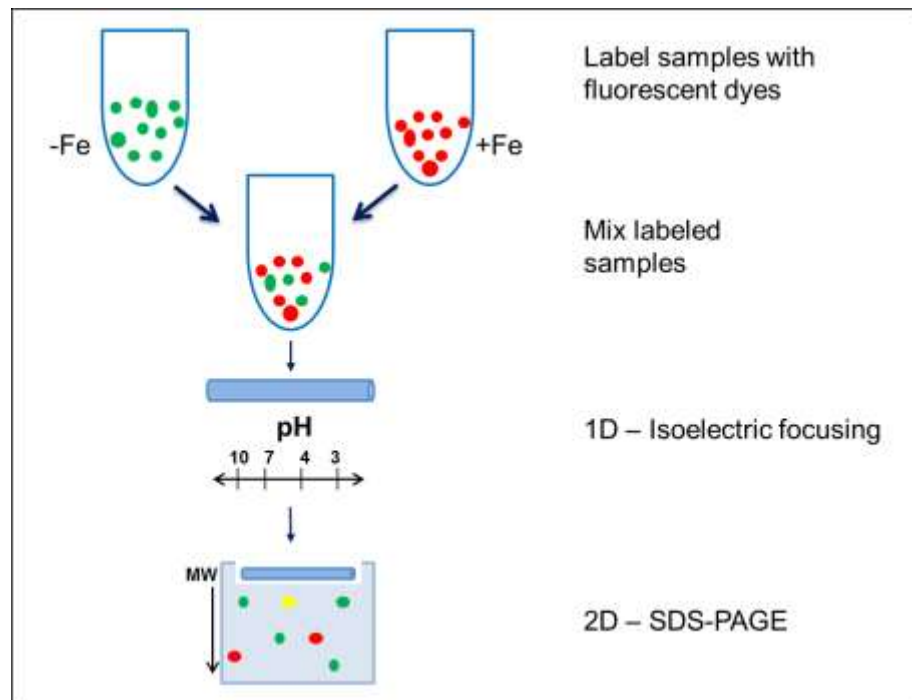
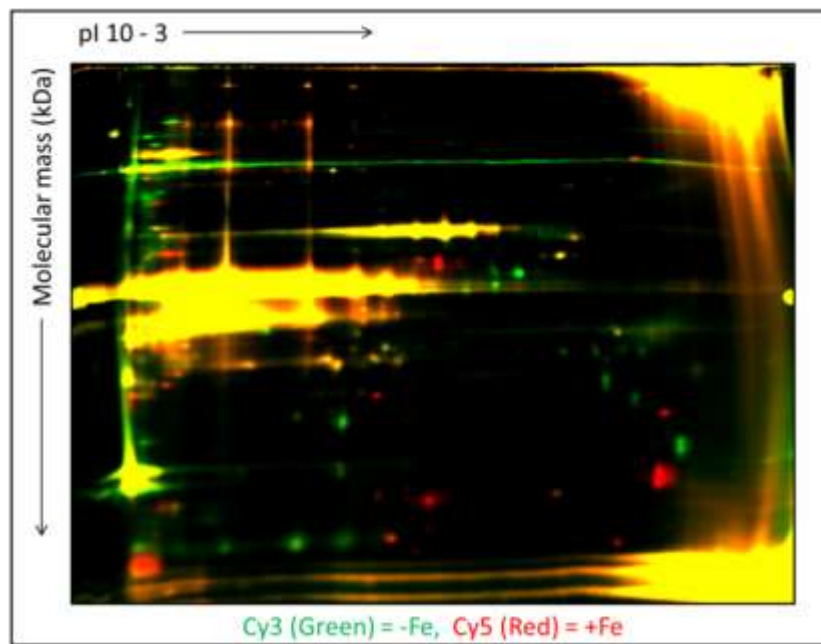


Figure 4. Work flow of 2D-DIGE analysis

Figure 5. 2D-DIGE gel of gonococcal Sarkosyl insoluble membrane preparations. (A). Representative gel image of FA1090 Sarkosyl insoluble OM from cultures grown under iron replete and iron deplete conditions. Image is oriented with the isoelectric focusing dimension horizontal and the SDS-PAGE dimension vertical. +Fe preps were labeled with Cy5 and –Fe preps with Cy3. Red spots indicate proteins up-regulated in high iron conditions whereas green spots indicate down-regulated proteins in comparison to iron restricted growth. (B) Final pick gel with multiple electrophoretic species denoted in color.

A



B

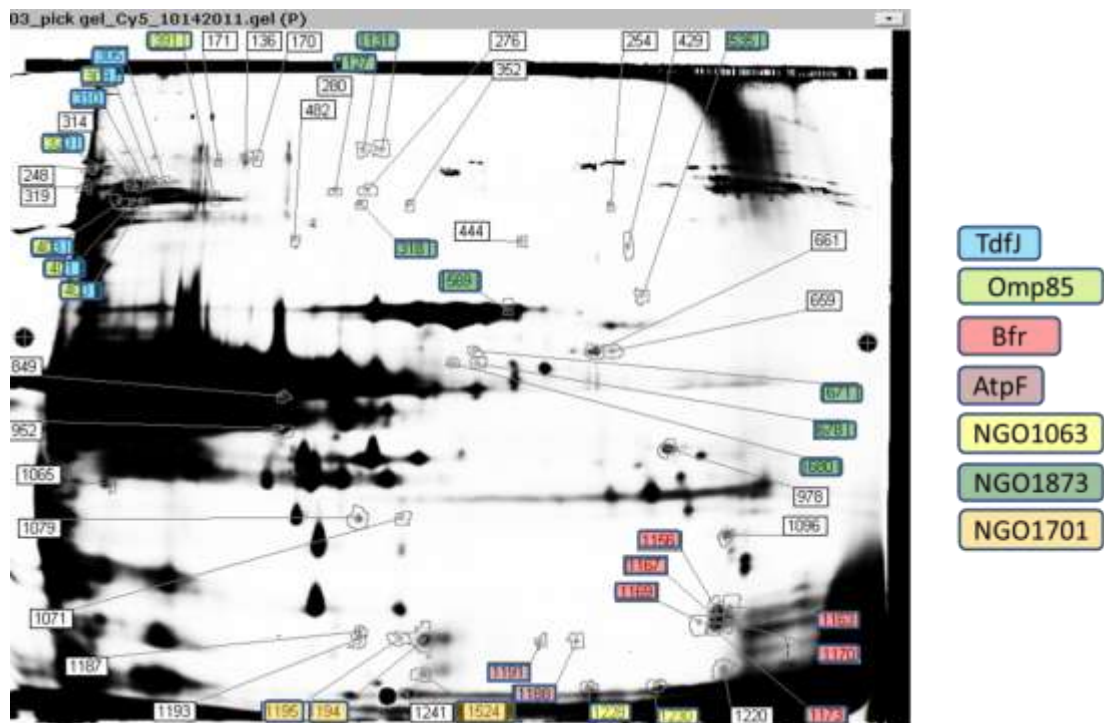


Figure 5. 2D-DIGE gel of gonococcal Sarkosyl insoluble membrane preparations

Table 4. Summary of iron induced OM proteins identified via 2D-DIGE

ORF No.	Name	Fold change range	MW (Da)	pI ^b
NGO0094	Pilus biogenesis and competence protein, PilQ	2.14	77,903	10.10
NGO0206	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	1.96	41,156	5.60
NGO0442	50S ribosomal binding protein L25, RplY	3.79	20,941	7.20
NGO0574	Carbonic anhydrase, Cah	3.13	28,067	9.90
NGO0794	Bacterioferritin A, BfrA	1.87 – 40.23	17,966	4.60
NGO0867	Hypothetical Protein	6.34	15,047	5.20
NGO1063 ^a	Hypothetical Protein	4.61 – 6.34	13,476	6.50
NGO1205 ^a	TonB-dependent receptor, TdfJ	1.84 – 4.25	85,648	9.80
NGO1494	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	1.96	41,231	5.70
NGO1513 ^a	Opacity protein D, OpaD	2.00	31,125	10.70
NGO1577 ^a	Outer membrane protein III, Rmp	2.31 – 3.14	25,524	8.90
NGO1701	Hypothetical Protein	5.11 – 21.3	15,970	7.20
NGO1715	LPS assembly protein, LptD	2.2 – 2.4	87,487	7.70
NGO1729	Hypothetical Protein	8.71	11,745	6.90
NGO1801 ^a	Outer membrane protein, Omp85	2.04 – 2.6	87,888	9.80
NGO1802	Outer membrane protein, possible OmpH	8.7	21,240	9.60
NGO1812	Major outer membrane protein porin, PorB	1.84 – 4.25	37,165	9.60
NGO1873	Hypothetical Protein	2.84 – 4.25	49,922	7.1
NGO2139	Putative lipoprotein	2.24	31,318	5.20
NGO2146	ATP synthase F0F1 subunit B, AtpF	2.45 – 2.97	17,128	5.50

^aIron induced proteins identified from FA1090 1D SDS-PAGE analysis

^bTheoretical molecular mass and pI as reported at STDGEN (<http://stdgen.northwestern.edu/>)

C. iTRAQ analysis of *N. gonorrhoeae* OM proteins.

The number of proteins identified by 2D analysis is limited to those of appropriate pI, molecular weight, and of sufficient abundance for detection. To overcome the obstacles encountered in resolving membrane proteins in gels and to obtain a complete catalogue of iron responsive outer membrane proteins, we utilized a gel free method of global proteomics to analyze the samples. Isotope tagging for relative and absolute quantification isobaric (iTRAQ) followed by liquid chromatography and tandem mass spectrometry (LC/MS/MS) was our technique of choice. This technique is advantageous over other heavy isotope labelling techniques in that up to four samples can be analyzed simultaneously, thereby reducing the amount of mass spectrometry time needed for analysis. In this procedure, outer membrane samples prepared as described for previous analyses were trypsinized and the peptides labelled with isobaric mass tags. The labeled samples were then combined for LC MS/MS analysis. Since the tags are isobaric, identical peptides from different samples co-elute and are analyzed by MS. During peptide fragmentation, these tags are also fragmented releasing reporter ions with distinct m/z ratios. Comparison of the intensity of reporter labels allows for relative quantification of identical peptides. Two biological replicates were analyzed in a four-plex assay (in which up to four differentially labeled samples can be analyzed together) and the third biological replicate was run in duplicate in a second four-plex assay. A summary of all proteins detected is listed in Table 5. A number of proteins showed varied expression between biological replicates and this limited our ability to definitely determine the level of iron regulation of these proteins. However, we were able to detect proteins with extreme pIs that were outside the limit of detection of the preceding 2D-DIGE analysis. A large number of these proteins were ribosomal proteins.

Figure 6. Schematic of iTRAQ analysis. Sarkosyl insoluble outer membranes from cultures grown under iron replete and iron deplete conditions were reduced, alkylated and digested. Peptides were labelled as depicted for a four-plex assay. Biological sample 3 was analyzed in duplicate in a second four-plex assay.

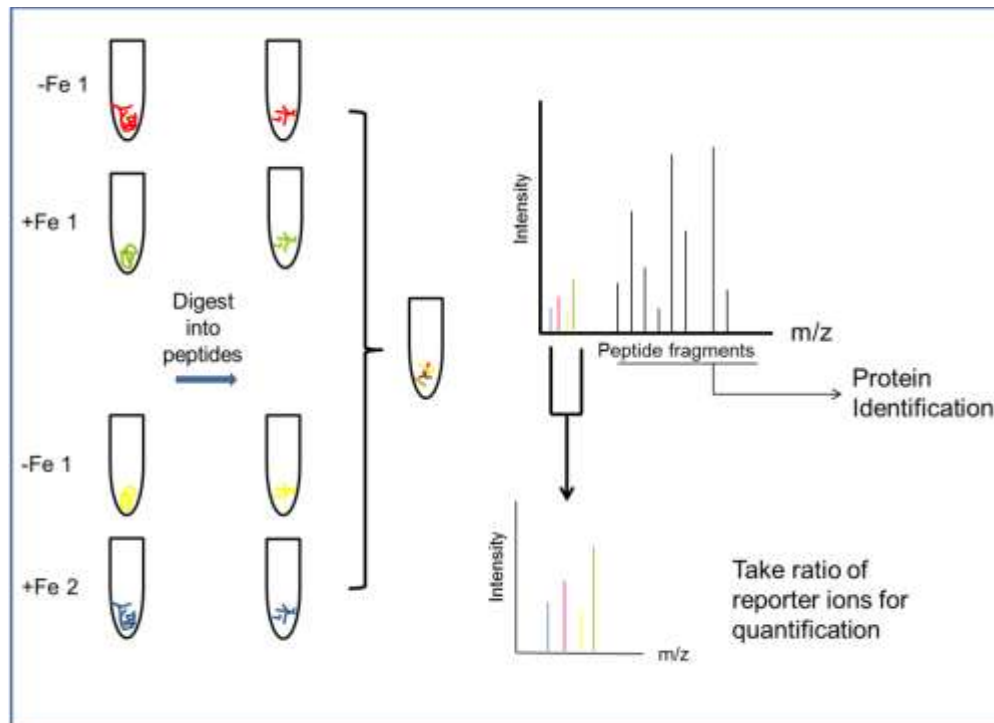


Figure 6. Schematic of iTRAQ analysis

Table 5. Proteins identified by iTRAQ analysis. +Fe/-Fe ratios expressed. Proteins that meet ≥ 1.8 fold increase or decrease ($P \leq 0.05$) are categorized as iron regulated

Gene	Name	N	+Fe/-Fe	pI
Iron induced				
NGO0265	Tetrapac protein	1	4.1	9.9
NGO0297	50S Ribosomal Protein L35, RpmL	3	5	12.3
NGO0298	50S Ribosomal Protein L20, RplT	1	4	12.2
NGO0795	Bacterioferritin B, BfrB	3	1.9	4.5
NGO1205	TonB dependent receptor, TdfJ	3	1.8	9.8
NGO1286	Initiation factor, InfB	1	4.3	9.7
NGO1492	Putative phospholipase, PldA	2	3.1	9.6
NGO1676	50S Ribosomal Protein L21, RplU	1	2.8	10.9
NGO1680	50S Ribosomal Protein L28, RpmB	1	7.4	12.2
NGO1833	50S Ribosomal Protein L22, RplV	1	13.6	11
NGO2146	ATP synthase F0F1 subunit B, AtpF	3	2.7	
NGO2182	50S Ribosomal Protein L34, RpmH	3	2.7	5.5
Not altered by iron				
NGO0026	prolyl endopeptidase	2	0.93	5.2
NGO0094	Pilus biogenesis and competence protein, PilQ	1	1	10.1
NGO0571	Hypothetical protein	2	0.96	10.8
NGO0678	Putative lipoprotein	3	1.16	10.2
NGO0861	Hypothetical protein	3	1.19	10.6
NGO0952	TonB dependent transporter, TdfH	2	1.5	10.2
NGO0994	Lipid modified azurin protein, Azu	2	1.27	4.5
NGO1043	Hypothetical protein	2	1.48	4.4
NGO1056	Uncharacterized lipoprotein	2	1.33	10.3
NGO1067/NGO1972	Adhesin, MafA 1/4	3	0.77	7.8
NGO1363	Multi-drug efflux pump channel protein, MtrE	3	0.92	10
NGO1393/NGO1584	Adhesin MafA 2/3	3	1.09	9.9
NGO1494	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	2	1.52	5.7
NGO1577	Outer membrane protein III, Rmp	3	1.39	8.9
NGO1812	Major outer membrane protein porin, PorB	3	1.02	9.6
NGO1832	30S ribosomal protein S3, RpsC	3	1.21	11.2
NGO1836	50S Ribosomal Protein L23	1	1.12	10.3

NGO1873	Hypothetical protein	3	1.21	7.1
NGO1956	Hypothetical protein	2	1.24	8.7
NGO2139	Genome derived Neisserial antigen 1946	2	1.35	5.2
Iron repressed				
NGO0275	IgA specific metallopeptidase, IgA	2	0.48	10.1
NGO1495	Transferrin-binding protein, TbpA	3	0.17	10.2
NGO2093	Iron-regulated outer membrane protein, FetA	3	0.07	10.5
Inconclusive				
NGO0070	Opacity protein, OpaB	3	v	10.5
NGO0168	Periplasmic binding protein, MntC	3	v	7.7
NGO0217	Ferric binding protein, FbpA	3	v	10.4
NGO0233	Outer membrane protein, NspA	3	v	10.6
NGO0494	Phage associated protein	2	v	9.6
NGO1513	Opacity protein, OpaD	3	v	10.7
NGO1715	LPS-assembly protein, LptD	2	v	9.8
NGO1801	Outer membrane protein assembly factor, Omp85	3	v	9.8
NGO1949	Hypothetical protein	2	v	10.8
NGO2096	sodium-dependent transporter, PotE	2	v	8.8
NGO2105	Putative adhesion penetration protein, App	2	v	10

v = Proteins that showed variable expression between biological replicates

N = number of biological replicates in which the protein was detected

III. Discussion

Outer membrane proteins play a key role in cellular processes such as interactions with host cells, sensing and responding to environmental stimuli, and transport processes. In this study, we evaluate the differential expression of outer membrane proteins of *N. gonorrhoeae* in response to iron. In our preliminary analyses, we combined two methods of isolating outer membrane proteins to get a good representation of the surface proteome. Upon visual inspection of Coomassie Blue stained 1D SDS-PAGE gels, we observed a difference in protein profiles derived from membrane blebs versus proteins obtained by Sarkosyl extraction. In particular, the major OM protein porin, was less abundant in OMVs and there was an increased representation of other minor proteins compared to OM preparations. These results suggest that although OMVs are a good source of OM proteins, their composition may not be a true representation of the relative abundance of the proteins in the outer membrane. Recent studies in *B. fragilis* and *B. thetaiotaomicron* (55) and *N. meningitidis* (200) have also shown a difference in OM and OMV protein profiles. While OMVs likely contribute to interactions with host cells, it is yet to be determined if proteins are selectively sorted into these vesicles or if the event is a result of random disintegration of the outer membrane.

A summary of iron induced proteins identified in our analysis is given in Table 6. A list of all proteins identified in our FA1090 OM preparations and their predicted subcellular localization is reported in Table 7. This table also shows proteins identified by the different techniques. The broadest coverage of proteins was obtained via iTRAQ analysis (See Table 8). Many ribosomal proteins were identified in this analysis in comparison to 2D analysis. This is presumably due to the high pIs of these proteins as they would have fallen outside of the detection range for the 2D analysis. Other than ribosomal proteins, the most highly up-regulated

proteins were NGO1729, NGO1063, TdfJ, and a putative phospholipase (NGO1492). Iron induction of this putative phospholipase is interesting as it could provide a means of penetrating and damaging host cell membranes and result in increased virulence in response to iron. Overall, as would be expected, under abundant iron conditions, proteins involved in iron acquisition were repressed, including FetA and TbpA. *N. gonorrhoeae* also increases expression of the iron storage protein, bacterioferritin, in response to high iron conditions probably as a protective response as this protein is associated with defense against iron-mediated oxidative stress (36). Comparison of the gonococcal iron induced proteins to those detected during manganese induced oxidative stress (194) revealed that the hypothetical proteins NGO0571 and NGO1043 were also expressed under oxidative stress.

In a previous study of the gonococcal iron regulated proteome using traditional 2D electrophoresis with whole cell proteins, a large number of iron induced spots were detected (170). In our 2D-DIGE study, using OM proteins, 546 statistically significant spots were detected, 160 of which were manually confirmed to be true proteins by 3D image analysis. 53 of these were up-regulated and 36 down-regulated by at least 1.8 fold in both cases. We successfully identified 49 iron induced spots by mass spectrometry. These spots resolved to a total of 20 distinct proteins due to the detection of multiple electrophoretic species. As indicated in Table 4, 10 proteins were found to be present as multiple electrophoretic species including Omp85, TdfJ, Rmp, and PorB. Multiple species of Omp85, Rmp, and porin were also reported in a 2D analysis of meningococcal proteins (12). This heterogeneity was mainly due to variability in pI. For example, 2 spots with similar mass but different pIs were determined to be NGO1063 (Fig. 5). However, variability in protein mass was also observed, as in the case of NGO1873. These multiple species are suggestive of post-translational modifications (PTMs). PTMs could

potentially impact protein structure/function and result in a diversity of biological function of the protein. For example, phosphorylcholine and glycosyl modification of *N. meningitidis* pilin have been demonstrated to be required for pili to optimally engage platelet activating factor receptor (PAFr) and adhere to human airway cells (94). These modifications are phase variable; little adherence is observed in the absence of the modification. Consequently, potential iron-regulated post-translation modification could translate into an iron-regulated function of the protein in question.

The results of our proteomic analysis can be compared to the iron regulated transcriptome reported for *N. gonorrhoeae* strain FA1090 (90). While perfect correlation between the transcriptome and proteome is not expected due to post-transcriptional regulation, we were surprised to see such little correlation between our proteome and the published transcriptome studies. We compared protein expression data (which in our case was collected at 4 hours) to transcript expression data at 4 hours. Both analyses show consistency in the expression of iron acquisition and ribosomal proteins with iron acquisition proteins being down-regulated and ribosomal proteins being up-regulated in the presence of iron. However, many of the ORFs that we found to be iron induced proteins with the exception of the iron storage protein, bacterioferritin and a phospholipase, NGO1729, were reported to be down-regulated via in the microarray study (Table 7). Transcript analysis via qRT-PCR of some of the iron induced OM proteins from FA19 proteomic analysis revealed the same divergence. These results are a strong indicator for iron dependent post-transcriptional regulation.

Selective export and post-translational modifications could also explain why our proteomics data did not correlate with published gonococcal transcriptomics (47, 90). Omp85 which was one of the proteins identified as an iron induced protein in more than one of our

proteomic analyses, is involved in outer membrane protein assembly (183). It is possible that the iron induced expression of Omp85 leads to the selective export of proteins to the outer membrane in which case, iron status would play a role in post-translationally determining the OM protein composition.

Since more women seem to present with complicated gonococcal disease when infection is acquired around the time of menses, targeting iron induced proteins as well as other adhesins as therapeutic targets may prove beneficial in lessening the morbidity of disease in the advent of antibiotic resistant gonorrhea.

Table 6. Summary of iron induced proteins of gonococcal strain FA1090

Gene	Name
NGO0070	OpaB
NGO0094	Pilus biogenesis and competence protein, PilQ
NGO0206	Putative ABC transporter, periplasmic binding protein, polyamine, PotF
NGO0233	Outer membrane protein, NspA
NGO0265	Tetrapac protein
NGO0794	Bacterioferritin A, BfrA
NGO0795	Bacterioferritin B, BfrB
NGO0867	Hypothetical Protein
NGO1063	Hypothetical protein
NGO1205	TonB dependent receptor, TdfJ
NGO1492	Putative phospholipase, PldA
NGO1494	Putative ABC transporter, periplasmic binding protein, polyamine, PotF
NGO1513	Opacity protein, OpaD
NGO1701	Hypothetical protein
NGO1715	LPS assembly protein, LptD
NGO1729	Hypothetical protein
NGO1801	Outer membrane protein assembly factor, Omp85
NGO1802	Outer membrane protein, possible OmpH
NGO1873	Hypothetical protein
NGO2139	Genome derived Neisserial antigen 1946
NGO2146	ATP synthase F0F1 subunit B, AtpF

Table 7. Comparison of iron induced proteins to published microarray analysis of gonococcal strain FA1090

Gene	Name	Trascript (via microarray)
NGO0070	OpaB	Down-regulated
NGO0094	Pilus biogenesis and competence protein, PilQ	Down-regulated
NGO0206	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	NR
NGO0265	Tetrapac protein	NR
NGO0794	Bacterioferritin A, BfrA	Up-regulated*
NGO0795	Bacterioferritin B, BfrB	Up-regulated*
NGO0867	Hypothetical Protein	Down-regulated
NGO1063	Hypothetical protein	Down-regulated
NGO1205	TonB dependent receptor, TdfJ	NR
NGO1492	Putative phospholipase, PldA	NR
NGO1494	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	NR
NGO1513	Opacity protein, OpaD	Down-regulated
NGO1701	Hypothetical protein	NR
NGO1715	LPS assembly protein, LptD	Down-regulated
NGO1729	Hypothetical protein	Up-regulated
NGO1801	Outer membrane protein assembly factor, Omp85	Down-regulated
NGO1802	Outer membrane protein, possible OmpH	Down-regulated
NGO1873	Hypothetical protein	Down-regulated
NGO2139	Genome derived Neisserial antigen 1946	Down-regulated
NGO2146	ATP synthase F0F1 subunit B, AtpF	Down-regulated

ND = Not reported

*Bacterioferritin A/B were not detected at 4 hours but were down regulated at 2 and 3 hours

Table 8. Subcellular localization and protein function for all proteins identified in the outer membrane fractions

Gene	Name	1D	2D	iTRAQ
Outer membrane				
NGO0070	Opacity protein, OpaB	+	-	+
NGO0094	Pilus biogenesis and competence protein, PilQ	-	+	-
NGO0233	Outer membrane protein, NspA	+	-	-
NGO0952	TonB-dependent transporter, TdfH	-	-	+
NGO1067/NGO1972	Adhesin, MafA 1/4	-	-	+
NGO1205	TonB dependent receptor, TdfJ	+	+	+
NGO1363	Multidrug efflux pump channel protein, MtrE	-	-	+
NGO1393/NGO1584	Adhesin, MafA 2/3	-	-	+
NGO1492	Putative phospholipase, PldA	-	-	+
NGO1495	Transferrin-binding protein, TbpA	-	-	+
NGO1513	Opacity protein, OpaD	+	+	+
NGO1577	Outer membrane protein III, Rmp	+	+	+
NGO1715	LPS-assembly protein, LptD	-	+	+
NGO1801	Outer membrane protein assembly factor, Omp85	+	+	+
NGO1812	Major outer membrane protein porin, PorB	+	+	+
NGO1956	Hypothetical protein	-	-	+
NGO2093	Iron-regulated outer membrane protein, FetA	-	+	+
Periplasm				
NGO0168	Periplasmic binding protein, MntC	-	-	+
NGO0206	Putative ABC transporter, periplasmic binding protein, polyamine	-	+	-
NGO0217	ABC transporter, periplasmic binding protein, iron related, FbpA	-	-	+
NGO0265	Tetrapac protein	-	-	+
NGO0571	Hypothetical protein	-	+	-
NGO0678	Putative lipoprotein	-	-	+
NGO0861	Hypothetical protein	-	-	+
NGO0994	Lipid modified azurin protein, Azu	-	-	+
NGO1043	Hypothetical protein	-	-	+
NGO1056	Uncharacterized lipoprotein	-	-	+
NGO1494	Putative ABC transporter, periplasmic binding protein, polyamine, PotF	-	+	+

NGO1949	Hypothetical protein	-	-	+
Inner membrane				
NGO2146	ATP synthase F0F1 subunit B, AtpF	-	+	+
NGO2096	sodium-dependent transporter, PotE	-	-	+
Extracellular				
NGO1063	Putative lipoprotein	+	+	-
NGO0275	IgA specific metalloproteinase, IgA	-	-	+
NGO2105	Putative adhesion penetration protein, App	-	-	+
Unknown				
NGO1802	Hypothetical protein	-	+	-
NGO1873	Hypothetical protein	-	+	+
NGO2139	Genome derived Neisserial antigen 1946	-	+	+
Cytoplasmic				
NGO0026	prolyl endopeptidase	-	-	+
NGO0297	50S Ribosomal Protein L35, RpmL	-	-	+
NGO0298	50S Ribosomal Protein L20, RplT	-	-	+
NGO0494	Phage associated protein	-	-	+
NGO0794	Bacterioferritin A, BfrA	-	+	-
NGO0795	Bacterioferritin B, BfrB	-	-	+
NGO0867	Hypothetical Protein	-	+	-
NGO1286	Initiation factor, InfB	-	-	+
NGO1676	50S Ribosomal Protein L21, RplU	-	-	+
NGO1680	50S Ribosomal Protein L28, RpmB	-	-	+
NGO1832	30S ribosomal protein S3, RpsC	-	-	+
NGO1833	50S Ribosomal Protein L22, RplV	-	-	+
NGO1836	50S Ribosomal Protein L23, RplW	-	-	+
NGO2182	50S Ribosomal Protein L34, RpmH	-	-	+

CHAPTER 4: IMPACT OF HIGH IRON CONDITIONS ON *NEISSERIA GONORRHOEAE* INVASION OF CERVICAL EPITHELIAL CELLS

I. Background

Iron is an essential element for most microorganisms. In the human host, free iron is scarce as most iron available is bound by human iron binding proteins such as hemoglobin, transferrin, and lactoferrin (35, 187). Consequently, an important virulence mechanism for human pathogens is the ability to acquire iron. *N. gonorrhoeae* is a very well adapted obligate human pathogen that has evolved various efficient high affinity iron uptake systems that facilitate iron acquisition from human iron-binding proteins (19, 35) such as lactoferrin, transferrin and hemoglobin.

Due to this ability to utilize host iron-binding proteins, we propose that *N. gonorrhoeae* likely encounters high iron conditions during natural infection. Human transferrin and lactoferrin are present in semen (122, 169) and vaginal fluid (168) in high enough conditions to support gonococcal growth, while heme and hemoglobin are also utilizable sources of iron that are likely available at higher concentrations during menses in women (122). Interestingly, women with pelvic inflammatory disease (PID) due to *N. gonorrhoeae* are more likely to present symptoms during or just after the onset of menses (86) potentially indicating a more invasive disease pathology in the presence of abundant iron.

However, the contribution of iron to the gonococcal invasion process has not been well characterized. Shaw and Falkow, who developed a model for gonococcal invasion of immortalized human endometrial cells (HecIB), observed that supplementing media containing non-supportive fetal calf serum with Isovitallex enabled the invasion of HecBI cells and that iron appeared to be the necessary supplement (151). Chen *et al* subsequently confirmed that iron was indeed the necessary supplement (34, 83). These findings were not universally appreciated as most subsequent studies characterizing gonococcal invasion did not report supplementing the infection medium with iron. Fetal bovine serum (FBS) is usually added to tissue culture media to ensure the health of the eukaryotic cells. FBS however contains bovine transferrin. *N. gonorrhoeae* can only remove iron specifically from human transferrin, thus, bovine transferrin effectively chelates free iron in a form that is inaccessible to the gonococcus. These conditions therefore represent iron limiting conditions for the bacteria and as such, potentially significant contributions made by iron to the gonococcal invasion process have not been investigated.

We have previously demonstrated that gonococcal strain FA1090 required iron for intracellular survival (201). We hypothesized that high iron conditions signals changes in the gonococci that facilitates a more invasive phenotype. Herein, we investigated the possibility that iron augments the invasion process as well.

II. Results

A. Iron enhances gonococcal invasion of cervical epithelial cells

To determine the effects of iron on invasion, we quantified the number of viable intracellular gonococci recovered from cervical epithelial cells infected with bacteria in the presence or absence of iron. Excess iron was added to the +iron conditions to overcome chelation by bovine transferrin. Cervical epithelial cell (ME180) monolayers were infected for 4 hours at an MOI of 10 with gonococcal strain FA1090 in the absence of iron or with the addition of varying concentrations of ferric nitrate. The cells were treated with gentamicin to kill extracellular bacteria and then the gentamicin was washed off before lysing the epithelial cells for the enumeration of intracellular gonococci. While intracellular gonococcal cells were recovered in the absence of iron, addition of ferric nitrate during the infection period resulted in a more than 100-fold increase in invaded bacteria (Figure 7A). These results indicate that the presence of iron induces or enhances invasion of host cells.

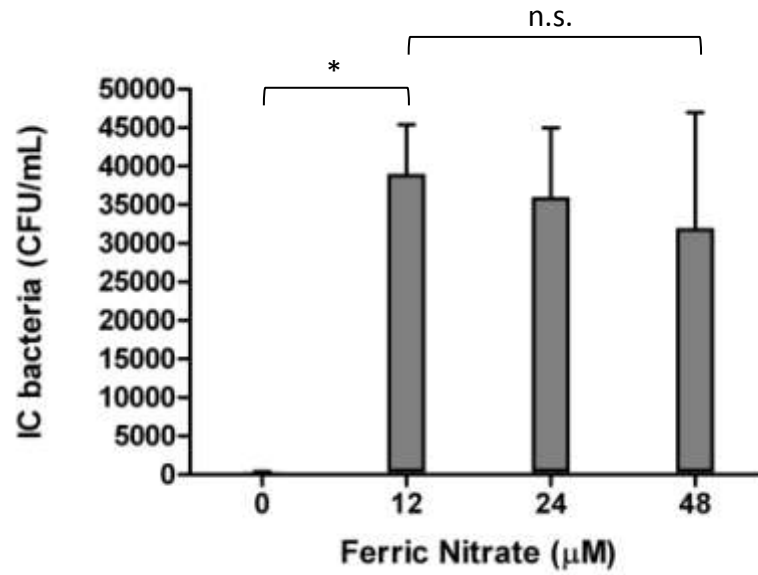
To check that the observed phenomenon was not simply due to non-specific electrostatic interactions, we conducted the assay using organic iron in the form of ferrated human transferrin as the sole iron source (Figure 7B). In this system, there is no free iron present as it is bound to transferrin. Again, over a 100-fold increase in intracellular bacteria recovered was observed under iron replete conditions suggesting that the phenomenon happens as a result of iron internalization by the gonococcus.

To confirm this hypothesis, we tested a *tonB* mutant (74), which is incapable of energizing iron transport through TonB-dependent transporters (15, 102), for iron induced invasion in the presence of human transferrin. Since the transferrin iron acquisition system requires TonB, this strain is unable to utilize iron from transferrin but can use inorganic sources

of iron such as ferric nitrate. Our experiments were repeated, comparing the invasion capacity of the *tonB* mutant to that of the wildtype strain in the absence of additional iron, as well as in the presence of iron in the form of either human transferrin or ferric nitrate. The *tonB* mutant and the wildtype were similarly capable of iron induced invasion in the presence of ferric nitrate indicating that inactivation of TonB did not inhibit the ability of gonococci to invade host cells. This mutant was however significantly defective when transferrin was the sole source of iron (Figure 7C) supporting the hypothesis that iron must be internalized in order to enhance invasion. These results suggest that the iron enhanced invasion phenotype requires iron internalization by the gonococcus resulting in replete internal supplies of iron.

Figure 7. Gonococcal invasion of ME180 cells as a function of iron. Intracellular gentamicin resistant wildtype strain FA1090 detected after 4 hours of infection at an approximate MOI of 10. Increasing concentrations of ferric nitrate (A) or human transferrin (B) were included in infection media as iron sources. (C) Invasion of the FA1090 *tonB* Ω strain compared to wildtype when ferric nitrate or transferrin is the sole source of iron. Data represents the mean and standard deviation of at least three independent experiments performed in duplicate. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant and designated with *. n.s. = not significant.

A



B

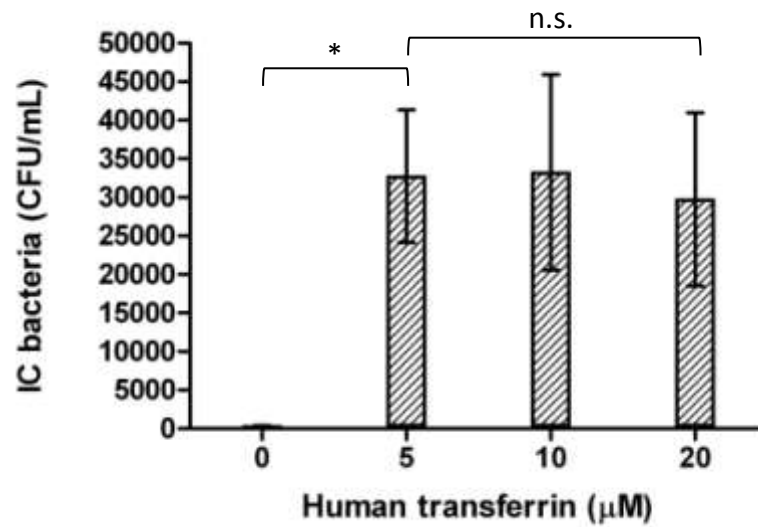


Figure 7. Gonococcal invasion of ME180 cells as a function of iron

C

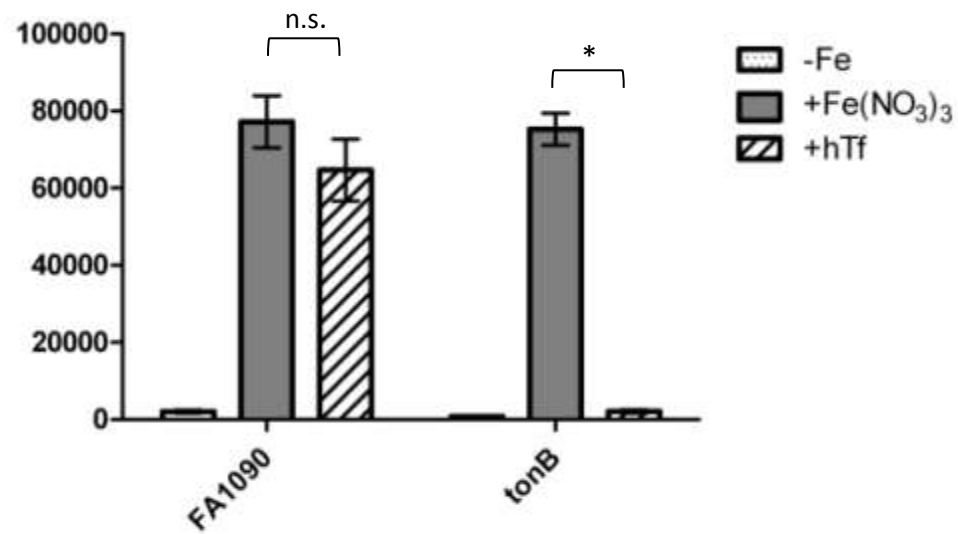


Figure 7. Gonococcal invasion of ME180 cells as a function of iron.

B. Iron induced invasion occurs regardless of Opa expression.

Opacity proteins have been well-characterized as invasins in the gonococcus, thus, we investigated whether this iron enhanced invasive phenotype was dependent on these proteins. We obtained, from Alison Criss Ph.D., at the University of Virginia, a partial Opa deletion strain (FA1090 Δ *opaKEB*, unpublished) which cannot express three transparent Opa proteins and a complete Opa deletion strain (FA1090 Δ *opaA-K*), which does not express any Opacity proteins (10). These strains were tested for iron induced invasion in comparison to wildtype FA1090 (Figure 8). Optically transparent colonies of each strain were used in the analysis. As seen with wildtype, the phenotypically Opa⁻ partial deletion strain, as well as the Opaless strain were both capable of iron induced invasion, displaying statistically similar and significantly increased invasion in the presence of iron. These data led us to conclude that iron-induced invasion is not dependent on Opa expression.

C. Iron does not increase the adherence capability of *N. gonorrhoeae*

Invasion processes typically occur subsequent to initial attachment of bacteria to host cell. Thus, we wanted to determine if this iron enhanced invasion phenotype observed is a result of iron enhanced adherence. Because OpaB/D was being expressed by our wildtype FA1090 strain, (see Figure 3, Table 3,4.), and we had previously determined the iron induced phenotype to be Opa-independent (Figure 8), we decided to conduct our analysis in the absence of these Opas. We quantified the number of attached and intracellular gonococci recovered from cervical epithelial cells infected with bacteria in the presence or absence of additional iron.

Figure 8. Comparison of iron induced invasion capacity of Opa negative variants. Intracellular gentamicin resistant strain FA1090 wt, *ΔopaKEB*, and *ΔopaA-K* detected after 4 hours of infection at an approximate MOI of 10. Iron source is ferric nitrate (24μM). Data represents the mean and standard deviation from four independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant and designated with *.

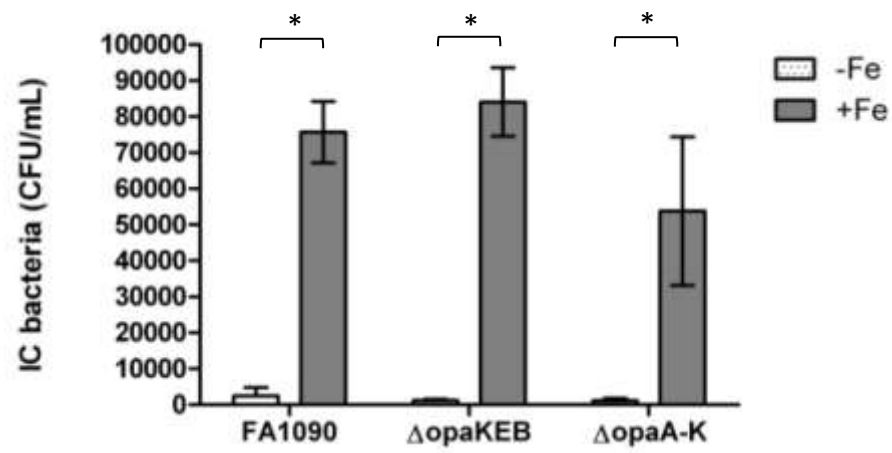


Figure 8. Comparison of iron induced invasion capacity of Opa negative variants

ME180 monolayers were infected for 2 hours with gonococcal strain FA1090 with or without the addition of iron. Monolayers were then washed multiple times to remove extracellular, unattached bacteria and the epithelial cells were lysed for enumeration of attached and invaded bacteria. We found that there was no significant increase in attachment of the gonococcus to host cells due to iron (Figure 9).

We also investigated whether iron induced invasion occurs at earlier time points and how adhesion compares to invasion. An invasion assay was carried out allowing for only 2 hours of infection before the collection of intracellular “gentamicin resistant” bacteria (Figure 10). We observed that gonococci are able to invade epithelial cells as early as 2 hours into infection and that while not as pronounced as at 4 hours, iron induced invasion is evident ($P = 0.04$) in the absence of iron induced adherence. Together, these data suggest that iron enhances invasion without enhancing adherence. Thus, factors contributing to iron induced invasion likely become relevant subsequent to attachment.

Figure 9. Effect of iron on adhesion of gonococci to cervical epithelial cells. Adherent and Intracellular gonococcal strain FA1090 *ΔopaKEB* detected after 2 hour infections at an approximate MOI of 10. 24μM ferric nitrate was included as a source of iron. Data represents the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant. n.s. = not significant.

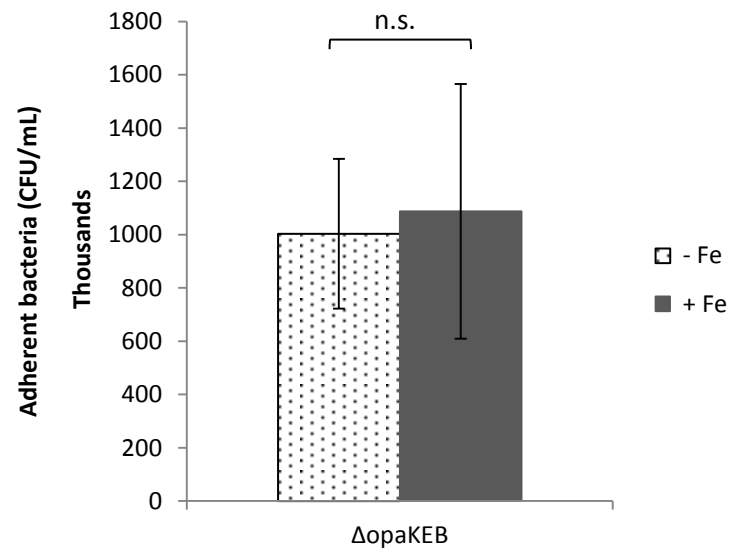


Figure 9. Effect of iron on adhesion of gonococci to cervical epithelial cells

Figure 10. Iron induced invasion at 2 hours. Intracellular gentamicin resistant strain FA1090 *ΔopaKEB* detected after 2 hours of infection at an approximate MOI of 10. Iron source is 24μM ferric nitrate. Data represents the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant.

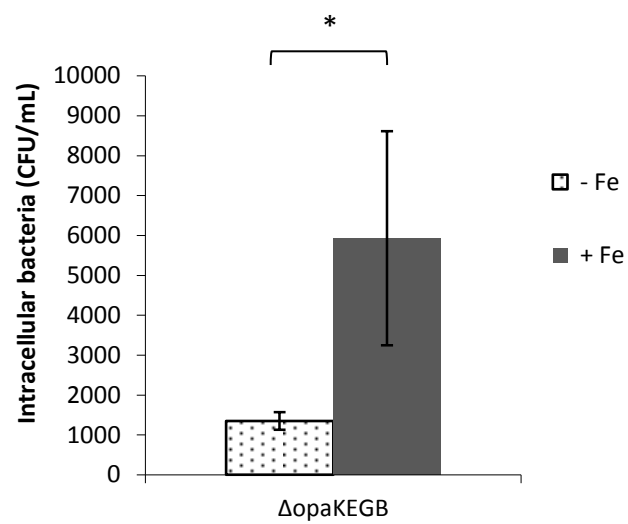


Figure 10. Iron induced invasion at 2 hours

III. Discussion

Iron availability poses a problem for invading bacteria as the amount of free iron is limited and insufficient to support replication and establishment of disease. The nutrient is sequestered intracellularly by ferritin, in the blood by heme or hemoglobin, or in serum and mucosal surfaces by the iron-binding proteins, transferrin and lactoferrin (128). *Neisseria gonorrhoeae* on the other hand, is such a well-adapted human pathogen that has evolved numerous high-affinity iron uptake systems that enable it to take up iron from multiple host iron binding proteins commonly encountered *in vivo*. Additionally, it is able to use certain siderophores produced by other organisms as iron sources (29, 162, 188). Therefore, it can be expected that the gonococci encounter certain stages during infection when iron is not limiting. As previously mentioned, transferrin and lactoferrin are present in semen, and vaginal fluid, while heme and hemoglobin are also utilizable sources of iron that are likely available at higher concentrations during menses in women.

Iron availability has been correlated with pathogenesis of other microorganisms. For example, elevated iron conditions have been documented to increase the adhesion, invasion, and intracellular survival of *Salmonella enteritidis* and other enteric pathogens (61, 107). Early *In vitro* studies using human endometrial cells showed that iron was a necessary factor to allow gonococcal invasion of endometrial cells (34, 151). However, follow up studies characterizing how iron aids gonococcal invasion have not been carried out. Accordingly, a more in depth look into the contributions of iron to the invasion process is warranted.

In the current study, we further investigated the role of iron in adherence and invasion by *N. gonorrhoeae*. We demonstrate that iron promotes gonococcal invasion of cervical epithelial

cells. We also found this phenotype to be dependent on the internalization of iron. This phenomenon is not dependent on the expression of the well characterized invasins of the pathogenic *Neisseria*, the Opacity proteins, suggesting the involvement of other factors. Further investigation of the effects of iron on host cell interactions revealed that iron did not enhance adherence indicating that iron specifically enables a process that triggers invasion. It is thus possible that iron could potentially act as a signal that activates factors alerting gonococci to switch from extracellular colonization to intracellular localization.

Studies have shown that women with PID due to *N. gonorrhoeae* are more likely to present symptoms during or just after onset of menses (86). Additionally, Opa-negative gonococcal variants were reported to be preferentially recovered from fallopian tubes of women with acute salpingitis (46) and in cervical isolates obtained during menses, in the fallopian tube and in genital, blood, and joint fluid obtained from patients with disseminated gonococcal infection leading to the suggestion that Opa negative organisms are typically associated with invasive disease. (54). We propose that during the natural infection process, the presence of iron triggers a switch to a more invasive phenotype.

To our knowledge, this is the first report of an Opa-independent, iron-responsive invasion pathway in *N. gonorrhoeae*. Considering the link of iron to ascending complicated disease, and Opa negative gonococci being associated with asymptomatic invasive disease, we propose that the mechanism observed here may be clinically relevant in asymptomatic invasive disease.

CHAPTER 5: IDENTIFYING PROTEINS THAT POTENTIALLY CONTRIBUTE TO IRON INDUCED INVASION

I. Background

It is well documented that Opa proteins confer bacterial entry into host cells by engaging heparin sulfate proteoglycans (HSPGs) and CEACAMS (Carcinoembryonic antigen related cell adhesion molecules) (179, 181). Interestingly, Opa-negative gonococcal variants were reported to be preferentially recovered from fallopian tubes of women with acute salpingitis (46) and CEACAM is not always expressed on female genital epithelial cells (163), suggesting that Opa-independent factors may be involved during invasive gonococcal infection in the upper female genital tract.

Given our previous findings that iron-induced invasion is Opa-independent, we hypothesized that novel invasins were responsible for the phenotype. To test this hypothesis, outer membrane proteins that were determined to be iron induced via outer membrane proteomic analysis were further studied to determine if they contributed to the iron induced gonococcal invasion pathway. Specifically, gonococcal proteins NspA, TdfJ and NGO1063 were selected for further investigation. NspA is an Opa-like conserved protein. TdfJ is a TonB-dependent transporter whose homologue, ZnuD, in *N. meningitis* has been reported to be involved in adhesion and invasion. Finally, NGO1063 is a hypothetical protein that contains a lysozyme inhibitor domain (NGO1063). In this

study, mutants unable to express the aforementioned proteins were generated and tested for defects in iron induced invasion.

II. Results

A. TdfJ as a potential iron induced adhesin/invasin

TdfJ is a poorly characterized TonB-dependent transporter. Although little is known about the function of gonococcal TdfJ, its meningococcal counterpart, denoted as ZnuD, was reported to be a zinc transporter (161) and to also possibly function in heme uptake (109). It was also reported to be involved in adherence, invasion, and intracellular survival (109). There are conflicting reports as to the iron regulation of ZnuD in *N. meningitidis*; Stork et al. saw no effect of iron on protein expression (161) while Kumar et al. reports iron induction of ZnuD (109). Nevertheless, gonococcal *tdfJ* transcript was reported to be up-regulated in the presence of iron by microarray (47). Our proteomic analysis identified TdfJ as an iron induced protein. Consequently, we sought to determine if gonococcal TdfJ contributes to iron induced invasion.

i. MCV407 is deficient in iron induced invasion

A *tdfJ* mutant was generated by disrupting the *tdfJ* gene with an Ω cassette, generating MCV407. A complement was generated by using the NCIS system (123). A wild-type copy of the gene along with 30 bp of upstream sequence was cloned into the NICS vector, pGCC4. This construct was used to transform the *tdfJ* mutant generating MCV413. The loss of TdfJ

expression and subsequent recovery in the presence of IPTG in the mutant and complemented strains, respectively, was confirmed via western blot analysis (Figure 11). To address the possibility that TdfJ could be an iron induced invasin, we investigated the capacity of MCV407 and MCV413 to invade cervical epithelial cells (Figure 12). Due to the positive effect of iron on growth, invasion efficiencies were also calculated for each strain as the gentamicin resistant counts divided by total bacterial counts (adherent and non-adherent) at the end of the 4 h infection (Figure 12). Compared to the parent strain, MCV407 was significantly defective for iron induced invasion. However, the complement (MCV413) did not invade any better than the mutant.

ii. TdfJ does not contribute to gonococcal invasion of epithelial cells

Kumar et al. reported that a *znuD* mutant was defective for invasion of human lung epithelial cells (109). It was possible that the inability of our complement strain to repair the invasion defect may have been due to the loss of native regulation of TdfJ expression. For example, iron regulation is abrogated in the TdfJ complement. Thus, we generated new *tdfJ* mutants and evaluated their capacity to invade ME180 monolayers (Figure 13). Our rationale for this experiment was that if the defect is TdfJ specific, newly created mutants would all display the same phenotype. Subsequent mutants showed no defect in invasion compared to the parent indicating that TdfJ does not significantly contribute to epithelial cell interaction.

Figure 11. TdfJ expression in mutant and complement strains. Western blot analysis of the solubilized whole cell lysates of FA1090 Δ KEB (parent), MCV407 (*tdfJ*) and MCV413 (*tdfJ*^c) in the absence of IPTG and in the presence of 0.25mM IPTG (for MCV413). Proteins were separated on a 7.5% acrylamide gel. Blots were probed with an anti-TdfJ antiserum. Ponceau S stain is included to show equal loading of total protein.

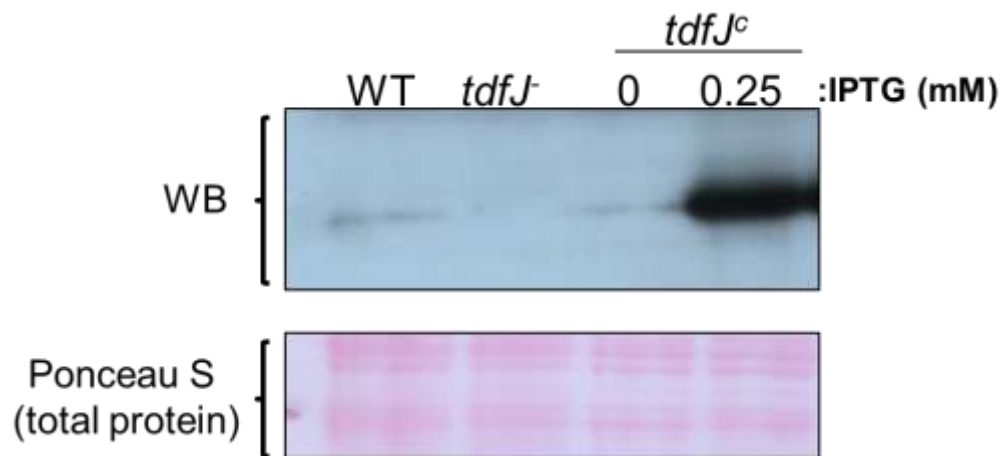
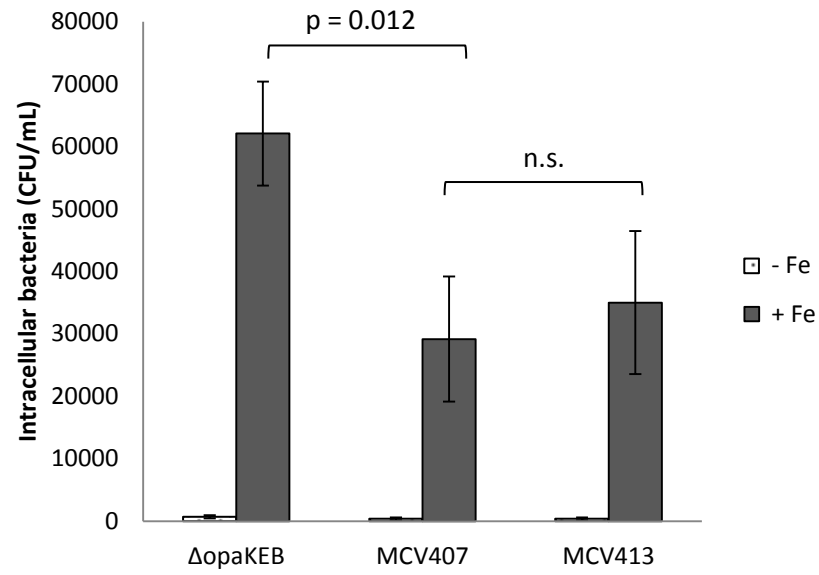


Figure 11. TdfJ expression in mutant and complement strains

Figure 12. Effect of TdfJ mutation on gonococcal invasion of epithelial cells. 4 hour infection of ME180 cells at an approximate MOI of 10 with indicated strains. (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Each condition was performed in duplicate and results represent the mean and standard deviation from four independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant. n.s. = not significant.

A.



B.

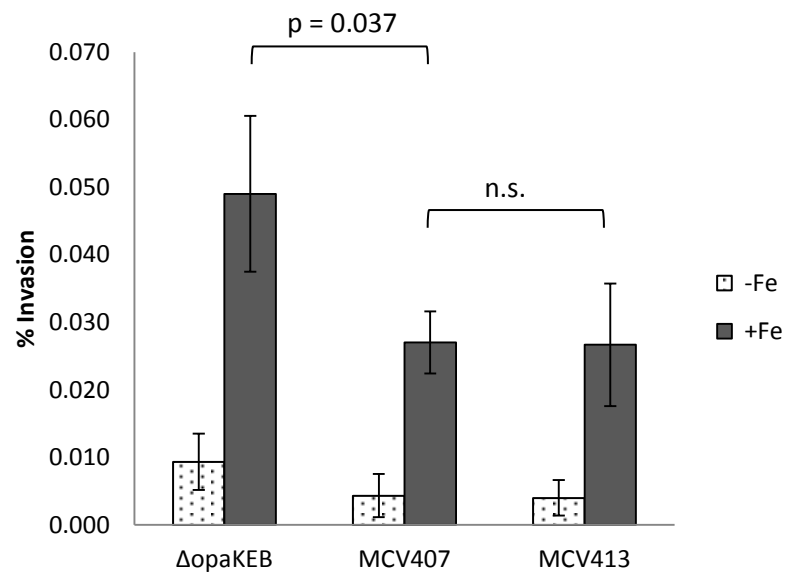
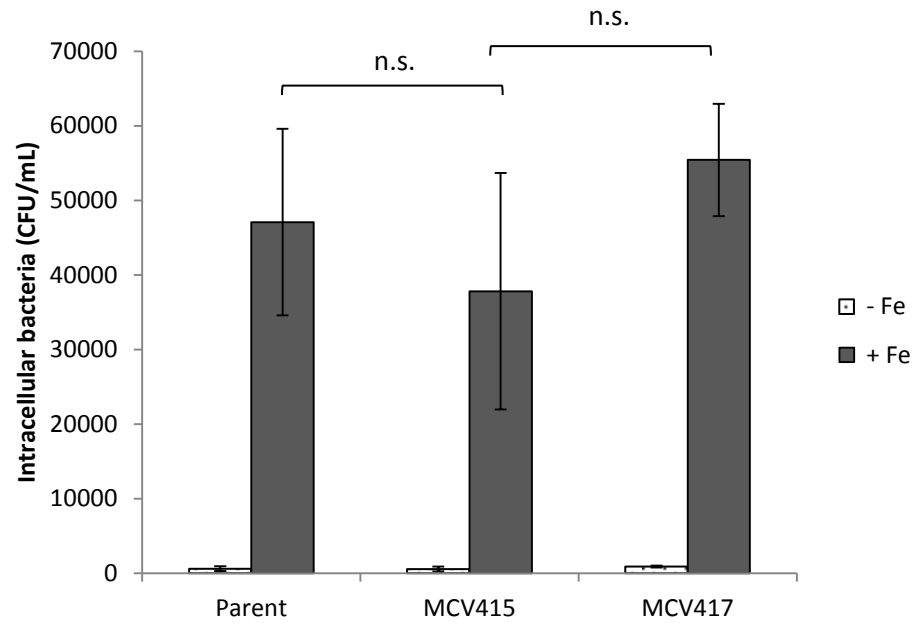


Figure 12. Effect of TdfJ mutation on gonococcal invasion of epithelial cells

Figure 13. Invasion capacity of TdfJ mutants. Evaluation of TdfJ mutants in invasion assay (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Each condition was performed in duplicate and results represent the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant. n.s. = not significant.

A.



B.

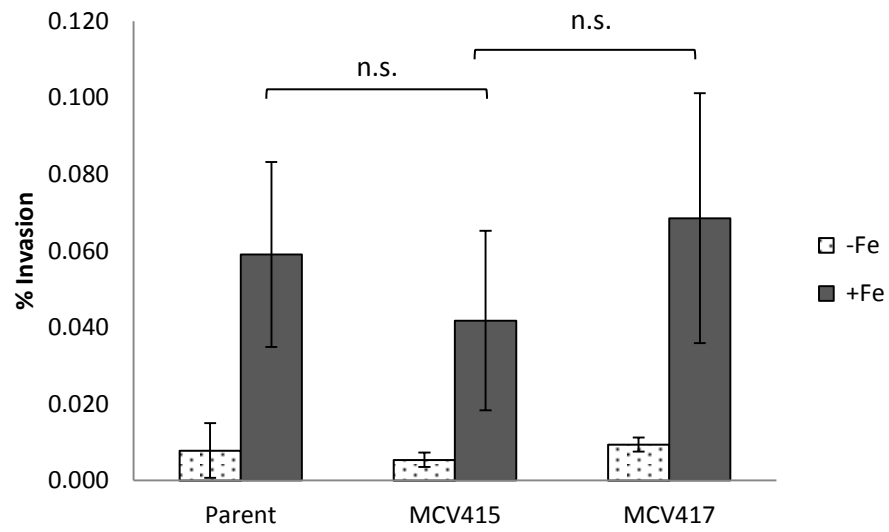


Figure 13. Invasion capacity of TdfJ mutants

B. NspA as a potential iron induced adhesin/invasin

NspA is an 18 kDa integral outer membrane protein of largely unknown function. NspA expressed by the closely related *N. meningitidis* has been characterized to be a homolog of the opacity proteins (180) which are known to mediate invasion of host cells. The protein consists of 8 transmembrane β -strands and four surface exposed loops that form a barrel in the outer membrane similar to the conformation expected for the opacity proteins (180). It is surface-exposed, highly conserved, and a current meningococcal vaccine candidate (118, 180). There is however no information in the literature about the role of NspA in adhesion or invasion. Meningococcal NspA has been demonstrated to bind Factor H and modulate the complement system (112). Gonococcal NspA, however, is not well characterized but has been reported to induce bactericidal and opsonic antibodies in mice (114). The *nspA* gene was reported to be iron induced in *N. meningitidis* (68) but iron repressed by gonococcal microarray studies (90). Due to its surface exposure and Opa-like structure, NspA seemed an ideal candidate to test for contributions to the iron induced invasion phenomenon.

i. NspA is iron induced

In our proteomics analysis, NspA was found to be an iron-induced protein by 1D SDS-PAGE analysis but we were unable to conclusively determine its expression via iTRAQ analysis. We confirmed our proteomics results by western blot analysis of whole cell lysates and outer membrane preparations from strains FA19 and FA1090 and found NspA to be iron induced (Figure 14).

Figure 14. NspA expression in response to iron. Expression level of NspA in wildtype FA19 and FA1090 strains grown for four hours with and without the addition of 24 μ M ferric nitrate. Whole cell lysates of FA19 grown in GCB and Sarkosyl extracted outer membranes of strain FA1090 grown in CDM were run on a 15% acrylamide gel, transferred on to nitrocellulose membranes, and probed with anti-NspA antisera.

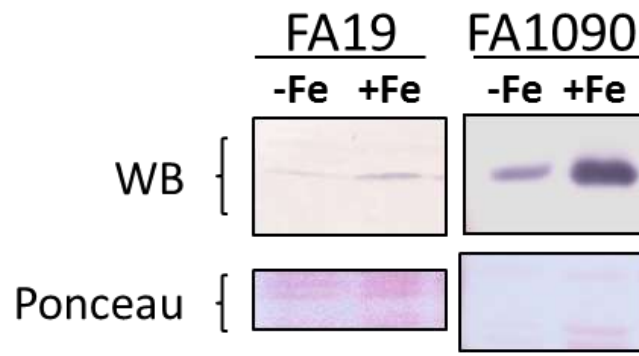


Figure 14. NspA expression in response to iron

ii. NspA does not function as an adhesin

We generated a gonococcal *nspA* mutant (MCV406, Table 2) by insertional inactivation of the *nspA* gene in the FA1090 Δ *opaKEB* background. Loss of wildtype *nspA* was confirmed by PCR and loss of protein expression was confirmed via western blot (Figure 15). The mutation was subsequently complemented using the NICS system (123). The wild-type *nspA* gene along with its native ribosomal binding site was cloned into the NICS vector, pGCC4, resulting in an IPTG-inducible gonococcal strain, MCV411. We determined that wild-type expression levels were obtained with 2mM IPTG (Figure 15). To investigate the potential role of NspA as an adhesin, we compared the adherence capacity of the mutant to parent in an adherence assay and found no difference between the two strains. The mutant showed no decrease in adhesion compared to its parent under either iron replete or depleted conditions (Figure 16). This suggests that NspA is not an adhesin.

Figure 15. NspA expression in mutant and complement strains. Western blot analysis of whole cell lysates from wildtype, mutant and complement strains in the absence of IPTG and in the presence of 0-2mM IPTG. Proteins were separated on a 15% acrylamide gel. Blots were probed with an anti-NspA antisera. Ponceau S stain is included to show equal loading of total protein.

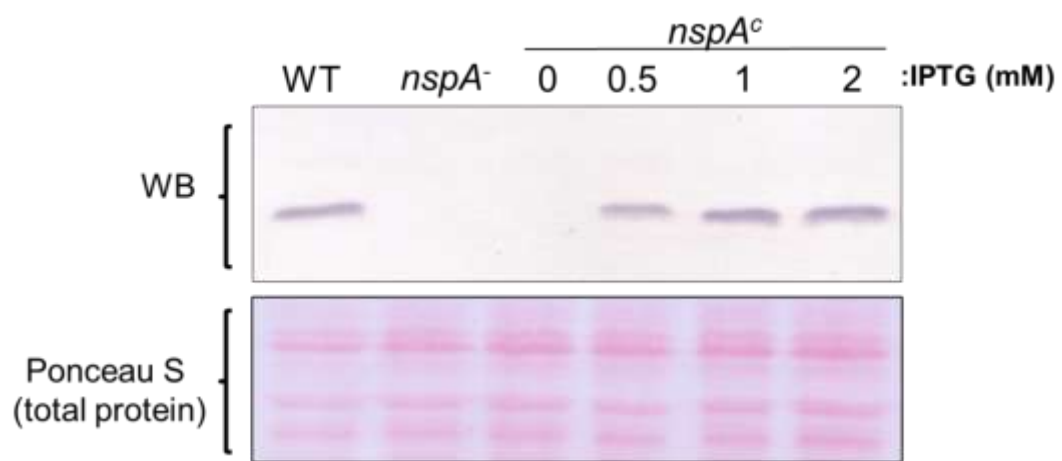


Figure 15. NspA expression in mutant and complement strains

Figure 16. Effect of *nspA* mutation on adhesion. Adherent FA1090 *ΔopaKEB* and MCV406 detected after 2 hour infections at an approximate MOI of 10. Iron source is ferric nitrate. Data represents the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis. *P* values < 0.05 were considered significant. n.s. = not significant.

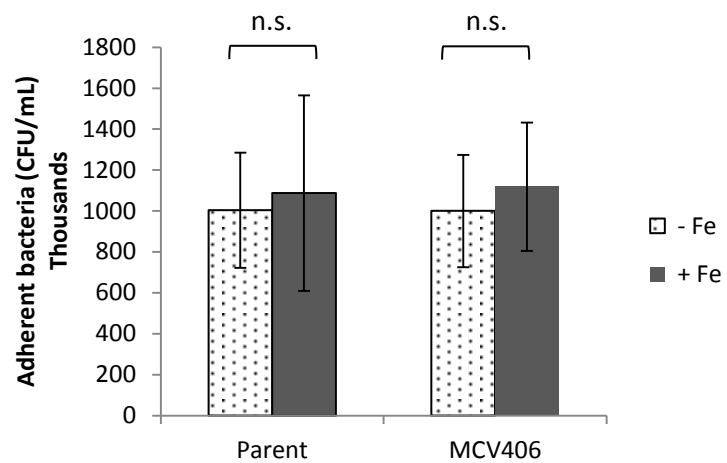


Figure 16. Effect of *nspA* mutation on adhesion

iii. MCV406 is deficient in iron induced invasion but phenotype is not recovered in complement

To determine if NspA plays a role in iron induced invasion of host cells, we analyzed the mutant and complemented strains for iron induced invasion in the invasion assay. A diminished number of intracellular MCV406 were recovered compared to the parent and complement (p values of 0.001 and 0.003 respectively) (Figure 17 A). Due to the effect of iron on growth, invasion percentages were calculated for each strain as the gentamicin resistant counts divided by total bacterial counts (adherent and non-adherent) at the end of the 4 h infection (Figure 17B). We found that although the *nspA* mutant exhibited a significant decrease in invasion efficiency compared to the parent control ($P = 0.03$). The complement did not fully recover wildtype efficiency and was not found to be significantly different from the mutant ($P = 0.06$). While it is possible that the incomplete recovery is due to artificial regulation of the *nspA* in the complement strain, it can also be indicative of other factors being involved to the defect observed.

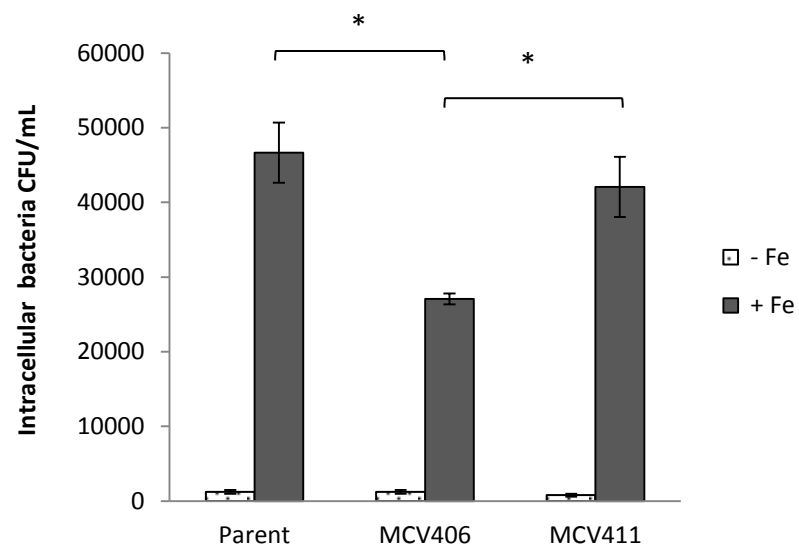
iv. NspA is co-transcribed with downstream genes

There are three ORFs, *NGO0232*, *apaH*, and *trkH*, located downstream of *nspA* (Figure 18A). To address the possibility that the incomplete recovery of wild-type phenotype in the complement strain is due to a polar mutation, we utilized endpoint RT-PCR to assess the co-transcription of these genes. Using primers designed to amplify regions from the end of one gene to the beginning of the succeeding one (Figure 18A), we determined that *nspA* is co-transcribed with these ORFs (Figure 19B). However, MCV406 retained the ability to express these genes (Figure

18C) indicating the presence of a downstream promoter(s) regulating expression of the other linked genes. We concluded that the *nspA* mutation was non-polar.

Figure 17. Effect of *nspA* mutation on invasion. 4 hour infection of ME180 at an approximate MOI of 10 with indicated strains. (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Each condition was performed in duplicate and results represent the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis. * indicates P values < 0.05. n.s. = not significant.

A.



B.

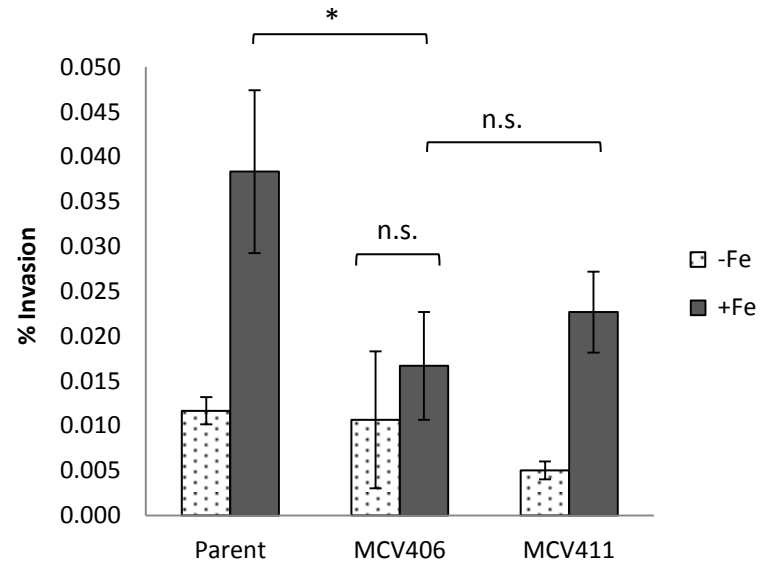
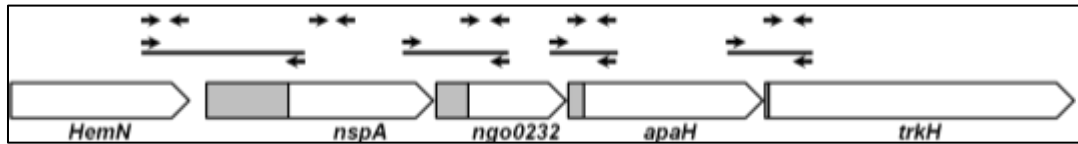


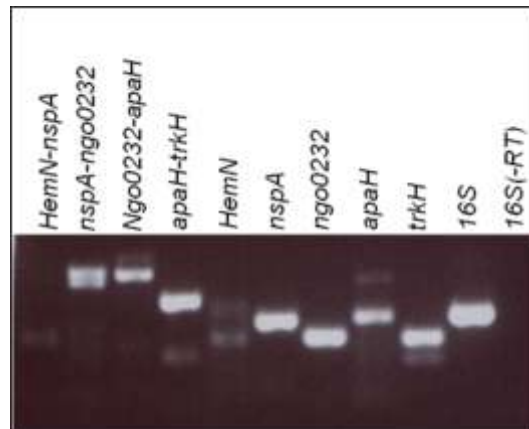
Figure 17. Effect of *nspA* mutation on invasion

Figure 18. Co-expression of *nspA* with downstream genes. (A) Schematic representation of the *nspA* genetic locus. Intergenic regions are denoted by grey bars. Small arrows indicate primer locations for co-transcription analyses. Black lines indicate amplicons generated from each primer set. (B) Co-transcription analysis of the *nspA* operon in FA1090. RNA was isolated under iron replete conditions. The *16S*rRNA gene was used as a positive control (*16S*) and expression of the same gene in the absence of reverse transcriptase [*16S*(-RT)] was used as a negative control. (C) RT-PCR analysis of downstream gene expression in parent and *nspA* mutant.

A.



B.



C.

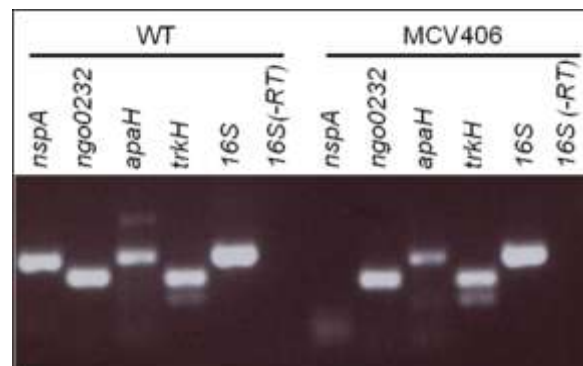


Figure 18. Co-expression of *nspA* with downstream genes

v. MCV406 and MCV411 express a different *pilE* sequence from the parent

Seifert et al. has shown that pilin variation occurs throughout the process of infection (150). The partial Opa deletion strain, FA1090 Δ OpaKEB, was created in an FA1090 background expressing the 1-81-S2 pilin sequence with a point mutation in the G-quadruplex region upstream of *pilE* (27) that was reported to abrogate pilin antigenic variation (FA1090 1-81-S2 P⁺_{nv}) to ensure all bacteria express the same pilin type (10). To determine if pilin variation was potentially contributing to the invasion defect seen in MCV406, we sequenced and compared the *pilE* region of FA1090 Δ OpaKEB, MCV406, and MCV411 (Figure 19). The mutant and complement strains were found to express a different *pilE* sequence from the parent. These nucleotide alterations resulted in missense changes in the codon sequence on the expected protein (Figure 20).

vi. Variants of the *nspA* mutant expressing the same *pilE* sequence as the parent do not show a significant defect in iron induced invasion.

To determine if the invasion defect seen in MCV406 could be partly due to *pilE* variation and also determine if NspA is a contributor to iron induced invasion, we isolated other NspA variants expressing the same pilin variant as the parent and tested these for defects in invasion (Figure 21). These variants showed no significant decrease in invasion compared to parent suggesting that the defect was likely due to *pilE* antigenic variation.

Figure 19. Alignment of *pilE* expression locus. The *pilE* expression locus was amplified and sequenced in FA1090 Δ *opaKEB*, MCV406 and MCV411. A. snapshot of the mismatched region is shown. Identical residues are shaded in yellow, residues found in only some of the strains are shaded in blue.

Parent	GGTTGAAGCCATTTGGCGGTAAACGACGCCCTTTGCGACTTCAAGTTTCTGAACATATTTGCCTTTGATTGTTGAAGCGGATGCCACGCCGGCAGAAGTGTGTCTTTCGGCCATATGCCGTGATTCAGG
MCV406	GGTTGAAGCCATTTGGCGGTAAACGACGCCGTTTGTGACCGTAACCGCTTTCAACATATTTGCCTTTGATGTCGGTGGGGTTTGCCACGCCGGCAGAAGTGTGTCTTTCGGCCATATGCCGTGATTCAGG
MCV411	GGTTGAAGCCATTTGGCGGTAAACGACGCCGTTTGTGACCGTAACCGCTTTCAACATATTTGCCTTTGATGTCGGTGGGGTTTGCCACGCCGGCAGAAGTGTGTCTTTCGGCCATATGCCGTGATTCAGG

Figure 19. Alignment of *pilE* expression locus

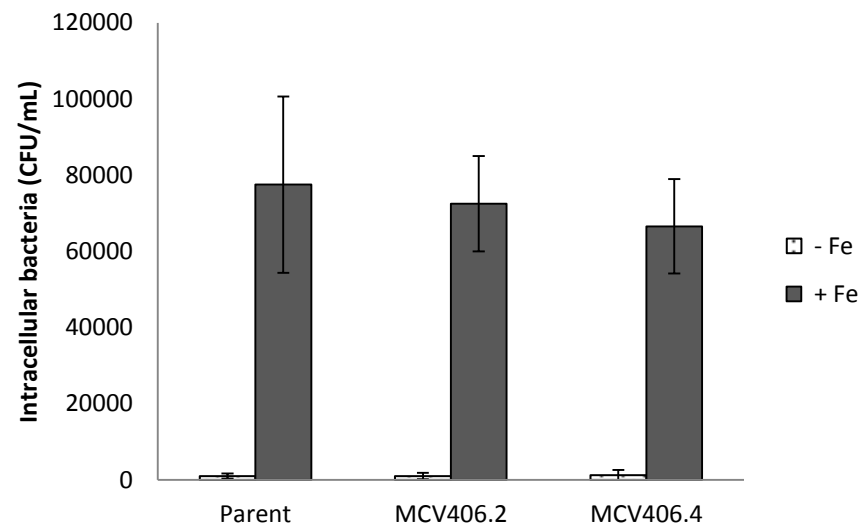
Figure 20. Alignment of deduced *pilE* protein sequence. Comparison of expected protein sequence of strains FA1090 Δ *opaKEB* (Parent), MCV406 and MCV411. Dots indicate residues identical to those shown on the top row for the parent strain. Sequences were deduced from the DNA sequence of *pilE* as determined in this study.

Parent	MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQVSEAILLAEGQKSAVTEYYL
MCV406
MCV411
Parent	NHGIWPKDNTSAGVASASTIKGKYVQKVEVAKGVVTAQMASTCVNKEIQDKKLSLWAKR
MCV406TPTD.....ES.T.TN.....K.....
MCV411TPTD.....ES.T.TN.....K.....
Parent	QDGSVKWFCGQPVTRTGDNDTVADANNAIDTKHLPSSCRDESSAT
MCV406
MCV411

Figure 20. Alignment of deduced pilE protein sequence

Figure 21. Invasion capacity of *NspA* mutants. (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Infection was carried out for 4 hours at an approximate MOI of 10 with indicated strains. Each condition was performed in duplicate and results represent the mean and standard deviation from three independent experiments. An unpaired student's *t*-test was used for statistical analysis.

A.



B.

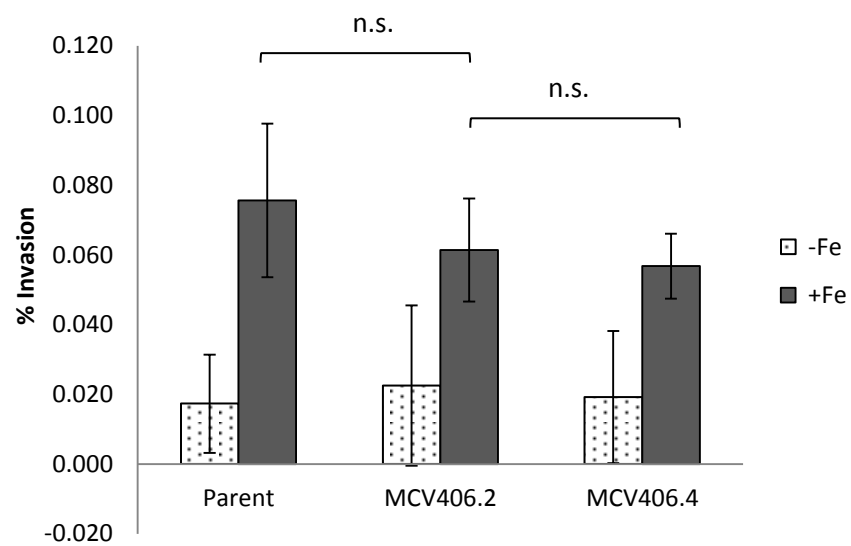


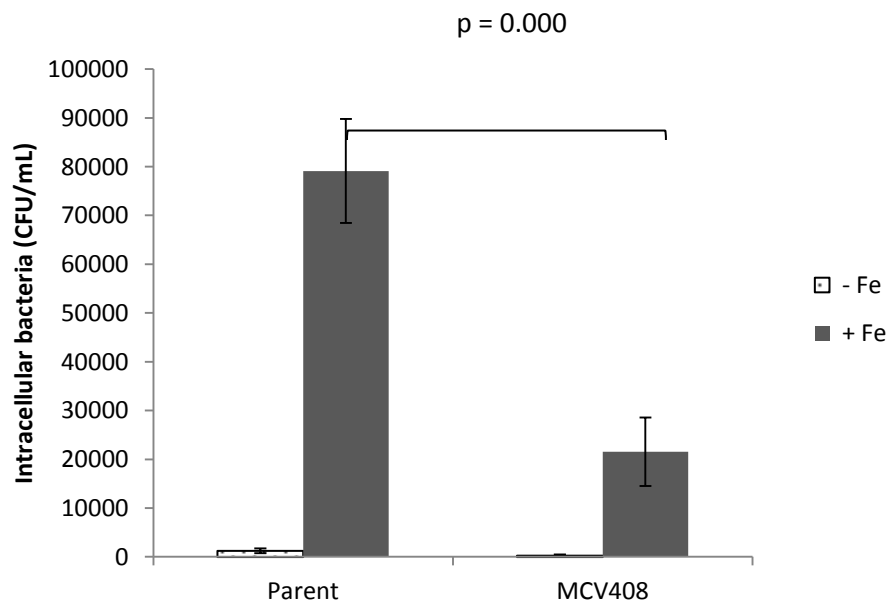
Figure 21. Invasion capacity of *NspA* mutants

C. NspA/TdfJ double mutant is defective for iron induced invasion

To explore the possibility that iron induced invasion occurs as a result of synergistic effects of multiple membrane proteins, we generated a mutant knockout strain that had lost the ability to express both NspA and TdfJ (MCV408) by knocking out *tdfJ* in the MCV406 (*nspA*⁻) background. When this mutant was tested for defects in iron induced invasion, we observed that this strain displayed significantly impaired iron induced invasion that was more severe than either single knock out alone (Figure 22). Upon further characterization of this strain, we determined that it expresses the same *pilE* as its immediate parent (MCV406) which is different from the original parent (FA1090ΔopaKEB).

Figure 22. Effect of *nspA/tdfJ* mutation on invasion. 4 hour infection of ME180 cells with MCV408 (FA1090 $\Delta opaKEB$ *nspA*⁻/*tdfJ*⁻) at an approximate MOI of 10. (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Each condition was performed in duplicate and results represent the mean and standard deviation from three independent experiments. Statistical significance was determined using an unpaired students *t*-test.

A



B

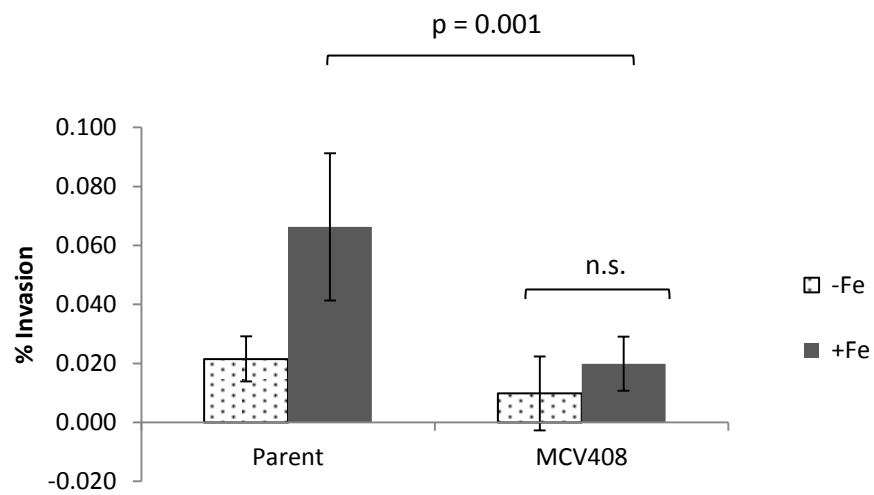


Figure 22. Effect of *nspA/tdfJ* mutation on invasion

D. NGO1063 as a potential iron induced adhesin/invasin

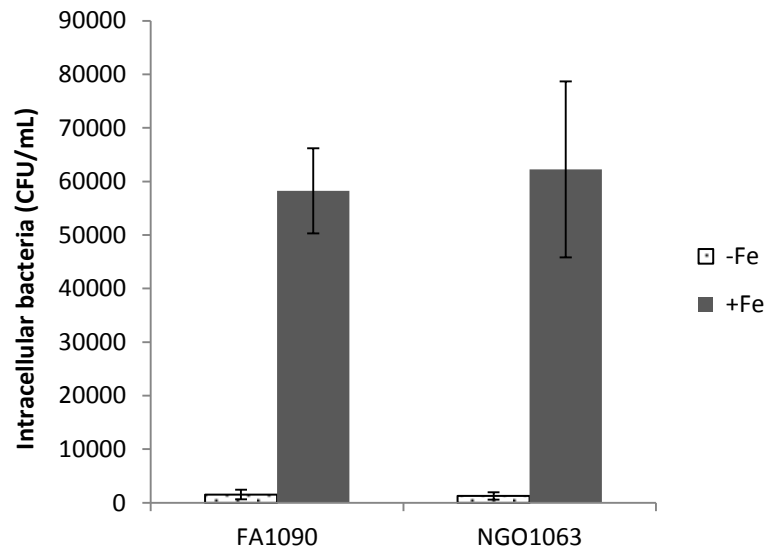
NGO1063 was one of the iron induced proteins identified via 1D SDS-PAGE and 2D DIGE proteomic studies. It is a putative lipoprotein that contains a conserved lysozyme-inhibitor domain found in MliC (membrane bound lysozyme inhibitor) of Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. Although these proteins are expected to localize to the periplasm, bioinformatics analysis of NGO1063 using CELLO 2.5 (195, 196), SOSUI_{GramN} (88), and PredictProtein (142), all predicted extracellular localization. A BLAST search on NCBI indicated that this protein is well conserved among the *Neisseria*. Consequently, we investigated whether this protein contributed to iron induced invasion.

i. NGO1063 mutation did not result in reduced invasion of epithelial cells

To determine if the iron induction of NGO1063 contributes to iron induced invasion, possibly by increasing the survival capacity of gonococci, we generated a mutant by insertional inactivation of the gene with the EZ Tn5 transposon. Mutants were confirmed by PCR. We then analyzed the mutant for iron induced invasion in the invasion assay (Figure 23). There was no difference in amount of intracellular bacteria recovered after 4 hours. A small decrease in invasion efficiencies was detected but the difference did not reach statistical significance ($p=0.09$).

Figure 23. Invasion capacity of *NGO1063* as compared to wildtype FA1090. (A) Intracellular gentamicin resistant bacteria recovered. (B) Invasion efficiencies calculated by dividing intracellular bacteria by total bacterial present in each well at the end of assay. Infection was carried out for 4 hours at an approximate MOI of 10 with indicated strains. Each condition was performed in duplicate and results represent the mean and standard deviation from three independent experiments. An unpaired student's t-test was used for statistical analysis. n.s. = not significant.

A.



B.

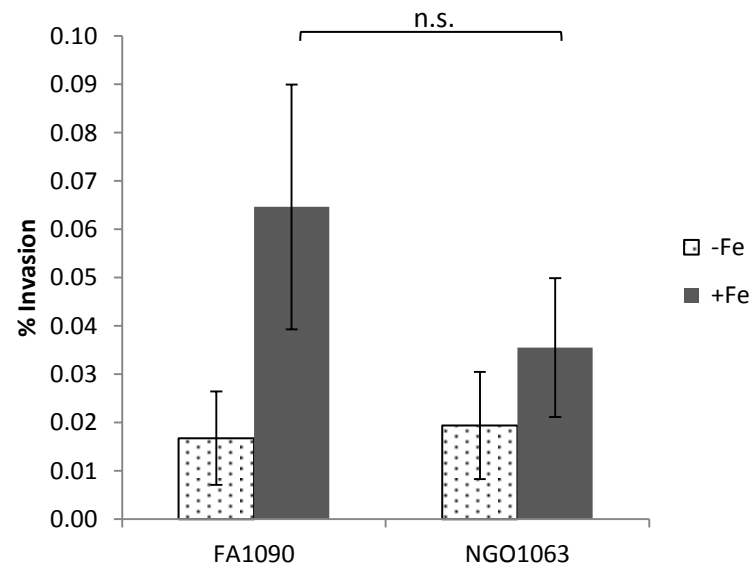


Figure 23. Invasion capacity of *NGO1063* as compared to wildtype FA1090

III. Discussion

The myriad of adhesins on the gonococcal cell surface likely contributes to the great success of this STI pathogen (see (87) for review). As previously discussed, Opa⁻ organisms have been associated with ascending infections. Thus, Opa-independent mechanisms may be clinically relevant in invasive disease. We identified an invasion mechanism that is iron induced, Opa-independent and likely distinct from other invasion mechanisms thus far described in the gonococcus. The aim of the present study was to identify iron induced surface proteins responsible for gonococcal iron induced invasion of human epithelial cells. Specifically, we investigated NspA, TdfJ, and NGO1063.

Gonococcal TdfJ has not been characterized, but the meningococcal homolog, ZnuD, was reported to be involved in adhesion, invasion, and intracellular survival (109). NspA is a conserved and surface exposed integral outer membrane protein (180) with similarity to the well described opacity proteins. We determined that both NspA and TdfJ are iron inducible in the gonococcal outer membrane and therefore, we further investigated their contribution to iron induced invasion.

Initially, TdfJ was thought to be involved in invasion; however, we were unable to complement the defect (Fig. 12) in the mutant (MCV407). While this could have been due to the absence of full native regulatory elements in the complement, subsequent newly-constructed mutants showed no defect in invasion. We have, so far, been unable to determine the exact cause of the defect as Opa protein and *pilE* gene sequence analyses showed no discrepancy between the parent and complemented strains (data not shown). Interestingly, while Kumar et al. described a defect in meningococcal adherence, invasion and intracellular survival in the *znuD* mutant (109), our studies did not detect a defect in intracellular survival (74), nor

adherence/invasion (current study). It is possible that this divergence could be due to experimental variability. More investigation will be required to determine if these proteins have similar functions in these two pathogens.

Any role for NspA as an adhesin has not been reported. Meningococcal NspA was recently discovered to bind factor H and attenuate the alternative complement pathway (113). Interestingly, factor H coated gonococci adhere to complement receptor 3 (CR3) on eukaryotic cells (2). We report here that unlike the Opas, NspA did not promote adhesion of gonococci to ME180 cells (Fig. 16). However, it has been reported that this cell line does not express CR3 (50) thus the adhesive potential of NspA could have been missed. We also cannot eliminate the possibility that multiple proteins are required for the iron induced invasion phenomenon. If this is the case, the adhesive capacity of functionally redundant proteins or stronger adhesins could mask the effect of the NspA mutation.

We initially suspected that NspA played a role in iron induced invasion (Fig. 17). However, although the mutation was made in a background strain with “locked” pilin expression (27), a screen for pilin variation revealed that the mutant and complemented strains expressed different *pilE* sequences from the parent (Figs. 19 and 20) indicating that antigenic variation still occurred in the strain. Further analysis determined that the defect seen was likely not NspA specific (Fig. 21) and probably due to pilin variation. This study underscores the importance of monitoring the many variable surface structures of gonococcus such as pili and Opa proteins when studying epithelial cell interactions. The decreased invasion capacity observed in this strain could indicate a role for pilin antigenic variation in modulating invasion.

A third interesting candidate was NGO1063. The function of this protein in the gonococcus is unknown but it contains a lysozyme inhibitor domain similar to other Gram negative bacteria. Protein localization prediction software predicted an extracellular localization suggesting a potentially different role for NGO1063. We investigated whether a mutant lacking expression of NGO1063 was impaired for invasion by the gonococcus. Although a slight decrease in invasion was seen, it did not reach the level of significance ($p=0.09$) (Fig. 23).

So far, we have been unable to definitively identify an adhesin or invasin that contributes to iron induced invasion but several mechanisms can be envisioned by which iron-induced invasion occurs. This challenge may be due functional redundancy of multiple adhesins/invasins. For instance, *S. gordonii*, *B. bronchiseptica*, and *Salmonella typhimurium* all express multiple membrane proteins that synergistically promote adhesion or invasion of host cells (52, 91, 175). To begin to investigate the possibility of such a scenario in *N. gonorrhoeae* iron induced invasion, a double mutant in which NspA and TdfJ were both insertionally inactivated was analyzed. Interestingly, this strain was severely defective for iron induced invasion (Fig. 22) however further analysis of the strain revealed that it expressed the same *pilE* variant as MCV406 (and different from the parent). Consequently, we are currently unable to indisputably conclude that the defect was due to the double mutation. The simplest mechanism for iron induced invasion may be that an iron induced surface protein directly (or via a bridge host protein as in the case of Opas binding HSPGs (179)) engages a receptor on the host cell which triggers engulfment of the gonococcus. In a more complex scenario, an interaction of multiple proteins could simultaneously engage a host cell receptor (as in the case for *S. gordonii* as described above), or a synergistic effect of more than one gonococcal protein binding different host cell receptors. While, we hypothesized that an iron induced protein is responsible for the iron enhanced invasive phenotype seen, it is possible that iron regulated

modification or export of a non-iron responsive surface protein could be responsible. All of these models are possible and warrant further investigation especially given that clinical manifestations indicate that an iron-influenced, Opa-independent mechanism may be very relevant in the human host.

CHAPTER 6: SUMMARY AND PERSPECTIVES

With the increasing threat of untreatable gonococcal infections in the near future, and the debilitating sequelae associated with untreated infections, the urgent need for alternative treatments and ideally preventive measures cannot be overstated. Because adherence and invasion of host epithelial cells are the critical first steps during gonococcal infection, studying these interactions is anticipated to aid in the development of therapeutics.

The ability to acquire iron is an important virulence factor for many pathogens as it is sequestered by host iron-binding proteins *in vivo*. Thus, for good reason, early studies on the effect of iron on gonococcal pathogenesis were focused on iron repressed proteins as they usually function as iron transport proteins. *N. gonorrhoeae* expresses a repertoire of high-affinity iron uptake systems that enable it to hijack iron from numerous host iron sources. Consequently, gonococci are well equipped to obtain this essential nutrient while in the host and likely experience phases of high internal iron pools. *In vitro* studies using human endometrial cells have shown that iron helps gonococcal invasion of these cells (34, 83). In addition, it has been reported that women with pelvic inflammatory disease (PID) due to *N. gonorrhoeae* are more likely to present symptoms during or just after the onset of menses (86) potentially indicating a more invasive disease pathology in the presence of iron. However, the contribution of iron to the invasion process has been grossly under-appreciated. We hypothesized that high iron conditions trigger increased surface expression of certain membrane proteins that mediate a more invasive phenotype.

In the hope of shedding light on clinically relevant gonococcal factors that would help in understanding gonococcal pathogenesis, we identified the gonococcal iron induced outer membrane proteome using SDS-PAGE, 2D-DIGE, and iTRAQ analyses. These results provide a bank of membrane proteins that could be potential drug or vaccine targets used in the development of therapeutics against gonococcal infections. Comparison of our proteomics data with published microarray data revealed that transcript expression levels do not universally equate to outer membrane protein expression levels. Iron induction of the membrane assembly protein, Omp85, could result in iron induced protein export and contribute to iron-dependent localization of proteins into the outer membrane. This could explain why there is an iron induction of outer membrane proteins in the absence of increased transcript levels. A mechanism like this will provide the bacterium a clever and rapid way to respond to environmental signal such as iron concentrations.

Furthermore, our 2D-DIGE analyses revealed the presence of multiple species of distinct proteins with varying mass and pI. These results suggest widespread post-translational modifications of gonococcal membrane proteins. PTMs could potentially impact protein structure/function and result in a diversity of biological functions for these proteins. Considering outer membrane proteins are an interface between host and bacteria, these modifications could potentially have a large impact on gonococcal - host cell interactions and immune evasion strategies. For instance, it has been reported that in *C. jejuni*, protein glycosylation affects host cell interactions (100, 167) and that the adhesin, CadF, is subject to proteolytic processing, which generates truncated forms that retain the ability to bind fibronectin, but are no longer recognized by patient serum (45). Likewise, *N. gonorrhoeae* is capable of modifying pilin with phosphorylcholine and glycan. These modifications of pilin have been demonstrated to be

essential for adherence of *N. meningitidis* to host airway cells (94). Consequently, the apparent iron induced post-translational modification of OM proteins may have substantial implications on gonococcal pathogenesis.

A potentially interesting protein that was determined to be iron induced is the putative phospholipase NGO1492 similar to *Campylobacter coli* and *Helicobacter pylori* outer membrane phospholipase A (OMPLA). Phospholipids are a major component of host cell membranes and thus phospholipases could aid in enzymatic disruption of the host cell membrane during invasion. Phosphatidylcholine-specific phospholipase C has been demonstrated to promote lipid hydrolysis and bacterial uptake in *N. gonorrhoeae* (66). Gonococcal phospholipase A was determined to be an autolysin (21). However, studies investigating its role in disrupting host cell membranes and aiding bacterial uptake have not been reported.

Another goal of our study was to characterize the role of iron in gonococcal-host cell interactions, and to identify potential contributions of iron induced surface proteins to the invasion of epithelial cells. Here, we present evidence of an Opa-independent invasion process in which *N. gonorrhoeae* is more invasive in high iron environments. Iron however, did not increase adherence indicating that the phenotype observed is specific to the invasion process. Interestingly, Opa-negative gonococcal variants were reported to be preferentially recovered from fallopian tubes of women with acute salpingitis (92) suggesting that Opa-independent factors may be involved during invasive gonococcal infection in the upper female genital tract. We propose that this iron induced mechanism observed is clinically relevant in ascending gonococcal infections.

With data from our proteomic analyses, we were able to investigate the potential role of iron induced proteins in Opa-independent iron induced invasion. We tested the Opa-like outer membrane protein, NspA, the TonB-dependent receptor, TdfJ, and the hypothetical protein, NGO1063. Despite the similarity between NspA and the Opacity proteins, NspA did not appear to contribute to iron induced invasion. We also observed that unlike the TdfJ homolog in *N. meningitidis*, ZnuD, which was reported to contribute to adhesion to, invasion of and intracellular survival in host cells (109), TdfJ does not seem to have this same function in *N. gonorrhoeae* host cell interactions even in the absence of iron. The difference may be due to discrepancies in experimental systems; however, it is possible that these homologous proteins have different functions in these similar yet distinct pathogens.

While we hypothesized that iron induced expression of outer membrane proteins is responsible for the observed iron induced invasion phenotype, it is also possible that iron-induced post-translational changes in membrane proteins or structures result in this phenotype. At this time, we do not know if this mechanism converges with other described pathways as the influence of iron on other Opa-independent mechanisms reported in the literature has not been evaluated. An Opa-independent pathway of interest is the LOS-dependent invasion pathway. The LNnt moiety on LOS was reported to facilitate invasion of epithelial cells without influencing adhesion (154) similar to our findings. In contrast to our findings though, the authors noted that the level of invasion observed was significantly less than that reported by others for Opa-dependent invasion. Further investigation will be required to determine whether iron influences this and other Opa-independent pathways.

Overall, we characterized the gonococcal iron induced outer membrane proteome, identified a novel iron induced Opa-independent invasion mechanism, and investigated potential invasins.

While our results suggest no role for NspA, TdfJ, and NGO1063 in iron induced invasion, it is possible that their effects were missed due to multiple proteins being involved in this process. For instance, in *S. gordonii*, multiple proteins bind to the same receptor (fibronectin) and act in concert to promote adherence (91). In this interaction, a certain set of proteins (Hsa and CshA/CshB) are the primary adhesins. SspA and SspB adhesins also form secondary interactions but this interaction is difficult to observe in the presence of Hsa and CshA/CshB. Defects in adherence in mutants of these secondary proteins only becomes visible in the absence of Has and CshA/B (91). To investigate the possibility of such a scenario in *N. gonorrhoeae* iron induced invasion, we analyzed a double mutant in which NspA and TdfJ were both insertionally inactivated. Interestingly, this strain was severely defective for iron induced invasion (Fig. 22) more so than either individual mutant. However, further analysis of the strain revealed that it expressed the same *pilE* variant as MCV406 (and different from the parent). One could argue that since the double mutant and its predecessor (MCV406) express the same PilE variant, they should be comparable to one another. In this comparison, the increased defect observed in the double mutant could be suggestive of a synergistic effect between NspA and TdfJ in iron induced invasion. Further studies are required to come to a definitive conclusion on the effect of these proteins to the phenomenon.

Iron induced invasion may be the result of an iron induced surface protein that engages a receptor on the host cell which triggers engulfment of the gonococcus. The phenomenon could also result from the interaction of multiple proteins that simultaneously engage a host cell receptor (as in the case for *S. gordonii* (91)), or a synergistic effect of more than one gonococcal protein binding different host cell receptors. While, we hypothesized that an iron induced protein is responsible for the iron enhanced invasive phenotype seen, it is possible that an iron regulated modification of a non-iron responsive surface protein could be responsible. All of these models are possible and

warrant further investigation especially given that clinical manifestations indicate that an iron-influenced, Opa-independent mechanism maybe very relevant in the human host.

We discovered a potential role for pilin variation on invasion in the absence of similar effects on adherence. The *pilE* sequence changes between FA1090 Δ *opaKEB* and MCV406 resulted in amino acid changes halfway into the pilin protein. These changes could potentially result in the loss of important epitopes or in distinct post-translational modification of pilin between the two strains. Because adherence was not affected, it is conceivable that the potential loss of this epitope abrogates interaction of the pilus with a nearby gonococcal surface structure whose interaction is expected to engage a host cell receptor. A similar phenomenon occurs in *N. gonorrhoeae* whereby gonococcal pili and porin co-operatively engage complement receptor 3 on primary cervical epithelial cells and mediate adhesion to and invasion of these cells (51). This could be a potentially interesting and novel phenomenon as while antigenic variation of pilin has been shown to regulate adhesin function (131), there have been no reports on the regulation of invasion in this manner.

Our study also underscores the importance of monitoring changes in pilin, Opa, and other variable surface structures when comparing wildtype and mutant strains. For instance, Seifert *et. al.* recently reported the misidentification of a virulence factor due to Opa phase variation between the strains (6). Importantly, the commonly used strain (FA1090 1-81-S2 P⁺_{nv}), which we used in our studies is thought to be locked for *pilE* variation. Yet, we observed changes in *pilE* sequence upon transformation of this strain (see Figures 19 and 20). Finally, the results presented herein reinforce earlier findings on the importance of iron in gonococcal invasion and lays a framework for further investigations on the effects of iron on gonococcal pathogenesis.

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Literature Cited

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VITA

Aminat Taiwo Oki was born in Lagos, Nigeria. She graduated from Queen's College, Yaba, Lagos in 2003. In the spring of 2008, she graduated from Shaw University with Bachelor of Science degrees in Biology and Chemistry *Summa Cum Laude*. Accomplishments and publications are listed below.

Honors and Awards:

Golden Key Honor Society, 2013

Virginia Commonwealth University Chapter

Phi Kappa Phi Honor Society, 2012

Virginia Commonwealth University Chapter

Igor Stojiljkovic Grant, 2012

International Pathogenic Neisseria Conference, Würzburg, Germany

Beta Kappa Chi Scientific Society, 2008

Shaw University Chapter

Suma Cum Laude Honors May 2008

Shaw University

Leadership and Academic Achievement Award, 2008

NC State University-Community Brotherhood Celebration

Outstanding Student Honoree, Chemistry, 2008

Shaw University

Outstanding Student Honoree, Biology, 2007

Shaw University

Presidential Scholarship Award 2004-2008

Shaw University

Honors Program, 2004-2008

Shaw University

Publications:

Michael Harwich, Joao Alves, Gregory Buck, Jerome Strauss, Jennifer Patterson, **Aminat Oki**, Philippe Girerd, and Kimberly Jefferson: Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC Genomics* 2010, **11**:375

Abstracts:

Characterizing the iron induced surface proteome of *Neisseria gonorrhoeae*. **Aminat T. Oki** and C.N. Cornelissen. Meeting of the VA Branch of the American Society for Microbiology. Blacksburg, VA. Nov 2011.

Iron induced invasion in *Neisseria gonorrhoeae*. **Aminat T. Oki**, and C.N. Cornelissen. Meeting of the 18th International Pathogenic Neisseria Conference. Würzburg, Germany. Sep. 2012.

Loss of p19^{ARF} accelerates the rate of proliferation in mouse prostate tumorigenesis. **Aminat T. Oki**, Yurong Song and Terry Van Dyke. National Association of African-American Honors Programs (NAAHP) 16th Annual Conference. University of Maryland Eastern Shore, Princess Anne, Maryland. Nov. 2007.

Exploring blunt telomere structures in *C. elegans*. **A. Taiwo Oki**, Mia Lowden, Shawn Ahmed. University of North Carolina Chapel Hill Research Symposium. Chapel Hill, NC. Jul. 2006.

Turtle population survey in harvested and “non-harvested” ponds of North Carolina. **A. Taiwo Oki**, Veronica Miller, Alvin Braswell. Shaw University Research Symposium. Raleigh, NC. Sep. 2005.