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ANALYSIS OF THE REGULATION OF THE TRANSFERRIN IRON ACQUISITION
SYSTEM IN NEISSERIA GONORRHOEAE

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

by

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List of Abbreviations

°C	degrees Celsius
Δ	delta, deletion
Ω	omega
α	alpha, anti
ABC	ATP binding cassette
AP	alkaline phosphatase
ATP	adenosine triphosphate
β	beta
bp	base pair
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CDM	chelexed defined media
CEACAM	carcinoembryogenic antigen-related cell associated molecule
CFU	colony forming units
CO ₂	carbon dioxide
Crp	cAMP receptor protein
DFO	Desferal
dH ₂ O	distilled water
DGI	disseminated gonococcal infection

DNA	deoxyribonucleic acid
<i>E.</i>	<i>Escherichia</i>
Fbp	ferric binding protein
Fe	iron
Fe(NO ₃) ₃	ferric nitrate
FNR	fumarate and nitrate reduction regulator
Fur	ferric iron uptake regulator
G4	guanine quadruplex
GCB	gonococcal growth media
GCU	gonococcal uptake sequence
H ₂ O ₂	hydrogen peroxide
Hb	hemoglobin
HIV	human immunodeficiency virus
hrs	hours
HS TBS	high salt Tris-buffered saline
IgA	immunoglobulin A
IgG	immunoglobulin G
kDa	kiloDalton
KU	klett unit
L	liter

LB	Luria Bertani <i>E. coli</i> growth media
Lbp	lactoferrin binding protein
Lf	lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LS TBS	low salt Tris-buffered saline
M	molar
mAMP	milliampere
min	minutes
mg	milligram
MgCl ₂	magnesium chloride
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MU	Miller units
<i>N.</i>	<i>Neisseria</i>
NaCl	sodium chloride
Na ₂ CO ₃	sodium carbonate
Na ₂ HCO ₃	sodium bicarbonate
NaNO ₂	sodium nitrite

NBT/BCIP	nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate
NMM	N-methyl mesoporphyrin IX
nM	nanometer
O ₂ ⁻	superoxide anion
OD	optical density
Opa	opacity protein
ONPG	2-nitrophenyl β-D-galactopyranoside
PBP	periplasmic binding protein
PID	pelvic inflammatory disease
PMNs	polymorphonuclear leukocytes
PCR	polymerase chain reaction
RBS	ribosomal binding site
ROS	reactive oxygen species
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOE	splicing by overlap extension
Tbp	transferrin binding protein
Tf	transferrin
Tween 20	polyoxyethylene sorbitan monolaurate

μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
w/v	weight per volume
WT	wild-type
X	times
Yops	<i>Yersinia</i> outer proteins

Abstract

ANALYSIS OF THE REGULATION OF THE TRANSFERRIN IRON ACQUISITION SYSTEM IN NEISSERIA GONORRHOEAE

By Rosuany N. Vélez Acevedo

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

Virginia Commonwealth University, 2009

Major Director: Cynthia Nau Cornelissen, Ph.D.
Professor of Microbiology and Immunology

The neisserial transferrin binding proteins (Tbps) comprise a bipartite system for iron acquisition from human transferrin. TbpA is a TonB-dependent outer membrane protein that forms the pore for iron internalization. TbpB is a surface-exposed lipoprotein that makes the iron-uptake process more efficient. Previous studies have shown that the genes encoding these proteins are arranged in a bicistronic operon, with the *tbpB* gene located upstream of *tbpA*. The operon is under the control of the ferric uptake regulator (Fur) protein. However, promoter elements necessary for the regulation of the operon have not been experimentally defined. In this study, putative regulatory motifs were confirmed

by mutagenesis. Further examination of the sequence upstream of these promoter/operator motifs led to the identification of two direct repeats. We hypothesized that these repeats may be involved in further regulation of the operon. Insertional mutagenesis of the repeats resulted in altered transcript and protein levels. These results confirmed that the region upstream of the operon serves as an extended regulatory region.

A comprehensive investigation of the expression of the operon in response to different environmental stimuli that gonococci might encounter upon infection was also conducted. Changes in osmolarity, carbon source, cAMP availability, and H₂O₂ stress did not alter expression of the operon at the transcript or protein levels. However, low oxygen levels resulted in decreased *tbpBA* transcript and protein. These results are biologically relevant, and provide new insights into the use of the transferrin binding proteins as vaccine candidates.

Lastly, the role of G4 DNA sequences identified in the vicinity of the *tbpBA* operon was investigated. We hypothesized that G4 DNA structures could be involved in the regulation of the operon. Results presented here indicate that interference with these sequences appears to have no effect on expression of the operon. However, identification of potential G4-forming sequences in the non-coding regions upstream and downstream of the operon suggests their importance, perhaps in mediating recombination which could lead to increased antigenic diversity.

CHAPTER 1: Introduction

I. The family *Neisseriaceae*

The family *Neisseriaceae* is comprised of four genera: *Neisseria*, *Kingella*, *Acinetobacter* and *Moraxella* (26, 136, 251). A Gram-negative classification is shared by all four genera, and differentiation from each other is based on characteristics such as cell morphology, production of acid from glucose, ability to reduce nitrite, presence of oxidase, catalase, thymidine kinase, and carbonic anhydrase, among others (Table 1) (26). The four genera encompass microorganisms that are of importance to humans as pathogens and as commensal species.

II. The Genus *Neisseria*

The genus *Neisseria* is named after Albert Neisser, who discovered *N. gonorrhoeae* in 1879 (251). Identification of closely related species soon followed with the isolation of *N. meningitidis* by Weischelbaum in 1887, *N. sicca*, *N. subflava*, and *N. mucosa* by von Lignelsheim in 1906, and *N. flavescens* by Branhman in 1930 (251). *Neisseria* species range from 0.6 to 1.5 μm in size. They are currently classified as aerobic or facultative aerobic microorganisms, and experience optimal growth between 35 and 37°C, in the presence of 5% CO₂. Species of this genus include both human pathogens and commensals, as outlined in Table 2, and differentiation amongst species is based on biochemical testing. The natural habitat of the

Table 1. Characteristics of Genera Assigned to the Family *Neisseriaceae*

	Genus			
Characteristic	<i>Neisseria</i>	<i>Kingella</i>	<i>Moraxella</i>	<i>Acinetobacter</i>
Cell Morphology	Diplococci *	Cocobacilli	Rods	Rods
Oxidase	+	+	+	-
Catalase	+	-	+	-
Acid from Glucose	+	+	-	+/-
Nitrite Reduction	+	+	-	-

* *N. elongate* is rod-shaped

Table adapted from Centers for Disease Control and Prevention (38)

commensals, as outlined in Table 2, and differentiation amongst species is based on biochemical testing. The natural habitat of the *Neisseria* species is the mucous membranes of the urogenital and respiratory tracts.

The genus contains two important human pathogens: *N. gonorrhoeae* and *N. meningitidis*. *N. gonorrhoeae* is the causative agent of the sexually transmitted infection gonorrhea, and *N. meningitidis* causes acute bacterial meningitis and severe sepsis. While these diseases are quite different, genetically, the pathogens are very closely related (243), and express some of the same virulence factors during infection.

III. Neisserial Diseases

A. Meningococcal Disease – Infection and Epidemiology

The first documented *Neisseria meningitidis* outbreak occurred in Switzerland in 1805 (29, 188). Various epidemics have been described since, implicating the meningococcus as the etiological agent. *N. meningitidis* infection is low in prevalence but causes high mortality; an estimated 500,000 cases of meningococcal disease are reported world-wide, with approximately 10% resulting in death (188).

Virulent meningococci are characterized into serogroups, based on their capsular polysaccharide structure. Although there are over 13 serogroups, only groups A, B, C and W-135 commonly cause invasive infection (101, 233), and along with groups X and Y, cause more than 90% of disease world-wide (29). Serogroup A is mostly associated with large epidemics along the “meningitis belt” in sub-Saharan Africa (29, 37, 233), where the rate of infection can be as high as 1000 per 100,000 population, resulting in approximately 75% mortality in children 15 years or younger (91, 101). Serogroups B, C, and Y cause the majority of the disease in the

Table 2. Characteristics of Human-Associated *Neisseria* Species

Species	Association w/ Humans	Acid From					Nitrate Reduction	Polysaccharide From Glucose
		Glucose	Maltose	Sucrose	Fructose	Lactose		
<i>N. gonorrhoeae</i>	Pathogen	+	-	-	-	-	-	-
<i>N. meningitidis</i>	(1)	+	+	-	-	-	-	-
<i>N. lactamica</i>	Commensal	+	+	-	-	+	-	-
<i>N. cinerea</i>	Commensal	-	-	-	-	-	-	-
<i>N. polysaccharea</i>	Commensal	+	+	-	-	-	-	+
<i>N. subflava</i>	Commensal	+	+	-	-	-	-	-
<i>N. sicca</i>	Commensal	+	+	+	+	-	-	+
<i>N. mucosa</i>	Commensal	+	+	+	+	-	+	+
<i>N. flavescens</i>	(2)	-	-	-	-	-	-	+
<i>N. elongata</i>	Commensal	-	-	-	-	-	-	-

(1) Occurs in carrier state. Some strains cause epidemics/pandemics.

(2) Isolated from outbreak of meningitis. Apart from the original description, there are no reliable isolations of this species; strains isolated as *N. flavescens* were probably *N. cinerea* or *N. polysaccharea*.

Table adapted from Centers for Disease Control and Prevention (38)

United States, with group B being the causative agent in the most cases involving small children (29, 79).

The upper respiratory tract is the main reservoir for the meningococci, and transmission commonly occurs through contact with large upper respiratory tract droplets or saliva (29, 37). Once in the respiratory tract, the interaction of the microorganism with the host can be transient, resulting in colonization and the carrier state or can lead to invasive disease (29, 232, 233). Symptoms can develop almost immediately, or within 3-7 days of exposure (29, 41), and can include headache, fever, stiff neck, nausea, photophobia, and altered mental status (39). In approximately 50% of patients with invasive disease, acute purulent meningitis develops (207). Meningococcal septicemia can also develop, and occurs in 5-20% of patients with invasive disease (29, 207). This disease is highly fatal, with mortality resulting from 20 to 80% of the cases. Disease manifestations include fever and a petechial or purpuric rash (29, 207).

Meningococcal infection can have long-term sequelae such as hearing loss, loss of limbs, or neurological disability (37, 207). Rapid diagnosis is essential and is usually done by culture of the microorganism from a sterile body fluid, such as the blood or the cerebrospinal fluid (37). Recommended treatments include the use of penicillin or ceftriaxone (29, 37, 233). Recently, however, resistance to penicillin has been documented (233). For prevention, two vaccines against *N. meningitidis* are available in the United States: MPSV4 (meningococcal polysaccharide vaccine) and MCV4 (meningococcal conjugate vaccine) (37). MPSV4 is a quadrivalent polysaccharide vaccine against serogroups A, C, Y, and W-135 which confers no long-term memory response (29). MCV4 consists of cell surface polysaccharides from serogroups A, C, Y, and W-135 linked to the diphtheria toxoid (29). Despite the available

methods for treatment and prevention, meningococcal infection continues to be a major world-wide health concern.

B. Gonococcal Disease – Infection and Epidemiology

Despite the rather recent identification and isolation of *N. gonorrhoeae*, references alluding to gonococcal infection can be found in biblical (229) and ancient Chinese texts (99), making gonorrhea one of the oldest diseases known to affect man (72). Currently, gonorrhea is the second-most commonly reported bacterial infection in the United States, preceded only by chlamydia (39, 40). In 2007 there were 355,991 cases of gonorrhea reported to the CDC, representing an overall rate of 118.9 cases per 100,000 population. In actuality, this number is thought to be at least twice as high, due to low-diagnosing and under-reporting. In the United States, approximately 75% of cases are reported in people in the 15-29 age group, and the highest rates of infection are found in 15-19 year old women and 20-24 year old men. At the beginning of the millennium, reports estimated that there were over 60 million cases of gonorrhea world-wide (83). This is of extreme importance, not only in terms of gonococcal disease, but also in the fact that gonococcal infections are commonly accompanied by chlamydial infection (41), and in the fact that it has been shown that infection with *N. gonorrhoeae* facilitates HIV transmission (39, 49, 142).

N. gonorrhoeae is primarily transmitted through close sexual contact with infected individuals, although transmission can also occur from an infected mother to her newborn during delivery. The primary sites of infection by *N. gonorrhoeae* are the urethral epithelium in males (6, 48) and the ecto- and endocervical epithelia in females (73), although infections of the rectal mucosa, the pharynx and the conjunctiva are also reported (72). Signs and symptoms usually appear 2-5 days after infection, but can take up to 30 days. In men, the disease can manifest as

urethritis, and upon ascension of the gonococci to the upper genital tract, can result in more complicated conditions such as prostatitis, epididymitis, or orchitis. In women, infection is usually asymptomatic, making it difficult to diagnose and treat. Up to 45% of infected women experience ascending gonococcal infection (21, 111, 134, 229), and if left untreated this can lead to more serious conditions such as pelvic inflammatory disease (PID), which can result in fallopian tube scarring and blockage, ectopic pregnancies, and infertility. It is estimated that 1 in 10 women suffers from PID (7, 72), and that 40% of the cases are caused by *N. gonorrhoeae* (240). Over 5 billion dollars are annually spent in the United States for the treatment of the PID (174).

Disseminated gonococcal infection (DGI) is less common, and results from the dissemination of the gonococci into the bloodstream. In women, menstruation is a major risk factor for acquisition, since approximately 50% of affected women show signs and symptoms of DGI within 7 days after the onset of menses (201). DGI can cause arthritis-dermatitis syndrome, endocarditis and in rare cases, meningitis (72, 201).

Gonorrhea is treatable, and antibiotic treatments for the disease have included fluoroquinolones, penicillins, and tetracyclines. However, increased resistance to these antibiotics has added a challenge to treatment. Plasmid-mediated resistance to tetracycline was first reported in 1986 (135), and by 2007, the CDC reported that over 22% of all isolates collected by the Gonococcal Isolate Surveillance Project (GISP) were resistant to tetracycline, penicillin, or both (39). Additionally, increased resistance to fluoroquinolones was also reported, causing the CDC to recommend only the use of 3rd generation cephalosporins as treatment for the disease (42). Having displayed plasmid-acquired and chromosomally-mediated antibiotic resistance mechanisms, it is possible that the gonococci could display resistance to the 3rd

generation cephalosporins in the near future. The need for more efficient or ideally preventative methods of treatment is evident.

IV. Gonococcal Virulence Factors

The host environment is not hospitable to the gonococci, and thus they must express proteins on their surface that aid in colonization, survival, and evasion of the immune response. Many of these surface proteins are subject to high frequency antigenic and/or phase variation, and are thus capable of providing several beneficial functions for the gonococci. The role of these surface proteins, or virulence factors, is essential in gonococcal pathogenesis.

A. Porin

Porins are the most abundant proteins in the gonococcal outer membrane (158). They form a trimeric, water-filled channel through which small molecules can traverse the outer membrane (72), although it is thought that they may also play a role in potentiating disease. *N. meningitidis* expresses two types of porins, PorA and PorB (90). In contrast, *N. gonorrhoeae* expresses only a PorB homolog, which is classified into two different serotypes termed PIA and PIB (90). Porin may be involved in the actin-mediated entry of the bacteria into epithelial cells, as it has been shown to act as an actin-nucleating protein in these cells (264). Porin may also play a role in immune evasion, particularly in the impairment of phagocyte function. It has been shown that gonococcal porin is able to translocate into eukaryotic cell membranes (19, 72, 151, 260) where it can form a voltage-gated channel that is modulated by ATP and GTP in the host cell (209), which can prove detrimental for host cells. Moreover, porin can also inhibit phagosome maturation (168), and it can down regulate IgG, Fc γ receptors, and complement receptors 1 and 3 on the surface of PMNs (17). Porin has also been implicated in conferring

serum resistance, as porin expressing gonococci are capable of binding Factor H, and thus down-regulating the alternative pathway of the complement cascade (194). Additionally, in studies with HeLa carcinoma cells, selective transport of porin to the mitochondria (169) resulted in initiation of apoptosis by induction of calcium influx, calpain and caspase activity (72, 170), implicating porin in yet another immune evasion mechanism.

B. Lipooligosaccharide

Many gram-negative bacteria have lipopolysaccharide (LPS) on their outer membranes. The *Neisseria* however, possess a shorter version of this molecule, that lacks the repetitive O-antigen sugar that makes up the polysaccharide chain of LPS, and is thus named lipooligosaccharide (LOS) (156, 250). LOS is subject to high frequency antigenic variation. Glycosyl transferases are involved in the addition of sugar groups to the α -chain during LOS synthesis. Poly G tracts within the genes encoding the glycosyl transferases enable slip-strand mispairing, making them subject to phase variation, and resulting in the turning “on/off” of these genes (32, 61, 121). Presence or absence of the glycosyl transferases leads to changes in LOS structure and size, which is also limited by the availability of substrates for the enzymes (72).

External modification of LOS by sialic acid deposition is also known to occur in vivo and is thought to confer a protective effect to the bacteria. Sialylation is mediated by a membrane-bound sialyltransferase with host-derived 5'-mono-phospho-N-acetylneuraminic acid (CMP-NANA) as the sialyl donor (156, 157). LOS sialylation can be beneficial for the gonococcus by contributing to serum resistance, as it can bind factor H, thus inhibiting complement mediated killing via the alternative pathway (195). However, LOS sialylation can also impair entry into

some host cells, suggesting that LOS sialylation could provide a mechanism that allows the gonococci to convert between an invasive and a serum-resistant phenotype (249).

C. Type IV Pilus

Neisserial pili are long ($< 6 \mu\text{m}$) filamentous structures that protrude from the gonococcal surface (90). The pilus is composed of two main protein subunits, PilE and PilC. PilE is the major structural subunit protein (also known as pilin), and through interaction with other PilE molecules forms the pilus fiber. PilC is an adhesin found at the tip of the pilus.

The pilus is subject to antigenic and phase variation. Pilin antigenic variation is the result of high frequency gene conversion between one of many silent *pilS* loci and *pilE*, the single expressed pilin locus, via a RecA-dependent pathway (97, 163, 164, 221). Phase variation occurs during DNA replication, is RecA-independent, and is the result of frame-shifting that can occur within a poly C tract located at the beginning of the *pilE* gene (139). It has been shown that this event occurs at a frequency of 10^{-4} per cell per generation (139, 273). Additionally, pilin can also be modified post-translationally by glycosylation (254), phosphorylcholine (107, 262), and phosphoethalonamine (107), which may aid in blocking pilus-specific antibodies.

Pilus plays a critical role in the initial adhesion of the gonococci to host cells by aiding in overcoming electrostatic repulsion between host cells and the surface of the gonococci (106). In previous studies, piliated gonococci were able to initiate infection in a human male challenge model, while non-piliated gonococci were not (127, 128). Cell tropism is also a contribution of the pilus, as it is able to bind human epithelial and endothelial cells, macrophages, granulocytes and erythrocytes (138, 210, 215). Additionally, the importance of pili has been demonstrated in twitching motility, perhaps providing a mechanism by which the non-motile gonococci are capable of ascending to the upper genital tract and colonize mucosal surfaces there (256).

Thus far, CD46 (complement regulatory protein) is the only putative receptor identified for the gonococcal pilus (125), although this finding is controversial. It has been suggested that the tip adhesin, PilC, does not bind or recognize CD46 (132), and that CD46-independent pilus binding to human epithelial cells is possible (131).

D. Opacity (Opa) proteins

Opacity proteins were originally named and identified by the color change and opacity they conferred to gonococcal colonies (72). They are integral outer membrane proteins, found in greater copy numbers in the pathogenic *Neisseria* species, and their predicted protein structure is comprised of eight membrane spanning domains, with four surface-exposed loops (155). Their importance is illustrated by the fact that isolates recovered from patients with diagnosed gonococcal infection express at least one Opa and that Opa expressing gonococci are recovered from volunteers infected with Opa⁻ phase variants (122, 238).

Gonococci possess as many as 11 unlinked Opa alleles, of which up to 4 can be expressed at any given time (16, 18) as a result of phase variability. This process is RecA-independent and results from the alteration of the number of CTCTT coding repeats in the signal-sequence encoding region of the genes. During replication slipped-strand mispairing can occur, by the addition or deletion of CTCTT repeats by DNA polymerase. The number of repeats present can lead to translational frame-shifting, altering the number of Opas that are ultimately expressed (172).

Although initial contact with host cells is mediated by pilus, a tight secondary association with epithelial tissues is mediated by the Opa proteins (89). Opa binding to two host cell surface molecules: heparin-sulfate proteoglycans (HSPG) and carcinoembryonic antigen-like molecules (CEACAMs) has been shown (63, 72, 253). Opa binding of CEACAMs on professional

phagocytic cells leads to activation of Src tyrosine kinases in the host cell (227) followed by activation of Rac which results in cytoskeletal rearrangements and internalization of the gonococci (104). Additionally, studies have shown induction of host cell death can be a result of the Opa-CEACAM interaction between gonococci and neutrophils (44).

E. IgA Protease

IgA proteases are common in both gram-negative and gram-positive human pathogens (247). This protease is capable of cleaving human IgA1, but not IgA2 (186), and since IgA1 is thought to be the primary antibody found in mucosal surfaces of the body, secretion of the protease may aid the gonococci by impairing local mucosal immune function. The protease can cleave IgA1 into F_(ab) and F_c fragments. The F_(ab) fragments can still bind to gonococcal surface antigens, and it is thought that they are capable of blocking immunodominant epitopes that could be targets for IgG-mediated complement activation (90).

Despite its very specific nomenclature, recent studies have shown that IgA protease may also be involved in more than IgA cleavage. For example, the protease has been shown capable of cleaving human LAMP1, a major integral membrane glycoprotein of lysosomes (10, 103, 149), aiding the intracellular survival of the gonococci. Additionally, the role of the protease in gonococcal transepithelial trafficking (112), and in inhibition of TNF- α mediated apoptosis (13) has also been reported.

F. Reduction Modifiable Protein (Rmp)

The reduction modifiable protein is an outer membrane protein identified in all gonococcal strains tested to date, and differs from other outer membrane proteins by exhibiting a high degree of inter- and intra-strain conservation with respect to molecular weight, structure and immunology (152). The protein was named for the shift in molecular weight that was observed

after reduction and SDS-PAGE analysis (86), and it shares partial homology with the OmpA protein of *E. coli* (86). Rmp is found on the outer membrane complexed with porin and LOS (109, 159). Studies have shown that antibodies against Rmp, generated in response to enterics or by infection with *Neisseria*, are capable of blocking deposition of antibodies against porin and LOS, due to steric hinderance (20). Thus, expression of Rmp may aid in disguising other important surface immunogens, and may aid in increasing host susceptibility to gonococcal infection.

V. Iron Availability and Utilization in the Human Host

Pathogenic bacteria must obtain nutrients from their human hosts in order to survive. While sources of organic carbon, nitrogen, phosphate, sulfate, potassium, magnesium, manganese, zinc and other nutrients are freely available in bodily fluids and tissues, iron is not (196). In aerobic environments, iron is primarily found in its oxidized ferric form, FeIII, with a solubility of 1.4×10^{-9} M at neutral pH (196), and tends to aggregate into insoluble oxy-hydroxide polymers (257). When reduced, FeII can activate the Fenton reaction ($\text{FeII} + \text{H}_2\text{O}_2 \rightarrow \text{FeIII} + \text{OH}^- + \text{OH}$) leading to the production of hydroxyl radicals that can have detrimental effects on most macromolecules (257). To avoid such effects, iron in the human host sequestered by carrier proteins.

In humans, after being assimilated from dietary components by the mucosal cells of the jejunum, iron passes to the blood stream for transport, attached to the glycoprotein transferrin (Tf) (196). There are two major classes of transferrins of importance to humans: serum transferrin and lactoferrin, the latter of which is found in lymph and mucosal secretions (196,

257). Both of these molecules have an approximate molecular weight of 80 kDa, with two homologous lobes that can bind iron (196, 257).

Iron can also be bound by the protoporphyrin ring in hemoproteins. Heme, hemoglobin, haptoglobin-hemoglobin and hemopexin are part of this group of iron-binding molecules and proteins. Heme is a prosthetic group that is necessary for the function of many enzymes, but due to its high toxicity is rarely found free (257). Hemoglobin is located in red blood cells, where it functions primarily as an oxygen transporter. This is a tetrameric protein that is capable of binding a single heme molecule within each of its subunits. Haptoglobin-hemoglobin is a serum glycoprotein that results when haptoglobin binds hemoglobin that is released by hemolysis. The binding affinity between these two proteins is so high that the complex does not dissociate, but is degraded after clearance in the liver (257). Hemopexin is capable of binding heme that is released into plasma. The heme-hemopexin complex is transported to the liver where hemopexin is recycled after heme is discharged (257).

Additionally, iron in the host can also be bound by ferritins. These are a broad superfamily of spherical, shell-like iron-storage proteins composed of 24 subunits assembled into a hollow sphere that can be filled with more than 4000 atoms of FeIII (196, 257). These cytoplasmic proteins can store iron in case of iron shortage, but also protect the eukaryotic cells from toxic effects of iron accumulation.

Eukaryotic cells rely on the iron transported and/or stored by these proteins for a wide range of metabolic and signaling functions. For example, iron incorporated into iron-sulfur clusters or in heme, serves as a catalytic center for enzymes involved in processes such as electron transport, peroxide reduction, amino acid, nucleoside, and DNA synthesis (257).

Similarly, pathogenic bacteria also require iron to maintain some of the same cellular processes to enable survival within the human host.

VI. Iron Acquisition Systems in Bacteria

As illustrated above, bacteria encounter iron-limited conditions when in the human host. In order to overcome this difficulty, many bacteria have developed iron-acquisition mechanisms that allow them to take advantage of the various sources of iron that they may encounter. Bacteria have developed two main systems for iron acquisition: siderophore production and host-iron binding protein receptors.

Many but not all bacteria are capable of secreting siderophores. Siderophores are low-molecular weight iron chelators. These chelators are capable of binding iron directly from host proteins based on their high iron-binding affinities (196). Siderophore production has been characterized in gram-negative and gram-positive bacteria, yeasts and fungi, and to date, a total of over 500 different siderophores have been identified (173, 196, 257). Although their structure is similar, siderophores are classified into one of three main types based on their iron ligation groups: hydroxamates (e.g. aerobactin), catechol rings (e.g. enterobactin), and hydroxyacids (e.g. pyochelin) (173, 257).

Siderophores are secreted by bacteria to scavenge iron. Once loaded, ferrisiderophores are recognized by cell surface receptors, and the entire iron-siderophore complex is internalized into the bacterial cell. Internalization of these complexes is an energy-dependent process, the energy for which is provided by the TonB-ExbB-ExD complex (28). Some well characterized siderophore receptors in *E. coli* include FhuA (ferrichrome receptor), IutA (aerobactin receptor), and FepA (enterobactin receptor) (257). Additionally, bacteria are capable of utilizing

siderophores produced by other microorganisms. For example, *E. coli* has been shown capable of using ferrichrome, and coprogen, both of which are fungal siderophores (196).

Bacteria are also capable of obtaining iron from the host by expressing host-iron binding protein receptors on their surface. Receptors for lactoferrin, transferrin, and/or hemoglobin have been identified in bacteria as diverse as *Haemophilus* spp. (259), *V. cholerae* (234) and *P. aeruginosa* (176). These iron acquisition systems are discussed in more detail below, for the pathogenic *Neisseria*.

VII. TonB-Dependent Transport

Iron internalization is an energy-dependent process. TonB-dependent transporters rely on the TonB-ExbB-ExbD complex to provide this energy. The complex is capable of harnessing the energy generated by the proton motive force at the cytoplasmic membrane and transducing it to the outer membrane (133, 189).

Characterized TonB-dependent transporters exhibit similar structures. They are composed of two domains: a C-terminal β -barrel is embedded in the outer membrane and composed of 22 antiparallel- β -strands, and the N-terminal plug domain. The plug domain in the characterized FhuA and FepA TonB-dependent transporters is located within the β -barrel (77) and occludes the pore. Close to their N-termini, TonB-dependent transporters have a conserved sequence motif, named the TonB box, that is implicated in TonB-complex interaction and function (27, 35, 257).

Once iron is internalized, it is bound by a periplasmic binding protein that transports the iron to the cytoplasmic membrane. Iron entry into the cytoplasm is mediated by a complex of one or two cytoplasmic membrane proteins with an associated ATP-binding cassette (77).

TonB-dependent transport has been characterized in Gram negative microorganisms, not only for siderophore- iron utilization (77, 257), but also for use of host-iron binding proteins (53, 147, 219, 224).

VIII. Iron Acquisition Systems in Pathogenic *Neisseria*

Neisseria produce no known siderophores (266), although they are capable of utilizing aerobactin and enterobactin. However, they do express several host-iron binding protein receptors on their surface, enabling them to acquire iron from hemoglobin, lactoferrin and transferrin (146, 165, 217). All three systems are structurally similar, consisting of an outer membrane TonB-dependent transporter and an accessory lipoprotein.

The identified receptor complex for the utilization of hemoglobin and hemoglobin-haptoglobin is HpuAB. In this system HpuB is the TonB-dependent outer membrane transporter, and HpuA is the accessory lipoprotein and both proteins are required for iron internalization (147). Additionally, *N. meningitidis* expresses the HmbR hemoglobin receptor, but *N. gonorrhoeae* does not. HmbR is comprised of a single outer membrane protein that can bind hemoglobin but not hemoglobin-haptoglobin (144). The lactoferrin receptor proteins are named LbpA and LbpB. LbpA is the TonB-dependent transporter and LbpB is the lipoprotein. In this system, LbpA is required for iron internalization, but LbpB is not. The transferrin binding receptor complex is discussed below in more detail.

IX. Gonococcal Transferrin Receptor

The gonococcal transferrin receptor complex is a bipartite system for iron acquisition from this human iron-transport protein. It is composed of two proteins, TbpA and TbpB, that are

preferentially expressed under iron-restricted conditions (56, 218). TbpA is the integral outer membrane protein capable of transporting iron across the outer membrane of gonococci (53), making it essential in the process of iron uptake from transferrin. Mutants that do not express TbpA have been shown incapable of growing on transferrin as a sole iron source, even though they are still capable of binding some transferrin (53, 55). TbpA is highly conserved among pathogenic *Neisseria* (51) and shares sequence similarity with other TonB-dependent outer membrane proteins (53). TonB-dependent receptors are energized by the TonB-ExbB-ExbD complex (124, 190). A TonB box has been identified at the N-terminal domain of TbpA. A gonococcal TonB box mutant was capable of binding transferrin, but not of internalizing transferrin-bound iron (52).

TbpB is the second component of the gonococcal transferrin receptor. It is a lipid-modified surface-exposed protein (4, 145) that is also capable of binding transferrin. However, unlike TbpA, TbpB is capable of discriminating between holo- and apo- transferrin (25, 200). Although TbpB is not essential for iron acquisition (4), it is thought to make the process more efficient (4) due to its ability to recognize holo-transferrin, and its ability to affect rapid association and dissociation of transferrin (67). Gonococcal TbpB mutants exhibit decreased iron-uptake efficiency, but can still grow on transferrin as a sole iron source (4).

The *tbpA* and *tbpB* genes are arranged in a bicistronic operon, with the *tbpB* gene located upstream of *tbpA* (53, 145, 206). The genes are separated by an 86 base-pair region which potentially forms a stem loop in the mRNA. The genes are co-transcribed (206) and the operon is under the control of the Fur protein, resulting in preferential expression of Tbps under iron-limited conditions. However, the genes are differentially expressed. Using a variety of RT-PCR

and fusion techniques, it has been shown that *tbpB*-specific transcripts are approximately two-fold more prevalent than *tbpA*-specific transcripts under iron-stressed conditions (206).

The Tbps are exposed on the outer membrane, and are capable of binding transferrin. Once transferrin is bound, TbpA is charged by the TonB-ExbB-ExbD complex through its TonB box. Iron is internalized, while the transferrin molecule is not. Once in the periplasm, iron is bound by the periplasmic binding protein FbpA, which shuttles the iron across the periplasm to the FbpB-FbpC permease complex in the cytoplasmic membrane. Iron is internalized through the permease in to the cytoplasm in an ATP dependent step (Figure 1).

Although it has been suggested that TbpA and TbpB work together as a complex, it has been found that *tbpB*-specific transcripts are twice as abundant as *tbpA* transcripts (206), making the stoichiometry of the complex unclear. Characterization of the TbpB/TbpA complex in *N. meningitidis* by photon correlation spectroscopy showed that there were approximately two TbpA proteins for every TbpB protein (25). Alternatively, studies with equilibrium-phase transferrin binding assays have shown that the TbpB:TbpA stoichiometry can range from 3:1 to 5:1 in gonococci (55). Differences of these results are likely due to their approaches, in the detection of complexed and uncomplexed TbpB. Still, the stoichiometry of the complex is unresolved.

X. Gonococcal Vaccine Development

Gonococcal vaccine development has proven unsuccessful thus far. Porin has been evaluated as a vaccine candidate with no success (267). Because porin is not subject to phase or antigenic variation, it was considered as a good antigen. However, LOS sialylation and antibodies directed against Rmp are capable of blocking porin antibodies, thus inhibiting their

bactericidal activity (202), and making porin a weak vaccine candidate. Pilus has also been evaluated as a vaccine antigen (22) to no avail, presumably because it is subject to high frequency antigenic variation.

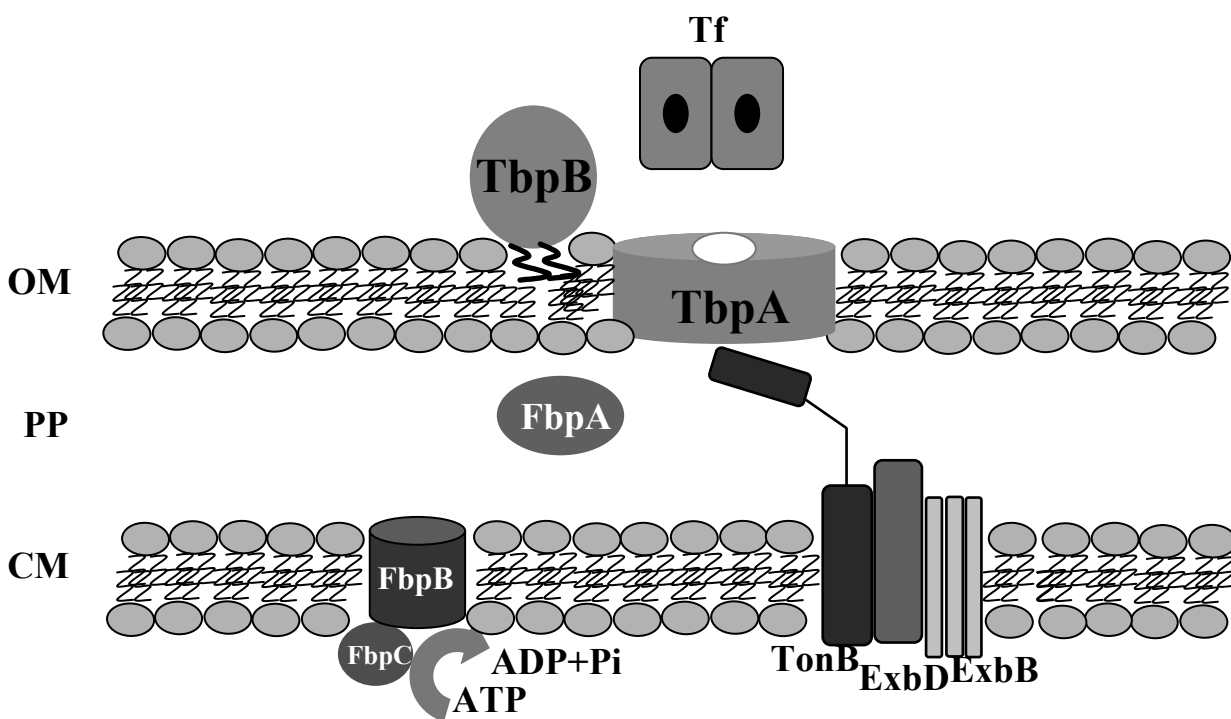
With increased antibiotic resistance, and only one method of treatment currently recommended by the CDC, the need for a gonococcal vaccine is evident. The transferrin iron binding proteins are attractive vaccine candidates because they are surface exposed, are not subject to high frequency phase or antigenic variation, are expressed by all strains tested to date (165), and they have been shown necessary for the establishment of infection in a human male infection model (54). It has recently been shown that recombinant Tbp proteins conjugated to the cholera toxin B subunit are capable of inducing antibody responses in the serum and the genital tract of female mice (191), suggesting that these antigens could be components of an efficacious vaccine.

XI. Research Objectives

The potential of the transferrin binding proteins as vaccine candidates makes understanding their regulation imperative. Previous studies on this receptor complex have focused on deciphering the structure-function relationship of its components. The genes encoding these proteins are encoded in a bicistronic operon, with the *tbpB* gene located upstream of the *tbpA* gene. The operon is under the control of ferric uptake regulator (Fur) resulting in preferential expression of the Tbps under iron-deplete conditions. However, little is still known about the mechanisms that coordinately regulate the expression of these proteins. The overall goal of the studies presented here was to elucidate the mechanisms of coordinate regulation of the transferrin binding proteins in order to determine their efficacy as possible vaccine targets.

First, we sought to characterize the promoter for the operon, extending our focus to possible regulatory sequences identified far upstream of the operon. Secondly, we wanted to examine differences in expression of the operon in response to varied environmental stimuli that the gonococci might encounter upon infection. Lastly, the possible role of non-B DNA forms in the vicinity of the operon in regards to its regulation was studied.

Figure 1: Schematic of Transferrin Iron Uptake in *N. gonorrhoeae*. Human transferrin can complex two atoms of Fe. The transferrin binding proteins, TbpA and TbpB are surface exposed, and each is capable of binding transferrin. TbpA forms the pore for iron internalization, an energy-dependent process. Energy is supplied by the TonB-ExbD-ExbB complex through TonB-TbpA interaction. Once internalized, Fe is complexed by FbpA, which shuttles it across the periplasm, to the FbpB/FbpC permease complex of the inner membrane. In an ATP-dependent step, Fe is internalized into the cytoplasm.



CHAPTER 2: Materials and Methods

I. Bacterial Growth Conditions

Neisseria gonorrhoeae strains were routinely maintained on GC medium (Difco) agar with Kellogg's supplement I (129) and 12 μM $\text{Fe}(\text{NO}_3)_3$ (Supplement II) at 37°C in a 5% CO_2 atmosphere. When required, iron-stress was imposed by overnight growth on GCB medium agar with Kellogg's supplement I and 10 μM Desferal (DFO) (Sigma) plates. All glassware was washed with nitric acid and rinsed extensively with deionized water to remove residual iron. To examine transferrin-iron utilization, agarose plate containing Chelexed Defined Media (CDM) (167, 265) were supplemented with 30% iron-saturated human transferrin (Sigma) as a sole iron source. Single bacterial colonies were streaked onto each plate, and growth was monitored after 24 to 48 hours. Selection for gonococcal *lacZ*-fusion transformants was accomplished by growth on GC agar supplemented with 1 $\mu\text{g}/\text{ml}$ erythromycin. *E. coli* strains carrying plasmids of interest were grown in Luria-Bertani broth (15, 212) supplemented with 100 $\mu\text{g}/\text{ml}$ erythromycin.

II. PCR Amplification of Promoter Regions, Sequencing and Alignment

A. PCR Amplification

Wild-type gonococcal strains MS11 and F62 were propagated on GCB/supplement I/ supplement II (129) agar plates. Single colonies were picked and resuspended in 100 µl of distilled water. Samples were boiled at 100°C for 10 minutes. PCR amplification was conducted using primers oVCU151 and oVCU153 (Table 4) and Platinum *Taq* DNA polymerase (Invitrogen). The size of the PCR amplicon was confirmed by gel electrophoresis visualization.

B. Cloning, Sequencing and Alignment

PCR amplicons were cloned into pCR2.1-TOPO (Invitrogen), following manufacturer's instructions. Sequencing was completed by the Nucleic Acids Research Facility at Virginia Commonwealth University, using universal primer M13. Alignment of sequenced products with the FA1090 genome sequence (GenBank accession # AE 004969) was completed using Vector NTi (Invitrogen) using the default settings.

III. Western Blot Analysis

A. Generation of Iron Stress Conditions for Whole Cell Lysate Preparation

Neisseria gonorrhoeae strains were cultivated on plates as described above. For Fe-stressed cultures, colonies were picked from the GCB/supplement I/ 10 µM DFO plates and used to inoculate GCB broth containing Kellogg's supplement I only. Cultures were grown at 37°C, 5% CO₂ and vigorous (220 rpm) shaking to allow for one mass doubling. After the desired cell density was achieved, the cultures were supplemented with 50 µM DFO. Cultures were allowed to grow for 4 hours in the conditions stated above. Final cell densities were measured after 4 hours of growth, and all samples were standardized to a constant cell number. Samples were

centrifuged for 10 min at 13,000 rpm. Pellets were resuspended in Laemmli solubilizing buffer (141) and stored at -20°C.

B. SDS-PAGE and Protein Transfer

Prior to analysis, 5% β -mercaptoethanol was added to the whole cell lysates. Lysates were then heated for 2 minutes at 100°C, drawn through a 28-gauge syringe, to decrease viscosity, and centrifuged for 30 seconds at 13,000 rpm. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel. Proteins were transferred to nitrocellulose membranes (Schleicher and Schull) in 20mM Tris Base, 150 mM glycine, and 20% methanol in a submerged transfer apparatus (Biorad) at a constant current of 28 mAmps for 18-20 hours. To confirm that similar amounts of protein were loaded in each lane, membranes were stained with Ponceau S solution (0.1% w/v Ponceau S, 5% acetic acid) for 10 minutes prior to probing, and excess stain was rinsed off with distilled water.

C. TbpA Detection

For TbpA detection, membranes were blocked with 5% non-fat dry milk (Biorad) in high-salt Tris buffered saline (TBS) and 0.05% Tween 20 (Sigma). Membranes were probed with primary anti-TbpA polyclonal antibodies (55) at a 1:5000 dilution, washed with high salt TBS and 0.05% Tween 20, followed by incubation with a goat-anti-rabbit alkaline phosphate conjugated (Biorad) secondary antibody at a 1:5000 dilution. Blots were developed using NBT/BCIP (Sigma) in buffer containing 100 mM Tris (pH 9.5), 50 mM MgCl₂, and 100 mM NaCl.

D. TbpB Detection

For TbpB detection, membranes were blocked with 5% non-fat dry milk (Biorad) in low-salt Tris buffered saline. Membranes were probed with primary anti-TbpB polyclonal antibodies

(192) at a 1:10,000 dilution, washed with low salt TBS and 0.05% Tween20, followed by probing with a goat-anti-rabbit alkaline phosphatase conjugated (Biorad) secondary antibody at a 1:5000 dilution. Blots were developed using NBT/BCIP colorimetric developing system as stated above.

IV. Generation of Transcriptional *lacZ* fusions

A. Plasmids

Plasmids pVCU108 and pVCU109 (Table 4) were previously generated in this laboratory by Dr. C. Ronpirin. The *E. coli* strains carrying these plasmids were grown at 37°C, with vigorous shaking, overnight in LB broth with erythromycin (100 µg/ml), resistance to which is encoded by a gene adjacent to the *lacZ* cassette. Plasmids were purified from the overnight cultures with a QIAprep Spin Miniprep kit (Qiagen). Purified plasmids were linearized and used to transform the appropriate *N. gonorrhoeae* strains.

B. Spot Transformation

Linearized plasmid was added to a spot of approximately 10 CFU of pilated MCV113 or MCV114 (Table 3). Cells and DNA were then incubated at 37°C and 5% CO₂ for 24 hours on non-selective media. Subsequently, single colonies were passed onto plates containing erythromycin (1 µg/ml) to select for transformants with the *lacZ-ermC'* cassette. Growth on plates containing streptomycin (100 µg/ml) was used to confirm the retention of the Ω cassette by recipient strains. Resultant strains are listed in Table 3.

TABLE 3. *Neisseria gonorrhoeae* Strains

Strain	Phenotype	Source
FA19	Wild Type (TbpB ⁺ /TbpA ⁺)	(165)
F62	Wild Type (TbpB ⁺ /TbpA ⁺)	
MS11	Wild Type (TbpB ⁺ /TbpA ⁺)	(239)
FA6815	TbpB ⁻ (<i>tbpB</i> :: Ω)/ TbpA ⁻	(4)
MCV108	<i>tbpA-lacZ</i> fusion (TbpB ⁺ /TbpA ⁻)	(206)
MCV109	<i>tbpB-lacZ</i> fusion (TbpB ⁻ /TbpA ⁺)	(206)
MCV113	FA19 <i>tbpB</i> $\nabla \Omega_{-465}$	(205)
MCV114	FA19 <i>tbpB</i> $\nabla \Omega_{-114}$	(205)
MCV117	-10 promoter element replaced by <i>SmaI</i> site	This lab
MCV118	-35 promoter element replaced by <i>BamHI</i> site	This lab
MCV119	Fur box element replaced by <i>SmaI</i> site	This lab
MCV120	<i>tbpA-lacZ</i> fusion in MCV113	This study
MCV121	<i>tbpB-lacZ</i> fusion in MCV113	This study
MCV122	<i>tbpA-lacZ</i> fusion in MCV114	This study
MCV123	<i>tbpB-lacZ</i> fusion in MCV114	This study

TABLE 4. Plasmids and Oligonucleotides

Plasmids		Description	Reference
pCR2.1-TOPO		Amp ^r Kan ^r	Invitrogen
pVCU108		<i>lacZ-ermC'</i> insertion in the <i>Mlu</i> I site of <i>tbpA</i>	(206)
pVCU109		<i>lacZ-ermC'</i> insertion in the <i>Pml</i> I site of <i>tbpB</i>	(206)

Oligonucleotide Name		Sequence (5'- 3')	Target
oVCU151	GCAACACCATAGCAGCCTGATTCACC	<i>tbpB</i> upstream region	
oVCU153	GCGGGAGAGAGTTAGAGAGAGGGCAACAAG	<i>tbpB</i> upstream region	

C. Liquid Transformation

Transformation of the *thpB::lacZ* fusion into the MCV113 recipient strain was unsuccessful with the spot transformation method, thus a liquid transformation technique was employed. Gonococci were suspended in GCB/MgCl₂ media and mixed with linearized pVCU109. Cells and DNA were incubated for 30 minutes at 37°C with 5% CO₂. Fresh GCB/MgCl₂ media was added and the mixture was incubated for an additional 5 hours under the same conditions as previously stated. Gonococci were plated on GC agar plates containing erythromycin (1 µg/ml) to select for the *lacZ-ermC'* insertion. Subsequently transformants were grown on GC agar plates containing streptomycin (100 µg/ml) to confirm the retention of the Ω cassette by the transformants. The resultant strain was named MCV121 as listed in Table 3.

V. β -galactosidase Assays

A. Generation of Iron-Stress Conditions for Maximal *thp* Expression

Gonococcal strains were propagated on GCB/ Supplement I/ Supplement II plates and cultures were inoculated as described above. Cultures in GC broth with Supplement I were grown at 37°C in a 5% CO₂ atmosphere and vigorous (220 rpm) shaking, and allowed to replicate through approximately one mass doubling, in an effort to allow all cultures to uniformly enter the same growth phase. Fresh GC broth was added to standardize each culture to the same cell density. Desferal (50 µM) was added to the iron-stress cultures and reagents used to mimic other environmental stress conditions were added to cultures as stated below.

B. Generation of Iron-Stress and Modified Osmolarity (NaCl) Conditions

GC broth used in the laboratory to achieve optimal *thp* expression contains 86 mM NaCl. In order to modulate the osmolarity of the growth media, the amount of NaCl added to the GC

broth was adjusted to achieve the desired concentrations. Cultures were grown in the NaCl-modified GC broth as stated above for the generation of iron-stress conditions.

C. Generation of Iron-Stress and Modified Osmolarity (Glucose) Conditions

GC broth used in the laboratory to achieve optimal *tbp* expression is supplemented with Supplement I, making the final concentration of glucose added to the media equal to 22 mM. In order to generate osmolarity changes in the growth media, the final glucose concentration to which the gonococci were exposed was altered. Broth was made in which final concentrations of the remaining components of Supplement I (co-carboxylase and L-glutamine) were unchanged. A 50% (w/v) glucose solution was used to modify the final concentration of glucose. Cultures were grown as indicated above for the generation of iron-stress conditions, except additional Supplement I was not added.

D. Generation of Iron-Stress and Increased cAMP Availability Conditions

Current growth conditions in the laboratory to achieve maximal levels of *tbp* expression do not include supplementation with cAMP. To study the effect of increased cAMP, GCB broth was supplemented with 20mM cAMP (Sigma). Cultures were grown as indicated above for the generation of iron-stress conditions.

E. Generation of Iron and Oxidative Stress Conditions

Oxidative stress conditions were generated by addition of a 3% hydrogen peroxide solution, to the desired final concentrations. Hydrogen peroxide was added simultaneously with Desferal, and cultures were grown as indicated above for the generation of iron-stress conditions.

F. Generation of Iron-Stress and Low Oxygen Conditions

Maximal *tbp* expression is achieved in the laboratory in high aeration conditions. Flasks are capped loosely, but are shaken vigorously at 37°C. In order to generate a low oxygen

environment during growth, the oxygen scavenger Oxyrase for broth (Oxyrase, Inc.) was added to GC broth at a concentration of 1 ml/ 50 mls of GC broth 30 minutes prior to inoculation. Following inoculation and Supplement I addition, flasks were capped and the tops parafilmed in order to minimize the entry of oxygen. Additionally, when required, the growth media was supplemented with 2mM NaNO₂, to provide an alternate electron acceptor. Cultures were incubated at 37°C, without shaking and allowed to replicate through approximately one mass doubling. Fresh Oxyrase-supplemented GC broth was added to standardize each culture to the same cell density. Desferal (50 µM) was added to iron-stress cultures.

G. Generation of Iron-Stress and Supplementation with N-Methyl Mesoporphyrin

IX

To assess the potential regulatory effects of the G4-DNA motifs located upstream of the operon, we studied disruption of their function by addition of N-Methyl Mesoporphyrin IX (NMM) (Frontier Scientific). NMM was diluted in sterile dH₂O to a concentration of 2M. Gonococcal strains that had been propagated on GCB/Supplement I/ Supplement II plates were grown in GC broth with Supplement I, for one mass doubling. Cultures were subsequently treated with 50 µM Desferal and NMM to the desired concentrations.

H. β-galactosidase Assay

Following the addition of fresh GC media, desferal, and any other required components as listed above, three 500 µl aliquots were removed from each culture (Figure 2B). Two 50 µl samples were taken from each 500 µl aliquot and used to assay transcript levels (each strain was analyzed in triplicate, with duplicate OD readings taken for each triplicate set at every time point). Each 50 µl aliquot was mixed with 950 µl of Z-buffer (166). Cells were lysed with 0.1% SDS and chloroform, and vortexed for 10 seconds before incubating at 25°C for 5 minutes. Five

hundred microliters of 2-Nitrophenyl β -D-galactopyranoside (ONPG) (Sigma), at a concentration of 4 mg/ml were added to each sample and shaken vigorously for nine minutes. Sodium carbonate (Na_2CO_3) was added to stop the reaction, and optical density readings were taken at 600 nm, 420 nm, and 550 nm in accordance with the Miller method (166) formula used to determine β -galactosidase activity. The procedure was repeated at one hour intervals, for the time points indicated in each experiment.

VI. Whole Cell Lysate Preparation of Environmental Stress Samples

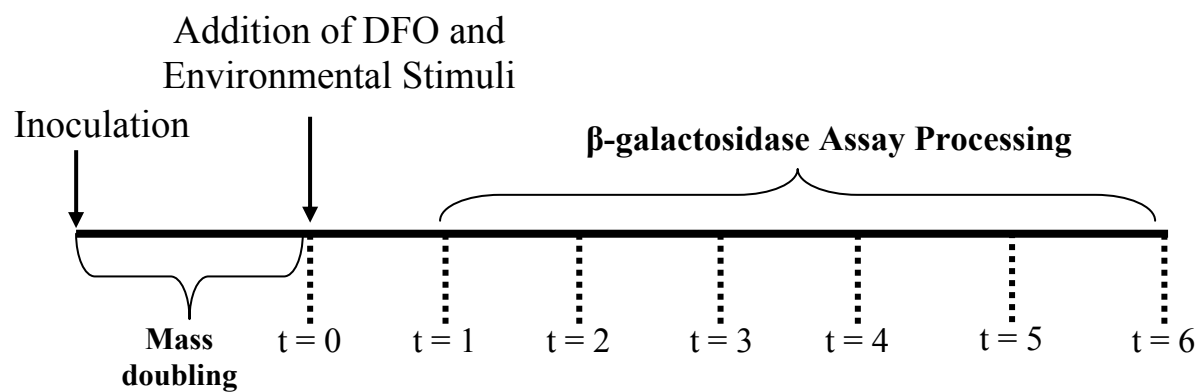
In order to correlate results at the transcriptional and protein levels, generation of whole cell lysates for western blot analysis was altered slightly from the procedure stated above. In conditions where exogenous reagents were added to mimic environmental changes (addition of cAMP, H_2O_2 , NMM,) these were added, at the desired concentrations, at the same time that the DFO was added. Cultures were shaken vigorously (220 rpm) at 37°C in a 5% CO_2 atmosphere for 2 (NMM samples) or 4 hours. In conditions where the growth media was modified by altering the amount of one of its core components (NaCl, and glucose) or where the media was made anaerobic, whole cell lysate preparation was conducted as indicated above for Fe-stress sample whole cell lysate preparation. Final cell densities of the cultures were measured after 2 or 4 hours of growth, and all samples were standardized to a constant cell number. Samples were centrifuged for 10 min at 13,000 rpm. Pellets were resuspended in Laemmli solubilizing buffer (141) and stored at -20°C. TbpB and TbpA detection was conducted as indicated above.

VII. Statistical Analysis

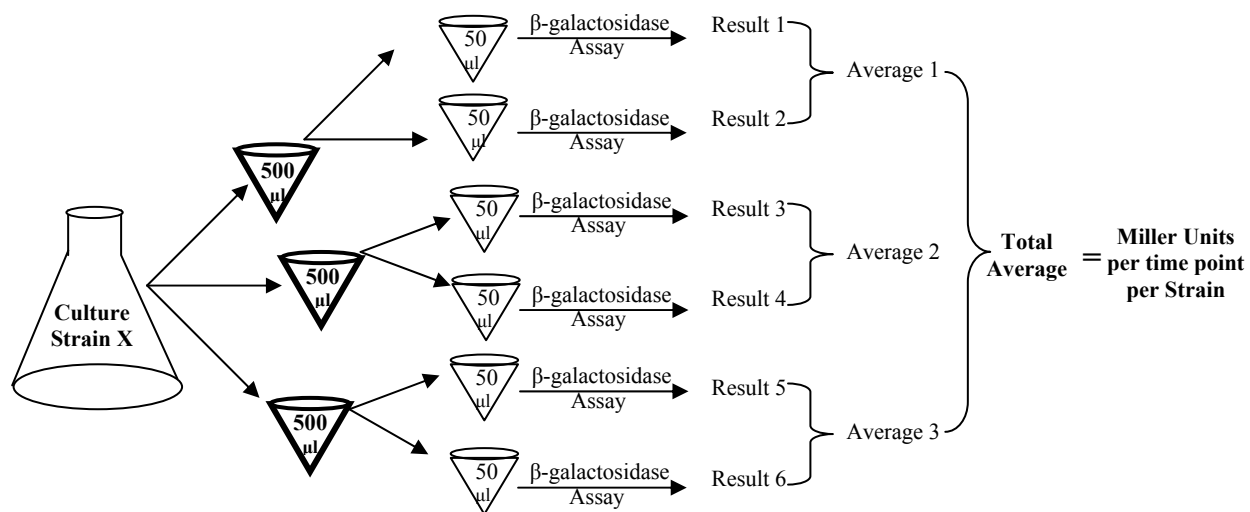
Statistical significance of *tbp* transcript levels was evaluated using a two-tailed unpaired Student's *t* test. Statistical significance is noted when $P \leq 0.05$. Specific *P*-values are included for each data set.

Figure 2: Schematic of β -galactosidase Assays. (A) Time line of gonococcal culture growth. Cultures were inoculated with the appropriate gonococcal strains and allowed to double in mass. Desferal (DFO) and components required to mimic environmental stimuli were added at $t = 0$. Aliquots to be analyzed by β -galactosidase activity assay were taken at 1 hour intervals after the addition of Desferal. (B) Schematic of β -galactosidase assay sample processing. At every time point, three 500 μ l aliquots were taken from each culture. Two 50 μ l aliquots were taken from each 500 μ l sample and processed as stated in β -galactosidase assay procedure described in Materials and Methods. β -galactosidase activity was calculated using the Miller Method formula and is expressed in Miller Units. Miller units for each of the 50 μ l sample were averaged to determine β -galactosidase activity for each strain at every time point.

A.



B.



CHAPTER 3: Promoter Mapping and the Region Upstream of the *tbpBA* Operon

I. Introduction

Expression of the transferrin binding proteins has been shown to be essential for survival and establishment of infection by *N. gonorrhoeae* (4, 53). Previous studies have focused mainly on elucidating the structure-function relationships of the proteins; however, little is still known about the mechanisms that regulate their expression. It is known that the genes encoding the proteins are arranged in a bicistronic operon with the *tbpB* gene located upstream of *tbpA* (54, 145, 206), and separated from it by an 86 base pair region, containing an inverted repeat, which potentially forms a stem loop in the mRNA.

Previous studies have confirmed that the genes are co-transcribed (206) and that the operon is under the control of the Fur protein, resulting in preferential expression of Tbps under iron-limited conditions. However, the genes are differentially expressed. Using a variety of RT-PCR and fusion techniques, it has been previously shown that *tbpB* transcripts are approximately two-fold more prevalent than *tbpA*-specific transcripts under iron-stressed conditions (206).

A transcriptional start site for the operon had been previously identified in our laboratory (205), as well as a putative promoter (4, 205), but the latter has never been experimentally defined. Thus, in order to fully understand the regulation of the *tbpBA* operon it is necessary to begin with the identification and characterization of promoter elements and a Fur operational motif.

Sequence analysis shows that there is a 1905 base pair region between the translational start site of TbpB and the next proximal gene upstream (NGO1499). This region is present in the pathogenic *Neisseria*, but absent in commensal species. This region contains only two small, atypical open-reading frames (ORFs), which are not known to encode proteins. The entire gonococcal genome is 2.154 Mb (GenBank, STDGEN). It is highly improbable that the gonococci would have such a significant amount of sequence with no known function. Therefore, we hypothesize that this region could be part of an extended regulatory region for the operon.

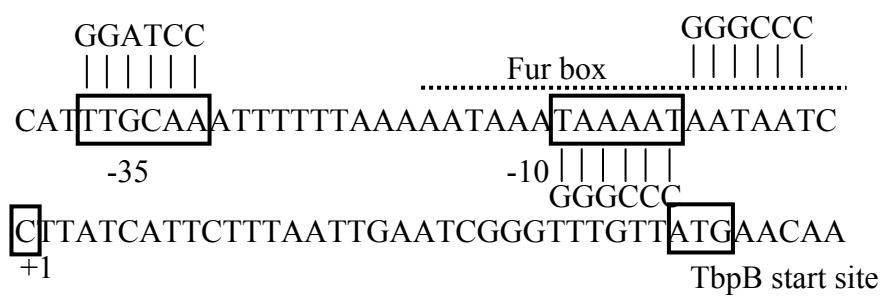
In order to define the iron-regulated promoter for the operon, putative promoter elements were mutagenized and their effect assessed at the protein level. Additionally, an approximately 600 base pair portion of the entire 1905 bp region upstream of the operon was analyzed to begin to define the extent of the promoter required for optimal expression. Possible extended regulatory regions were mutagenized and their effects analyzed at the transcript and the protein levels.

II. Results

A. Promoter Mapping

Identification of putative promoter components was accomplished by comparison with *E. coli* consensus sequences with the upstream region of *N. gonorrhoeae* wild-type strain FA19 (Figure 3). The -10 region was putatively located 6 base pairs upstream of the identified transcription start site, and matched the *E. coli* consensus sequence (5'-TATAAT-3') at five of six positions. A putative -35 region matched the *E. coli* consensus sequence (5'-TTGACA-3) at

Figure 3. Sequence and Mutagenesis of the *thpBA* Promoter Region. The identified transcriptional start site (+1) appears boxed. Identified putative promoter elements -10 and -35 also appear boxed, and the Fur binding site is highlighted by the dashed line. The translational start site (ATG) is shown. Sequences shown either above or below the boxed promoter elements designate mutagenized residues.



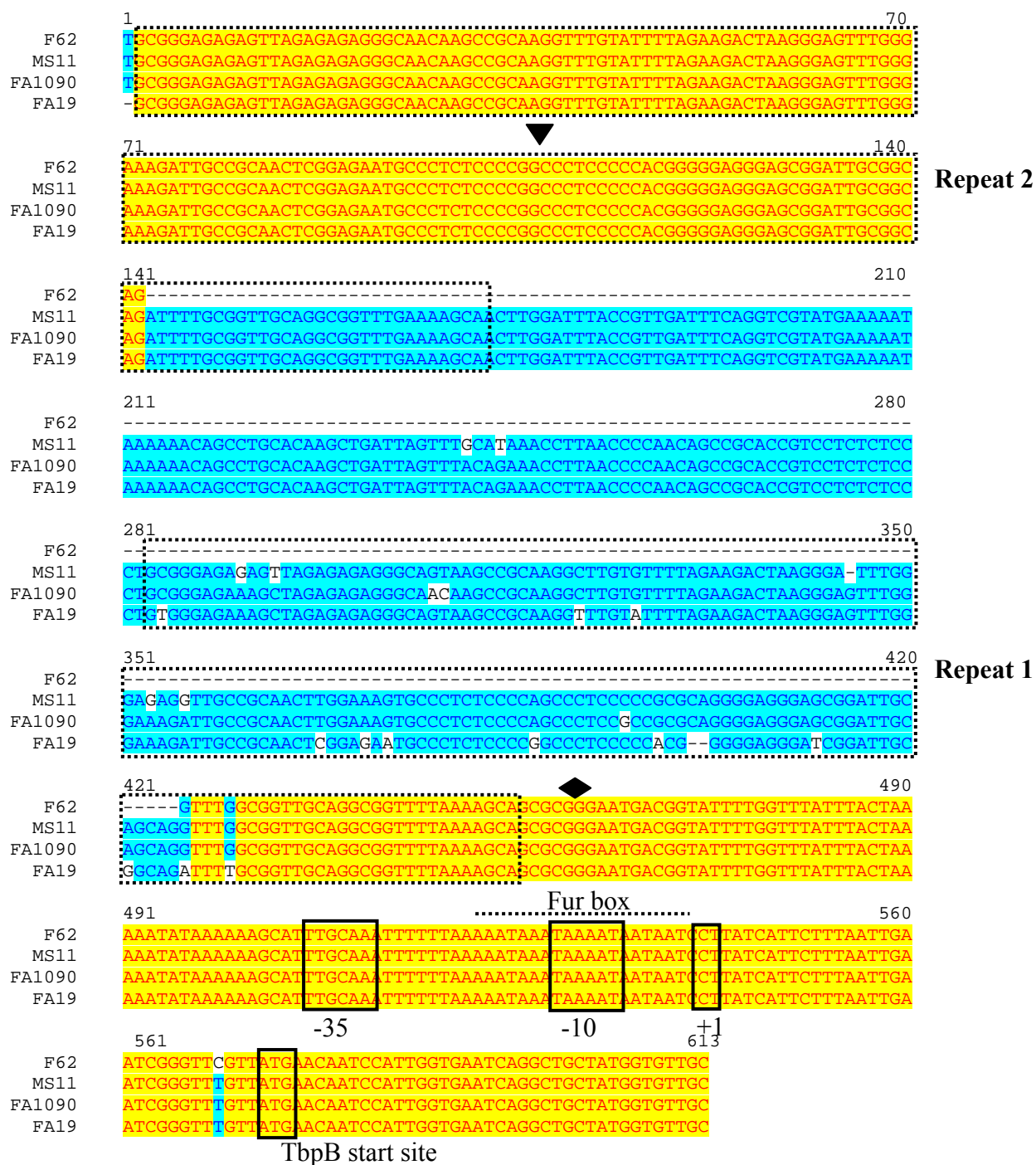
four of six positions. These two motifs are separated by 17 nucleotides, similar to the spacing found in the *E. coli* consensus promoter. The Tbps are preferentially expressed in low iron conditions, and we also identified a Fur binding site (Fur box, Figure 3) which overlaps the identified -10 sequence, consistent with transcriptional regulation in response to iron. The Fur box matched the *E. coli* consensus sequence (5'-GATAATGATAATCATTATC-3') at thirteen of nineteen positions.

We expected that if the identified sequences actually comprised the promoter for the operon, they would be conserved among neisserial strains. A 600 base pair portion of the region upstream of the *tbpBA* operon was amplified by PCR with primers specified in Table 4 in wild-type strains FA19, MS11 and F62. The PCR products were cloned into pCR2.1-TOPO and sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University. The software program Vector NTi (Invitrogen) was used generate an alignment of sequenced products. Alignment of the region approximately 600 base pairs upstream of the TbpB start site in these three strains with the same region of the previously sequenced strain FA1090 (GenBank accession # AE004969) revealed that the putative promoter elements were completely conserved (Figure 4).

B. Characterization of the *tbpBA* Promoter

To confirm that the identified -10, -35 and Fur box elements were indeed responsible for promoter activity, mutational analysis was conducted. Using PCR amplification with mutagenic primers, the putative promoter elements were mutagenized (Figure 3) (completed by Dr. Ronpirin). The putative -10 region was changed to a *SmaI* site, as were 6 residues in the Fur Box region. The putative -35 region was changed to a *BamHI* site. Mutant strains were grown in GCB with the addition of iron ($\text{Fe}(\text{NO}_3)_3$) or with the addition of the iron chelator Desferal

Figure 4. Alignment of the Region Upstream of the *tbpBA* Operon in Several *Neisseria gonorrhoeae* Strains. Approximately 600 base pairs upstream of the operon were amplified and sequenced in strains FA19, MS11 and F62. FA1090 represents the sequenced strain (NC 002946). Identical residues are shaded in yellow, residues found in only some of the strains are shaded in blue and dashes (-) represent missing residues. The TbpB start site and the transcriptional start site (+1) are shown. Conserved promoter elements appear boxed. The Fur box is shown highlighted by the dashed line. Repeats found in the upstream region are shown boxed in dashed lines. The direct repeats are 162 nucleotides in length and are differ from each other in 20 base pairs (bp) in MS11, 19 bp in FA1090, and 11 bp in FA19. ▼ denotes the position of the Ω cassette insertion in strain MCV113. ◆ denotes the position of the Ω cassette insertion in strain MCV114.



for four hours. Cultures were standardized to a constant cell number and whole cell lysates prepared. The effect of the promoter region mutations on protein expression was verified by western blot analysis (Figure 5). As expected, TbpA and TbpB were detected in the wild-type strain under iron-deplete conditions. Mutagenesis of the -10 region abolished expression of the Tbps even under low iron conditions. Mutagenesis of the -35 region almost completely abrogated expression of both Tbps, although a very faint band was detected in the blot probed with the α -TbpA antibody. Mutation of the Fur box region resulted in expression of both Tbps regardless of iron availability during growth.

The promoter region mutants were also tested for growth on human transferrin as a sole iron source (Figure 6). In this assay gonococcal strains are plated on CDM (chelexed defined media) agarose plates supplemented with 30% iron-saturated human transferrin. Growth of the strains is monitored after 24 to 48 hours, and will occur if gonococci are capable of TbpA expression. Wild-type strain FA19 grew, as expected. Strain FA6815, the negative control, was not able to grow as this strain is TbpA deficient. The -10 region mutant (MCV117) showed no growth after 48 hours, and the Fur box mutant (MCV119) was capable of growth on transferrin. The -35 region mutant (MCV118) grew on the plates after 24 hour incubation. The latter result demonstrated that a very low level of TbpA expression was accomplished by this mutant and was sufficient to support transferrin-dependent growth. Taken together these data suggest that the identified putative promoter elements do indeed control the expression of the *tbpBA* operon. The operon is under the control of the Fur repressor, thus leading to de-repressed expression of the Tbps under high iron conditions in the Fur box mutant.

Figure 5. Tbp Expression in the Promoter Region Mutants. Expression of Tbps in the promoter-region mutants. Gonococci grown under iron replete (+) or iron deplete (-) conditions were lysed, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed with α -TbpA or α -TbpB antibodies as labeled to the left. Ponceau stain is shown to establish equal loading.

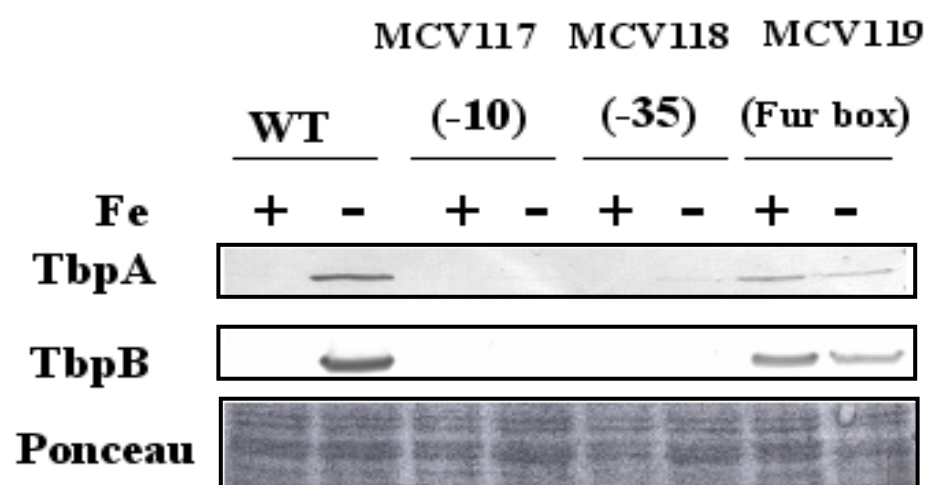
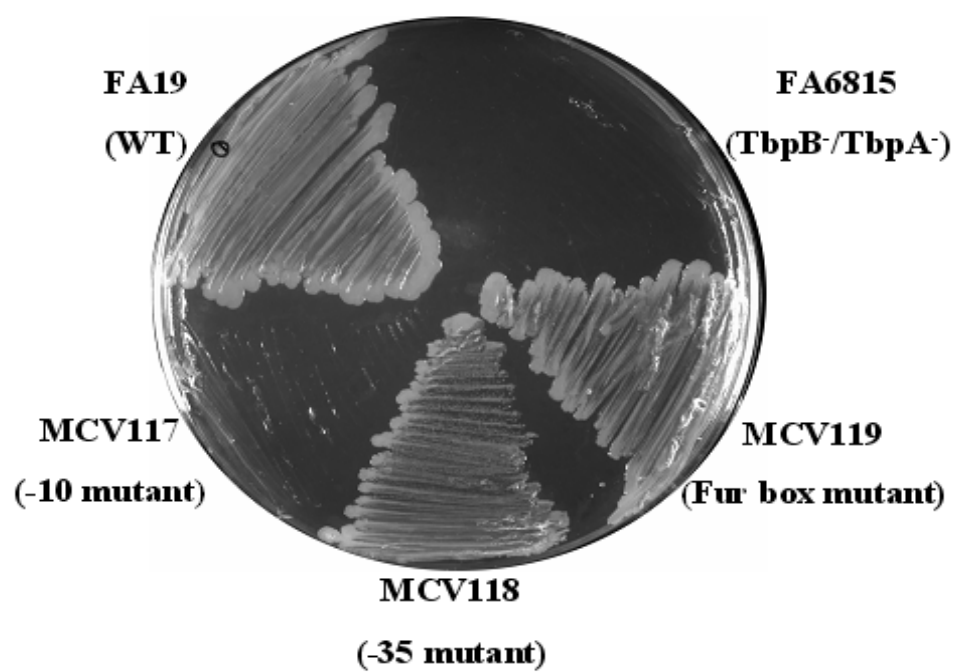


Figure 6. Transferrin Iron Utilization by the Promoter Region Mutants. Gonococcal mutants were plated onto CDM medium containing 30% saturated Tf. Strains are labeled according to strain name and the phenotypes appear in parenthesis. FA19 serves as a positive control (TbpA⁺), and FA6815 serves as a negative control (TbpA⁻).



C. Identification of Extended Promoter Regions

The confirmed promoter extends 66 bp upstream of the TbpB start site. However, the entire region between the operon and the next proximal gene upstream is comprised of approximately 1.9 Kb and contains no known coding sequences. Two nearly perfect direct repeats were identified in the 600 base pairs directly adjacent to the start site for the operon (Figure 4). These repeats are 162 base pairs in length, and differ from each other by only 20 bp (13% difference) in strain MS11, 19 bp (12% difference) in strain FA1090, and 11 bp (7% difference) in strain FA19. Such a high degree of similarity between repeat 1 and repeat 2 in each strain gives us a hint as to the origins of these repeats and that one of the repeats probably evolved from the other rather than being acquired at different times. Direct repeats have been identified as sites involved in transcriptional regulation in phage (62) yeasts (242), eukaryotes (43, 182) and bacteria (65, 175), thus the possibility that these sequences may form part of an extended promoter for the *thpBA* operon was explored. We rationalized that if the repeats were involved in the regulation of the operon, they should be highly conserved. Sequence alignment revealed that the repeat found further away from the operon (labeled as Repeat 2 in Figure 4) was perfectly conserved in all the examined strains for the majority of its sequence, while the repeat found closer to the operon (labeled as Repeat 1 in Figure 4) was slightly less well conserved (contained more base pair variations amongst the strains), and was absent from wild-type strain F62. It is important to note that the identification of the length of the repeats was hindered by the sequence available on strain FA19. Preliminary sequence analysis of the 1.9 Kb region upstream of the operon in strain FA1090 (the only sequenced strain) suggests that the repeats may actually be longer than our analysis has determined, and that the repeat sequence

may also be found at least once more in the upstream region. Additionally, each repeat appears to be flanked by at least one inverted repeat of sequence CTCTCTCCC(N₃)GGGAGAGAG.

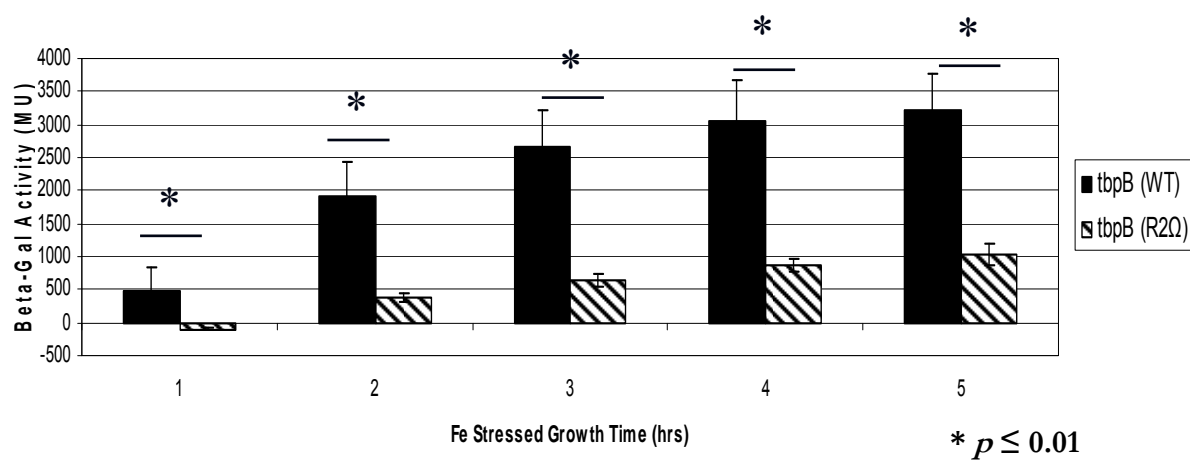
D. Disruption of Repeat 2 Affects Transcript and Protein Levels

To elucidate whether repeat 2 was involved in the regulation of the operon, it was disrupted by the insertion of an Ω cassette at position 465 base pairs upstream of the TbpB start codon (strain MCV113, Table 3, Figure 4) (205). In order to assess the effects of the Ω cassette insertion at the transcriptional level, we also generated transcriptional *lacZ* fusions for each *tbp* gene in the Ω_{-465} insertion background (MCV120 and MCV121, Table 3). Plasmids pVCU109 and pVCU108 contain a promoterless *lacZ* gene fused to either *tbpB* or *tbpA*, respectively. The *E. coli* strains carrying these plasmids were grown in LB broth overnight, supplemented with erythromycin (resistance to which is encoded in a gene adjacent to *lacZ*). Subsequently, plasmids were purified, linearized by digestion, and used to transform gonococcal strain MCV113. Incorporation of the *lacZ* cassette was confirmed by gonococcal growth on plates containing erythromycin. Gonococcal growth on plates with streptomycin was used to confirm that the original Ω cassette insertion remained.

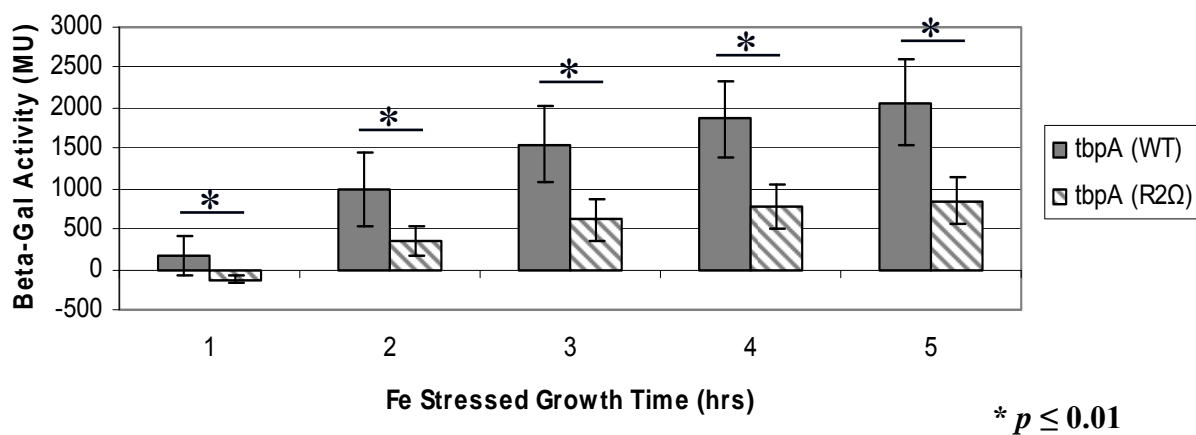
Gonococcal strains MCV108 and MCV109 were previously generated (206) and contain *lacZ* insertions into the *tbpA* and *tbpB* genes, respectively. These fusions have undisturbed upstream regions, and were thus used as positive controls for *tbp* transcript levels. All strains were grown in GCB media supplemented with Kellogg's supplement I. Desferal was added to all cultures in order to generate iron-deplete conditions. Samples were removed at one hour intervals after the addition of Desferal. Cells were lysed open with Z-buffer (141) and ONPG was added to generate the color change. Reactions were allowed to develop for 9 minutes and subsequently stopped with Na₂CO₃. β -galactosidase activity assays showed that *tbpB* transcript

Figure 7. Transcriptional Analysis of the Repeat 2 (Ω_{465}) Mutant. β -galactosidase assays were used to determine the effects of the disruption of upstream repeat 2. β -galactosidase activity is expressed in Miller Units. (A) Comparison of *tbpB* transcript levels. The WT strain (MCV109) is shown in the solid black bars. The repeat 2 Ω mutant (MCV121) is shown in the black striped bars. (B) Comparison of *tbpA* transcript levels. The WT strain (MV108) is shown in the solid grey bars. The repeat 2 Ω mutant is shown in the striped grey bars. (C). Comparison of overall *tbp* transcript levels. The WT transcript levels are shown in the solid bars, while the repeat 2 mutant strains are shown in the striped bars. Errors bars represent the standard deviation of the means of at least 3 experiments done on different days. Asterisks denote statistical significance, $P \leq 0.01$.

A.



B.



C.

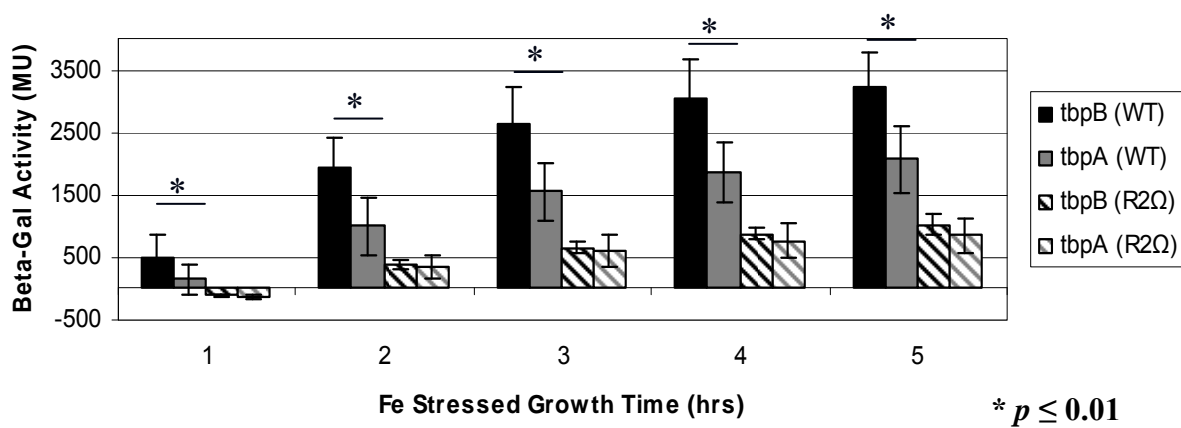


Table 5. *tbpB/tbpA* Ratios in the Wild-Type, Repeat 1 and Repeat 2 Upstream Mutants

Fe Stressed Growth Time (hrs)	WT (MCV109/MCV108)	Repeat 1 Mutant (MCV123/MCV122)	Repeat 2 Mutant (MCV121/MCV120)
1	3.1	1.6	.8
2	1.9	1.3	1.1
3	1.7	1.3	1.1
4	1.6	1.3	1.1
5	1.6	1.2	1.2

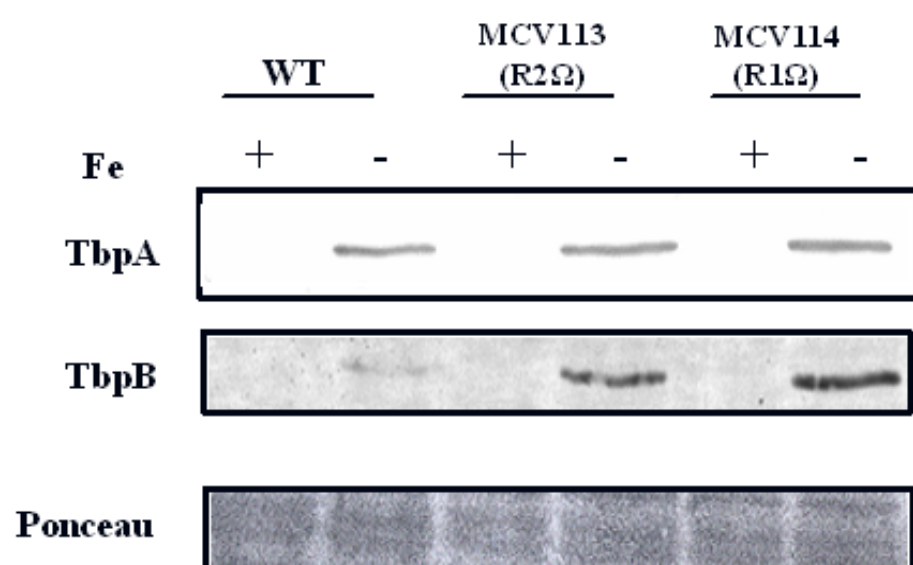
levels were decreased in the Ω_{-465} insertion mutant, as compared to the control strain (Figure 7A). *tbpA* transcript levels were similarly decreased relative to the wild-type *tbpA* levels (Figure 7B). Additionally, while in the strain with an intact upstream region the *tbpB:tbpA* transcript ratio is close to 2:1, in the strain with the Ω cassette disruption this ratio decreased closer to 1:1 (Figure 7C and Table 5). These results were not an artifact due to the orientation of the polar Ω cassette and its internal termination signals, as determined by evaluating the cassette in the opposite direction, which resulted in the same outcome (data not shown).

We also assessed the effect of the disruption of repeat 2 at the protein level. Strains FA19 (WT) and MCV113 (Ω_{-465}) were grown under iron replete and iron deplete conditions as indicated above. Again, as expected, the Tbps were only detected under iron-deplete conditions in both the wild-type and mutant strains. However, TbpA protein levels appeared unchanged in the mutant relative to the wild-type (Figure 8). Interestingly, TbpB protein levels increased in the mutant relative to the wild-type. Taken together these data show that the repeat 2 region does appear to exert some regulatory control over the *tbpBA* operon.

E. Insertional Mutagenesis of Repeat 1 Affects Transcript and Protein Levels

We also sought to determine the role of repeat 1 in the regulation of the operon. An Ω cassette was inserted 114 base pairs upstream of the TbpB start site (strain MCV114, generated previously). Although this insertion does not directly impact the sequence of the repeat, it does affect its spacing relative to the start site. In order to assess the effects of this mutation on the operon at the transcriptional level, transcriptional *lacZ* fusions were generated in this background. These fusions were generated in the same manner indicated above for the MCV113 transcriptional fusions. β -galactosidase assays were employed to determine transcript levels in the repeat 1 mutant, and strains MCV109 (*tbpB-lacZ*) and MCV108 (*tbpA-lacZ*) were used as the

Figure 8. Tbp Expression in the Upstream Insertion Mutants. Gonococcal strains FA19 (WT), MCV113 (Ω_{-465} ; Repeat 2 mutant) and MCV114 (Ω_{-114} ; Repeat 1 mutant) were grown under iron-replete (+) and iron-deplete (-) conditions. Western blot was probed with anti-TbpA polyclonal antibody (top panel) and anti-TbpB polyclonal antibody (second panel). Ponceau stain is shown (bottom panel) to establish equal loading in all lanes. Results are representative of at least 3 experiments conducted on different days.



wild-type, control strains. β -galactosidase activity assay results show that *tbpB* transcript levels appear decreased in the Ω_{-114} (R1 Ω) mutant relative to the control strain (Figure 9A). *tbpA* transcript levels in the R1 Ω mutant appear unchanged relative to the wild-type strain (Figure 9B), as there was no statistically significant difference at any of time points tested. However, comparison of the *tbpB:tbpA* ratio in this mutant also appear decreased and closer to a 1:1, demonstrating a change from what is observed in the wild-type strain (Figure 9C and Table 5).

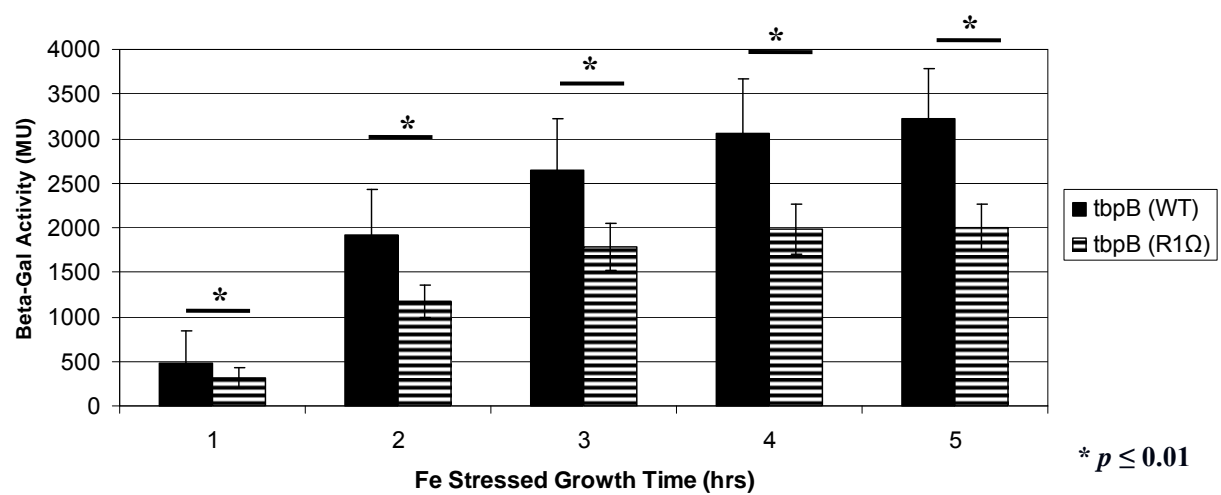
Effects of the repeat 1 Ω mutation were also assessed at the protein level. Strain MCV114 was grown under iron-stressed conditions for 4 hours and whole cell lysates were prepared as described above. Results show (Figure 8) that TbpA expression appears unchanged in the mutant (top panel), while TbpB expression appears increased (second panel) relative to Tbp expression in wild-type strain FA19. Together, these data show that disruption of repeat 1 in relation to its position to the operon also affects expression of the *tbp* genes.

F. Disruption of Repeat 2 Results in More Profound Effects on *tbp* Expression

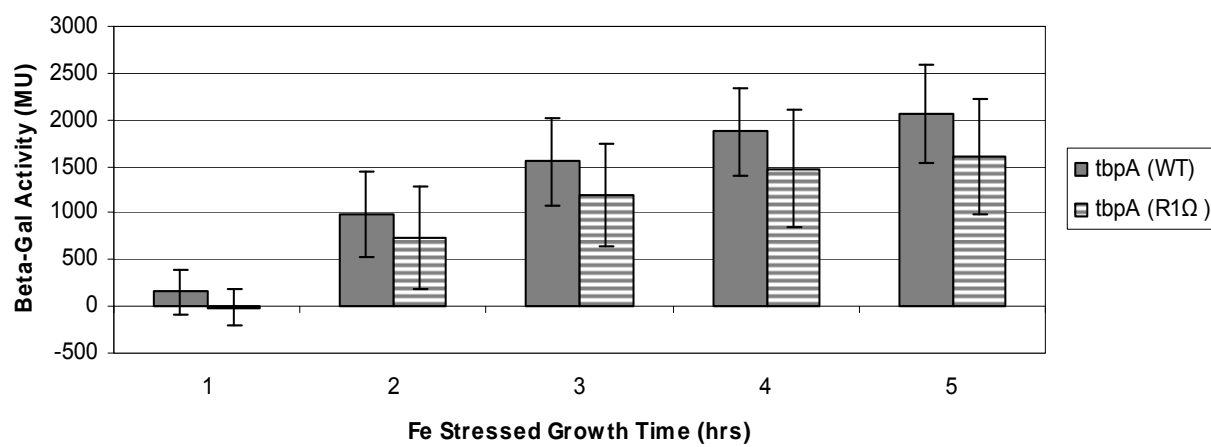
Although alteration of both repeats results in changes in expression of the operon, comparison of *tbpB* transcript levels in both of the upstream insertion mutants, relative to wild-type (Figure 10) shows that disruption of repeat 2 (the one located further away from the operon and better conserved one) results in a more drastic decrease in detectable transcript levels than does the disruption of repeat 1. Similarly, comparison of overall *tbpA* transcript levels results in decreased transcript detection for the repeat 2 mutant, while the repeat 1 mutant does not show any statistically significant difference with the control strain (Figure 11). These data suggest that the repeats are important in the regulation of the operon, and that the sequence integrity plays a more significant role than spacing of the sequence in relation to the operon.

Figure 9. Transcriptional Analysis of the Repeat 1 (Ω_{114}) Mutant. β -galactosidase assays were used to determine the effects of the disruption of upstream repeat 2. β -galactosidase activity is expressed in Miller Units (MU). (A) Comparison of *tbpB* transcript levels. The WT strain (MCV109) is shown in the solid black bars. The repeat 1 Ω mutant (MCV121) is shown in the black striped bars. (B) Comparison of *tbpA* transcript levels. The WT strain (MV108) is shown in the solid grey bars. The repeat 1 Ω mutant (MCV122) is shown in the striped grey bars. (C). Comparison of overall *tbp* transcript levels. Wild-type transcript levels are shown in the solid bars, while repeat 1 transcript levels are shown in the striped bars. Error bars represent the standard deviation of the means of at least 3 experiments done on different days. Asterisks denote statistical significance, $P \leq 0.01$.

A.



B.



C.

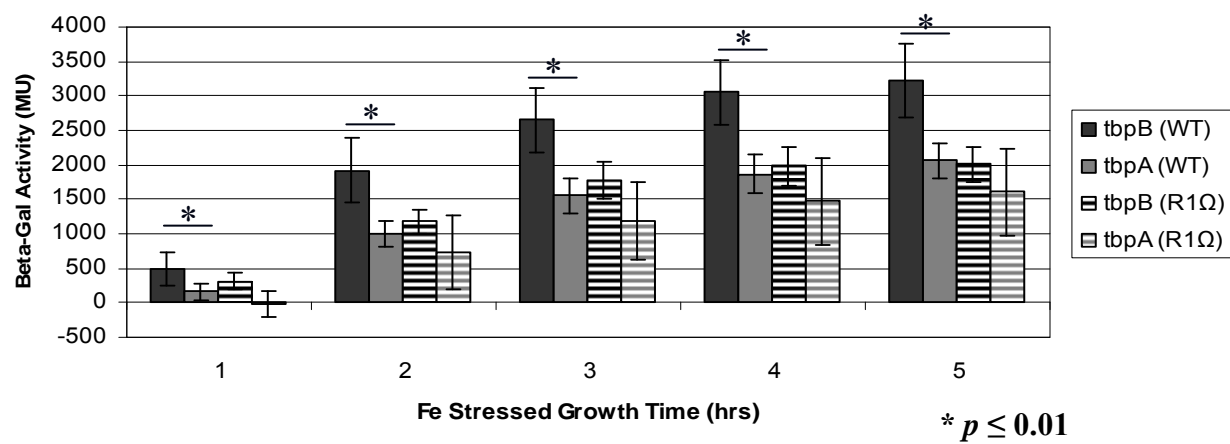


Figure 10. Comparison of *thpB* Transcript Levels in the Upstream Insertion Mutants. Transcriptional activity was assayed by β -galactosidase assays. β -galactosidase activity is expressed in Miller Units. Wild-type *thpB* transcript abundance is shown in the solid black bars. Ω -insertion *thpB* transcript abundance is shown in the striped bars. Error bars represent the standard deviation of the means of at least 3 experiments done on different days. Asterisks denote statistical significance in the levels of transcript detected in each of the upstream insertion mutants relative to the WT strain.

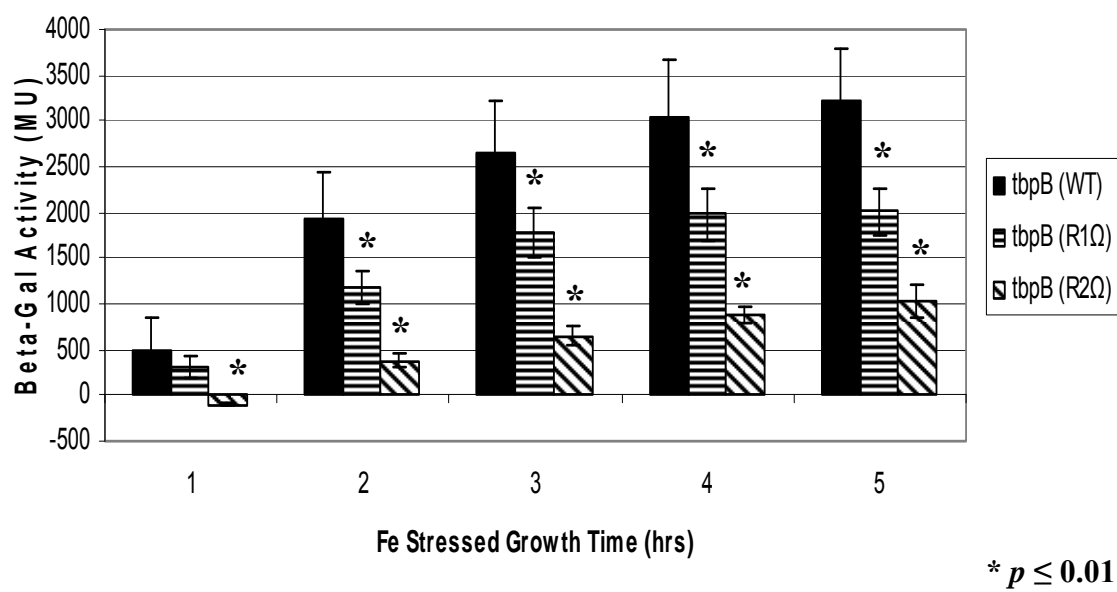
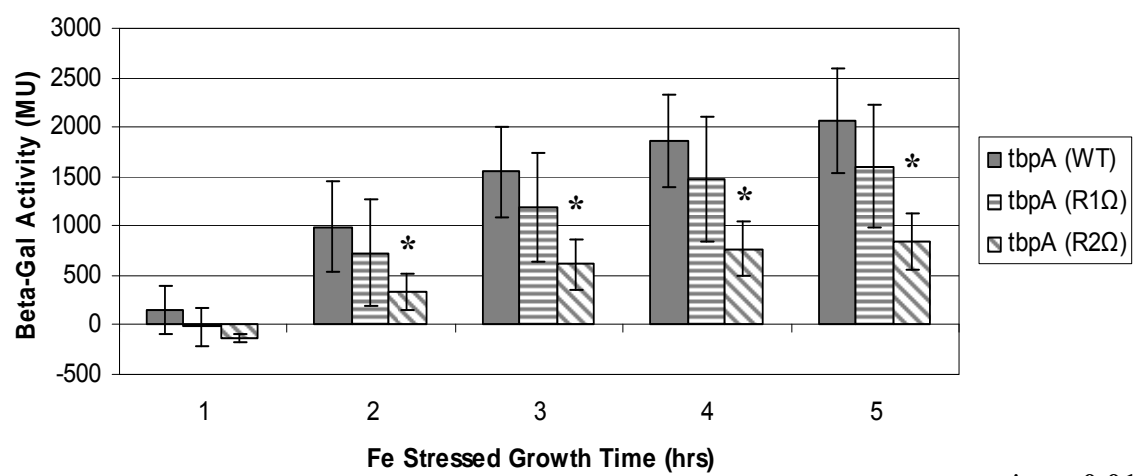


Figure 11. Comparison of *tbpA* Transcript Levels in the Upstream Insertion Mutants. Transcriptional activity was assayed by β -galactosidase assays. β -galactosidase activity is expressed in Miller Units. Wild-type *tbpA* transcript abundance is shown in the solid grey bars. Ω -insertion *tbpA* transcript abundance is shown in the striped bars. Error bars represent the standard deviation of the means of at least 3 experiments done on different days. Asterisks denote statistical significance in the levels of transcript detected in each of the upstream insertion mutants relative to the WT strain



* $p \leq 0.01$

III. Discussion

Studies on the gonococcal transferrin receptor have focused mainly on deciphering the structure-function relationships of its components, and little is known about the mechanism that coordinately controls expression of these proteins. A putative promoter for the operon had previously been identified (4), but never experimentally defined. The purpose of this study was to characterize the extent of the functional promoter for the operon. Using primer extension analysis, the start site of *tbp* transcription was mapped at a position 31 base pairs upstream of the *tbpB* start codon (205). The C residue, representing the transcription start site in the *tbp* promoter was preceded by a C at position -1 and followed by a T residue at position +2. These nucleotides are the most common found at positions -1 and +2 in *E. coli* promoters (100, 105). Tbp expression was abolished or severely decreased when promoter elements were mutagenized, confirming that these regions are necessary for transcription of the operon. Although mutagenesis of the -35 promoter element did not completely abrogate expression of the Tbps, it did lead to a severe decrease in the detectable levels of the proteins. In the transcription machinery, the -35 region serves as a point of initial contact between RNA polymerase and the DNA, but it is the -10 region where the DNA starts to unwind to initiate transcription. It is possible that the mutagenesis of the -35 element was enough to disturb gene expression but not eliminate it entirely, since the -10 region was still intact. Additionally, the Fur binding site motif was similarly identified by homology and mutagenized. The resulting mutant exhibited Tbp expression regardless of iron availability during growth. This is the first report to experimentally define the promoter for the *tbpBA* operon. Thus, our results confirm and extend previous findings that the genes are co-transcribed from a common upstream promoter and that the operon is under the control of the Fur protein (206).

Table 6. Summary of Results from Studies with Upstream Ω Insertion Mutants

	<u>MCV114 (R1Ω)</u>	<u>MCV113 (R2Ω)</u>
TbpB western blot	Increased protein	Increased protein
TbpA western blot	No apparent change	No apparent change
<i>tbpB-lacZ</i> fusion	Decreased transcription	Decreased transcription
<i>tbpA-lacZ</i> fusion	Unchanged transcript levels	Decreased transcription

Analysis of the region upstream of the *tbpBA* operon revealed approximately 1.9 Kb of sequence containing only two short hypothetical ORFs which are not known to encode proteins. In the 600 base pairs directly upstream of the TbpB start site we identified two direct repeats. Direct repeats have been shown to be involved in the regulatory mechanisms of the arabinose and maltose operons in *E. coli*. The AraC protein is able to bind to direct repeat sequences to positively and negatively regulate the arabinose operon (31). Similarly, the direct repeat sequences in *malT* serve to facilitate positive transcriptional activation (242). Of the two repeats present in the *tbpBA* operon upstream region, the repeat situated closer to the operon appeared to be less well conserved in several wild-type gonococcal strains analyzed. Moreover, disruption of the spacing between this repeat and the operon resulted in altered transcript and protein amounts. Results show that the repeat located furthest upstream was perfectly conserved in its majority in several *N. gonorrhoeae* strains and that disruption of this repeat resulted in decreased transcript levels and increased TbpB protein levels relative to the wild type strain. Additionally, we noted that the *tbpB:tbpA* transcript ratio had decreased from 2:1 to near 1:1.

Having an inverse correlation between transcript levels and protein expression (Table 6) seemed counterintuitive. Since the upstream repeats do appear to be exerting a regulatory function on expression of the operon, we propose a model in which additional transcription is initiated from an unknown point further upstream than the identified transcriptional start site (+1, Figure 4). It is possible that due to the limits of the primer extension analysis used for the identification of the transcriptional start site, the detection of transcripts that originate further upstream could have been missed. We propose that in the wild-type strain, there could be transcript initiation further upstream, which could explain the increase in transcript levels that are observed in this strain when compared to the Ω insertion mutant strains (Figure 12A). However,

it is also possible that in this extended transcript, the repeats are capable of promoting RNA folding and occluding the ribosomal binding site (RBS), resulting in the decreased Tbp protein levels observed in the wild-type strain in comparison to the Ω insertion mutants. Additionally, it is possible that the generation of this transcript/structure could interfere with the translatability of the transcript generated from the identified +1 through an unknown mechanism. Conversely, in the Ω insertion mutants, since the upstream region has been disrupted, we are no longer able to detect the transcripts that are originated upstream of the previously identified transcriptional start site (+1), which could explain the decreased transcript levels observed in these mutants. Additionally, because the upstream region has been mutagenized, the possible interference with the translatability of the transcript generated from the +1 could be alleviated, resulting in the increased Tbp protein expression that is observed in these mutants relative to the wild-type strain. Moreover, if the RNA is prone to folding, this could have also contributed to the impeded detection of any transcriptional start sites that are in the upstream region with the primer extension analysis.

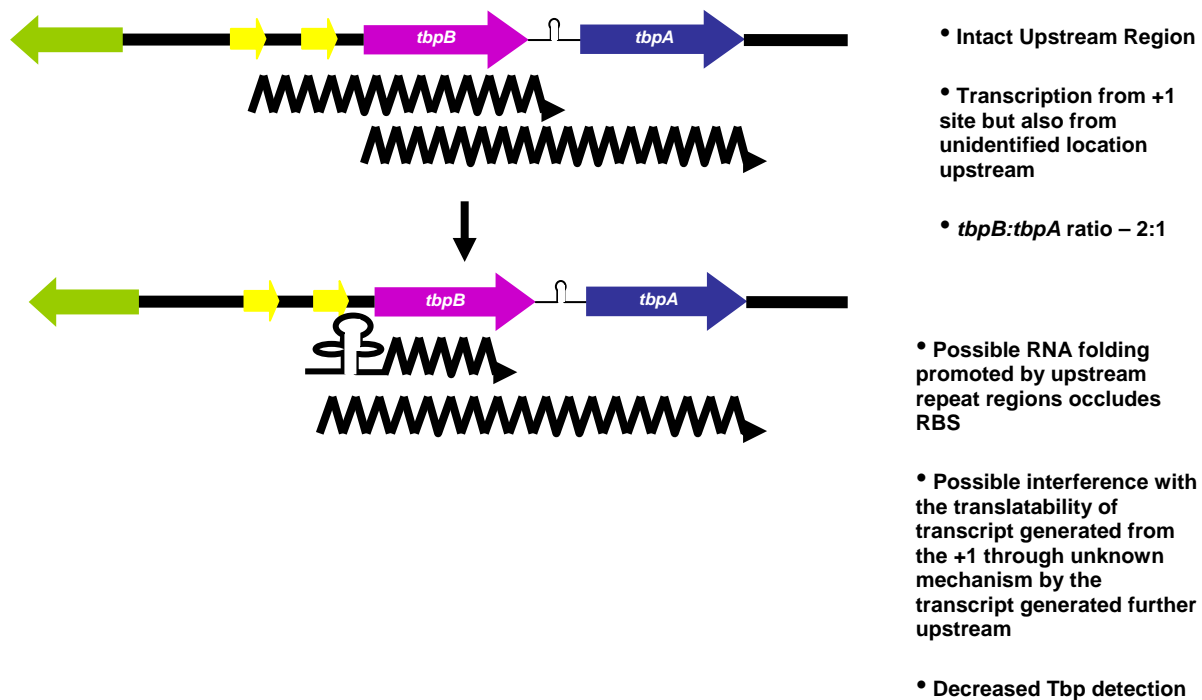
Although further investigation is necessary to provide support for this model, preliminary searches with several RNA-folding prediction web-based programs have shown that there is a high potential of folding in the region upstream of the operon. A similar mechanism has been described in the transcriptional and translational regulation of the α -acetolactate decarboxylase of *Lactobacillus lactis* (87). Goupil-Feuillerat *et al.* have shown that in this system, while the gene is actively transcribed, significant protein levels are not always detected. Their results show that RNA folding results in occlusion the ribosomal binding site for the gene. Mutagenesis of the RNA-folding structure and translational fusions were used to determine that the folded structure actually results in a major decrease in translational activity. In our system, translational

regulation provided by the presence of the upstream repeats could ensure that there is a tight control on expression of this iron-acquisition system during periods of iron-starvation, when the Fur protein no longer transcriptionally represses the operon. Further studies to determine the validity of this proposed mechanism would include detection of transcripts that originate far in the upstream region, and the use of translational fusions to determine how translation is affected when the upstream repeats are disrupted.

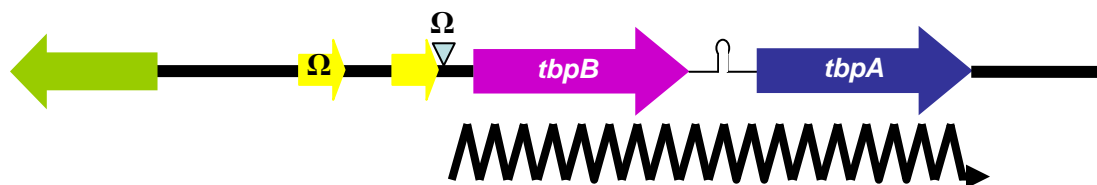
Overall, these studies confirm the identity of the promoter elements and the Fur operator motif that are involved in transcriptional regulation of the operon. Additionally, they confirm that the region upstream of the operon is also involved in the regulation of the operon.

Figure 12: Proposed Model for the Role of the Repeats Upstream of *tbpBA*. The arrangement of the operon is shown. The repeats found in the upstream region are denoted by the yellow arrows. (A) We propose that in the Wild-type strain, there is transcription occurring not only from the identified transcriptional start site for the operon, but also from an as of yet unknown location upstream, resulting in increased *tbpBA* transcript levels, denoted by the black zigzag lines. Possible RNA folding promoted by the presence of the repeats could occlude the RBS of the transcript generated in the upstream region. Additionally, generation of this transcript/structure could possibly interfere with the translatability of the transcript generated from the identified +1, resulting in decreased Tbp expression. (B) In the upstream insertion mutants, mutagenesis of the upstream region, denoted by Ω , potentially disrupts the generation of transcripts originated in the far upstream region, explaining the decreased transcript levels observed in these mutants relative to the Wild-type strain. Additionally, mutagenesis of the upstream region could alleviate the possible interference of the translatability of the transcript generated from the +1, resulting in the increased Tbp expression observed in these mutants relative to the Wild-type strain.

A. Wild-type Strain



B. Upstream Insertion Mutants



- Disrupted Upstream Region
- *tbpB:tbpA* ratio – 1:1- transcript not generated from upstream region
- Possible interference of transcript generated further upstream with translatability of transcript generated at +1 alleviated
- Increased Tbp detection

CHAPTER 4: Regulation of the *tbpBA* Operon in Response to Environmental Stimuli

I. Introduction

Neisseria gonorrhoeae is an obligate human pathogen. Upon entering the host, gonococci must not only compete with the host for essential nutrients, but must also adapt in response to the host environment. Modification of structure and metabolism are imperative for survival upon encountering sudden, intense, and sometimes threatening environmental conditions.

It is well documented that bacterial pathogens are capable of regulating virulence factors in response to environmental stimuli as varied as phosphate (95, 143), carbon starvation (177, 274), oxygen (12, 24, 50, 180), nitrite (59, 226), and many others. All these regulatory networks work in unison to provide bacteria with an advantage, or at least a coping mechanism, when exposed to the harsh host environment. Hence, the goal is to maintain appropriate levels of all nutrients and necessary gene products to avoid potentially dangerous situations, such as poisoning by reactive oxygen or nitrogen species. Additionally, maintaining this balance also provides an energetic advantage by limiting the amount of energy spent in generating components that are not required or used only under specific situations.

Expression of the *Tbps* is known to be regulated by iron availability (206). However, iron starvation is not the only environmental condition that the gonococci encounter upon infection. Here, we analyze several biologically relevant environmental stress conditions that the gonococci might be subject to. We hypothesize that multiple environmental stimuli are involved

in the regulation of the *tbpBA* operon, as a tight control between iron acquisition and utilization is absolutely necessary in order to guarantee the survival of the gonococci.

II. Results

Maximal *tbp* expression in the laboratory is achieved by culturing the gonococci in GC broth with Kellogg's supplement I (129) and 50 μ M DFO, at 37°C in a 5% CO₂ atmosphere with vigorous shaking (220 rpm). Results from the diverse environmental stimuli studies are compared against transcript and protein levels from gonococci cultured under these conditions.

A. Osmolarity Studies

It has previously been reported that expression of some TonB-dependent transporters in *E. coli* are controlled by changes in osmolarity (96). It is likely that the gonococci experience osmolarity changes when transitioning from the male to the female genital tract or from the genital tract to the serum. It is likely that osmolarity differences between the sera and the seminal fluid (187, 208) and extracellular vs. intracellular milieu could also impact gene expression patterns. Thus, the effects of osmolarity fluctuations on expression of the *tbpBA* operon were investigated. Osmolarity variations were generated in two different ways: by altering the amount of NaCl added to the GC broth or by altering the amount of glucose used to supplement the growth media.

A.1. Changes in Osmolarity Generated by NaCl Fluctuations

Standard GCB media used to routinely culture gonococci in the laboratory contains 86 mM NaCl. The NaCl content in the GCB media was adjusted to 43 mM, 129 mM, 172 mM, and 214 mM, representing .5X, 1.5X, 2X and 2.5X, respectively, of the NaCl concentration found in standard GCB media. First, the effects of osmolarity fluctuations on

transcription of the operon were assayed. Transcriptional *lacZ* fusions MCV109 (*tbpB-lacZ*) and MCV108 (*tbpA-lacZ*) were used to determine changes in transcript levels. β -galactosidase assays were conducted as previously stated in Methods. Results show that there was no statistically significant change in *tbpB* transcript levels detected in any of the osmolarity-modulated GCB media as compared to the standard (86 mM NaCl) GCB media (Figure 13A). Similarly, when *tbpA* transcript levels were assayed, no statistically significant changes were observed in any of the NaCl-modulated GCB media (Figure 13B).

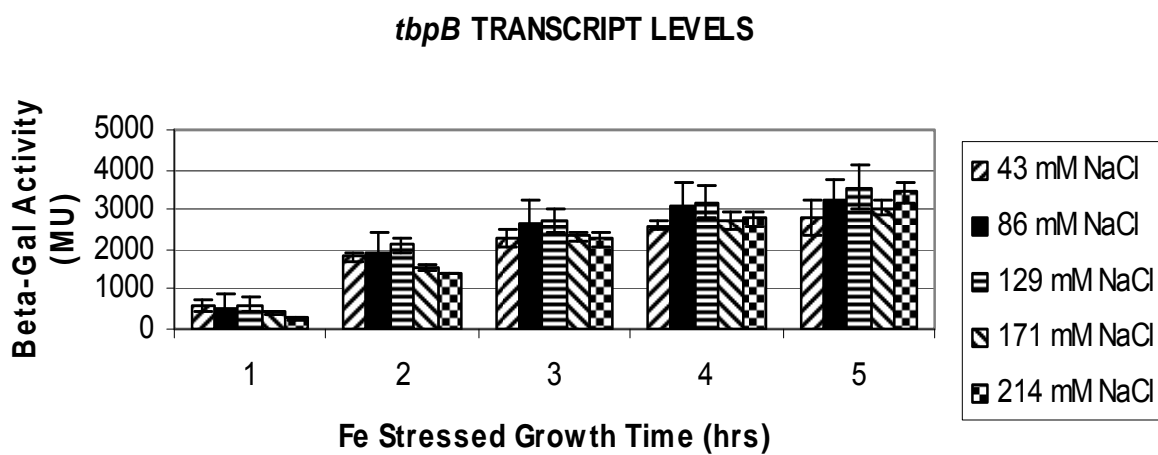
The effects of osmolarity changes in NaCl-modulated media were also assayed at the protein level. Gonococcal strain FA19 (wild-type) was cultured in the various NaCl-modified media under iron-stress conditions for 4 hours. Cell lysates were prepared and SDS-PAGE analysis was conducted as described in Methods. Results show that there were no detectable changes in either TbpA or TbpB expression in any of the NaCl-modulated media as compared to the standard GCB media (Figure 14). These results support the effects seen at the transcript level.

A.2. Changes in Osmolarity Generated by Glucose Fluctuations

Osmolarity changes experienced by gonococci during natural infection could also result from fluctuations in glucose concentrations. Thus, the effects of osmolarity changes generated by glucose fluctuations on expression of the *tbpBA* operon were similarly assayed. Standard GCB media utilized for maximal *tbp* expression is supplemented with 22 mM glucose, equivalent to 0.4%. A 50% (w/v) glucose solution was used to adjust the concentration of glucose added to the growth media. Final concentrations of 56 mM, 139 mM, and 277 mM were assayed, corresponding to 1%, 2.5%, and 5% glucose, relative to standard GCB media. Results show that increases in glucose availability, and thus, changes in osmolarity, yielded no

Figure 13. *tbpBA* Transcript Levels in Response to Osmolarity Fluctuations in NaCl-Modified GCB Media. Both panels show gonococci grown in iron-deplete conditions. Osmolarity fluctuations were generated by modulating the NaCl content in the GCB media growth media. NaCl content in normal GC broth is denoted 86 mM NaCl. (A) *tbpB* transcript levels. NaCl content in normal GCB media is shown in the solid black bars. (B) *tbpA* transcript levels. NaCl content in normal GCB media is shown in the solid grey bars. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days.

A.



B.

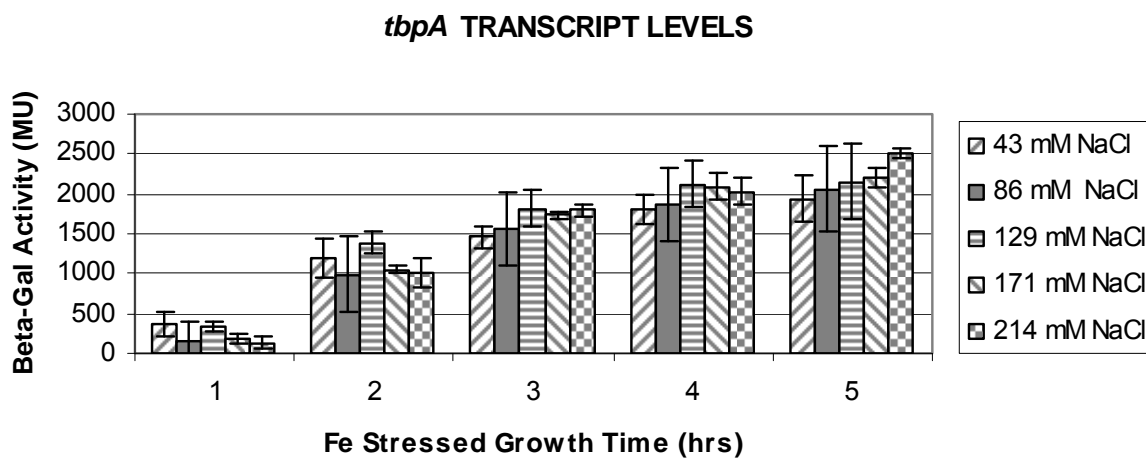
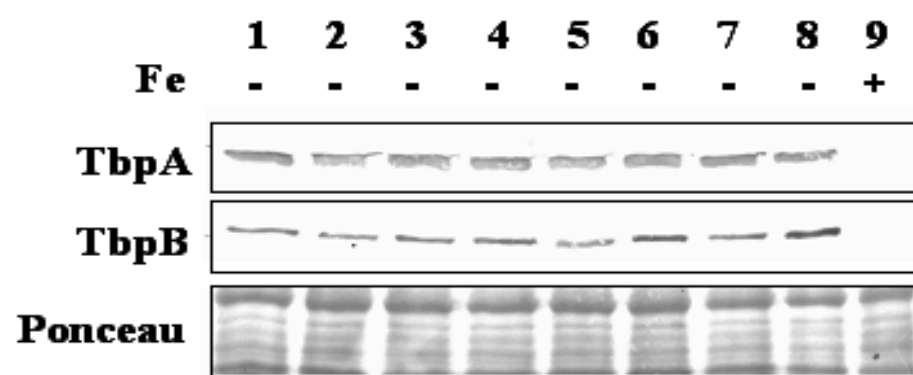


Figure 14. Tbp Protein Expression in Response to Osmolarity Fluctuations in NaCl-Modified GCB Media. Whole cell lysates were prepped from cultures subjected to 4 hours of iron and NaCl stress. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish equal loading. Lanes 1, 3, 5, and 7 show gonococci cultures grown in GCB media containing 86 mM NaCl. Lanes 2, 4, 6 and 8 show gonococci grown in GCB media grown in 43 mM, 129 mM, 171 mM and 214 mM NaCl, respectively. Lane 9 shows gonococcal culture grown in iron-replete conditions and GCB media containing 86 mM NaCl, and serves as a negative control. Results are representative of at least 3 experiments done on different days.



statistically significant changes in *tbpB* transcript levels when compared to the levels detected with the standard GC media (Figure 15A). Similarly, no statistically significant changes were detected in *tbpA* transcript levels (Figure 15B). Effects of osmolarity changes modulated by glucose were also assayed at the protein level, with wild-type strain FA19 cultured in the different glucose-modulated GCB media under iron-stress for four hours. Results show that there was no detectable change in either TbpB or TbpA expression in any cultures grown with the osmolarity-altered GCB media (Figure 16). These results support the effects seen at the transcript level.

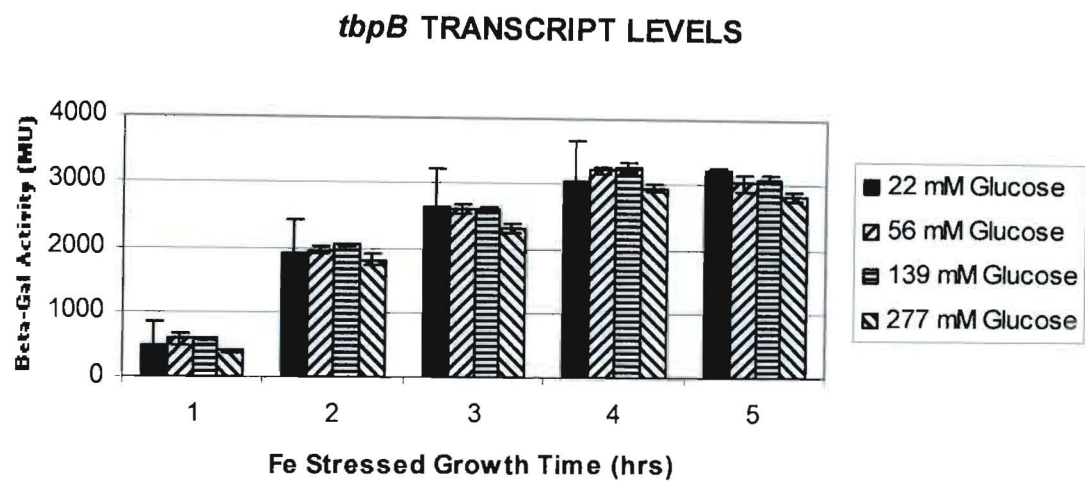
Additionally, glucose-modulation of the GCB media served a two-fold purpose, as these results could also be interpreted as changes in carbon source availability. Thus, it can be extrapolated that increases in carbon availability do not affect expression of the *tbpBA* operon.

B. cAMP Availability

Cyclic adenosine monophosphate (cAMP) is an important secondary messenger involved in many essential cell processes. cAMP is known to be involved in the regulation of some virulence factors in bacteria, many of which are mediated through the cAMP receptor protein (Crp) (75, 277). Crp is a transcriptional factor whose conformation and activity are dependent on cAMP. In the presence of cAMP, Crp is able to complex with cAMP and adopts a conformation that allows for binding to the Crp binding site in the DNA of target genes (14, 30, 60, 85). Crp is capable of acting as an activator (248) and repressor, and some of the Crp-regulated genes in *E. coli* are involved in catabolite repression, flagellum biosynthesis, enterotoxin production and iron acquisition (23). It has been shown that in *E. coli*, maximal expression of the *cir* gene, which encodes the receptor for the toxin colicin I but can also serve as

Figure 15. *tbpBA* Transcript Levels in Response to Osmolarity Fluctuations in Glucose-Modified GCB Media. Both panels show gonococci grown in iron-deplete conditions. Osmolarity fluctuations were generated by modulating the glucose content in the GCB media growth media. Glucose content in normal GC broth is denoted 22 mM glucose. (A) *tbpB* transcript levels. Glucose content in normal GCB media is shown in the solid black bars. (B) *tbpA* transcript levels. Glucose content in normal GCB media is shown in the solid grey bars. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days.

A.



B.

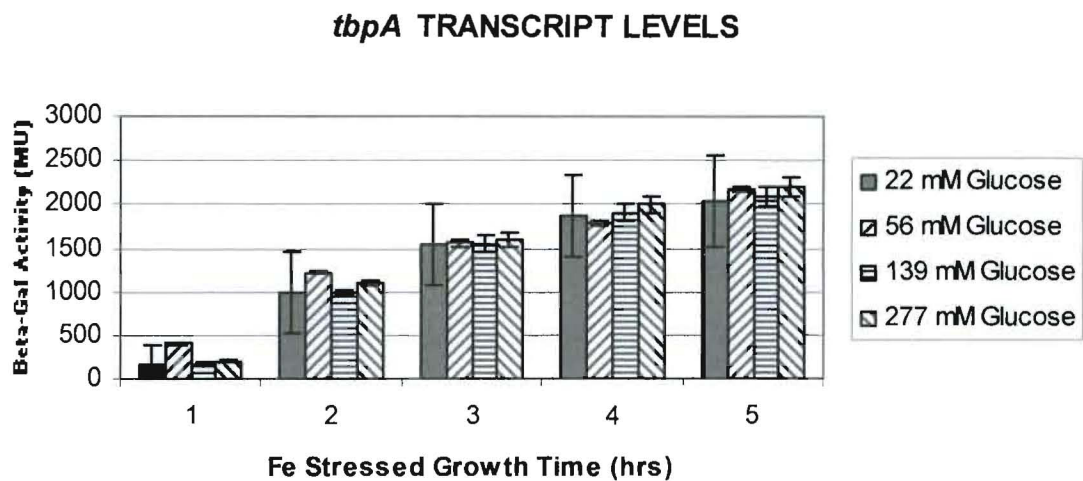
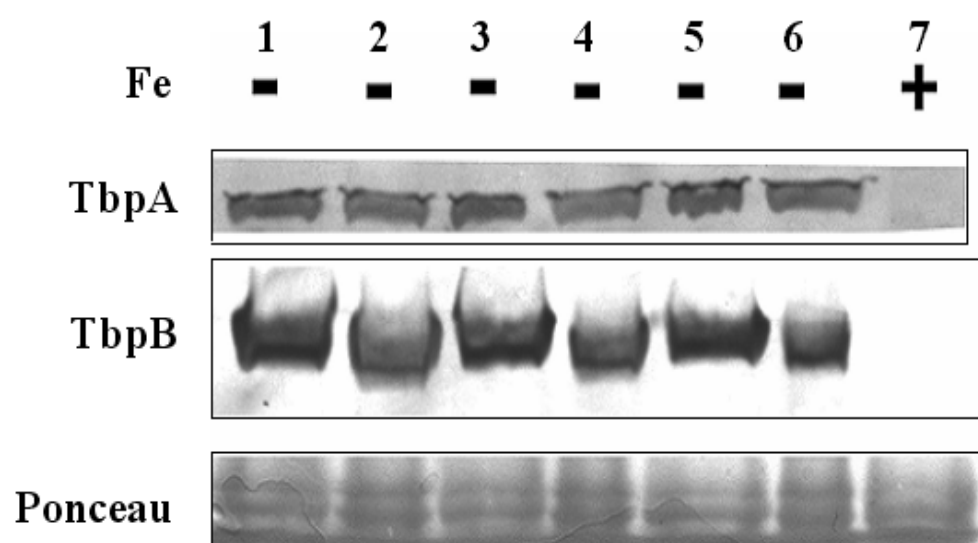


Figure 16. Tbp Protein Expression in Response to Osmolarity Fluctuations in Glucose-Modified GCB Media. Whole cell lysates were prepped from cultures subjected to 4 hours of iron and glucose stress. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish equal loading. Lanes 1, 3, and 5 show gonococci cultures grown in GCB media containing 22 mM glucose. Lanes 2, 4, and 6 show gonococci grown in GCB media grown in 56 mM, 139 mM, and 277 mM glucose, respectively. Lane 9 shows gonococcal culture grown in iron-replete conditions and GCB media containing 86 mM NaCl, and serves as a negative control.

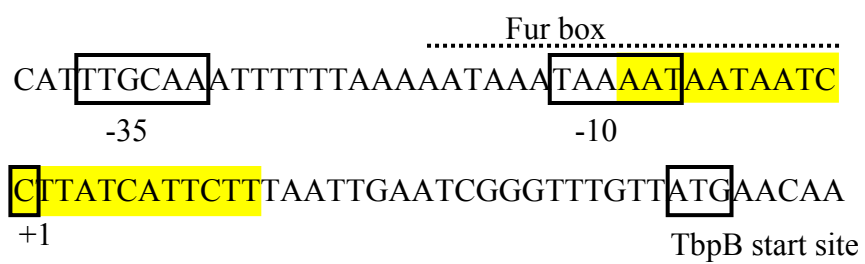


a siderophore receptor, is dependent on a critical concentration of cAMP, regardless of the simultaneous state of repression of the gene by iron (94). Additionally, Zhang *et al.* (274) have demonstrated that there are important interactions between the carbon and iron utilization regulators (Crp and Fur) and that some iron transport genes are subject to dual control by both of these regulators. Moreover, previous studies have shown that Crp is capable of modulating *fur* transcription (66).

Functional interactions between these regulons are important in the coordination of activities of the different metabolomes so that abundance of nutrients match, in a manner that ultimately benefits the cell. Thus, we sought to determine the effects of cAMP availability on expression of the operon. In order to consider cAMP regulation a possibility for the operon, identification of a Crp binding site in the vicinity of the operon was necessary. The Virtual Footprint software (171) was utilized for this purpose. This is a new suite for recognizing single or composite DNA patterns. It was especially designed to analyze potential or consensus transcription factor binding sites in whole bacterial genomes and their underlying regulatory networks. The genome sequence of gonococcal strain FA1090 (ATCC7008825) was utilized for this analysis. A potential Crp binding site was identified in the region upstream of the operon (Figure 17). This site matched the *E. coli* consensus sequence (5-AAATGTGAN₆TCACATTT-3') at 9 of 16 bases. Interestingly, the potential site also overlapped with the portions of the Fur box and the -10 region, and extended past the transcriptional start site (+1) for the operon.

Gonococcal strains MCV108 (*tbpA-lacZ*) and MCV109 (*tbpB-lacZ*) were grown in GCB media under iron stress, as stated previously in Methods, and supplemented with 20 mM cAMP (Sigma). β -galactosidase assays results show that there were no statistically significant changes

Figure 17. Identification of Potential Crp Binding Site. A portion of the region upstream of the operon is shown. The previously identified promoter elements are shown boxed (TbpB start site, -10, and -35) or highlighted by the dashed line (Fur box). The potential Crp binding site was identified using the Virtual Footprint (171) software and is shown highlighted in yellow.



in *tbpB* transcript detection when the media was supplemented with 20mM cAMP (Figure 18A). Similarly, when *tbpA* transcripts were assayed, there was no statistically significant difference in the abundance of transcripts detected in the presence of 20 mM cAMP. Gonococcal strain FA19 was subjected to 4 hours of iron stressed growth in the presence or absence of 20 mM cAMP, and its effects assayed at the protein level. Detection with anti-Tbp antibodies showed no difference in the abundance of either TbpA (Figure 19, top panel) or TbpB (Figure 19, second panel) with or without the addition of 20 mM cAMP, which correlates with the results of the transcriptional analysis.

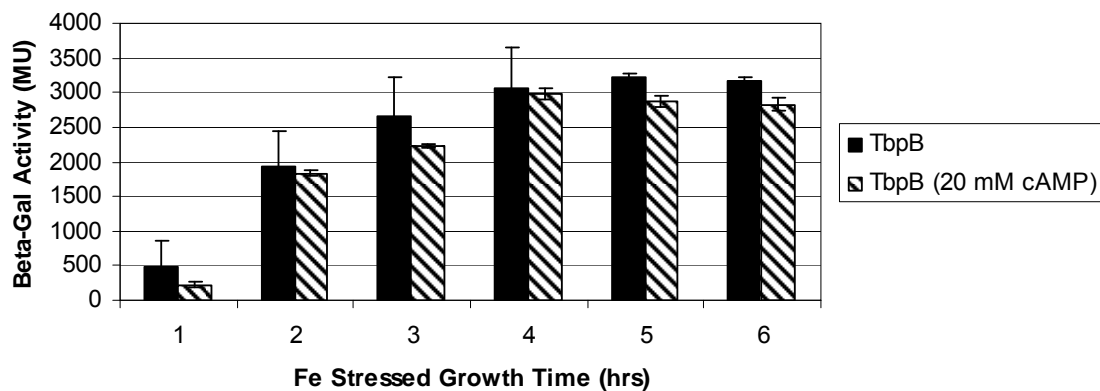
These results also correlate with the results obtained with the glucose-modulated media (Figures 15 and 16). Increase in glucose levels leads to a decrease in cAMP levels (154, 185). Thus, by increasing the glucose concentration of the media (56 mM, 139 mM and 277 mM glucose), we have tested several concentrations that lower cAMP availability. Addition of 20 mM cAMP thus allowed for the testing of the other side of the spectrum, increase in cAMP availability. Although the identified Crp binding site is a good match and overlaps several key promoter elements, we conclude that the operon is not under the control of Crp, and therefore, is not responsive to cAMP fluctuations.

C. Hydrogen Peroxide Stress

The primary sites of infection by *N. gonorrhoeae* are the urethral epithelium in males (6, 48) and the ecto- and endocervical epithelia in females (73). There, gonococci are exposed to a variety of oxidants, which may result from its own metabolism and from host defense mechanisms. Activated polymorphonuclear leucocytes, which are involved in the innate immune response against *N. gonorrhoeae* (8), can generate superoxide (O_2^-) and H_2O_2 as part of their bactericidal mechanism (33, 98). It is also important to remember that the gonococci will

Figure 18. *tbpBA* Transcript Levels in Response to Increased cAMP Availability. Both panels show gonococci grown in iron-deplete conditions with (striped bars) or without (solid bars) 20 mM cAMP. (A) *tbpB* transcript levels. (B) *tbpA* transcript levels. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days.

A.

***tbpB* TRANSCRIPT LEVELS**

B.

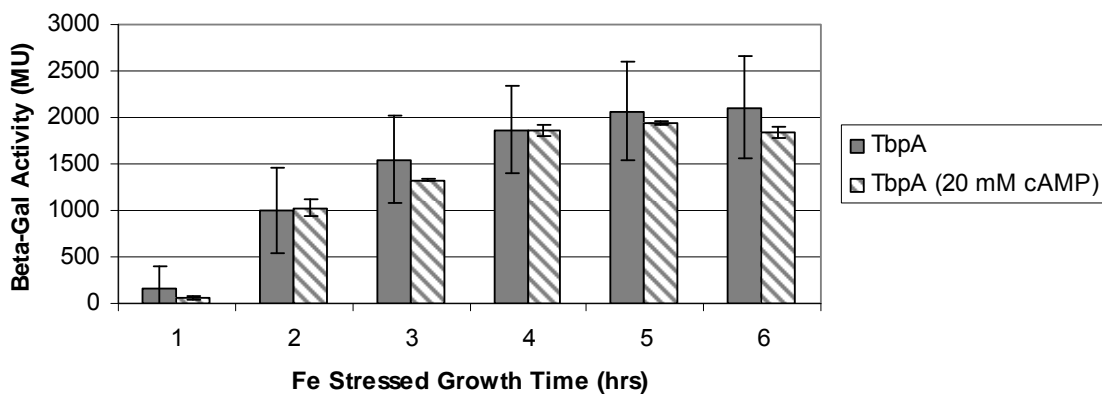
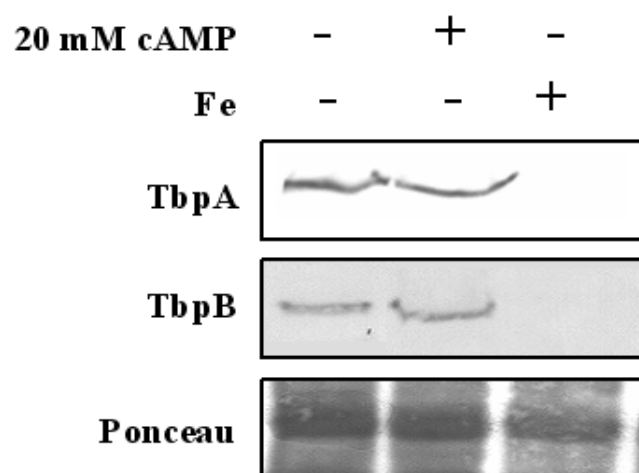
***tbpA* TRANSCRIPT LEVELS**

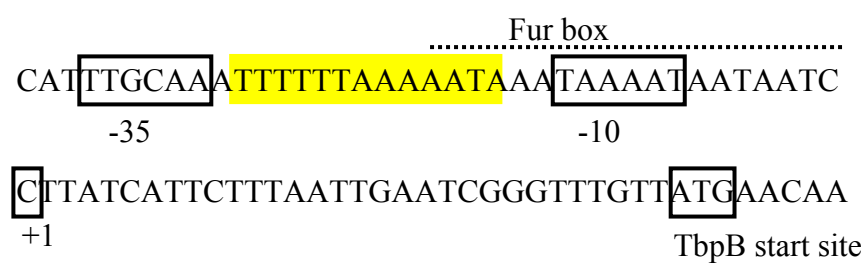
Figure 19. Tbp Protein Expression in Response to Increased cAMP Availability. Whole cell lysates were prepped from cultures subjected to 4 hours of iron stress and with or without addition of 20 mM cAMP. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish loading. Results are representative of at least 3 experiments done on different days.



not be the only microorganisms inhabiting these surfaces. For example, Lactobacilli are the most numerous microorganisms that form part of the indigenous vaginal flora in healthy, premenopausal women (5, 181, 198). Studies have demonstrated an inverse correlation in vaginal colonization between gonococci and H₂O₂-producing *Lactobacillus* species (5, 269), and some of these Lactobacilli commonly isolated from the vagina and the cervix have been shown to inhibit gonococci in vitro (5, 230), with H₂O₂ identified as the significant inhibitory factor (230, 275).

The oxidants, or reactive oxygen species (ROS), that the gonococci are exposed to can damage DNA, lipids and proteins (118). Bacteria must regulate gene expression in response to these environmental factors to minimize dangerous effects. Hydrogen peroxide is sensed by OxyR, a member of the LysR family of DNA-binding transcriptional modulators (45). In *N. gonorrhoeae* OxyR is known to repress catalase (*kataA*) expression in response to low levels of H₂O₂ (246). A study was published by Stohl *et al.* (235) in which the gonococcal transcriptome response to hydrogen peroxide was examined. Their results show that the *tbps* were amongst the genes that were upregulated in response to hydrogen peroxide. However, it should be noted that the experimental conditions for this study exposed the gonococci to hydrogen peroxide stress for only 15 minutes, and thus, there is a possibility that changes in gene expression and mRNA stability may have been overlooked due to the short exposure time, and the single time point analysis (222). Nonetheless, it is reasonable to postulate that iron-acquisition genes may be regulated in response to H₂O₂ stress. Lowering iron uptake during oxidative stress could reduce potential damage caused by Fenton chemistry. The effect of H₂O₂ stress on expression of the *tbpBA* operon was examined at the transcriptional level (with transcriptional *lacZ* fusions MCV108 and MCV109) and at the protein level.

Figure 20. Identification of Potential OxyR Binding Site. A portion of the region upstream of the operon is shown. The previously identified promoter elements are shown boxed (TbpB start site, -10, and -35) or highlighted by the dashed line (Fur box). The potential OxyR binding site was identified using the Virtual Footprint (171) software and is shown highlighted in yellow.

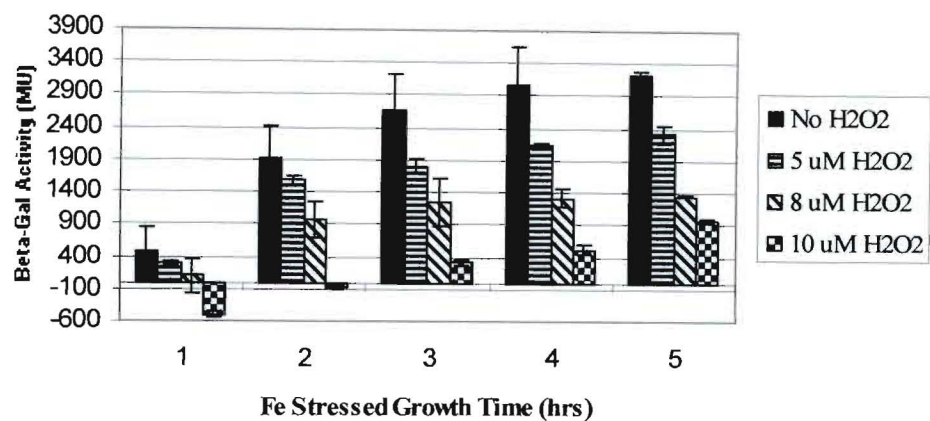


Identification of a potential OxyR binding site in the proximity of the operon was accomplished using the Virtual Footprint program (171). The identified binding site appeared to fit the pattern of the generic motif used to identify binding sites for members of the LysR family of transcriptional regulators, T-N₁₁-A (84, 140) (Figure 20). The potential OxyR box was located between the -35 and -10 regions and overlapped with the identified Furbox at 3 base pairs, which meant that OxyR could potentially have transcriptional control of the operon.

The effect of H₂O₂ stress on transcription of the operon was investigated by the use of β -galactosidase assays. Cultures were iron stressed and hydrogen peroxide was added. Previous studies in *Neisseria* have utilized H₂O₂ concentrations ranging from 200 μ M (93) to 5mM (235) but only for very short periods of time. In this study, several concentrations of H₂O₂ were tested: 5 μ M, 8 μ M and 10 μ M (Figure 21). Addition of 5 μ M H₂O₂ resulted in no statistically significant changes in *tbpB* or *tbpA* transcripts between the hydrogen peroxide-stressed cultures and the untreated cultures. Addition of 8 μ M and 10 μ M H₂O₂ resulted in significantly decreased *tbp* transcript levels (Figure 21), but these concentrations also resulted in severely decreased gonococcal growth over time (Figure 22). Although decreased transcripts and growth were detected, the effects of hydrogen peroxide stress were assayed at the protein level. Gonococci were grown in the presence of 8 μ M hydrogen peroxide and iron stressed for 4 hours. This concentration was chosen as it exhibited decreased growth and transcript detection over time. Western blot analysis showed that there was no detectable change in either TbpA (Figure 23, top panel) or TbpB (Figure 23, middle panel) expression in the presence or absence of hydrogen peroxide. Even though whole cell lysates are standardized to a constant cell number and the β -galactosidase assay takes into consideration cell density, and it is highly probable that the decreases in transcript levels observed by means of the β -galactosidase assay are due to

Figure 21. *thpBA* Transcript Levels in Response Oxidative Stress. Both panels show gonococci grown in iron-deplete conditions with (striped bars) or without (solid bars) hydrogen peroxide. (A) *thpB* transcript levels. (B) *thpA* transcript levels. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments conducted on different days.

A.

tbpB TRANSCRIPT LEVELS

B.

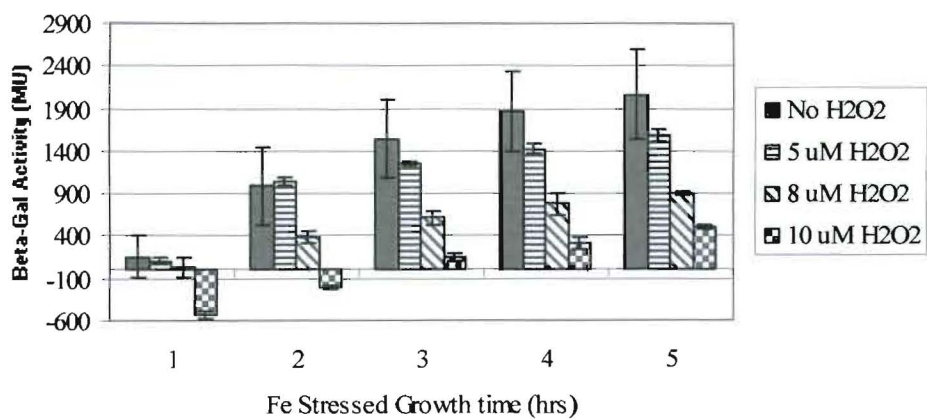
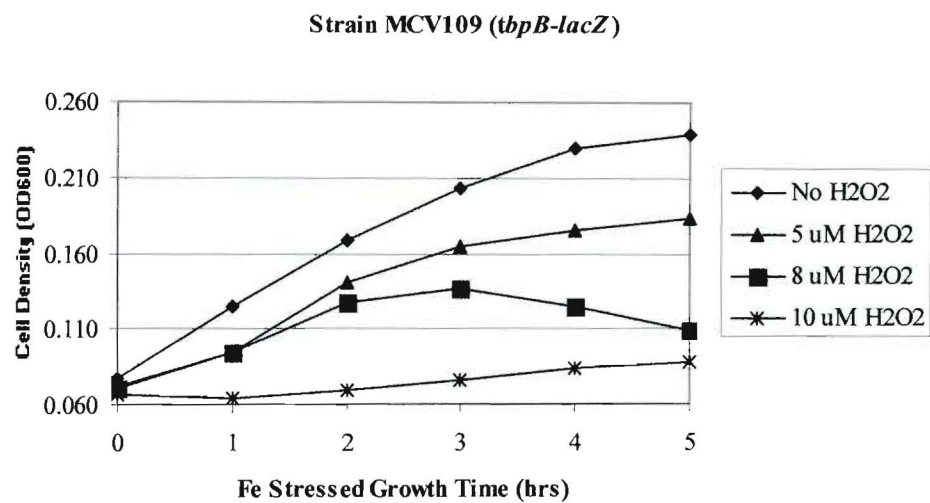
tbpA TRANSCRIPT LEVELS

Figure 22. Growth Curves of Gonococcal Cultures Grown With or Without Hydrogen Peroxide. Strains MCV109 (*tbpB-lacZ*) and MCV108 (*tbpA-lacZ*) were subjected to iron-stressed growth with and without H₂O₂ at the concentrations shown. Growth was monitored by optical density (OD) readings at 600 nanometers. Results represent the means of at least 3 experiments done on different days.

A.



B.

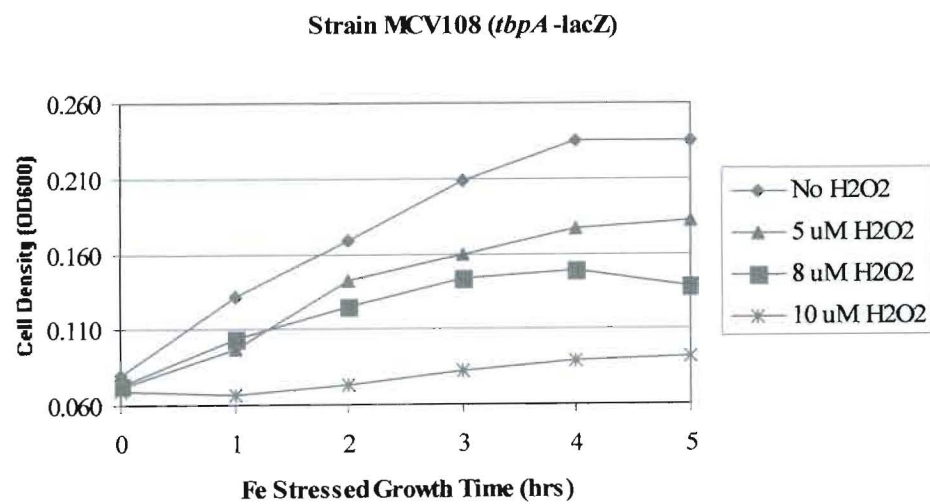
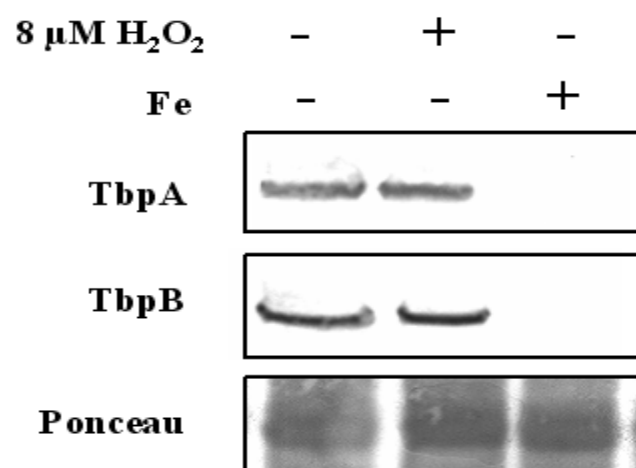


Figure 23. Tbp Protein Expression in Response to Hydrogen Peroxide. Whole cell lysates were prepped from cultures subjected to 4 hours of iron and with or without addition of 8 μ M H₂O₂. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish loading. Results are representative of at least 3 experiments done on different days.



differences in growth rates in the presence of H_2O_2 rather than to actual OxyR regulation of the operon.

Taken together, these data suggest that the operon is not under the regulation of OxyR. Further experiments that would confirm these findings would include mutagenesis of the identified OxyR box. Additionally, more subtle increases in the concentration of H_2O_2 to which the gonococci are exposed could help identify a concentration at which growth is not inhibited and effects on the operon could be more clearly discerned.

D. Oxygen Availability

Although generally classified as an aerobic microorganism (252), *N. gonorrhoeae* is capable of surviving and proliferating in the genitourinary tract with other obligate anaerobes (228). The first steps of gonococcal infection include adherence to epithelial cells (239) and internalization (160). The mucosal layer of the urogenital tract provides a barrier that the gonococci must penetrate in order to adhere to the underlying epithelial cell layer (162). *N. gonorrhoeae* is not capable of growing anaerobically by fermentation (120, 270), but it can grow anaerobically if nitrite is present as an alternate electron acceptor (137).

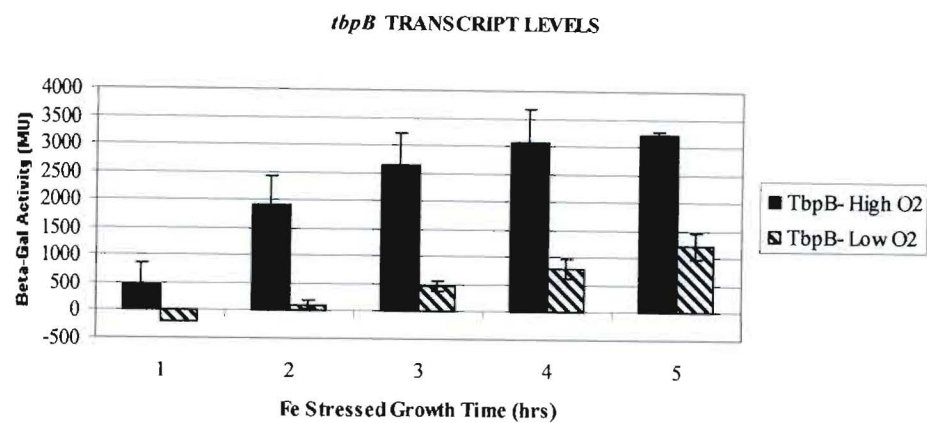
It has been previously shown that exposure to anaerobic conditions can alter the expression of several gonococcal outer membrane proteins (46). One of these proteins, and the most documented case, is *aniA*, which encodes a nitrite reductase (162). *aniA* expression is under the control of the fumarate and nitrate reduction transcriptional regulator (FNR) (46). FNR is a global transcription factor that is known to mediate the transition from aerobic to anaerobic environments in many bacteria. In *E. coli*, FNR is known to sense oxygen deprivation and respond by altering transcription of many genes (50, 88, 126, 211), acting as both an activator and a repressor (57, 58, 123, 216).

Western blot analyses with sera from patients with confirmed gonococcal infection have demonstrated an antibody response against AniA (47), thus suggesting that AniA is expressed in the host and that anaerobic conditions are encountered by the gonococci during in vivo infection. Adaptation to oxygen-limiting conditions appears to be important in the virulence of *N. gonorrhoeae*. Control of iron acquisition genes in response to oxygen availability would be important, not only as a way of limiting excess iron acquisition that could end up unused inside the gonococci and available for detrimental Fenton chemistry reactions, but also from an energetic point of view, limiting the energy spent on synthesizing proteins that have a decreased functionality under such environmental stimuli.

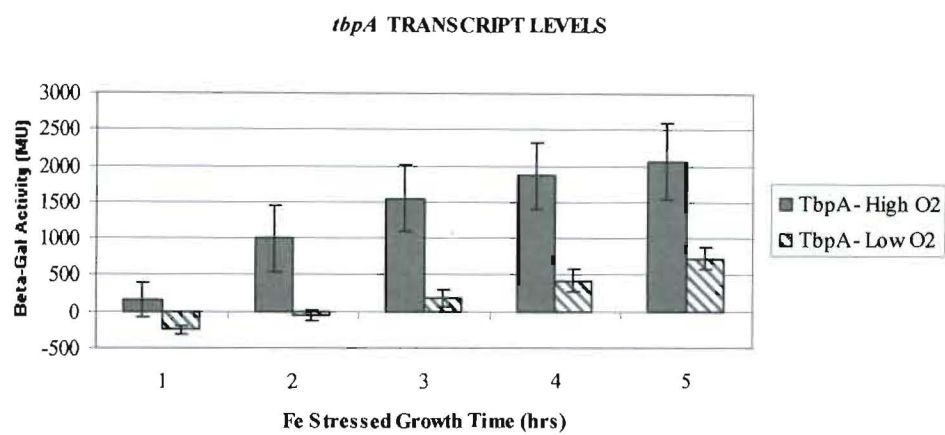
With so much evidence supporting the concept that gonococci do encounter oxygen-limiting conditions during infection and the importance of iron acquisition control during inhabitation in such an environment, we sought to determine the effects of oxygen limitation on expression of the *tbpBA* operon. Maximal *tbp* expression has been achieved in the laboratory by subjecting gonococcal cultures to vigorous shaking at 37°C in a 5% CO₂ atmosphere. In order to generate low oxygen conditions GCB media was supplemented with the oxygen scavenger Oxyrase® for broth (Oxyrase Inc.) as suggested by the manufacturer. Oxyrase for broth is a commercially available biocatalytic agent that is capable of removing oxygen from liquid media (1) and has been used to generate anaerobic conditions in other studies (193). Additionally, Wade and Graver have designed a growth media that allows for the anaerobic growth of *Neisseria gonorrhoeae* by the addition of Oxyrase (255). Shaking was eliminated, and the tops of the culture flasks were parafilmed to reduce entry of oxygen. Because there is still head space available in the culture flasks, we refer to the addition of oxyrase as low oxygen conditions rather than completely anaerobic.

Figure 24. *tbpBA* Transcript Levels in Response to Low Oxygen. Both panels show gonococci grown in iron-deplete conditions. Low oxygen conditions were generated by addition of an oxygen scavenger to the growth media. (A) *tbpB* transcript levels. Maximal *tbpB* expression obtained under high oxygen conditions is shown in the solid black bars. (B) *tbpA* transcript levels. Maximal *tbpA* expression under high oxygen conditions is shown in the solid grey bars. (C) Comparison of *tbpB:tbpA* transcript ratios under varying oxygenic conditions. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days. Asterisks denote statistical significance between *tbpB* and *tbpA* transcripts under one same condition at the indicated time point.

A.



B.



C.

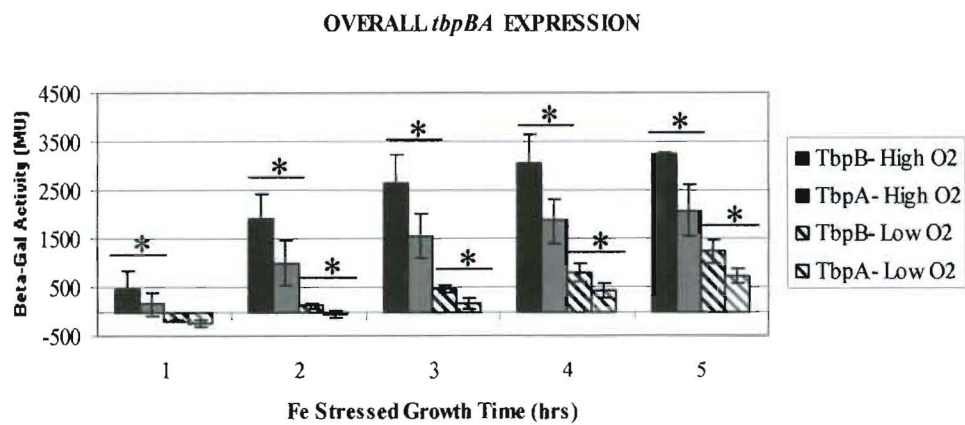
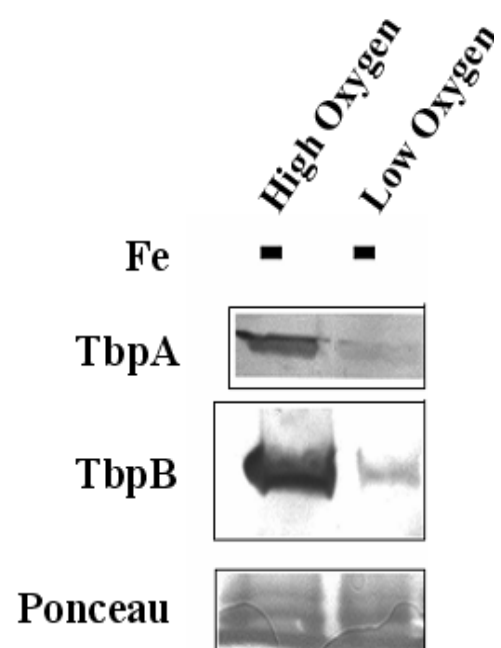


Figure 25. Tbp Protein Expression in Response to Low Oxygen. Whole cell lysates were prepped from cultures subjected to 4 hours of iron stress and high or low oxygen conditions. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish loading. Results are representative of at least 3 experiments done on different days.



First, the effects of low oxygen conditions on transcript levels were assayed by employing the transcriptional *lacZ* fusions (MCV108 and MCV109). Results show that there was decreased transcript abundance for both *tbpB* (Figure 24A) and *tbpA* (Figure 24B) in low oxygen conditions relative to high oxygen conditions. This represents an overall decrease in transcript levels for the operon by 2 - 5 fold, depending on the gene and the time point considered. The 2:1 *tbpB:tbpA* ratio previously detected under iron stress remained unchanged under the low oxygen conditions (Figure 24C). These results were corroborated at the protein level by subjecting the wild-type strain FA19 to low or high oxygen conditions during growth. Western blot analysis shows that there is a marked decrease in TbpB and TbpA protein when the gonococci are exposed to a low oxygen environment (Figure 25).

Because gonococcal anaerobic growth has been shown to be coupled to nitrite reduction, we also wanted to confirm that the results described above were not due to growth defects. For this purpose, the transcriptional *lacZ* fusions were grown under low oxygen conditions, as described in Methods, in addition to supplementing the growth media with 2 mM NaNO₂ (110). Supplementation of the media with NaNO₂ resulted in similar growth curves and rates for all cultures (Figure 26). Additionally, decreased transcript abundance was detected for both *tbpB* (Figure 27A) and *tbpA* (Figure 27B) in low oxygen plus NaNO₂ growth conditions when compared to the high oxygen conditions. As seen previously, this represented an overall decrease in transcript levels for the operon, and the 2:1 *tbpB:tbpA* ratio remained unchanged (Figure 27C). Although growth with low oxygen and NaNO₂ resulted in decreased cell density as compared to the high oxygen growth conditions, the β -galactosidase assay is normalized to final optical density of the cultures. Additionally, shown in Figure 26, it is clear that growth rates for the high oxygen cultures are very similar those that of the low oxygen and NaNO₂

cultures, all owing us to conclude that decreased transcript levels were not solely due to decreased growth rates.

III. Discussion

Gene regulation in response to environmental stimuli is necessary for the survival of microorganisms. This study was designed to analyze how the gonococci modulate expression of the Tbps, important in iron acquisition, survival and establishment of infection, in response to environmental stimuli.

To our knowledge, no studies have been conducted on how changes in osmolarity affect gene expression in the gonococci. However, it is highly likely that the gonococci do experience changes in osmolarity when transitioning in between the different microenvironments found in the human host. Reports have been published elucidating the differences in osmolarity between the sera and the seminal fluid (187, 208). Osmolarity is also bound to be altered as the gonococci travel from the lower to upper genital tract, from the female to the male genital tract, and from the extracellular to the intracellular milieu. Additionally, no two hosts of the same sex are likely to present the exact same osmolarity patterns, for example, people with diabetes and/or with hyperosmolar, hyperglycemic nonketotic syndrome have been shown to have a different sera and urine osmolarity than that of people without the disease because of altered sugar levels in both of these bodily fluids (64, 80, 81, 236). Although the gonococci are prone to experience more subtle changes in osmolarity than other human pathogens that can survive outside of the host, such as *E. coli* or *V. cholerae*, evidence suggests that variable conditions are likely encountered during natural infection. Our studies with osmolarity modulation, either by altering

Figure 26. Growth Curves of Gonococcal Cultures Grown in High Oxygen Conditions or in Low Oxygen and 2 mM NaNO₂ Conditions. Strains MCV109 (*tbpB-lacZ*, shown as TbpB) and MCV108 (*tbpA-lacZ*, shown as TbpA) were subjected to iron-stressed growth in a high oxygen environment or low oxygen and 2 mM NaNO₂ environment. Growth was monitored by optical density (OD) readings at 600 nanometers. Results represent the means of at least 3 experiments done on different days.

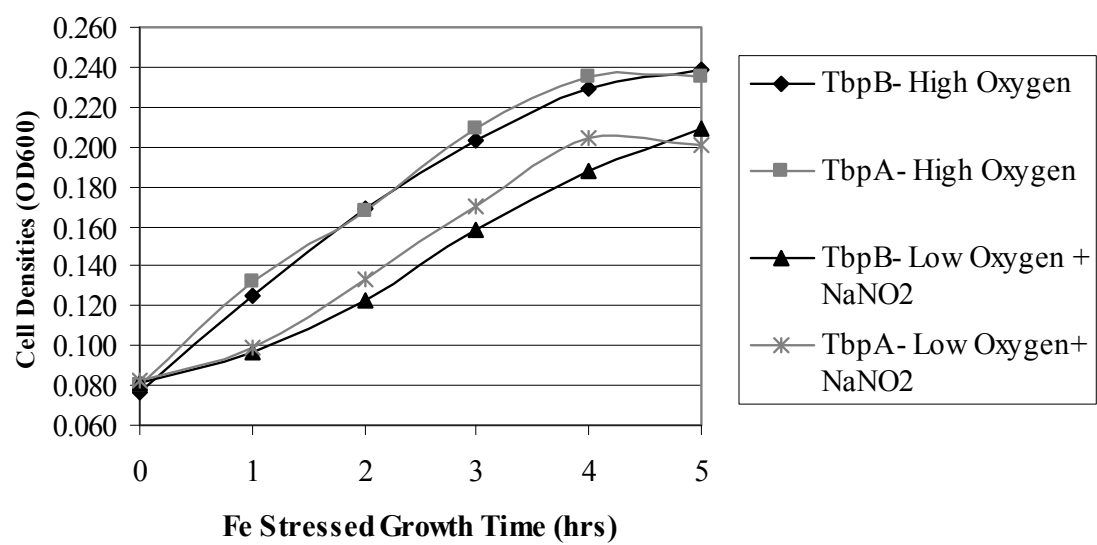
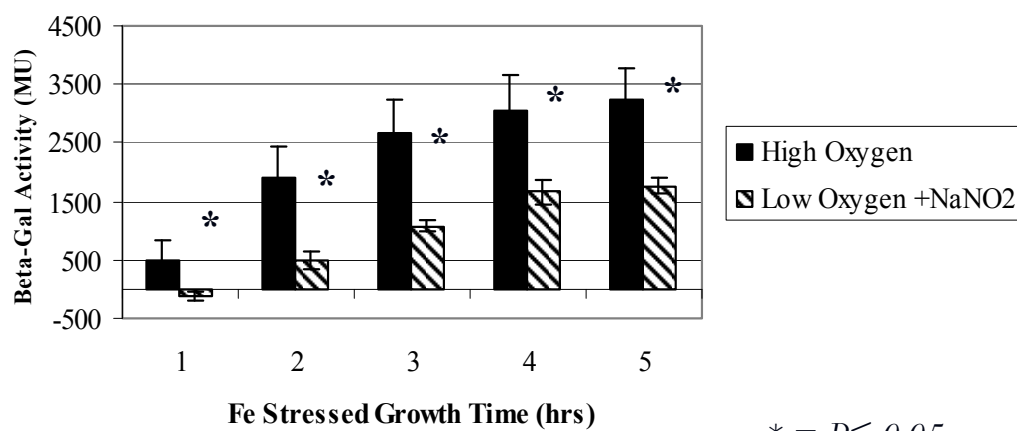
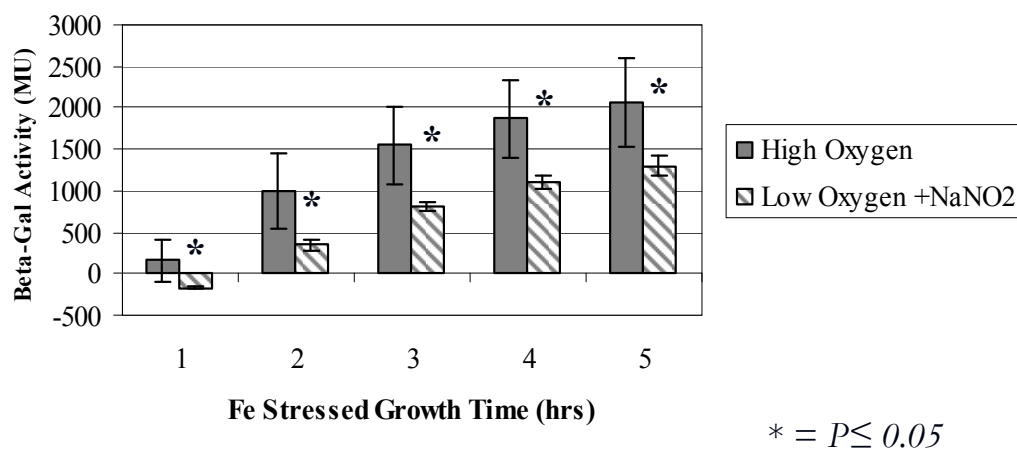


Figure 27. *tbpBA* Transcript Levels in Response to Low Oxygen and 2mM NaNO₂. Both panels show gonococci grown in iron-deplete conditions. Low oxygen conditions were generated by addition of an oxygen scavenger to the growth media and 2 mM NaNO₂. (A) *tbpB* transcript levels. Maximal *tbpB* expression obtained under high oxygen conditions is shown in the solid black bars. (B) *tbpA* transcript levels. Maximal *tbpA* expression under high oxygen conditions is shown in the solid grey bars. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days.

A.

***tbpB* TRANSCRIPT LEVELS**

B.

***tbpA* TRANSCRIPT LEVELS**

C.

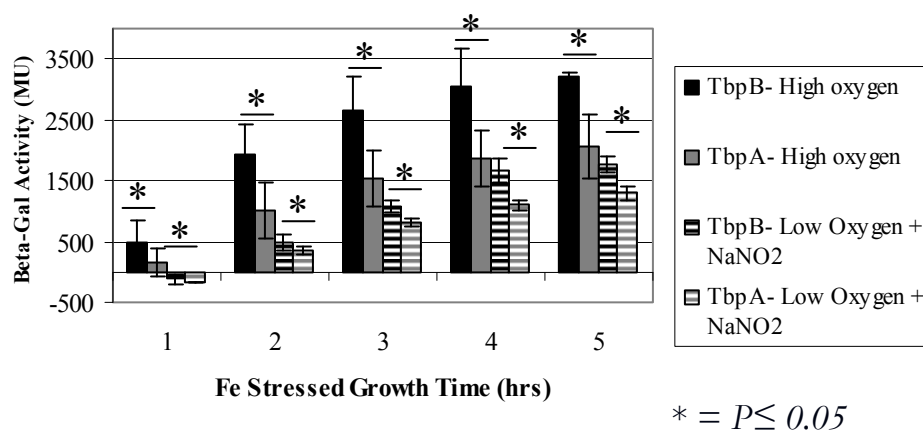
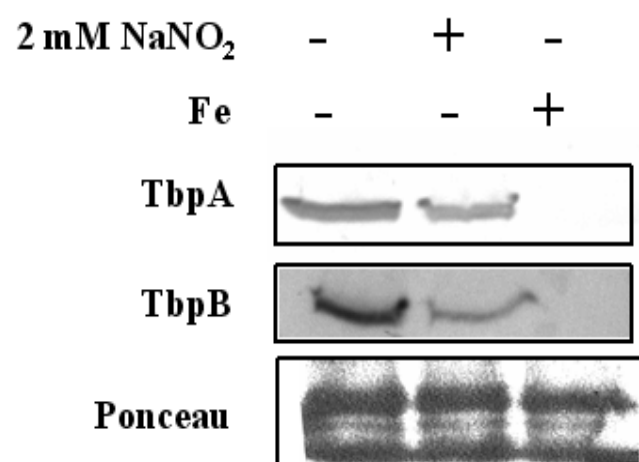
OVERALL *tbpBA* EXPRESSION

Figure 28. Tbp Protein Expression in Response to Low Oxygen and 2 mM NaNO₂. Whole cell lysates were prepped from cultures subjected to 4 hours of iron stress and high oxygenic conditions or low oxygenic conditions supplemented with 2 mM NaNO₂. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish loading. Results are representative of at least 3 experiments done on different days.



the NaCl or the glucose concentration during growth, show that *tbp* expression is not altered by increases or decreases in osmolarity. Although TbpA does form a pore in the outer membrane, it is highly specific for transferrin iron internalization, and its N-terminal plug domain is predicted to fold up within the β -barrel domain to occlude the pore. Thus, it is conceivable that expression of TbpA does not change in response to osmolarity. It would be interesting to elucidate if expression of other outer membrane proteins, such as porins, which do not have a specific ligand and that are important in virulence (17, 168, 261), are altered in response to this environmental signal as they are in *E. coli*.

The studies done with glucose modulation served a two-fold purpose, for they not only allowed for elucidation of the effects of osmolarity changes, but also allowed us to determine that changes in carbon source availability did not affect Tbp expression. These results were complemented by the results of the increased cAMP availability study. Because of the inverse correlation between glucose and cAMP levels (154, 185), we have tested a wide range of settings when dealing with carbon source availability. In either end of spectrum, high glucose and low cAMP (277 mM glucose) or high cAMP and low glucose (20 mM cAMP) there were no changes in *tbp* expression detected.

The effects of hydrogen peroxide on expression of the operon were also analyzed. It was important to consider this environmental stimulus in relation to iron acquisition, as Fenton chemistry reactions between iron and hydrogen peroxide can lead to more toxic reactive oxygen species. Since H_2O_2 can penetrate membranes, H_2O_2 stress arises inside cells whenever it is present in the extracellular habitat (117). Thus, it is very probable that iron acquisition genes could be regulated in response to the presence of the oxidant. Our studies with exposure to hydrogen peroxide showed that the gonococci are highly sensitive to this oxidant. Even at

concentrations lower than those used in other published studies (93, 235), gonococcal cultures to which H₂O₂ had been added showed decreased survival over extended periods of time.

However, lowered *tbpBA* transcripts were detected in response to H₂O₂ stress. When protein levels were assayed, no change in expression of either Tbp was detected in response to oxide stress. Because whole cell lysates are standardized to constant cell number, and the β -galactosidase assays take into consideration cell density, it is highly likely that the decrease in transcripts detected were due to differences in growth rates of the cultures, rather than to regulatory effects in response to the oxidant. Our results are in disagreement with those published by Stohl *et al.* (235) in which *tbp* expression was found to be increased in response to hydrogen peroxide. In their study, the group exposed gonococci to 5 mM H₂O₂ for fifteen minutes before isolating RNA to be used in microarray studies. Although this time frame was enough to detect changes in the mRNA, it does not correlate to the in vivo infection scenario, where gonococci might be exposed to prolonged periods of time under H₂O₂ stress.

Additionally, it is hard to consider what amount of hydrogen peroxide is physiologically relevant, because although reports have been published on the importance of hydrogen peroxide in the immune response (33, 98) and in the survival of competing microorganisms in the vaginal flora (5, 269), it is unknown what concentrations of the oxidant the gonococci are exposed to when all of these components come together simultaneously.

Decreased transcripts and proteins were detected as a result of exposure to oxygen limited conditions. These results were not due to growth defects, as addition of NaNO₂ as a final electron acceptor did improve growth of the gonococci in low oxygen conditions, but still yielded the same results. Ample research has been conducted in the area of gonococcal anaerobic growth. It is known that anaerobic growth is coupled to nitrite reduction (137), and

that there are several outer membrane proteins whose expression is regulated in response to oxygen limitation (46). To date, the Tbps have not been identified as membrane proteins that are regulated in response to oxygenic variations, likely due to the fact that they were not maximally expressed (under iron limitation) in previous studies (46). In our current study we are probably mimicking the environment inside the host more authentically, as the gonococci (and bacteria in general) are likely subjected to more than one source of environmental stress at any given time, and limitation of both iron and oxygen are known to occur in the genital tract. Regulation of the Tbps in response to oxygen-limiting conditions would be important for the gonococci, as it would be beneficial to decrease iron acquisition while the nutrient is not needed in great demand if the respiratory pathway is shut down. Diminishing unused iron pools greatly reduces the risk of Fenton chemistry reaction by-products which could result in detrimental effects to protein, DNA, and numerous other cellular components.

Our results could also prove relevant in the natural infection process as *Neisseria gonorrhoeae* has been shown capable of forming biofilms (communities of bacteria intimately associated with each other and included with in an exopolymer matrix)(92), and the presence of gonococcal biofilms has been detected in biopsy specimens obtained from women with culture-proven gonococcal infection (231). Elevated AniA expression was detected in these biofilms, suggesting that gonococcal biofilms grow anaerobically or microaerobically (76). Biofilm formation may be a special adaptation to growth in the cervical environment (76); thus it is plausible to think that while embedded in the biofilm, gonococci are “protected” from the host environment, their survival not threatened, and thus do not have an increased need for iron and can downregulate *tbp* gene expression.

In an attempt to begin to decipher the mechanism by which oxygen regulation of the *Tbps* occurs, a potential FNR binding site has been identified upstream of the operon (Figure 29) by comparison with the putative FNR box found upstream of the *thpBA* operon in the closely related pathogen *N. meningitidis* (93). FNR is a well documented transcriptional regulator that mediates the transition from aerobic to anaerobic environments in many bacteria. The identified FNR binding site matched the FNR consensus sequence (5'-TTGATnnnnATCAA-3') (74) at 7 of 10 bases, and it overlaps the Fur box. A previous study that attempted to analyze the FNR regulon in *N. gonorrhoeae* did not include the *tbps* amongst their findings (268). However, the authors of this study did mention that they had trouble with transcript accumulation and were not able to detect genes that have been well characterized to be regulated by FNR. In addition, as in other studies, these were not conducted under iron stress conditions needed to maximize *Tbp* expression. Mutagenesis of the identified FNR binding site upstream of the operon could serve as a stepping stone in elucidation of a possible FNR-mediated regulatory mechanism of the *thpBA* operon.

Overall, these studies provide insight into the regulation of the *tbps* in response to the surrounding environment. The potential of these proteins as vaccine candidates makes understanding the conditions under which they are maximally expressed important, as a vaccine preparation that would include immunogens that are only expressed under laboratory-designed conditions would not be beneficial.

Figure 29. Identification of Potential FNR Binding Site. A portion of the region upstream of the operon is shown. The previously identified promoter elements are shown boxed (TbpB start site, -10, and -35) or highlighted by the dashed line (Fur box). The potential FNR binding site was identified by comparison with a potential FNR binding site found upstream of the *tbpBA* operon in the closely related pathogen *Neisseria meningitidis* (93).

Fur box
.....
CATTTGCAAATTTTTTAAAAATAAAATAAAATAATAATC
-35 -10
CTTATCATTCTTTAATTGAATCGGGTTTGTTATGAACAA
+1 TbpB start site

CHAPTER 5: Insights into the Role of Guanine Quadruplexes Found in the Non-Coding Regions Flanking the *thpBA* Operon

I. Introduction

Double stranded DNA, commonly referred to as the B-form DNA, is the main genetic molecule in most biological systems (263). However, DNA is capable of adopting several secondary structures, (197) and individual helical segments can exist in different conformations owing to the flexibility and plasticity of the molecule (263). The importance of non-B DNA motifs has been the subject of extensive research, and their role in replication, recombination, and predominantly in regulation of gene expression has been shown (11, 183, 184, 197, 225). Evidence shows that the formation of these structures is not coincidental, and that the DNA actually encodes these spatial structures, in addition to protein sequences, and *cis*-acting regulatory elements (197). This provides a mechanism for the utilization of DNA, in which sequence information has a minimal role per se, other than facilitating formation of the structural motifs (197). The role of non-B DNA in gene regulation has been shown to occur in both eukaryotes (11, 203) and prokaryotes (102).

A particular type of non-B DNA that has recently been recognized as an important regulatory motif is the G-quadruplex structure (G4, G-tetrads, or G-quartet). The formation of these secondary structures was first elucidated more than 50 years ago (82), but was thought more of as nuisance than a structure of scientific importance (34). G-quadruplexes are the result of stacking of a core element composed of four guanine bases that form hydrogen bonds with each other to generate a planar structure (245). G4 DNA forms in sequences containing at least 4

guanine runs, each of which is at least 3 nucleotides in length (although longer G-runs promote the formation of more stable structures) that are separated by other nucleotides that form the intervening loops (153). These structures present great variety in respect to the orientation of the nucleic acid strands, and the loops connecting the G-tetrads (both in orientation, and in number of intervening nucleotides) (78, 114, 245) .

G4 structures have been shown to be highly stable under physiological pH and salt conditions (108, 153) and it is predicted that there are more than 300,000 sites with G-quadruplex forming potential (QFP) in the human genome (116, 244). Some of the more researched examples of chromosomal regions that possess sequences capable of forming G-quartets include immunoglobulin heavy chain switch regions, where it is thought that these structures are involved in DNA recombination, and telomeres, where they are thought to be involved in telomere maintenance (69, 71, 115, 179). Additionally, there is evidence to support that the G4 structures are involved in the regulation of the *c-myc* oncogene (220, 223). Studies with *Saccharomyces cerevisiae* have shown that G-quadruplexes are involved in the transcriptional regulation of several genes in this microorganism (108). Until recently, detection of quartet formation in vivo had not been successful. Using G4-specific antibodies and electron microscopy, Paeschke *et al.* (178) have been able to confirm the formation of G4 quadruplexes in vivo in the telomeric DNA of the ciliated protozoan *Stylonychia lemnae*.

In prokaryotes, study of G4 DNA has been mostly limited to studies in *E. coli* where the existence of G4 DNA in vivo has been confirmed (70), and the role of RecQ helicase in the unwinding of G4 motifs has been elucidated (271). Rawal *et al.* used a pattern recognition program to search 18 prokaryote genomes for G4 DNA and analyzed their distribution and association with genes (197). Their results indicated enrichment of G4 DNA in putative

promoter regions, and detailed analysis of *E. coli* G4 sequences suggested a role for these motifs in activation of transcription during growth phase.

Recently, a study was published by Cahoon *et al.* (36), in which G4 DNA was found to be necessary for pilin antigenic variation in *N. gonorrhoeae*. Pilin antigenic variation is the result of high frequency gene conversion between one of many silent *pilS* loci and *pilE*, the single expressed pilin locus (97, 163, 164, 221). In their study, the group was able to identify a G4-forming sequence in the region upstream of *pilE* and using circular dichroism, were able to confirm that the predicted structure does indeed form in the gonococcus. They were also able to determine that mutation of any of the 12 guanine-cytosine (GC) base pairs in the identified 16 base pair G4- forming sequence inhibited pilin antigenic variation. To investigate the role of the G4 structure in live gonococci, Cahoon *et al.* added N-methyl mesoporphyrin IX (NMM) to GC media plates and assayed the effects on pilin antigenic variation. NMM is a porphyrin derivative that has been shown to be able to enter live cells and specifically bind G4 DNA but not double-stranded DNA (3, 9, 148, 199, 271). Bacterial growth on NMM resulted in decreased pilin antigenic variation, and further investigation demonstrated that the nicks necessary to initiate homologous recombination are the result of the G4 structure. Addition of NMM inhibited the detection of nicks in the DNA strand that contains the G4-forming sequence. Overall, the group concluded that an intact G4-forming sequence is required to produce nicks in the G4 DNA, and in turn, that the G4 structure is a specialized recombination initiation sequence/structure. This study provides evidence to the fact that the G4 DNA phenomenon does occur in bacteria, other than *E. coli*. Additionally, it supports the idea that the function of such regions/sequences is not limited to one type of molecular process, as their involvement in recombination, transcription and telomere maintenance has now been elucidated.

There are 46 predicted G4-forming sequences in the *N. gonorrhoeae* chromosome (36). It was suggested to us by Dr. Hank Seifert and Laty Cahoon (authors of the gonococcal study) that potential G4-forming sequences were found in the vicinity of the *thpBA* operon. The intergenic region between the operon and the next proximal upstream gene encompasses approximately 1.9 Kb, with no known coding sequences. Formation of G4 quartets would provide a function for this region, where the sequence information would be of little importance, other than to ensure the generation of these structures. We were interested in identifying the potential G4-forming sequences and potential regulatory effects on the *thpBA* operon.

II. Results

A. Identification of G4-forming sequences in the region upstream of the operon

Increased interest in G4 DNA research has lead to the development of several web-based servers for the prediction of quadruplex-forming G-rich sequences in nucleotide sequences. Quadfinder (213), QGRS Mapper (130) and QuadBase (272) are three examples that have been used previously for the successful identification of G4-forming sequences that have been confirmed to materialize in vivo (36). All three programs identify putative G-quadruplexes using the motif $G_xN_{y1}G_xN_{y2}G_xN_{y3}G_x$, where x is the number of guanines in the G-stretch, and $y_1y_2y_3$ is the length of the gaps (the length of the loops connecting the guanine tetrads). The programs have set limits restricting the maximal and/or minimal lengths of G tetrad, the intervening loops, or the entire G4-forming sequence in order to ensure the best results. Under these restrictions, the 1905 base pair region upstream of the *thpBA* operon (starting from the TbpB translational start site) in the sequenced wild-type gonococcal strain FA1090 was searched using all three programs with the same parameters: the G-stretch length was limited to

a minimum of 2 ($x = 2$), the length of the intervening loops was allowed to range from 1 to 7 nucleotides ($y = 1$ to 7), and when prompted to enter a limit for the length of the entire G4-forming sequence, this was set to 30 nucleotides, conforming to reports in the literature (116, 244) and to the default parameter of the QGRS Mapper search engine. Several G4-forming sequences were identified in the region upstream of the operon, some of which are highlighted in Figure 30. The QGRS Mapper program evaluates the likelihood that the identified sequences have of forming a stable G-quadruplex by assigning it a score, known as the G-score. Criteria used in generating the score include the length and symmetry of the loops, and the number of guanine tetrads (130). As a reference, the sequence GGGGGGTGGGGGGTGGGGGGTGGGGGG is assigned a G-score of 105, and represents the highest possible score attainable. G-scores for the potential quadruplex forming sequences upstream of the operon ranged from 17 to 42. The G4-forming sequence found upstream of *pilE* had generated a G-score of 41 in the QGRS Mapper program (personal communication with L.A. Cahoon). Since it was confirmed that this sequence does form a G-quadruplex structure in vivo, this number was used as a minimum value, i.e, the lowest score possible, in identifying the other 45 G4-forming sequences predicted to occur in the gonococcal genome. This also indicated that some of the potential G4-forming sequences upstream of the operon have a higher probability of forming G-quadruplexes in vivo than others. It is important to note that both of the sequences that scored 20 were perfect matches of each other, and the two sequences that scored 42 were perfect matches of each other as well. Moreover, it was found that the location of two of the potential G-forming quartet sequences overlapped with the repeats found upstream

Figure 30: Identification of Potential G4-Forming Sequences Upstream of the *tbpBA* Operon in the Coding Strand. The 1905 base pair region between the operon and the next proximal upstream gene (NGO1499) in the sequenced gonococcal strain FA1090 was searched for potential quadruplex DNA forming sequences using the QuadBase (272), Quadfinder (213), and QGRS Mapper (130) web-based search programs. Promoter elements are labeled for orientation, the repeat sequences previously identified in the 600 base pair region upstream of the operon appear boxed in red (repeat 1) and blue (repeat 2). An additional repeat was identified further upstream and appears boxed in green (repeat 3). Potential G4- forming sequences are highlighted in yellow with key guanine (G) residues underlined. The potential G4-forming sequences overlapping repeats 3 and 2 generated G-scores of 20, while the G4-forming sequence overlapping repeat 1 had a G-score of 17 (Table 7).

```

1  TGTTTAAAAT TTCGAAAAAT TTAAAGATGT TTCCAGTATA TGTTTACGCC GTGTATATAT CAAGGATATA TGTTTAAAAA TTTGGCTTTT GTAAATATAT 100
101 GGGCGGTAAA ACCGCCCCGA AAAGTCAATT TGCATGGGGT TAATTCAAAA TCCGCCCTTC ATCTGCCCCG TCCCCTAAAA AAACCGTATA TATATAACTG 200
201 TCCGCATTCT ATCGCTCCGG CGACGATACC CATATTTCCA AGTTTGTGTA TCAAAATTGT ATATCGGGCA TAGACTATTT CGGCGAGGAC GAAGATATAG 300
301 ATTTCCACGA TTGAATACAT GGAGGCCAAG TACGTCTATC AACACTATAT TAAAACACAG CTTTTTTTTG AGGTTTCGGG TAACTTTTAA ACCGTCATTC 400
401 CCGTGAAAAC AGAAAAATTA AAACAGAAAC CTCAAATCCC GTCATTCCCG CGAAGGCGGG AATCCGGACC TTCAATGCTA AGGCAATTTA TCGGGAATGA 500
501 CTGAAACTCA AAAAACCAGA TTCCCACTTT CGCGGGAATG ACGGCAGAGC GGCTTCTGTT GCTCCCGATA AATGCCGCAA TCTCAAATCC CGTCATTCCC 600
601 GCGCAGGCGG GAATCCGGAC CTTCATGCT AAGGCAATTT ATCGGGAATG ACTGAAACTC AAAAACCAGG ATTCCCCTT TCGCGGGAAT GACGGGATGC 700
701 AGGTTTCCGT ATGGATGGAT TCGTCATTCC CGCGCAGGCG GGAATCCGAA CCTGTCGGAA CAGAACTTA TCGGATAAAA CGGTTTCTTG AGATTTGACG 800
801 TTCTAGATTC CCACTTTCGT GGGAATGACG ATTCAGATAT TCCTAATAGC AAGCCGCCGG TGGGCATCAT AGTCCTCTCT CCCCCTGGGA GAGAGTTAGA 900
901 GAGAGGGCAG CAAGCCGTAT TAGTTTGCAT AAACCTTAAC CCAACAGCC ACCCGTCCT CTCTCCCTGC GGGAGAGAGT TAGAGAGAGG GCAACAAGCC 1000
1001 GCAAGGTTTG TATTTTAGAA GACTAAGGGA GTTTGGGAAA GATTGCCGCA ACTCGGAGAA TGCCCTCTCC CCGGCCCTCC CCCACGGGGG AGGGAGCGGA 1100
1101 TTGCGGCAGA TTTTGCAGTT GCAGGCGGTT TGAAAAGCA CTTGGATTTA CCGTTGATT T CAGGTCGTAT GAAAAATAAA AAACAGCCTG CACAAGCCGT 1200
1201 ATTAGTTTGC ATAAACCTTA ACCCAACAG CCACCCGTC CTCTCTCCCT GCGGGAGAGA GTTAGAGAGA GGGCAGCAAG CCGTATTAGT TTGCATAAAC 1300
1301 CTTAACCCCA ACAGCCAC TC CGTCCTCTCT CCCTGCGGGA GAGAGTTAGA GAGAGGGCAA CAAGCCGCAA GGTTTGATT TTAGAAGACT AAGGGAGTTT 1400
1401 GGGAAAGATT GCCGCAACTC GGAGAATGCC CTCTCCCCGG CCCTCCCCCA CGGGGGAGGG AGCGGATTGC GGCAGATTTT GCGGTTGCAG GCGGTTTGAA 1500
1501 AAGCAACTTG GATTTACCGT TGATTTCAGG TCGTATGAAA AATAAAAAAC AGCCTGCACA AGCTGATTAG TTTACAGAAA CCTTAACCCC AACAGCCGCA 1600
1601 CCGTCCTCTC TCCCTGCGGG AGAAAGCTAG AGAGAGGGCA ACAAGCCGCA AGGCTTGTGT TTTAGAAGAC TAAGGGAGTT TGGGAAAGAT TGCCGCAACT 1700
1701 TGGAAAGTGC CCTCTCCCA GCCCTCCGCC GCGCAGGGGA GGGAGCGGAT TGCAGCAGG TTGGCGGTTG CAGGCGGTTT TAAAAGCAG CCGGGGAATGA 1800
1801 CCGTATTTTG GTTTATTTAC TAAAAATATA AAAAAGCATT TGCAAATTTT TTAAAAATA ATAAATAAT AATCCTTATC ATTCTTTAAT TGAATCGGGT 1900
1901 TTGTTATG

```

-35

-10

+1

TbpB start site

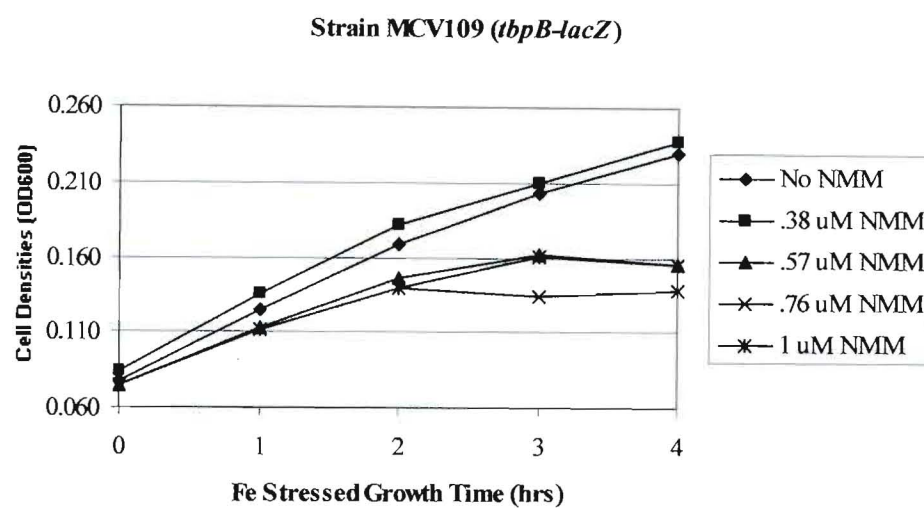
of the operon (refer to Figure 4). Because the location of the identified quadruplex-forming sequences overlapped with extended promoter elements shown to be involved in the regulation of the operon, we investigated their potential role in controlling expression.

B. Effect of NMM Addition on Transcription

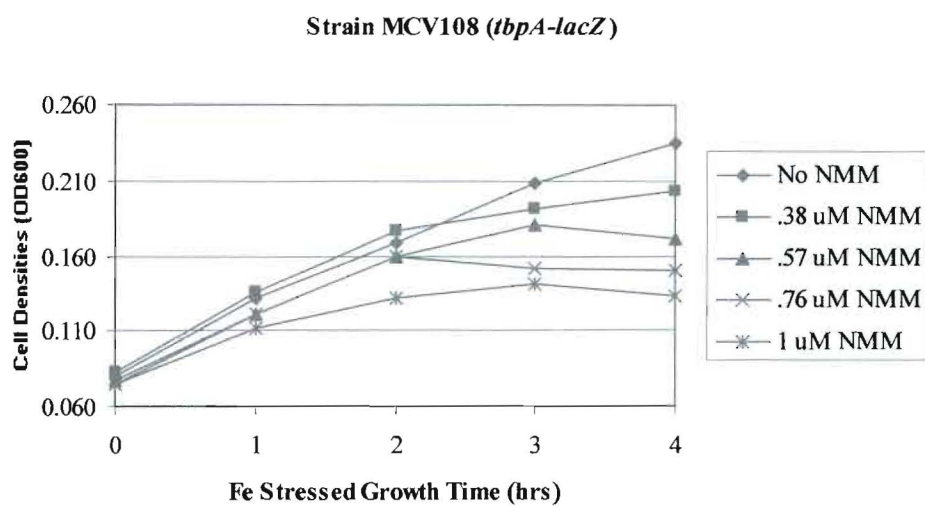
In order to determine if the identified G4-forming sequences were involved in the regulation of the operon, we analyzed the effect of G-quadruplex interference by NMM on transcription of the operon. NMM interference with G4 DNA has been used by Cahoon *et al.* in *N. gonorrhoeae* (36) and in other studies in eukaryotes (108, 113, 271). In fact, Hersherman *et al.* were able to utilize NMM addition to detect changes in transcript levels of genes predicted to contain G4 structures in their upstream regions in *S. cerevisiae* by microarray (108). In the previous *Neisseria* study, GCB plates were supplemented with 0.38 μ M NMM (Frontier Scientific), which resulted in no growth defects, but in noticeable effects on pilin antigenic variation. Thus, we began our studies with the same concentration. Gonococcal strains MCV108 and MCV109 (transcriptional *lacZ* fusion strains, Table 3) were cultured in GCB media with Kellogg's supplement I (129) at 37°C, in a 5% CO₂ atmosphere with vigorous shaking. After an approximate mass doubling, cultures were diluted with fresh GCB media to a standard cell density. Desferal was added at this point, as well as NMM. Cultures were returned to the incubation conditions stated above, and samples were removed at one hour intervals to examine transcript levels. Addition of 0.38 μ M NMM had no obviously detrimental effects on the growth of the experimental cultures over time when compared with cultures where no NMM had been added (Figure 31). Additionally, β -galactosidase assays demonstrate that this concentration of N-methyl mesoporphyrin IX resulted in no detectable changes in either *tbpB* or *tbpA* transcript levels in comparison with no addition of NMM (Figure 32).

Figure 31: Growth Curves of Gonococcal Cultures Grown in the Presence or Absence of NMM. Strains MCV109 (*tbpB-lacZ*, panel A) and MCV108 (*tbpA-lacZ*, panel B) were subjected to iron and N-methyl mesoporphyrin IX (NMM) stressed growth at the indicated concentrations. Growth was monitored by optical density (OD) readings at 600 nanometers. Results represent the means of at least 3 different experiments done on different days.

A.



B.



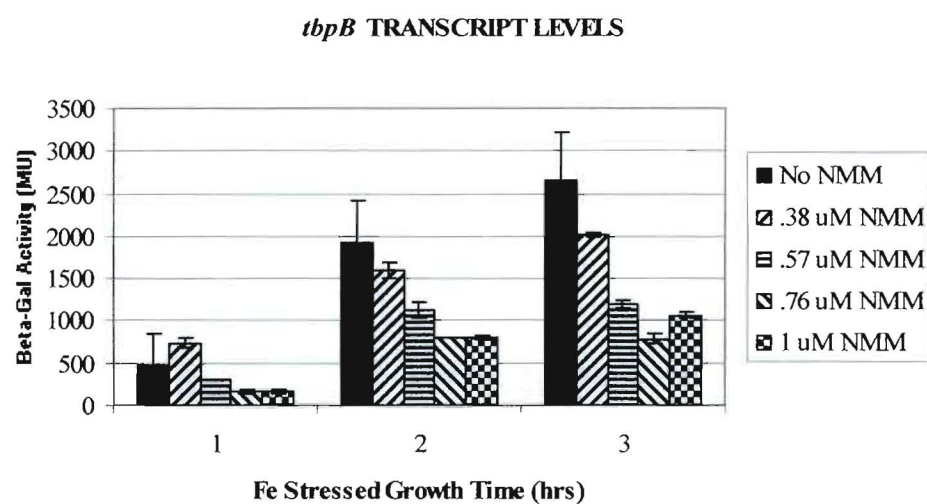
Since there are 46 predicted G4-forming sequences in the gonococcal chromosome (36) we rationalized that perhaps 0.38 μ M NMM was not abundant enough to effectively bind to the predicted G4-forming sequences upstream of the operon in our assay, where the gonococci are replicating at a much faster rate than compared to growth on plates. The concentration of NMM was increased to 0.57 μ M, 0.76 μ M, and 1 μ M NMM and effects assayed at the transcript level. Addition of higher concentrations of NMM resulted in decreased transcript levels for both *tbpB* (Figure 32A) and *tbpA* (Figure 32B). However, increasing the NMM concentration proved to be toxic for gonococcal growth, as cultures to which NMM had been added to exhibited inhibited growth over time (Figure 31 A and B). Even though transcript levels showed a statistically significant decrease at several time points and NMM concentrations tested, they were not labeled as such (asterisk denomination) because of the growth defects that ensued in response to NMM.

C. Effect of Increased NMM Concentrations on Tbp Expression

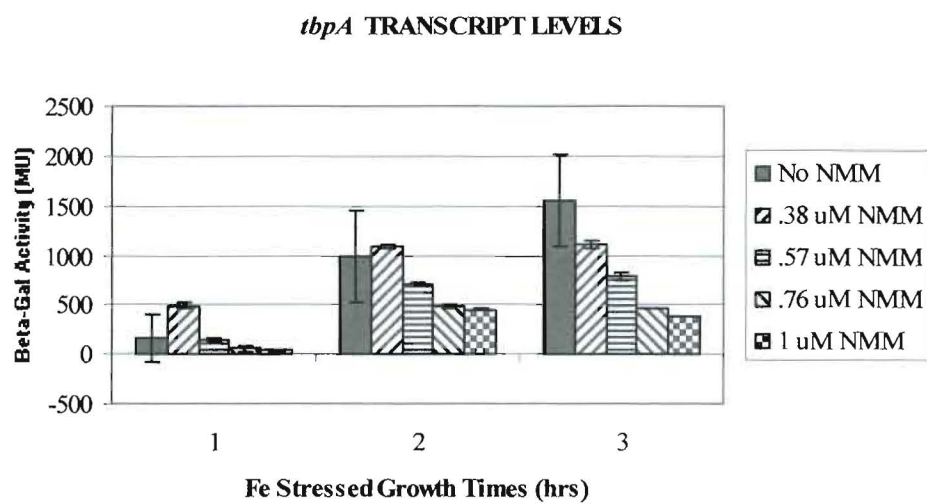
In order to determine if decreased transcript levels detected in the presence of 0.57 μ M, 0.76 μ M, and 1 μ M NMM were due to growth defects or not, wild-type gonococcal strain FA19 was subjected to 2 hours iron and NMM stressed growth. This time point was chosen as it was the point at which all cultures showed the least amount of variation in cell densities. Whole cell lysates were prepped standardizing to a constant cell number and used in western blot analysis. Our results show that at this time point no change in either TbpA (Figure 33, top panel) or TbpB (middle panel) protein were observed. Thus, it is more than likely that changes in transcript abundance detected in the β -galactosidase assays are due to growth rate differences, rather than to direct effects of NMM interference of the potential regulatory role of G4 DNA upstream of the operon.

Figure 32: *tbpBA* Transcript Levels in the Presence of Varying Concentrations of NMM. Both panels show gonococci grown in iron-deplete conditions. N-methyl mesoporphyrin IX (NMM) was added to gonococcal cultures at the indicated concentrations. (A) *tbpB* transcript levels. Maximal *tbpB* expression in the absence of NMM is shown in the solid black bars. (B) *tbpA* transcript levels. Maximal *tbpA* expression in the absence of NMM is shown in the solid grey bars. β -galactosidase activity is expressed in Miller Units (MU). Error bars represent the standard deviation of the means of at least 3 experiments done on different days.

A.



B.

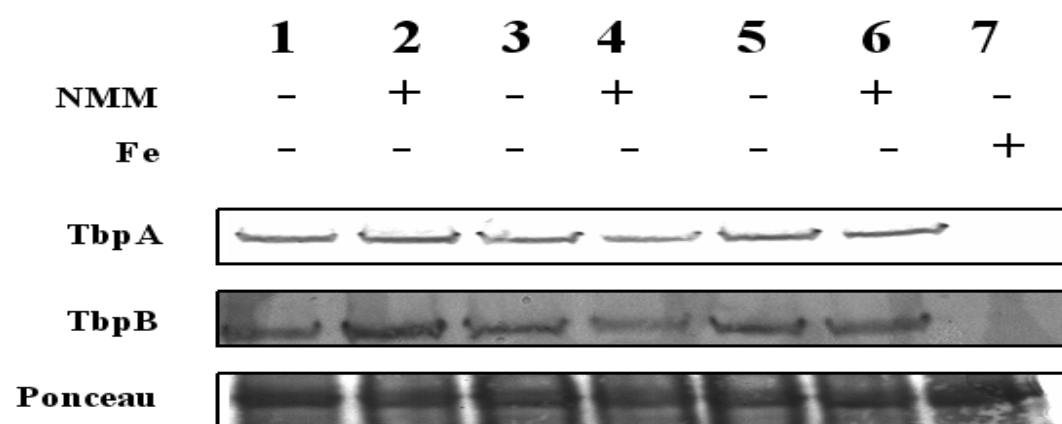


III. Discussion

G4-DNA structures have recently been identified as non-B DNA forms in which the DNA sequence has a minimal role, other than for the formation of these structures (197). Research has shown that these quadruplex structures are found in eukaryotes and prokaryotes and that they are involved in the molecular processes as varied as recombination, transcriptional regulation, and telomere maintenance (36, 69, 71, 116, 179, 197, 223). Recently it was found that a G-quadruplex structure was necessary for pilin antigenic variation in *N. gonorrhoeae* (36). Using several web-based programs several potential G4-forming sequences were identified in the region upstream of *tbpBA*. Studies with NMM, in order to disrupt the function of these potential structures, showed that increased concentrations of NMM had detrimental effects on gonococcal growth. Taking this into consideration, we were not able to detect any changes in *tbp* transcript or protein expression. Because NMM is known to bind G4 DNA, it is possible that addition of high amounts of NMM (1 μ M compared to 0.38 μ M) could have led to interference with the DNA and replication. It is known that RecQ family helicases can unwind G4-DNA (237), and that in the presence of NMM the helicase can become trapped in the NMM-G4-DNA complex and is unable to unwind DNA or even dissociate (113). Thus, it is possible that increasing the concentration of NMM may have disrupted processes that are essential for survival. It would be interesting to determine if mutation of the potential G4-forming sequences would generate the same results on expression of the operon, without affecting growth.

Alternatively, the predicted G4-forming sequences could prove important in elucidating a mechanism for recombination by which the *Neisseria* species have acquired the transferrin binding protein genes. It is thought that neisserial species have evolved from a common ancestor

Figure 33: Tbp Protein Expression in Response in the Presence of Varying Concentrations of NMM. Whole cell lysates were prepped from cultures subjected to 2 hours of iron and NMM stress at the indicated concentrations. (A) Blots were probed with α -TbpA (top panel) or α -TbpB (middle panel) polyclonal antibodies. Ponceau stain (bottom panel) is shown to establish loading. Lanes 1, 3, and 5 show gonococci grown in iron-deplete conditions and no NMM addition. Lane 2 shows addition of 0.57 μ M NMM. Lane 4 shows addition of 0.76 μ M NMM. Lane 6 shows addition of 1 μ M NMM. Lane 7 shows gonococci grown in iron-replete conditions and no NMM addition. Results are representative of at least 3 different experiments done on different days.



(214). However, all *Neisseria* species do not express the same Tbps. For example, it is known that *N. meningitidis* possesses two isotypes of TbpB (204). Horizontal gene transfer is common in *Neisseria*, and it is known that there is frequent recombination between commensal *Neisseria* species and *Neisseria meningitidis* (150), due to their proximity in the mucosa of the nasopharynx. Studies have shown that *tbpB* genes that are acquired from *N. lactamica* by the meningococcus may give short-term within the host advantage, but ultimately renders them less efficient for transmission amongst hosts (276). Thus, it is plausible to consider the possibility that the G4-forming sequences could be involved in enhanced recombination of the neisserial *tbp* genes, not only for evolution purposes, but also perhaps at times to enable survival within the host.

It is of interest to note that region downstream of the operon is comprised of approximately 1.6 Kb with no known coding sequences, similar to the situation found in the region upstream of the operon. Analysis of downstream region in the both of the DNA strands with Quadfinder, QuadBase, and QGRS Mapper resulted in the identification of several predicted G4-forming sequences. Two sequences were identified in the region downstream of the operon that generated G-scores of 42 (Table 7). Thus, conforming to the previously established criteria, the two sequences do have a high probability of forming G-quartet structures in vivo. Similar to what was observed with the upstream sequences, the downstream sequences were perfect matches of each other. Moreover, the predicted upstream and downstream quadruplex-forming sequences are almost identical, the only difference in three nucleotides that are involved in forming one of the intervening loops in the tetrad

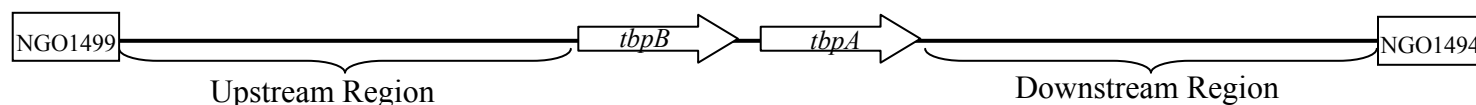
(GGGGGAGGG**CCG**GGGAGAGGG vs. GGGGGAGGG**TTT**GGGAGAGGG) (Table 7).

Identification of these sequences downstream and upstream of the operon strengthens the possibility that they could be involved in recombination events. Further studies need to be completed to elucidate if G4 DNA is of importance in the recombination events surrounding the operon. It would be of interest to determine if such quadruplex-forming sequences are present in other *Neisseria* species, in relation to the operon. Additional studies to elucidate the potential role of the G4- quadruplexes in recombination could include break site mapping, and confirming recombination of a gene of interest, led by the quadruplexes. Deciphering the function of the predicted G4-forming sequences identified in the vicinity of the operon could provide insights into the evolution of the *Neisseria* species.

Table 7. Potential G4-Forming Sequences in Non-Coding Regions Flanking the *tbpBA* Operon

Position in Relation to <i>tbpBA</i>	Strand ⁽¹⁾	Start Position ⁽²⁾	Length (Nucleotides)	G4-Forming Sequence	G-Score ⁽³⁾
Upstream	Coding	1086	21	<u>GGGGGAGGGACGGGATTGCGG</u>	20
Upstream	Coding	1452	21	<u>GGGGGAGGGACGGGATTGCGG</u>	20
Upstream	Coding	1763	17	<u>GGTTTGGCGGTTGCAGG</u>	17
Upstream	Non-coding	7361	21	<u>GGGGGAGGGCCGGGGAGAGGG</u>	42
Upstream	Non-coding	6995	21	<u>GGGGGAGGGCCGGGGAGAGGG</u>	42
Downstream	Non-coding	1239	21	<u>GGGGGAGGGTTTGGGAGAGGG</u>	42
Downstream	Non-coding	741	21	<u>GGGGGAGGGTTTGGGAGAGGG</u>	42

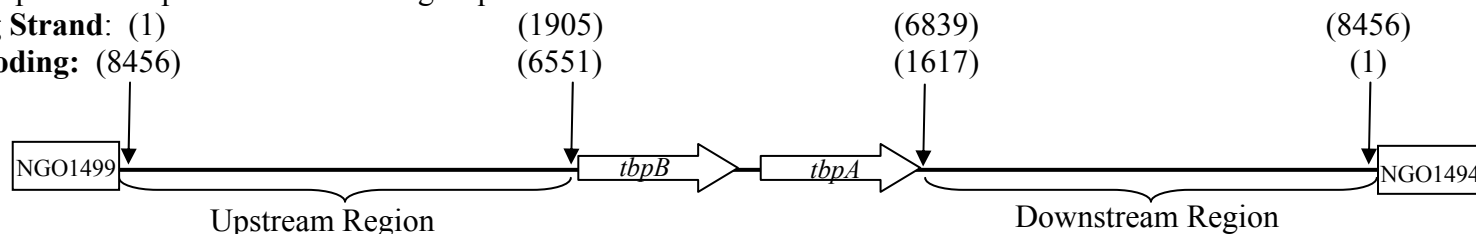
(1) Position in relation to the operon as show below: the entire sequence is a total 8456 bp



(2) Start position of potential G4-forming sequences as numbered below:

Coding Strand: (1)

Non-Coding: (8456)



(3) G-scores as generated by QGRS Mapper Program (130)

CHAPTER 6: Summary and Perspectives

The sexually transmitted infection gonorrhea is the second-most commonly reported infectious disease in the United States, with an estimated 356,000 cases reported to the CDC in 2007 (39). Although infection is treatable, the CDC currently recommends only the use of 3rd generation cephalosporins (42) for treatment, due to rise in resistance to previously effective antibiotics (119, 241, 258). The need for new treatments or ideally preventative methods is evident. Vaccine development efforts against gonorrhea have been unsuccessful thus far. The transferrin binding proteins (Tbps) are attractive vaccine candidates because they are expressed by all gonococcal strains tested to date (161), they are surface accessible, have conserved sequences, and have been shown necessary to establish infection in a human male experimental model (51, 52, 54). The potential of the transferrin binding proteins in *Neisseria gonorrhoeae* as vaccine candidates makes understanding the regulation of their expression imperative.

The gonococcal transferrin receptor complex is a bipartite system for iron acquisition. Transferrin binding protein A (TbpA) is an integral outer membrane protein that forms the pore for the internalization of iron, and has been shown to be essential in the iron acquisition process (53). TbpB is a surface exposed lipoprotein that although not essential, makes the iron acquisition process more efficient (4, 68). The genes encoding these proteins are arranged in a bicistronic operon (53, 145, 206), and although they are co-

transcribed, the genes are differentially expressed (206). Previous studies on the gonococcal transferrin receptor have mostly centered on deciphering the structure-function relationships of its components and little is known about the mechanism that controls their expression. The goal of this study was to define the mechanisms of coordinate regulation of the *tbpBA* operon.

A putative promoter for the operon had previously been identified (4, 205), but never experimentally defined. The studies shown here demonstrated that mutagenesis of putative promoter elements resulted in modified Tbp expression. Additionally, the Fur box operator motif was confirmed. These results are in agreement with previous reports that the genes are co-transcribed from a common promoter upstream of *tbpB* and that the operon is under the control of the Fur protein (205). Thus, maximal *tbp* expression is achieved under iron-starvation conditions.

The confirmed promoter extends to approximately 66 base pairs upstream of the translational start site for TbpB. Two long direct repeats were identified in a 600 base pair region directly upstream of the operon. We hypothesized that this upstream region serves as an extended regulatory region for the operon, as it is approximately 1.9 Kb in length and contains no known coding sequences. We were able to confirm that one of the repeats was perfectly conserved in several gonococcal strains, but the other one was not. Mutagenesis of the repeats resulted in altered amounts of both transcripts and proteins. Taken together, these data show that the repeats do exert regulatory functions on expression of the operon. We have proposed a mechanism by which the repeats are controlling expression of the operon by promoting RNA folding. Further studies are needed in order to elucidate the

mechanism by which this regulation occurs, including detection of any transcripts generated in the upstream region. It is noteworthy to mention that our analysis of the upstream region was limited by the sequence length of 600 base pairs obtained from one of the wild-type strains. It is very possible that the identified repeats are actually more numerous than what we have proposed. Further studies will aim at obtaining sequence data further upstream than 600 base pairs in order to extend the direct repeat analysis.

Identification of the Fur box confirmed that expression of the operon is controlled by iron availability. However, upon entering the host, iron starvation is not the only environmental condition that the gonococci are exposed to. We have conducted an extensive study to determine how *tbp* gene expression is modulated in response to environmental stimuli that the gonococci might encounter in natural infection. We have considered conditions that are of biological significance: changes in osmolarity (which varies amongst bodily fluids (187, 208) and hydrogen peroxide stress (which is encountered by the mounted innate immune response (8, 33, 98) and is also generated by other microorganisms that are part of the vaginal flora (5, 269). Our results show that alteration of osmolarity, carbon source availability, cAMP, and hydrogen peroxide levels did not have a detectable effect on expression of the operon. However, *tbp* transcript and protein were decreased in response to low oxygen availability. These results are also biologically relevant as anaerobic conditions are encountered by the gonococci during natural infection (47, 137, 228), and it would be beneficial for the gonococci to regulate iron intake under conditions where the aerobic respiratory chain is turned off. Reducing the amount of unused intracellular iron would minimize potential damage to cellular

components by reactive oxygen species, which could be the result of Fenton chemistry reactions. We propose that regulation of the operon in response to oxygen is mediated by the fumarate and nitrate reduction regulator (FNR), and to this effect, we have identified a potential FNR binding site upstream of the operon. Further studies to confirm an FNR mechanism of control will include mutagenesis of the putative FNR box. Because excess iron can be extremely detrimental, it is feasible that expression of iron acquisition proteins is tightly regulated by more than one mechanism. Transcriptional control of a single gene by various regulators in response to diverse environmental stimuli is not an uncommon occurrence. For example, the *E. coli yhjA* gene, which encodes a predicted cytochrome c peroxidase, has been shown to be regulated by both FNR and OxyR (180) in response to anaerobic conditions and peroxide stress, respectively. Additionally, multiple mechanisms of regulation could confer an energetic advantage to the gonococci by limiting the amount of energy spent in synthesizing proteins that are not absolutely required under certain conditions.

Lastly, we have investigated the possible role of G4 DNA sequences found in the vicinity of the operon. Our results show that the sequences do not appear to be directly involved in regulating the expression of the operon. However, G4-forming sequences with a high potential of forming quadruplexes *in vivo* were identified in the regions upstream and downstream of the operon. Cahoon *et al.* (36) have shown that a G4-forming sequence is essential to mediate recombination events necessary for pilin antigenic variation in *N. gonorrhoeae*. Thus, it is possible that these sequences are involved in recombination events with regards to the *tbpBA* operon. Determining the role of the G4-forming

sequences found in the vicinity of the operon could enhance our understanding of the acquisition of the *tbp* genes, and provide further insights into the evolution of virulence factors in the *Neisseria* species. Further studies are needed to determine the role of the G4-forming sequences, which could include identification of G4 sequences in several pathogenic and commensal neisserial strains, and break site mapping to determine nicks generated in these or around these sequences.

Overall, the studies presented here provide insights into the mechanisms of coordinate regulation of the transferrin binding proteins in *Neisseria gonorrhoeae*. Elucidation of such mechanisms is essential in exploiting their potential as vaccine candidates. An efficacious vaccine preparation must include antigens that will generate the maximal protective effect. Price *et al.* (192) have shown that recombinant Tbp proteins conjugated to the cholera toxin B subunit are capable of inducing antibody responses in the serum and in the genital tract of female mice, suggesting that these antigens could be the components of an efficacious vaccine. The results presented here suggest that although the Tbps meet numerous criteria that qualify them as good immunogens, their expression does appear to be altered in response to environmental stimuli encountered during infection. These results could have an impact on development of a Tbp-based vaccine against *N. gonorrhoeae*, by suggesting that because there are certain conditions in which the gonococci might not maximally express Tbps, their protective effect might not be optimal. Perhaps the best vaccine preparation would be one that targets an entire class of proteins involved in a particular pathogen function, such as the components of the several iron acquisition systems, rather than focusing on just one specific protein. In a recent study

published by Alteri *et al.* (2) a similar approach was taken in the development of a vaccine against uropathogenic *E. coli*. In their study, the group studied the protective effects generated by six proteins, all involved in iron acquisition. Their conclusions state that development of a subunit vaccine that would incorporate several of these antigens could have a broader efficacy within the urinary tract and across uropathogenic isolates. A similar approach in the development of a gonococcal vaccine could have the same implications, not only in the genital tract, but perhaps also, in being cross-protective against other *Neisseria* pathogens.

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VITA

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PUBLICATIONS

Vélez Acevedo, R.N., C. Ronpirin, and C.N. Cornelissen. 2009. Identification of Regulatory Elements and Environmental Stimuli Controlling Expression of the *tbpBA* operon in *Neisseria gonorrhoeae*. (submitted)

ABSTRACTS AND PRESENTATIONS

Rosuany N. Vélez Acevedo and Cynthia Nau Cornelissen. 2009. Environmental Conditions Regulating the Expression of the *tbpBA* Operon in *Neisseria gonorrhoeae*. 5th Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, Virginia.

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AWARDS AND HONORS

Travel Grant February 2009

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Nominee December 2008

Presidential Management Fellows Program

3rd Place Best Student Presentations November 2008

Virginia ASM Branch Meeting, James Madison University, Harrisonburg, Virginia

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