Foreward

The work presented in this thesis was performed by the author with the following exceptions. Lori Wright was responsible for creating all plasmids with the designation pWL (Table 3). Kenneth Barth was responsible creating plasmid pKB1 (Table 3) and for performing nitric oxide reductase assays in chapter 1. John Lapek was responsible for performing the mass spectroscopic analysis of purified NsrR protein in chapter 2. The gonococcal fur mutant was provided by Caroline Genco. Matthew Kennedy helped to pour and run the 8% TBE-Urea sequencing gels used in the DNaseI footprinting assays.
Introduction

*Neisseria gonorrhoeae: incidence and disease*

*Neisseria gonorrhoeae* is the causative agent of the sexually transmitted disease gonorrhea, one of the most prevalent diseases in the world. As an obligate human pathogen, this organism has a long history of co-evolution with its host, and descriptions of gonococcal urethritis date back to the Old Testament (Lev. 15:1-15:19) (54, 156). Gonococcal disease remains a considerable global health threat, with 62 million cases reported annually and an average of 22 million cases at any given time (69). In the 1960’s and 1970’s, as the so-called “sexual revolution” was taking place, the incidence of gonococcal infection rose dramatically in the United States, resulting in over 1 million reported cases per year, with an estimated 2 to 2.5 million actual cases per year. As AIDS became a recognized problem in the 1980’s, governmental awareness programs and safer sex practices led to a general decline in gonococcal disease, and incidence rates stabilized at approximately 300,000-350,000 reported cases per year (102, 104). In 2006, the Centers for Disease Control and Prevention (CDC) reported 358,366 cases of gonococcal infection in the United States. The reported disease rate, 121 per 100,000 people, marked an increase for the third consecutive year, and gonorrhea is still the second most frequently reported communicable disease in the country. Prompt identification and treatment are essential to prevent permanent host damage, however, the initial infection during the course of gonococcal disease may oftentimes be asymptomatic, hampering effective
control. In addition, the prevalence of antibiotic resistant \textit{N. gonorrhoeae} strains is becoming an increasing concern (66, 199, 211).

\textit{N. gonorrhoeae} is extremely well adapted for growth on a variety of surfaces of the mucosal epithelium. The urogenital tract is the predominant site of gonococcal colonization, though infections of the conjunctiva, pharynx, and rectum have been reported (54, 199). Gonococcal disease is generally transmitted through sexual contact, though pregnant women can transmit the bacterium to their offspring during delivery. Specifically, the urethra and uterine cervix are the predominant sites of urogenital infection in men and women respectively. Disease severity and persistence is largely a function of the host immune response to the pathogen, and gonococcal infections can be uncomplicated or complicated.

In men and women, uncomplicated gonococcal infection remains confined to the genital mucosa, and urethritis or cervicitis will develop. In men, a strong inflammatory response is directed at the pathogen, which includes increased production of the pro-inflammatory cytokines IL-6, IL-8, and TNF-\(\alpha\) in the urethral lumen (178). The hallmark symptoms of uncomplicated gonococcal infection in men are a purulent yellow discharge and dysuria, and these symptoms are largely a result of massive polymononuclear leukocyte (PMN) influx and the shedding of epithelial cells in the urethra (70). Conversely, cervical secretions from women with uncomplicated gonococcal infections show no antibody response and no increase in IL-1, IL-6, or IL-8 compared to uninfected women (92). While the majority of infected males (90-95\%) will develop uncomplicated gonococcal urethritis, the
majority of females with an infection of the lower genital tract (50-90%) will remain asymptomatic (46, 133, 178, 201).

Asymptomatic infection is associated with the more serious sequelae of gonorrhea (22, 199). In women, asymptomatic infection ultimately leads to a prolonged lack of treatment and the onset of complicated disease (22). Ascending infection of the female urogenital tract can occur in up to 45% of infected women, which can further progress to pelvic inflammatory disease (PID) (4, 191, 223). PID can cause chronic pain, infertility, and ectopic pregnancy, the latter of which can be fatal (70). Permanent host damage may occur, and manifests itself in the form of extensive scarring within the uterus, fallopian tubes, and the pelvic cavity. Ascending infection is, in part, facilitated by the ability of the gonococcus to exhibit twitching motility concurrently with hormonal changes in the host that can alter the expression of specific gonococcal receptor proteins and complement factors (54).

Disseminated gonococcal infection (DGI) can also occur as a result of untreated asymptomatic disease, and DGI is thought to occur in 1-3% of all gonococcal infections (70). Interestingly, the incubation period for the onset of DGI can vary greatly, from 7 days, particularly in menstruating females, to several months in elderly individuals (67, 180). DGI is more common in females than males, and can lead to dermatitis and migratory polyarthritis. Rarely, gonococci can disseminate to internal organs, causing more serious complications (70, 143).
Gonococcal morphology and physiology

*N. gonorrhoeae* is a fastidious gram-negative diplococcus requiring enriched media and CO$_2$ (3-10%) for growth *in vitro*. Growth of this organism can occur in a pH range of 6.0 to 8.0, though optimum growth has been observed between 7.0 to 7.5 (154). As the gonococcus is a well adapted human pathogen, optimum growth temperature is in the narrow range of 36-39 °C, as would be encountered *in vivo* (153). The cell has a fairly typical gram-negative structure, consisting of an inner cytoplasmic membrane and an outer membrane with a periplasmic space in between. The cytoplasmic membrane contains many proteins involved in solute and electron transport. The peptidoglycan cell wall (PG) is located in the periplasmic space, and has the same composition as other gram-negative bacteria, however, gonococcal PG is more resistant to lysozyme due to O-acetylation of muramic acid residues facilitated by the peptidoglycan acetylase, PacA, and periplasmic protein, PacB (47, 153).

Due to the lack of the sigma factor RpoS, *N. gonorrhoeae* has no true stationary phase and quickly undergoes autolysis upon encountering conditions non-conducive to growth (65). Autolysis rates can be affected by variables such as changes in pH, temperature, or the presence of cations. Divalent cations are thought to be capable of stabilizing cell membranes, improving integrity and thus reducing autolysis rates, while the opposite is true for the monovalent cation, K$^+$, which enhances autolysis rates (29, 57). The role of autolysis during gonococcal infection is not known, but possible advantages of autolysis include the provision of nutrients to a
starving population, modulation of the host immune response by released cellular components, and donation of DNA for natural transformation (65, 85). Gonococcal autolysis is an enzymatic process facilitated, at least in part, by the N-acetylmuramyl-l-alanine amidase, AmiC (65).

Though autolysis is a factor in the large level of PG turnover, the gonococcus is also capable of releasing PG during active growth and division, and PG turnover may have large effects on the host (188). PG turnover may thwart a host immune response by preventing localization of cellular, chemical, or protein effectors to a specific area. Gonococcal PG can also lower serum levels of complement through a phenomenon known as complement consumption (173, 188). Interestingly, in a fallopian tube organ culture model of pelvic inflammatory disease, PG fragments were shown to be capable of causing ciliated cell death, while the same fragments were shown to elicit an inflammatory response in other models of infection (49, 73, 94, 148).

The gonococcal outer membrane is somewhat unique in that it contains phospholipid bilayer regions that make it more sensitive to fatty acids and steroids. During exponential growth, production of cell wall and outer membrane can become unbalanced, resulting in protein-containing outer membrane blebs (153). The lipid polysaccharide component of the gonococcal outer membrane consists of shorter, branched chains of oligosaccharides (LOS), unlike that of E. coli and other enteric bacteria that contain longer, linear polysaccharide units (LPS). Gonococcal LOS lacks O-antigen and can act as a virulence factor through recognition of its lipid A
portion by the TLR-4/MD2 complex. The carbohydrate moiety of gonococcal LOS can be variable, and will be discussed in more detail later, as will other virulence factors of the outer membrane (3).

*N. gonorrhoeae* is also a naturally competent organism capable of DNA uptake in cells expressing a type IV pilus (150). Genetic exchange in the gonococcus occurs frequently, and is specific to the neisserial genus, as donor DNA must be recognized by a 10 bp uptake sequence present mostly in the intergenic regions of pathogenic and commensal *Neisseria* spp. (5’-GCCGTCTGAA). Incorporation of donor DNA must occur through homologous recombination (85). These requirements minimize the incorporation of non-neisserial DNA. Furthermore, the 10 bp uptake sequence frequently appears in parts of transcriptional terminators, ensuring that the donor DNA is a real gene (26, 150). Piliated gonococci are competent during all phases of growth, though the type IV pilus itself is phase variable (85). This natural competence is thought to be a major contributor to the high degree of genetic diversity that exists in *N. gonorrhoeae*, and is likely responsible for the persistence of this pathogen among the human population. This high rate of DNA exchange also makes antibiotic resistance a major concern.

**Gonococcal virulence factors**

Evolution of the gonococcus with its obligate human host has allowed the acquisition and maintenance of multiple mechanisms to avoid clearance by the immune system. Some of the major gonococcal virulence factors are highlighted in
Table 1. Unlike many bacterial pathogens, the gonococcus does not produce classical toxins, nor does it encode any type III secretion systems. Most of the described gonococcal virulence factors instead function to evade or suppress the immune system.

One of the most dynamic factors involved in gonococcal virulence is the type IV pilus, which can carry out a number of important functions during infection. As discussed previously, this pilus is responsible for gonococcal transformation through its ability to recognize and import donor DNA. Thus, expression of pili may function in the rapid spread of advantageous mutations involved in thwarting host immunity. PilE makes up the major subunit of the type IV pilus, and two alleles exist for the tip adhesin, PilC. PilC plays a critical role in the initial attachment of the gonococcus to the host epithelial cell surface through interaction with CD46, a widely distributed human complement regulatory protein (54, 63, 120, 193, 208). Furthermore, the gonococcus is able to utilize this pilus for twitching-based motility, facilitating ascension upwards through the urogenital tract and allowing colonization at locations distant from the initial site of infection (149).

The *pilE* gene is subject to antigenic variation due to the presence of multiple copies of the silent pilin gene, *pilS*. Recombination events between *pilS* and *pilE* result in the transfer of silent pilin fragments into the *pilE* locus. Because there are many copies of *pilS* that can repeatedly transfer DNA into the actively transcribed *pilE* locus, the sequence variation within *pilE* can be vast. This mechanism can also lead to phase variation if the recombination leads to a non-functional *pilE* gene.
Table 1: Gonococcal virulence factors

<table>
<thead>
<tr>
<th>Designation</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PilE/PilC</td>
<td>fimbrial proteins</td>
<td>Initial binding to epithelial cells</td>
</tr>
<tr>
<td>P.II (Opa)</td>
<td>outer membrane protein</td>
<td>contributes to invasion</td>
</tr>
<tr>
<td>P.I (Por)</td>
<td>outer membrane porin</td>
<td>may prevent phagolysosome formation in neutrophils and/or reduce oxidative burst</td>
</tr>
<tr>
<td>LOS</td>
<td>outer membrane lipooligosaccharide</td>
<td>elicits inflammatory response, triggers release of TNF (mimicry; sialic acid)</td>
</tr>
<tr>
<td>P.III (Rmp)</td>
<td>outer membrane protein</td>
<td>elicits formation of ineffective antibodies that block bacteriocidal antibodies against P.I, LOS and complement protection</td>
</tr>
<tr>
<td>Tbp1</td>
<td>outer membrane receptors for transferrin</td>
<td>iron acquisition for growth</td>
</tr>
<tr>
<td>Tbp2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lbp</td>
<td>outer membrane receptor for lactoferrin</td>
<td>iron acquisition for growth</td>
</tr>
<tr>
<td>Iga</td>
<td>IgA Protease</td>
<td>Cleaves heavy chain of IgA1</td>
</tr>
</tbody>
</table>
(i.e. frameshift or premature stop codon) (2, 86). Constant variation of the PilE phenotype acts to prevent an effective humoral response.

The Opa proteins (also referred to as P.II) are a second type of adhesin that contribute to the cellular trophisms exhibited by gonococci. The gonococcus encodes an average of 10 to 12 *opa* loci, and any strain may express none, one, or multiple *opa* variants at any given time. The Opa protein is believed to contain eight outer-membrane spanning domains that function to form a membrane-embedded \( \beta \)-barrel with four extracellular loops (139). The central two of these loops are the predominant sites of sequence diversity, and are termed hypervariable domain 1 and 2 (HV-1 and HV-2). New Opa variants are constantly emerging due to a high frequency of point mutations within HV-1 and HV-2 and the modular exchange of domains through recombination events between different *opa* genes (101).

Intriguingly, these highly variable regions have been shown to be the site of interaction between the gonococcus and human cells, contrary to what would be expected to achieve a functional recognition of a specific host receptor (23, 76). The extreme diversity among the Opa proteins guarantees that a portion of a gonococcal population will be well suited in the event of an environmental change.

Opa proteins exhibit two major binding patterns. Opa\(_{50}\) variants interact with host heparin sulfate proteoglycan receptors (HSPG) on the surface of epithelial cells. It is believed that the Opa\(_{50}\)-HSPG interaction results in cytoskeletal rearrangements in the host, ultimately leading to endocytosis of the gonococci (54, 77). The second variant class of Opa, Opa\(_{52}\), mediates interaction with host carcinoembryonic antigen
receptors (CEACAM) found on both epithelial cells and professional phagocytes (54, 222). Opa-CEACAM engagement has been shown to result in Rac activation, resulting in cytoskeletal rearrangement and internalization (91).

The Opa proteins are subject to a high degree of phase variation, explaining their heterogenous expression pattern in gonococcal strains. A pentameric repeat region is located in the coding region of the amino-terminal leader peptide for the *opa* gene, and slipped strand mispairing can occur with high frequency at these repeat sequences during DNA replication. Such mispairing can lead to a frameshift mutation, resulting in a phase-off phenotype (90).

The porin protein, Por (also called P.I), is the major outer membrane protein of *N. gonorrhoeae* and is encoded by an essential single-copy gene (113). The Por protein is antigenically diverse among strains, though its expression is thought to be stable within a given strain. Two structural Por variants exist, and any strain can express either the P.IA or P.IB isotype. The gonococcal porin has the interesting ability to translocate into host cell membranes where it can function as a calcium channel. *In vitro*, porin was shown to induce apoptosis in HeLa cells, suggesting a role in the cytotoxic effects involved with epithelial cell shedding during symptomatic infection (158). Conversely, porin was shown to inhibit apoptosis in a primary urethral epithelial cell line, and thought to provide a mechanism for the bacteria to proliferate in a protective intracellular environment (16-17). Porin treatment of PMNs was also shown to inhibit phagosome maturation and degranulation, and was implicated in the downregulation of the immunologically important cell surface
receptors FcγR II and III, complement receptor 1, and complement receptor 3, thus acting to block opsonin-dependent phagocytosis (18, 54). Porin also appears to be able to inhibit killing by the alternate complement pathway by directly binding the complement regulatory protein, Factor H (138).

Porin is somewhat unique for a gonococcal outer membrane protein in that it is one of the few that is not subject to antigenic or phase variation, yet it is extremely immunogenic (54). However, the gonococcus is able to combat the immunogenicity of porin through the action of another outer membrane protein, reduction-modifiable protein (Rmp or P.III). Rmp is intimately associated with porin on the gonococcal cell surface, and this protein is able to elicit the formation of ineffective antibodies that block bacteriocidal antibodies directed against porin (54-55, 114). Thus, an efficacious immune response against porin is prevented.

As stated above, gonococcal LOS can be immunogenic when recognized by the TLR4 pathway. However, the oligosaccharide unit can be highly variable in carbohydrate length and content, as LOS composition is largely dependent on the availability of substrate (31). Furthermore, phase shifting of genes encoding some of the enzymes involved in LOS biosynthesis can occur, as well as there is considerable inter-strain diversity within the coding region of some of the LOS biosynthesis genes (31, 75). The side chains of gonococcal LOS can mimic host glycosphingolipids, thus limiting an immune response. The most common gonococcal LOS phenotypes mimic paragloboside antigens found on most human cells (140). In addition, gonococci produce a 2,3 sialytransferase that can sialylate galactose residues of
gonococcal LOS using host CMP-\textit{N}-acetylneuraminic acid as a sialic acid donor (169). Sialylation of LOS blocks phagocytosis by PMNs, and complement-mediated killing by inhibiting the action of C3b (179, 224).

Almost all pathogens require iron for growth, and \textit{N. gonorrhoeae} has evolved a repertoire of iron acquisition systems that facilitate iron uptake. The concentration of free iron in the host is extremely low, and far too low to support bacterial growth (\(~10^{-18}\)M). Iron sequestration is considered to be a host defense mechanism, and excess host iron is complexed with the major iron binding proteins transferrin, lactoferrin, and ferritin (219). The gonococcus does not produce siderophores, but does express receptors that allow the hijacking of siderophores produced by other bacteria. Gonococci also express iron-repressible transferrin receptors, TbpA and TbpB, as well as a lactoferrin receptor, Lbp, which act to bind host transferrin or lactoferrin, remove the complexed iron, and transport that iron into the cell (176). Gonococci also contain a TonB receptor system that is involved in the transport of iron from siderophores, transferrin, lactoferrin, and hemoglobin. In addition, gonococci express cell surface receptors for heme and hemoglobin, thus gonococci can utilize almost all forms of host iron (68).

Secretory IgA antibodies can function in mucosal immunity by a phenomenon known as “immune exclusion,” where antibodies can bind to the mucosal surface, preventing bacterial access and thus colonization (119, 174). \textit{N. gonorrhoeae} encodes an IgA protease, Iga, which facilitates the cleavage of IgA1-type antibodies in the hinge region of the heavy chain. This mechanism may enhance gonococcal
colonization. Iga protease may also be an important determinant of virulence in internalized gonococci, as this protease was shown to be able to cleave the phagosomal protein Lamp1, thus acting to prevent phagosomal maturation (84).

**Gonococcal gene regulation**

All bacteria have evolved mechanisms to regulate the expression of genes in response to environmental stimuli. Often times such regulation relies on DNA-binding proteins that can bind to the promoter region in the upstream region of genes and act to activate or repress transcription. Other mechanisms of regulation exist that act on a post transcriptional level through modulation of cis-acting secondary structures in mRNA, trans-acting small RNAs, or effects on RNA turnover (147). As discussed previously, the gonococcus is also able to alter gene expression of many of its surface proteins by antigenic and phase variation, which is a mechanism of gene regulation independent of transcription altogether.

Sigma factors are a well-characterized class of DNA-binding proteins involved in genetic regulation. These factors associate with core RNA polymerase (RNAP) and impart promoter specificity to the RNAP holoenzyme complex (81). Compared to *E. coli*, the use of alternative sigma factors in the gonococcus is very limited. Aside from the housekeeping sigma, *rpoD*, only two other sigma genes exist, *rpoE* and *rpoH*. Not much is known about gonococcal RpoE, though in other organisms RpoE is important in the regulation of genes involved in extracytoplasmic and cell envelope stress (15). Gonococcal RpoH has been better characterized, and
has been shown to regulate genes involved in heat stress response as well as genes important for invasion of the mucosal epithelium (51, 129).

The annotated gonococcal genome contains far fewer open reading frames encoding putative DNA-binding transcriptional regulators compared to that of *E. coli*. Furthermore, the gonococcus utilizes very few two component regulatory systems, likely reflecting the restrictive environment in which this organisms resides (147). The study of genetic regulation in the gonococcus has largely focused on genes involved in anaerobic growth or genes induced or repressed in response to iron, as these genes are assumed to play an important role in virulence (80, 105-106, 109). The regulators involved in anaerobic growth, FNR, NarPQ, NsrR, and the iron responsive repressor, Fur, are probably the best characterized in the gonococcus, and will be discussed at length throughout the text.

The gonococcal IHF protein has also been shown to be an important trans-acting regulator involved in virulence. Gonococcal IHF is a heterodimeric protein encoded by *ihfA* and *ihfB* (98). The binding of IHF to its consensus sequence results in localized DNA bending, which is the mechanism of IHF-mediated regulation (99). In the current model of gonococcal *pilE* regulation, IHF binding upstream of the -35 promoter element appears to establish an appropriate promoter structure that allows RNAP access to an UP element, resulting in activation of *pilE* transcription (64, 147). IHF has also been shown to aid in the repression of FarAB, an efflux pump important in gonococcal resistance to antimicrobial agents found in mucosal secretions (131, 147). In this case, IHF binding to the FarAB upstream region results in promoter
bending, which facilitates the binding of two FarR dimers, subsequently causing repression of FarAB (147).

Additional regulatory mechanisms, such as the action of regulatory small RNAs, have only recently begun to garner significant attention in the bacterial world. In the pathogenic *Neisseria*, one sRNA has been discovered and characterized thus far (52, 146). NrrF is a Fur-repressed sRNA involved in repression of succinate dehydrogenase during times of iron restriction. In the future, additional sRNA molecules will likely be discovered in pathogenic *Neisseria*, as a mutation in *hfq*, the RNA chaperone important in the regulatory function of sRNA, was shown to have global effects on gene expression (147).

**Anaerobic growth and denitrification in *N. gonorrhoeae***

*N. gonorrhoeae* was long considered to be an obligate aerobe until it was discovered that anaerobic growth was possible when nitrite was used as a terminal electron acceptor (125). Anaerobic growth is presumed to be a physiologically significant state during gonococcal infection, as the gonococcus is oftentimes recovered from infected individuals in co-culture with obligate anaerobes such as *Peptidococcus* and *Bacteroides* spp. (163). Furthermore, gonococci induce and repress the expression of several genes in response to anaerobiosis, and antibody to AniA, the major anaerobically induced protein, can be found in sera from infected women, demonstrating that this protein is expressed during the course of gonococcal infection (37).
Microbial denitrification is a regulated process catalyzing the stepwise reduction of nitrate (NO₃⁻) to nitrogen (N₂) (234-235). Gonococci can perform the middle two reactions of the denitrification pathway that convert the oxyanion nitrite (NO₂⁻) to nitric oxide (NO) gas, followed by reduction of NO to nitrous oxide (N₂O) gas. However this organism does not encode functional enzymes that allow the reduction of nitrate or nitrous oxide (Figure 1) (36). Interestingly, pseudogenes for the enzyme complex involved in the final step in microbial denitrification (Nos) exist in both N. gonorrhoeae and N. meningitidis. In N. gonorrhoeae, premature stop codons exist in the nosR, nosZ, and nosD coding regions, while in N. meningitidis, a four kilobase deletion extends from the middle of nosR into the middle of nosD, completely eliminating nosZ. Thus the pathogenic Neisseria have utilized two distinct mechanisms to inactivate this last step of denitrification. This is perplexing, as the only steps of denitrification presumed to allow coupling of anaerobic respiration to ATP synthesis are the nitrate reductase, of which there is no gonococcal or meningococcal ortholog, and the nitrous oxide reductase (36, 234-235). Thus, elimination of nitrous oxide reductase activity in the pathogenic Neisseria spp. has likely resulted in some type of selective advantage, though the reasons remain elusive.

The aniA gene encodes a nitrite reductase, and expression of this gene is only induced under anaerobic conditions. AniA is a copper-containing nitrite reductase (CuNir) that can catalyze the one electron reduction of nitrite to nitric oxide. Crystallographic data of the soluble domain of AniA reveal that it is similar to the
Figure 1. The denitrification pathway in *Neisseria gonorrhoeae*. The enzymatic stages of microbial denitrification are depicted. The class of reductase complex involved with each step in denitrification is displayed above each arrow (Nar: nitrate reductase, Nir: nitrite reductase, Nor: nitric oxide reductase, Nos: nitrous oxide reductase). Red arrows depict reactions that occur in *N. gonorrhoeae*, while grey arrows represent the stages of denitrification that do not occur in the gonococcus.
Figure 1. The denitrification pathway in *Neisseria gonorrhoeae*.
CuNirs of soil bacteria, with a conserved type I copper center presumed to be the site of electron transfer, and a conserved type II copper center presumed to be the site of nitrite reduction (24). Like other CuNirs, AniA is functional as a trimer, with the enzymatic copper centers located between the individual AniA monomers (24, 127). A unique feature of neisserial AniA is the fact that it is predicted to be a lipoprotein, and to be located in the outer membrane, whereas other CuNirs are reported to be soluble periplasmic proteins (36, 175, 233). The electron donor utilized by AniA is unknown, but it has been determined that it is not ubiquinol or cytochrome C. Some evidence suggests that azurin-like electron carriers may be involved (36).

The norB gene encodes a single subunit nitric oxide reductase, and shares high sequence identity with NorB from the denitrifying bacterium Ralstonia eutropha. Gonococcal NorB is located in the cytoplasmic membrane, contains 14 transmembrane domains, and has six conserved cysteine residues presumed to be responsible for the coordination of the heme and non-heme iron cofactors involved in nitric oxide reduction (40). The gonococcal NorB is categorized as a qNor-type, based on the fact that quinol is used as an electron donor rather than the cytochrome C-utilizing cNor-type encoded by most soil denitrifiers (212).

Gonococci can utilize these denitrification enzymes so that nitrite or nitric oxide may be used as a terminal electron acceptor. Although neither of these enzymes are directly coupled to ATP synthesis, the regeneration of NAD$^+$ via the reduction of nitric oxide maintains the oxidation/reduction balance of the cell. This NAD$^+$ is then available for use during glycolysis, specifically via the Entner-
Duoderoff pathway (Figure 2) (36). Under anaerobic conditions, the gonococcus is also capable of ATP production via coupling to NADH dehydrogenases (coupling site I).

Modulation of host response by nitric oxide reduction

Nitric oxide serves an important function in host defense. The primary role of NO is that of a signaling molecule. For a mammalian host, tonic signaling through the basal levels of NO generated by the constitutive nitric oxide synthases (eNOS and nNOS) are anti-inflammatory (44, 204). In mammals, bacterial infection generally activates the Toll-like receptor pathway, leading to cytokine production and activation of the inducible nitric oxide synthase (iNOS) (111, 136-137). Upon de novo synthesis of iNOS, micromolar concentrations of NO are produced (upwards of 5 µM), and NO plays a large role in the subsequent host response (83, 142, 204).

Although nitric oxide regulation of the mammalian immune response is complex and not completely understood, NO does have a multitude of recognized effects on NFκB, one of the primary mediators of the inflammatory response (Table 2, For a review of the role of NO on regulation of NFκB, see (1, 20-21, 83, 171, 202)).

As discussed previously, a majority of gonococcal infections in women are asymptomatic. Some evidence suggests that the gonococcal reduction of host-produced NO into the anti-inflammatory range may be responsible, at least in part, for the high incidence of asymptomatic disease (10, 34, 36). In vitro, N. gonorrhoeae
Figure 2. The role of denitrification in anaerobic growth of *N. gonorrhoeae*.

Denitrification in gonococci maintains the oxidation/reduction balance during anaerobic growth. *N. gonorrhoeae* is capable of using nitrite or nitric oxide as a terminal electron acceptor during anaerobic growth, and nitric oxide reduction regenerates NAD\(^+\), which is utilized in glycolysis via the Entner-Duoderoff pathway.
Figure 2. The role of denitrification in anaerobic growth of *N. gonorrhoeae*
Table 2. The effects of Nitric oxide on NFκB.

<table>
<thead>
<tr>
<th>Nitric Oxide Concentration</th>
<th>Source of Nitric Oxide</th>
<th>Immune System Status</th>
<th>Effects on Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (μM)</td>
<td>- iNOS</td>
<td>- Pro-inflammatory</td>
<td>- NFκB active</td>
</tr>
<tr>
<td></td>
<td>• Induction via inflammatory mediators</td>
<td></td>
<td>• TLR signalling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Lack of IκB-α stabilization and induction</td>
</tr>
<tr>
<td>Low/Moderate (nM)</td>
<td>- Constitutive NOS (eNOS, nNOS)</td>
<td>- Anti-inflammatory</td>
<td>- NFκB inactive</td>
</tr>
<tr>
<td></td>
<td>• Basal NO levels/Tonic signalling</td>
<td></td>
<td>• Induction of P_{IκB-α}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Stabilization of IκB-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Translocation of IκB-α to the nucleus</td>
</tr>
<tr>
<td>Absent</td>
<td>- NOS inhibition</td>
<td>- Pro-inflammatory</td>
<td>- NFκB active</td>
</tr>
<tr>
<td></td>
<td>- Bacterial metabolism of NO</td>
<td></td>
<td>• Increased basal activation of NFκB</td>
</tr>
</tbody>
</table>
was shown to be capable of setting a NO steady-state level in the anti-inflammatory range (34). In addition, gonococcal infection is often polymicrobial in nature, and co-infection with organisms such as *Chlamydia trachomatis* and *Trichomonas vaginalis* are common, however, local cytokine responses are not elevated in the cervical mucus secretions in gonococci-infected women even when these other organisms were present. This suggests that the lack of immune response is not solely due to antigenic variation of the surface antigens of this organism (34, 93). In addition, antibody levels were shown to be low in cervical mucus samples, and in women with concomitant rectal infection, the local rectal antibody response was poor despite the presence of organized lymphoid tissue (92). Furthermore, reduction of host NO by the other pathogenic *Neisseria* species, *N. meningitidis*, has been shown to have several effects in a human macrophage model. In these studies, it was determined that meningococcal NO reduction resulted in modulation of cytokine responses, enhanced intracellular survival, and inhibition of apoptosis within the macrophage (205-206, 216).

**Regulation of gonococcal denitrification**

The ability of pathogenic organisms to sense and respond to NO is likely an important virulence determinant during the course of infection. Understanding how the gonococcus is able to sense NO and affect an appropriate response is the goal of this work. At the time I entered the Clark laboratory, Tracy Householder had partially worked out the regulation of the nitrite reductase gene, *aniA*, (see chapter 1)
(106). However, the mechanism of regulation of the nitric oxide reductase gene, \textit{norB}, was unknown, save for the fact that this gene was induced by NO. This work attempts to continue the work of Tracy Householder by completely elucidating the regulation of the gonococcal denitrification pathway. The nitric oxide responsive transcriptional repressor, NsrR, is discovered, followed by its biochemical characterization. The regulators involved in anaerobic growth and nitric oxide metabolism are compared between the pathogenic and commensal \textit{Neisseria} spp. in order to examine potential differences in regulation. This work has helped to further depict the gonococcal NO stimulon, which is likely an important aspect of gonococcal disease.
Materials and Methods

Growth of gonococcal strains. All gonococcal strains were derived from strain F62 (Table 3) and were grown on Difco™ GC medium base (Becton, Dickinson and Co., Sparks, MD) plates with 1% Kellogg’s supplement (GCK) (122). When necessary, chloramphenicol, kanamycin, or erythromycin was added at 1, 150, or 2 µg ml⁻¹ respectively. Aerobic plate cultures were grown in a 37 °C incubator supplying 5% CO₂. Anaerobic cultures were incubated in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) at 37 °C for 20 h in an atmosphere of 85% N₂, 5% H₂, and 5% CO₂. Nitrite was provided for anaerobic cultures by placing 40 µl of a 20% (wt/vol) NaNO₂ solution on a sterile cellulose disk in the center of a plate inoculated for confluent growth (125). Cultures grown with nitrite grow in a characteristic halo around the nitrite disk.

PCR. Genomic DNA from gonococcal strain F62 was isolated for use as a PCR template. Promoter sequences for lacZ fusions and genes amplified for insertional inactivation or complementation were amplified with either iProof™ High Fidelity Polymerase (Bio-Rad, Hercules, CA) or GC-rich PCR system (Roche, Indianapolis, IN). Clones were screened by PCR for presence and orientation of the insert using Amplitaq® (Applied Biosystems, Foster City, CA).
Table 3. Bacterial strains used in this study.

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<th>Constructs</th>
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<td><strong>Plasmids</strong></td>
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<td>pEXT20</td>
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<td>pLES94/norB upstream from -150 to +9</td>
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<td>RUG7803</td>
<td>RUG7800, C103A, transformed with pEF1</td>
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Construction of *lacZ* fusions. Deletions and mutations of the *norB* upstream region were created by PCR. Translational *lacZ* fusions were constructed in pLES94 (200). Genomic DNA from gonococcal strain F62 was used as template. PCR fragments and pLES94 were cut with *BamHI*. Digested insert and plasmid were ligated and cloned into *E. coli* MC1061 or DH10B. Transformants were selected on LB medium plates containing chloramphenicol at 25 µg ml\(^{-1}\) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Invitrogen) at 40 µg ml\(^{-1}\). Plasmids were checked for the presence and orientation of the insert by PCR, and those plasmids that contained an insert in the correct orientation were used to transform F62. Colony PCR was performed on chloramphenicol resistant colonies to confirm the presence of the reporter construct. The PCR product was also sequenced to ensure the appropriate fusion was made.

Construction of mutants. RUG7600 was constructed by deleting 226 bp in *nsrR* and inserting an erythromycin resistance cassette within the gene (*erm*). Two fragments were amplified using GC Rich Taq (Roche, Indianapolis, Indiana). The 5’ fragment begins 378 bp upstream from the *nsrR* start site and includes 102 bp of the coding region, with a *PstI* restriction site on the 3’ end. The second fragment has an *XhoI* restriction site on the 5’ end and begins 328 bp into the gene and ends 495 bp downstream of the TAA stop codon. The two fragments, 490 bp (*PstI* cut) and 616 bp (*XhoI* cut) were ligated with a complementarily digested *erm* resistance cassette and transformed directly into RUG7500. Clones were verified by colony PCR. The
arsR mutant was constructed in a similar fashion, by deleting 162 bp in the gene and inserting a aph resistance cassette. The 5’ fragment begins 704 bp upstream of the arsR start site and includes 97 bp of the coding region, with a HindIII restriction site on the 3’ end. The second fragment has an XhoI site on the 5’ end and begins 251 bp into the gene and ends 924 bp downstream of the TAG stop codon. The fragments were cut with HindIII or XhoI and ligated with a complementarily digested kanamycin resistance cassette (aph) and transformed into RUG7526. The gonococcal fur mutant was generously provided by Caroline Genco, and was constructed as previously described for the generation of the meningococcal fur mutant (45).

**Complementation of ΔnsrR mutant.** To complement the nsrR mutation, RUG7605 was created by inserting a copy of nsrR into the nosX pseudogene. The nsrR gene was amplified from 415 bp upstream of the ATG start site to 174 bp downstream of the TAA stop codon (to preserve the gonococcal uptake signal sequence 5’-GCCGTCCTGAA-3’) (56). The fragment contained BamHI and EcoRI sites on the 5’ and 3’ ends respectively. The fragment was digested and a ligation was performed with this fragment, a 550 bp amplified fragment containing the 5’ end of the nosX coding region plus upstream sequence digested with BamHI, and to a 2.2 kb fragment containing a kanamycin resistance cassette along with the 3’ coding region of nosX and downstream sequence digested with EcoRI. Gonococcal strain F62 was transformed with this ligation mix and construction of the complemented mutant was confirmed by PCR.
**Gonococcal transformation.** For natural transformation, a light suspension of type I cells was prepared in 1 ml of GCK broth containing 0.042% NaHCO$_3$ and 10mM MgCl$_2$ (122). Purified plasmid DNA or ligation mixture was added, and 100 µl of the suspension was plated on two GCK plates and incubated between 6-9 hours at 37 ºC. Cells were then harvested from the plates and streaked on GCK plates containing the appropriate antibiotic for selection of clones. Clones typically took 2 days to become visible on antibiotic plates.

For transformation by electroporation, gonococcal cells were harvested from overnight plate cultures and washed three times in 0.3 M sucrose. After washing, 150 µL of cells were added to electrocuvettes, and electroporation was performed at 2.5 kV at 400 Ω in a Bio-Rad electroporator. Cells were resuspended in 1 mL GCP broth (proteose peptone #3 (15 g), soluble starch (1 g), KH$_2$PO$_4$ (4 g), K$_2$HPO$_4$ (1 g), NaCl (5 g)/L dH$_2$O), and 500 µL of cells were spread on a plate, and the plate was incubated right side up for 6-9 hours at 37 ºC. Cells were then harvested from the plate and streaked on GCK plates containing the appropriate antibiotic for selection of clones.

**β-Galactosidase assays.** Gene reporter activity was determined by β-galactosidase assays from cultures grown under various conditions (151). For gonococcal cultures, sterile swabs were used to harvest cells from overnight plate cultures and cells were resuspended in Z-buffer (151). When cells were grown anaerobically with nitrite disks, only the halo of gonococcal growth around the nitrite disk was used (106).
Cells were lysed with chloroform and 0.1% SDS and assayed as described (151). Activity is reported in Miller units and the reported results are the average of at least three assays performed in duplicate from each day the cultures were grown.

For *E. coli*, overnight broth cultures were resuspended to an OD$_{600}$ of approximately 0.05 in LB containing kanamycin at 50 µg ml$^{-1}$ to which 15 mM Diethylenetriamine/nitric oxide adduct (DETA/NO, Sigma) or 2 mM nitrite was added as indicated, and cultures grown to an OD$_{600}$ of approximately 0.5. Cells were spun down, resuspended in Z-buffer, and β-galactosidase activity measured as described above.

**Nitric oxide reductase (Nor) assays.** Aerobic and anaerobic nitric oxide reductase (Nor) activity of F62, the *nsrR* knockout, and the *nsrR* complemented mutant was measured by Ken Barth as previously described (34, 109).

**Repression by gonococcal NsrR in *E. coli.*** Plasmid pEXT22 (53) and promoter *lacZ* fusions were digested with restriction enzymes *XbaI* and *HindIII* and ligated so that the *lacZ* fusion was oriented in the same direction as the tac promoter, but is only induced by P$_{tac}$ when IPTG is added. Otherwise, the fusion is under control of its own promoter. The construct was transformed into *E. coli* strain MC1061 and selected on LB plates containing kanamycin at 50 µg ml$^{-1}$ and X-gal at 40 µg ml$^{-1}$. The plates were incubated overnight at 37 °C and plasmids were extracted from blue colonies and screened for the presence of the insert by PCR. Plasmid pEXT22 was
also digested with *BamHI* and *SstI* and *nsrR* insert (obtained by PCR from genomic gonococcal F62 DNA) was digested with *BglII* and *SstI*, ligated, and transformed into *E. coli* strain MC1061 and selected on LB plates containing kanamycin at 50 µg ml\(^{-1}\) and X-gal at 40 µg ml\(^{-1}\). The promoter *lacZ* fusion was then cloned into the *XbaI* and *HindIII* sites into this construct as before. Plates were incubated overnight at 37ºC and plasmids were extracted from blue colonies and screened for the presence of the insert by PCR. Chemically competent MC1061 was transformed with either plasmid and selected on LB plates containing kanamycin at 50 µg ml\(^{-1}\) and ampicillin at 100 µg ml\(^{-1}\).

**Isolation of ArsR by DNA affinity chromatography.** ArsR was isolated by a method based on that described for IscR with several modifications (230). The putative repressor binding site was amplified by PCR from RUG7526 (Figure 5) using an upstream primer located in the gonococcal proline region and a downstream primer ending at the -35 sequence making a 100 bp fragment. This fragment was cut with *BamHI* and cloned into pLES94. A clone containing a plasmid with two copies of the *BamHI* fragment was selected, and outside primers were used to generate a 320 bp affinity probe by PCR, with the downstream primer being end-labeled with biotin. This 320 bp fragment was purified and incubated with 25 µl of streptavidin coated agarose beads (Pierce) in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) with rotation for 1 h at room temperature. The beads were then spun down at 1000 x g in a microcentrifuge and washed with TBS to removed unbound DNA.
Gonococcal cultures (100 mL) of strain F62 were grown aerobically in a shaking incubator at 37 °C in GCK broth to an OD \(_{600}\) of 0.5. Cells were spun down, resuspended in 5 mL of TBS, and lysed by sonication (Branson 150D sonicator) to sheer the genomic DNA into approximate 0.5 kb pieces. Lysates were incubated with the DNA coated streptavidin beads for 1.5 h with rotation at 4 °C. The beads were spun down and washed four times with 5 mL TBS to remove non-specific proteins, then resuspended in 25 µl of SDS-PAGE running buffer (BIO-RAD) and incubated in boiling water for 10 min. The eluates were recovered and run on SDS-PAGE gels.

**Site specific mutagenesis of NsrR binding sites.** Splice overlap extension (SOE) PCR was used to change the 29 base pair inverted repeat sequence of the putative \(\text{norB}\) NsrR binding site in strain F62 to match consensus NsrR binding sites from other gonococcal genes (100). The primers used to generate the \(\text{norB}::\text{lacZ}\) in strain RUG7500 were used as outside primers in the PCR reaction, fragments were cut with \(\text{BamH}I\), and cloned as described earlier for the construction of \(\text{lacZ}\) fusions.

**Construction of an \(\text{nsrR}::\text{FLAG}\) fusion gene.** The bases encoding a FLAG epitope tag (Sigma) were linked in-frame to the 5’-end of primer that annealed to the 3’ end of the gonococcal \(\text{nsrR}\) gene and the wild type stop codon was removed (Figure 3). The complementary FLAG bases and a stop codon, TAA, were added to the 5’ end of a
Figure 3: Construction of an nsrR::FLAG fusion gene. The 5’ end of primer P2 contains nucleotides coding for a FLAG epitope tag. The 3’ end of P2 anneals to the 3’ end of the nsrR coding region and excludes the wild type stop codon. The nsrR gene and upstream region were amplified using primers P1 and P2. The 5’ end of primer P3 contains the codons of a FLAG tag that are complementary to those in P2 followed by the addition of stop codon TAA. The 3’ end of P3 anneals to the 5’ end of aph (Kan’ cassette). The aph gene was amplified using primers P3 and P4. After amplification, these fragments were spliced together in a SOE PCR reaction to make nsrR::FLAG-aph, which was subsequently cloned into an E. coli expression vector to make pV128. Alternatively, the region downstream of nsrR was amplified and ligated downstream of aph for making a fragment for transformation of a gonococcal strain in order to replace wild type nsrR with nsrR::FLAG. Small bent arrows indicate direction of transcription. Fragments are not drawn to scale.
Figure 3: Construction of an $nsrR::$FLAG fusion gene.

(1) Separate PCR amplification of each fragment
(2) Splice Overlap Extension (SOE) PCR
(3) Digest and clone into expression vector
   - IPTG inducible under $P_{tac}$
   - Make recombinant NsrR::FLAG in E.coli
(4) Clone the region downstream of $nsrR$ downstream of $aph$ to allow homologous recombination in GC, replacing wild type NsrR with the FLAG tagged version
primer that annealed to the 3’ end of *aph*. An *nsrR* fragment (beginning at -342 nucleotides relative to the *nsrR* start codon) and an *aph* cassette were separately amplified using these primers with their suitable pairs. These fragments were then spliced to make a single *nsrR::FLAG-aph* fragment using SOE PCR (100). This fragment was cloned into the *SstI* and *HindIII* restriction sites in the *E. coli* expression vector pEXT20 to make pVI28 (53). Cysteine mutations in the *nsrR::FLAG* gene were also constructed by SOE PCR using suitable primers and pVI28 as a PCR template.

**Construction of gonococcal strains expressing *nsrR::FLAG*.** An *nsrR::FLAG-aph* fusion gene was obtained by digestion of pVI28 with *SstI* and *HindIII*. PCR was used to amplify another fragment that contained the 915 bp chromosomal region immediately downstream of the *nsrR* stop codon, and was joined to the free end of the kanamycin resistance cassette by restriction with *HindIII* and subsequent ligation. This fragment was used to transform the gonococcal strain RUG7500. Cysteine mutants of the *nsrR::FLAG* gene were used to transform RUG7500 in a similar manner. Presence of fusion gene, as well as insertional mutagenesis of the wild-type *nsrR* gene, was confirmed by PCR. Gene reporter activity was determined by β-galactosidase assays from cultures grown aerobically and anaerobically with nitrite (151).
**Chromatin immunoprecipitation.** 10 mL gonococcal cultures expressing nsrR::FLAG were grown aerobically in a shaking incubator at 37°C in GCK broth to an OD_{600} of approximately 0.5. In vivo cross-linking of bacterial nucleoprotein was initiated by addition of formaldehyde to cultures up to a final concentration of 1%. After 15 minutes, cross-linking was quenched by the addition of 0.125 M glycine, and cultures were incubated an additional five minutes. Cultures were then harvested by centrifugation, and cell pellets were washed three times in TBS. Pellets were resuspended in 1 mL of a solution containing buffer A (TBS, 10mM EDTA, 0.5% Triton X-100, and 0.4 mM Pefabloc SC (Roche)) and were sonicated (Branson 150D) to shear DNA to an average size of 1,000-1,500 bp. Cell debris was removed by centrifugation, and the supernatant was retained for the immunoprecipitation reaction.

800 µL of sonicate was aliquoted into clean microfuge tubes and incubated with 60 µL of a 50% slurry of 6% agarose beads for one hour on a rotating wheel to preclear the sonicate. Preclearing beads were spun down, and the supernatant was recovered and incubated with 35 µL of M2 affinity gel 50% slurry (Pierce), or an equal amount of 6% agarose beads as a control, for one hour with rotation. Beads were then washed twice in buffer A and one time in high salt buffer (50 mM Tris pH 7.4, 500mM NaCl, 5 mM EDTA, 1% Triton X-100) for five minutes in each wash in batch format before adding to Handee™ Spin-columns (Pierce) and spinning down supernatant. Beads were then washed in the column once more with high salt buffer and twice with LiCl buffer (10 mM Tris pH 8, 250 mM LiCl, 1 mM EDTA). Beads were removed from column with fresh LiCl buffer and washed in batch format for
five minutes followed by two five minute washes in TBS before being spun down in a fresh spin-column. Beads were reusupended in 250 µL of elution buffer (50 mM Tris pH 7.4, 10 mM EDTA, 1% SDS) and incubated overnight in a 65 °C water bath to remove immunoprecipitated complexes from the M2 antibody and to reverse formaldehyde crosslinks. The supernantant was then run through a PCR purification kit (Qiagen) and DNA was suspended in 120 µL of water.

Following purification, PCR was used to analyze immunoprecipitated DNA; 1 µL DNA sample was used as template in a 50 µL reaction mix containing a 1 µM concentration of each oligonucleotide primer. PCR was performed with Amplitaq (Applied Biosystems), and the reaction was allowed to proceed for 27 cycles before visualization by electrophoresis on an agarose gel.

**Isolation and purification of NsrR::FLAG.** *E. coli* strain DH10B was transformed with pVI28, the expression vector containing the *nsrR::FLAG* fusion gene under control of the IPTG-inducible *P*<sub>tac</sub> promoter (53). A 20 mL volume of an overnight culture of DH10B harboring pVI28 was used to inoculate 500 mL of LB containing kanamycin at 50 µg ml<sup>-1</sup> and cells were grown shaking at 37 °C. When the culture reached an OD<sub>600</sub> of 0.5, 1 mM IPTG was added and cells were grown out for 4 hours, at which point cells were harvested and the pellet was frozen overnight at -20 °C. The cell pellet was resuspended in 10 mL of TBS, 0.4 mM PEFA-BLOC (Roche), 1 mM DTT, and DNaseI at 200 µg/mL (Sigma, D-5025). Cells were lysed in a French pressure cell press (SLM Instruments), and the resulting lysate was spun
at 20,000 x g twice to remove the insoluble fraction. A 90 µL volume of M2 affinity resin (Sigma) was added to the cleared lysate, followed by rocking incubation for 2 h at 4 °C. M2 affinity resin was collected by centrifugation at 500 x g and subsequently washed four times with 10 mL of TBS. Bound resin was added to Handee Spin-columns (Thermo) and incubated for 15 minutes with 400 µL 3X FLAG peptide (Sigma) in TBS at 1 mg/mL before centrifugation to collect released protein. Recovered FLAG fusion protein was stored in TBS, 1 mM DTT, and 50% glycerol at -20 °C.

**Electrophoretic Mobility Shift Assays (EMSA).** EMSAs were performed with a LightShift Chemiluminescent EMSA kit (Thermo). Briefly, PCR was used in conjunction with a biotin end-labeled primer (Invitrogen) and unlabeled primer pair to generate biotin end-labeled targets to measure protein binding. Binding reactions contained 3.5 fmol biotin labeled DNA, 2 µl 10x binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), 1 µl 50% glycerol, 1 µl 1% NP-40, 1 µl MgCl₂ (100 mM), 0.5 µg poly (dI•dC), varied NsrR::FLAG concentrations, and dH₂O up to a total volume of 20 µl. Binding reactions were incubated at room temperature for 15 min and run on 6% 0.5x TBE gels. Gels were transferred to nylon membrane, crosslinked in a UV crosslinker (Stratagene), and biotin was detected following manufacturer’s protocol (Thermo). For EMSAs performed with the long half-life NO donor DETA-NO, binding reactions were increased to 1 hour. For supershift reaction, 1 µl of M2 antibody (Sigma, F1804) was added to the binding reaction.
Quantitation of shifted DNA was calculated by spot densitometry using a FlourChem IS-5500 imager (Alpha Innotech).

**DNaseI footprinting.** NsrR::FLAG extract was used to generate DNaseI footprints from the upstream regions of *norB* (nucleotides -92 to +13 relative to the translation start site) and *nnrS* (-143 to -60 nucleotides relative to the translation start site). Briefly, plasmids containing the relevant upstream regions were cut singly with one restriction enzyme, treated with cow intestine alkaline phosphatase (CIP, New England Biolabs), and radiolabeled with $\gamma^{32}$P-ATP using T4 polynucleotide kinase (Fermentas). After phenol/chloroform extraction, single end-labeled fragment of these upstream regions were released by digestion at a second restriction site, and fragments were gel purified. Binding reactions were performed as described for EMSA except that 20,000 cpm of radiolabeled DNA was used rather than biotin-labeled DNA. After 15 minutes of preincubation, 1 µl of DNaseI at 40 µg/mL (Sigma; D5025), suspended in TBS plus 50 mM CaCl$_2$, was added to each reaction. After 90 sec incubation, reactions were stopped by the addition of 20 µl of 2X stop loading buffer (80% dimethylformamide, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol FF, 2% SDS, and 40 mM EDTA, pH 8.0 in 0.5X TBE) and immediately incubated at 95 °C for 20 minutes. A+G sequencing ladders were generated as previously described (145). A volume of 5 µl of each reaction was loaded onto 8% TBE-Urea sequencing gels. Reaction products were visualized by phosphorimaging (Bio-Rad).
Mass spectroscopy. Undigested samples were analyzed on an AutoflexIII TOF/TOF MALDI mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Spectra were collected in linear positive ion mode (1800 shots accumulated in m/z range 3000-20000). An external calibrant, Protein1CalibStandard (Bruker Daltonics, Billerica, MA, USA), was used to ensure mass accuracy. This calibrant ranged from m/z 5734-16952. Spectra were processed by baseline subtraction and analyzed with flexAnalysis (Bruker Daltonics, Billerica, MA, USA). SequenceEditor (Bruker Daltonics, Billerica, MA, USA) was used to predict the m/z of the proteins used in this experiment.

BLAST analysis of denitrification proteins and regulatory components. Basic local alignment search tool (BLAST) was used to compare amino acid sequences of transcriptional regulators involved in controlling expression of the denitrification pathway (FNR, NarP, NarQ, NsrR) and the upstream regions of the denitrification genes (aniA and norB) in various Neisseria spp. N. gonorrhoeae strain FA1090 sequences were obtained from the STDgen database (http://stdgen.northwestern.edu/), while other neisserial sequences were obtained from the National Center for Biotechnology Information (NCBI). For determining protein similarity, annotated genomes were surveyed using the blastp program using a protein query against the protein database. For those bacterial genomes that have not been annotated, N. gonorrhoeae FA1090 amino acid sequences were used to perform tblastn analysis with the query set as protein against the genomic database. The genomic sequence of
the appropriate contig from the sequencing project was analyzed in the Clone Manager Professional software program. The translate tool was utilized to provide amino acid sequence. The blastn program was used to retrieve upstream sequence of the denitrification genes, and alignments were performed in MultAlin (38).

**Oligonucleotide and DNA sequencing.** All synthesized oligonucleotides were obtained from Invitrogen, and confirmatory DNA sequencing was performed at ACGT Inc. (Wheeling, IL).

**Molecular biology techniques.** Cloning and PCR techniques were performed in accordance to standard protocols (6-7, 194). Plasmid preparations were obtained with Wizard Plus SV Minipreps kits (Promega Corp., Madison, Wis.). DNA fragments were purified with QIAquick PCR Purification or QIAquick Gel Extraction kits (QIAGEN, Valencia, CA).
Chapter 1

Genetic analysis of \textit{norB} regulation by Fur, ArsR, and NsrR, and elucidation of the NsrR regulon

Introduction

\textit{N. gonorrhoeae} is a facultative anaerobe possessing the \textit{aniA} gene, which encodes a copper-containing nitrite reductase, AniA. This reductase is localized to the outer membrane and is only expressed under anaerobic conditions (36, 106, 125). Another gene, \textit{norB} (\textit{norZ}), was identified by members of the Clark laboratory as encoding a single component nitric oxide reductase whose expression is induced by the presence of nitric oxide (107). Both AniA and NorB are essential for anaerobic growth, and the gonococcus is capable of anaerobic respiration using nitrite (NO$_2^-$) or nitric oxide (NO) as a terminal electron acceptor (36, 106-107, 125).

As an obligate human pathogen, that fact that anaerobically grown gonococci both induce and repress the expression of several outer membrane proteins is indicative that anaerobic growth is a physiologically significant state for this organism (35, 184). This is further supported by the presence of antibody to AniA in sera from women with gonococcal infections, which demonstrates that this anaerobically induced protein is indeed expressed \textit{in vivo} (37).

NO can be toxic to bacterial cells via reactions with molecular oxygen, superoxide radicals, or the metal centers in various enzymes. In mice and humans, NO has also been demonstrated to be a modulator of cellular events comprising the
immune response (44, 204). Therefore, the ability of a pathogenic organism to reduce host-produced NO can have a profound effect on the nature of the resulting host response.

A comprehensive understanding of gonococcal NO metabolism in *N. gonorrhoeae* requires that the complete mechanism of *norB* regulation be elucidated. At the time that I entered the Clark laboratory, it was reported that *aniA* expression was activated by a gonococcal FNR homolog in response to anaerobiosis and by NarP in response to nitrite, however, the mechanism of *norB* regulation was still completely unknown (Figure 4) (106, 134). Unlike the case in some other denitrifying bacteria, the gonococcal *norB* gene was shown not to be regulated by either FNR or NarP, though induction of the gene could be achieved in the presence of NO (181). In *N. meningitidis*, it was suggested that the ferric uptake regulator (Fur) protein was responsible for direct transcriptional activation of *norB*, despite the fact that Fur is generally thought to act as a repressor (45).

In this chapter I present an analysis of the nucleotide sequence upstream of *norB* to thoroughly characterize elements involved in its transcription and regulation. I identify *nsrR*, a gene encoding an Rrf2-type transcriptional repressor that controls *norB* regulation in *N. gonorrhoeae*. I also show that NsrR regulates *norB* through repression, and is responsive to the presence of NO. Furthermore, I describe a novel mechanism of Fur activation, where Fur indirectly activates *norB* expression by preventing binding of ArsR, a repressor of *norB* whose binding site overlaps that of
the Fur binding site. Finally, I identify other members of the gonococcal NO stimulon by identifying other genes in the regulon of NsrR.
**Figure 4. Previous model of regulation of gonococcal denitrification.** This figure depicts the established regulation of the divergently transcribed \textit{aniA} and \textit{norB} genes before the undertaking of these studies. FNR acts as a class-II activator of \textit{aniA} in response to anaerobiosis, and NarP acts as a class-I activator of \textit{aniA} in response to nitrite. Class-II-mediated activation generally involves regulator binding at a position centered at -42.5 with respect to transcription start site. In class-II activation, three activating regions on the regulator make positive contacts with the $\alpha$CTD, $\alpha$NTD, and $\sigma$ subunit of RNA polymerase (36). Class-I activation generally occurs further upstream from the -35 element and mediates activation by a single positive interaction between an activating region on the regulator with the $\alpha$CTD of RNA polymerase. Expression of gonococcal \textit{norB} is induced in response to NO by an unknown mechanism, though some evidence suggests that meningococcal \textit{norB} is activated by Fur.
Figure 4. Previous model of regulation of gonococcal denitrification.
Results

Sequence upstream of norB. An intergenic sequence spanning 367 bp separates the divergently transcribed denitrification genes, norB and aniA (107). As the nucleotide sequence 150-bp upstream of the translational start site of norB contained several interesting motifs, it was selected for further study (Figure 5). A Fur binding motif located between -130 and -150 relative to the translation start site (-75 to -95 relative to the -10 element) suggested a potential role for Fur in transcriptional activation of norB, as has been proposed in the taxonomically close relative, Neisseria meningitidis (45). The -35 region contains a poor match to the E. coli -35 consensus, 5'-TTGACA, while there is a TG dinucleotide at position -15,-14 relative to the –10 sequence. This architecture is typical of extended –10 promoters that do not have a requirement for a strong -35 sequence for efficient transcription (121). The extra TG dinucleotide greatly stabilizes open complex formation by providing additional contacts with region 3.0 of RNA polymerase holoenzyme (81, 159).

Mutational analysis of the norB promoter. Thorough analysis of the norB promoter was performed in order to define the regulatory and transcriptional elements therein. Translational lacZ fusions were constructed by cloning different segments of the norB upstream sequence into vector pLES94 followed by chromosomal integration into the proAB pseudogenes by homologous recombination, creating a single-copy reporter system (200). These fusions contained the ribosome binding site and first three codons of norB.
Figure 5. Sequence of the *norB* promoter/operator region. The numbers below the sequence are in reference to the ATG translation start site. The C at position –53 is the *norB* transcription start site identified in *N. meningitidis* (45), indicated by an asterisk above the sequence, and the putative -10 and -35 sequences are based on this site. The inverted repeat described in the text is underlined, while the putative NsrR binding site predicted by Rodionov et al. (185) within this repeat is highlighted in light gray. A 6 base pair conserved motif present in one copy in forward and two copies in inverted orientation are highlighted in dark gray, and this sequence is predicted to bind ArsR. The ribosome binding site (RBS) is indicated by a double underline. The deletions and the site specific mutations in the *norB::lacZ* fusions described in the text are indicated under the sequence.
Figure 5. Sequence of the norB promoter/operator region.
I first wanted to determine if the TG dinucleotide at -15, -14 was indeed a true extended -10, as this type of promoter had not been described in *Neisseria* species previously. Site-specific mutagenesis was performed on the TG dinucleotide, changing it from 5’-TGCTACAAT-3’ to 5’-GTCTACAAT-3’ (RUG7513 in Figure 6). Alteration of the dinucleotide resulted in a large decrease in promoter activity under both aerobic and anaerobic conditions (20-fold and 80-fold respectively; p < 0.005, Figure 6), though the promoter was still induced in the presence of NO (cells grown anaerobically with nitrite). These results demonstrate the importance of the TG dinucleotide in *norB* expression, and provide the first evidence that Neisserial RNA polymerase is capable of recognizing extended -10 promoters.

To locate regulatory elements in the *norB* promoter, a series of deletions from the 5’ end of the *norB* upstream region were constructed (Figure 6). A deletion that eliminated the Fur binding site (RUG7512) had no effect on *norB::lacZ* expression or induction by NO (Figure 6). This initial result suggested that Fur is not involved in *norB* activation. A 5’-end deletion of the *norB* upstream region up to, but not including, the -35 sequence (RUG7531) also had no effect on *norB* regulation, however, a deletion that eliminated the -35 element, leaving only the extended -10 sequence (RUG7523), caused an approximate 10-fold reduction in promoter activity (p < 0.005). These data show that even with its weak consensus, the -35 element is important for *norB* expression, though its absence has no effect on induction by NO. These results indicate that *norB* is not regulated by an activator that binds upstream of the extended -10 element.
Figure 6. Expression of norB::lacZ fusions in gonococcal strain F62. Schematic representations of the norB::lacZ constructs described in Figure 5 are shown to the left (Constructs are not drawn to scale). β-galactosidase activity from these strains was measured from cells grown aerobically (blue) or anaerobically with nitrite (red). Results are presented as the average of 6 determinations plus one SD.
Figure 6. Expression of norB::lacZ fusions in gonococcal strain F62.
To determine if the region downstream of the extended -10 motif was involved in \textit{norB} regulation, a \textit{lacZ} fusion was constructed that replaced \textit{norB} sequence downstream of the –10 element with promoter sequence from \textit{aniA} (RUG7508 in Figure 6). Previous studies have demonstrated that the \textit{aniA} sequence used for this replacement contains no regulatory elements (106). This sequence merely acts to maintain spacing and provide a ribosome binding site for \textit{lacZ} translation. The mean level of aerobic \textit{norB::lacZ} activity from strain RUG7508 (1831 ± 294 Miller units) was higher than that observed with RUG7500 grown anaerobically (1460 ± 177 Miller units) (Figure 6). This result demonstrates three important points: [1] the \textit{norB} sequence required for NO induction is found downstream of the extended -10 sequence, [2] the transcriptional regulator is very likely to be a repressor, and [3] gonococcal \textit{norB::lacZ} is not fully derepressed during anaerobic growth.

**Identification of NsrR as a transcriptional repressor of \textit{norB}**. Examination of \textit{norB} sequence immediately downstream of the extended -10 element was performed using Regulatory Sequence Analysis Tools (RSAT) (http://rsat.ulb.ac.be/rsat). This analysis revealed a region of dyad symmetry with an imperfect inverted repeat sequence of 5’-TTTAACATTCAATTTTGTTGAATTTTAAA (Figure 5). To determine whether this inverted repeat sequence was responsible for the apparent repression of \textit{norB}, a \textit{lacZ} fusion was created in which \textit{norB} sequence downstream of this repeat was replaced with that of \textit{aniA} (RUG7520 in Figure 6). The reporter fusion in strain RUG7520 restored repression of \textit{norB} expression and displayed levels
of norB::lacZ activity comparable to that of RUG7500 (Figure 6). These data show that the DNA sequence involved in norB regulation is, or is contained within, this inverted repeat.

In 2004, Beaumont et al. discovered what was believed to be a nitrite-sensitive repressor protein involved in the regulation of nitrite reductase in Nitrosomonas europaea. This protein was termed NsrR (nitrite sensitive repressor) (14). In 2005, Rodionov et al. used a comparative genomics/in silico approach to identify regulators of the denitrification pathway within all divisions of the bacterial kingdom (185). Using this approach, Rodionov predicted that the gonococcal norB gene would be regulated by an NsrR homolog. His work also yielded a 19 bp NsrR consensus sequence that overlapped the 29 bp inverted repeat discovered in the RSAT analysis (Figure 5). Though the annotated gonococcal genome (http://stdgen.northwestern.edu) did not include an NsrR homolog, BLAST analysis of the gonococcal genome using the NsrR protein from Nitrosomonas europaea as a query returned NGO1519, a conserved hypothetical protein with 46% identity (63% similarity) to N. europaea NsrR.

NGO1519 was insertionally inactivated in N. gonorrhoeae to determine its role in norB regulation (Figure 7A). The aerobic expression of norB in the NGO1519 mutant (RUG7600; 1542 ± 89 Miller units) was higher than the level expressed under NO generating conditions in the wild type strain (RUG7500; 1317 ± 56 Miller units; p < 0.005). While insertional inactivation of this gene suggests that it is the repressor of norB, the gonococcal genome annotation reveals that NGO1519 is completely
**Figure 7. Construction and complementation of a ∆nsrR mutant.** (A) Wild type (RUG7500), nsrR mutant (RUG7600), and complemented nsrR mutant (RUG7605) harboring a norB::lacZ fusion were grown aerobically (blue) or anaerobically with nitrite (red) as described, and β–galactosidase activity was measured. Results are presented as the average of 6 determinations plus one SD. (B) Aerobic (blue) and anaerobic (red) nitric oxide reductase activities were determined in gonococcal strains F62, F62 ∆nsrR, and, F62 ∆nsrR complemented as described in Methods. Results are presented as the average of 3 determinations.
Figure 7. Construction and complementation of a \( \Delta \text{nsrR} \) mutant.
contained within another putative ORF, NGO1518, which is transcribed in the opposite direction. This ORF would also be inactivated in a NGO1519 knock-out. To complement the NGO1519 mutation in single-copy in *N. gonorrhoeae*, the NGO1519 ORF, but not the 5’ and 3’ ends of the NGO1518 ORF, was used to transform RUG7600. The NGO1519 ORF was inserted into the non-functional *nosX* gene through homologous recombination (RUG7605; see Methods) and assayed for β-galactosidase activity from the *norB::lacZ* fusion (Figure 7A). This complementation restored the wild-type phenotype and repressed the aerobic expression of *norB*, proving that NGO1519 and not NGO1518 is responsible for regulating *norB* expression. NGO1519 will henceforth be termed *nsrR*.

Nitric oxide reductase activity was assayed directly in strain F62 and in the ΔnsrR mutant. Activity was determined by the addition of the NO-donor DEA/NO to gonococcal cultures followed by measuring the rate of NO disappearance. The aerobic nitric oxide reductase activity of the ΔnsrR mutant was found to be 16-fold higher than aerobic activity levels in the wild-type strain (Figure 7B). In agreement with results of the *norB::lacZ* fusions, these data further confirm the role of NsrR in *norB* regulation.

**NsrR regulation of norB in E. coli.** To ensure that NsrR is involved in direct regulation of *norB*, we constructed a system to recapitulate repression of *norB* in *E. coli*. The *norB::lacZ* fusion harbored by RUG7500 was amplified and cloned into the low copy plasmid, pEXT22, to make pVI1 (53). The entire *nsrR* gene was cloned, including 415 upstream from the ATG start site, upstream of *norB* in pVI1 to make
pVI18. β-Galactosidase activity was determined aerobically in strains carrying pVI1 and pVI18. The presence of nsrR in the strain carrying pVI18 significantly repressed norB::lacZ expression (p < 0.05; Figure 8). Thus NsrR directly regulates norB and does not act through other gonococcal regulatory proteins.

I next sought to determine the signaling molecule that NsrR was responsive to, as NsrR from N. europaea was reported to be sensitive to nitrite, yet the gonococcal norB gene was responsive to NO and not nitrite (14, 107). Expression of norB::lacZ in the E. coli strain carrying pVI18 (nsrR) was induced to levels comparable to that of pVI1 by the addition of 15mM DETA/NO, a long half-life NO donor (Figure 8). This data shows that NsrR is directly responsive to nitric oxide and that norB becomes derepressed in its presence. Derepression of the norB::lacZ fusion could not be achieved by addition of nitrite at any concentration tested (up to 2mM). The addition of NO to the strain harboring pVI1 did not increase norB expression levels, suggesting that E. coli NsrR did not repress gonococcal norB, and supporting the prediction by Rodionov et al. that the neisserial NsrR binding site was different from that found in other bacteria (185). I also supported this by demonstrating that construction of a non-polar mutation of the E. coli nsrR gene did not affect expression of gonococcal norB in the pVI1 strain (data not shown).

**norB expression in a gonococcal ΔnsrR mutant.** In order to determine whether there were any other trans-acting factors regulating norB that respond to NO or anaerobiosis, the plasmids containing the norB::lacZ constructs analyzed in wild-type strain F62 were used to transform a ΔnsrR mutant (all 7600 constructs in Figure 9).
Figure 8. Recapitulation of norB repression by gonococcal NsrR in E.coli. β-galactosidase activity was measured from E. coli strains harboring pVI1 (norB::lacZ) or pVI18 (norB::lacZ, nsrR+). Cells were treated with no addition (blue), 2 mM nitrite (red), or 15 mM DETA/NO (green). Results are presented as the average of 6 determinations plus one SD.
Figure 8. Recapitulation of norB repression by gonococcal NsrR in E.coli.
Figure 9. Expression of *norB::lacZ* fusions in gonococcal strain F62 ΔnsrR.

Schematic representations of the *norB::lacZ* constructs described in Figure 5 are shown to the left (Constructs are not drawn to scale). All strains contain the ΔnsrR mutation. β-galactosidase activity from these strains was measured from cells grown aerobically (blue) or anaerobically with nitrite (red). Results are presented as the average of 6 determinations plus one SD.
Figure 9. Expression of norB::lacZ fusions in gonococcal strain F62 ΔnsrR.
The mean levels of aerobic β-galactosidase activity from these constructs were high and even exceeded the anaerobic expression level observed in a wild type background (Figure 9). The lower norB expression when the -35 consensus or the extended -10 dinucleotide were deleted or mutated in the ΔnsrR mutant (RUG7623 and RUG7613, respectively) is attributable to a decrease in promoter strength rather than a lack of regulation. The promoter activity from strains RUG7508 and RUG7600 showed nearly identical levels of both aerobic and anaerobic expression. Thus it appears that the elimination of the repressor binding site (RUG7508) has the same effect on norB expression as the inactivation of the repressor (RUG7600), and suggests that there are no other trans-acting factors regulating norB that are responsive to NO or anaerobiosis.

**Indirect regulation of norB by Fur.** Elimination of the Fur binding site in RUG7512 immediately suggested that Fur had no direct role in regulation of norB, despite reports to the contrary in both N. gonorrhoeae and N. meningitidis (45, 80, 198). I therefore wished to investigate the expression of various norB::lacZ fusions in a Δfur mutant (Figure 10). Though the deletion of the Fur binding site had no apparent effect on norB expression, a Δfur mutant in reporter strain RUG7500 (RUG7550) displayed a 60% reduction in anaerobic expression compared to the wild type (p < 0.005). This decrease in expression was independent of nsrR, as a norB::lacZ fusion strain in a ΔnsrR Δfur double mutant (RUG7650) and the norB::lacZ fusion strain lacking the NsrR binding site in a Δfur mutant (RUG7558) were decreased in expression by 65% and 72% respectively compared to the wild
type (p < 0.005). In reporter strains containing deletions of the norB promoter that eliminated the Fur box (RUG7512 and RUG7531), the effect of the fur mutation (RUG7562 and RUG7581) was to increase norB expression by 41% and 36% respectively compared to the wild type (P < 0.005). At this point, it did become evident that Fur was acting as a regulator of norB, though it would seem to be acting in an indirect manner.

The paradoxical effect of Fur on norB regulation could be explained by the presence of a second repressor protein that binds in the upstream region of the norB promoter whose effect was decreased by Fur binding. Upon closer examination of the norB upstream region it was noted that there are three copies of a 6 bp conserved motif that partially overlaps the Fur box (Figure 5). Since the norB::lacZ fusion in RUG7512 lacks part of the first repeat, we constructed a norB::lacZ fusion that deletes all but 2 base pairs of the Fur box and retains this inverted repeat (RUG7526 in Figure 10). Anaerobic expression of this fusion was only 18% of that observed with RUG7500 (p < 0.005), and the ∆fur mutation had no effect. These results suggest that norB is regulated by a repressor that binds upstream of the -35 promoter. The binding site for this repressor partially overlaps the Fur binding site, and the apparent activation of norB expression by Fur may be due to its inhibition of binding by this second repressor. This would be a novel mechanism for indirect activation by Fur, whereby Fur and a second repressor compete for operator binding in the norB upstream region.
Figure 10. Anaerobic expression of norB::lacZ constructs in wild type and fur mutant strains of N. gonorrhoeae F62. Schematic representations of the norB::lacZ constructs described in Figure 5 are shown to the left (Constructs are not drawn to scale). Gonococci were grown anaerobically, and β-galactosidase activity was determined. The lacZ fusion strains were in a wild type background (orange) or in a fur mutant (brown). The fur mutation resulted in decreased norB::lacZ expression in the wild type and nsrR background, making it appear as though Fur was acting as an activator. However, this apparent activation by Fur was no longer observed in fusion constructs with deletions in the 5’ end of the norB promoter, suggesting that another repressor was responsible for these effects. Similar effects of the fur mutation were observed in aerobically grown cells (data not shown). Results are presented as the average of 6 determinations plus one SD.
Identification of ArsR as a repressor of norB. In attempt to isolate the putative repressor of norB that binds between the -35 promoter and the Fur binding site, a fragment containing the three inverted repeat sequences located in that region (Figure 5) was amplified with a biotin end-labeled primer and used to coat streptavidin beads (see Methods). After incubation in a gonococcal lysate, the eluate recovered from DNA coated beads was analyzed by SDS-PAGE. A negative control, consisting of streptavidin beads coated with biotin labeled non-specific DNA, was run in parallel. Only one enriched protein was found in the eluate from beads containing the inverted repeats compared to the control (Figure 11A). The enriched band was approximately 10.5 kDa. Examination of an annotated list of putative gonococcal regulatory proteins revealed only one protein of that size. NGO1562 encodes an arsR family transcriptional regulator with a predicted molecular mass of 10.6 kDa. Genome wide RSAT analysis of gonococcal intergenic regions was performed with query 5’- CATATAnnTATTTG, the first two inverted repeats in Figure 5. This analysis revealed one other hit, upstream of NGO1411, which encodes a conserved protein homologous to an ArsB efflux pump. These two facts, along with the high degree of similarity between this putative binding site and the E. coli chromosomal ArsR binding site suggested that this ArsR protein may bind to the norB upstream region (Figure 11B) (228). This 10.6 kDa arsR homolog was insertionally inactivated in strain RUG7526, creating RUG7726 (Figure 12). This mutation alleviated the observed repression of norB::lacZ in RUG7526, and restored high level anaerobic
Figure 11. Identification of ArsR binding to the norB upstream region. A) Streptavidin agarose beads were coated with biotinylated DNA containing the putative repressor binding site, or with non-specific DNA. After incubation with a sonicated gonococcal lysate, the beads were boiled, and the eluate analyzed by SDS-PAGE. Streptavidin monomers were removed from the agarose beads during boiling, and were used as an internal control for molecular weight. Only one protein (~10.5 kD) was shown to be enriched in beads containing the inverted repeat sequence compared to the control. B) A segment of the gonococcal norB upstream region is depicted. The Fur binding site is underlined, and the partially overlapping putative repressor site is compared to the E. coli ArsR binding site (228).
Figure 11. Identification of ArsR binding to the *norB* upstream region.
Figure 12. β–galactosidase activities of *norB::lacZ* fusion strains in the wild type and Δ*arsR* mutant. RUG7500, RUG7526, and RUG7726 (RUG7526/Δ*arsR*) were grown aerobically (blue) and anaerobically with nitrite (red), and β–galactosidase was measured. The mutation in *arsR* alleviates the observed repression in RUG7526. Results are presented as the mean of 6 determinations plus one SD.
Figure 12. β-galactosidase activities of norB::lacZ fusion strains in the wild type and ΔarsR mutant.
expression of \textit{norB::lacZ} in the presence of nitrite (Figure 12), confirming that ArsR represses \textit{norB} in the absence of bound Fur.

**Lack of signal recognition by gonococcal ArsR.** At this point it had become clear that \textit{norB} regulation in the gonococcus is a more complex process than that observed in other denitrifiers (185, 234). The discovery of a novel Fur/ArsR regulatory mechanism in the \textit{norB} upstream region means that \textit{norB} expression may not only be regulated in response to NO (NsrR) and iron (Fur), but also to a third signal if ArsrR is capable of sensing a ligand. ArsR-type regulators generally respond to the presence of metal ions, causing derepression of the regulated gene (32, 172). Several conserved motifs exist within the ArsR family of proteins that confer metal ligand recognition (172). However, alignment of the gonococcal ArsR protein with characterized ArsR proteins from other bacterial species revealed that the gonococcal ArsR protein contained none of these motifs (Figure 13). Regardless, I wished to determine if ArsR could respond to a metal ligand. To achieve this, a \textit{norB::lacZ} promoter fusion strain was created that was lacking both the NsrR and Fur operators so that \textit{norB} activity in this strain was only regulated by ArsR (RUG7538 in Figure 14). Promoter activity in this strain was assayed in parallel with an unregulated control strain, RUG7518, in the presence of various metal ions, however, none of these metal ions were able to induce \textit{norB} expression at the concentrations tested.

**Other members of the NsrR regulon.** As these studies progressed in the Clark laboratory, NsrR was shown to be a NO-responsive regulator in several other organisms, including \textit{E. coli}, \textit{Bacillus subtilis}, \textit{Salmonella typhimurium}, and \textit{N.
Figure 13. Amino acid sequence alignment of ArsR-family proteins. ArsR-family proteins from various bacterial species were aligned using clustalW (213). Residues known or predicted to be involved in ligand binding in the α3N or α5 metal sensor domains are shown in color. ArsR proteins are involved in recognition of As(III) and Sb(II), CadC proteins are involved in recognition of Cd(II), Pb(II), and Bi(III), SmtB and ZiaR proteins are involved in recognition of Zn(II), CzrA proteins are involved in recognition of Zn(II) and Co(II), and NmtR is involved in recognition of Ni(II) and Co(II) (32). The gonococcal ArsR homolog contains none of the conserved residues involved in metal sensing.
Figure 13. Amino acid sequence alignment of ArsR-family proteins.

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| **N. gonorrhoeae**   | α5 site                |                      |                        |
| E. coli              |                        |                      |                        |
| B. subtilus          |                        |                      |                        |
| S. aureus pI258      |                        |                      |                        |
| S. maltophilia       |                        |                      |                        |
| S. agalactiae        |                        |                      |                        |
| B. Firmus            |                        |                      |                        |
| L. monocytogenes     |                        |                      |                        |
| N. meningitidis      |                        |                      |                        |
Figure 14. Gonococcal ArsR is not responsive to metal. A) Schematic representation of the constructs used to measure β-galactosidase activity (constructs are not drawn to scale). RUG7518 contains the -10/-35 region from norB, with no regulatory elements upstream of the -35, and a replacement downstream of the -10 that removes the NsrR binding site. This is a constitutive, unregulated control for lacZ expression. RUG7538 contains the same sequence as RUG7518, except for the presence of the putative ArsR binding site upstream of the -35. B) β-galactosidase activity from aerobically grown RUG7518 (green) and RUG7538 (blue) was measured from plates supplemented with no addition, 50 µM Fe, 50 µM Cu, 25 µM Zn, 25 µM Mn, 1 mM Ca, 1 mM Mg, 15 µM AsO₄, 15 µM Co, or 50 µM EDTA. Results are the mean of 2 determinations.
Figure 14. Gonococcal ArsR is not responsive to metal.
meningitidis, and was confirmed to function in *N. gonorrhoeae* (19, 72, 160, 168, 184). In *N. gonorrhoeae*, Overton *et al.* demonstrated NsrR regulation of *aniA* and *dnrN* under microaerobic conditions by using an *aniA::*lacZ fusion and quantitative real time PCR, respectively (168). In that same work, he provided evidence that gonococcal NarQ, the histidine sensor kinase of the NarQP two component system, is degenerate in its ability to sense nitrite (168). Gonococcal NarQ appears to be locked in its “on” state and is constitutively phosphorylating NarP (36, 109, 168). Thus NarP would always be poised to activate *aniA* expression, irrespective of the availability of nitrite (168).

We confirmed regulation of *aniA* by NsrR in anaerobically grown cells using several *aniA::*lacZ fusion strains (Figure 15). The effect of the Δ*nsrR* mutation in the *aniA::*lacZ reporter strain RUG7001 (RUG7601) was to increase anaerobic *aniA* expression in both the absence and presence of nitrite (p < 0.005). This effect was also seen in the Δ*nsrR* mutant of reporter strain RUG7045 (RUG7645) which lacked the NarP binding site (106). The apparent activation of *aniA* expression in the presence of nitrite is due to derepression by NsrR rather than stimulation of the NarQP two component system by nitrite. There was a slightly higher mean level of expression of *aniA::*lacZ in the Δ*nsrR* mutant in the absence of nitrite versus it presence. This effect is likely due to a partial inhibition of FNR by the nitric oxide produced through nitrite reduction (39, 41). Aerobic levels of *aniA::*lacZ expression in the Δ*nsrR* mutant were also extremely low, demonstrating that FNR is required for *aniA* transcription, regardless of the presence or absence of the NsrR repressor (106).
Figure 15. Lack of nitrite induction and NsrR regulation of *aniA::lacZ*.

Translational lacZ fusions to *aniA* were constructed in the wild type and Δ*nsrR* mutant, and β-galactosidase activity was determined in cells grown aerobically (blue), anaerobically without nitrite (green), and anaerobically with nitrite (red). RUG7001 contained the full length *aniA* promoter sequence, while RUG7045 contained a truncated sequence lacking the NarP binding site. Results are presented as the mean of 6 determinations plus one SD.
Figure 15. Lack of nitrite induction and NsrR regulation of *aniA::lacZ*. 
The gonococcal *dnrN* gene is homologous to the *ytfE* gene in *E. coli*, the *norA* gene in *Ralstonia eutropha*, and the *scdA* gene of *Staphylococcus aureus*. The *ytfE* gene was shown to be regulated by the *E. coli* homolog to NsrR, and both *norA* and *scdA* expression are induced by NO (19, 167-168, 207). *E. coli* strains with a mutation in the *ytfE* gene demonstrated increased sensitivity to NO (19, 118). It has also been suggested that YtfE, DnrN, and ScdA proteins may play an important role in the repair of stress-damaged Fe-S clusters in *E. coli*, *N. gonnorhoeae*, and *S. aureus* respectively (116-117, 167). In *R. eutropha*, *norA* was suggested to be a cytoplasmic NO-binding protein that regulates gene transcription by lowering the free cytoplasmic concentration of NO (207). Regulation of gonococcal *dnrN* by NsrR was confirmed, as aerobic expression of a *dnrN::lacZ* promoter fusion increased 11-fold in the ∆nsrR mutant (Figure 16A). However, there was an approximate 4-fold higher level of expression in the *dnrN::lacZ* fusion in the ∆nsrR mutant when grown anaerobically versus aerobically. This suggests that other transcription factors may also control this gene.

In 2006, Overton *et al.* reported that there was an NsrR binding site in the upstream region of gonococcal *narQP*, but that NsrR did not function to regulate those genes (168). Examination of the gonococcal genome revealed that this putative binding site lies in the intergenic region between the divergently transcribed *narQP* and *mobA* genes. Inspection of this intergenic region for putative transcriptional elements (*i.e.* -10 and -35), revealed that this putative NsrR site was in a much better location to be involved in regulation of *mobA* rather than *narQP*. A gonococcal
mobA::lacZ promoter fusion was created and used to transform wild type and the ∆nsrR mutant to investigate NsrR regulation of mobA. Expression of mobA::lacZ was shown to be induced when the cells were grown anaerobically with nitrite, immediately suggesting a potential role of NO in gene regulation (Figure 16B). Aerobic β-galactosidase activity of the fusion in the ∆nsrR mutant was 11-fold higher aerobically than was observed in the wild type, demonstrating repression of mobA by NsrR (Figure 16B).

The nnrS gene encodes a putative copper-heme protein found in many denitrifying bacteria. This gene is highly conserved, and its expression is induced under denitrifying conditions, however, its function is largely unknown (13, 103, 128, 132). In Shewanella oneidensis, nnrS was shown to be regulated by NsrR (132). A gonococcal nnrS::lacZ promoter fusion was created and transformed into the wild type and ∆nsrR mutant to determine if gonococcal nnrS was subject to regulation by NsrR. Aerobic β-galactosidase activity of the fusion in the ∆nsrR mutant was two-fold higher than was observed in the wild type, demonstrating a weak regulation by NsrR (Figure 16C). Anaerobic expression of nnrS::lacZ was increased four-fold in the wild-type strain, suggesting that other regulators may act on this gene, as is the case in several other organisms (13, 132). Surprisingly, there was a small but recurring decrease in anaerobic expression of nnrS::lacZ in the ∆nsrR background compared to the wild-type, suggesting that nnrS regulation may be quite complex.

Analysis of the NsrR binding site. Rodionov et al. predicted a 19 bp consensus sequence to be involved in NsrR binding, though the deletion analysis we used to
Figure 16. NsrR regulation of dnrN::lacZ, mobA::lacZ, and nnrS::lacZ.

Translational lacZ fusions to dnrN (A) mobA (B) and nnrS (C) were constructed in the wild type and ΔnsrR mutant, and β-galactosidase activity was determined in cells grown aerobically (blue) and anaerobically with nitrite (red). Results are presented as the mean of 6 determinations plus one SD.
Figure 16. NsrR regulation of $dnrN::lacZ$, $mob\Delta:: lacZ$, and $nnrS:: lacZ$. 
examine the F62 norB upstream region identified a larger, imperfect inverted repeat sequence spanning 29 bp and completely overlapping the site predicted by Rodionov et al., extending it by 5 bp on each side (185). In order to further characterize the NsrR binding site and thus determine bases likely to be important in protein-DNA interaction, we analyzed the 29 bp putative NsrR binding sites from genes known to be regulated by NsrR (norB, aniA, dnrN, mobA, and nnrS). Site directed mutagenesis was used to clone these putative NsrR binding motifs into the norB::lacZ fusion, replacing the wild-type NsrR binding sequence and allowing for determination of the strength of the different binding sites by measuring β-galactosidase levels in the context of the norB promoter (Figure 17). The putative NsrR binding site from the norB upstream region in strain FA1090 had the best match to an inverted repeat sequence and also displayed the lowest anaerobic norB::lacZ activity, presumably because the presence of the near perfect inverted repeat was ideal for high strength interaction between NsrR and the DNA. The NsrR binding site from norB in gonococcal strain F62 contains a single base pair change from that of FA1090 at the second base (T→A), and this single change, making the inverted repeat less perfect, was enough to allow a 28% higher level of anaerobic expression, suggesting derepression was easier to achieve at this less perfect sequence. This also demonstrated that bases outside of the 19 bp consensus predicted by Rodionov et al. were important for binding (185). The NsrR binding sites from dnrN, aniA, and mobA lacked similarity to the norB FA1090 inverted repeat sequence at various locations. These NsrR binding sites were not as capable as the FA1090 norB or F62
Figure 17. Alignment of the putative NsrR binding sites of NsrR-regulated genes. The 29 base pair inverted repeat was aligned with the upstream regions from *aniA*, *dnrN*, *mobA*, and *nrrS* using Multalin (38). Bases conserved in all putative NsrR binding sites are highlighted in yellow, and bases conserved with respect to the FA1090 *norB* NsrR binding site are highlighted in light gray. Stars above the sequence represent bases making up an inverted repeat in the FA1090 NsrR binding site. In strain F62, the 29 bp wild type NsrR binding site in a *norB::lacZ* fusion was replaced with the *norB*FA1090, *aniA*, *dnrN*, *mobA*, or *nrrS* NsrR binding site using site directed mutagenesis. β-galactosidase activity was measured aerobically and anaerobically with nitrite. The fold induction presented is the ratio of β-galactosidase activity (-O₂/NO₂)/+O₂ within each strain. Results are presented as the mean of 6 determinations plus one SD.
Figure 17. Alignment of the putative NsrR binding sites of NsrR-regulated genes.
*norB* NsrR binding sites at repressing *norB* expression aerobically and also displayed higher levels of anaerobic expression, probably due to lower strength binding by NsrR. The NsrR site from the *nrrS* gene showed the poorest similarity to that of the *norB* FA1090 inverted repeat sequence, but still retained some ability to repress *norB* expression aerobically by approximately four-fold (Figure 17).
Discussion

This work has helped elucidate the complex regulatory mechanism of the genes involved in gonococcal denitrification, *aniA* and *norB* (Figure 18). During aerobic growth, *aniA* and *norB* exhibit extremely low basal levels of expression due to the inactivity of FNR in the presence of oxygen and the activity of NsrR in the absence of NO respectively. When cells are shifted to an anaerobic environment in the absence of nitrite, FNR becomes active and *aniA* is partially induced by both FNR and NarP, while *norB* is still repressed due to the lack of NO. When nitrite is added to anaerobic cells, nitrite reductase (AniA) reduces nitrite to NO, which in turn inactivates NsrR. This results in a higher level of *norB* expression and an increase in expression of *aniA* as NsrR-mediated repression is relieved. This model also proposes that while *aniA* cannot be significantly expressed aerobically due to the absolute requirement for FNR (36, 106), *norB* can be expressed aerobically in the presence of NO, as might be encountered *in vivo* during an infection. The *norB* gene is subject to a second level of regulation by Fur and a gonococcal ArsR homolog. In the presence of iron, Fur binds to its operator and inhibits the binding of ArsR. However, when iron is limiting, Fur may not be bound to its operator, allowing ArsR access to its overlapping binding site and resulting in *norB* repression. Fur has been extensively studied as an iron-responsive repressor of gene transcription in both Gram positive and Gram negative bacteria. Fur regulates the transcription of genes whose proteins take up and metabolize iron to maintain homeostasis within
Figure 18. Schematic representation of *aniA* and *norB* regulation. In this model for regulation of *aniA* and *norB*, “+” indicates activation while “-” indicates repression. Figure is not drawn to scale. See discussion for detailed explanation.
Figure 18. Schematic representation of *aniA* and *norB* regulation.
the cell. Beyond this function, for which its role in regulation was initially defined, Fur has been implicated in the regulation of genes important in oxidative stress and acid resistance, as well as in virulence (87). Fur acts to repress transcription by binding to a 19 bp consensus sequence (Fur box) and blocking RNA polymerase entrance to the promoter or promoter clearance (58, 165, 232). Though generally thought of as a repressor, there are a few examples showing that Fur acts as an activator of transcription (58, 87). However, this activation function in *E. coli* was subsequently shown to be an indirect result of the repression of an antisense regulatory RNA (144). There have been several reports suggesting that *norB* is activated by Fur aerobically (79-80, 198), however most of the data supporting a role for Fur in activation of *norB* is based on demonstration that Fur can bind to the site. In an *in vitro* transcription assay, Delany *et al.* provide evidence that Fur may directly activate the meningococcal *norB* promoter (45). However, the *in vivo* translational fusion analysis of the gonococcal *norB* upstream region I performed showed no direct Fur activation, and the fact that the meningococcal and gonococcal *norB* upstream regions are 100% identical upstream of the -10 element suggests that Fur acts indirectly in both organisms (see Chapter 3).

The ArsR family of transcriptional regulators generally controls the expression of genes required for metal ion detoxification and efflux (32, 172). Unlike Fur, ArsR family proteins bind to their operators in the absence of their inducing metal ligands (32). However, this gonococcal ArsR homolog contains little similarity to any of the proposed metal binding sites in functionally characterized ArsR proteins
from other organisms (32, 172), and, in vivo, was shown to be unresponsive to various metal ions typically recognized by ArsR family proteins (This study). It is likely that gonococcal ArsR has become degenerate in its metal sensory domain and merely functions as an adapter protein that links positive regulation by Fur and iron and allows a decrease in norB expression when iron is limiting. However, it would be expected that ArsR plays an important role in regulating norB during infection, when iron is limiting and Fur would not always be bound to its operator (203).

It is becoming increasingly clear that NsrR controls the expression of nitric oxide detoxification systems in several pathogenic and environmental organisms, and that genes in the NsrR regulon contribute to resistance to nitrosative stress (61, 72, 132, 168, 184-186). There is a great deal of overlap between the currently defined NsrR regulon of E. coli and N. gonorrhoeae. The E. coli NsrR regulon is larger than that of N. gonorrhoeae, however, additional NsrR-regulated genes in E. coli are not present in the gonococcus (36, 61).

Aside from aniA and norB, less is known about the function of the other members of the known gonococcal NsrR regulon. Some very recent work has shown that gonococcal DnrN is involved in repair of proteins containing oxidatively damaged Fe-S clusters, but the precise mechanism of action is still not clear (167). The mobA gene (NGO0754) encodes a protein required for molybdopterin guanine dinucleotide (MGD) synthesis, an essential cofactor for many bacterial molybdoenzymes (82, 195). MobA catalyzes the synthesis of MGD from molybdopterin and GTP. In E. coli, molybdopterin is synthesized in a complicated
enzymatic process involving the gene products of the moa, moe, and mog operons (197). None of the sequenced Neisserial species contain orthologs to any of these operons, so it is a mystery why mobA, utilized in the very last step of MGD synthesis, is maintained in the genome and is regulated. Perhaps these bacteria possess some novel mechanism to hijack molybdopterin from the host or from other commensal organisms. Another possibility is that the Neisserial mobA ortholog has evolved a novel function. Regulation of this gene by NO, and the lack of Neisserial proteins with homology to molybdoenzymes from other organisms, alludes to this possibility. In any case, further work is needed to characterize the role of this protein on anaerobiosis or NO metabolism.

The nnrS and nsrR genes are transcribed from overlapping divergent promoters, and I had originally reported that the NsrR binding site located in this intergenic region was responsible for regulating nsrR (109). However, the gonococcal strain purportedly containing the nsrR::lacZ fusion was induced over 60-fold in the presence of NO, while, when cloned downstream of the norB -10, the weak NsrR binding site in the nsrR upstream region was only induced four-fold by NO (Figure 17). Upon re-sequencing of this nsrR::lacZ clone, it was found to actually harbor the norB::lacZ fusion, indicating that there was a strain mix-up at some point. Further study of the true nsrR::lacZ fusion strain revealed that activity from this promoter was low, and was not subject to autoregulation. Subsequently, it was discovered that this NsrR binding site was actually involved in regulating the divergently transcribed nnrS gene.
The *nnrS* gene is extremely widespread among bacteria, and *nnrS* expression is induced under denitrifying conditions. Still, very little is known about this gene’s function, and mutation of the *nnrS* gene in several organisms has displayed no phenotype (13, 103, 128, 132). In *Rhodobacter sphaeroides*, a Δ*nnrS* mutation resulted in a slightly altered chemotactic response towards nitrate (103), however, *N. gonorrhoeae* contains no flagellar machinery and does not possess any genes required for nitrate utilization. A gonococcal Δ*nnrS* mutant displayed no growth defects aerobically or anerobically (data not shown), though NnrS may play a role in denitrification and/or nitrosative stress resistance in the much different environment of the human host. Regulation of *nnrS* by NsrR was shown to be weak, with only a two-fold increase in expression in a Δ*nsrR* mutant. Unlike *dnrN*, *mobA*, and *norB*, whose NsrR binding sites are located immediately downstream of their respective -10 elements, the NsrR binding site in the *nnrS* upstream region is located immediately upstream of the -35 element, suggesting a different mechanism of repression, such as blocking access of RNA polymerase α-CTD to a bacterial UP sequence or an activator protein. The location of the NsrR binding site also explains the difference in fold induction between the *nnrS* gene (two-fold) and induction of *norB* when the NsrR binding site of *nnrS* was cloned upstream of the *norB* -10 (four-fold).

An alignment of the putative NsrR binding sites from the NsrR regulated genes, *norB*, *aniA*, *dnrN*, *mobA*, and *nnrS*, shows a high degree of similarity. In Figure 17 it was observed that differences in the sequence of NsrR binding sites had a large effect on the ability of NsrR to regulate transcription aerobically or in the
presence of NO. Degeneration within the NsrR binding site allows for a dynamic range of regulation by NsrR; the more perfect the repeat, the higher the degree of regulation (i.e. fold induction by NO). Rodionov et al. predicted a 19 base pair NsrR binding site that would be centered within the aforementioned 29 base pair sequence proposed (185). Another Rrf2 transcriptional regulator, IscR, has been shown to protect a conserved 25 bp inverted repeat sequence as well as a more redundant 26 bp sequence (71, 230), suggesting that the NsrR binding site may be bigger than that predicted by Rodionov et al., as well as leaving open the possibility that NsrR may be able to recognize two dissimilar binding sites (185).

It would be ideal to determine the effects of gonococcal NO metabolism during active infection, and growing evidence suggests that the gonococcal reduction of host produced NO may, in part, account for asymptomatic gonococcal infection (10, 34, 36). Elucidation of NsrR function may help to reveal other NO-regulated genes that could potentially be involved in virulence. In order to understand the gonococcal NO stimulon, it is necessary to further investigate the NsrR protein, including the interaction with its operator and with its ligand, NO.
Chapter 2

Biochemical Analysis of Gonococcal NsrR, a Nitric Oxide-Sensing, Rrf-2 Type Transcriptional Repressor

Introduction

As an obligate human pathogen, the gonococcus is extremely well adapted for growth on a variety of mucosal surfaces (54, 70). As described above, *N. gonorrhoeae* is a facultative anaerobe capable of nitrite respiration under anaerobic conditions and nitric oxide respiration in the presence of NO (125, 168). The gonococcal denitrification pathway is subject to regulation by NsrR, a nitric oxide sensing Rrf2-type transcriptional repressor in the winged helix superfamily. Beyond this function, NsrR has also been reported to be involved in regulation of nitric oxide detoxification systems and nitrosative stress resistance in several organisms (19, 61, 72, 109, 168, 184-186, 199).

NsrR homologs in *Bacillus subtilis, Salmonella typhimurium*, and *E. coli* have all been shown to regulate the expression of the gene encoding flavohemoglobin (Hmp), one of the most extensively studied NO-detoxifying proteins (72, 168, 186). Hmp is present in a broad range of bacterial species, and *hmp* mutations in most bacteria severely compromise bacterial survival in the presence of NO (72). However, *N. gonorrhoeae* does not contain an *hmp* ortholog, and unlike most bacteria, gonococcal *norB* mutants incubated anaerobically in the presence of nitrite (NO-generating conditions) are still able to survive (66, 107, 233). This suggests that
N. gonorrhoeae has additional NO detoxification pathways, and that these pathways are novel (12). This also underscores the importance of further characterizing NsrR.

Though little is known about this family of transcriptional regulators, a closely related Rrf2 protein, IscR, has been more fully characterized in E. coli and is reported to sense the Fe-S cluster status of the cell by reversible binding of a [2Fe-2S] cluster (71, 184, 196, 230). NsrR contains three conserved cysteine residues that, in IscR, coordinate this [2Fe-2S] cluster. It is predicted that NsrR also coordinates an Fe-S cluster, and that interaction of this cluster with NO perturbs the ability of the protein to bind DNA (184). Secondary structure analysis of gonococcal NsrR and E. coli IscR reveals a great deal of similarity (Figure 19) (30, 115). Both proteins are predicted to contain the customary N-terminal tri-helical bundle, with the third helix presumed to be the recognition helix responsible for the principal interactions with DNA through insertion into the major groove (5, 27). The two strand β-hairpin immediately downstream of the presumed recognition helix makes up the “wing,” and is predicted to provide an additional interface for DNA contact by interacting with the minor groove of the DNA through charged residues in the hairpin (5). In both of these regulators, the three conserved cysteine residues presumed to be involved in Fe-S binding are located in an unstructured region outside of the domain presumed to be involved in DNA binding (C90, C97, and C103 in NsrR, and C92, C98, and C104 in IscR), with a nearly identical cysteine spacing in both proteins.

The potential pathogenic outcome of the bacterial response to NO sensing, as well as the relative lack of information available on the Rrf2 class of transcriptional
Figure 19: Secondary structure analysis of the gonococcal NsrR and *E. coli* IscR proteins. The PSIPred protein structure prediction server was used to predict the secondary structure of NsrR from *N. gonorrhoeae* and IscR from *E. coli*. The top row represents the confidence of the structure prediction. In the second and third row, cyclinders or (H) represent predicted α-helical regions, and arrows or (E) represent predicted β-strands respectively. The last row represents the amino acid sequence.
Figure 19: Secondary structure analysis of the gonococcal NsrR and *E. coli* IscR proteins.
regulators, gave cause to further analyze NsrR. In this chapter I describe the preparation of an epitope-tagged gonococcal NsrR extract for use in vitro. A thorough analysis of NsrR/DNA interaction with the upstream regions of norB, aniA, and nnrS was completed in order to calculate binding affinities for the NsrR operators controlling expression of those genes. NsrR binding sites were identified in the upstream regions of norB and nnrS by generating DNaseI footprints. Mass spectroscopy was used to analyze the iron-sulfur cluster content of purified NsrR extracts. Finally, the effects of alanine substitution of conserved cysteine residues in NsrR are reported.
Results

**NsrR::FLAG purification.** Site directed mutagenesis was used to add the codons of a FLAG peptide tag to the 3’ end of the gonococcal nsrR gene. This fusion gene was overexpressed in *E. coli,* and NsrR::FLAG protein (17 kD) was subsequently isolated with M2 antibody (α-FLAG) to generate an extract for use *in vitro.* The recovered extract was shown to be very pure, as no contamination was observed in a silver stained sample and no cross-reactive species were observed by Western blot analysis (Figure 20). In a gonococcal strain containing a *norB::lacZ* translational promoter fusion (RUG7500), the wild-type nsrR gene was replaced with a copy of *nsrR::FLAG* (RUG7800). Comparison of β-galactosidase levels in the wild-type and *nsrR::FLAG* strain confirm that NsrR::FLAG is still functional as a repressor and is capable of regulating expression of *norB::lacZ* under NO-generating conditions (Figure 21A). *In vivo* functionality of NsrR::FLAG was also confirmed by chromatin immunoprecipitation (Figure 21B). In a strain expressing *nsrR::FLAG,* ChIP analysis demonstrated enrichment of DNA containing the *aniA/norB* intergenic region and *dnrN* upstream region, verifying that NsrR::FLAG can bind to its operator *in vivo.*

NsrR is predicted to coordinate a [2Fe-2S] cluster involved in NO-sensing (160, 184). Purification of NsrR::FLAG by immunoprecipitation, though resulting in an extremely pure extract, was not amenable for the production of protein in the concentrations necessary for observation of an iron-sulfur cluster by electron paramagnetic resonance (EPR) or UV-visible spectroscopy. In order to confirm that this NsrR::FLAG extract contained a [2Fe-2S] cluster, mass spectroscopic analysis of
Figure 20: Analysis of NsrR::FLAG extract. Affinity purified NsrR::FLAG was analyzed by SDS-PAGE and silver stained (left) or probed with M2 antibody in Western blot analysis (right). No contaminating proteins or cross-reactive species are evident.
Figure 20: Analysis of NsrR::FLAG extract.
**Figure 21. In vivo functionality of NsrR::FLAG.** (A) Wild type (RUG7500) and nsrR::FLAG expressing (RUG7800) gonococcal norB::lacZ reporter fusion strains were grown aerobically (blue) and anaerobically with nitrite (red), and β-galactosidase activity was measured. These data are the mean of 6 determinations plus one SD. B) Chromatin immunoprecipitation was performed on gonococcal cells expressing nsrR::FLAG. Enrichment of DNA in the norB/aniA intergenic region and the dnrN region was observed when immune complexes were isolated with M2 antibody (α-FLAG) compared to non-specific antibody (goat α-mouse). Enriched DNA was observed by performing PCR for 27 cycles.
Figure 21. *In vivo* functionality of NsrR::FLAG.
undigested protein was performed in linear positive ion mode (Figure 22). The predicted mass of a [2Fe-2S] cluster is 175.8 Daltons, while the predicted mass of NsrR with the attached octapeptide FLAG tag is 17,038.8 Daltons. Combining the mass prediction of NsrR::FLAG with that of [2Fe-2S] gives approximately 17,214 Daltons, which was the observed mass peak of the NsrR::FLAG sample. The data also suggests that the extract is fully loaded with [2Fe-2S], as there is no peak visible at 17,038.8 Daltons. The case is different for IscR, where the purified protein was observed to be 26-66% occupied with iron-sulfur cluster (70). The observed discrepancy in iron-sulfur cluster content between purified NsrR and IscR may simply be due to the different expression and purification schemes used in each case, or conversely, it may be due to the fact that physiologically, apo-IscR is a functionally relevant state, whereas apo-NsrR has yet to be shown to be involved in gene regulation in vivo (19, 71, 109, 168, 184).

**NsrR-specific binding of the norB upstream region.** An Electrophoretic Mobility Shift Assay (EMSA) was performed using a 120 bp biotin end-labeled fragment of the norB upstream region (-90 to +30 relative to the translation start site). Labeled norB target DNA was shifted to a higher molecular weight upon addition of NsrR::FLAG (Figure 23). DNA binding was shown to be NsrR-specific by supershift with M2 antibody. Binding was shown to be sequence-specific by shift inhibition upon the addition of a 500 fold higher concentration of unlabeled norB target to the binding reaction.
Figure 22. Mass Spectroscopic analysis of NsrR::FLAG extract. Mass spectrum of undigested NsrR::FLAG in linear positive ion mode. 1800 laser shots were summed to obtain this spectrum. The observed mass peak is equal to the predicted mass of the protein plus the mass of a [2Fe-2S] cluster.
Figure 22. Mass Spectroscopic analysis of NsrR::FLAG extract.
Figure 23. In vitro analysis of NsrR::FLAG extract. EMSA analysis was performed on a biotin end-labeled fragment of the *norB* promoter: Lane (1) No NsrR addition, (2) 50 nM NsrR::FLAG, (3) 50 nM NsrR::FLAG + M2 antibody, (4) 50 nM NsrR::FLAG + 500X concentration (1750 fmol) of unlabeled *norB* fragment.
Figure 23. *In vitro* analysis of NsrR::FLAG extract.

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<td>αFLAG antibody:</td>
<td>-</td>
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<tr>
<td>500X unlabeled norB:</td>
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**Binding affinity at the NsrR operator.** In the previous chapter, I partially characterized the gonococcal NsrR binding site *in vivo*, by analysis of the ability of NsrR to regulate promoter-*lacZ* reporter fusions containing variations of the NsrR operator. In order to determine important bases of the 29 bp inverted repeat sequence presumed to make up the NsrR binding site, I determined the *in vitro* binding affinity of NsrR for its binding sites in the *norB*, *aniA*, and *nnrS* upstream regions. EMSA analysis of the 120 bp biotin end-labeled *norB* fragment with increasing concentrations of NsrR::FLAG allowed for the estimation of dissociation constant (K\(_d\)) between NsrR and its operator in *norB*, which was equal to the concentration of NsrR::FLAG at which half of the biotinylated DNA was bound. NsrR was found to interact with the *norB* operator with an estimated K\(_d\) of 7 nM (Figure 24). This measurement was verified by shifting half of the *norB* fragment with NsrR::FLAG at its estimated K\(_d\) in a subsequent EMSA (Figure 24).

In the previous chapter I described the characterization of putative NsrR operators by cloning these sites upstream of the *norB* -10 element and measuring the ability of these sites to regulate *norB* in response to NO. To estimate the K\(_d\) between NsrR::FLAG and its operator in *aniA*, a 120 bp biotinylated fragment, identical to the one used to measure NsrR/*norB* interaction except for the 29 bp operator replacement, was used. NsrR::FLAG was shown to interact with the *aniA* operator with an approximate K\(_d\) of 19 nM (Figure 24). These results show that NsrR has a different affinity for the promoters of each of the denitrification genes.
The putative NsrR binding site from the *nnrS* upstream region has a low level of similarity to the site found in *norB* and was shown to only weakly regulate *nnrS* (Figure 17). The *nnrS* gene is also transcribed divergently with the *nsrR* gene, and although *nsrR* was shown not to be autoregulated by β-galactosidase activity in a Δ*nsrR* mutant, and there was no other sequence with similarity to an NsrR binding site in this intergenic region, *E. coli* IscR was found to be autoregulated and demonstrated to recognize two distinctly different classes of binding sites (71, 230). To determine if a second NsrR site existed in this intergenic region, an EMSA was performed with a 380 bp biotin end-labeled fragment of the *nnrS/nsrR* intergenic region (+180 to +30 relative to the translation start site of *nnrS* and *nsrR*, respectively; Figure 24). No higher order shifted bands were detected in this assay, suggesting that there is only one NsrR binding site in this intergenic region. The 35 nM Kₐ affinity measurement observed with this fragment was higher than that observed for *norB* or *aniA*, and consistent with the weaker interaction expected for the weakly regulated *nnrS* gene. A 120 bp fragment of the *norB* upstream region in which the 29 bp *norB* NsrR operator was replaced with that from *nnrS* displayed a Kₐ comparable to that seen in the 380 bp fragment, further suggesting that this low similarity operator was the only functional regulatory site in this region (Figure 25).

In comparison to the NsrR binding site of *norB*, the NsrR binding site of *nnrS* shows greater similarity to only a half-site (Figure 24). This suggested that NsrR may recognize only a half-site in the *nnrS* non-coding region. To test whether NsrR can recognize a half site, I removed the last 14 bp of the 29 bp *norB* NsrR binding site by
Figure 24. **Binding affinity at the NsrR operator.** (A) Increasing concentrations of NsrR::FLAG were used to shift 120 bp biotin end-labeled fragments containing the *norB, aniA* and *norB* half-site NsrR operators, as well as a 380 bp fragment of the *nnrS/nsrR* intergenic region. Panels to the right show fragments shifted to their estimated $K_d$ in subsequent experiments. (*) Positive control containing full length *norB* NsrR binding site (B) Spot densitometry was used to quantify the ratio of shifted DNA from the EMSA analysis in (A). $K_d$ estimations were calculated by extrapolating the concentration of NsrR::FLAG where 50% of the labeled fragment was shifted. Legend shows the sequence of the NsrR operators from *norB, aniA, nnrS*, and the *norB* half-site. Bases shaded in grey are common to the NsrR operator of *norB*.
Figure 24A. Binding affinity at the NsrR operator.
Figure 24B. Binding affinity at the NsrR operator.
**Figure 25.** The NsrR binding site of *nrrS* has the same affinity for NsrR as the *nrrS/nrrS* intergenic region. NsrR displays a similar affinity for the 380 bp biotinylated fragment of the *nrrS/nsrR* intergenic region (left) as it does for a 120 bp fragment of the *norB* region in which the 29 bp *norB* NsrR operator was replaced with that from *nrrS* (right).
Figure 25. The NsrR binding site of *nnrS* has the same affinity for NsrR as the *nnrS/nnrS* intergenic region.
substitution with an arbitrary sequence, leaving only the first 15 bp from the original sequence (5’-TTTAACATTCAATATT). NsrR::FLAG was unable to cause a significant shift of a labeled target fragment containing this site (Kd > 1 µM; Figure 24), suggesting that NsrR cannot bind to a half-site.

**DNaseI footprinting of the norB and nsrR promoters.** Rodionov *et al.* used computational analysis to predict that NsrR recognized a 19 bp sequence in neisserial genes, while I used genetic analysis that suggested that the NsrR binding site spanned a larger 29 bp sequence (109, 185). To resolve these differences, DNase I footprinting was performed on the *norB* and *nsrS* upstream regions, which contained the highest and lowest affinity NsrR binding sites respectively.

As expected, the footprint created by binding of NsrR::FLAG to the *norB* upstream region spanned the 29 bp inverted repeat sequence, protecting nucleotides -31 to -59 relative to the translation start site (Figure 26A). These data definitively show that this inverted repeat sequence is the NsrR binding site. The footprint generated upon the interaction of NsrR::FLAG with the *nsrS* upstream region also protected the previously identified region, spanning nucleotides -107 to -133 relative to the translation start site (Figure 26B). Though this low affinity binding site reveals only a half-site in comparison to the site in *norB*, NsrR still retains the capacity to bind DNA across an extended region.

**NsrR responds directly to Nitric Oxide.** It has previously been shown that NsrR derepresses the genes in its regulon in response to NO *in vivo* (109, 184). To show that NO directly inhibits the ability of NsrR to bind to its operator *in vitro*, I
Figure 26. DNaseI footprint analysis of the *norB* and *nnrS* upstream regions.

(A) A DNaseI footprint was generated from a radiolabeled fragment of the *norB* upstream region using increasing concentrations of NsrR::FLAG. Binding by NsrR::FLAG was shown to protect nucleotides -31 through -59 relative to the translational start site. In the sequence data, the large bracket above the sequence represents nucleotides that make up the predicted NsrR binding site (109), and the small bracket makes up the consensus predicted by Rodionov *et al.* (185). Underlined bases represent nucleotides protected from DNaseI digestion. (B) DNaseI footprint analysis of the *nnrS* upstream region using increasing concentrations of NsrR::FLAG. Protein binding was shown to protect nucleotides -107 through -133 relative to the translational start site. Brackets above the sequence show the predicted NsrR binding site. Underlined bases represent nucleotides protected from DNaseI digestion. (NA) No addition.
Figure 26. DNaseI footprint analysis of the norB and nnrS upstream regions.
performed an EMSA with increasing concentrations of the long half-life (~20 h) NO donor DETA-NO. Increasing concentrations of DETA-NO in the binding reactions inhibited the shift of the biotin-labeled norB target DNA, showing that NsrR senses NO by a direct mechanism (Figure 27).

**Role of conserved cysteine residues in NsrR function.** The gonococcal NsrR protein contains three conserved cysteines that correspond to residues suggested to coordinate a [2Fe-2S] cluster in IscR (230). To investigate the role of these cysteine residues in NsrR function, C90A, C97A, and C103A mutations were constructed in the \( nsrR::\text{FLAG} \) gene in gonococcal strain RUG7800. These strains were monitored for their ability to repress \( \text{norB}::\text{lacZ} \) expression in the absence of NO. A C90A substitution resulted in a level of \( \text{norB}::\text{lacZ} \) expression comparable to that seen in a \( \Delta nsrR \) mutant (RUG7600), illustrating the importance of this residue in NsrR function and indicative of a potential role in Fe-S coordination (Figure 28A). Expression of \( \text{norB}::\text{lacZ} \) in the C97A and C103A mutants were 52% and 74% of that observed in the \( \Delta nsrR \) mutant respectively, showing that these residues are also likely important in the presumed coordination of Fe-S.

In order to further investigate the cysteine mutants of NsrR::FLAG, purified extracts of each modified protein were isolated and used in EMSA analysis in order to determine their capacity to bind to the \( \text{norB} \) upstream region. A 50 nM concentration of wild type NsrR::FLAG was enough to shift all DNA in the binding reaction (Figure 28B). The cysteine mutants of NsrR::FLAG were not as effective as the wild type at shifting the \( \text{norB} \) fragment. The C97A mutant displayed very little binding at
Figure 27. Nitric Oxide sensing by purified NsrR::FLAG using long half-life NO donor DETA/NO. EMSA analysis was performed on a biotin end-labeled fragment of the norB promoter. Lane (1) contains DNA only. All other lanes contain 25 nM NsrR::FLAG with increasing concentrations of the long half-life (~20 h) NO donor DETA/NO. (NA) No addition.
Figure 27. Nitric Oxide sensing by purified NsrR::FLAG using long half-life NO donor DETA/NO.
Figure 28. Role of conserved cysteine residues in NsrR function. (A) Single C90A, C97A, and C103A substitutions were created in the appropriate codons of the nsrR::FLAG gene. These mutants were monitored for their ability to repress norB::lacZ aerobically in gonoccocal reporter fusion strains. Cells were grown aerobically and β–galactosidase activity was measured and compared to parental nsrR::FLAG expressing strain (RUG7800) and ΔnsrR strain (RUG7600). These data are the mean of 6 determinations ± one SD. (B) Purified NsrR::FLAG containing C90A, C97A, or C103A substitutions were monitored for their ability to bind to a biotin end-labeled fragment of the norB promoter.
Figure 28. Role of conserved cysteine residues in NsrR function.
50 nM and only shifted the DNA completely when the NsrR concentration was in the micromolar range. The C90A and C103A mutants only showed a measureable shift of the norB fragment at a concentration of 5 µM NsrR::FLAG, far in excess of what would be encountered in vivo. The NsrR C90A mutant protein was also incapable of shifting a fragment containing the nnrS/nsrR intergenic region at concentrations up to 5 µM (data not shown).

To confirm that the loss of norB regulation and decreased binding affinity associated with the C90A mutation was concomitant with lack of [2Fe-2S] coordination, mass spectroscopy of undigested NsrR::FLAG C90A protein was performed in linear positive ion mode (Figure 29A). Unlike the wild type protein, which displayed a 175.8 Dalton increase above the predicted mass attributable to the presence of [2Fe-2S] (Figure 22), the observed mass peak of NsrR::FLAG C90A matched the predicted mass of 17,006 Daltons, verifying the absence of [2Fe-2S], and confirming that C90A is the apo-form of NsrR.

High NsrR::FLAG C90A concentrations were able to shift a biotinylated fragment of the norB promoter (Figure 28B). However, unlike the wild type NsrR::FLAG protein, the mobility shift observed with NsrR::FLAG C90A was not inhibited by the addition of a NO donor (Figure 29B). The NO insensitivity of NsrR::FLAG C90A shows that the ability of NsrR to sense and respond to NO is dependent upon the presence of [2Fe-2S].
Figure 29. NsrR cysteine residues coordinate a NO-sensitive [2Fe-2S] cluster.

(A) Mass spectrum of undigested NsrR::FLAG in linear positive ion mode. 1800 laser shots were summed to obtain this spectrum. The observed mass peak is equal to the predicted mass. (B) Purified NsrR::FLAG (wild type or C90A) was monitored for its ability to bind to a biotin end-labeled fragment of the norB promoter with and without the presence of long half life NO-donor DETA-NO. (NA) No addition.
Figure 29. NsrR cysteine residues coordinate a NO-sensitive [2Fe-2S] cluster.
Discussion

This work has helped to advance the understanding of the biochemistry of Rrf2 type proteins, a subfamily of winged helix regulators about which relatively little is known (5). Comparison of gonococcal NsrR and the related IscR protein in *E. coli* illustrate that several similarities and differences exist between these two regulators.

Iron-sulfur clusters are essential cofactors in many different types of proteins, and these clusters can be involved in any number of cellular processes, including electron transfer, nitrogen fixation, and gene regulation to name just a few (112). In *E. coli*, IscR is predicted to utilize three cysteine residues to coordinate a labile Fe-S cluster capable of sensing the Fe-S status of the cell by reversible binding of a [2Fe-2S] cluster (71, 196). IscR has also been demonstrated to regulate genes involved in biogenesis and repair of Fe-S clusters in times of oxidative stress (71, 108, 177, 230). This observation comes as no surprise, as reactive oxygen species are known to damage or destroy Fe-S clusters, explaining the need for their high turnover rate under stressful conditions (71). There is a great deal of similarity in secondary structure between NsrR and IscR (Figure 19), and there is likely some functional redundancy as well. There are various examples of crosstalk between the regulons of each regulator. NO is capable of causing damage to Fe-S clusters, and IscS, a cysteine desulfurase in the IscR regulon, has been demonstrated to play a role in the repair of Fe-S clusters damaged by nitrosative stress (229). IscR, like NsrR, is
expected to sense NO by a direct mechanism, and microarray data shows that genes in the IscR regulon are indeed regulated in response to NO (108, 177). Also, the NsrR protein of several organisms, including Neisseria spp. and E. coli, has been shown to regulate dnrN, a second protein demonstrated to repair the Fe-S clusters of proteins damaged by reactive oxygen or nitrogen (97, 109, 167).

There are several differences between NsrR and IscR. IscR is capable of interacting with one class of operator when coordinating an Fe-S cluster and to a second class in its apo-form, while only one class of NsrR binding site has been identified in both Neisseria spp. and E. coli to date (19, 71, 97, 109-110, 170, 230). While binding of the [2Fe-2S] cluster in IscR has been shown to be reversible, there is no evidence that this is the case in NsrR, and recent studies have suggested that the Fe-S center in NsrR is nitrosylated in response to NO, but remains protein-bound (215, 231). IscR was also shown to act as a direct activator of the suf operon in its apo-form (230). NsrR does not seem to be as broad acting as IscR, and there is no evidence that NsrR is able to act as a direct activator of gene transcription at any promoter (36, 109-110, 170). Microarray studies comparing nsrR+ and ΔnsrR strains in N. meningitidis have suggested that the Neisserial NsrR regulon is small, though it should be noted that this study would not have taken the role of apo-NsrR or NO-treated NsrR into account, if these species are in fact relevant, and would not be able to discern genes requiring NsrR for activation if such genes do exist (97).

IscR was shown to autoregulate its own expression, and DNaseI footprinting of the E. coli iscR promoter revealed two copies of a class I IscR binding site
overlapping the -35 region (71). This was not the case for gonococcal NsrR.

Footprinting of the gonococcal nsrR/nnrS intergenic region revealed just a single low affinity NsrR binding site that was genetically determined to regulate nnrS, and not to be involved in autoregulation of nsrR. By gel shift analysis, the nsrR upstream region could not be shifted by NsrR::FLAG C90A, where [2Fe-2S] was shown to be absent, or by NO-treated NsrR, showing that these species are not involved in binding to the nsrR upstream region, and further supporting that gonococcal nsrR is not autoregulated (data not shown). The footprint of iscR also revealed a second region of protection that was unique to this promoter, however no additional regions of protection were observed in the nsrR footprint (71). The NsrR footprints of norB and nnrS fail to show any bases that are hypersensitive to DNase cleavage, whereas in E. coli, the IscR footprints of several promoter regions contained bands that corresponded to hypersensitivity, suggesting that binding by IscR may have a larger effect on DNA structure and cause more DNA bending than NsrR (71, 110).

The dissociation constants estimated from EMSA analysis using the norB, aniA, and nsrR upstream regions may not be the same as that observed in vivo, under differing ionic conditions and in the limiting environment of the cell. These constants should be taken as relative values for comparison between each other. The actual concentration of NsrR in vivo is unknown, however, it is expected to be quite low as the gonococcal NsrR regulon is likely small, and β-galactosidase activity from the nsrR promoter is low. The use of a streptavidin-purified biotinylated primer for generating an EMSA target DNA made it possible to perform this assay.
Radiolabeling is not as efficient and cannot guarantee that all target DNA used in the binding reaction is labeled, whereas the biotin labeled primer ensures that all target DNA is labeled and can be easily quantitated. As expected from the previous genetic analysis, the *nnrS* promoter displayed the least affinity for NsrR while the *norB* promoter displayed the highest affinity. The DNaseI footprint generated in the *nnrS* upstream region shows resistance to DNaseI cleavage that overlaps some of the -35 element (Figure 26), suggesting that the mechanism of NsrR repression at this promoter is the inhibition of RNA polymerase binding. As for *norB*, footprint analysis displayed DNaseI resistance downstream of the extended -10 element, suggesting the mechanism of repression at this promoter could be either inhibition of binding or prevention of promoter clearance by RNA polymerase.

During the course of this study, I attempted to use mass spectroscopy to observe the nature of the interaction between NsrR and NO. Treatment of purified NsrR with NO resulted in conversion of the protein from a charge of +1 (Figure 22) to a charge of +3, and the resultant mass peak was a broad triplet of which an accurate mass could not be determined (data not shown). It is not known if this charge increase was due to the intrinsic characteristics of a NO-modified NsrR or the addition of chemical adducts to NsrR that are hyper-reactive to matrix-assisted laser desorption/ionization. A possible cause of the charge increase was the addition of dinitrosyl adducts to each Fe atom in the [2Fe-2S] cluster, and two recent studies have shown that the interaction of NsrR with NO in other organisms does result in such adducts (215, 231). The formation of dinitrosyl-iron complexes is also the
mechanism of NO modification of SoxR, another [2Fe-2S] cluster containing transcription regulator (48). Dinitrosyl addition to Fe in analogs of the iron sulfur complex cause a +1 increase in charge (88), and dinitrosyl iron is a NO-derived intermediate with the redox equivalence of NO$^+$ (218). However, D’Autréaux et al. have shown that the presence of dinitrosyl iron in purified Fur did not cause a charge increase in the protein, though the active site Fe in Fur is not part of an Fe-S cluster (42).

In conclusion, this study extends recent work dealing with the NO-sensitive repressor protein NsrR. The response to NO sensing in some organisms is beginning to emerge as an instrument of virulence, and it will be important to be aware of exactly what changes occur when a pathogen encounters NO (i.e. as part of the human immune response). Future work will attempt to elucidate the overall response of *N. gonorrhoeae* upon encountering NO, and to determine what that response means for the host.
Chapter 3
Genomic Analysis of the Denitrification Regulation in the Neisseria Genus

Introduction

Twelve species and biovars in the Neisseria genus can be isolated from humans, but only two of these species, N. gonorrhoeae and N. meningitidis, are frequently pathogenic (221). Differentiation of Neisseria spp. can be accomplished by characteristics such as acid production from carbohydrates, nitrate reduction capacity, and polysaccharide production using sucrose as a carbohydrate source (124). Human Neisseria spp. can be divided into two major groups, the first of which includes N. gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea, N. flavescens, and N. polysacharea. Generally, members of this group grow as nonpigmented, translucent colonies, with the exception of the yellow-pigmented N. flavescens. The second group includes the saccharolytic commensal strains, N. subflava (including the N. subflava biovars perflava and flava), N. sicca, and N. mucosa. Colonies of these species are generally opaque and yellow-pigmented (124).

N. gonorrhoeae is considered to be pathogenic at all times, and the gonococcus is extremely well adapted for infection of mucosal surfaces including the cervix, urethra, rectum, and, occasionally, the nasopharynx (122, 199). Serious complications of gonococcal infection are believed to be due to the fact that the initial infection can oftentimes be asymptomatic, especially in women (12, 34). Unlike N. gonorrhoeae, not all strains of N. meningitidis are able to cause disease in healthy
individuals, rather pathogenicity is associated with specific serogroups (50, 162).

Encapsulated meningococcal strains belonging to the serogroups A, B, C, and W-135 have most often been associated with epidemic disease (124). Meningococcal strains may be carried asymptptomatically in the oro- or nasopharynx, with carriage occurring in 3-30% of healthy persons in geographic areas that are non-epidemic (74, 124, 155).

Sporadic isolation of commensal *Neisseria* spp. from disseminated sites, blood, and cerebrospinal fluid has been reported, though no correlation has been established between any of these species and disease. However, some commensal species are capable of opportunistic pathogenicity in immunocompromised individuals, though this occurrence is rare (95, 130, 155). The commensal *Neisseria* spp. share the same ecological niche as *N. meningitidis*, occupying the nasopharynx and/or oropharynx where they exist as part of the normal flora. The carriage rate of *N. lactamica* in children is high, and it is estimated that 59% of children have been colonized by *N. lactamica* by 4 years, though only 1% of adults were shown to harbor this species in a separate study (74, 124). In studies investigating the carriage rates of several other commensal *Neisseria* spp., it was found that 40-96 % of adults carried *N. subflava-perflava*, 45% of adults carried *N. sicca*, 29% carried *N. cinerea*, 11-26% carried *N. subflava-flava*, and 25% carried *N. mucosa* (124).

As described previously, *N. gonorrhoeae* and *N. meningitidis* are both cable of anaerobic growth using nitrite and NO as alternative electron acceptors. The commensal *Neisseria* spp. are also capable of denitrification, and some species are able to utilize a more complete denitrification pathway, containing genes encoding for
nitrate and/or nitrous oxide reductases (11). In recent years, bacterial denitrification has been implicated to play a role in virulence in several bacteria, including *Brucella* spp. and *Pseudomonas aeruginosa* (8, 217). Denitrification has also been implicated in the pathogenicity of *N. meningitidis*, where active denitrification has been linked to modulation of host cytokine responses, enhanced intracellular survival, and inhibition of apoptosis in a macrophage model (205-206, 216). In the gonococcus, inactivation of the denitrification pathway hinders formation of biofilms and may prevent the establishment of anti-inflammatory nitric oxide steady state levels (34, 60, 206).

Due to the fact that *N. gonorrhoeae* is an obligate human pathogen, it has been a challenge to establish an animal model that would allow direct testing of the involvement of denitrification as it relates to virulence. Comparative genomics may be a useful method to evaluate the role of specific proteins in neisserial pathogenesis, as only two neisserial species are capable of causing disease in healthy individuals. Recent completion of genome sequencing projects for the commensal *Neisseriaceae* has allowed for a genomic survey of the denitrification pathway, allowing a comparison of pathogenic (*N. gonorrhoeae*, *N. meningitidis*) to commensal *Neisseria* species (*N. lactamica*, *N. cinerea*, *N. subflava*, *N. sicca*, *N. mucosa*, and *N. flavescens*).

Previous members of the Clark laboratory have explored the genetic organization and amino acid sequence of each of the four reductases involved in denitrification in *Neisseria* spp. (Nar, Nir, Nor, Nos, Figure 30) (11). In this work, it was shown that there was high sequence conservation within the catalytic domains of
Figure 30. Extent of denitrification in Neisseria species. The enzymatic steps of the denitrification pathway are shown in conjunction with intermediate NO$_x$ substrates. Enzyme-complex names are given above each arrow, and gene names below. All neisserial species contain enzymes for nitrite and nitric oxide reduction, while only one commensal, *N. mucosa*, contains a functional nitrate reductase gene and only some commensal species contain a functional nitrous oxide reductase.
Figure 30. Extent of denitrification in *Neisseria* species.
the reductases involved in denitrification, while some unique differences existed in regards to the presence of nitrate or nitrous oxide reductases, changes within specific protein domains of the nitrite reductase, and the mechanisms of gene inactivation within the nitrous oxide reductase regulon. In chapter 1, I described the regulation of gonococcal denitrification and showed that five regulatory proteins were involved. FNR, NarP, and NsrR act to regulate *aniA*, while Fur, ArsR, and NsrR regulate *norB* (Figure 18). In this chapter I use comparative genomics to examine potential differences in the regulation of the neisserial denitrification pathway in regards to analysis of the regulators involved in controlling expression of denitrification genes, as well as the analysis of the upstream regions of those genes in the pathogenic and commensal *Neisseriaceae*. 
Results

Comparison of neisserial FNR. Research has shown that genes involved in denitrification and/or adaptation to anaerobic growth in the Neisserial species includes the transcriptional regulators FNR, NsrR, ArsR, and the two component system, NarQP (36, 109). FNR is a member of the FNR-CRP superfamily of transcriptional regulators, and it is the only regulator in the pathogenic Neisseria known to respond directly to anaerobic environmental conditions. The oxygen sensing capability of FNR is coupled to a labile, oxygen-sensitive [4Fe-4S] cluster coordinated by four cysteine residues (36, 126, 166). All four of these cysteine residues are essential for FNR function (78, 166). Upon exposure to oxygen, the [4Fe-4S] cluster dissociates into a [2Fe-2S] cluster, and after prolonged exposure, this [2Fe-2S] cluster dissociates from the protein altogether (123). Gonococcal FNR, active as a dimer, binds to the same inverted repeat sequence as E. coli FNR (5’-TTGAT-N₄-ATCAA), and it was shown that gonococcal FNR could repress transcription from FNR-repressible promoters in E.coli. However, gonococcal FNR could not activate transcription of E.coli promoters, likely due to poor alignment of FNR activating regions and their cognate regions in E. coli RNA polymerase (166). In E. coli, FNR was also shown to be responsive to nitric oxide, where the [4Fe-4S] cluster reacts with NO to form a dinitrosyl-iron complex, rendering FNR inactive. This mode of response has been shown to be important in regulation of the E. coli hmp gene, but it is unclear if NO-inactivated FNR is a player in the gonococcal NO stimulon (41).
FNR activation is a requirement for efficient transcription of \textit{aniA}, and \textit{fnr} mutations in both the meningococcus and gonococcus result in the inability of these strains to reduce nitrite (36, 106). Sequence alignments show that FNR is very highly conserved across all members of the Neisserial genus, with the most distant FNR orthologs, expressed by \textit{N. mucosa} and \textit{N. sicca}, containing 95\% similarity to the FNR sequence of \textit{N. gonorrhoeae} FA1090 (Table 4, Figure 31). All Neisserial species contain the four Fe-S coordinating cysteines (166). Furthermore, amino acid sequence is conserved in and around areas where mutations have been found to increase the oxygen stability of gonococcal FNR (L22H and D148A) (105, 166). This suggests that FNR likely functions in a similar, oxygen-dependant manner in all Neisserial strains. The high level of conservation of this anaerobic regulator across the genus also implies that anaerobiosis is not only a physiologically significant state for the pathogenic \textit{Neisseria} spp., but for commensal \textit{Neisseria} spp. as well (35, 124, 182-183).

**Comparison of neisserial NsrR.** As discussed previously, NsrR is a nitric oxide-sensing Rrf2-type transcriptional repressor, and although the discovery of this protein has been somewhat recent, extensive research has characterized its regulatory role in several bacterial species. In \textit{N. gonorrhoeae} and \textit{N. meningitidis}, NsrR negatively regulates \textit{aniA} and \textit{norB} and derepression occurs in the presence of NO (36, 109-110). Sequence alignments of the NsrR protein across the Neisserial genus illustrate that this regulator is well conserved, with the most distant NsrR ortholog, encoded by \textit{N. mucosa}, containing 88\% similarity to that of \textit{N. gonorrhoeae} FA1090 (Table 4,}
Table 4. Sequence similarity between regulators of the neisserial denitrification pathway

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* % Identity (% similarity) as compared to *N. gonorrhoeae* FA1090
Figure 31. Amino acid sequence alignment of neisserial FNR. The FNR protein from pathogenic and commensal *Neisseria* spp. was compared with Multalin. Cysteine residues, in green, are presumed to be involved in binding a [4Fe-4S] complex. A S18F mutation, highlighted in yellow, increases the ability of gonococcal FNR to function in *E. coli*. The mutations L22H and D148A, highlighted in blue, increase the oxygen stability of gonococcal FNR (166).
Figure 31. Amino acid sequence alignment of neisserial FNR.
Figure 32). Furthermore, most of the sequence differences are contained within the C-terminal end of the protein, which is not predicted to play a role in protein function (110). In chapter 2, I provided evidence that the three conserved cysteine residues in gonococcal NsrR coordinate a NO-sensitive [2Fe-2S] cluster. All Neisserial strains analyzed here show conservation of these key cysteine residues (Figure 32). It may of interest to note that in N. gonorrhoeae, N. lactamica, and N. cinerea, the amino acid in position 93, which is located between the first two cysteines (Cys90 and Cys97), is proline, whereas in N. meningitidis MC58, N. mucosa, N. subflava, N. flava, and N. sicca, the amino acid at position 93 is a negatively charged Glu or Asp residue. Since these amino acids would be predicted to be within the Fe-S cluster coordination pocket of NsrR (110, 215), it may be interesting to speculate that these amino acid differences may affect the chemical reactivity of the [2Fe-2S] cluster towards NO, and thus potentially change the sensitivity of the protein to its ligand.

Regardless, both meningococcal and gonococcal NsrR have been shown to be capable of responding to NO (110, 184)

**Comparison of the neisserial NarQP two component system.** The NarQP two component regulatory system is involved in regulation of the Neisserial denitrification pathway, and is known to activate aniA in both N. gonorrhoeae and N. meningitidis (36, 106). Originally reported to respond to nitrite, NarQ appears to be constitutively active in N. gonorrhoeae, and does not appear to require a stimulus for activation (phosphorylation) of NarP (168). As in E. coli, the gonococcal NarP protein recognizes a 7-2-7 inverted repeat sequence, though the actual consensus
Figure 32. Amino acid sequence alignment of neisserial NsrR. The NsrR protein from pathogenic and commensal Neisseria spp. was compared with Multalin. Cysteine residues, in green, are involved in binding a [2Fe-2S] complex.
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Figure 32. Amino acid sequence alignment of neisserial NsrR.
between these two organisms appears to differ slightly. The first discovered gonococcal NarP binding site, 5'-TAGTTAT-N2-AGTATTA, is centered 95.5 bp upstream of the transcription start site of *aniA* where it acts to amplify *aniA* expression as a class-I activator (36, 105, 168). Since that time, investigation of the NarP regulon on a genomic scale revealed that the gonococcal NarP regulon is small, with only four additional genes confirmed to be subject to NarP regulation (168). NarP is highly conserved among the Neisserial genus, with the most distant NarP ortholog, expressed by *N. mucosa*, containing 90% similarity to gonococcal FA1090 NarP (Table 4, Figure 33). Interestingly, *N. mucosa* contains two copies of *narP*, both of which are highly similar to each other, but are not adjacent to each other on the chromosome. *N. mucosa* is also the only neisserial strain believed to contain a functional Nar complex (11). It may be fascinating to speculate that this second NarP gene product could be involved in regulation of a subset of genes involved in nitrate utilization and/or reduction as NarL is in *E. coli*. Although *N. mucosa* contains only one ortholog of NarQ, in *E. coli*, NarQ is capable of phosphorylating both NarP and NarL (164). All Neisserial NarP orthologs contain the conserved aspartate residue presumed to be the phosphorylation target by the sensor kinase NarQ (105, 168). Despite the similarity between Neisserial NarP orthologs, large differences exist within the sequence of NarQ (Table 4, Figure 34).

NarQ is the sensor kinase of the NarQP two component system and is anchored in the bacterial inner membrane (210). In *E. coli*, NarQ senses the presence of nitrite and relays this signal, via phosphorylation, to NarP, its cognate response
Figure 33. Amino acid sequence alignment of neisserial NarP. The NarP protein from pathogenic and commensal *Neisseria* spp. was compared with Multalin. The Aspartate residue, in green, is the phosphorylation target by the sensor kinase NarQ.
### Figure 33. Amino acid sequence alignment of neisserial NarP

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regulator (89). Since the gonococcal NarQ homolog appears to be insensitive to nitrite and constitutively active, nitrite sensing in the gonococcus, at least at the level of aniA regulation, has apparently been taken over by NsrR in a complex regulatory mechanism whereby the product of nitrite reduction, NO, causes derepression by NsrR, which in turn allows access of NarP to RNAP, thus leading to activation (109, 168). To date, no ligand has been found for gonococcal NarQ. Contrary to this finding, aniA expression was shown to be nitrite but not nitric oxide dependent in N. meningitidis, suggesting that meningococcal NarQ may still respond directly to nitrite (184).

Because NarQ is not as well conserved as is cognate response regulator NarP, it suggests that environmental sensing may be different between species and thus elicit a different regulatory output. The most divergent NarQ sequence, encoded by N. mucosa, contains 73% similarity and only 60% identity to that of the N. gonorrhoeae FA1090 NarQ protein (Table 5). Many sensor kinase proteins contain periplasmic sensing domains on their N-terminus (210, 226). It is possible that sequence differences in the N-terminus of neisserial NarQ affect the ability to sense and respond and/or halt a response to nitrite.

A conserved domain search of neisserial NarQ proteins revealed a composition of four key domains, including a HAMP, GAF, HisKA_3, and HATPase_c domain, all of which are predicted to be located on the cytoplasmic face of the inner membrane (Table 5) (141, 210). The HAMP domain, through rotation of a four α-helical bundle, is implicated in transduction of a perceived environmental
signal (210). The HATPase_c and HisKA_3 domains comprise the catalytic core of the protein, where ATP is hydrolyzed and kinase activity is utilized in the transfer of phosphate to the response regulator respectively (210, 226). The GAF domain is thought to play a role in environmental sensing at the cytoplasmic face of the inner membrane (210). Interestingly, whereas the HAMP, HATPase_c, and HisKA_3 domains are very well conserved across the neisserial genus, the GAF domain is particularly divergent (Table 5, Figure 34) (141). GAF domains from *N. gonorrhoeae* and *N. lactamica* NarQ display the weakest conservation compared to other neisserial species (Table 5, Figure 34). It is perhaps interesting to speculate that divergence within this GAF domain may impact upon the structure of NarQ in such a way as to alter its signal perception properties. In the case of the *N. gonorrhoeae*, the GAF domain may have evolved this way to maintain NarQ in a constitutively active state, perhaps due to the fact that the gonococcus would not be expected to encounter much nitrite in its own environmental niche. The NarQ protein from other neisserial species show greater conservation of this GAF domain, and due to this fact, may possibly be capable of sensing nitrite, as was observed in the meningococcus (184).

**Genomic comparison of the neisserial norB upstream region.** Interesting observations can be made when an alignment of the norB upstream region in *Neisseria* spp. is analyzed (Figure 35). All species contain the TG dinucleotide making up the extended -10 element, which was shown to be essential for high level expression (see chapter 1). However, in some of the commensal strains (*N. mucosa, N. sicca, N. subflava*, and *N. flavescens*), the NsrR binding site, located immediately
Figure 34. Amino Acid sequence alignment of neisserial NarQ. The NarQ protein from pathogenic and commensal Neisserial spp. was compared with Multalin. The Histidine residue, in green, is the site of phosphotransfer to the response regulator NarP. The GAF, HAMP, HATPase_c, and HisKA_3 domains are bracketed above the sequence.
NarQ

NarQ_NGO_FA1090
NarQ_NGO_NCCP1
NarQ_N.lactamica
NarQ_NMC_FAM18
NarQ_NMB_MC58
NarQ_N.cinerea
NarQ_NMC_22491
NarQ_N.subflava
NarQ_n.flavescens
NarQ_N.mucosa
NarQ_N.sicca

|------- HAMP Domain -----------------|

Figure 34. Amino Acid sequence alignment of neisserial NarQ.
Figure 34. Amino Acid sequence alignment of neisserial NarQ.
Figure 34. Amino Acid sequence alignment of neisserial NarQ.
Table 5. Domain homologies of neisserial NarQ

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<td>6.9</td>
<td>2e-09</td>
<td>2e-09</td>
</tr>
<tr>
<td>N. cinerea ATCC 14685</td>
<td>2e-05</td>
<td>0.27</td>
<td>5e-10</td>
<td>9e-10</td>
</tr>
<tr>
<td>N. flavescens NRL 30031</td>
<td>4e-07</td>
<td>0.003</td>
<td>9e-10</td>
<td>1e-10</td>
</tr>
<tr>
<td>N. subflava NJ9703</td>
<td>8e-07</td>
<td>0.013</td>
<td>1e-09</td>
<td>1e-10</td>
</tr>
<tr>
<td>N. mucosa ATCC 25996</td>
<td>1e-05</td>
<td>0.024</td>
<td>2e-09</td>
<td>1e-13</td>
</tr>
<tr>
<td>N. sicca ATCC 29256</td>
<td>2e-05</td>
<td>9e-05</td>
<td>7e-10</td>
<td>7e-12</td>
</tr>
</tbody>
</table>

Domain homologies obtained from conserved domain database (CDD) available at NCBI (141).
downstream of the -10 element, deviates by 2-5 bp from the inverted repeat in *N. gonorrhoeae*. In the first two chapters of this work, it was shown that deviation from this repeat sequence resulted in decreased affinity for NsrR and a concomitant decrease in regulation (measured in terms of fold induction in response to NO). Interestingly, the deviation in NsrR binding sites in these commensal species occurs only in the 3’ half-site, while the 5’ half-site is perfectly conserved (Figure 35). For this reason, it is likely that NsrR has a decreased affinity for these promoters rather than the alternative explanation, that NsrR recognizes a different site in these species.

The gonococcal *norB* gene illustrates a novel mechanism of indirect activation by Fur, through inhibition of ArsR binding. In this way *N. gonorrhoeae* can regulate the expression of *norB* in response to iron concentration in addition to NO. Interestingly, *N. mucosa, N. sicca, N. subflava,* and *N. flavescens,* and the pathogenic strain *N. meningitidis* Z2491, contain a 5’ deletion in the *arsR* gene (Table 4, Figure 35). Analysis of the *norB* upstream region in the commensal strains that have lost the functional *arsR* gene also reveals a loss of the putative ArsR binding site and a less conserved Fur binding site (Figure 35). Taken together, degeneration of the NsrR binding site and deletion of *arsR* suggest that the *norB* gene is less regulated in response to NO and insensitive to regulation by iron in some commensal species of *Neisseria*.

**Genomic comparison of the neisserial aniA upstream region.** The promoter architecture of the *aniA* upstream region in *Neisseria* spp. is fairly well conserved, though there is considerable sequence divergence and a longer leader sequence in
Figure 35. Nucleotide sequence alignment of the neisserial norB upstream region. The norB upstream region from pathogenic and commensal Neisseria spp. was compared with Multalin. The extended -10 element is highlighted in red. Nucleotides making up the inverted repeat of the N. gonorrhoeae FA1090 NsrR binding site are shown in red underlined text. The putative ArsR binding site is shown in purple underlined text. The Fur binding site is shown in highlighted yellow text. The ribosome binding site is underlined. The numbers above the sequence correspond to distance from the translational start site in N. gonorrhoeae FA1090.
Figure 35. Nucleotide sequence alignment of the neisserial norB upstream region.
both *N. mucosa* and *N. sicca* (Figure 36). All species of *Neisseria* contain a putative FNR binding site centered 22.5 bp upstream of the -10. Gonococcal strain RUG7645 is a ΔnsrR strain containing an *aniA::lacZ* reporter fusion lacking the NarP binding site (Figure 15). This reporter fusion is solely under the control of FNR, and in chapter 1 it was reported that this strain was induced 35-fold when grown anaerobically with nitrite. In an unrelated experiment, a hybrid promoter fusion was generated that contained the -10 element and FNR binding site of the most highly induced FNR activated gene in *N. gonorrhoeae* (a small transcript upstream of ORF NGO0797) and arbitrary *aniA* sequence downstream of the -10 (as described for RUG7508 construction) (225). It should be noted that both reporter fusions were under the exclusive control of FNR, and that both FNR binding sites were centered at exactly -32.5 with respect to the -10. The strain harboring the hybrid promoter was induced nearly 260-fold when grown anaerobically with nitrite (34 ± 7 Miller units vs. 8830 ± 475 Miller units). The key difference between these fusion strains was the FNR binding site, where the hybrid promoter was a 10/10 match for the paradigm *E. coli* consensus (5’-TTGAT-N₄-AACTA) compared to the 8/10 match for gonococcal *aniA* (5’-TTGAC- N₄-ATTAA) (Figure 36). Interestingly, in the *aniA* upstream region, all meningococcal and commensal species were at least a 9/10 match for the *E. coli* consensus, except for *N. flavescens*, which was a 10/10 match, suggesting FNR may be more proficient in activation of *aniA* expression at these sites.

In *N. gonorrhoeae*, NsrR represses *aniA* transcription by sterically blocking the ability of FNR to bind its operator. As is the case for *norB*, several of the
**Figure 36. Nucleotide sequence alignment of the neisserial aniA upstream region.** The *aniA* upstream region from pathogenic and commensal *Neisseria* spp. was compared with Multalin. The -10 element is highlighted in red. Nucleotides making up the inverted repeat of the *N. gonorrhoeae* FA1090 NsrR binding site are shown in red underlined text. The class-II FNR binding site is shown in green highlighted text. The NarP binding site is shown in highlighted blue text with respect to the FA1090 binding site. The ribosome binding sites are underlined. The numbers above the sequence correspond to distance from the translational start site in *N. gonorrhoeae* FA1090.
Figure 36. Nucleotide sequence alignment of the neisserial aniA upstream region.
commensal species contain putative NsrR binding sites that are more divergent from the high affinity \textit{norB} FA1090 inverted repeat compared to \textit{N. gonorrhoeae} and \textit{N. meningitidis} (Figure 36). This suggests that \textit{aniA} expression in commensal \textit{Neisseria} spp. is more dependent on anaerobiosis than on NO concentration. Alternatively, it is possible that the NarQP system is responsive to nitrite in the commensal \textit{Neisseriaceae}, and therefore control of \textit{aniA} by NO is not necessary. All commensal species except for \textit{N. subflava} appear to contain a NarP binding site in the correct orientation to act as a class-I activator, though the paradigm consensus of the neisserial NarP binding site is unknown.
Discussion

The human salivary glands actively take up nitrate in a plasma concentration dependent manner, and this nitrate is reduced to nitrite by anaerobic bacteria within the oral cavity, generating nitrite levels ranging from 10 \( \mu \text{M} \) to > 1 mM (135). High nitrite concentrations, in concert with a microaerobic niche, may afford opportunities for Neisseria spp. that reside in the nasopharynx to utilize denitrification as a route of electron transfer. N. gonorrhoeae primarily colonizes the genital mucosa, a niche that would not be predicted to encounter high nitrite levels in the female. Though the gonococcus may encounter an intermittent supply of nitrite through the flushing of urine in men, nitrite levels in urine and the urogenital tract in healthy individuals are extremely low (152, 157). While the gonococcal NarQP system was determined to be insensitive to nitrite, aniA expression in a meningococcal \( \Delta \text{nsrR} \) mutant was shown to be upregulated in the presence of nitrite. This suggests that the NarQP system in N. meningitidis is able to sense nitrite (168, 184). Using this comparative genomics approach, some evidence has been provided to support the idea that although nitrite sensing capacity has likely degenerated in N. gonorrhoeae, it may still be possible in other Neisseria spp. where nitrite is of physiological significance.

The anatomical sites inhabited by Neisseria spp. are competent at host generated NO production, providing an additional electron acceptor for energy production (20, 204). Analysis of the norB upstream region in Neisseria spp. showed that many commensal species had degenerate NsrR binding sites compared to gonococcal strain FA1090 and had lost the ability to be indirectly activated by Fur by
virtue of an \textit{arsR} deletion. The fact that the pathogenic \textit{Neisseria} spp. have retained
the ability to regulate \textit{norB} in response to iron may be advantageous \textit{in vivo}, and will
be discussed further in the final conclusions of this work. The fact that the \textit{norB} gene
from \textit{Neisseria gonorrhoeae} appears to be more regulated in response to NO is
interesting. It suggests that expression of this gene is more sensitive to changes in
NO concentration, and this fact may be important for the suspected
immunosuppressive effect of NO reduction by the gonococcus (10-11, 34).

There was some divergence in the \textit{aniA} FNR binding site between \textit{Neisseria
gonorrhoeae} and the rest of the \textit{Neisseria} spp., and small changes in this site were
shown to have a huge effect on gene induction capacity (35-fold in RUG7645 vs.
260-fold in NGO0797/\textit{aniA} hybrid promoter fusion) The higher match to the strongly
activating FNR operator in these species would suggest that FNR may activate \textit{aniA}
expression to a much higher level than that observed in the gonococcus. However,
this would seem unlikely, as previous studies have shown that the enzymes of
denitrification are under respiratory control, so that increased \textit{aniA} expression would
not be beneficial, and that nitrite reductase activities in commensal and
meningococcal \textit{Neisseria} spp. are not significantly different than levels observed in
\textit{N. gonorrhoeae} (11). It is perhaps interesting to speculate that the sequence
divergence within the FNR binding site of \textit{Neisseria} spp. is a reflection of the
ecological niche in which these species reside. The strongly activating FNR operator
would be expected to have a higher affinity for FNR, therefore, it is possible that
FNR is able to achieve proficient activation at this site under microaerobic rather than
anaerobic conditions, or that a higher basal level of \textit{ani}A transcription is valuable in the oro- or nasopharynx. Conceivably, the gonococcal FNR binding site in the \textit{ani}A upstream region may have evolved this divergence for a reason. Perhaps in the unique niche of the urogenital tract, this pathogen occupies sites that are more anaerobic, and this weaker binding site prevents expression of \textit{ani}A to levels beyond that necessary for dentirification. Alternatively, as \textit{N. gonorrhoeae} would not be expected to encounter a steady supply of nitrite at appreciable levels \textit{in vivo}, this FNR site may maintain basal \textit{ani}A expression at extremely low levels as AniA is immunogenic in the gonococcus (37).
Conclusions

This study has significantly increased our knowledge of the mechanism by which *N. gonorrhoeae* regulates the genes involved in denitrification. Importantly, this study has identified that the gonococcus is able to sense nitric oxide in a direct manner, through NsrR, and respond by altering gene expression accordingly. As an obligate human pathogen, the fact that *N. gonorrhoeae* has this ability suggests that NO sensing and the NO stimulon have important consequences on gonococcal pathogenicity, as nitric oxide plays an important role in shaping the human immune response.

In its natural environment as an obligate human pathogen, *N. gonorrhoeae* will be presented with a variety of environmental factors that may act as stimuli for gene expression. Regulation of the gonococcal denitrification pathway is a prime example of a gene whose expression is regulated by the integration of multiple signals. importantly, this demonstrates that the bacterial response to varying stimuli in vivo does not simply result in an on or off phenomena, but actually involves a more complex network of gene regulation and the ability to “fine-tune” gene expression. In chapter 1, the complex regulation of *norB* was elucidated, and this mechanism is revisited in Figure 37. NsrR binds to its operator immediately downstream of the RNAP extended -10 element. At this location it is believed that NsrR functions as a simple dimeric repressor, either inhibiting binding by RNAP or preventing clearance of RNAP from the promoter, or both (36, 215). In the presence of NO, *norB* becomes
Figure 37. Model of transcription regulation at the gonococcal norB promoter.

The \( \alpha, \beta, \beta' \) and \( \sigma \) subunits of RNA polymerase are shown along with the NsrR, ArsR, and Fur proteins and their binding sites. In the absence of nitric oxide, \( p^{norB} \) is inactive as NsrR prevents RNAP binding. In iron-limiting conditions and in the presence of nitric oxide, NsrR is inactivated and allows RNAP binding. The ArsR repressor presumably partially inhibits RNAP binding through an unknown mechanism. When iron is abundant, Fur likely binds to its operator and prevents ArsR binding. Maximal induction of \( p^{norB} \) occurs in the presence of NO and Fe.
Figure 37. Model of transcription regulation at the gonococcal norB promoter.
derepressed so that the genes involved in NO metabolism can be induced, and the adverse effects of NO can be prevented.

The competition for operator binding between Fur and ArsR modulates \( \text{norB} \) transcription (Figure 37). In the model proposed here, binding of Fur and ArsR is mutually exclusive, and the presence of bound Fur in the \( \text{norB} \) upstream region prevents binding of ArsR, thus preventing ArsR-mediated repression. The iron content of the gonococcal NorB protein is unknown, however, the closely related single subunit NorB protein from the denitrifying bacterium \( \text{Ralstonia eutropha} \) has been purified, and was shown to contain two heme B cofactors and an additional non-heme iron atom per enzyme molecule (40). Perhaps the high level of \( \text{norB} \) expression in the presence of NO is excessively taxing on the available supply of iron at certain times or in certain environments where iron may be limiting, and iron can be conserved through this Fur-dependent regulation of \( \text{norB} \).

The mechanism of ArsR repression remains unclear, though the location of its putative binding site would appear to be too far upstream to prevent the binding of RNA polymerase. It is doubtful that ArsR would mediate repression by binding over an UP signal sequence, as the putative ArsR binding site is too far upstream, and deletion of the \( \text{norB} \) upstream region up to the -35 had no effect on \( \text{norB} \) expression, suggesting that this gene does not contain an UP sequence (RUG7531, Figure 6) (59, 189-190). Potentially, ArsR could repress \( \text{norB} \) through a direct interaction with the polymerase that physically tethers RNAP to the promoter to prevent efficient clearance (187). Another possibility is that ArsR mediates bending or looping of
DNA that hinders RNAP interaction with its promoter, as is the case with bacterial nucleoid-associated proteins (i.e. IHF, HU, and H-NS) or the lac repressor (28, 62, 209). Interestingly, the upstream regions of norB and NGO1411 (the putative ArsB efflux pump that contains the putative ArsR binding site) both contain three short 6 bp repeats upstream of the -35 element. In both cases, the first two repeats are inverted and separated by 2 bp, however, the third repeat is located further downstream and the spacing and orientation of this repeat is not conserved. Conceivably, this operator architecture may allow for complex binding and DNA bending. A recent study of the E. coli ferritin gene (ftnA) demonstrated a regulatory mechanism similar to the indirect Fur activation observed in gonococcal norB. In E. coli, H-NS mediated repression of ftnA is alleviated when Fur binds to its operator, which is upstream of the ftnA -35 sequence and overlapping with H-NS binding sites (161).

In other organisms, ArsR acts as a simple dimeric repressor that prevents RNAP binding (32, 227). The gonococcal ArsR protein appears to act in a novel fashion. This regulator lacks the conserved residues necessary for ligand sensing and represses gene expression by an unknown mechanism through binding upstream of the norB -35 element. For these reasons, the gonococcal ArsR protein is an attractive target for future study. Such future work should aim to illuminate the regulatory mechanism and complete regulon of ArsR. Assays that monitor Protein/DNA interactions such as EMSA and DNaseI footprinting will greatly help in elucidating the repressive mechanism of ArsR, while ChIP and/or microarray studies will help
determine the regulon. It is interesting that the arsR gene is present in all gonococcal strains sequenced, but has undergone a deletion in several commensal species and in one strain of *N. meningitidis* (Chapter 3). For this reason, it is reasonable to speculate that genes in the ArsR regulon could play a role in gonococcal pathogenicity in the unique niche of the urogenital tract.

The fact that norB is regulated by Fur is intriguing. Fur functions as a global regulator in several pathogenic organisms, and has been shown to upregulate the production of virulence determinants in times of iron-restriction, including the diphtheria toxin in *Corynebacterium diphtheriae*, α-hemolysin and verocytotoxin in *E. coli*, Shiga toxin in *Shigella dysenteriae*, and exotoxin A by *Pseudomonas aeruginosa* (87, 203). Fur also has a role in regulating genes involved in oxidative stress and acid resistance in a broad range of bacteria (87). In *E. coli* and *S. typhimurium*, expression of hmp, encoding the NO-detoxifying protein flavohemoglobin, was shown to be regulated, in part, by Fur (43, 96). Though NorB, like flavohemoglobin, plays a role in NO detoxification, the pathogenic *Neisseria* spp. are the first example demonstrating regulation of genes involved in denitrification by Fur.

In *E. coli*, Fe$^{3+}$-loaded Fur was shown to be directly responsive to NO, forming an FeFur-NO complex incapable of DNA binding (25, 43). Interestingly, while the hmp genes from *E. coli* and *S. typhimurium* are repressed by FeFur in the absence of NO, the gonococcal norB gene is activated. If the gonococcal Fur protein can sense NO, this regulatory mechanism would seem counterintuitive, as norB
would be more repressed in the presence of its target molecule. Furthermore, in Figure 6 it was shown that there was no difference in expression between the wild-type norB promoter and a promoter lacking both the Fur and ArsR binding site in the presence of nitric oxide (RUG7500 vs. RUG7512). For this reason, it appears that either the gonococcal Fur protein is incapable of sensing NO or that the gonococcus is capable of effectively reducing NO levels beneath a threshold necessary for inactivation of Fur. This implies that the regulation of gonococcal norB by Fur is likely an iron-responsive rather than a NO-sensing mechanism.

In pathogenic bacteria, iron availability does not necessarily have to be perceived as a nutritional signal, as was suggested above. As human pathogens have adapted to survive within a host, iron can be perceived as an environmental signal, allowing bacteria to mount an appropriate response in the context of their location within the human host (9, 192, 203, 214). Perhaps N. gonorrhoeae can exploit this iron-responsive tailoring of norB expression to maintain host-produced NO at levels that can best counteract the host immune response in the specific environments that this pathogen will encounter.

Regulation of the gonococcal nitrite reductase, aniA, was also shown to be complex, and expression of this gene is responsive to both anaerobiosis and NO (Figure 38). Unlike norB, the expression of aniA requires an anaerobic or microaerobic environment for expression due to the requirement for activation by the oxygen-sensitive FNR protein. It was previously discussed that nitrite-sensing in the gonococcus through the NarQP two component system appears to have degenerated,
**Figure 38. Model of transcription regulation at the gonococcal aniA promoter.**

The α, β, β' and σ subunits of RNA polymerase are shown along with the FNR, NarP and NsrR proteins and their binding sites. In the presence of oxygen, p^aniA is inactive as FNR cannot bind. It is not known whether NarP or NsrR are bound to the promoter. In the absence of both oxygen and NO_2^-, FNR is bound and contacts the σ subunit of RNAP. NsrR is also bound, and blocks interactions between FNR and αCTD. Upon exposure to nitrite, which is converted to NO by AniA, NsrR is inactivated and not bound to the promoter, allowing FNR-αCTD interactions. Thus, nitrite control is actually mediated by NsrR sensing NO and controlling the ability of FNR to interact with RNAP.
Figure 38. Model of transcription regulation at the gonococcal *aniA* promoter.
and that \textit{aniA} expression is derepressed in response to NO by NsrR rather than activated in the presence of nitrite by NarP. At the \textit{aniA} promoter, NsrR does not function as simple repressor, as in \textit{norB}, but instead appears to interfere with \textit{aniA} activation rather than blocking RNAP directly. The NsrR binding site in \textit{aniA} is immediately adjacent to the FNR binding site, suggesting that NsrR acts to sterically interfere with FNR binding when NO is absent. NarP likely acts as an amplifier of \textit{aniA} expression, but is not capable of \textit{aniA} activation without FNR. It is interesting that the gonococcus has evolved to regulate the expression of \textit{aniA} by NO rather than nitrite, and this fact underscores the importance of NO sensing in this bacteria.

Nitrite can be formed by the reaction of NO with oxygen (\textit{i.e.} conditions that may be encountered during an inflammatory response), therefore, the reduction of nitrite may also play a role in detoxification as well as respiration under certain circumstances (36).

Chapter 2 described the biochemical analysis of the gonococcal NsrR protein. In these studies, the putative NsrR binding sites were validated at the strong NsrR operator upstream of \textit{norB} as well as the weak operator upstream of \textit{nnrS}. Furthermore, these experiments showed that NsrR contained a [2Fe-2S] cluster that was necessary for both DNA binding and recognition of NO. Since the time that these studies were performed, the nature of the molecular interaction between NO and NsrR was described in the gram positive soil bacterium, \textit{Streptomyces coelicolor}, and this interaction is likely the same in \textit{N. gonorrhoeae}. The interaction of NO with the [2Fe-2S] cluster in \textit{S. coelicolor} NsrR resulted in the formation of dinitrosyl-iron...
complexes (DNICs), as is the case with the NO-sensitive [4Fe-4S] clusters in aconitase and FNR, as well as the [2Fe-2S] cluster of SoxR (33, 41, 48, 215). The [2Fe-2S] cluster is destroyed upon interaction with NO, and the iron atoms remain protein-bound to form thiolates on the cysteine residues responsible for Fe-S coordination (Figure 39). Two molecules of NO are bound by each Fe atom (215, 220). In NO-treated NsrR from *S. coelicolor*, a mixture of dinuclear and mononuclear DNICs were formed by the reaction of NsrR [2Fe-2S] with NO. In a dinuclear DNIC, both Fe atoms from [2Fe-2S] are bound by two NO molecules, while in a mononuclear DNIC, only one Fe atom is bound by two NO molecules (215). Though the formation of DNICs is concomitant with the destruction of [2Fe-2S], it is uncertain if the repair of this cluster can be accomplished *in situ*, or requires *de novo* production of [2Fe-2S] that is inserted into a damaged protein.

Nitric oxide is a key intermediate involved in modulating the human immune response. Therefore, the ability of pathogenic organisms to sense and respond to NO is likely an important determinant of virulence. Though the function of the genes involved in denitrification are known (anaerobic/microaerobic growth), the effect of gonococcal denitrification/NO reduction on the host is largely unknown. Unfortunately, without an adequate animal model, determining the presumed immunomodullary effects of gonococcal denitrification on the host will be difficult to determine. Future studies should employ a genome-wide approach to determine all of the genes in the gonococcal NsrR regulon and NO stimulon. It will be interesting to see if the NO stimulon is larger than the NsrR regulon. Further research will be
Figure 39. Formation of dinitrosyl iron complexes in NsrR in response to NO.

The top panel depicts the [2Fe-2S] cluster of NsrR coordinated by three cysteine residues, and a fourth coordination point, represented by a (?), predicted to be a positively charged residue. The bottom panel represents the formation of a dinuclear dinitrosyl iron complex in NsrR in response to nitric oxide. Upon encountering NO, the sulfur atoms from [2Fe-2S] are lost and each iron atom is ligated by two molecules of NO (215, 220). The iron atoms remain bound to residues responsible for [2Fe-2S] coordination. In *S. coelicolor* NsrR, over 90% of NO-treated NsrR was shown to form dinuclear dinitrosyl iron complexes as shown, while the remaining NO-treated NsrR contained mononuclear dinitrosyl iron complexes. In the case of mononuclear dinitrosyl iron complexes, one of the two iron atoms is lost, resulting in only one dinitrosyl iron group per NsrR molecule (215). It is unknown if the formation of dinitrosyl complexes increase the overall charge of the protein.
Figure 39. Formation of dinitrosyl iron complexes in NsrR in response to NO
necessary to determine the function of those genes, as they may reveal novel determinants of bacterial pathogenesis.
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