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Defining the MtrR Regulon Beyond the *mtrCDE* Efflux Pump Operon

By

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Graduate Division of Biological and Biomedical Sciences
Program in Microbiology and Molecular Genetics
Doctor of Philosophy

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B.S. Biology, North Georgia College & State University, 2002

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An abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Neisseria gonorrhoeae, the causative agent of gonorrhea, is an important public health problem as it causes ~95 million cases of this sexually transmitted disease each year. As with other bacterial infections, previously successful antibiotic treatments have become less effective over time in treating gonorrhea. One way in which *N. gonorrhoeae* resists these antimicrobial agents is through the expression of efflux pump systems such as the MtrC-MtrD-MtrE efflux pump. Additionally, this pump and its regulators have recently been recognized as being important pathogenic determinants. Specifically, mutations that occur in the regulators of the MtrC-MtrD-MtrE efflux pump system alter the ability of the gonococcus to establish infections in *in-vivo* mouse studies. Loss of the repressor of this system, MtrR, results in an early fitness advantage followed by a loss of advantage in later stages of infection. Therefore, this work was designed to determine the MtrR regulon in order to further understand its role in gene regulation of the gonococcus given its central importance in efflux and pathogenesis. It was determined that MtrR is a global regulatory protein of significant importance in the gonococcus as it regulates at least 70 genes, including genes involved in pathogenesis, antimicrobial efflux, transport, stress response, and biosynthetic pathways. Given the breadth of these systems, these studies focused on the regulation of the gene encoding the alternative sigma factor RpoH, and select members of its regulon, as well as the glutamine biosynthetic pathway, *glnE* (glutamine synthetase adenylyltransferase) and *glnA* (glutamine synthetase). This work demonstrated that MtrR was responsible for repressing *rpoH* expression, and subsequently RpoH- activated genes, while inducible expression of MtrR could affect resistance to H_2O_2 via its affects on *rpoH*. Further, MtrR was found to repress *glnA* by its effects upon the DNA binding of a second transcriptional regulator, FarR, upstream of *glnA* as well as by its repression of *farR* expression. Additionally, MtrR was found to activate *glnE*. The disparate regulatory actions of MtrR identified in this research provide new insights regarding the contributions of this regulatory protein with respect to the physiology and pathogenic mechanisms of the gonococcus.

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Acknowledgements

I would like to thank my mentor, Bill Shafer. I am grateful for his support, insight, assistance, and patience over the years. There were many good times and there were many trying times. Through these trying times I would not have made it without his continual support, encouragement, feedback, understanding, and humor. Thank you Boss.

To my committee members, I would like to thank you for your generous suggestions and guidance: Gordon Churchward, Charlie Moran, Phil Rather, and Yih-Ling Tzeng.

I would also like to thank my mother Dr. Kathleen Tatti, my brother Joseph, and my father Paul for their help and support over the years.

Lastly, I have to thank my wife Angela for all of the years of support, and companionship through this process. Thank you dear.

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Chapter 1: Introduction

I. *Neisseria gonorrhoeae*

The genus *Neisseria* is currently composed of 12 species that colonize the various mucosal surfaces of the human body. Of these 12, only two members of the *Neisseria* genus that commonly colonize or infect the human body typically cause disease, *Neisseria gonorrhoeae* and *N. meningitidis*. It is these two members that present a persistent threat and a modern public health burden that has only become more severe with the expression of elevated antimicrobial agent resistance. *N. gonorrhoeae* was the first identified member of the *Neisseria* genus when it was described by Albert Neisser as the causative agent of gonorrhea in 1879 (182). However, the disease gonorrhea has been known to mankind since antiquity with varying references to disease states that are similar to that of a classic urogenital infection caused by *Neisseria gonorrhoeae* appearing in many ancient texts including the book of Leviticus (5). In the second century AD, the Greek physician Galen finally put a name to this ancient disease. The name he gave it, gonorrhea, comes from the Greek words *gonos* (seed) and *rhoia* (flow) for the misunderstood belief that the discharge caused by the infection in males was semen leaking from the body (188). While clearly we know today this is not the case, the name has remained and the gonococcus has continued to challenge mankind.

A. Selected virulence factors of the gonococcus

The gonococcus is a Gram-negative, fastidious diplococcus of which mankind is its only known natural host. However, related *Neisseria* species, such as *Neisseria*

macacae, can naturally colonize our close primate relatives, while *Neisseria canis*, *Neisseria mucosa,* and other *Neisseria* spp. are natural colonizers of other mammals (24, 270, 271). It is further possible to experimentally infect our closest primate relatives, such as chimpanzees, with *N. gonorrhoeae,* but more distant primates, such as baboons, have been shown to be unsuitable for gonococcal colonization (66, 159). Recently, a mouse model for studying *N. gonorrhoeae* has been developed to supplant the previous non-human primate models and this model provides an excellent resource for studying this elusive human pathogen in conjunction with the human male volunteer model (49, 118). While typically found extra- and intracellularly as a diplococcus in both human and animal models, the gonococcus can also be found in higher order groupings as it is capable of forming biofilms *in vitro* and *in vivo* (94). The *in vivo* formation of biofilms by *N. gonorrhoeae,* particularly on the cervix, may lead to a persistent asymptomatic infection in women and increased resistance to antimicrobial agents, giving it a distinct survival advantage in the human body (245). Besides the ability to form biofilms, the gonococcus possesses many features that make it a successful human pathogen that does not seem to induce natural immunity to reinfection (24), such as the antigenic variable Type IV pilus, outer membrane proteins (OMP), opacity-associated proteins (Opa), and production of multiple lipooligosaccharide (LOS) species. It also has efficient iron uptake mechanisms, can resist neutrophil killing systems, and can display resistance to host antimicrobial agents that bathe mucosal surfaces (discussed in Section II).

i. Pilus

The antigenic variable pilus produced by the gonococcus is a Type IV pilus. This pilus is encoded by the main structural gene, *pilE*, and a variable number of silent copies of this gene, *pilS*, which readily recombine within *pilE* leading to the antigenic variable characteristic (100, 173, 225). In the gonococcus, the Type IV pilus is responsible for initial attachment to cells, and is necessary for the establishment of a sustained infection (131, 132, 166, 169, 251, 255). There is some evidence that this attachment is mediated by the CD46 receptor (128, 129). However, CD46-independent binding of the gonococcal pilus has also been demonstrated, making the exact mechanism of initial attachment somewhat unclear, but it does not change the importance of the pilus itself in an infection model (135, 259). Type IV pilus is necessary for the natural competence exhibited by gonococci (26). The gonococcus is naturally transformable and readily takes up exogenous DNA from other *Neisseria* spp. and closely related pathogens, giving it a ready pool of new genes for recombination beyond its own species (27, 73). This characteristic leads to horizontal gene flow in the gonococcus, complicating any effort to eradicate this ancient pathogen. Among the notable effects of this capability is the acquisition of plasmids containing beta-lactamase genes from *Haemophilus* spp. (145), as well as the acquisition of *penA* genes encoding Penicillin Binding Protein 2 (PBP-2), which are less inhibited by penicillin, from the commensal *Neisseria* spp. (240). The horizontal gene flow among *Neisseria* spp. and strains is also evident in the mosaic nature of genes other than *penA*, such as those encoding the IgA proteases (105).

ii. Opa

The gonococcus can encode approximately 11 transmembrane phase and antigenic variable opacity related proteins (Opa). The expression of these Opa proteins is independent of one another (143, 252, 253) and the phase variable nature is mediated by at least two mechanisms. The first mechanism by which expression is mediated involves a 5 bp repeat sequence (CTCTT) that exists in the leader region of the Opa genes. This repeat sequence is present in varying numbers in a classical signal-peptide region of the Opa genes and transcription of these genes is seemingly constitutive, indicating that the regulation of expression of Opa proteins is mediated at the level of translation (247). However, the transcriptional strength of Opa promoters during the switch from Opa+ to Opa- has been indicated as another level of control in phase variation (20). The antigenic variable nature of the Opa proteins is due to the presence of two hypervariable regions found in the coding sequences which can readily recombine with one another (50). This process leads to the variation in expressed Opa proteins and contributes to the varying antigenic profiles presented by the gonococcus.

Of these 11 Opa proteins, 10 have been shown to bind CD66. These 10 proteins that bind CD66 can mediate efficient adherence and transcytosis of *N. gonorrhoeae* (273). This process involves the recruitment of CD66 receptors, the activation of cellular microvilli, and presence of the Type IV pilus for optimal cellular invasion (95). Like the Type IV pilus, the Opa proteins of the gonococcus are also important in transformation efficiency, as Opa+ gonococci show higher rates of transformation than Opa- strains (110). The mechanisms by which Opa proteins enhance transformation however, remain unclear.

iii. LOS

While the pilus is necessary for initial attachment to host cells, and Opa proteins are necessary for efficient transcytosis, the production and modification of gonococcal LOS aides in a number of capacities important for infection. Among these capacities are attachment and invasion of host cells in strains that do not express Opa in a process most likely mediated through the asialoglycoprotein receptor (107, 238). Sialylation of LOS, however, further complicates the invasion process by hampering invasion (along with lessening infectivity) while making the cell more resistant to complement dependent killing (223, 278). The presence of certain gonococcal LOS chemotypes also reduces binding of certain antimicrobial peptides to their target on the bacterial membrane. The structure of LOS is also important in mediating high levels of resistance to certain agents recognized by the MtrC-MtrD-MtrE efflux pump (158, 230). LOS is further responsible for the toxicity that gonococci exhibit when incubated with human fallopian tube mucosa (93). This toxicity and damage is specific for human fallopian tube mucosa, as opposed to other mammals, and may be part of the human-specific nature of gonococcal infections (92, 122).

As with the gonococcal pilus and the gonococcal Opa proteins, the LOS produced by the gonococcus is highly variable due to phase variation (222). The phase variable nature of LOS production is mediated by the presence of differing length poly-G tracts in several genes responsible for the production of the alpha chain and a poly-C tract in the *lgtG* gene responsible for production of the beta chain (15, 59, 91). These alpha chain synthesizing genes include *lgtA*, *lgtC*, and *lgtD*, which have 17 bp, 10 bp, and 11 bp poly-G tracts respectively, and encode glycosyl transferases responsible for the production and

attachment of the lacto-*N*-neotetraose side chain to inner core. In addition, the locus *lsi-2,* which is almost identical to *lgtA,* contains a 12bp poly-G tract, which can result in phase variation as well (59, 91). Single nucleotide insertions or deletions in these poly-G tracts via slipped-strand mispairing results in the rapid on/off switching of these genes and antigenically differing forms of LOS present on the surface of the gonococcus (59). Additionally, the LOS produced by *N. gonorrhoeae* can vary antigenically in a phase variation independent manner when recombination occurs between members of the *lgt* gene cluster forming hybrid genes, or when loss of a gene occurs during homologous recombination (260). Further, this antigenic variation can be influenced by the multiple promoters found in this gene cluster (29). Like the *lgtA, lgtC,* and *lgtD* genes the *lgtG* gene encodes a lipooligosaccharide glycosol transferase that is responsible for initiation of production of the beta side chain of gonococcal LOS. This gene is phase variable due to an 11 residue poly-C tract which allows for slipped-strand mispairing (15). LOS variation that affects the pathogenicity of the gonococcus is further mediated through the varying chain lengths mediated by the glycosol transferases, the use of varying sugar moieties, and the addition or loss of PEA (12, 87, 151, 163, 190, 283).

iv. Porin

Porins in the outer membrane of the gonococcus are the channels by which many important nutrients enter the cell from the environment. Given this pivotal location as surface exposed proteins of the outer membrane, and their role in the regulation of molecules crossing the gonococcal membrane, porins represent a key factor in a range of resistance and pathogenesis-related determinants which aide in the gonococcus' ability to

be a well adapted human pathogen. In the closely related meningococcus, there are two major porin proteins, PorA and PorB, that are part of these pathways. The gonococcus, however, expresses only one functional porin protein, termed PorB, as the gonococcus's *porA* is a pseudogene (77). However, the gonococcus' functional porin is represented by two distinct alleles, *porB1A* (P1A) and *porB1B* (P1B), which each confer different advantages and disadvantages upon the bacteria. Gonococci harboring the P1A type porin are common among disseminated infections (36). This is perhaps due to the ability of this porin type to mediate serum resistance through the binding of Factor H and/or C4bp (206-208). Additionally, P1A can provide a selective advantage during infection and/or transmission, due to the P1A allele's ability to facilitate cellular invasion, which is also found in those gonococcal strains that cause disseminated infections (85, 90, 265). Once the gonococcus has established an infection or finds itself inside a phagosome, the presence of P1A instead of P1B can arrest phagosome maturation, hampering the body's ability to fight the disease and allowing the bacteria to become disseminated (17, 177).

In addition to necessary nutrients, porins can also let in undesirable compounds such as antimicrobial agents that are detrimental to the cell. It is in this regard that the P1B porin type, that is most often associated with local infections, becomes an advantage to the gonococcus (31). The P1B porin type confers elevated resistance to antibiotics such as rifampicin, thiamphenicol, and tetracycline when compared to P1A (34, 37). This low level alteration in antibiotic resistance may be due to the differing charge imparted to the cell by the P1A or P1B protein (254). In addition to the mere presence of the P1B porin type, alterations to the P1B porin can further increase the resistance of gonococci to antimicrobial agents. One such mechanism for doing so is the *penB*

mutation. The *penB* mutation is due to alterations in the porin channel on loop 3 at positions 101 and 102 that may block the uptake of compounds such as penicillin, tetracycline, ciprofloxacin, and nalidixic acid (88). This reduced uptake of antimicrobial agents combined with the intrinsic P1B resistance level, and other mutations such as the *mtr* mutation discussed later, may be one factor that aides strains' harboring the P1B porin type in effectively establishing local infections as the mucosal surfaces on which the gonococcus resides are bathed in host derived antimicrobial agents.

v. Iron Acquisition

Iron is a scarce nutrient in the gonococcus's human host, as the vast majority is locked tightly away intracellularly, as ferritin or haem, and extracellularly, as transferrin and lactoferrin. As such, the acquisition of iron is vital for growth and for the virulence displayed by the gonococcus (198-200). While many bacteria secrete low molecular weight siderophores in order to acquire iron from host sources, the gonococcus instead relies upon TonB-dependent receptors that transport host proteins saturated with iron for use by the bacterium (189, 213, 277). These receptors are expressed at generally high levels, but certain ones, such as FetA, are subject to phase variation due to slipped-strand mispairing in a poly-C tract that exists in between the -10 and -35 regions. This process can reduce their expression significantly (39). Additionally, a large number of these iron transporters, in addition to TonB, are regulated by the global transcriptional regulator ferric uptake regulator (Fur) in a negative manner in the presence of excess iron (10, 116, 224).

Gonococci that are defective in their ability to take iron from transferrin due to mutations in, or loss of, the transferrin receptor are particularly at a disadvantage during infection as the transferrin receptor is necessary for sustained infection in humans (52). Further, gonococci that lack the iron transporter TdfF and/or TonB are deficient in intracellular replication (101). However, in mice, the ability to acquire iron from transferrin or hemoglobin is only of importance in experimental subcutaneous chamber infections and during simulated disseminated gonococcal infections, but not in the lower genital tract infection model (51, 86, 120). The influence of iron availability is further important to the gonococcus as low iron conditions, such as those found on the genital mucosa, increase the efficiency of transformation and recombination, thereby providing increased genetic diversity, which may provide survival advantages during infection (229).

vi. Resistance to Oxidative Killing

The ability of gonococci to resist killing by oxidative stress is imperative, as throughout the infection process gonococci encounter large amounts of oxidative stress. The sources of this stress are varied, and sometimes controversial in the case of intracellular survival of gonococci in PMN's, but they include the gonococcus's own respiration, release of reactive oxygen species (ROS) from PMN's during the "oxidative burst", and intracellularly in the periplasm/phagosome of epithelial cells (56, 179, 237). The induction of the oxidative burst results in exposure to superoxide (O_2-) and hydrogen peroxide (H_2O_2), while PMN's constitutively and inducibly produce nitric oxide (NO) (227). Exposure to these, and other oxidative stresses, cause damage to proteins, DNA,

and ultimately can result in cell death if the bacterium is unable to resist their action or repair the damage they cause (115).

In order to resist this potent mechanism of killing, the gonococcus has a number of mechanisms that have evolved somewhat differently from those found in the closely related meningococcus (226). The response to oxidative stress in gonococci is robust and in the case of hydrogen peroxide includes the change in transcription of more than 150 genes (248). Classes of genes covered by this robust response to hydrogen peroxide exposure include thioredoxin, cytochrome c peroxidase, Fur, heat shock proteins, and protein chaperones (226, 227).

Among these classes of genes, thioredoxin and the thioredoxin-like proteins have been identified as key components in resistance to hydrogen peroxide and other oxidative killing means. *trxA* of *N. gonorrhoeae*, which encodes thioredoxin, is upregulated in the presence of hydrogen peroxide along with *grxC*, which encodes an annotated glutaredoxin. Both of these are also involved in the response to hydrogen peroxide induced oxidative stress in *E. coli* (202, 226, 290). The thioredoxin-like protein TlpA is important in a number of aspects of gonococcal biology, as loss of this protein results in decreased resistance to hydrogen peroxide and paraquat. While this loss does not affect the bacteria's ability to bind to cervical epithelial cells, it does reduce the bacteria's intracellular survival (9). Additionally, the loss of the thioredoxin reductase encoded by *trxB* leads to sensitivity to nitric oxide, as well as reduced biofilm formation and survival in cervical epithelial cells (204). Certainly, thioredoxin and other thioredoxin-like genes may play further roles in gonococcal resistance to oxidative stress, as there are other

annotated members of this class; however, genes such as cytochrome c peroxidase also function in neisserial resistance to oxidative stress.

In the gonococcus, cytochrome c peroxidases (CCPs) are important in a number of cellular processes relevant to its lifestyle as a strict human pathogen (226). These processes include resistance to various reactive oxygen species. One such neisserial CCP, encoded by the *cycP* locus, helps protect the cell against nitric oxide induced damage that is produced during the switch to oxygen limited growth (264). Additionally, the CCP encoded by the *ccp* locus has been identified as being upregulated during the transition from aerobic to anaerobic growth and as being important in resistance to hydrogen peroxide (154, 263). Further, inactivation of the *ccp* gene results in a much attenuated biofilm phenotype, indicating that CCP also plays an important role in *in vitro* and possibly *in vivo* fitness (76). Interestingly, CCP is also up-regulated in the presence of the host derived antimicrobial peptide LL-37 (Shafer, Personal Communication).

The presence of hydrogen peroxide leads to the up-regulation of a number of heat shock response and chaperone protein genes, including *grpE, dnaK, dnaJ,* and *clpB* in *N. gonorrhoeae* (248). A large number of these genes identified as up-regulated in the presence of hydrogen peroxide are also upregulated in the general stress response mechanisms of gonococci regulated by RpoH (144). In *E. coli*, this function is much the same, but a functioning robust heat shock response system also contributes to resistance to lysis mediated by certain beta-lactam antibiotics (205). *grpE* and *clpB* have been shown to be upregulated both in the presence of hydrogen peroxide and under general stress induced by elevated temperature in *N. gonorroheae*, and also function in resistance to beta-lactams and response to heat shock in *E. coli*. (96, 144, 248). GrpE is primarily a

co-chaperone that improves the rate of protein disaggregation mediated by DnaK (22). GrpE accomplishes this by acting as a nucleotide exchange factor that exchanges ADP for ATP while further increasing the rate of peptide release from the DnaK protein binding pocket (106). ClpB is a co-chaperone that was first identified in *E. coli* as heat shock protein F84.1. ClpB is necessary for survival of *E. coli* at elevated temperatures, as ClpB null mutants have been found to have slower growth rates at mildly elevated temperatures and significantly reduced cell survival at more extreme elevated temperatures (241). Like GrpE, ClpB functions in conjunction with DnaK in a stepwise mechanism (8). ClpB binds to protein aggregates induced via stress upon the bacterium, classically defined by heat shock, and aides in the dissagreggation of these proteins (149). Further, in *E. coli*, *clpB* expression is regulated by RpoH (Sigma 32) (136).

Taken together, these selected mechanisms, which aide the gonococcus in resistance to oxidative stress, clearly show an organism well adapted to its unique environment as it possess a number of duplicate and complimentary methods for dealing with oxidative stress. The closely related meningococcus also must resist oxidative stresses in the nasophaynx, but the two closely related organisms do not posses all of the same mechanisms for resisting these stresses (226, 227). Further, the resistance to oxidative stress in the gonococcus is closely intertwined with other characteristics that are of central importance to survival in the human host, making this important to pathogenesis (76). Once the gonococcus is inoculated into the human host it can establish a wide range of infection types with a variety of different manifestations, which will be the focus of the next section.

B. Disease

The classical infection caused by *N. gonorrhoeae* is defined as a local mucosal colonization most commonly in the columnar epithelia of the urogenital tract, including the urethra of men and the endocervix (and/or urethra) of women (188). However, the gonococcus is a well-adapted human pathogen and it can cause a range of other localized infections including proctitis, peritonitis, perihepatitis and conjunctivitis (188). Further complications can arise if the gonococcus initiates the complex and multistep process of crossing the epithelium and subepithelial tissue, which allows entry into the bloodstream followed by the establishment of disseminated infections including arthritis, dermatitis, endocarditis, and meningitis. The disseminated gonococcal infection (DGI) can be quite severe and life threatening; however, it is relatively rare, accounting for just 0.5% to 3% of reported gonococcal cases each year (58, 134). The more common classical urogenital infection has a mean incubation period of 8.3 days and is characterized in men by white, yellow, or green discharge, pain/burning during urination, and/or swollen/painful testicles (236). In women, the early classical urogenital infection, when symptomatic, can be characterized by yellow or bloody discharge, bleeding during intercourse, pain/burning during urination followed by more severe symptoms if the disease is left untreated. Despite these very apparent infections caused by *N. gonorrhoeae*, reported cases of gonorrhea are almost certainly low due to under-reporting and the occurrence of subclinical or asymptomatic infections, which are common particularly in women, but also occur in men (3). On the extreme end of this asymptomatic spectrum are those people that can be lifelong carriers of *N. gonorrhoeae* without ever being symptomatic. However, gonorrhea infections, particularly those in women, who bear the greatest

burden of this disease, can be devastating beyond the classical urogenital tract infection. The occurrence of pelvic inflammatory diseases (PID) resulting from untreated gonoccocal infections is well documented and the possible results of PID (damage to the female reproductive tract, scarring, and sterility) are severe and can be permanent/life-altering. Additionally, gonorrhea can play a role in the transmission of HIV as well as often co-infecting with chlamydia (48, 60, 165). Further, transmission of urogenital tract infections from mother to child can result in severe conjunctivitis termed gonococcal opthalmia neonatorum (GON), sepsis, meningitis, or rhinitis. GON is the most common of these manifestations, and if not properly treated can lead to perforation of the corneas and loss of vision in the infant (279). Since the ramifications of gonococcal infections are significant beyond the classical urogenital infection, and feed directly into other important public health concerns such as HIV transmission, continued attention to the disease is warranted via surveillance, detection, prevention, and treatment on all levels and through all age groups.

C. Epidemiology

The Center for Disease Control and Prevention (CDC) identifies gonorrhea as the second most reportable/notifiable disease in the United States, trailing Chlamydia (*Chlamydia trachomatis),* with which it commonly co-infects. The epidemiological history of gonorrhea in the United States has been largely one of declining numbers over the last 30 years following the establishment of the National Gonorrhea Control Program; however, this progress is tempered by the rise in antibiotic resistance seen in the remaining cases, and the plateau in reported cases that makes it unlikely to hit program

goals of an infection rate of just 19 cases per 100,000 people by 2010 (43). In 2007, the reported rate of gonorrhea in the United States was still 118.9 cases per 100,000 or approximately ten-fold the target for 2010 (43). The local infection rates across the United States, however, show considerable variability, with the South still having the highest incidence rate. Prior to the mid 1990's, men bore the greatest reported infection rate (45). This trend has changed in recent years, as the reported incidence of gonorrhea infection rates among women has surpassed that of men (45). Among women, the highest disease burden has been concentrated among those women in the 15-24 year old age group (45).

Gonorrhea represents a significant public health burden, as annually there are approximately 95 million cases (2007) reported worldwide (272). The burden this disease represents is significant, particularly in developing countries, where the necessary antibiotics for treatment of modern drug resistant strains are unavailable (or prohibitively expensive) and treatment/prevention is scarce. However, even western industrial nations still struggle with gonorrhea incidence, as is seen in Sweden (and other parts of Europe), where reported cases of gonorrhea have increased over the last 10 years. Gonorrhea incidence in Sweden in 1996 was 2.4 cases per 100,000 people, however, by 2008 the incidence of gonorrhea had increased to 7.8 cases per 100,000 (272). This resurgence of gonorrhea incidence in European nations follows decades of decline and is concurrent with the increase in gonoccocal resistance to antimicrobial agents, making new infections more difficult to treat. Further, the emergence of high level antibiotic resistance in *N. gonorrhoeae* to every known class of antibiotics makes this issue dire for non-industrialized (or poorer) nations where the newest and most effective antibiotics are not

widely available. However, even in industrialized nations with modern medical infrastructure, the gonococcus is becoming more difficult to treat successfully as there few universally effective choices left for treatment. This reality is highlighted by the addition of *N. gonorrhoeae* to the CDC's list of "superbugs".

D. Treatment and Prevention

The occurrence and incidence of STI's (Sexually Transmitted Infection) such as gonorrhea represent not only large public health burdens, but often devastating outcomes to the individual who has contracted the infection. While general incidence of reported cases of gonorrhea has been on the decline in the United States, worldwide there are still ~95 million cases reported a year, and the incidence of antibiotic resistance in gonorrhea cases has been increasing since modern antibiotic therapies first appeared (272). This rise in antibiotic resistance makes the proper treatment of gonorrhea both necessary as in the absence of a vaccine chemotherapy remains the mainstay for reducing the spread of gonorrhea and difficult as many formerly effective treatments choices are no longer suitable for use in treatment (Figure 1).

Early chemotherapy-based treatments for gonorrhea were crude, at best, and often based on silver preparations or potassium permanganate coupled with irrigation, as suggested by Jules Janet and others (1, 2). The silver preparations used included silver nitrate, which still finds wide use in the prevention of neonatal gonococcal conjunctivitis, and Protargol (142). Protargol supplanted potassium permanganate as the standard treatment for gonorrhea around 1897 and was commonly used until the development of sulfanomides in the early to mid 1900's (266). Upon the development and demonstrated

efficacy of early sulphanomides, such as sulphathiazole and sulphapyridine, they became the front line treatment for gonorrhea beginning in 1937 (63). In less than 6 years, wide-scale treatment failure due to the development of resistance to these antimicrobial agents was being reported (35). Then, by 1949, sulphanomides were no longer recommended for clinical use in treating gonorrhea (71). At this time the standard course of treatment for gonorrhea was supplanted by the use of penicillin, to which *N. gonorrhoeae* had proven to be exceptionally susceptible when first assayed (6). The first penicillin- and streptomycin-resistant strains of the gonococcus appeared in 1958 and by 1987, penicillin was no longer available for use in treating gonorrhea (40, 44, 55).

This difficulty in treating gonorrhea was further complicated by the loss of tetracycline, azithromycin, erythromycin, and fluoroquinolones, until finally in 2007 the CDC added *Neisseria gonorrhoeae* to their list of "superbugs" (Figure 1) (42). This resulted in the third generation cephalosporins, such as ceftriaxone and cefixime, being the only class of remaining antibiotic therapy recommended for treatment of this common disease (46). However, resistance to even these third generation cephalosporins in conjunction with previous multidrug resistance phenotypes is rapidly developing in clinical cases (62). The current recommended regimen for the treatment of uncomplicated gonococcal infections is a 125 mg intramuscular dose of ceftriaxone or a 400 mg oral dose of cefixime, with the alternative regimen being a 2 g intramuscular dose of spectinomycin (47). However, spectinomycin is not currently available in the United States and cefixime has just become available again in April of 2008 (41).

While the management of the closely related meningococcus has been greatly simplified by the development of vaccines against some of the most common serotypes,

the management of gonococcal infections has further been complicated by the lack of a viable vaccine against the gonococcus. The surface of the gonococcus is the obvious place to look for vaccine targets; however, due to the variability of many surface exposed proteins and the ability to produce multiple LOS chemotypes, there are few conserved stable targets available to be used in a vaccine. The gonococcal pilus accounts for considerable amounts of surface exposed protein; however, it undergoes frequent antigenic and phase variation, making it a poor target for elucidating immune response, though PilC may provide a viable target (228). For much the same reason, LOS is a poor candidate for vaccine targets, however there is some promising work that indicates that certain conserved regions may be able to be used as targets (183). The Opa proteins also represent a large number of cell surface exposed targets that have been found to be unsuitable for vaccine candidates due to their high copy number and variable expression (119). Of some promise is the use of iron acquisition targets such as transferrin binding proteins, as these have shown early positive results in animal models (258). This work, however, likely remains years away from providing a suitable vaccine.

Given the current circumstances of high antibiotic resistance and lack of vaccine availability, the prevention and treatment of gonorrhea remains an extremely important public health concern. Further work in understanding the mechanisms by which the gonococcus becomes resistant to antibiotics along with vaccine development and public health monitoring (and education) all must be maintained or increased in order for us to attempt to effectively combat this newest “superbug”. Further, because Multidrug-Resistant *Neisseria gonorrhoeae* (MDR-NG) isolates are quite common, we must be intelligent and deliberate in our use of currently available antimicrobial therapies lest

Extensively Drug-Resistant *Neisseria gonorrhoeae* (XDR-NG) become widespread, as has happened with other pathogens such as *Mycobacterium tuberculosis* (257). It is further imperative that we continue to study the methods by which this elusive human pathogen has been able to become resistant to every course of antibiotic treatment developed to date, as the lessons learned from the gonococcus will provide us with key insights into emerging human pathogens for which we do not have such extensive research experience.

II. Antimicrobial resistance mechanisms exhibited by *Neisseria gonorrhoeae*

Bacteria exist in almost every known niche of our planet and in each of those environments the bacterial cell is bombarded with agents which will likely damage or kill the cell. Bacteria that inhabit the human body must resist the naturally derived human antimicrobial agents, as well as those agents used therapeutically. In doing so, bacteria employ a wide range of mechanisms to resist the actions of these antimicrobial agents, including intrinsic and acquired modes of resistance (109). Among these mechanisms, the four main methods utilized by *N. gonorrhoeae* are entry exclusion, drug metabolism or modification, target modification, and drug efflux.

A. Entry exclusion

One of the first lines of defense a bacterium has to resist antimicrobial compounds is to prevent their entry altogether. As a Gram-negative bacterium, the outer membrane (OM) of *N. gonorrhoeae* itself provides a formidable barrier that can aide in excluding antimicrobial compounds from the cell, giving the cell a baseline intrinsic resistance.

However, the OM contains a number of pores, channels and transporters that allow compounds necessary for the cell's survival and growth to enter into the cell. These entry points can also allow influx of unwanted products, including antimicrobial compounds. Because of this, any mutation that alters the permeability of the outer membrane can impart significant resistance to antimicrobial agents encountered by the gonococcus. There are several prominent mutations that exist in gonococcal porins that alter the permeability of the outer membrane and lead to a significant abrogation of this unwanted entry. These mutations include the *penB* and *penC* mutations.

The *penB* mutation was first identified in 1975 and as part of the stepwise acquisition of chromosomally-mediated penicillin resistance that occurs at the Por1B locus (239). Entry inhibition of penicillin and tetracycline occurs when mutations at positions 120 and/or 121 cause an amino acid substitution on loop 3. These amino acid substitutions are hypothesized to obstruct the porin channel (191). There is further preliminary evidence that the *penB* mutation is involved in the growing resistance of *N. gonorrhoeae* to third generation cephalosporins by a currently unknown, though likely related, mechanism (153, 288). The *penC* mutation occurs in the *pilQ* locus and, unlike the *penB* mutation, has wide ranging effects on the cell. The single point mutation at nucleotide 1996 changes the glutamic acid to a lysine residue. This change prevents the multimerization of the *pilQ* products which form the outer membrane pore through which the gonococcal pilus extrudes from the cell. This loss of stable pore not only reduces entry into the periplasm of penicillin and tetracycline, but also blocks pilus extrusion and reduces competence of the cell (289).

B. Drug modification

If an antimicrobial agent does manage to cross the outer membrane of a Gram-negative bacterium, the cell can produce a number of enzymes, which can alter toxic compounds, providing the cell another potent method of resistance. This modification can be as simple as methylation or acylation of the target drug in order to reduce the net positive charge, as is the case with aminoglycosides, or as radical as the cleavage of the molecule by an enzymatic product of a resistance determinant. These antimicrobial resistance determinants are widespread among bacteria and pass from species to species with relatively high frequency (61). One such example of enzymatic cleavage of an antibiotic target is the now almost ubiquitous beta-lactamase enzymes, which target beta-lactam antibiotics such as penicillin and were first described in 1940 (7).

Penicillin is perhaps the best known of all antibiotics due to its early discovery, long term use, efficacy, and generally low toxicity (195). Penicillin disrupts cell wall synthesis when it becomes covalently linked to the active site of penicillin-binding proteins (PBPs). To achieve this binding and action, penicillin's lactam ring mimics the structure of a D-Ala-D-Ala motif, which then prevents the cross-linking of *N*-acetylglucosamine with *N*-acetylmuramic acid and peptide crosslinks (276). While the beta-lactam ring of penicillin is a perfect fit for unaltered PBP's, it also is the target of beta-lactamases, which confer resistance to beta-lactam antibiotics by direct hydrolysis of the beta-lactam ring (61).

Prior to 1976, resistance to beta-lactams in *N. gonorrhoeae* was largely believed to be chromosomally mediated and not due to beta-lactamase production (18, 239). While not mediated by beta-lactamase production at that time, penicillin resistance was

still a significant problem, as by 1972 the recommended does of penicillin for uncomplicated gonorrhea cases had risen to 4,800,00 units (4). This situation worsened in 1976 when a number of reports began to emerge of beta-lactamase producing *N. gonorrhoeae* in Great Britain and the United States (13, 201). Following the isolation of the African and Asian R plasmids in *N. gonorrhoeae*, it was determined that the gonococcus could, albeit not commonly, carry TEM-1 beta-lactamase producing plasmids (25, 74, 211). These plasmids were most likely acquired from *Haemophilus* spp. and represent antibiotic resistance through horizontal gene transfer between *Haemophilus* spp. and *N. gonorrhoeae* (145). These discoveries were soon followed by a number of other isolates identified as harboring beta-lacatamase producing plasmids (67).

C. Target modification

Recognition, binding, and effecting a change on cellular targets are the keys to the function of any antibiotic, and this process represents another area in which resistance mechanisms by bacteria can be effective. Different classes of antibiotics target various cellular processes (beta-lactams target cell wall synthesis and elongation, macrolides and aminoglycosides interfere with protein synthesis, quinolones interfere with DNA replication), in order to either stop/slow bacterial growth, or kill cells all together. All classes of antibiotics' effectiveness can be impaired by mutations in their target proteins encoding gene. Given the wide range of these targeted processes, a bacterium may easily have numerous mutations in different pathways, giving rise to multiple antibiotic resistances in several chromosomally encoded determinants.

The gonococcus possesses a wide range of these chromosomally-mediated target modification methods for resisting antimicrobial agents including penicillin. The *penA* mutation occurs in PBP 2 and was first identified, but not completely characterized, in 1974 (221). It is one step in producing clinically significant chromosomally mediated non beta-lactamse dependent penicillin resistance. This mutation is due to the insertion of an additional aspartic acid residue at position 345 (Asp-345a), and results in a lower acylation rate by penicillin, conferring additional resistance to beta-lactams (28). Additionally, the *ponA* gene, which encodes PBP 1, can also possess a mutation that raises the MIC (minimum inhibitory concentration) of *N. gonorrhoeae* to penicillin and is a significant step in accumulating chromosomally mediated penicillin resistance that is high enough to result in clinical failure of treatment. The mutation responsible for this change is a T to C transition resulting in a substitution of proline for leucine at position 421. This change results in a lower affinity for penicillin (214). Of growing concern and unclear mechanism are the findings that the *penA* mutation in particular, but also the *ponA* mutation, are also involved in the growing resistance to cefixime and ceftriaxone (153).

Resistance to two classes of polyketide antibiotics used in the treatment of *N. gonorrhoeae* can also be mediated by alterations to the targeted structure, in this case the ribosome and ribosome-associated proteins. The first class of polyketide antibiotics that can be resisted by alterations to the targeted structure, the ribosome, are the macrolides. In the gonococcus at least two methods of resisting the actions of macrolides exist. The first is a simple point mutation at position 2599 in domain V of the 23S rRNA subunit, which is a C to T transition that likely alters the secondary structure of this domain, and

confers high level resistance to both erythromycin and azithromycin. Alternatively, the presence of rRNA methylase genes *ermB*, *ermC*, and *ermF* in clinical isolates has been reported. These genes confer resistance to macrolides such as erythromycin by methylating a specific residue on the 23S rRNA subunit, resulting in decreased binding of the target by the drug (212).

High level resistance to tetracycline in Gram-negative bacteria is often mediated by the TetA efflux pump; however, in *N. gonorrhoeae* it can also be mediated by the acquisition of the *tetM* determinant from *Streptococcus* spp. on a conjugative plasmid or a combination of mutations occurring in the *mtrR* locus, *penB* locus, and the *rpsJ* locus (14, 176, 262). *tetM* encodes a product whose mechanism of action mimics translation elongation factors and prevents antibiotic binding to the ribosome or promotes the release of bound antibiotic (33, 209, 220). *rpsJ* encodes the S10 ribosomal protein, which is responsible for binding t-RNA's to the ribosome. Mutations that occur at position 57, which change the valine to methionine, leucine, or glutamine, increase resistance to tetracycline likely by interfering with its binding to the 30S subunit of the ribosome complex (113).

Following the downfall of penicillin and tetracycline as treatments for uncomplicated gonorrhea, fluoroquinolones became the first line of therapy (46). Much like with the alterations to PBP's of *N. gonorrhoeae* that, coupled with other stepwise factors lead to the emergence of chromosomally mediated penicillin resistance, alterations to other targets, such as *parC* and *gyrA* along with other factors, confer resistance to fluoroquinolones. *parC* encodes topoisomerase IV, and mutations that map to amino acids 88 and 91 can result in lower binding of fluoroquinolones to

topoisomerase IV, giving rise to significant resistance (21, 68). *gyrA* encodes the A subunit of DNA gyrase and mutations at amino acids 91 and 95 provide significant resistance to fluoroquinolones by a similar mechanism as *parC* mutations (21, 68). *gyrB* encodes the B subunit of DNA gyrase, yet no mutations have been identified to date that confer significant resistance to fluoroquinolones. However, there is limited evidence that an aspargine to aspartic acid mutation at position 419 in the *N. gonorrhoeae* GyrB is one method by which *N. gonorrhoeae* can resist nalidixic acid (246).

D. Drug Efflux

One active method of resisting antimicrobial agents found among almost all bacteria is the use of antimicrobial efflux pumps. The first efflux pump correctly identified in such a context, however, was described not in bacteria but rather in man. The P-glycoprotein (PgP) was first identified in tumor cell lines that showed resistance to chemotherapy agents in 1976 and represents a prototypical member of the ATP Binding Cassette Superfamily (ABC) (124, 125). Increased production of this 170-kDa plasma membrane-associated protein was originally found to correlate to multidrug resistance (MDR) in mammalian cells (19, 89) and was later found to be sufficient to induce this phenotype as well as transport a wide range of non-chemotherapy drugs (reviewed in (181)). The ability of Pgp to recognize and export a large variety of structurally diverse substrates is mediated by its large, multifaceted, binding pockets and this is a characteristic that it shares in common with many efflux pump systems found in bacteria (11).

The first bacterial efflux pump to be identified was the Tet pump of *E. coli* in the late 1970's, however, the *acr* mutation of *E. coli* identified in 1965 would later be identified as the AcrAB efflux pump system (14, 150, 170, 171, 180). The Tet pump identified is a plasmid encoded efflux pump of the major facilitator superfamily (MFS) that exports tetracycline and closely related antibiotics (172, 197). Following the identification of the Tet pump in *E. coli*, a number of other efflux pumps were discovered including the QacA-QacB pump of *S. aureus* (MFS), EmrB of *E. coli* (MFS), AcrA-AcrB of *E. coli* (RND), MexA-MexB of *P. aeruginosa* (RND), and others (117, 155, 162, 196, 197, 203). In fact, one survey of the *E. coli* genome alone found at least 37 efflux transporters belonging to all 5 classes of efflux pumps (187) while the *P. aeruginosa* genome also posses a large number of efflux pumps (approximately 65) (249). With the rapidly expanding ability to sequence whole genomes quickly and inexpensively the future identification of more efflux pumps is almost without doubt, even in some of these well investigated species. However, even with a limited number of efflux pumps bacteria can successfully resist the action of a wide range of structurally dissimilar agents. The method of resistance imparted to the bacteria by these efflux pumps may be constitutive, inducible, or both. This method of antibiotic resistance in *N. gonorrhoeae*, along with its regulation, is wide-ranging and the focus of the next section.

III. Major Efflux Pumps in *Neisseria gonorrhoeae*

The movement of small molecules across cellular membranes by living organisms is common across the entirety of living organisms. The mechanisms of this cross membrane transport vary in complexity and form, but the necessity and function of this

transport remains the same in resisting toxic compounds found in the environment, along with the movement of certain vital small molecules (simple sugars, amino acids, metabolites, anions, cations, etc).

The outer membrane of Gram-negative bacteria provides more of an obstacle to effective efflux and the majority of efflux pumps from the multidrug and toxic compound extrusion (MATE) and ATP-binding cassette (ABC) families of efflux pumps, found almost exclusively in Gram-negative bacteria, move antimicrobial compounds to the periplasmic space (140). There are a number of clinically significant exceptions to this rule in the MATE and major facilitator superfamily (MFS). However, in Gram-negative bacteria, antimicrobial efflux across both cellular membranes is dominated by the resistance-nodulation-division (RND) transporters, which can successfully form a channel spanning the periplasm encompassing both inner and outer membrane components, which along with a membrane fusion component provide a single unobstructed pathway for removing antimicrobial compounds from the cell (140). The major examples of these systems in *N. gonorrhoeae* will be discussed in this section (Figure 2). Other examples, such as the MFS member *mef*, can be found in *N. gonorrhoeae*, but they are not as well characterized or as ubiquitous (53, 54, 160).

A. NorM

The multidrug and toxic compound extrusion (MATE) family of efflux pumps are the newest recognized class of multidrug efflux pumps, but the MATE family represents an ancient system common to many organisms as examples of the MATE family are found across all three kingdoms of life (30). The MATE family transporters are

characterized by 12 putative hydrophobic transmembrane domains and couple Na+ import to drug export, making them Na+/drug antiporters. The drugs recognized by these antiporters are varied between members of the MATE family, with the most common class of drugs recognized being the fluoroquinolones and to a lesser extent ethidium bromide and kanamycin (141). These drugs are structurally dissimilar, indicating that the MATE system, like other multidrug efflux systems, has a wide range of substrate specificity. This wide range of specificity makes the MATE family of efflux pumps clinically relevant in the case of MepA of *Staphylococcus aureus* and NorM of *N. gonorrhoeae*.

The *mepA* gene of *S. aureus* confers resistance to a wide range of drugs and biocides including, but not limited to fluoroquinolones, ethidium bromide, quaternary ammonium compounds, crystal violet, and tigecycline (127, 168). The *mepA* gene is further under the control of the MarA family transcriptional regulator, MepR, which shows varying affinity for the *mepR* and *mepA* operators in the presence of MepA substrates, resulting in increased expression of the *mepA* gene when a MepA substrate is present (126).

The *norM* gene of *N. gonorrhoeae* is homologous to the prototypical member of the MATE family, NorM of *Vibrio parahaemolyticus*, and represents one of at least 4 distinct efflux pumps found in the gonococcus. Like other members of the NorM family of MATE transporters, the gonococcal NorM exports compounds such as ethidium bromide and berberine, while over expression of the pump mediated by a single base pair mutation upstream of the coding region of *norM* results in the other characteristic resistance for the NorM family members, resistance to fluoroquinolones (217).

B. MacAB

In eukaryotic research, ATP-binding cassette (ABC) transporters have long been identified as a major mediator of drug efflux in cancer biology; however, their role in drug efflux in Gram-negative bacteria was not identified until the discovery of the MacAB-TolC system in *E. coli* (124, 138). Previously, ABC transporters in *E. coli* had been identified in a wide range of cellular functions, including protein export and more recently the secretion of heat-stable entertoxin II by the MacAB-TolC system (282). The MacAB system is composed of three separate parts: the *macA* gene encoding the membrane fusion protein, the *macB* gene that is the ABC protein, and *tolC* which encodes the multifunctional outer membrane channel. The protein products of these three genes work together to export a number of macrolide class antibiotics such as erythromycin, clarithromycin, oleandomycin, and azithromycin from *E. coli* (138). Beyond merely exporting antibiotics, the homologous MacAB system of *Salmonella enterica* plays a significant role in virulence, as a *macAB* deletion mutant is less virulent, possibly due to the loss of export of some factor similar to that of the *E. coli* MacAB system or removal of other compounds encountered during infection (ie the pumps natural targets) (186). Additionally, the *macAB* locus in *S. enterica* is under the control of the two-component regulatory system PhoP-PhoQ, in which PhoP directly binds to the region upstream of *macAB* and represses transcription from this locus (185).

Similar to the MacAB system of *E. coli*, *N. gonorrhoeae* and *N. meningitidis* possess a MacAB efflux pump system which can recognize and export macrolides (218). This is of particular clinical importance, as azithromycin and other macrolides have been

used for the treatment of gonococcal infections, and strains of *N. gonorrhoeae* that are highly resistant to macrolides have been reported due to known and unknown mechanisms (72, 83, 284, 287). The expression of the MacAB system in *N. gonorrhoeae* is not the dominant mechanism for macrolide resistance, as a promoter mutation exists in *N. gonorrhoeae* that results in very low expression of this efflux pump. However, a single nucleotide substitution in the -10 region of the *macAB* promoter is sufficient to increase transcription from this locus and increase resistance to macrolides recognized by the pump (218).

C. FarAB

The Major Facilitator Superfamily (MFS) is the largest grouping of transporters yet defined, as it contains at least 17 distinct families of transporters (194). The majority of these families are responsible for transport of sugars, metabolites, amino acids and other peptides. However, a number of families are also responsible for drug efflux (194). The most common MFS families that export drugs are H+/Drug antiporters with 12 or 14 transmembrane domains. They transport a wide range of drugs that share only hydrophobicity as a common trait for recognition by the system (194). While capable of exporting many antimicrobial agents, few of the MFS members are major contributors to drug resistance at normal physiological expression levels; however, under over expression conditions many such systems export clinically relevant antimicrobial agents. One example, MdfA in *E. coli*, can export ethidium bromide, chloramphenicol, tetracycline, neomycin, and various fluoroquinolones (78). A clinically relevant exception to this pattern is the EmrAB efflux system of *E. coli* (and its homologs), which

recognizes and exports a range of hydrophobic compounds at clinically significant levels under normal expression levels (78). Among these hydrophobic compounds are the antibiotics nalidixic acid and thiolactomycin (82). The EmrAB system is able to export a range of diverse structures through its EmrAB arrangement and the characteristic large hydrophobic binding pockets of MFS systems (256). The expression of this system is tightly regulated by a number of factors and is inducible by its substrates. Among these methods of control are the presence of a promoter driving transcription of both the *emrA* gene and another driving the transcription of the *emrB* gene, which are repressed by the MarR like repressor, EmrR (156). This transcriptional repressor directly binds the site upstream of *emrA* and the ability of EmrR to bind to this region can be mitigated by the inducers of the pump (281).

The gonococcus possesses a MFS family efflux pump encoded by the *farAB* locus (148). This efflux pump is similar to the *E. coli* EmrAB system and is composed of FarA, which is the membrane fusion component, and FarB, which serves as the inner membrane component. Further, the FarAB system uses the TolC-like outer membrane channel MtrE for export of long chain fatty acids (oleic acid, palmitic acid, and linoleic acid), to which it confers resistance (148). Regulation of the *farAB* locus is complicated as at least three mechanisms are involved. The *farAB* locus is directly repressed by the action of the MarR type repressor FarR, which binds to a sequence upstream of the *farAB* promoter. This region also contains binding sites for integration host factor (IHF), which is required for FarR repression of the *farAB* locus, and is capable of repressing transcription of *farAB* by itself (146). Lastly, *farAB* transcription is indirectly activated

by MtrR, as MtrR binds to the region upstream of *farR* and represses its transcription (147).

D. MtrCDE

The Resistance Nodulation Cell Division (RND) family of transporters are an ancient line of drug transporters found in eubacteria, archaebacteria and eukaryotes. The RND pumps are tripartite efflux pumps consisting of a membrane transport protein with 12 transmembrane domains (or 6 in *Mycobacterium jannaschii*), a membrane fusion protein, and an outer membrane channel protein complex (commonly TolC) (261). Substrates recognized by the RND efflux pumps are actively pumped out of the cell by a substrate/H+ antiport mechanism, which make these pumps functionally coupled to the cellular membrane's proton motive force. As such, agents that disrupt this proton motive force, such as carbonyl cyanide-m-chlorophenylhydrazone (CCCP), disrupt the function of these efflux pumps, including the export of antimicrobial agents (232), and also dampen the formation of biofilms (114). Among the structurally diverse molecules recognized by the RND transporters are compounds such as antibiotics, host derived antimicrobial peptides, bile salts, dyes, and detergents. The ability of the RND family of efflux pumps to recognize such a diverse set of substrates is largely mediated by the flexibility of the substrate binding cavity and the large hydrophobic binding pockets present in the membrane transport component of the efflux pump (184, 285).

Unlike the MFS family of transporters, the RND family is responsible for a large portion of the intrinsic resistance to clinically significant antibiotics in Gram-negative bacteria (140). The two most well studied RND systems are the AcrA-AcrB-TolC

system of *E. coli* and the MexA-MexB-TolC system of *Pseudomonas aeruginosa*. In the AcrA-AcrB-TolC system of *Escherichia* coli and its homologs, AcrA is the membrane fusion protein, AcrB is the membrane transport protein, and TolC serves as the outer membrane channel (23, 81, 139). Together, these three components are capable of exporting a host of antibiotics (ciprofloxacin, erythromycin, oxacillin, minocycline), detergents (dodecylsulfate), dyes (berberine, acridine, and ethidium), long chain fatty acids, and bile salts in *E. coli* (140, 152). In *Klebsiella pneumoniae*, the system also expels antimicrobial peptides such as HBD-1 and HBD-2 (192). The AcrA-AcrB-TolC expression is indirectly inducible by the effect of certain bile salts and fatty acids on the transcriptional regulator Rob (216). More locally, transcription from the *acrAB* locus is under the direct negative control of the TetR type transcriptional regulator AcrR, which also represses its own transcription, and the transcriptional repressor AcrS (111, 161). Further control over this system occurs in a global AcrR-independent manner involving the transcriptional regulator MarA (16). This regulation by MarA can result in elevated resistance to certain antimicrobial agents such as tigecycline (130).

Among the efflux pumps *N. gonorrhoeae* possesses, the RND family MtrC-MtrD-MtrE pump is perhaps the best characterized and has been the target of intense study in our laboratory for many years. The MtrC-MtrD-MtrE efflux pump was first identified in 1973 as a single outer membrane mutation deemed *mtr*, multiple transferable resistance, that bestowed resistance to a wide range of antimicrobial agents upon the strain of *N. gonorrhoeae* harboring the mutation (98, 164). It was thought originally that this mutation altered the permeability of the outer membrane, due to the observed reduction in uptake of certain agents and the alterations that were seen in the cell envelope in these

strains (98, 99). Since then, the mechanism, structure, regulation, and physiological functions of this system have been more finely investigated as new methods became available and through the focused efforts of a number of laboratories. It was first determined that the *mtr* system consisted of more than a single mutation in 1994 when the *mtr* locus was cloned and sequenced. At that time the *mtr* locus was found to encode a putative transcriptional repressor deemed MtrR as well as a portion of a homolog of the *E. coli envC* gene deemed *mtrC* (193). Soon after, the remainder of the *mtr* locus was cloned and found to additionally contain the gene *mtrD* and the gene *mtrE*. These three components, MtrC-MtrD-MtrE, are the main structural components of the efflux pump and loss of any of these three results in a loss of function for the pump (64, 269). In this system, the 12 transmembrane domain RND family member MtrD is the transporter located in the inner membrane of the cell and is responsible for recognizing the substrates of the pump (102). MtrE is a TolC-like protein that forms the outer membrane channel through which substrates recognized by the pump are expelled from the cell (64). These two components are brought together by the MtrC gene and it serves as the membrane fusion protein of the system resulting in active export by the system (103, 158). As with the homologous AcrA-AcrB-TolC efflux system, the MtrC-MtrD-MtrE system recognizes a wide range of antimicrobial compounds, including erythromycin, azithromycin, crystal violet, Triton X-100 (t-Octylphenoxypolyethoxyethanol), LL37, PG-1, nonoxynol-9, and penicillin (103, 232, 267). This is accomplished much the same way as with the aforementioned AcrA-AcrB-TolC system in which the AcrB component is able to recognize a wide range of structurally diverse compounds that only have stretches of hydrophobicity in their structure as common factors. In doing so, the MtrC-

MtrD-MtrE system forms a channel in which captured antimicrobial agents are exported directly out of the cell conferring high levels of both intrinsic and inducible resistance to *N. gonorrhoeae*. However, in order for this system to operate to its full potential the accessory protein MtrF must be present (268). The expression of this gene, like that of *mtrCDE*, is inducible and subject to repression by MtrR, as well as being subject to repression by the transcriptional regulator MpeR in an additive independent mechanism (80).

IV. MtrCDE Transcriptional Regulators (MtrR/MtrA)

The regulation of the MtrC-MtrD-MtrE efflux pump is tightly and intricately controlled by both a repressor and an activator. The repressor of this system is MtrR, a TetR/QacR family transcriptional regulator (193). Positive regulation of the system is mediated by the AraC like transcriptional regulator MtrA (219).

MtrR is the originally identified resistance determinant deemed *mtr* (164). It is transcribed opposite of the structural genes of the MtrC-MtrD-MtrE efflux pump and features a promoter that overlaps the promoter for the *mtrCDE* locus. The product of *mtrR* is a 210-amino-acid-residue ~23k Da protein. This protein has a N-terminal helix-turn-helix motif that is 57% identical to the 60 amino acid residues of the helix-turn-helix motif of AcrR, a member of the TetR/QacR family of transcriptional regulators (193). MtrR binds as two homodimers to the 250 bp intergenic region between *mtrR* and *mtrCDE* (112). This region encompasses a number of key features related to MtrR binding and regulation, including a 15 bp inverted repeat and a region encompassing 22-26 bp of DNA on both the coding and non-coding strands that MtrR can bind (104, 112,

157) (Figure 3). Mutations in *mtrR* are quite frequent in clinical isolates and are one of the reasons for elevated antimicrobial resistance among gonococci (65, 123, 167, 231, 286). A few of these relevant mutations descriptions follow. There are significant numbers of clinically isolated mutations that map to the helix-turn-helix motif of MtrR. These included mutations at position 45, resulting in a change from glycine to aspartic acid, and at positions 39 or 40, which results in a histidine to threonine change. These changes result in *in vitro* increases in antimicrobial resistance deemed to be of intermediate level, due to their MIC, possibly resulting from their lower binding affinity for the *mtrR-mtrCDE* intergenic region (231, 286). Other clinically significant mutations can occur at position 105 where the histidine residue is changed to a tyrosine residue leading to an increase in penicillin resistance (193). However, the overall clinical significance of these mutations may also be increased due to a lower binding affinity for the *farR* promoter. As stated earlier, the FarAB system is repressed by MtrR and is responsible for resistance to long chain fatty acids (148). Additional mutations outside of the helix-turn-helix motif exist that result in slightly elevated levels of antimicrobial resistance. Some of these mutations map to the C-terminus, including residue 202, and may alter MtrR dimerization or may interfere with substrate recognition (275).

Alterations to the *mtrR* promoter region that decrease transcription of the *mtrR* gene and/or increase the transcription of the *mtrC* gene are responsible for highly elevated antimicrobial resistance in *N. gonorrhoeae* and are often found clinically (54, 280, 287). Among these promoter mutations that lead to high level antimicrobial resistance are mutations that affect the spacing between the -10 and -35 region. These mutations can either be insertions, such as a dinucleotide insertion in the inverted repeat

that increase spacing from the optimal 17 nucleotides to 19, or these mutations can be deletions, such as a single nucleotide deletion in the inverted repeat that decrease spacing from an optimal 17 nucleotides to 16 nucloetides (104, 287). Lastly, a recently identified mutation deemed mtr_{120} results in the generation of a new promoter outside the typical *mtrCDE* promoter leading to increased antimicrobial resistance. This promoter is a naturally occurring element and is found in the laboratory strain MS11 as well as from strains recovered from *in-vivo* experiments (275) (Johnson and Ohneck, unpublished results).

Activation of the *mtrCDE* operon is achieved not only by the absence of MtrR but also the presence of a second transcriptional regulator, MtrA (219). MtrA is a 301 amino acid AraC/XylS family transcriptional regulator. It shares ~61% similarity with MarA and SoxS over the 100 amino acid C-terminal helix-turn-helix motif that defines AraC/XylS family transcriptional regulators (219). As with these regulators, there is a large N-terminal domain which may act as an effector binding domain that is critical for activity of the activator (219). In *N. gonorrhoeae*, MtrA is not an essential gene as many strains, including the original sequence strain FA1090, have 11bp deletions that render the protein non-functional (219). When MtrA is present, *mtrCDE* transcription is activated and is additionally inducible when the bacteria are grown on sub-lethal concentrations of certain antimicrobial compounds. This inducible phenotype results in high levels of resistance to agents recognized by the MtrC-MtrD-MtrE efflux pump including TX-100 and the commonly used spermicidal lubricant nonoxynol-9 (219). The exact mode of action of MtrA is not currently understood; however, some early evidence indicates that it activates *mtrCDE* transcription by directly binding to the *mtrCDE*

promoter and by doing so with greater affinity when certain pump substrates are present (Zalucki and Shafer, unpublished results).

V. MtrCDE/TetR/Efflux Pump Regulators Beyond the Efflux Pumps

The classically defined role of regulators of antimicrobial efflux pumps has typically been constructed as regulating genes involved in the expression of the efflux pumps alone. However, recent work on TetR/QacR family regulators similar to MtrR, and other classes of transcriptional regulators of efflux pumps, has suggested that these regulators once thought to be local regulators may indeed be responsible for controlling wide-ranging cellular functions and may act as global transcriptional regulators (108). This global regulatory scheme ties the mechanisms of antibacterial resistance to some of the basic biological functions of bacteria, indicating the vital importance of these transcriptional regulators in bacterial cellular processes.

A. TetR family regulators

The *vanT* gene of *Vibrio anguillarum* encodes a TetR family transcriptional regulator that is responsible for regulating a range of diverse genes/cellular processes not typically related to traditional TetR targets. Among the processes that it regulates are pigmentation, biofilm production, and serine metalloprotease production (57). Further, recent work in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* has identified a predicted regulon of 74 genes and 83 genes respectively for the TetR/QacR family regulator Rv3574 (133). The TetR family regulator AmtR in *Corynebacterium glutamicum*, an industrially significant source of amino acid production, is responsible

for regulating either directly or indirectly at least 36 genes mostly involved in the production of amino acids and cellular transport (32). More over, in *Campylobacter jejuni*, the repressor of the CmeA-CmeB-CmeC multidrug efflux pump, CmeR, is responsible for regulating a number of genes beyond the *cmeABC* locus. In total, 28 genes have been identified as being differentially regulated, including membrane transporters, lipoproteins, and genes involved in capsule biosynthesis. Further, CmeR mutants are deficient in colonization of their natural hosts, chickens (97).

B. MtrR

As was indicated by CmeR, the role of these efflux pumps and their regulators during an infection can be vital. The MtrC-MtrD-MtrE system of *N. gonorrhoeae* is crucial during an infection *in vivo* when examined in the female BALB/c mouse model. In the female genital tract infection model of BALB/c mice, it was found that inactivation of either *mtrD* or *mtrE* resulted in lower fitness and less recovery (121). Further, mutations that occur in the *mtrR-mtrCDE* overlapping promoter region can increase gonococcal fitness *in vitro* and *in vivo* when they result in de-repression of or over-expression of the MtrC-MtrD-MtrE efflux pump. Mutations occurring in the *mtrR* gene itself such as the alanine to threonine substitution at residue 39, the glycine to aspartic acid change at position 45, and the glutamic acid to glycine change at position 202 are also responsible for increased fitness compared to wild type strains in the experimental mouse model. Conversely, mutations in the *mtrA* gene that render it non-functional, result in lower fitness during an experimental infection (274, 275). While the exact mechanisms for MtrR mutants' increased fitness in an infection model are still being

investigated (though it is likely due at least in part to elevated expression of the MtrC-MtrD-MtrE efflux pump), there is other evidence that MtrR acts as a transcriptional regulator beyond the MtrC-MtrD-MtrE efflux pump. The first locus that was identified beyond the MtrC-MtrD-MtrE efflux pump as regulated by MtrR was the *farR* locus (Figure 3). MtrR indirectly activates transcription of the FarAB efflux pump by repressing the transcription of *farR*, the repressor of this system (147). MtrR is also responsible for differentially regulating a pair of divergently transcribed genes outside of the scope of efflux pumps. The *ponA-pilMNOPQ* locus of *N. gonorrhoeae* strain FA19 encodes PBP-1 (*ponA*) and components of the Type 4 pilus secretion system (*pilMNOPQ*) (Figure 3). At this locus, MtrR binds specifically and represses the transcription of *pilMNOPQ* while activating the transcription of *ponA* (79). These two operons are of vital importance in resistance to penicillin, as PonA is a direct target of penicillin and PilQ forms a pore through which antimicrobial agents such as penicillin have been shown to enter the gonococcus (214, 215, 289).

As the reported cases in which transcriptional regulators of efflux pumps, including members of the TetR/QacR family, have been shown to be global transcriptional regulators has increased, we sought to determine the scope of the regulons controlled by the regulators of the MtrC-MtrD-MtrE efflux pump system. This work was further focused on the TetR/QacR like repressor of the system, MtrR, as it had previously been shown to regulate genes outside of the MtrC-MtrD-MtrE pump, indicating that it may serve as a global transcriptional regulator of significance in the gonococcus (79, 147).

Among these global regulatory targets of significance that MtrR may serve to regulate are components of the oxidative stress response systems, which are of particular importance to gonococcal survival during infection, as well as pathways importance in the acquisition of and production of high energy nitrogen donors such as glutamine. These two targets are discussed below.

rpoH encodes the alternative sigma factor (Sigma 32) in the gonococcus that is responsible for general stress response. General stress response in the gonococcus, relative to RpoH, has been previously defined as growth at elevated temperatures and cellular contact (69, 70, 96). However, in addition to stress response, *rpoH* appears to be an essential gene as it is required for growth even under normal conditions (69, 96). During growth at elevated temperatures, or other stress conditions that induce the heat shock response, RpoH positively regulates the expression of a variety of heat shock proteins (HSPs) including ClpB, GrpE, DnaK, and GroES as well as a MarR homolog (96). The increase in HSP production is thought to be due to the increase in RpoH production at the level of translation, and the increased stability of RpoH which is freed from the mitigating effects of DnaK by the accumulation of misfolded proteins during growth at elevated temperatures, for which DnaK has a greater affinity (84, 178). As misfolded proteins are removed from the cell, DnaK once more begins to destabilize RpoH resulting in a reduction of the heat shock response and providing an autonomous self regulating response circuit (84). However, constitutive expression of RpoH can also result in a robust heat shock response in the absence of DnaK (178, 250). In addition to this DnaK-mediated control of RpoH expression, the majority of gamma proteobacteria also mediate *rpoH* translation by way of a thermosensor in the mRNA (175). Lastly,

minor regulation of *rpoH* expression can be further modulated by different promoters that exist in the *rpoH* upstream region (75). The expression of *rpoH* in *N. gonorrhoeae* is mediated slightly different from that of the gamma proteobacteria as a 172 bp leader sequence exists in its transcript that mediates a mitigation in RpoH production (144).

glnA encodes the glutamine synthetase gene of *N. gonorrhoeae*. Glutamine synthetase catalyzes the reaction NH3+glutamate+ATP → glutamine+ADP+Pi and consists of 12 identical subunits. These 12 subunits are adenylated or deadenylated by the adenylyltransferase encoded by *glnE* (242). When a *glnA* subunit is adenylated it becomes inactive and further sensitizes the enzyme to feedback inhibition (233-235) (243, 244). The adenylyation and deadnylylation ability of GlnE is independent of the other regulatory proteins in the glutamine synthesis cascade and are the functions of 2 different sites on the enzyme (210). In addition to glutamine being a vital high energy nitrogen donor in the cell, members of the glutamine synthesis pathway are also important in virulence and general cell health. Glutamine synthetase (*glnA*) mutants of Salmonella are less fit *in vivo* but not less virulent compared to wild type (137). Additionally, in *Mycobacterium tuberculosis* glutamine synthetase is involved in resistance to killing by macrophages and *glnE* is an essential gene (38, 174). Further, glutamine is abundant in the blood but is limiting on mucosal surfaces and in phagolysosomes so its acquisition and production may be important during infection.

As such, the following specific aims were addressed in this work:

1. To ascertain the MtrR regulon of *N. gonorrhoeae*.
2. To characterize MtrR's linking of oxidative and non-oxidative resistance mechanisms in the gonococcus via RpoH regulation.
3. To understand the ability of MtrR to regulate genes involved in glutamine biosynthesis.

VI. Major Conclusions

The goal of this research product was to determine the extent of the MtrR regulon and to characterize the involvement of MtrR in the biology of the gonococcus beyond its traditional scope of regulating the *mtrCDE* locus. Additional work identifying the regulons of MtrA and MpeR is presented in the Unpulished Results section. This work however, was not the focus of this project and as such only the major conclusions concerning MtrR and its regulation of genes in the gonococcus follows.

By far the most global in scope of the regulators examined during the microarray analysis was MtrR, which repressed 47 genes at the mid-log phase of growth and activated 22 other genes. During the late-log phase of growth, MtrR repressed 24 genes while activating 23 genes. Since MtrR was found to regulate such a wide range of genes this work was focused on two particular candidate systems, oxidative stress and metabolism, as they were significantly divergent from the classically defined MtrR regulon and are of central importance to the gonococcus's biology.

RpoH is an alternative sigma factor (Sigma 32) which has traditionally been categorized as a crucial part of the heat shock response and was one of the genes identified in the microarray as being repressed by MtrR during the mid-log phase of growth. This regulation was confirmed by translational *lacZ* fusions as well as by direct DNA binding assays which established a MtrR binding site in the promoter region of RpoH. Further work found that the MtrR indirectly regulates the RpoH regulated gene *grpE*. *grpE* is a protein chaperone that RpoH up-regulates during heat shock, or other inducing conditions of stress response. Further examination of these stress conditions were done by looking at response to exposure to hydrogen peroxide, a potent component of the oxidative response in neutrophils, as a number of members of the RpoH regulon that were found to be regulated by MtrR as well are part of the response to hydrogen peroxide in *E. coli*. It was found that when exposed to hydrogen peroxide, both RpoH and GrpE expression were increased and overepression of MtrR could reduce RpoH expression while increasing sensitivity to hydrogen peroxide.

Among the genes identified in the MtrR microarray data were a number of genes involved in glutamine biosynthesis (*glnA* and *glnE*) and glutamine transport (*glnM* and *glnQ*). As glutamine serves as a vital high energy nitrogen donor we set out to characterize MtrR's regulation of the genes involved in glutamine biosynthesis that had been identified in the microarray data. Translational *lacZ* fusions, in addition to EMSA and DNAse1 footprint analysis, confirmed that MtrR directly represses the expression of *glnA*. However, a second level of control appears to exist at this locus that involves MtrR indirectly regulating *glnA* expression through the transcriptional regulator FarR. Through the same set of assays it was determined that FarR directly activates expression of *glnA*.

Additionally, the loss of *glnA* by insertion of a non-polar kanamycin cassette results in an altered growth profile characterized by a long lag phase and lower overall density but a similar to wild type growth rate during exponential phase. Further, disruption of the *glnA* locus results in altered susceptibility to selected antimicrobial agents recognized by the MtrC-MtrD-MtrE efflux pump system. Additionally, MtrR activates expression of the *glnE* locus as seen during the microarray. To determine if this activation was mediated directly, direct DNA binding experiments with MtrR were conducted on the upstream region of *glnE*. It was found that MtrR specifically binds the region upstream of *glnE* indicating that this regulation is indeed direct in nature and not mediated through MtrR's affects on a second transcriptional regulator.

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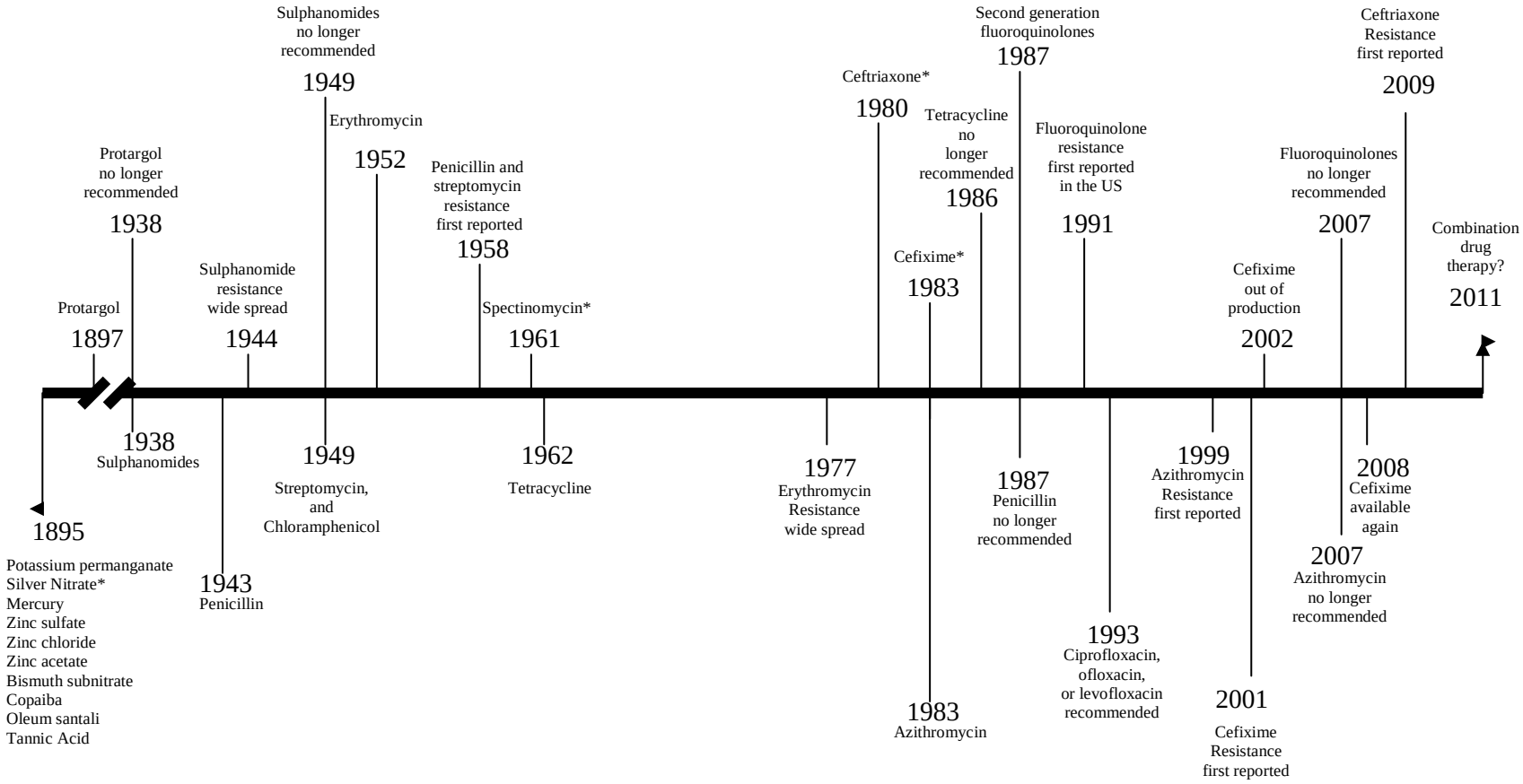
Figure 1. Antimicrobial Usage Timeline for *Neisseria gonorrhoeae.*

Timeline indicating the discovery/introduction of antimicrobial agents used to treat gonorrhea and the subsequent emergence of resistance and loss of effective use of these agents.



*Antimicrobial agent still recommended and/or in front line use

Figure 2: Major Efflux Pumps of *Neisseria gonorrhoeae.*

The major efflux pumps possessed by Neisseria gonorrhoeae include NorM (MATE family), MacAB (ABC Superfamily), FarAB and mef (Major Facilitator Superfamily), and MtrCDE (RND family). These efflux pumps recognize and export a number of diverse antimicrobial agents including quaternary compounds (NorM), macrolides (MacAB and mef), long chain fatty acids (FarAB), and hydrophobic agents (MtrCDE).

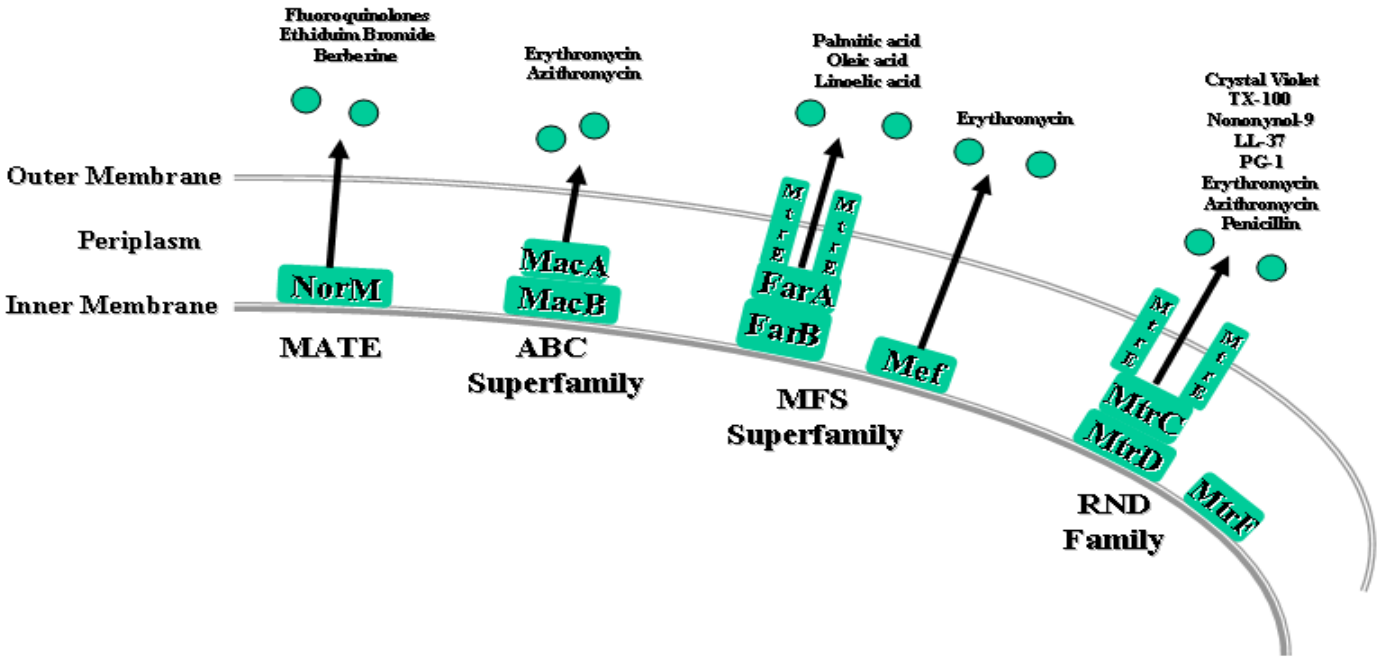
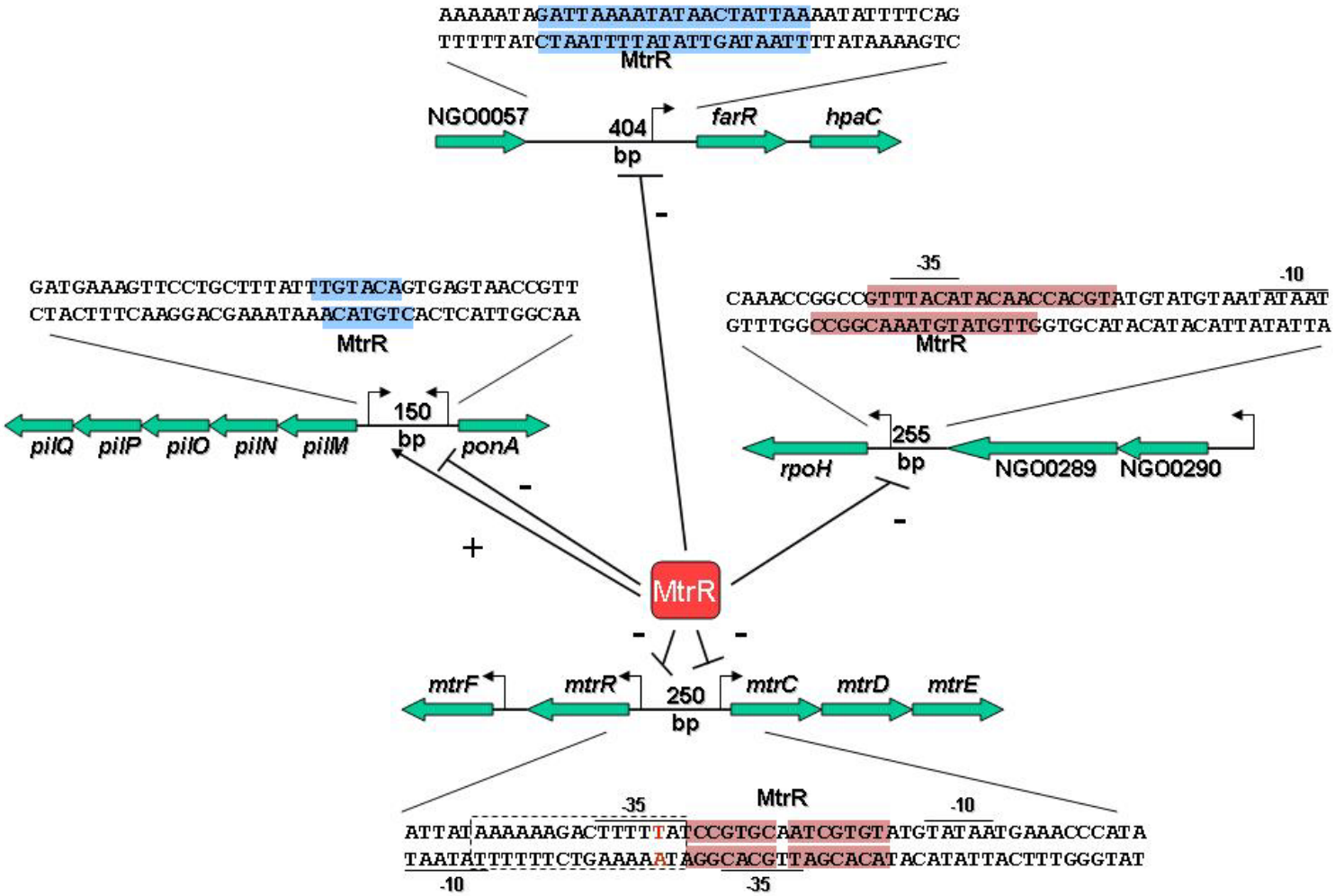

Fluoroquinolones
Ethiduim Bromide
Berberine
Erythromycin
Azithromycin
Palmitic acid
Oleic acid
Linoelic acid
Erythromycin
Crystal Violet
TX-100
Nonanynol-9
LL-37
PG-1
Erythromycin
Azithromycin
Penicillin
Outer Membrane
Periplasm
Inner Membrane
NorM
MATE
MacA
MacB
ABC
Superfamily
MtrE
FarA
FarB
MFS
Superfamily
Mef
MtrC
MtrD
MtrF
RND
Family

Figure 3: MtrR Binding and Regulation of Directly Controlled Genes.

The binding sites of MtrR and activation or repression of genes previously identified as being regulated by MtrR are indicated. MtrR binding sites identified by DNAse1 protection assays are identified in red while areas defined as MtrR binding sites by other means (mutational analysis of binding site and EMSA or competitive EMSA plus bioinformatics examination) are indicated in blue. Regulation of these genes is indicated by an arrow and a + sign for activated genes while repressed genes are identified by a blocked line and a – sign.


AAAAATAGATTAAAATATAACTATTAAAATATTTTCAG
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MtrR
NGO0057
404
bp
farR
hpaC
-
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CTACTTTCAAGGACGAAATAAACATGTCACTCATTGGCAA
MtrR
150
bp
pilQ
pilP
pilO
pilN
pilM
ponA
-
+
-35
-10
CAAACCGGCCGTTTACATACAACCACGTATGTATGTAATATAAT
GTTTGGCCGGCAAATGTATGTTGGTGCATACATACATTATATTA
MtrR
255
bp
rpoH
NGO0289
NGO0290
-
MtrR
- -
mtrF
mtrR
250
bp
mtrC
mtrD
mtrE
MtrR
-35
-10
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Chapter 2: MtrR Modulates *rpoH* Expression and Levels of Antimicrobial Resistance in *Neisseria gonorrhoeae*

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Published in
Journal of Bacteriology, 2009
Vol. 191, No. 1
p.287-297

This manuscript was written by first authors, Jason P. Folster and Paul J. T. Johnson with editorial changes made by William M. Shafer.

The MtrR transcriptional-regulatory protein is known to repress transcription of the *mtrCDE* operon, which encodes a multidrug efflux pump possessed by *Neisseria gonorrhoeae* that is important in the ability of gonococci to resist certain hydrophobic antibiotics, detergents, dyes, and host-derived antimicrobials. In order to determine whether MtrR can exert regulatory action on other gonococcal genes, we performed a whole-genome microarray analysis using total RNA extracted from actively growing broth cultures of isogenic MtrR-positive and MtrR-negative gonococci. We determined that, at a minimum, 69 genes are directly or indirectly subject to MtrR control, with 47 being MtrR repressed and 22 being MtrR activated. *rpoH*, which encodes the general stress response sigma factor RpoH (sigma 32), was found by DNA-binding studies to be directly repressed by MtrR, as it was found to bind to a DNA sequence upstream of *rpoH* that included sites within the *rpoH* promoter. MtrR also repressed the expression of certain RpoH-regulated genes, but this regulation was likely indirect and a reflection of MtrR control of *rpoH* expression. Inducible expression of MtrR was found to repress *rpoH* expression and to increase gonococcal susceptibility to hydrogen peroxide (H2O2) and an antibiotic (erythromycin) recognized by the MtrC-MtrD-MtrE efflux pump system. We propose that, apart from its ability to control the expression of the *mtrCDE*-encoded efflux pump operon and, as a consequence, levels of gonococcal resistance to host antimicrobials (e.g., antimicrobial peptides) recognized by the efflux pump, the ability of MtrR to regulate the expression levels of *rpoH* and RpoH-regulated genes also modulates levels of gonococcal susceptibility to H2O2.

Neisseria gonorrhoeae is a gram-negative strict human pathogen and the causative agent of the sexually transmitted disease gonorrhea. Worldwide, over 60 million cases of gonorrhea occur each year (7, 33). Although the incidence of disease in the United States has decreased since the 1970s, there has since been an increase in antibiotic-resistant strains reported in the United States and elsewhere (25, 33). Indeed, the prevalence of gonococci resistant to penicillin, tetracycline, and/or fluoroquinolones became so significant over the past 25 years that in 2007 the Centers for Disease Control and Prevention added *N. gonorrhoeae* to the list of "superbugs".

The capacity of gonococci and other bacteria to employ drug efflux pumps to recognize and export antibiotics has attracted considerable attention, as these pumps can impact the efficacy of antibiotic therapy of infections (18, 19, 25, 30, 34, 35). Moreover, since certain efflux pumps recognize host-derived antimicrobial agents, such as antimicrobial peptides (24), there is increasing suspicion that they provide bacteria with a mechanism to escape innate host defenses (13, 21). The gonococcal MtrC-MtrD-MtrE efflux pump can export a variety of antibiotics (17, 28), including penicillin (30) and macrolides (35), and antimicrobial peptides, such as human LL-37 (24).

The *mtrCDE* operon is negatively regulated by the MtrR repressor (10, 16, 23), which is encoded by a gene (*mtrR*) immediately upstream of, but transcriptionally divergent from, *mtrCDE* (19). Two homodimers of MtrR bind to a DNA sequence within the *mtrCDE* promoter (12). Point mutations in the MtrR-binding site (16), a single-base-pair deletion within a 13-bp inverted-repeat sequence in the *mtrR* promoter (10), or

missense mutations that cause radical amino acid replacements within the helix-turn-helix motif of MtrR all can result in derepression of *mtrCDE* expression (23). Derepression of *mtrCDE* results in decreased antimicrobial susceptibility of gonococci (10, 11, 23) and increased fitness of gonococci in an experimental murine lower genital tract infection model (31).

Consequently, it is important to define the regulatory properties of MtrR. DNA-binding proteins such as MtrR that negatively regulate bacterial efflux pump genes have been considered to be "local" gene regulators, although there is increasing evidence that they can directly or indirectly influence the expression of other genes (6, 15). In order to define the genes in gonococci regulated by MtrR, we employed a microarray analysis of total RNA extracted from mid-logarithmic-phase broth cultures of isogenic MtrR-positive and MtrR-negative gonococci. Through this analysis, we found that MtrR can directly or indirectly regulate 69 genes, which are distributed throughout the genome. We identified 47 MtrR-repressed genes (including the previously described *mtrCDE* operon) and 22 MtrR-activated genes. An MtrR-repressed gene of particular interest, which was the subject of this investigation, was *rpoH*. *rpoH* encodes the alternative stress response sigma factor RpoH (sigma 32), which appears to be essential for gonococcal viability even under normal growth conditions (3, 8). Earlier studies showed that *rpoH* expression is increased during exposure of gonococci to certain stress conditions, such as growth at elevated temperatures (8), and during bacterial contact with cultured epithelial cells (2, 3). Moreover, certain RpoH-regulated genes are differentially expressed during exposure of gonococci to H_2O_2 (29). Thus, apart from its regulation of *mtrCDE* and levels of

gonococcal resistance to hydrophobic antimicrobial agents (20, 21, 24, 30), MtrR regulation of *rpoH* expression may have significance for gonococcal survival in vivo, particularly at sites rich in neutrophils producing large quantities of H_2O_2.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) or strain DH5α mcr was used in all cloning experiments. *E. coli* strains were grown in Luria-Bertani broth or on Luria-Bertani agar plates at 37°C. *N. gonorrhoeae* strain FA19 was used as the primary gonococcal strain (22). *N. gonorrhoeae* strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, MI) containing glucose and iron supplements (22) at 37°C under 3.8% (vol/vol) CO_2 or in GCB broth with supplements and sodium bicarbonate as described previously (22). The plasmids and oligonucleotide primers used in this investigation are listed in Tables 1 and 2. All chemicals were purchased from Sigma Biochemical (St. Louis, MO).

RNA isolation. RNA was isolated from 50-ml GCB broth cultures of strains FA19 and JF1 (the same as FA19 but with *mtrR* deleted [Δ*mtrR*] [5]) grown to mid-log phase using a hot-phenol method as previously described (4). Samples were then DNase treated on column (Qiagen DNase kit), and the RNA was recovered using the Qiagen RNeasy Mini kit. RNA was quantified by Nano-

Drop1000 (NanoDrop Technolgies), and the RNA integrity was analyzed on an Agilent bioanalyzer (Agilent Technologies). The RNA samples were stored at 80°C until further use.

Microarray design. A custom multipathogen Affymetrix GeneChip, MPAUT1a520274F, was utilized for the microarray experiments in the present study. Probe pairs were designed for each gene, consisting of 11 probe pairs (22 features) with a perfect match and a single-nucleotide mismatch. The *N. gonorrhoeae* genome probe set (1,925 genes) was represented on the array.

cDNA labeling and hybridization. Sample preparation and hybridization were done at the University of Iowa DNA Facility (http://dna-9.int-med.uiowa.edu/) according to the Affymetrix Genechip Expression Analysis technical manual prokaryotic protocol. Briefly, biotinylated cDNA was generated from 10 µg of total RNA and fragmented prior to hybridization to the custom chip at 45°C for 16 h. The arrays were stained using streptavidin-phycoerythrin (ProkGEWS2v3_450 protocol; Affymetrix, Inc.), and the scanned images (Affymetrix GeneChip Scanner 3000) were processed using Genechip Operating Software version 1.4 (Affymetix, Inc.). Hybridizations were done on three separate biological replicates of each isolate.

Array analysis. Data files generated by Genechip Operating Software (Affymetix, Inc.) were imported into GeneSpring GX 7.3.1 (Agilent Technologies) and normalized per chip by dividing each measurement by the 50th percentile of all the measurements in

that sample and per gene by dividing each gene by the median of its measurements across all samples. The cross-gene error model was operating based on replicates. The normalized data from all samples were filtered on genes flagged as present or marginal, with the resulting gene list used for further gene expression analysis and clustering. Change was expressed as the ratio of gene expression of the wild-type FA19 over the same gene expression for the mutant. Genes with a differential expression of ≥1.5-fold and a *P* value of 0.05 were selected.

Construction of *mtrR*-complemented strains, inducible expression of *mtrR*, detection of MtrR, and antimicrobial susceptibility in induced cultures. The construction of pJF1, which contains the wild-type *mtrR* coding sequence and promoter sequence from strain FA19 cloned into pGCC3 (27), and strain JF6 (like JF1 but with this *mtrR* sequence, including its promoter, located between *aspC* and *lctP*) has been described by Folster and Shafer (5). In order to engineer an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible copy of *mtrR*, the *mtrR* coding sequence with its ribosome binding site was PCR amplified from chromosomal DNA prepared from wild-type strain FA19 using primers 5'*mtrR* and 3'*mtrR* (Table 2). The resulting DNA fragment was inserted into the PmeI and PacI sites of pGCC4, which contains the *lacZ* promoter (27) (kindly provided by A. Jerse and H. Seifert) to produce pJF2, and the correct orientation and nucleotide sequence were confirmed by DNA sequencing. pJF2 was digested with ClaI, and the fragment containing the gonococcal *lctP* gene, *mtrR*, *ermC* (an erythromycin [Ery] resistance cassette), and *aspC* was purified and used to

transform gonococcal strain FA19 Δ*mtrR*. Transformations were performed as previously described (9). Transformants were selected on GCB agar containing 1 μg/ml of Ery, and the resulting strain was named JF7 (like JF1, but *mtrR*+). For inducible expression of *mtrR*, strain JF7 was grown on GCB agar as described above. The overnight growth was then used to inoculate 50 ml of GCB broth, and the culture was grown at 37°C with shaking (200 rpm) until the optical density at 600 nm reached 0.2, at which time it was split into two equal samples. One sample received IPTG (1 mM), while the other received an equal volume of sterile distilled water. These cultures were grown for 1 h, and samples were then harvested by centrifugation. MtrR was detected in solubilized cell extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting as described previously (6). MtrR was detected using a rabbit anti-MtrR antiserum (1:5,000 dilution) prepared by Protein Tech Group, Inc. (Chicago, IL), with purified MtrR fused to maltose-binding protein (MBP) (15) as the antigen and was visualized on the blot using goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (1:5,000 dilution). IPTG-induced and control samples were also evaluated for their susceptibility to Ery (0.5 μg/ml) by an efficiency-of-plating assay described previously (22), using agar plates with or without 1 mM IPTG. H2O2 susceptibility was determined using a disk diffusion assay containing filter disks (1 cm) presoaked in 3% (vol/vol) H2O2 that were placed on freshly inoculated GCB agar plates that contained or lacked 1 mM IPTG.

Construction and analysis of *rpoH-lacZ*, *grpE-lacZ*, and *mtrR-lacZ* fusions. Translational *lacZ* fusions were constructed as previously described (5, 6). In brief, the promoter sequence of *rpoH* was amplified by PCR from strain FA19 using primers 5'PrpoH and 3'PrpoHB (Table 2). The resulting DNA fragment was inserted into the BamHI site of pLES94 (26), and the recombinant plasmid was introduced into *E. coli* DH5α mcr by transformation. The correct orientation of the insertion was confirmed by PCR analysis and DNA sequencing. The plasmid was used to transform strains FA19, JF1, and JF6 to allow insertion into the chromosomal *proAB* gene, and transformants were selected on GCB agar containing 1 μg/ml of chloramphenicol. Representative transformants obtained with the three recipient strains were termed JF8, JF9, and JF10, respectively (Table 1). Strains bearing a *grpE-lacZ* fusion were prepared essentially as described above, but the oligonucleotide primers 5'PgrpE and 3'PgrpE (Table 2) were used to PCR amplify a 300-bp fragment encompassing the upstream sequence of *grpE* and the first two codons. The strains bearing the *grpE-lacZ* fusion were termed JF11 (FA19 *grpE-lacZ*), JF12 (FA19 Δ*mtrR grpE-lacZ*), and JF13 (FA19 Δ*mtrR mtrR*+ *grpE-lacZ*), respectively (Table 1). For construction of an *mtrR-lacZ* fusion in strain FA19, oligonucleotide primers mtrC1 and pmtrR (Table 2) were used to PCR amplify *mtrR*; the strain bearing the *mtrR-lacZ* fusion was termed JF16 (FA19 *mtrR-lacZ*). β-Galactosidase (β-Gal) assays were conducted as previously described (5, 6).

Deletion of the *rpoH* extended leader sequence. PCR mutagenesis was used to delete the 172-bp *rpoH* leader sequence and was performed using the overlapping

primers 5'ΔPrpoH and 3'ΔPrpoH (Table 2). First, two fragments were amplified by PCR from FA19 chromosomal DNA using the primer set pairs 5'PrpoH/3'ΔPrpoH and 3'PrpoHB/5'ΔPrpoH. The resultant fragments were purified after agarose gel electrophoresis using the Qiaquick purification kit (Qiagen Inc., Valencia, CA), and they then served as both primers and templates for a second PCR. After eight reaction cycles, primers 5_PrpoH and 3_PrpoHB were added to the PCR mixture, and amplification continued for an additional 25 cycles. The resulting DNA fragment containing the deletion was purified and served as the template for the last PCR using primers 5'PrpoH and 3'PrpoHB. The resulting DNA fragment was inserted into the BamHI site of pLES94, resulting in the *rpoH*Δ-*lacZ* construct. The recombinant plasmid was introduced into DH5α mcr by transformation. Correct insertion and orientation were confirmed by PCR analysis, and DNA-sequencing analysis confirmed the mutation of the MtrR-binding site (data not presented). The plasmid was used to transform strains FA19 and JF1 to allow insertion into the chromosomal *proAB* gene. Transformants were selected on GCB agar containing 1 μg/ml of chloramphenicol; representative transformants obtained with recipient strains FA19 and JF1 were termed JF14 and JF15, respectively (Table 1).

EMSA and DNase I protection. Electrophoretic mobility shift assays (EMSAs) using purified MBP fused to MtrR were performed as previously described (5, 6, 15). In brief, the 184-bp promoter region of *rpoH* lacking the leader sequence was amplified by PCR using 5'PrpoH and 3'PrpoHB from strain JF14 (Table 1). The 184-bp nonspecific probe was PCR amplified from FA19 chromosomal DNA using primers 5'ΔPrpoH and 3_PrpoHB (Table 2). The resulting PCR products were end labeled with [γ-32P]dATP

using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled DNA fragments (10 ng) were incubated with purified MtrR in 30 µl of reaction buffer [10 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% (vol/vol) glycerol, 1 mM MgCl2, 50 mM NaCl, poly(dI-dC) (0.05 µg/ml), salmon sperm (200 ng/ml)] at 4°C for 20 min. Samples were subjected to electrophoresis on a 6% (wt/vol) polyacrylamide gel at 4°C, followed by autoradiography. DNase I protection assays were performed as previously described (16). Target DNA sequences for DNase I footprints were generated by PCR using the oligonucleotide primers 5'PrpoH and 3'PrpoHB (Table 2) to generate a 184-bp promoter region of *rpoH* lacking the leader sequence from strain JF14. Target DNA was labeled at the 5' end of one strand as described for EMSA. Purified MBP-MtrR protein was allowed to bind in the binding buffer as in the EMSA before CaCl2 and MgCl2 were added to final concentrations of 2.5 mM and 5 mM, respectively. Five units of DNase I (Promega, Madison, WI) were then added to the reaction mixture, which was incubated at room temperature for 1 min. Digestion was stopped by the addition of NaCl to 250 mM, and the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) before precipitation with 100% ethanol for 30 min at -20°C. The pellet was washed four times with 70% (vol/vol) ethanol, vacuum dried, and resuspended in gel loading buffer, which consisted of 0.1 M NaOH-formamide (1:2 [vol/vol]), 0.1% (vol/vol) xylene cyanol, and 0.1% (vol/vol) bromophenol blue. Regions protected from DNase I digestion were resolved on a 6% denaturing polyacrylamide gel, which was dried and exposed to X-ray film for autoradiography.

Microarray data expression number. Gene expression data for all microarray experiments can be retrieved from the Gene Expression Omnibus (GEO) database at NCBI (http://www.ncbi.nih.gov/geo/) (accession number GSE12686).

RESULTS AND DISCUSSION

Identification of MtrR-regulated genes in gonococci. To identify MtrR-regulated genes in *N. gonorrhoeae*, a microarray analysis was performed that permitted a comparison of gonococcal transcript levels in total RNA prepared from mid-logarithmic-phase cultures of wild-type strain FA19 and its isogenic *mtrR* deletion mutant, strain JF1 (Table 1). Using an expression difference of 1.5-fold ($P \leq 0.05$), analysis of the microarray results allowed us to establish an MtrR regulon that at a minimum consists of 47 MtrR-repressed and 22 MtrR-activated genes (Table 3); these MtrR-regulated genes were distributed throughout the genome (Fig. 1). Four previously identified MtrR-repressed genes (*mtrC*, *mtrD*, *mtrE*, and *mtrF*) were confirmed as being MtrR repressed by the array, which validated the array as a means to identify MtrR-regulated genes. Based on the annotation of the FA1090 genome sequence (GenBank accession no. AE004969), the majority of MtrR-regulated genes were predicted to encode hypothetical proteins, but several genes encoding proteins with known or putative functions were identified (Table 3). In particular, we noted that expression of *rpoH*, which encodes the stress-related sigma factor RpoH, and some RpoH-regulated genes (*grpE*, *clpB*, and *marR* [8, 29]) were increased in strain JF1 relative to the MtrR-positive parental strain FA19, suggesting that they are MtrR repressed. Since RpoH expression in gonococci is increased during

exposure to certain environmental stresses (8) when gonococci bind to cervical epithelial cells (2, 3) and is essential for viability (3, 8), we studied the ability of MtrR to regulate *rpoH* expression in *N. gonorrhoeae*.

Expression of *rpoH* and certain RpoH-regulated genes is repressed by MtrR. To study the capacity of MtrR to regulate the expression of *rpoH*, a translational *lacZ* reporter fusion system was engineered. This translational fusion consisted of 365 bp of DNA sequence upstream of *rpoH*, which included the promoter region (14), the first two codons of *rpoH* (Fig. 2), and a promoterless *lacZ* gene. This construct was transformed into isogenic strains FA19 and JF1 to create the transformant strains JF8 (FA19 *rpoH-lacZ*) and JF9 (FA19 Δ*mtrR rpoHlacZ*), respectively, resulting in a single copy of the *rpoH* promoter fused translationally to *lacZ* within the *proAB* chromosomal locus. β-Gal activity in cell lysates from these strains indicated that expression of *rpoH* increased more than eightfold in the MtrR-negative strain JF9 compared to the MtrR positive strain JF8 (FA19 *rpoH-lacZ*) (Fig. 3A). To confirm that these results were due to deletion of *mtrR* and not a polar effect on other genes, strain JF9 was complemented with the wild-type *mtrR* gene from strain FA19, which was inserted at a secondary site within the gonococcal chromosome (between the *lctP* and *aspC* genes) to create strain JF10 (FA19 Δ*mtrR mtrR+ rpoH-lacZ*) (Table 1). Using this strain, we found that complementation of the *mtrR* deletion in JF9 (FA19 Δ*mtrR rpoH-lacZ*) with the wild-type *mtrR* gene restored *rpoH* repression to a level similar to that of JF8 (like FA19, but *rpoH-lacZ*) (Fig. 3A).

The sequence of the gonococcal *rpoH* promoter, as well as the transcriptional start point and ribosome binding site, were defined previously (8) (Fig. 2). This region contains an extended 172-bp RNA leader sequence, which was previously observed to be involved in *rpoH* repression during growth under normal conditions (14). To determine if the leader sequence was involved in MtrR repression of *rpoH*, an internal deletion was engineered within the *rpoH-lacZ* construct and transformed into strains FA19 and JF1 (FA19 Δ*mtrR*) to create strains JF14 (FA19 *rpoH-lacZ*) and JF15 (FA19 Δ*mtrR rpoHlacZ*) (Table 1), respectively, and expression was measured. We found that *rpoH-lacZ* expression from the wild-type promoter and the promoter with the leader deleted did not differ in either the MtrR-positive (JF14) or MtrR-negative (JF15) background (Fig. 3B), indicating that MtrR-mediated repression of *rpoH* is independent of the leader sequence.

Gunesekere et al. (8) identified a panel of RpoH-regulated genes in gonococci, and Stohl et al. (29) showed that some of these were upregulated during bacterial exposure to H2O2. Since our microarray analysis (Table 3) suggested that a subset of these RpoH-regulated genes (*clpB, grpE*, and *marR*) is also subject to MtrR regulation, we examined the ability of MtrR to control the expression of RpoH-regulated genes in gonococci and used *grpE* for this purpose. We examined its expression in isogenic MtrR-positive and MtrR-negative gonococci utilizing a *grpE-lacZ* construct introduced into strains FA19, JF1 (FA19Δ*mtrR*), and JF6 (FA19 Δ*mtrR mtrR*+); the transformed strains were termed JF11, JF12, and JF13, respectively (Table 1). The results indicated that expression of *grpE* (Fig. 4) was elevated in the absence of MtrR (see strain JF12) and

restored to near-wild-type levels (strain JF11) by complementation with the intact *mtrR* gene (strain JF13).

As expression of RpoH-regulated *grpE* is induced during exposure of gonococci to low levels of H2O2 (29), we next evaluated whether expression of *mtrR*, *rpoH*, and *grpE* was altered when gonococci were exposed to the oxidizing agent. Using the *lacZ* fusion strains JF8 (FA19 *rpoH-lacZ*), JF11 (FA19 *grpE-lacZ*), and JF16 (FA19 *mtrR-lacZ*) (Table 1), we monitored the expression of *rpoH*, *grpE*, and *mtrR* during a brief (15-min) exposure of gonococci to 1.5 mM H2O2; this concentration of H2O2 did not reduce viability (data not presented). As is shown in Fig. 5, we found that under these conditions, expression of both *rpoH* and *grpE* was increased significantly ($P < 0.0001$ and 0.002, respectively), while *mtrR* expression was slightly but significantly ($P = 0.049$) dampened. In order to determine if H2O2 induction of *rpoH* expression would be enhanced in the absence of MtrR, we next compared its expression when strains JF8 (FA19 *rpoH-lacZ*) and JF9 (like JF8, but Δ*mtrR*) were exposed to 1.0 mM H2O2 for 15 min. Although *rpoH* expression was nearly 15-fold greater in strain JF9 than in JF8, the changes in expression after H2O2 exposure were nearly identical (data not presented). We propose that although MtrR can control constitutive levels of *rpoH* and *grpE* exposure, it is not necessarily involved in their H_2O_2-inducible expression. Alternatively, under the experimental conditions employed, *rpoH* expression in JF9 reached maximal levels, and induction above the observed level was not possible or detectable.

MtrR directly binds to the *rpoH* promoter region. Initial EMSA experiments showed that MtrR bound to the DNA sequence upstream of *rpoH* encompassing its promoter

region in a specific, concentration-dependent manner and did not require the 172-bp leader sequence (data not presented). In order to determine if MtrR directly or indirectly regulates the RpoH-regulated *grpE* gene, an EMSA was performed using MtrR and a 305-bp probe containing the upstream DNA (including a putative promoter element) of the *grpE* coding sequence. Unlike its binding to the DNA sequence upstream of *mtrC* and *rpoH*, MtrR failed to bind the *grpE* target DNA sequence (data not presented). Accordingly, we believe that the ability of MtrR to regulate *grpE* is indirect and due to its capacity to repress *rpoH*.

In order to identify the MtrR-binding site within the *rpoH* promoter region, a DNase I protection assay was performed. The *rpoH* promoter probe described above was radiolabeled separately at its 5' and 3' ends and utilized in DNase I protection assays that employed increasing amounts (0 to 20 μg) of MtrR (Fig. 6). With increasing amounts of MtrR, an area of protection of seventeen nucleotides on the sense strand (Fig.2) could be identified as being involved in binding MtrR. A second area of protection of sixteen nucleotides was observed on the antisense strand (Fig. 2), which overlapped the area observed on the sense strand. The region of protection indicates that MtrR binds to the *rpoH* promoter at an area that overlaps the -35 hexamer sequence of the *rpoH* promoter. Using the nucleotide sequence of the MtrR-binding site on the noncoding strand within the *mtrCDE* promoter (5'-GGCACGTTAGCACATA-3') defined by Hoffman et al. (12), we were able to identify a region of similarity (73.3% identity) (Fig. 2) within the *rpoH* promoter region, which was also found by DNase I protection to bind MtrR.

Modulation of MtrR expression and levels of antimicrobial resistance. In order to further connect MtrR expression with levels of *rpoH* expression and to assess the biologic consequence of MtrR gene control in gonococci, we next inserted an IPTG-inducible copy of the wild-type *mtrR* gene from strain FA19 into its transformant strain JF9 (FA19 Δ*mtrR rpoH-lacZ*) to create strain JF7 (FA19 Δ*mtrR mtrR+ rpoH-lacZ*). Strain JF7 was grown in GCB broth to mid-log phase and split into two samples, one receiving 1 mM IPTG and one left untreated. After 1 h of induction, MtrR was clearly produced by the IPTG-treated culture (Fig. 7B), and *rpoH* expression was decreased by nearly twofold (Fig. 7C) compared to the untreated sample. In order to determine whether induction of *mtrR* expression resulted in biological changes of gonococci, we next examined the susceptibility of the IPTG treated and control cultures to Ery and H2O2. Ery susceptibility analysis was performed because MtrR repression of the *mtrCDE*-encoded efflux pump increases gonococcal susceptibility to this antibiotic and other hydrophobic antimicrobial agents (10, 11, 19). In support of this, we found that inducible expression of *mtrR* resulted in increased susceptibility of gonococci to Ery (Fig. 7D). H_2O_2 assays were performed because loss of RpoH production was found to enhance the susceptibility of *Brucella melitensis* (1) to the oxidizing agent. Since we (unpublished observations) and others (3, 8, 14) have been unsuccessful in obtaining an *rpoH* null mutant, we hypothesized that under conditions where *rpoH* expression is dampened due to the repressive action of MtrR, gonococcal susceptibility to H_2O_2 would increase. Indeed, we found that inducible expression of *mtrR* increased gonococcal susceptibility to H_2O_2 (Fig. 7E).

We believe that the MtrR regulon defined in this study represents the minimum number of gonococcal genes under MtrR control, as other genes (*farR*, *ponA*, and *pilMNOPQ*) known (6, 15) to be controlled by MtrR were not identified as being regulated by ≥1.5-fold in the microarray data set. In this respect, the MtrR-repressed *farR* (15) and *pilM* (6) genes were measured as being repressed by 1.44- and 1.40-fold, respectively, in the microarray data set, while the MtrA-activated *ponA* gene was measured to be 1.24-fold activated. Other MtrR-controlled genes might be uncovered under different growth conditions or by exposure of gonococci to stress conditions or antimicrobial agents.

Although the microarray results indicated that expression of *rpoH* was modestly repressed by MtrR, the level of repression (1.58-fold) was only slightly less than those of the *mtrCDE* genes (1.7- to 2.2-fold). Since loss of MtrR repression of *mtrCDE* in strain JF1 (FA19 Δ*mtrR*) can have important consequences for certain gonococcal properties (levels of antimicrobial susceptibility and in vivo fitness [31]), we elected to use *rpoH* as a model gene outside of the *mtrCDE* locus to study the global regulatory properties of MtrR. The combined translational fusion data that examined *rpoH* expression in the presence or absence of MtrR, especially those involving the inducible production of MtrR (Fig. 7), the increased expression of three RpoH-activated genes (*clpB*, *marR*, and *grpE*) in the MtrR-deficient strain JF1, and the ability of MtrR to bind within the *rpoH* promoter (Fig. 6), led us to conclude that MtrR acts as a direct repressor of *rpoH*. As EMSA experiments failed to show MtrR binding to *grpE*, we also conclude that the ability of MtrR to control the expression of this RpoH-regulated gene is likely indirect and is most easily explained by the ability of MtrR to dampen *rpoH* expression.

What is the biological significance of MtrR regulation of *rpoH*? We considered the possibility that because *rpoH* expression can enhance bacterial resistance to killing by H_2O_2 (1) and because certain genes under RpoH control are upregulated in gonococci during bacterial exposure to H_2O_2 (29), MtrR expression in gonococci would impact the levels of such resistance. The results presented here support this hypothesis. Moreover, based on these results, we suggest that MtrR is a general regulator of levels of gonococcal susceptibility to antimicrobial agents of the innate host defense, particularly the nonoxidative and oxidative killing systems of neutrophils. Thus, by repressing *mtrCDE* expression, MtrR can enhance gonococcal susceptibility to LL-37, which is produced by neutrophils, epithelial cells, and certain organs (e.g., testis) (24) and is a mediator of nonoxidative killing of bacteria. As MtrR production also increases bacterial susceptibility to H_2O_2, its control of gene expression, along with other resistance systems, may be important in the ability of gonococci to survive oxidative killing by neutrophils. We do not yet know if the observed decrease in H_2O_2 susceptibility in *mtrR* mutants directly or indirectly involves RpoH, but the ability of MtrR to control *rpoH* expression suggests that this regulatory scheme is important in the general stress response. We also noted that *hsp33* expression is also subject to MtrR repression (2.24-fold) (Table 3). This is of interest because Hsp33 has been implicated in bacterial responses to heat and peroxide (32). Unlike, *grpE*, MtrR regulation of *hsp33* may be direct, as a potential MtrR binding site consisting of 31 bp is positioned from nucleotides -110 to -141 (data not presented); this putative binding site is 61.3% identical to the 31-bp region upstream of *mtrCDE* shown by Lucas et al. (16) to bind MtrR. Taking these data together, we propose that naturally occurring *mtrR* mutants (22, 23, 34, 35) may have an advantage during

infection, based on their increased resistance to LL-37 (24) and H_2O_2 (Fig. 7). This linkage of bacterial resistance to distinct mediators of innate host defense through MtrR may help to explain the recent observation of Warner et al. (31) that *mtrR* mutants have a competitive advantage over otherwise wild-type gonococci in a female mouse lower genital tract infection model. The potential roles of other transcriptional regulators similar to MtrR in modulating bacterial resistance to innate host defense mechanisms should be considered.

Against this background, it should be asked why *mtrR* mutants are not the dominant strain in the community. At first glance, it seems to be a disadvantage for gonococci to have maintained *mtrR*, as its presence would downregulate the expression of genes needed for resistance to antimicrobials. We propose that because MtrR can also transcriptionally activate certain genes (Table 3) involved in metabolism (e.g., *abpE*, *glnE*, and *rfbB*), the need to resist host-derived antimicrobials through constitutive MtrR-repressible systems may be essential only at those sites rich in these agents. For most strains and infections, the need to have expression of MtrR-activated genes is more critical and overrides any advantage afforded by *mtrR* mutations, particularly since the *mtrCDE* efflux operon can be transcriptionally induced by MtrA in the presence of sublethal levels of antimicrobials (20), even in the presence of a functional MtrR.

ACKNOWLEDGMENTS

We thank L. Pucko for help in manuscript preparation and submission, N. Kamal and Y. Zalucki for critical reading of the paper, M. Apicella for facilitating access to the microarray slides, and the staff at the University of Iowa DNA Facility for sample preparation and hybridizations.

This work was supported by NIH grants AI-21150 (W.M.S.), RR-015564 (J. Iandolo and D.W.D.), and RR016478 (F. Waxman and D.W.D.) and by a VA Merit Award to W.M.S. W.M.S. was supported by a Senior Research Career Scientist Award from the Department of Veterans Affairs Medical Research Service.

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Table 1. Gonococcal strains, plasmids used.

Strain	**Relevant Genotype**	**Source**
FA19	wild type	P. F. Sparling
JF1	as FA19 but Δ*mtrR*	ref. 5
JF6	as JF1 but *mtrR*$^{+\mathbf{A}}$	this study
JF7	as JF1 but *mtrR*$^{+\mathrm{B}}$	this study
JF8	as FA19 but *rpoH-lacZ*	this study
JF9	as JF1 but *rpoH-lacZ*	this study
JF10	as JF6 but *rpoH-lacZ*	this study
JF11	as FA19 but *grpE-lacZ*	this study
JF12	as JF1 but *grpE-lacZ*	this study
JF13	as JF6 but *grpE-lacZ*	this study
JF14	as FA19 but *rpoH1-lacZ*$^{\mathbf{C}}$	this study
JF15	as JF1 but *rpoH1-lacZ*$^{\mathbf{C}}$	this study
JF16	as FA19 but *mtrR-lacZ*	this study

Plasmid	**Remarks**	**Source**
pLES94	cloning vector containing promoterless *lacZ* for insertion of gonococcal genes between *proA proB* (26)	V. Clark
pGCC3	NICS vector (25) used for insertion of *mtrR* between *lctP* and *aspC*	A. Jerse
pGCC4	same as pGCC3 with IPTG-inducible *lacZ* promoter	A. Jerse
pJF1	as pGCC3 but *mtrR*$^{+}$	this study
pJF2	as pGCC4 but *mtrR*$^{+}$	this study

Table 2. Oligonucleotides used.

Oligonucleotide primer name	**Sequence (5' →3')**
5'PrpoH	GGGATCCGGCGAAACGCCCTATATGAA
3'PrpoHB	GGGATCCCGATTCATTTGGGCATTTCCTTT
5'ΔPrpoH	GGACAGGATGAGTTGTTTGG
3'ΔPrpoH	TATCGGCGGTTGTAAACCTGATAGCTCAATTC
5'mtrR	GGTTAATTAACCGCCCTCGTCAAACCGA
3' mtrR	GGGTTTAAACTTATTTCCGGCGCAGGCAG
mtrC1	CGGGATCCCGAGCCATTATTTATCCTATCTGTC
pmtrR	GGATCCTCTCATAATGGCGTTTT CGTTTCG
5'PgrpE	GGGGATCCACACGGTTTGGTGCAAAAAAC
3'PgrpE	GGGGATCCGGCTCATATCGTATCCCTCAAA

[A] *mtrR* from pJF1 is under the control of its own promoter in this strain.
[B] *mtrR* from pJF2 is under the control of the *lacZ* promoter in this strain.
[C] this fusion lacks the 172 bp *rpoH* leader sequence

Table 3. MtrR-regulated genes[A] in *Neisseria gonorrhoeae*

A. MtrR-repressed genes

Gene classification	Common name	Change (fold)	Functional
NGO0006	*leuS*	1.59	leucyl-tRNA synthetase
NGO0018	NGO0018	1.65	unknown
NGO0195	*potH*	1.56	putative ABC-type polyamine transporter
NGO288	***rpoH***	**1.58**	**alternative sigma factor**
NGO0302	NGO0302	1.98	unknown
NGO0308	*bioA*	1.59	putative aminotransferase
NGO0309	*bioD*	1.53	dithiobiotin synthetase
NGO0332	*rmlD*	1.76	putative reductase
NGO0658	*pdxH*	1.51	pyrodoxamine 5'-phosphate oxidase
NGO0756	NGO0756	1.53	unknown
NGO0795	*brfB*	1.98	bacterioferrritin B
NGO0846	*umpA*	1.54	putative prolipoprotein diacylglycerol transferase
NGO0863	NGO0863	1.71	unknown
NGO0891	NGO0891	1.92	unknown
NGO0916	*accB*	1.58	dihydrolipoamide acetyltransferase
NGO0927	NGO0927	1.51	unknown
NGO1002	NGO1002	1.54	unknown (putative phage-associated)
NGO1029	*fumC*	1.57	fumarate hydratase
NGO1046	***clpB***	**2.10**	**heat shock chaperone**
NGO1069	NGO1069	1.63	unknown
NGO1084	NGO1084	1.75	unknown
NGO1189	*hsp33*	2.24	heat shock chaperonin
NGO1244	**NGO1244**	**1.52**	**MarR-family regulator**
NGO1293	NGO1293	2.11	unknown
NGO1294	*lrp*	1.77	AsnC-family transcriptional regulator
NGO1314	NGO1314	1.8	putative protease
NGO1325	*gcvP*	2.35	glycine dehydrogenase
NGO1363	***mtrE***	**2.20**	**Mtr efflux pump protein component**
NGO1364	***mtrD***	**2.40**	**Mtr efflux pump protein component**
NGO1365	***mtrC***	**1.78**	**Mtr efflux pump protein component**

NGO1368	***mtrF***	**2.21**	**accessory protein of Mtr efflux pump**
NGO1418	*nqrF*	1.74	subunit F, NADH-quinone reductase
NGO1422	***grpE***	**1.83**	**heat shock protein chaperone**
NGO1494	*potF*	1.58	putative periplasmic, polyamine binding protein
NGO1520	NGO1520	1.55	unknown
NGO1600	*glnA*	1.63	glutamine synthetase
NGO1699	NGO1699	1.56	unknown
NGO1738	*nuoM*	1.52	NADH dehydrogenase subunit M
NGO1741	*nuoK*	1.80	NADH dehydrogenase kappa subunit
NGO1742	*nuoJ*	1.56	NADH dehydrogenase I chain J
NGO1749	*nuoC*	1.57	NADH dehydrogenase subunit C
NGO1751	*nuoA*	1.78	putative NADH dehydrogenase I chain A
NGO1765	*pglA*	1.63	glycosyltransferase
NGO1890	*gltS*	1.66	putative glutamate permease
NGO2011	*glnM*	1.55	putative ABC-type polyamine transporter
NGO2013	*glnQ*	1.75	putative ATP-binding protein
NGO2014	*cjaA*	2.00	amino acid periplasmic binding protein

B. MtrR-activated genes

Gene classification	Common name	Change (fold)	Functional
NGO0113	*rng*	1.57	putative ribonuclease G/ cytoplasmic axial filament protein
NGO0150	NGO0150	1.61	unknown
NGO0290	NGO0290	1.57	unknown
NGO0481	NGO0481	1.85	unknown (putative phage-associated)
NGO0739	NGO0739	1.62	putative DNA helicase
NGO0876	NGO0876	1.83	unknown
NGO1024	NGO1024	1.57	unknown
NGO1059	NGO1059	1.77	unknown
NGO1062	NGO1062	1.71	unknown
NGO1097	NGO1097	1.70	unknown (phage-associated)
NGO1420	*abpE*	1.65	thiamine biosynthesis
NGO1460	NGO1460	1.72	unknown
NGO1488	NGO1488	1.53	unknown
NGO1511	NGO1511	1.67	unknown
NGO1722	*recQ*	1.67	recombination
NGO1728	NGO1728	1.60	unknown
NGO1758	*glnE*	7.04	glutamine synthetase adenylyltransferase
NGO1759	NGO1759	148.7	unknown
NGO1760	NGO1760	1.73	unknown
NGO1874	NGO1874	1.58	unknown
NGO1897	*rfbB*	1.73	dDTP-D-glucose 4,6 dehydratase
NGO2135	NGO2135	1.63	putative transglycosylase

[A] Genes of interest relative to this investigation are highlighted in bold.

Figure Legends

Figure 1. Chromosomal map position of MtrR-regulated genes. The position of MtrR-regulated genes that were identified by the microarray analysis (Table 2) is shown on the circular map of the gonococcal chromosome (strain FA1090). MtrR-activated genes are shown in red while MtrR-repressed genes are shown in green.

Figure 2. Nucleotide sequence upstream of *rpoH* and identification of the MtrR-binding site. The nucleotide sequence of the DNA upstream of *rpoH* is shown with the 172 bp leader sequence indicated by the dashed lines and the first two codons encodoing methionine (M) and asparagine (N) are also shown in bold. The position of the transcriptional start point (8) is shown, by the bolded A nucleotide downstream of the -10 sequence. The -10 and -35 sequences of the *rpoH* promoter identified earlier (8) is shown with the -10 and -35 hexamers and is highlighted by a single line under the sequence. The 4 bp ribosome binding site (RBS) sequence is shown by an underline. The MtrR-binding site identified by DNase I protection (Figure 5) is shown by the wide bars above the coding strand and below the non-coding strand. The boxed region shows a 15 nucleotide sequence on the coding strand (5'-**GGC(-) CGTT**TA**CA**TA**C**A-3') that is 73.3% identical (identical nucleotides are bolded in above sequence) to the MtrR-binding sequence (5'-GGCACGTTAGCACATA-3') upstream of *mtrCDE* on the noncoding strand (12); the (-) in the sequence above represents a single nucleotide gap in the *rpoH* sequence compared to the *mtrCDE* bound by MtrR.

Figure 3. MtrR regulation of *rpoH* expression. A. Shown is the level of β-gal activity (specific activity expressed as nanomoles of ONPG hydrolyzed per mg or protein) in strains JF8 (as FA19 but *rpoH-lacZ*), JF9 (as JF1 but *rpoH-lacZ*) and JF10 (as JF6 [Table 1] but *rpoH-lacZ*). Complementation of the *mtrR* deletion mutation resulted in reduced *rpoH* expression. The results are shown as average values (±SD) from three independent assays. The difference in β-gal activities between strains JF8 vs JF9 was significant ($p<0.001$) as was that that between JF9 vs JF10 ($p<0.001$). B. MtrR-repression of rpoH was independent of the 172 bp leader sequence upstream of *rpoH* as shown by similar levels of β-gal production between isogenic pairs (MtrR-positive strains JF8 and JF14 and MtrR-negative strains JF9 and. JF15) with (strains JF8 and JF9) or without (JF14 and JF15) the 172 bp leader sequence. The differences in β-gal activities between strains JF8 vs JF 14 and JF9 vs. JF15 were not statistically significant.

Figure 4. MtrR regulation of the RpoH-regulated *grpE* gene. Shown are the β-gal specific activities expressed in *grpE-lacZ* fusion strains JF11 (MtrR-positive), JF12 (MtrR-negative) and the *mtrR*$^+$ complemented strain JF13. The results are shown as average values (±SD) from three independent assays. The differences between strains JF11 vs. JF12 and JF 12 vs. JF13 were significant ($p<0.001$ and 0.003, respectively).

Figure 5. H_20_2 induction of *rpoH* expression. Shown in the graph are the β-gal specific activities in *rpoH-lacZ* fusion strain JF8 after a 15 minute exposure to 1 mM H_20_2. The results are shown as average values (±SD) from three independent assays; the

difference was significant ($p < 0.00001$). The insert shows the fold change in expression for *mtrR, rpoH* and *grpE* when fusion strains were exposed to H_20_2.

Figure 6. Identification of the MtrR-binding site within the *rpoH* promoter. The MtrR-binding site (see nucleotide sequence in Figure 2 and accompanying legend) was identified by DNase I protection using increasing amounts of MtrR (0, 5, 10 and 20 μg) in assays that employed sense and anti-sense probes. The site of protection on both strands is shown by the vertical bar adjacent to the protected region. The nucleotide sequencing reaction for each strand is shown adjacent to the lanes with the protection reactions.

Figure 7. Inducible production of MtrR represses *rpoH* expression and modulates antimicrobial susceptibility levels in gonococci. Strain JF7 was grown in the presence or absence of 1 mM IPTG as described in Materials and Methods and Methods and cell lysates before induction (t=o) or after 1 hr of incubation in absence or presence of IPTG were solubilized and subjected to SDS-PAGE using a 12.5 % (w/v) SDS-PAGE gel with the separated proteins stained by Coomassie Brilliant Blue (**Panel A**); the position of the molecular weight markers are shown to the left of the gel and the approximate position of MtrR as determined by immunoblotting with detection using anti-MtrR antiserum (**Panel B**) are shown. **C**. Shown are the specific activity values for β-gal levels in the control (-IPTG) and induced (+IPTG) cultures. The results are shown as average values (±SD) from three independent assays. The difference was significant ($p = 0.0012$). **D.** The EOP of the control and IPTG-induced cultures on GCB agar with or without 0.5 μg/ml of Ery

is shown. **E.** The H_20_2-susceptibility of the control and IPTG-induced cultures were assessed by a disk diffusion assay and the difference in growth inhibition between these cultures was significant ($p<0.001$).

Figure 1

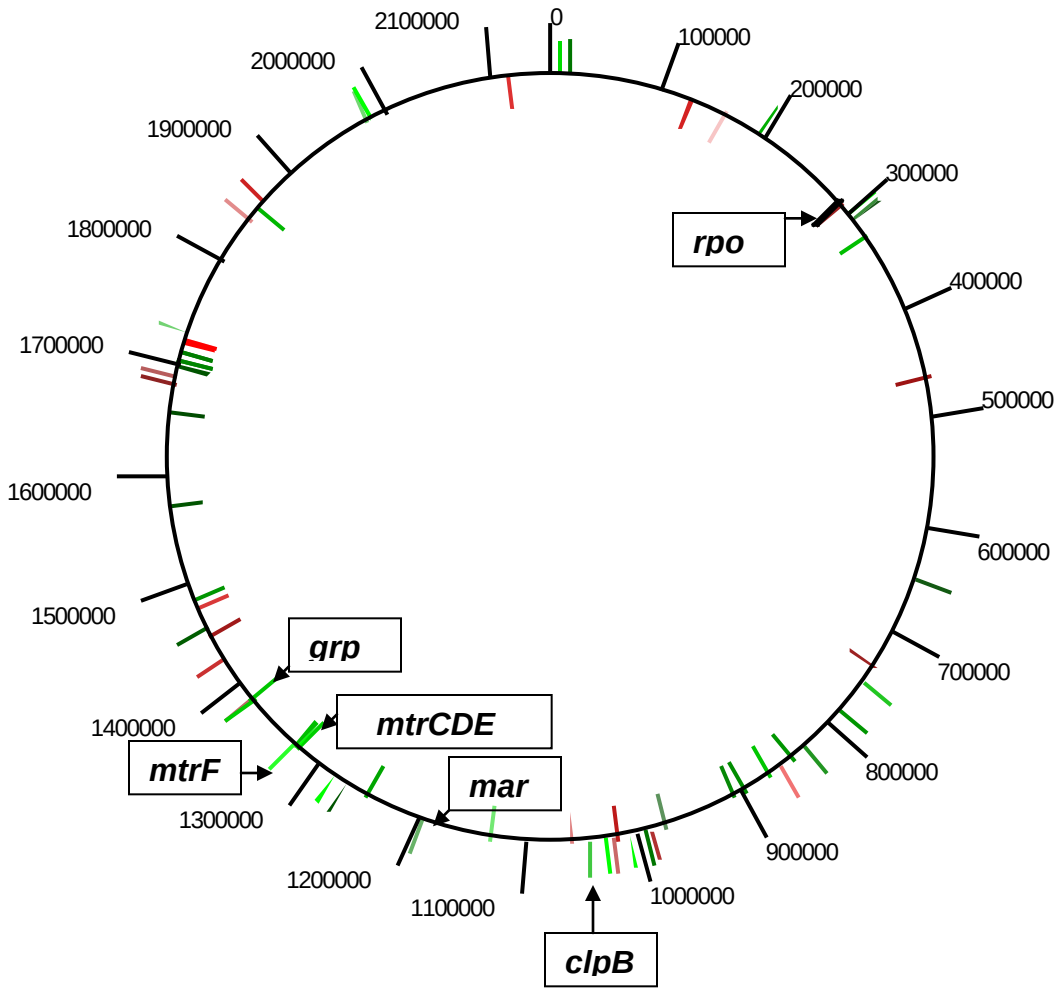
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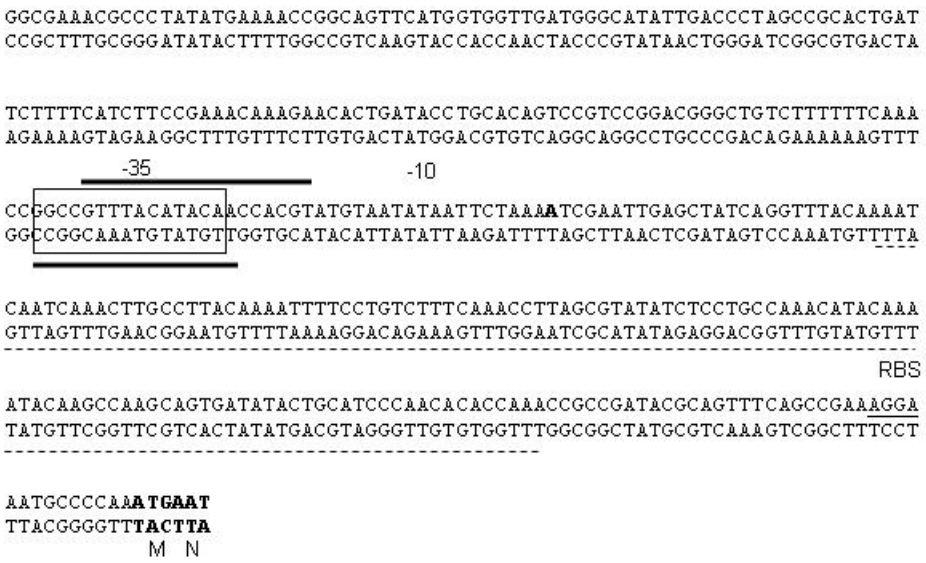
Figure 2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-35
-10
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RBS
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TATGTTCGGTTCGTCACTATATGACGTAGGGTTGTGTGGTTTGGCGGCTATGCGTCAAAGTCGGCTTTCCT
AATGCCCCAAATGAAT
TTACGGGGTTTACTTA
M N

Figure 3

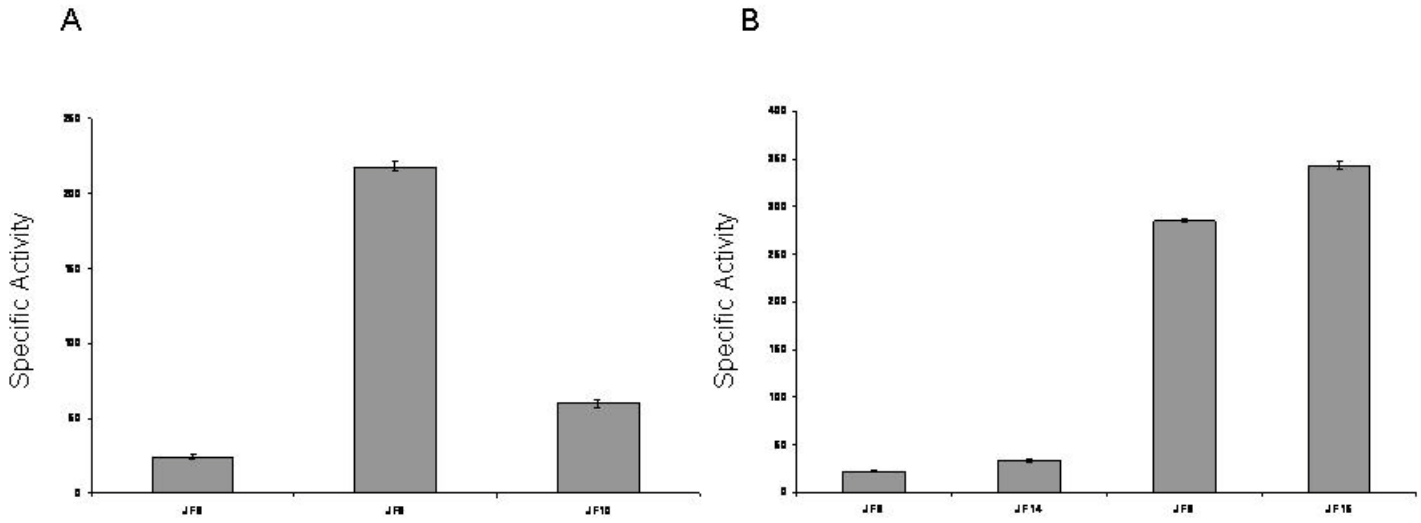


Figure 4

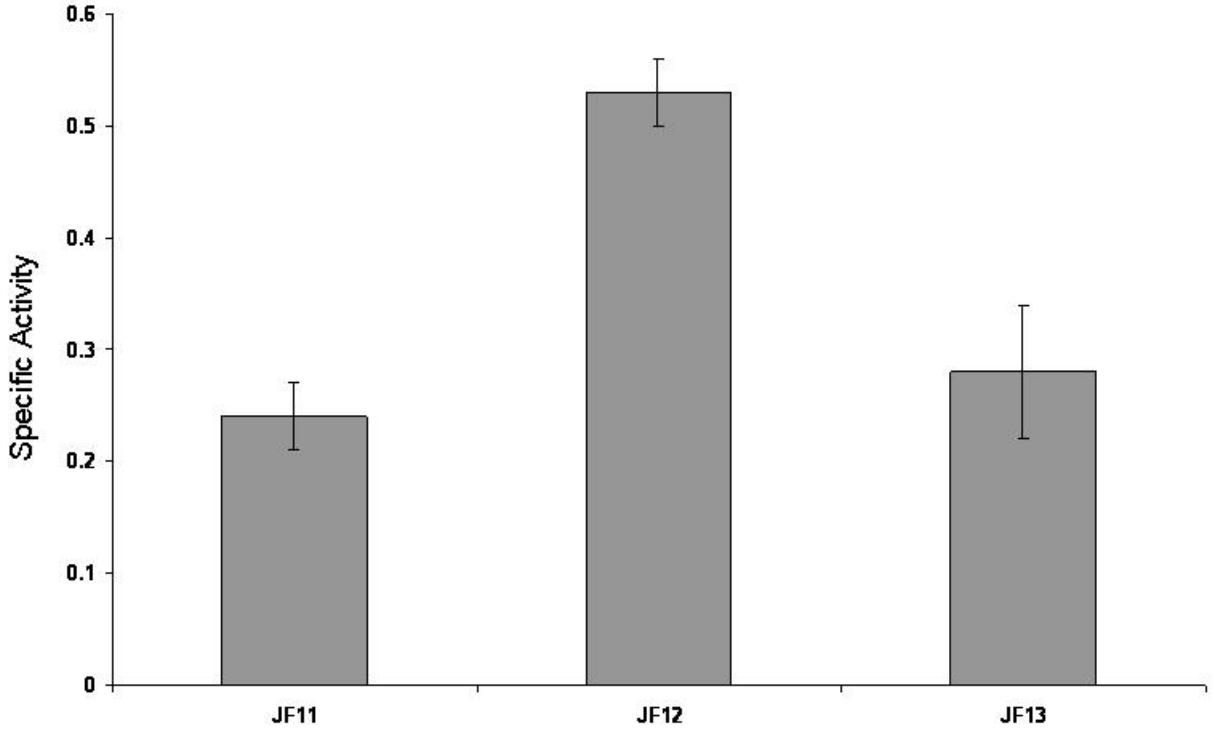


Figure 5

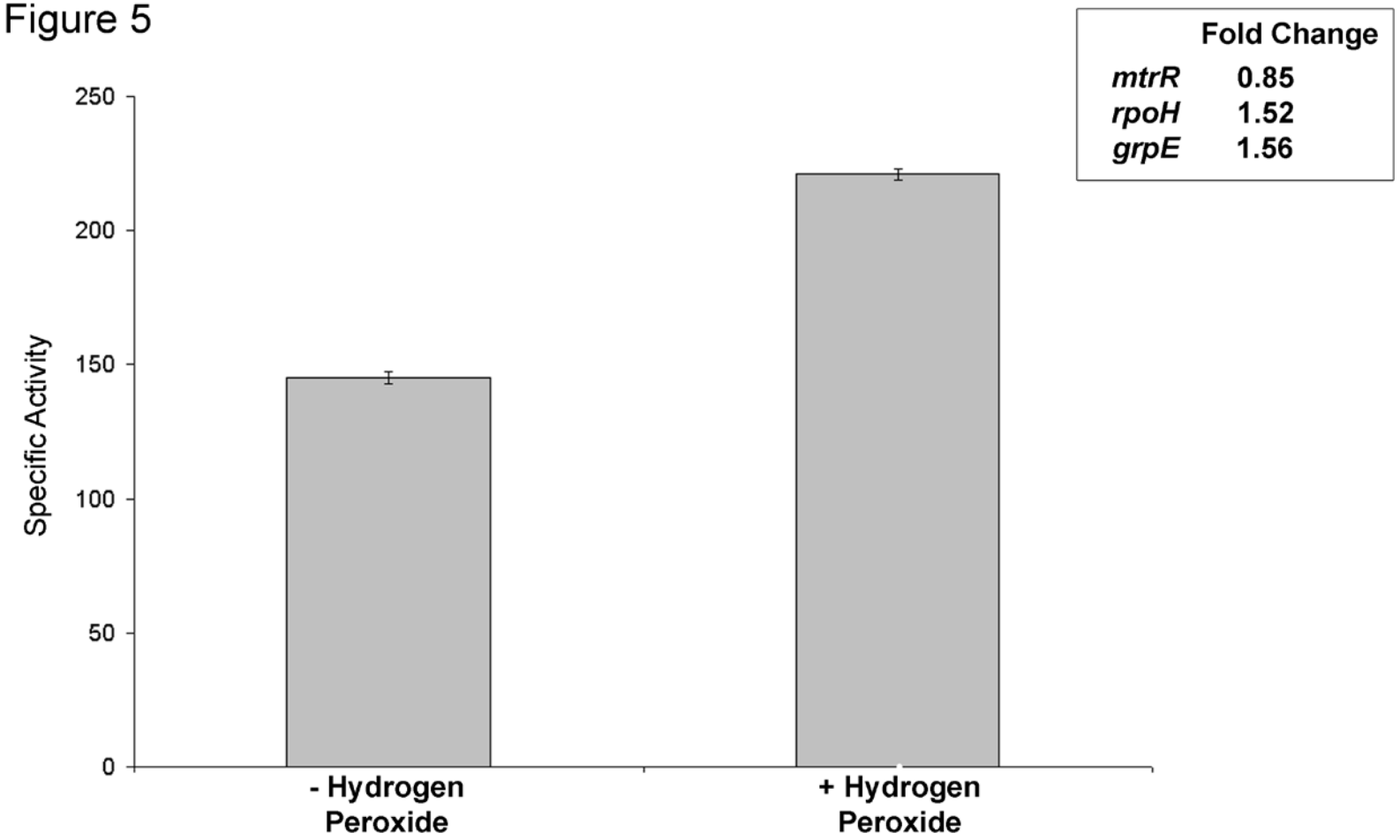

Fold Change
mtrR 0.85
rpoH 1.52
grpE 1.56
Specific Activity
250
200
150
100
50
0
- Hydrogen Peroxide
+ Hydrogen Peroxide

Figure 6

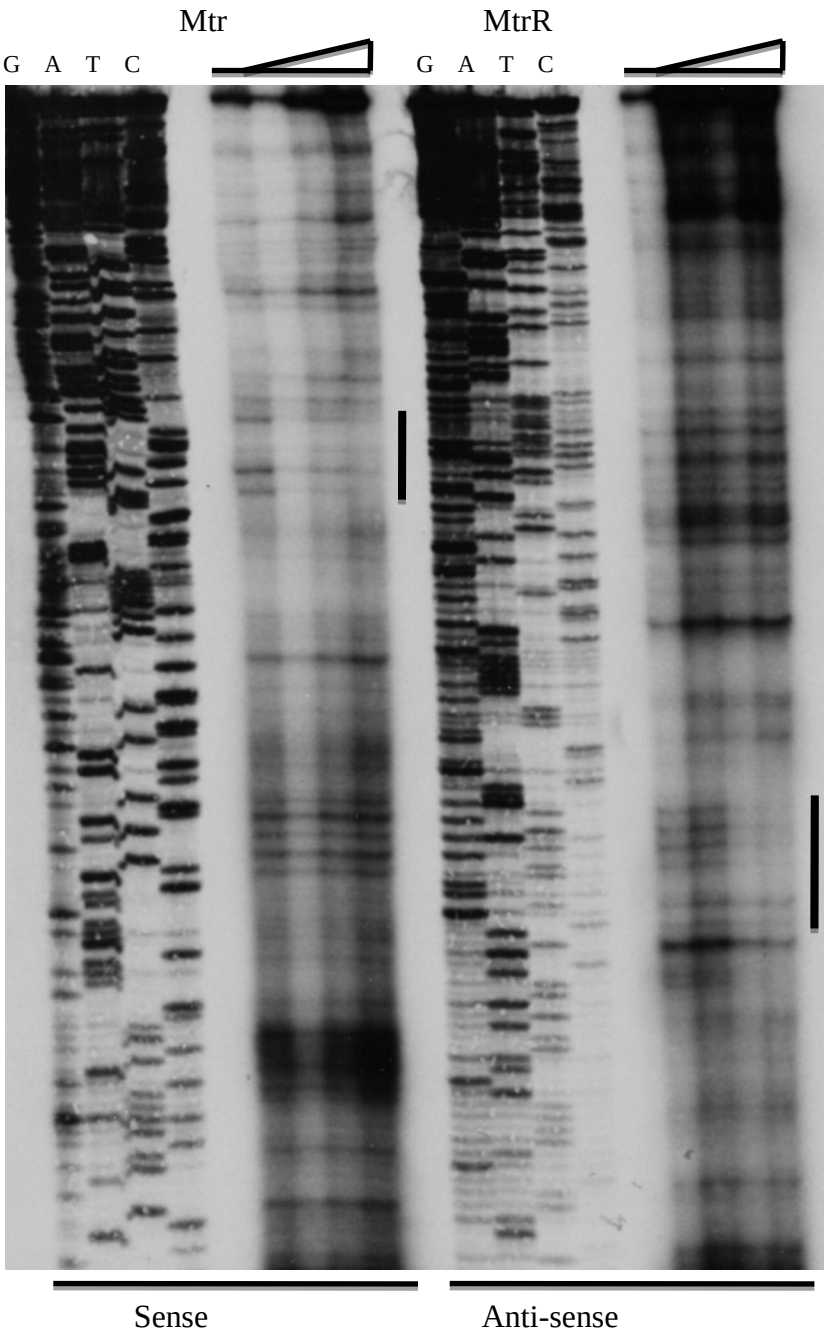
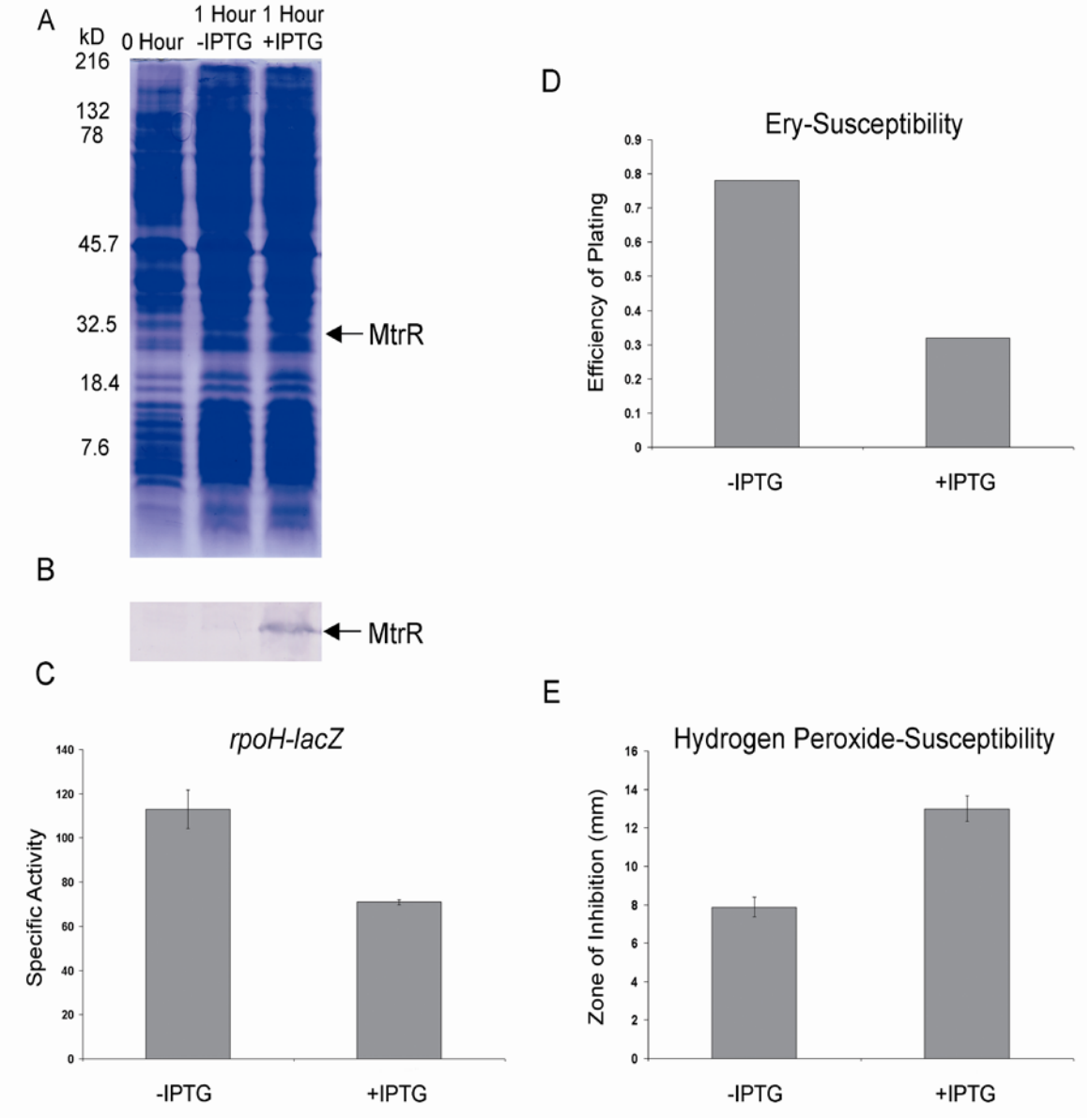


Figure 7


A
kD
0 Hour
1 Hour -IPTG
1 Hour +IPTG
216
132
78
45.7
32.5
18.4
7.6
MtrR
B
MtrR
C
rpoH-lacZ
Specific Activity
140
120
100
80
60
40
20
0
-IPTG
+IPTG
D
Ery-Susceptibility
Efficiency of Plating
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-IPTG
+IPTG
E
Hydrogen Peroxide-Susceptibility
Zone of Inhibition (mm)
16
14
12
10
8
6
4
2
0
-IPTG
+IPTG

Chapter 3: Differential Regulation of Glutamine Biosynthesis Genes *glnA* and *glnE* in *Neisseria gonorrhoeae* by MtrR

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Manuscript submitted to
Molecular Microbiology

This manuscript, in preparation for submission to *Molecular Microbiology*, is written by Paul J. T. Johnson with editorial changes made by William M. Shafer.

Summary

MtrR is a DNA-binding protein that was initially recognized for its ability to repress transcription of the *mtrCDE*-encoded efflux pump operon in the strict human pathogen *Neisseria gonorrhoeae*. Recent microarray studies revealed that in addition to its repressive action on *mtrCDE*, MtrR can dampen the expression of nearly fifty genes as well as enhance expression of over twenty genes. Interestingly, two genes encoding enzymes involved in glutamine biosynthesis were identified as being differentially controlled by MtrR with *glnA* (encoding glutamine synthetase [GS]) being subject to MtrR repression and *glnE* (encoding an adenylase/deadenylase [PII] that regulates GS activity) being MtrR-activated. Herein, we confirm the capacity of MtrR to repress *glnA*, which is also positively controlled by the product of an MtrR-repressed gene (*farR*). FarR binding to its target DNA sequence upstream of *glnA* was significantly impacted by the presence of competing MtrR *in vitro*, suggesting that MtrR-mediated repression of *glnA* occurs by reducing the gene activating action of FarR. In contrast, we found that expression of *glnE*, which has a characteristic extended -10 promoter element, was enhanced by the presence of MtrR and that MtrR could bind upstream of *glnE* in a specific manner.

Introduction

By their capacity to export toxic antimicrobials, which include clinically useful antibiotics, man-made or naturally-occurring biocides and host compounds that participate in innate immunity, bacterial efflux pumps assist microbes to survive hostile environments that they encounter (Nikaido, 1996; Paulsen *et al.,* 1996; Putman *et al.,* 2000; Neykahf, 2001; Saier & Paulsen, 2001; Shafer *et al.,* 2009). There is growing evidence that bacterial efflux pumps can, by themselves or in concert with other resistance mechanisms, result in clinically significant levels of antibiotic resistance and provide bacteria with an ability to escape the antimicrobial action of mediators of innate host defense that perform important protective effects during early stages of infection (Shafer *et al.,* 1998; Warner *et al.,* 2008; Bailey *et al*., 2010).

Over the past two decades, considerable progress has been made in characterizing the biochemistry, genetics, regulation and function of bacterial efflux pump systems. Prior to the availability of genome sequence information, many efflux pumps were identified by the isolation of laboratory-derived mutants that expressed decreased susceptibility to antibiotics and other antimicrobials (Nikaido, 1996). These mutations frequently mapped to genes encoding DNA-binding proteins that normally function to transcriptionally dampen efflux protein-encoding genes. In this respect, the identification of the Mtr system of *N. gonorrhoeae* by Pan and Spratt (1994) was made possible by their cloning and sequencing of DNA fragments harboring mutant alleles of the *mtrR* gene, which encodes a repressor (MtrR) of the transcriptionally divergent *mtrCDE* efflux pump-encoding operon (Pan & Spratt, 1994; Hagman & Shafer, 1995). MtrR is a member of the TetR/QacR family of repressors (reviewed in Grkovic *et al.,*

2002), which are known to perform important roles in controlling expression of a number of bacterial efflux genes (Ramos *et al.,* 2005).

Mutations in the *mtrR* coding sequence (Pan & Spratt, 1994; Hagman *et al.,* 1995) or in its promoter (Hagman & Shafer, 1995) can enhance *mtrCDE* expression, resulting in decreased susceptibility of gonococci to hydrophobic antibiotics (e.g., macrolides [Zarantonelli *et al.,* 2001] and certain beta-lactams [Veal *et al.,* 2002]), biocides (e.g., the spermicide nonoxynol-9 [Rouquette *et al.,* 1999]) and mediators of human innate immunity (e.g., antibacterial peptide LL-37 [Shafer *et al.,* 1998], bile salts [Hagman *et al.,* 1997] and progesterone [Warner *et al.,* 2008]). As assessed by a murine vaginal competitive infection model, *mtrR* mutations also increase fitness of gonococci *in vivo* (Warner *et al.,* 2007; Warner *et al.,* 2008) and such mutants can be selected in this experimental infection model (Warner *et al.,* 2008). Due to its ability to modulate levels of *in vivo* fitness of *N. gonorrhoeae* and the frequent occurrence of *mtrR* mutants in clinical isolates (Shafer *et al.,* 1995; Zarantonelli *et al.,* 1999; Xia *et al.,* 2000), it is important to understand how MtrR controls *mtrCDE* and other genes that may be important in gonococcal pathogenicity, antibiotic resistance or basic metabolism. With respect to repression of *mtrCDE,* two homodimers of MtrR bind the DNA sequence that overlaps the promoter used for *mtrCDE* transcription (Lucas *et al.,* 1997; Hoffmann *et al.,* 2005) and a pseudo-direct repeat element (CCGTGCA and TCGTGTA), separated by a single nucleotide, within this binding site is important for MtrR recognition (Hoffmann *et al.,* 2005).

While transcriptional regulators of efflux pump genes have significance for controlling levels of bacterial resistance to antimicrobials, they also likely participate in

the regulation of other genes that are important in the overall biology of the cognate bacterium (Shafer *et al*., 2009; Bailey *et al*., 2010). In this respect, results from a recent microarray study defined the MtrR regulon of *N. gonorrhoeae* strain FA19 as consisting of at least 47 repressed and 22 activated genes (Folster *et al.*, 2009). In order to further study MtrR as a global transcriptional regulator in gonococci that can both activate and repress gene expression, we evaluated its ability to modulate expression of genes involved in glutamine biosynthesis. We analyzed this metabolic pathway because certain genes within it appeared to be MtrR-repressed (e.g., *glnA*, encoding glutamine synthetase [GS]) or MtrR-activated (e.g., *glnE*, encoding an adenyltransferase/deadenylase [PII] that positively and negatively controls GS activity). We were especially interested in MtrR regulation of these genes because glutamine is a scarce amino acid at mucosal surfaces and within phagocytic phagolysosomes (Klose & Mekalanos, 1997), sites that would harbor gonococci during infection (Shafer & Rest, 1989), and *glnE* has been reported to be an essential gene in other pathogens, such as *Mycobacterium tuberculosis* (Parish & Stoker, 2000). Thus, intracellular pools of glutamine in gonococci during infection may rely to a large extent on its biosynthesis and the regulatory systems that control biosynthesis. Hence, understanding how glutamine biosynthesis genes are regulated is important, especially since levels of intracellular glutamine can impact concentrations of other amino acids (e.g., arginine and proline) that are produced from the same precursor, glutamate. Moreover, regulatory pathways that modulate glutamine biosynthesis could influence gonococcal survival or fitness during infection as they might, as a consequence, impact levels of certain by-products of amino acid metabolism that have important cellular functions (e.g. polyamines, Tabor and Tabor [1984]). Since *glnA* in

Corynebacterium glutamicum has been recently shown (Buchinger *et al.,* 2009) to be negatively controlled by another member of the TetR/QacR family (AmtR), we thought that studies with MtrR would, in general, provide important insights regarding regulation of glutamine biosynthesis.

We now report that MtrR indirectly represses *glnA* by reducing the binding of a second DNA-binding protein (FarR) that activates *glnA* expression. In contrast to its repressive action on *glnA,* MtrR binding to the DNA sequence upstream of *glnE* was associated with activation of its expression. Using the glutamine biosynthesis gene system as a model, we propose that the ability of MtrR to directly or indirectly impact genes outside of the *mtr* efflux locus has importance for the basic physiology and metabolism of *N. gonorrhoeae*.

Results

Since results from our recent microarray study (Folster *et al.,* 2009) that identified the MtrR regulon in strain FA19 suggested that MtrR can repress *glnA* yet activate *glnE,* we used these genes as a model system to study how a single DNA-binding protein can differentially control genes involved in the same biosynthetic pathway in gonococci. Furthermore, since MtrR represses *farR* (Lee *et al.,* 2003), which encodes a DNA-binding protein that represses *farAB* expression in gonococci, we also asked if MtrR controls *glnA* and *glnE* through a FarR-dependent pathway.

Regulation of *glnA* in gonococci by MtrR and FarR

Using quantitative real-time reverse transcription – polymerase chain reaction (qRT-PCR), we confirmed that *glnA* expression was increased in an MtrR-deficient transformant (strain JF1) of strain FA19; the fold change of expression was 1.9 (data not presented) compared to the 1.6 value obtained by microarray (Folster *et al*., 2009). Further confirmation of MtrR regulation of *glnA* was obtained using isogenic MtrR-positive and –negative genetic derivatives (strains PJ9 and PJ10, respectively) of strain FA19 with a *glnA-lacZ* translational fusion that consisted of 500 bp of DNA upstream and the first two codons of *glnA* (Figure 1). As is shown in Figure 2, the difference in *glnA* expression between strains PJ9 and PJ10 was significant (p= 0.005). Importantly, when the *mtrR* deletion mutant (strain PJ10) was complemented with an ectopically expressed wild type *mtrR* gene (strain PJ12), *glnA* expression was restored to wild type levels ($p < 0.005$ for both PJ9 vs PJ10 and PJ12 vs. PJ10). Using the *glnA-lacZ* translational fusion in isogenic derivatives of strain FA19 containing a wild type (strain PJ9) or an insertionally-inactivated *farR* gene (strain PJ11), we found (Figure 2) that expression of *glnA* was significantly ($p = 0.007$) higher in the FarR-positive strain. As this result suggested that FarR is a positive regulator of *glnA* expression, we tested if complementation of the *farR* null mutant with the wild type *farR* gene expressed ectopically (strain PJ14) would restore levels of *glnA* expression and found this to be the case; the difference in *glnA expression* between strains PJ11 vs. PJ14 was significant (p= 0.002). Due to these results, we next examined *glnA-lacZ* expression in an MtrR- FarR- double mutant (strain PJ13). In PJ13, *glnA* expression mimicked (Figure 2) that of the single mutant that only lacked FarR (strain PJ11). Moreover, complementation of the

double mutant with a wild type *farR* gene (strain PJ15), but not *mtrR* (strain PJ22), expressed ectopically enhanced *glnA* expression to a level that resembled the MtrR- FarR+ strain PJ10 (Figure 2); the difference in *glnA* expression between strains PJ13 and PJ15 was significant ($p < 0.0001$).

MtrR and FarR binding to *glnA*

Since maximal *glnA* expression occurred in an MtrR- FarR+ background (Figure 2), we hypothesized that MtrR and FarR have opposing regulatory properties and therefore examined their DNA-binding ability. In order to test whether MtrR and/or FarR can bind to the DNA sequence upstream of *glnA*, we performed electrophoretic mobility shift assays (EMSA) that employed the DNA sequence shown in Figure 1 minus the second *glnA* codon. Preliminary results from EMSA experiments showed that both MtrR and FarR could bind in a specific manner to this DNA target (data not presented). We next tested their ability to bind to three non-overlapping regions of this target DNA and these sections are highlighted in different colors in Figure 1. Using EMSA, we found that MtrR only bound section I in a specific manner (data not presented and Figure 3A), In contrast, FarR only bound section II in a specific manner (data not presented and Figure 4A).

Based on the MtrR-binding site sequence upstream of *mtrCDE* (Lucas *et al.*, 1997; Hoffmann *et al.*, 2005), we detected a potential MtrR-binding site upstream of *glnA*, which is shown in the red box in Figure 1. Similarly, using the FarR-binding site sequence positioned upstream of *farAB* (Lee *et al.*, 2003) as a guide, we detected a

possible FarR-binding site (see black box in Figure 1), within section II, which was separated from the predicted MtrR-binding site by 96 bp. In order to experimentally identify the sites that bind MtrR and FarR in sections I and II, respectively, we used DNase I protection on both strands of the target DNA probes. We detected two closely linked regions (termed A and B), separated by three nucleotides, on the *glnA* sense strand in section I that were protected by MtrR (Figure 3B and summarized in Figure 1). On the anti-sense strand, two sites (A' and B'), separated by nine nucleotides, gave evidence of being protected by MtrR. Interestingly, although the protected regions on the sense and anti-sense strands were not complementary, Sites B and A' were separated by only 4 bp (see Figure 1).

For detection of FarR-binding sites upstream of *glnA,* we first attempted to use section II (Figure 1) as the target DNA as it was the only section in the full length probe that bound FarR in a specific manner. However, in several preliminary experiments we could not observe consistent protection from DNase I with the section II probe. Accordingly, we used the full length region (Figure 1) to experimentally identify FarR-binding sites. With this target, we identified (Figure 4B) three sites protected by FarR on the sense strand, all three of which had adjacent DNase I hypersensitive nucleotides. Importantly, only one of the FarR-protected sites was within section II (shown by the # sign next to the vertical bar in Figure 4B), which bound FarR in a specific manner (Figure 4A). On the anti-sense strand, only a single predominant region gave evidence of protection. This protected region was complementary to a 28 nucleotide stretch on the sense strand in section II that was also protected by FarR (summarized in Figure 1). Overall, this area of protection is located nearly 200 bp upstream of the previously

annotated *glnA* promoter, which we verified (data not presented) by primer extension analysis.

The relative positions of the FarR- and MtrR-binding sites suggested a model for MtrR control of *glnA* expression that involves, in part, its ability to impact FarR binding to its target DNA sequence. In order to test this model, we first examined the importance of the MtrR-binding site in controlling *glnA* expression. For this purpose, a translational fusion that lacked section I of the full length *glnA-lacZ* fusion (see Figure 1) was created. We then examined and compared expression of *glnA* from this fusion to that of the full length fusion in isogenic MtrR-negative and –positive strains PJ20 and PJ21, respectively. As is shown in Figure 5, we found that in strain PJ21, deletion of section I resulted in enhanced expression of *glnA* when compared to $MtrR^+$ strain PJ12, which contained the full-length *glnA-lacZ* fusion (p=0.009). In contrast, when the full length or truncated *glnA* fusions were expressed in an MtrR-deficient background (strains PJ10 and PJ20, respectively), no significant (p=0.8) difference in *glnA-lacZ* expression was detected.

Although these results, along with those from the EMSA and DNase I protection studies, indicated that MtrR could bind upstream of *glnA*, it did not allow for conclusions to be drawn as to whether repression of *glnA* by MtrR was direct or indirect. Given the distance of the MtrR-binding sites from the *glnA* promoter (Figure 1), where such binding would more likely result in direct repression, and their relative location to the downstream FarR-binding sites, we hypothesized that MtrR influences the formation and/or stability of FarR::DNA complexes. In order to test if FarR::DNA complexes can be impacted by MtrR, target DNA containing binding sites for both MtrR and FarR was

pre-incubated with a fixed concentration of one protein and then incubated with increasing concentrations of the second protein. The results (Figure 6) showed that in the absence of competing protein, MtrR and FarR gave distinct shifts of the probe (see arrows in Figure 6). We found that as the amount of FarR was increased after the DNA had been pre-incubated with a fixed amount of MtrR (0.1 µg), the lower of the two FarR::DNA complexes appeared at 0.25 µgs of competing FarR. Importantly, the electrophoretic mobility of the preformed MtrR::DNA complex showed only minor changes in mobility. In sharp contrast to these results, the pattern of the FarR-specific shifts was significantly changed by the addition of increasing amounts of MtrR. Specifically, the slower migrating FarR-specific shift was lost and higher molecular weight complexes became evident and seemed to predominate; one of these complexes co-migrated with the MtrR-specific shift. The complexes that had a slower electrophoretic mobility than that of the MtrR-specific shift likely represent multimers of MtrR bound to the target DNA as they were observed (data not presented) in other EMSAs that used high amounts of MtrR ($\geq$ 0.5 µgs), but not FarR. Taken together, we suggest that MtrR can either impact FarR binding to its target and/or displace FarR from the protein::DNA complex.

MtrR regulation of *glnE*

In contrast to *glnA*, the earlier microarray work by Folster *et al*. (2009) indicated that MtrR significantly (seven-fold difference; $p \leq 0.05$) enhances *glnE* expression in strain FA19. In confirmation of this report, using qRT-PCR with RNA extracted from MtrR-positive strain FA19 and its MtrR-negative deletion mutant strain JF1, we found a

nine-fold difference in the *glnE* transcript level between these isogenic strains (data not presented). Results from further experiments with *glnE-lacZ* fusion derivatives of strain FA19 with or without *mtrR* or *farR* mutations, confirmed that MtrR enhances *glnE* expression and that complementation of an *mtrR* deletion mutant with a wild type copy of *mtrR* restored such expression (data not presented). In contrast to *glnA*, expression of *glnE* was independent of FarR because a *farR* null mutant expressed levels of *glnE-lacZ* similar to that of the wild type parent (data not presented).

In order to determine if MtrR can bind upstream of *glnE*, we first used EMSA to detect its binding to a 307 bp sequence (Figure 7). Based on the published MtrR-binding site sequence upstream of *mtrCDE* (Lucas *et al.,* 1997; Hoffmann *et al.,* 2005) and *rpoH* (Folster *et al*., 2009), we detected four potential MtrR-binding sites on the sense and anti-sense strands that ranged in sequence identity from 67% to 52% for regions on the sense and anti-sense strands, respectively. We confirmed that the DNA sequence upstream of *glnE* could bind MtrR (Figure 8A), but not FarR (data not presented), in a specific manner.

In order to experimentally locate MtrR-binding sites upstream of *glnE*, we used DNase I protection on both the sense and anti-sense strands. On the sense strand, we detected a relatively long, contiguous sequence that had regions protected by MtrR (Figure 8B). This 82 nucleotide region T-rich (48.8%), encompasses two of the predicted MtrR binding sites that were ≥ 50% identical to previously identified sites, and overlapped (16 nucleotides) the predicted MtrR-binding site with the highest identity to previously identified binding sites (Figure 7). Additionally, we detected DNase I hypersensitive regions on the sense strand, which are near the annotated *glnE* promoter.

While no clear protection was evident at these DNase I hypersensitive sites, their presence suggest that DNA-protein interactions are occurring in or near these regions resulting in alterations in the local DNA structure. On the anti-sense strand, we observed (Figures 7 and 8B) two protected sites (labeled as A´ and B´ in Figures 7 and 8B), one of which straddles the annotated *glnE* promoter (site A´), while the other is located further downstream (site B´). Site A´ is notable as it overlaps the 27 bp region that was identified as being a possible MtrR- binding site. Of interest, we also noted a DNase I hypersensitive C residue in site A´ (see asterisk in Figures 7 and 8B) that became apparent in the presence of MtrR; this site overlaps the annotated promoter region on the complementary strand. Examination of the nucleotide sequence surrounding this DNase I hypersensitive C nucleotide revealed the presence of an imperfect seven nucleotide indirect repeat element (TAGCTTC/CAACGAT) that intervenes the annotated -35 and -10 hexamers.

Further examination of the MtrR protected region identified two interesting features regarding the putative *glnE* promoter. First, we noted the presence of a potential extended -10 motif (Keilty & Rosenberg 1987) (TGATATAGT), which has been previously observed for other -10 sequences in *N. gonorrhoeae* (Isabella *et al.*, 2008), that could mitigate the impact of a poor -35 element that was identified in the annotated sequence. Second, we identified an alternative -35 hexamer sequence (TTCAGA) that is more consistent with the sigma-70 consensus -35 hexamer sequence (TTGACA) in *E. coli*. This alternative -35 region would change the spacing between the annotated -10 hexamer from an optimal 17 nucleotides to a suboptimal 21 nucleotides or 18 nucleotides from the proposed extended -10 element. For either -10 element, the binding of MtrR to

this region could enhance interaction of the promoter with RNA polymerase leading to increased transcription of *glnE*. In this respect, it is of interest that BmrR of *Bacillus subtilis* activates transcription of the *bmr*-encoded efflux transporter gene by binding to an imperfect inverted repeat element within the respective promoter that also has sub-optimal spacing (19 nucleotides) between the -10 and -35 hexamers (Ahmed *et al.,* 1994; Grkovic *et al.,* 2002).

Discussion

DNA-binding proteins that control efflux gene expression in bacteria have been termed "local regulators" (Ma *et al.,* 1995), as their encoding gene is frequently located adjacent to their respective target genes that encode efflux pump proteins. While the ability of such regulators to control expression of efflux pump genes is certainly important in modulating levels of bacterial susceptibility to antimicrobials, they may, based on our work with MtrR described herein and elsewhere (Lee *et al.,* 2003; Folster *et al.,* 2007; Folster *et al.,* 2009), as well as work with *Salmonella enteritica* serovar Typhimurium (Bailey *et al*., 2010), have a more global action and control expression of genes that contribute to important physiological processes. In this respect, our collective work on the transcriptional regulatory action of MtrR has shown that it can negatively or positively control genes that are scattered throughout the chromosome (Lee *et al.,* 2003; Folster *et al.,* 2007; Folster *et al.,* 2009). The products of these MtrR-regulated genes participate in important and diverse functions such as peptidoglycan biosynthesis (*ponA*), type IV pilus secretion (*pilMNOPQ*), stress response (*rpoH*, and RpoH-regulated genes),

resistance to hydrogen peroxide (*ccp)*, transcriptional regulation (*farR* and *marR*) and amino acid biosynthesis (*glnA* and *glnE*).

We propose that MtrR represses *glnA* by its ability to both reduce expression of *farR* (Lee *et al.,* 2003) and by negatively influencing binding of FarR to its target site upstream of *glnA* or decreasing stability of such complexes (Figure 6), which would normally result in activation of *glnA.* The ability of a transcriptional regulator in the TetR/QacR family to repress *glnA* is not without precedent as AmtR of *Corynebacterium glutamicum* was recently shown to negatively control *glnA* expression (Buchinger *et al.,* 2009) by a yet to be defined mechanism. In contrast to *glnA,* our results suggest that binding of MtrR to the DNA sequence upstream of *glnE* has a positive effect on transcription as opposed to its repressive action on other genes (Lucas *et al.,* 1997; Lee *et al.,* 2003; Folster *et al.,* 2007; Folster *et al.,* 2009). Against this background, we propose three consequences of MtrR binding to gonococcal DNA with respect to gene control: a direct repressive action when it binds to a promoter sequence (e.g., *mtrCDE* and *farR*), an apparent repressive action when it binds upstream of a DNA sequence also bearing a site for a transcriptional activator and displacing or inhibiting the binding of that activator (e.g., FarR activation of *glnA*), and a direct activating property when it binds upstream of a gene (e.g., *glnE*) in the absence of other discernible transcriptional factors. In the case of MtrR activation of *glnE,* we do not yet know if all of the binding sites identified by DNase I protection are important. However, given the gene activation model for BmrR (Ahmed *et al.,* 1994), we hypothesize that binding Site A, which contains the imperfect indirect repeat element (Figure 7) between the -35 and -10 domains, is important in *glnE* activation by MtrR. Moreover, since the -10 element of the *glnE* promoter is similar to

extended -10 element bearing promoters in gonococci (Isabella *et al.,* 2008) and *E. coli*, (Keilty & Rosenberg, 1987), the ability of MtrR to bind to this region could facilitate interactions of RNA polymerase with the promoter.

The repertoire of gonococcal genes controlled by MtrR, either directly or indirectly, and the diverse nature of the role of these regulated genes, raises questions regarding the evolution of MtrR as a global regulator of gene expression in gonococci with respect to its role in modulating *in vivo* fitness (Warner *et al.,* 2007; Warner *et al.,* 2008). In this regard, it is important to understand why naturally occurring mutants lacking a functional MtrR were not selected over the millennia to predominant in the community, particularly since loss of MtrR is known to increase gonococcal fitness *in vivo* (at least as assessed by the murine vaginal infection model [(Warner *et al.,* 2007; Warner *et al.,* 2008)]). While *mtrR* mutants can be isolated from patients (Eisenstein & Sparling, 1978; Morse *et al.,* 1982; Shafer *et al.,* 1995; Xia *et al.,* 2000; Zarantonelli *et al.,* 2001), particularly those with rectal gonococcal infections where levels of certain efflux pump substrates (e.g., fecal fatty acids and bile salts) can be elevated, they actually represent <30 percent of all gonococcal isolates regardless of the type of infection (Shafer *et al.,* 1995). We propose that in early stages of infection, the repressive action of MtrR might decrease gonococcal fitness if levels of the MtrC-MtrD-MtrE pump are insufficient to remove toxic hydrophobic antimicrobials that bathe mucosal surface. As infection progresses, possession of MtrR would become advantageous due to its ability to activate genes that encode products important for metabolism, such as biosynthesis of glutamine (e.g., *glnE*). In this case, *mtrR* mutations could decrease gonococcal fitness *in vivo*.

Is the capacity of MtrR to regulate *glnA* and *glnE* important for gonococcal survival *in vivo*? We have hypothesized this to be the case since glutamine, while abundant in the bloodstream, is at low levels at mucosal surfaces and in phagolysosomes (Klose & Mekalanos, 1997), both sites that harbor gonococci during infection. Accordingly, gonococci may rely on glutamine synthetase (GS) for fulfilling its glutamine requirement during infection. In order to address the importance of *glnA* and *glnE*, we attempted to construct non-polar mutations in these genes and assess the growth of such mutants. Despite repeated attempts, we were unable to construct a *glnE* null mutant in strain FA19 even though it was possible to construct a *glnA* null mutant in the same experiments (data not presented). Interestingly, the *glnA* mutant had a severe growth defect, characterized by an extended (nearly 12 hours) lag phase in GCB broth supplemented with glutamine. Hence, for at least strain FA19, the glutamine biosynthesis pathway is important for optimal growth in laboratory media and probably can not be replaced by glutamine transport systems without a significant fitness cost. The reason(s) why *glnE* appears to be an essential gene in gonococci is unclear, but a similar requirement has been reported (Carroll *et al.*, 2008) for *M. tuberculosis.* In general, loss of the *glnE*-encoded PII enzyme would result in dysregulation of GS and this could have a significant, negative impact on the intracellular pool of glutamate with subsequent downstream effects on levels of other amino acids (e.g., arginine and proline) and the synthesis of end-products of amino acid metabolism that contribute to important cellular functions (e.g., polyamines derived from arginine (Tabor & Tabor, 1984). Against this background, we propose that MtrR activation of *glnE* could have a beneficial influence on gonococcal survival and fitness at sites of infection deficient in glutamine and other

amino acids. This hypothesis is supported by the observation of Warner *et al*. (2007) that the fitness advantage afforded by loss of MtrR decreased after day seven of infection in the mouse vaginal model.

Outside of the context of regulating levels of drug efflux pump proteins and modulating resistance to antibiotics, it is clear from this and other work that the transcriptional regulatory properties of MtrR can significantly influence the basic physiology and metabolism of the gonococcus. By defining the function of MtrR-regulated genes, it should be possible to better understand how MtrR influences gonococcal fitness and survival during different stages of infection.

Experimental Procedures

Strains used and growth conditions

All of the strains of *N. gonorrhoeae* employed (Table 1) are genetic derivatives of strain FA19 and were constructed in previous investigations (Folster & Shafer, 2005; Lee *et al.,* 2003; Folster *et al.,* 2009) or for this study. Gonococci were routinely cultured as non-piliated, opacity-negative variants on GCB agar with defined supplements I and II (Shafer *et al.,* 1984) at 37°C under 3.8% (v/v) CO_2; transformation experiments used piliated variants. Gonococci were also cultured in GCB broth with supplements I and II and 0.048% (w/v) sodium bicarbonate with shaking at 37°C. *E. coli* strain DH5α was routinely cultured in Luria Bertani (LB) broth or on LB agar with antibiotics (100 μg/ml of ampicillin or kanamycin).

qRT-PCR

The essentials of the qRT-PCR protocol employed have been described previously (Katzif et al., 2005). In order to confirm the micro-array data reported by Folster *et al*. (2009), a portion of the RNA used in that study was employed. cDNA was synthesized in an RT reaction with random hexamer primers and Superscript II RT (Invitrogen). The specific primers for *glnA*, *glnE* and 16S RNA used in qRT-PCR are listed in Table 2. qRT-PCR was performed on an iCycler iQ real-time PCR detection system (Bio-Rad). iQSYBR Green Supermix (Bio-Rad) was employed in a reaction volume of 25 µl with 200 nM of 5' and 3' primers and five-fold dilutions of RT reaction mixtures. 16S RNA cDNA was employed as the internal control.

β-galactosidase assays

Translational *lacZ* fusions were constructed using pLES94 by transformation into strains FA19, JF1, JF6, EL24 or their derivatives and β-galactosidase assays were performed as described by Folster and Shafer (2005). Plasmids pJF3 and pJF4 that contained *glnA-lacZ* or *glnE-lacZ* fusions were prepared essentially as described by Folster *et al*. (2009). The strains bearing translational fusions are described in Table 1. The primer pairs (Table 2) used to construct the fusions were: 5′pglnA and 3′pglnA (strains PJ9, PJ10, PJ11, PJ12, PJ13, PJ14, PJ15, and PJ16), 3′pglnA and glnAtrunc (strains PJ20 and PJ21), as well as 5′plgnE and 3′pglnE (strains PJ17, PJ18, and PJ19). Briefly, the promoter sequence of *glnA* was amplified by PCR from strain FA19 using primers 5′pglnA and 3′pglnA (Table 2). Following amplification by PCR the DNA sequence was cloned into the BamHI cloning site of pLES94 (Silver & Clark, 1995), and

the plasmid was then transformed into *E. coli* DH5α and transformants were recovered by ampicillin selection. The cloned fragment in the resulting transformant was identified by PCR analysis and DNA sequencing. This plasmid (pJF3) was then transformed into strains FA19, JF1, JF6, and EL24 with insertion occurring at the nonessential *proAB* locus of the recipient strains. Transformants were selected on GC agar supplemented with 1 μg/ml of chloramphenicol. The resulting transformants (Table 1) were then used in β-galactosidase assays as previously described (Folster *et al.,* 2009). *glnE-lacZ* bearing strains were constructed in a similar manner, but utilized the 5′pglnE and 3′pglnE primers to prepare the upstream fusion DNA. The resulting transformants (Table 1) were obtained with plasmid pJF4 and after verification, were used in β-galactosidase assays. Construction of a *glnA* truncated promoter *lacZ* fusion was conducted in the same manner as for *glnA-lacZ* fusions, but utilized primers glnAtrunc and 3′pglnA in order to omit the MtrR binding site in section I (Figure 1). pPJ1 was used to transform strains JF1 and JF6 and the resulting transformants (strains PJ21 and PJ29, respectively; Table 1) were then used in β-galactosidase assays along with PJ12 (MtrR+) and PJ10, both of which contained the full length *glnA-lacZ* fusion (Table 1).

Protein purification and DNA-binding studies

The production and purification of the maltose-binding protein (MBP)-MtrR and the histidine (His)-tagged FarR fusion proteins used in this study have been described previously (Lee *et al.,* 2003; Folster *et al.,* 2007). These proteins were used in EMSA and DNase I protection studies using previously described methods (Lee *et al.,* 2003; Folster *et al.,* 2009). The DNA probes for EMSAs consisted of PCR products that were obtained

using sets of oligonucleotide primers (Table 2); the generation of specific PCR products is described in the relevant figure legend. These PCR products were end-labeled with [γ-^{32}P] dATP using T4 polynucleotide kinase (New England Biolabs) as described previously (Lee *et al.*, 2003). The labeled PCR generated products were incubated with purified MtrR-MBP, or FarR-His, or both in a final reaction volume of 30 µl consisting of the reaction buffer [10 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% (vol/vol) glycerol, 1 mM $MgCl_2$, 50 mM NaCl, poly(dI-dC) (0.05 µg/ml),] and diH_2O at 25 °C for 30 min. Following incubation, the reactions were subjected to gel electrophoresis utilizing a 6% (wt/vol) polyacrylamide gel at 4°C, dried, and autoradiography was performed for visualization. Competitive EMSA's were performed in the same manner, but with the addition of unlabeled specific competitor, generated from the same sequence as the target, or non-specific competitor, generated from the *rmp* gene using primers rmpF and rmpR (Table 2), in amounts up to 100× that of the target sequence. DNase I protection assays were performed essentially as described by Folster *et al*. (2009). PCR generated target DNA sequences were synthesized using primers glnAsec1F, glnAsec1R, glnAsec2F, glnAsec2R, glneF, and glnER was labeled at the 5′ end of one strand as described for EMSA. Purified MtrR-MBP, or FarR-His was then incubated with the target DNA sequence under the same binding conditions as used in the EMSA for 15 minutes at 37°C. Following the addition of $MgCl_2$ (5mM) and $CaCl_2$ (2.5mM), DNase1 was added and the reactions were incubated at 37°C for 1 minute. The reactions were stopped with DNase1 stop buffer [95% ethanol, 7.5mM ammonium acetate and nuclease free H_2O], snap frozen on dry ice for 15 minutes, and then precipitated at -80°C overnight. The pellet was then washed in 70% (vol/vol) ethanol,

dried, and resuspended in gel loading buffer (Epicentre). Reactions were then loaded on 8% denaturing polyacrylamide gel, subjected to gel electrophoresis, dried and autoradiography was performed for visualization.

Acknowledgments

We thank C. Moran and Y. Zalucki for critical reading of the manuscript prior to submission, J. Folster and V. Dhulipala for preparing pJF3 and pJF4 and L. Pucko for help with manuscript preparation. This work was supported by NIH grant R37 AI021150-25 and a VA Merit Award from the Department of Veterans Affairs, both to W.M.S. W.M.S. is the recipient of a Senior Research Career Scientist award from the Department of Veterans Affairs.

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Table 1. Gonococcal strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or remarks	Source or reference
Strain		
FA19	Wild type	P.F. Sparling
EL24	as FA19 but *farR::kan*	Lee *et al*., 2003
EL28	as EL24 but *farR*+	Lee *et al*., 2003
JF1	as FA19 but Δ*mtrR*	Folster & Shafer, 2005
JF6	asJF1 but *mtrR*+	Folster *et al*., 2009
PJ9	as FA19 but *glnA::lacZ*	This Study
PJ10	as JF1 but *glnA::lacZ*	This Study
PJ11	as EL24 but *glnA::lacZ*	This Study
PJ12	as JF6 but *glnA::lacZ*	This Study
PJ13	as PJ10 but *farR::kan*	This Study
PJ14	as EL28 but *glnA::lacZ*	This Study
PJ15	as PJ14 but *farR*+	This Study
PJ16	as PJ12 but *farR::kan*	This Study
PJ17	as FA19 but *glnE::lacZ*	This Study
PJ18	as JF1 but *glnE::lacZ*	This Study
PJ19	as JF6 but *glnE::lacZ*	This Study
PJ20	as JF1 but *glnA::lacZ* truncated	This Study
PJ21	as JF6 but *glnA::lacZ* truncated	This Study
PJ22	as PJ13 but *mtrR*$^{+}$	This Study

Plasmid		
pLES94	Cloning vector containing promoterless *lacZ* for insertion of gonococcal genes between *proA* and *proB*	V. Clark
pJF3	as pLES94 but *glnA::lacZ*	This Study
pJF4	as pLES94 but *glnE::lacZ*	This Study
pPJ1	as pLES94 but *glnA::lacZ* truncated	This Study
pGCC3	NICS vector used for insertion of gonococcal genes between *lctP* and *aspC*	H. Seifert

Table 2. Oligonucleotides used in this study

Oligonucleotide primer name	Sequence (5′→3′)
5′pglnA	GGGGATCCCATAAAGGCGGGGCGGTC
3′pglnA	GGGGATCCCGGGACATCTTCAGCTCCTGAA
5′pglnE	CAAGTGAGGAGCTGCATGAA
3′pglnE	CGGGACATTTCGGATTCCGTTTG
glnA1	GCAACCGCCTGTTTCAAAAAATG
glnA2	GGACATCTTCAGCTCCTGAAAAAG
glnasec1F	TAACGTTTGCCCCGCAACC
glnasec1R	CCCCCGCTACGCCGTTTTC
glnasec2F	GGGCGTGCATAGTCATATTC
glnasec2R	GCGTCAAATTCCAAGCCGG
glnasec3F	AAACCGGTTTCAGACGGCAT
glnasec3R	GGACATCTTCAGCTCCTGAA
glnatrunckf	CATGGATCCGATGAATCTGCGGCGATTTG
rmpF	CATGTTTCTACAGCGGCCTG
rmpR	CGGCAAGATATTACCTAGCCT
glnERTR	ACTTCCCGCCACAATTTCCT
glnERTF	CGACGAATTGCTGTCCCATT
glnARTR	GGCTTTGGCGTGTTTGATG
glnARTF	TCCGATACCGCGCTCTACTAC

A: primers with ending letters RTR or RTF were used in qRT-PCR for the 16S *rRNA*, *glnE* and *glnA* transcripts

Figure Legends

Figure 1: The nucleotide sequence upstream of *glnA* and identification of the MtrR and FarR-binding sites. The nucleotide sequence of the DNA upstream of *glnA* is shown including the first two codons encoding methionine (M) and serine (S); the translational start codon is identified by the bent arrow. This 506 bp sequence is divided into three sections that were used in EMSA experiments with section I shown in green, section II in yellow and section III in blue. The −10 and −35 hexamer sequences of the *glnA* promoter (section III) are identified with the −10 and −35 notation as well as a line under the sequences. The start point of transcription is shown by the † symbol and was determined by primer extension analysis as described previously (Hagman *et al.*, 1995). The MtrR-binding site in section I that is boxed in red was predicted based on similarity to the binding site upstream of *mtrCDE* (Lucas *et al.*, 1997; Hoffmann *et al.*, 2005). The MtrR binding sites on the sense and anti-sense strands are identified by DNase I protection (Figure 3), indicated by heavy underlines with sites A and B on the sense strand (see text) and A' and B' on the anti-sense strand (see bold red letters). The FarR-binding site predicted by sequence similarity with that upstream of *farAB* (Lee *et al.*, 2003) is shown in the black boxed area in section II and the binding sites identified by DNase I protection (Figure 4) are noted by the dotted line above the sense strand and below the anti-sense strand.

Figure 2: MtrR and FarR regulation of *glnA* expression. The specific activity of β-galactosidase (expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per mg of protein) in strains PJ9 (FA19 *glnA::lacZ*), PJ10 (JF1 *glnA::lacZ*),

, PJ12 (JF6 *glnA::lacZ*), PJ11 (EL24 *glnA::lacZ*), PJ14 (PJ11 *farR*$^{+}$ *glnA::lacZ*), PJ13 (PJ11 Δ*mtrR glnA::lacZ*), PJ13 (PJ14 *farR*$^{+}$),and PJ22 (PJ13 *mtrR*$^{+}$). The results are shown as an average value (± standard deviation) from three independent experiments.

Figure 3: Identification of the MtrR-binding site upstream of *glnA*. (A) The area of MtrR specific binding was identified using three sections of the full length DNA sequence described in Figure 1. The full length probe was PCR-amplified from DNA of strain FA19 using primers 5'pglnA and 3'pglnA (Table 2), while binding site I was amplified with primers glnAsec1F and glnAsec1R. The nonspecific probe was prepared using primers rmpF and rmpR. MtrR was found to specifically bind only (data not presented) to section I since specific competitor DNA, but not non-specific competitor (up to 100×), was able to abrogate the shifting of section I *glnA* DNA in EMSA; this result is shown in the figure. (B) The MtrR-binding site within section I was identified by a DNase I protection assay that employed increasing amounts of purified MtrR-MBP (0, 5, 10, and 15 μg) with both sense and anti-sense target DNA probes. The protected regions are identified in the figure by the black bars and denoted as A and B (sense strand) and A′ and B′ (anti-sense strand). The sequencing reactions for each probe are adjacent to the DNase I protection reactions and the lanes are oriented as G, A, T, C.

Figure 4: Identification of the FarR-binding site upstream of the *glnA* promoter. (A) The area of FarR specific binding was first identified using the subsections of the full length *glnA* sequence shown in Figure 1. The full length probe was PCR-amplified using primers 5'pglnA and 3'pglnA, while binding site II was amplified with primers

glnAsec2F and glnAsec2R. The nonspecific probe was prepared using primers rmpF and rmpR. FarR was found to only (data not presented) bind section II in a specific manner as determined by competitive EMSA (shown in figure). (B) The FarR-binding sites on the sense and anti-sense strands were identified by DNase I protection assays that employed increasing amounts of purified FarR-His (0, 1, 5, and 10 μg). The protected regions on each probe are identified by the black bars with the protected region on the sense strand within section II being denoted by a # next to the bar to distinguish it from the other two protected regions that lie within section I. DNase I hypersensitive sites are shown by an *. The sequencing reactions for each probe are adjacent to the DNase I protection reactions and oriented G, A, T, C.

Figure 5: MtrR regulation of *glnA* expression is dependent on the MtrR binding site. The specific activity of β-galactosidase (see Figure 2 legend) in $MtrR^+$ vs. $MtrR^-$ strains containing the full length *glnA-lacZ* fusion (PJ12 and PJ10, respectively) or truncated *glnA-lacZ* fusion (PJ21 and PJ20, respectively) lacking section I (see Figure 1) are indicated. Deletion of section I containing the MtrR binding site resulted in increased *glnA* expression in $MtrR^+$ strain PJ21 compared to expression of the full length sequence in $MtrR^+$ strain PJ12 (p=0.009). There was no significant change in expression of these two fusions (p=0.8) when they were expressed in $MtrR^-$ strains PJ10 and PJ20.

Figure 6: MtrR influences FarR::DNA complexes. Shown are the results from an EMSA experiment that evaluated the binding of MtrR and FarR to the ^{32}P-labeled full length *glnA* probe (Figure 1) that was prepared as described in legend 3. The lane

assignments are: 1, probe alone; 2, probe plus 0.1 µgs of MtrR-MBP; lanes 3-8 are the same as lane 2, but with increasing amounts of FarR-His (0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 µgs); 9, probe alone; 10, probe plus 0.5 µgs of FarR-His; lanes 11-16 are the same as lane 10, but with increasing amounts of MtrR-MBP (0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 µgs). The positions of MtrR and FarR shifted bands in the absence of competing protein are shown.

Figure 7: The nucleotide sequence upstream of *glnE* and MtrR-binding sites. The 301 bp sequence of the DNA upstream of *glnE* and the first two codons (M and S) is shown with the annotated −10 and −35 hexamer sequences of the *glnE* promoter identified with a line under the sequences. The putative extended -10 element is shown in blue. The alternative -35 hexamer is shown by the dashed line above the sequence. The boxed regions represent predicted MtrR binding sites that were identified based on sequence similarity to that of a site upstream of *mtrCDE* (Lucas *et al.*, 1997; Hoffmann *et al.*, 2005) or *rpoH* (Folster *et al.*, 2009). The grey box represents a sequence with 53% identity to the region upstream of *rpoH* (Folster *et al.*, 2009) while the yellow, black, and red boxes represent sequences with 55%, 67%, and 52%, respectively, identity to regions upstream of *mtrCDE* (Hoffmann *et al.*, 2005). The MtrR-binding sites identified by DNase I protection (Figure 8) are noted by the solid line above the sense strand or below the anti-sense strand; the two sites on the anti-sense strand are denoted as A´ and B´ with the DNase I hypersensitive site in A´ shown by an *. The adjacent seven nucleotide imperfect inverted element is shown in green and red.

Figure 8: Identification of the MtrR-binding site in the *glnE* upstream DNA. (A) The binding specificity of MtrR for the DNA shown in Figure 8 was determined by competitive EMSA as described above. The probe was prepared by PCR using primers 5'pglnE and 3'pglnE (Table 2). (B) The MtrR-binding sites within this sequence were identified by DNase I protection assays that employed increasing amounts of purified MtrR-MBP (0, 5, 10, and 15 μg) with both sense and anti-sense probes. The protected regions on each probe are identified by the black bars and the two sites on the anti-sense strand are labeled as A´ and B´. Regions containing DNase I hypersensitive sites, which could contain more than one nucleotide, on the sense and anti-sense strands are denoted by *. The sequencing reactions for each probe are adjacent to the DNase I protection reactions and oriented G, A, T, C.

Figure 1

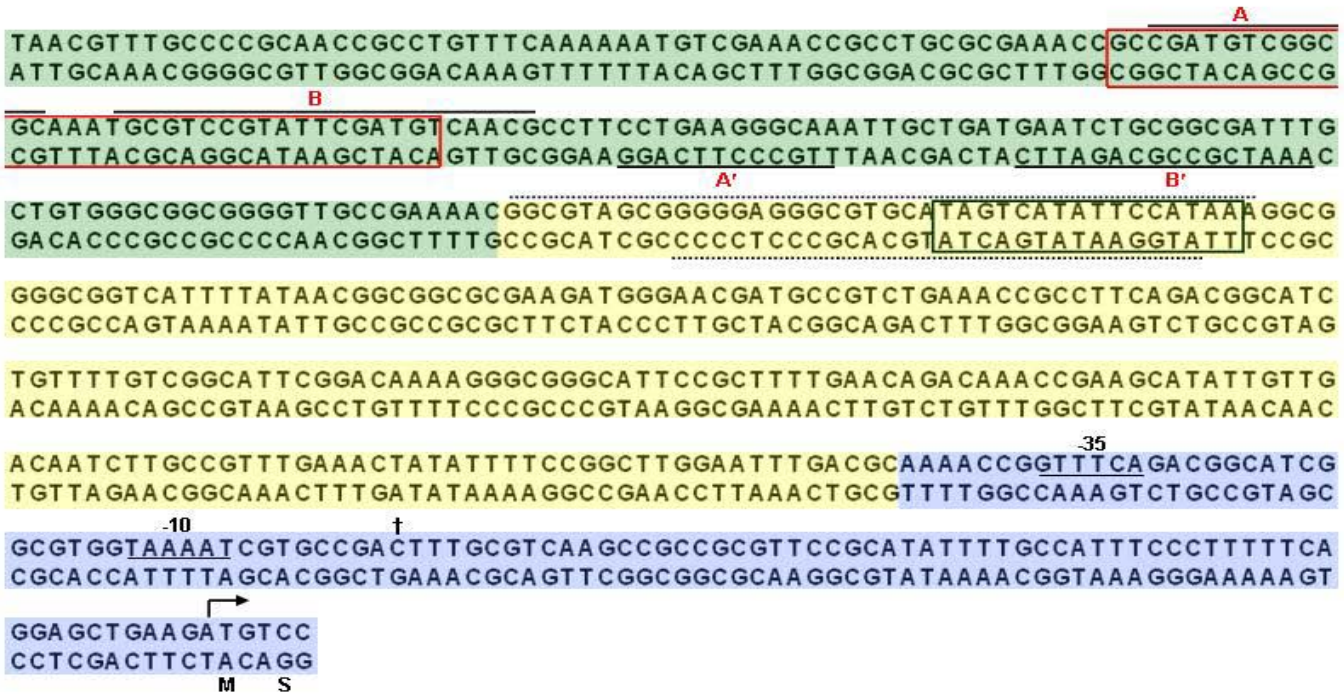


Figure 2

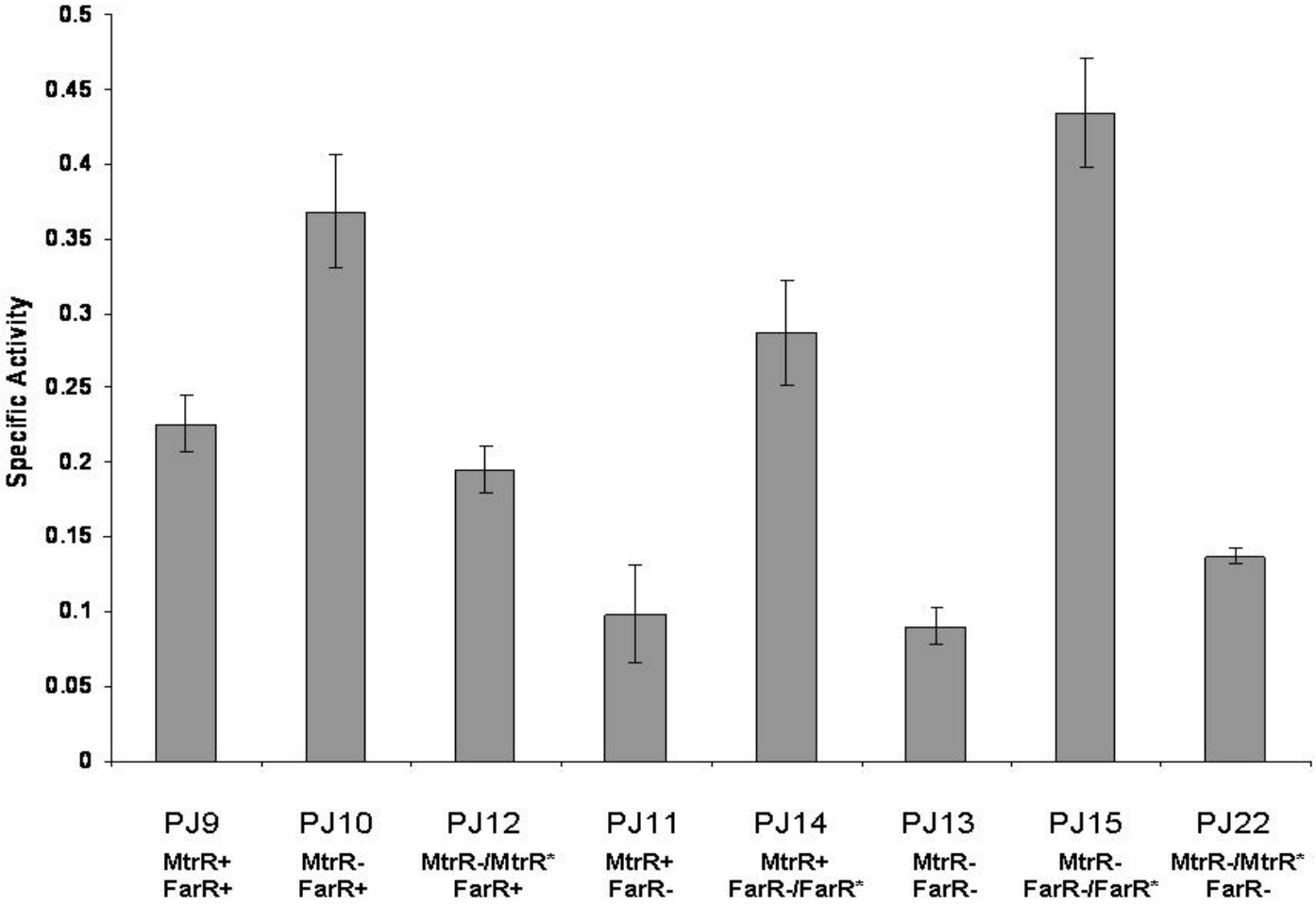

Specific Activity
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PJ9
MtrR+
FarR+
PJ10
MtrR-
FarR+
PJ12
MtrR-/MtrR*
FarR+
PJ11
MtrR+
FarR-
PJ14
MtrR+
FarR-/FarR*
PJ13
MtrR-
FarR-
PJ15
MtrR-
FarR-/FarR*
PJ22
MtrR-/MtrR*
FarR-

Figure 3

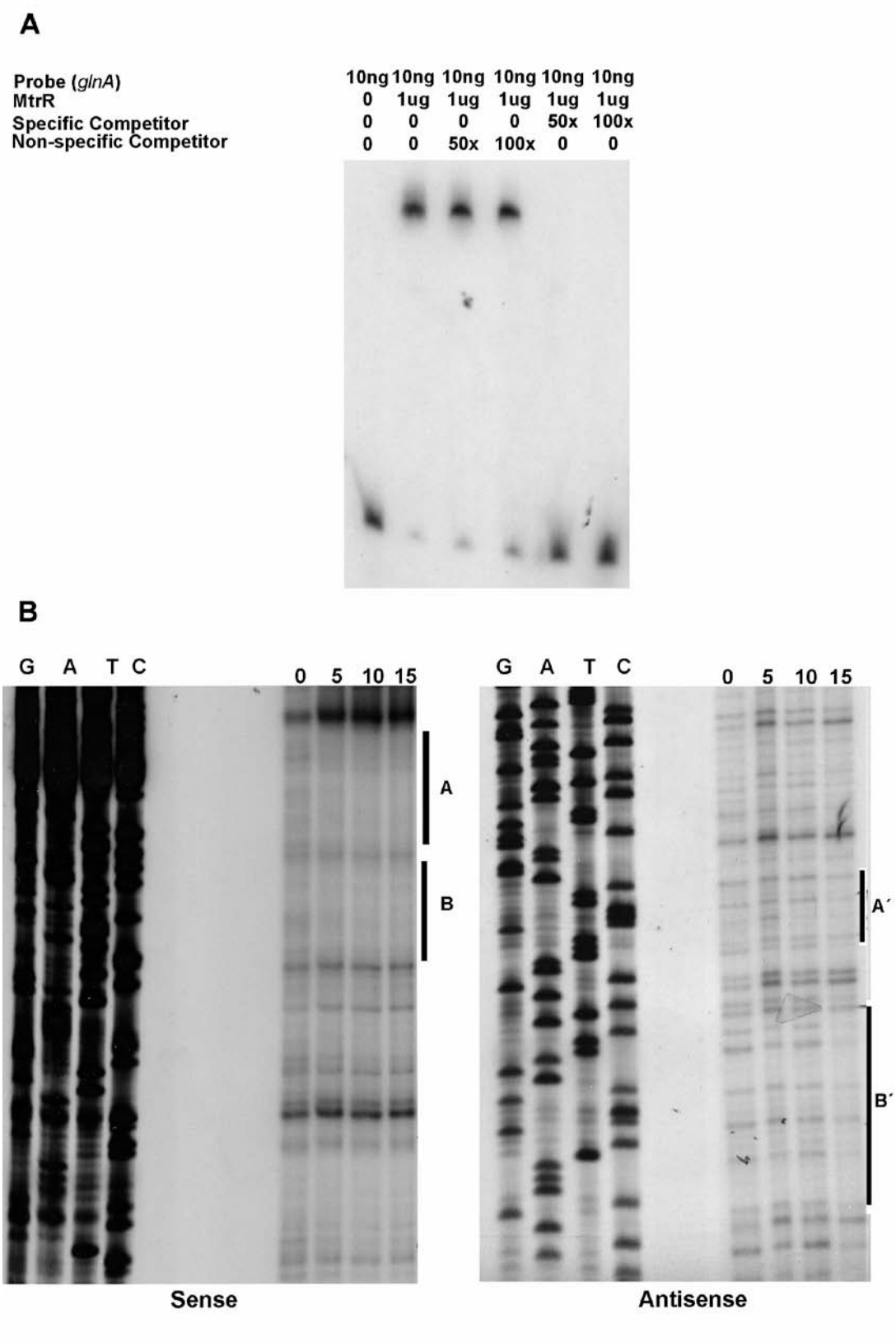

A
Probe (glnA)
MtrR
Specific Competitor
Non-specific Competitor
10ng 10ng 10ng 10ng 10ng 10ng
0 1ug 1ug 1ug 1ug 1ug
0 0 0 0 50x 100x
0 0 50x 100x 0 0
B
G A T C
0 5 10 15
A
B
G A T C
0 5 10 15
A´
B´
Sense
Antisense

Figure 4

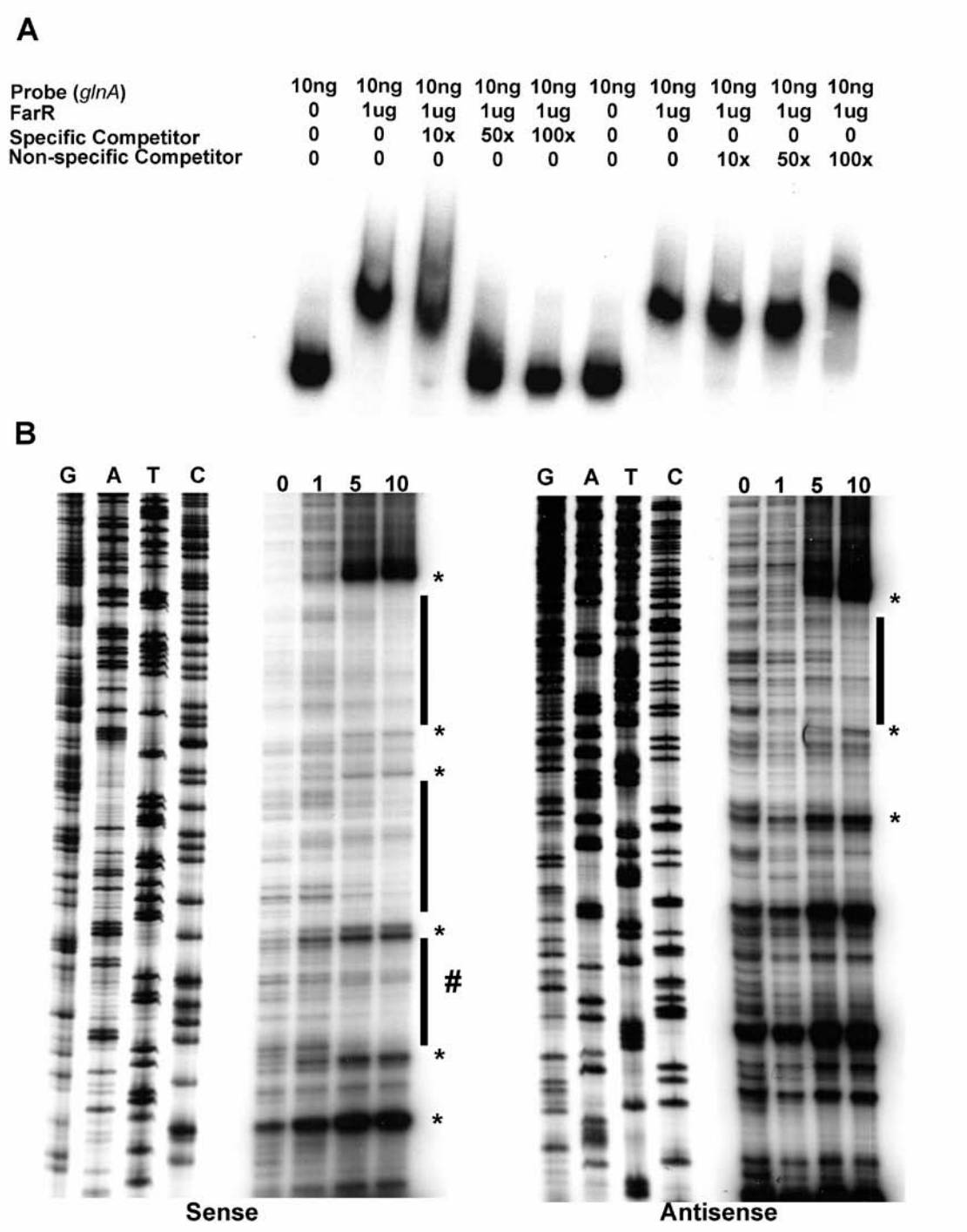

A
Probe (glnA)
FarR
Specific Competitor
Non-specific Competitor
10ng 0 0 0
10ng 1ug 0 0
10ng 1ug 10x 0
10ng 1ug 50x 0
10ng 1ug 100x 0
10ng 0 0 0
10ng 1ug 0 0
10ng 1ug 0 10x
10ng 1ug 0 50x
10ng 1ug 0 100x
B
G A T C
0 1 5 10
*
*
*
*
#
*
*
Sense
G A T C
0 1 5 10
*
*
*
Antisense

Figure 5

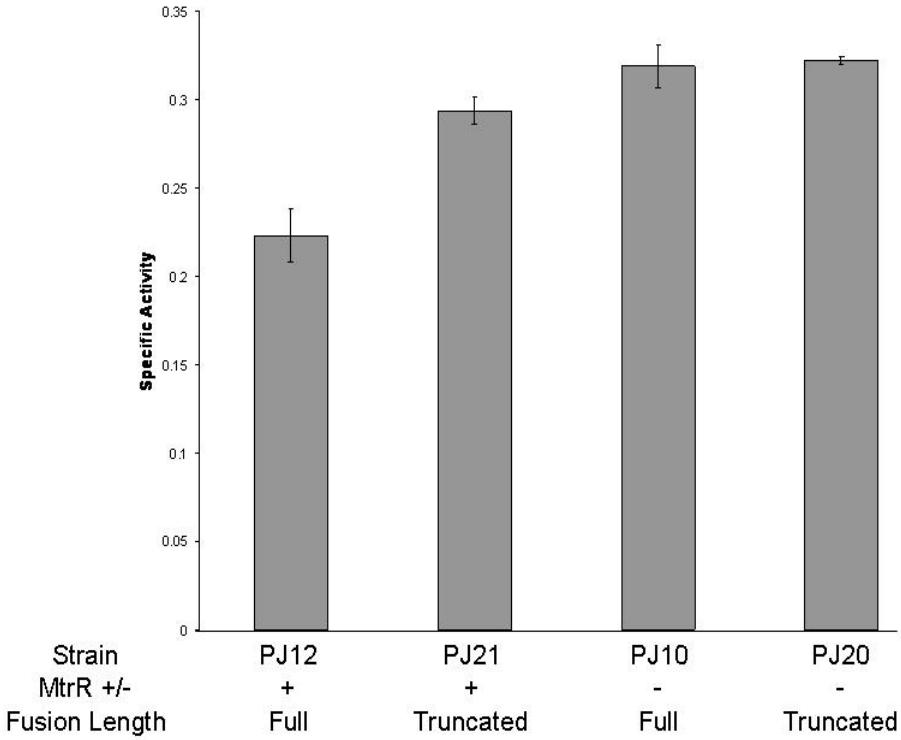

Specific Activity
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Strain
MtrR +/-
Fusion Length
PJ12
+
Full
PJ21
+
Truncated
PJ10
-
Full
PJ20
-
Truncated

Figure 6

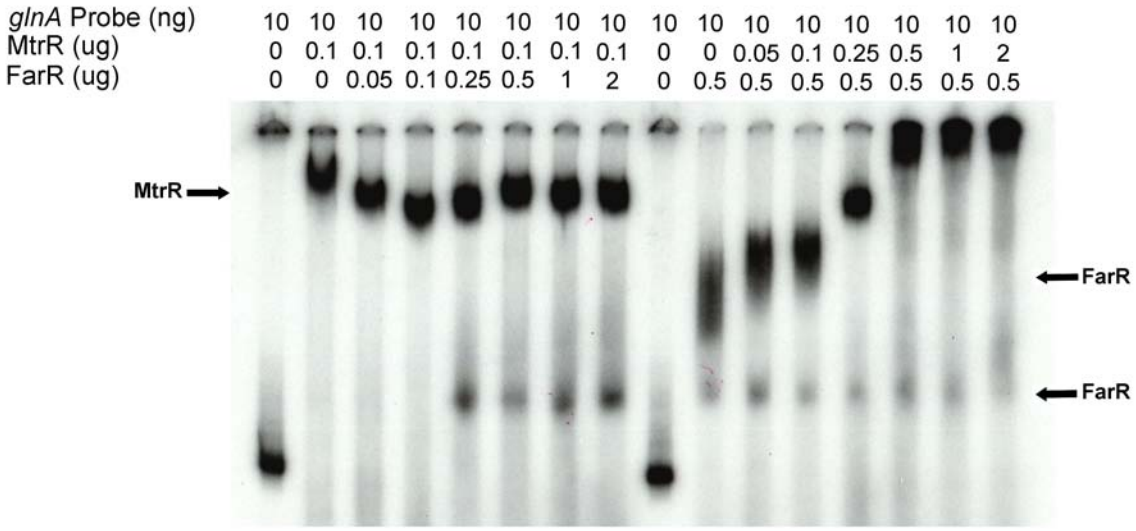
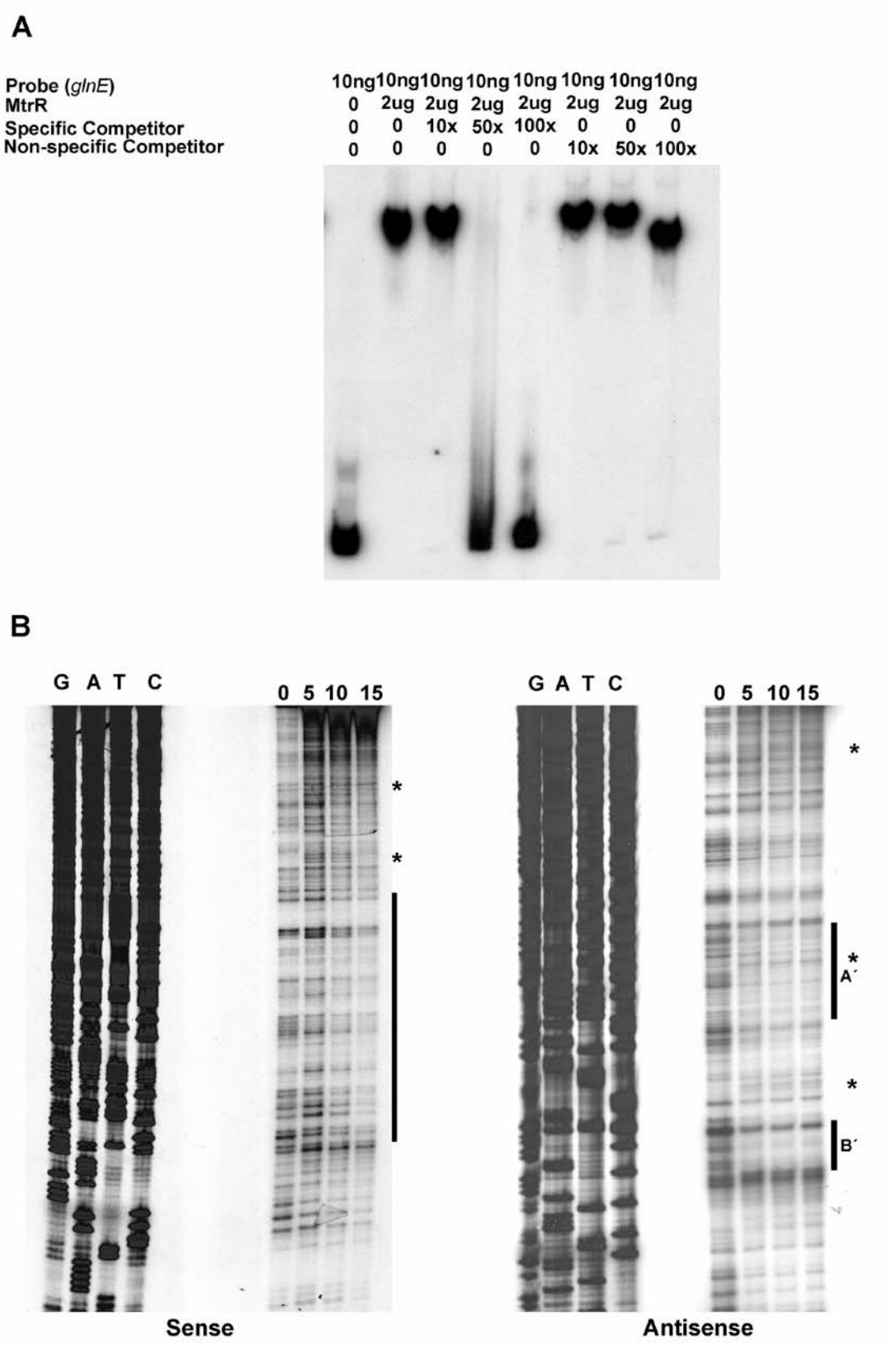

glnA Probe (ng)
MtrR (ug)
FarR (ug)
10 10 10 10 10 10 10 10 10 10 10 10 10 10 10 10
0 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0 0 0.05 0.1 0.25 0.5 1 2
0 0 0.05 0.1 0.25 0.5 1 2 0 0.5 0.5 0.5 0.5 0.5 0.5 0.5
MtrR
FarR
FarR

Figure 7


CAAGTGAGGAGCTGCATGAAATCGTAAAGAGTGATGGTTTTTTTTAAAAATTCCACACGGGAGAAAGGTGC
GTTCACTCCTCGACGTACTTTAGCATTTCTCACTACCAAAAAAATTTTTAAGGTGTGCCCTCTTTCCACG
GGCTTATGCTGTTTTAAAAGAGCGTGGCGAGGTTTGAACGGGAAACGGCGGCATTTGCCGCTGTTTTTTT
CCGAATACGACAAAATTTTCTCGCACCGCTCCAAACTTGCCCTTTGCCGCCGTAAACGGCGACAAAAAAA
-35
-10
ATCGGCAGGCATCCGTCCGAATATCGGGGCAAGGTTTCAGACGGCATCGAAGGTTGCTATGATATAGTGG
TAGCCGTCCGTAGGCAGGCTTATAGCCCCGTTCCAAAGTCTGCCGTAGCTTCCAACGATACTATATCACC
*
A'
CTTGACTTTAAACCGGTACGGCTCCGCCTTGCCCTGATTTGAAGTTCATCCGCTATCACATTCCCGCCAT
GAACTGAAATTTGGCCATGCCGAGGCGGAACGGGACTAAACTTCAAGTAGGCGATAGTGTAAGGGCGGTA
B'
CCTTTCAAACGGAATCCGAAATGTCC
GGAAAGTTTGCCTTAGGCTTTACAGG
M
S

Figure 8


A
Probe (glnE)
MtrR
Specific Competitor
Non-specific Competitor
10ng 10ng 10ng 10ng 10ng 10ng 10ng 10ng
0 2ug 2ug 2ug 2ug 2ug 2ug 2ug
0 0 10x 50x 100x 0 0 0
0 0 0 0 0 10x 50x 100x
B
G A T C
0 5 10 15
G A T C
0 5 10 15
*
*
*
A´
*
B´
Sense
Antisense

Chapter 4: Unpublished Results

I. Microarray Analysis of MtrR, MtrA, and MpeR

As previous work with the MtrC-MtrD-MtrE efflux pump and its regulators had indicated a central importance in determining gonococcal fitness *in vivo* (16, 17), as well as recent evidence of MtrR regulating genes outside of the MtrC-MtrD-MtrE efflux pump (3), the possibility that these systems were more involved in global gene regulation than previously thought became apparent. As such, I set out to determine to what extent this gene regulation exists in the gonococcus in order to better understand the involvement of the regulators of the MtrC-MtrD-MtrE efflux pump in basic gene regulation of the gonococcus.

The regulators studied by microarray analysis include the previously described MtrR, MtrA, and MpeR. Briefly, MtrR is a QacR/TetR family transcriptional regulator that is most commonly associated with its role as a repressor of the *mtrCDE* operon (5, 11). MtrR has been extensively studied in our lab and by other labs. Thus, it has shown that MtrR is a transcriptional regulator of importance in regulation of antimicrobial resistance expressed by gonococci. MtrA is a transcriptional activator of the MtrC-MtrD-MtrE efflux pump system that previously has had no described function beyond its regulation of the *mtrCDE* operon (13). This AraC-like transcriptional regulator acts as an activator of the *mtrCDE* operon under inducing growth conditions, which are defined as exposure to sublethal concentrations of substrates of the efflux pump. Inducible high level resistance to pump substrates is only seen in strains that have a functional *mtrA* gene (13). MpeR is another AraC-like protein that was identified as a transcriptional

regulator that controls expression of the MtrC-MtrD-MtrE efflux pump accessory gene *mtrF* (4). MtrF is necessary for high level resistance mediated by MtrC-MtrD-MtrE efflux pump by a currently unknown mechanism (15).

In order to analyze the differential expression of genes in transformants of strain FA19 with mutations in *mtrR*, *mtrA*, or *mpeR*, versus FA19, we utilized a custom Pan-STD Affymetrix GeneChip, (MPAUT1a520274F). This chip contains not only a *N. gonorrhoeae* genome probe set (1,925 genes), but also probe sets for *Treponema pallidum*, *Chlamydia trachomatis*, *Haemophilus ducreyi*, HSV 1, HSV 2, HIV 1, and HIV 2. Each gene on the array was represented by 11 probe pairs (22 features) consisting of a perfect match probe and a single-nucleotide mismatch probe.

RNA was isolated by hot-phenol extraction (2)from three separate biological replicates of FA19 and isogenic strains with *mtrR* (JF1[Δ*mtrR*]), *mtrA* (CR1[*mtrA::kan*), or *mpeR* (JF5[*mpeR::kan*]) mutations. These strains were grown in GCB until the mid-log phase of growth (OD600=0.6) and the late-log phase of growth (OD600=0.9) (2). These samples were then examined for DNA contamination by PCR and DNase treated to remove contaminating DNA. Once RNA free of DNA contamination was obtained, it was quantified by spectrophotometry (NanoDrop1000 by NanoDrop Technolgies) and RNA quality was determined using an Agilent bioanalyzer (Agilent Technologies). Following isolation and quality control, biotinylated cDNA was generated from 10 μg of total RNA, fragmented, and then hybridized at 45°C for 16 h. The arrays were then stained with streptavidin phycoerythrin, followed by anti-streptavidin antibody and Goat IgG. Finally, the arrays were stained again with streptavidin phycoerythrin for

visualization, scanned (Affymetrix GeneChip Scanner 3000), and the scanned images were processed using Genechip Operating Software version 1.4 (Affymetix, Inc.).

Data files generated by Genechip Operating Software (Affymetix, Inc.) were imported into GeneSpring GX 7.3.1 (Agilent Technologies), normalized, and analyzed. Fold changes were given as the ratio of gene expression of the wild-type strain FA19 over the same gene expression for the mutant strain. Genes with a differential expression of ≥1.5-fold and a *P* value of 0.05 were then selected for further evaluation.

This microarray analysis revealed that all three transcriptional regulators regulate a broad range of genes beyond the classically defined *mtr* system and these genes are scattered throughout the chromosome. These categories included genes involved in pathogenesis, virulence, and stress response as one would predict from previous *in vitro* and *in vivo* work. However, a large number of genes were also identified that are involved in basic cellular metabolism, transport, and nutrient acquisition that were not necessarily expected but also could be of importance during the establishment and maintenance of an infection by a strict human pathogen such as the gonococcus. The results of these microarrays not previously published follow.

MtrR Microarray Results

By far, MtrR was the most global in scope of the regulators studied; it was found to repress 47 genes at the mid-log phase of growth and activate 22 other genes (Chapter 2). Two particular candidate systems, oxidative stress and metabolism, were chosen for further study. MtrR regulated genes that belong to these two categories vary significantly

from the classically defined MtrR regulon and are of central importance to the gonococcus's biology. This work was previously discussed in Chapters 2 & 3.

During the late-log phase of growth, MtrR repressed 24 genes while activating 23 genes (Figure 1). Among these genes were a number of interesting components of various antimicrobial resistance, stress response, and biosynthetic systems. These genes include the genes that encode the MtrC-MtrD-MtrE efflux pump (NGO1363-NGO1365), which were shown in previously published work (Chapter 2) to be repressed at the mid-log phase of growth, and whose repression by MtrR has been studied in our lab in great detail for many years. The inclusion of these genes here at late-log phase is therefore not unexpected and serves to further validate the microarray results obtained. Moreover, MtrR was shown to repress *dhpS* (dihydropteroate synthetase), which is involved in the synthesis of the folic acid derivative tetrahydrofolate and is a target of sulfanomide antibiotics (6, 12). This repression is interesting in that, if direct, it would indicate yet another way in which MtrR may directly affect the antimicrobial resistance of the gonococcus by acting upon a gene involved in a biosynthetic pathway.

Among the genes identified as being activated by MtrR at the late-log phase of growth was *glnE* (glutamine adenylyltransferase), which was also identified by microarray analysis as being activated by MtrR in the mid-log phase of growth. This is not unexpected, as continued regulation of a critical pathway such as glutamine biosynthesis would be necessary for growth of the cell, particularly when nutrients may start to become limited in later phases of growth. The regulation of *glnE* is, in part, the topic of Chapter 3.

MtrA Microarray Results

MtrA was found to repress 14 genes at the mid-log phase of growth while activating 6 genes (Figure 2). During the late-log phase of growth, MtrA directly or indirectly activated 10 and repressed 20 genes (Figure 3). Among these genes were a number that were identified in the MtrR microarray. Interestingly, one of these genes was *glnE,* which was found to be MtrA activated by ~5-8 fold at both mid-log and late-log phases of growth. These are similar to the results from the MtrR microarray. The mechanism of this regulation was not determined in this work; however, two possibilities exist. This regulation could occur via a direct MtrA mediated mechanism or an indirectly mediated MtrA mechanism. Further, regulation by MtrA may be due to the effects of other transcriptional regulators in the MtrR/MtrA/MpeR transcriptional regulatory circuit. Work in our lab has begun on this topic and results indicate that these transcriptional regulators can act upon one another to affect expression levels. Therefore, it may be of interest to follow up on this aspect of *glnE* regulation with DNA binding assays utilizing a purified form of MtrA in order to elucidate this mechanism.

Also of interest among the MtrA microarray results was the regulation of accessory components of the MtrC-MtrD-MtrE efflux pump, but not the structural genes themselves. Previous work with MtrA had not indicated a major role for MtrA in the activation of the MtrC-MtrD-MtrE efflux pump under normal growth conditions, however when a strain containing a wild-type *mtrA* was grown in the presence of sublethal concentrations of certain substrates of the efflux pump and compared to a *mtrA::kan* mutant (CR1) it was found that only the wild-type strain (FA19) could be

induced to elevated levels of antimicrobial resistance (13). The MtrA microarray results seem to confirm that observation since there were no significant changes in expression levels of the *mtrCDE* operon under the normal growth conditions. However, the microarray results implicate MtrA in a possible autoregulatory scheme (1.76 fold activation) at the mid-log phase of growth, which may be beneficial under inducing conditions *in vitro* or during similar types of exposure to host derived antimicrobial compounds during the early stages of colonization *in-vivo*. Further, the gene encoding MtrF, a recently characterized accessory protein of the MtrC-MtrD-MtrE efflux pump, is indicated as being repressed by MtrA at the mid-log phase of growth.

An MtrA-repressed gene of interest is *gltS*, which encodes an L-glutamate permease responsible for the transport of glutamate from the gonococcus's environment. L-glutamate is necessary for the production of glutamine by glutamine synthetase (GlnA) in the reaction NH3+glutamate+ATP → glutamine+ADP+Pi. Like *glnA*, *gltS* is repressed by MtrR at mid-log and in this case is also repressed by MtrA. This overlap in repression of another component of the glutamine biosynthetic pathway is of interest, as limiting the amount of glutamate taken up by the gonococcus could affect the production of glutamine and other amino acids for which glutamate is a precursor molecule such as arginine and proline.

MpeR Microarray Results

MpeR was found to repress 30 genes at the mid-log phase of growth while activating another 16 genes (Fgiure 4). During the late-log phase of growth, this

regulation shifted and 5 genes were activated while 16 genes were repressed (Figure 5). MpeR is also regulated by Fur and Fe3+ and the late-log phase of growth is likely to be iron-starved increasing the expression of MpeR. As such it was not surprising that the array profile would change at late-log. Among the genes repressed by MpeR are two genes that were identified and studied in the MtrR mid-log microarray, *glnA* and *clpB* as well as MtrF.

Previous work in our lab originally identified MpeR as an AraC-like protein that was an important transcriptional regulator of the MtrC-MtrD-MtrE efflux pump accessory gene *mtrF* (4). As such, the inclusion of *mtrF* in the microarray work is not unexpected. Further, its inclusion also helps to validate that the array was working properly as previously identified transcriptional regulatory targets by other, classical, means were also identified by the microarry.

glnA encodes glutamine synthetase and was identified as being MtrR repressed in the MtrR mid-log microarray as well as by translational beta-galactosidase fusion assays (Chapters 2 and 3). Further, the regulation exhibited by MtrR was found to be direct and indirect, via its effects on FarR, which was found to activate *glnA*. Therefore, the effects seen by MpeR could be due to a number of mechanisms that are either direct or indirect and may be of interest to pursue.

In addition to *glnA*, a number of genes involved in response to heat shock were identified in the MpeR microarrays. These genes include *groES*, NG1422, and *clpB*. *clpB* was found to be regulated indirectly by MtrR largely due to the effects of RpoH upon *clpB*. However, it remains possible that in addition to this regulation by RpoH, MpeR could further modulate the expression of *clpB* by direct or indirect means. Indeed,

it was found that MpeR can repress MtrR at the late log-phase of growth in this microarray as well. This could be tested, and may yield further insight into the mechanisms that link resistance to killing by oxidative and non-oxidative means mediated by the *mtr* system (inclusive of its transcriptional regulators) in the gonococcus. Also of interest here at late-log phase, we find that MpeR is shown to be repressing *mtrR* transcription by 1.74 fold, which introduces another level of possible transcriptional regulation to the classical MtrC-MtrD-MtrE efflux pump system.

Lastly, among the array data, a number of genes were identified at mid-log phase of growth as being co-regulated by two regulators studied. However, only 2 genes were found to be regulated by all three transcription regulators of the MtrC-MtrD-MtrE efflux pump system studied. Those two genes were NGO2013, an ABC transporter, and *mtrF*. Work on the regulation of MtrF by the transcriptional regulators examined in this study is ongoing in the laboratory.

II. Construction and Analysis of *glnA/glnE* Mutants

The regulation of *glnA* and *glnE* was discussed in detail in Chapter 3, and as these genes are differentially regulated by MtrR and encode important enzymes for glutamine biosynthesis, an attempt was made to create null mutations in them so biological studies could be conducted. This work was considered to be important as, in addition to glutamine being a vital high energy nitrogen donor in the cell, members of the glutamine synthesis pathway are also important in virulence and general cell health in other bacteria. Glutamine synthetase (*glnA*) mutants of *Salmonella* are less fit *in vivo* but not less virulent compared to wild type (7). Additionally, in *Mycobacterium tuberculosis*

glutamine synthetase is involved in resistance to killing by macrophages (10). Further, glutamine is abundant in the blood but is limited on mucosal surfaces and in phagolysosomes, so its acquisition and production may be important during infection.

glnE

glnE encodes the glutamine synthetase adenylyltransferase gene in *N. gonorrhoeae.* In order to examine its possible importance in the gonococcus's biology I set out to make a non-polar insertional mutation utilizing the *aphA-3* cassette encoding resistance to kanamycin. However, after extensive attempts to recover mutant strains of *N. gonorrhoeae* in the FA19 background with *glnE* disrupted in this manner, I was unable to recover viable cells. Given that *glnE* is an essential gene in *Mycobacterium tuberculosis*, this outcome is not completely without precedent (1). In order to attempt to examine this issue in the future, it may be possible to introduce a second copy of *glnE* under the control of an IPTG-inducible promoter at a second site in the gonococcal chromosome utilizing the pGCC4 complementation system (14). This may allow for disruption of the original copy of the *glnE* while leaving a copy of the gene intact so that expression could be manipulated in order to study its effects on gonococcal biology.

glnA

glnA encodes the glutamine synthetase gene of *N. gonorrhoeae* and is also of central importance to the gonococcus's cellular physiology. In order to examine this further, I attempted to make a non-polar insertional mutation in this gene utilizing the *aphA-3* cassette encoding resistance to kanamycin. Unlike the attempt to construct a

glnE mutant strain, I was able to recover a *glnA* mutant after numerous attempts. The *glnA* mutant isolated showed poor growth on GC agar containing Kellogg's supplements I and II. When the mutant's growth was further examined in GC broth containing Kellogg's supplements, which contains glutamine, it was found that the mutant exhibited an extended lag phase followed by a growth rate that was similar to that of wild-type strain FA19. However, the *glnA::kan* mutant strain did not grow to the same optical density as the wild-type strain FA19 (Figure 6).

Glutamine supplementation in laboratory growth conditions has been standard practice in the culture of *Neisseria* spp. since the 1940s, when it was found that 10-15% of isolates were incapable of being cultured in the laboratory without supplementation of glutamine (8, 9). As such, glutamine is present in Kellogg's supplement at a concentration of 0.03 M L-glutamine before addition to our growth media. I found that FA19 is capable of growing in liquid media and on solid media without glutamine supplementation. The *glnA::kan* mutant strain derived from FA19, however, is unable to grow in media that is lacking in glutamine supplementation. This observation indicates that disruption of *glnA* represents one place in the glutamine biosynthetic pathway in which a mutation may arise that prevents growth without glutamine supplementation similar to what has previously been described in certain blood-borne strains of *N. gonorrhoeae.*

In her studies on MtrF, Wendy Veal in our laboratory noticed that an *mtrF* null mutant had a reduced capacity to transport glutamine (Emory University, doctoral dissertation) and displayed increased susceptibility to antimicrobials recognized by the MtrC-MtrD-MtrE efflux pump. Moreover, the presence of glutamine in the growth

media resulted in enhanced expression of *mtrF* and enhanced the resistance of an *mtrF* null mutant to substrates of the pump. Since *glnA* is subject to MtrR repression and glutamine appeared from W. Veal's work to be important in determining antibiotic resistance, I asked if the *glnA* mutant had altered susceptibility to such antimicrobials. Interestingly, the mutant displayed decreased susceptibility to four antimicrobials (polymyxin B, erythromycin, crystal violet and chloramphenicol) that are pump substrates and this was reversed by complementation when the wild type *glnA* gene was expressed ectopically (Table 1). At first it was thought that such resistance might simply be due to the decreased growth rate of the mutant. Accordingly, to test this hypothesis wild type strain FA19 was grown in 0.25X GCB broth with defined supplements. In this media strain FA19 had a growth rate similar to the *glnA* mutant grown in full-strength broth (data not presented). Interestingly, this reduced growth did not change the susceptibility of strain FA19 to any of the antimicrobials tested (Table 1). Given the link between glutamine and antimicrobial resistance that was established by W. Veal, I asked if the resistance displayed by the *glnA* mutant required a functional MtrC-MtrD-MtrE efflux pump. In order to test this hypothesis, a null mutation was created in the *mtrD* gene. This mutation reversed the impact of the *glnA* mutation on antimicrobial resistance, but did not change the growth rate (data not presented).

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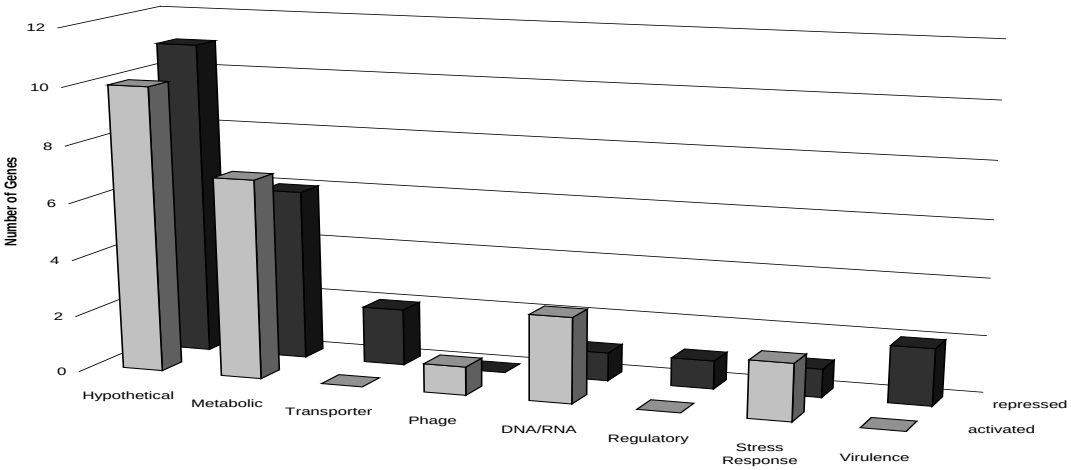
Figure 1. Genes differentially regulated by MtrR during the late-log phase of growth. Genes that are differentially regulated during the late-log phase of growth were determined by microarray analysis comparing FA19 (WT) to JF1 (*ΔmtrR*).



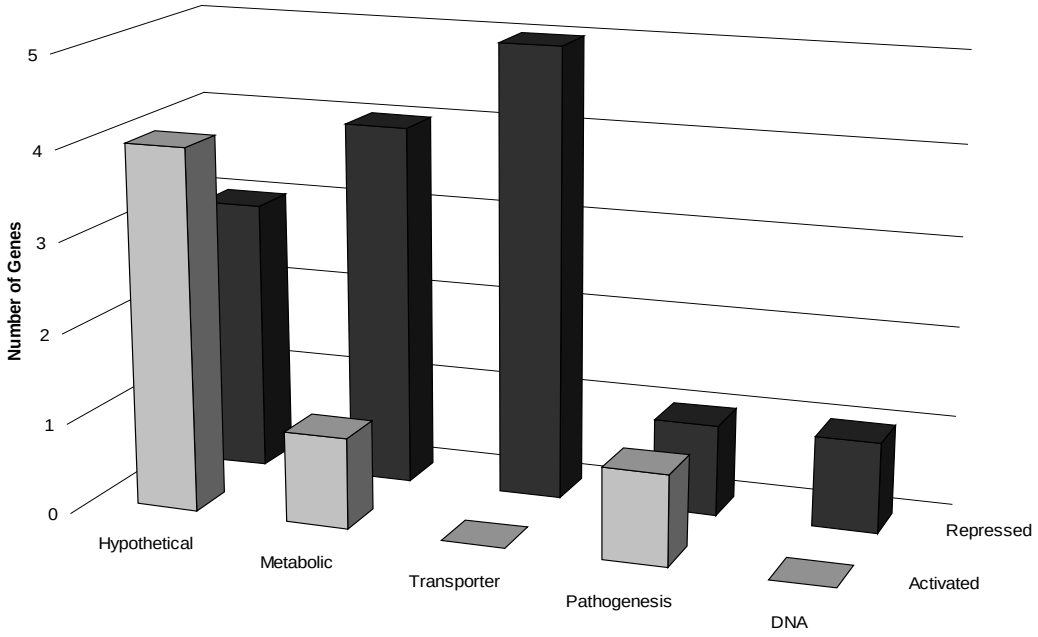
MtrR Repressed Genes

NGO1024		2.34	hypothetical protein
NGO1325		2.14	glycine dehydrogenase
NGO1363	*mtrE*	1.88	antibiotic resistance efflux pump component
NGO1365	*mtrC*	1.86	antibiotic resistance efflux pump component
NGO0924		1.74	hypothetical protein
NGO0308		1.73	putative adenosylmethionine-8-amino-7-oxononanoate aminotransferase
NGO1958		1.73	hypothetical protein
NGO1355		1.72	putative sodium dependent ion transport protein
NGO0545		1.71	putative type III restriction-modification system methyltransferase
NGO1294		1.71	putative AsnC-family transcriptional regulator
NGO1243		1.70	hypothetical protein
NGO1337		1.70	peptide chain release factor 1
NGO1051		1.68	hypothetical protein
NGO0288		1.68	RNA polymerase sigma factor
NGO0302		1.65	hypothetical protein
NGO1336		1.63	D-lactate dehydrogenase
NGO0927		1.62	hypothetical protein
NGO0594	*gcpE*	1.58	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
NGO1251		1.55	hypothetical protein
NGO1364	*mtrD*	1.55	antibiotic resistance efflux pump component
NGO1231		1.53	aconitate hydratase
NGO1443		1.53	hypothetical protein
NGO1342	*dhpS*	1.51	dihydropteroate synthetase
NGO1405		1.51	hypothetical protein

MtrR Activated Genes

NGO1758	*glnE*	8.88	glutamine adenylyltransferase
NGO0818		4.29	hypothetical protein
NGO1170		2.77	conserved hypothetical protein, putative phage associated protein
NGO1270		2.77	conserved hypothetical protein, putative phage associated protein
NGO1559		2.10	hypothetical protein
NGO1567		2.05	quinolinate synthetase
NGO1179		2.01	hypothetical protein
NGO1769	*ccpR*	1.93	cytochrome c peroxidase
NGO1381	Glr2	1.92	glutaredoxin 2
NGO0213		1.78	imidazole glycerol phosphate synthase subunit HisH
NGO0715		1.71	putative glucose 6-phosphate 1-dehydrogenase
NGO1881	*pykA*	1.64	pyruvate kinase II
NGO1258		1.60	phosphoglyceromutase
NGO2035		1.58	hypoxanthine-guanine phosphoribosyltransferase
NGO1594		1.57	hypothetical protein
NGO1907		1.54	hypothetical protein
NGO0367	*radA*	1.54	DNA repair protein
NGO0248	*trpA*	1.53	tryptophan synthetase
NGO1274		1.52	hypothetical protein
NGO1690		1.51	hypothetical protein
NGO0237	*ampD*	1.51	anhydro-N-acetylmuramyl-tripeptide amidase
NGO1932		1.51	hypothetical protein
NGO1608		1.50	hypothetical protein

Figure 2. Genes differentially regulated by MtrA during the mid-log phase of growth. Genes that are differentially regulated during the late-log phase of growth were determined by microarray analysis comparing FA19 (WT) to CR1 (*mtrA::kan*).



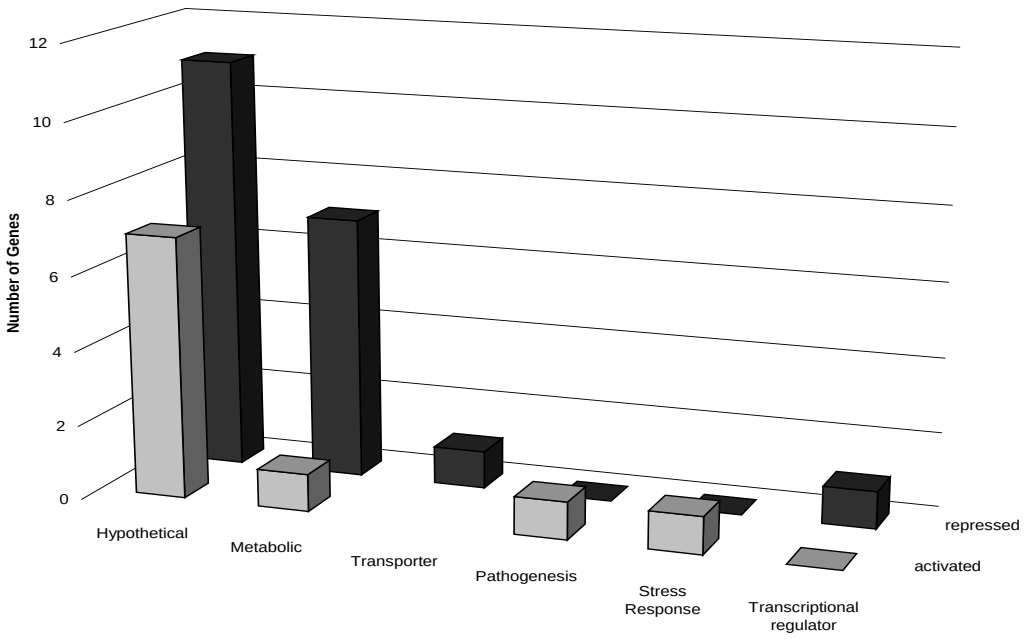
MtrA Activated Genes

NGO1758	*glnE*	5.15	glutamine adenylyltransferase
NGO1249		3.49	hypothetical protein
NGO1248		3.06	hypothetical protein
NGO1250	*mtrA*	1.76	mtrCDE transcriptional regulator, activator
NGO1728		1.51	hypothetical protein

MtrA Repressed Genes

NGO0377		2.79	putative transport protein
NGO1361		2.72	hypothetical protein
NGO1325		2.10	glycine dehydrogenase
NGO1368	*mtrF*	2.07	antibiotic resistance efflux pump component
NGO2011		1.97	putative ABC transporter, permease protein, amino acid
NGO0332		1.86	putative reductase
NGO0655		1.70	exodeoxyribonuclease VII large subunit
NGO1406		1.63	aminomethyltransferase
NGO1405		1.62	hypothetical protein
NGO2013		1.61	putative ABC transporter, ATP-binding protein, amino acid
NGO1552		1.61	putative sodium/proline symporter, proline permease
NGO2012		1.54	putative ABC transporter, permease protein, amino acid
NGO2042		1.51	hypothetical protein

Figure 3: Genes differentially regulated by MtrA during the mid-log phase of growth. Genes that are differentially regulated during the late-log phase of growth were determined by microarray analysis comparing FA19 (WT) to CR1 (*mtrA::kan*).



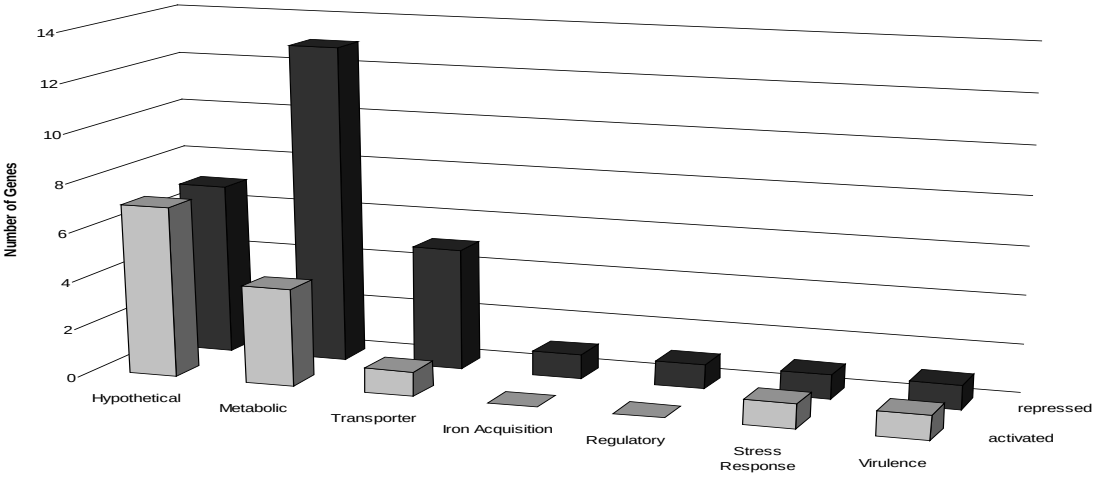
MtrA Activated Genes

NGO1758	*glnE*	8.13	glutamine adenylyltransferase
NGO1249		2.66	hypothetical protein
NGO1248		2.56	hypothetical protein
NGO0466		1.73	hypothetical protein, putative phage associated protein
NGO0225	*mafB1*	1.60	mafB related adhesin
NGO1139		1.56	conserved hypothetical protein, putative phage associated protein
NGO1166		1.56	conserved hypothetical protein, putative phage associated protein
NGO1264		1.56	conserved hypothetical protein, putative phage associated protein
NGO1645		1.56	conserved hypothetical protein, putative phage associated protein
NGO2124		1.54	putative thioredoxin

MtrA Repressed Genes

NGO1325		2.87	glycine dehydrogenase
NGO1024		2.04	hypothetical protein
NGO1384		2.02	hypothetical protein
NGO1176		1.90	hypothetical protein
NGO1231		1.85	aconitate hydratase
NGO2042		1.72	hypothetical protein
NGO0309		1.71	dithiobiotin synthetase
NGO2003		1.68	hypothetical protein
NGO2136		1.66	hypothetical protein
NGO0025		1.64	putative AraC-family transcriptional regulator
NGO1240		1.64	histidinol dehydrogenase
NGO1243		1.62	hypothetical protein
NGO0776		1.61	hypothetical protein
NGO1890	*gltS*	1.61	L-glutamate permease
NGO0308		1.59	putative adenosylmethionine-8-amino-7-oxononanoate aminotranferase
NGO1232	*otcC*	1.57	ornithine carbamoyltransferase
NGO0806		1.56	hypothetical protein
NGO0220		1.52	putative UTP--glucose-1-phosphate uridylyltransferase
NGO1087		1.51	hypothetical protein, putative phage associated protein
NGO0302		1.50	hypothetical protein

Figure 4. Genes differentially regulated by MpeR during the mid-log phase of growth. Genes that are differentially regulated during the late-log phase of growth were determined by microarray analysis comparing FA19 (WT) to JF5 (*mpeR::kan*).



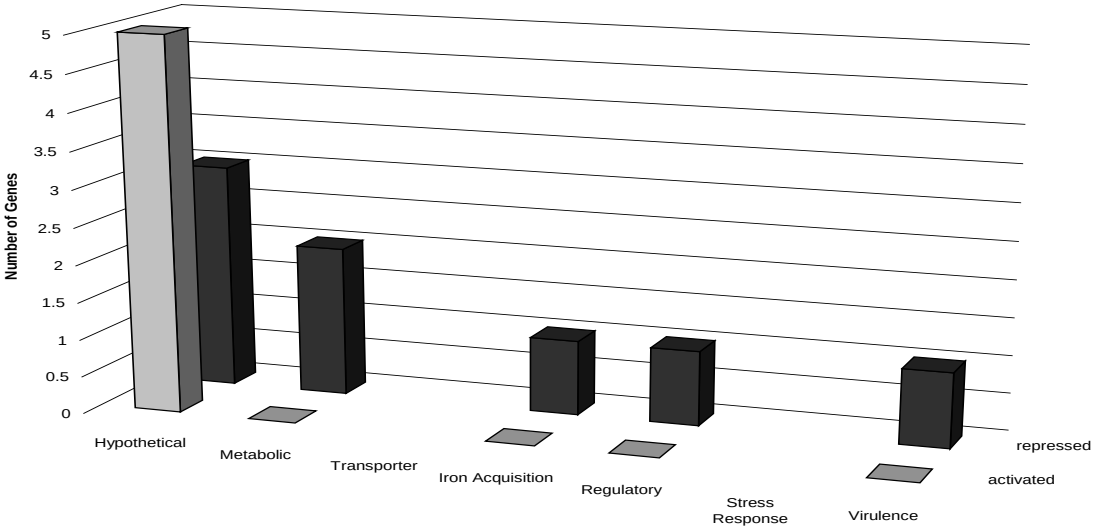
MpeR Activated Genes

NGO1059		2.17	hypothetical protein
NGO0876		1.90	hypothetical protein
NGO0820		1.75	hypothetical protein
NGO0567		1.69	putative hydrolase
NGO1058	*surE*	1.64	acid phosphotase
NGO0958		1.62	putative ribonuclease PH
NGO1810		1.62	hypothetical protein
NGO0694		1.62	hypothetical protein
NGO0527		1.58	hypothetical protein
NGO0606		1.58	putative sodium-dependent transport protein
NGO2132		1.58	hypothetical protein
NGO0221		1.57	putative deoxyribonucleotide triphosphate pyrophosphatase
NGO1406		1.55	aminomethyltransferase
NGO1488		1.53	hypothetical protein
NGO2167		1.51	hypothetical protein

MpeR Repressed Genes

NGO0795	*bfrB*	2.24	bacterioferritin B
NGO1368	*mtrF*	2.22	antibiotic resistance efflux pump component
NGO2014		2.15	putative ABC transporter, periplasmic binding protein, amino acid
NGO0891		1.95	hypothetical protein
NGO1600	*glnA*	1.95	glutamine synthetase
NGO2094	*groES*	1.84	heat shock protein
NGO2013		1.82	putative ABC transporter, ATP-binding protein, amino acid
NGO1416		1.80	NADH-ubiquinone oxidoreductase
NGO0373		1.76	putative ABC transporter, permease protein, amino acid
NGO0863		1.71	hypothetical protein
NGO1422		1.71	putative heat shock protein
NGO1494		1.71	putative ABC transporter, periplasmic binding protein, polyamine
NGO1418		1.70	Na(+)-translocating NADH-quinone reductase subunit F
NGO1685		1.68	hypothetical protein
NGO1046	*clpB*	1.66	heat shock chaperone
NGO1780		1.66	hypothetical protein
NGO0916		1.65	dihydrolipoamide acetyltransferase
NGO1665		1.62	branched-chain amino acid aminotransferase
NGO0205	*lolA*	1.60	outer membrane lipoprotein carrier protein
NGO0678		1.59	hypothetical protein
NGO0754		1.56	putative molybdopterin-guanine dinucleotide biosynthesis protein
NGO0018		1.54	hypothetical protein
NGO1770	*prlC*	1.54	oligopeptidase A
NGO1428		1.53	hypothetical protein
NGO0393		1.52	putative TetR-family transcriptional regulator
NGO1749		1.52	NADH dehydrogenase subunit C
NGO0679		1.51	isopropylmalate isomerase large subunit
NGO1273		1.51	hypothetical protein
NGO1765	*pglA*	1.51	glycosyltransferase
NGO1809		1.50	valyl-tRNA synthetase

Figure 5. Genes differentially regulated by MpeR during the late-log phase of growth. Genes that are differentially regulated during the late-log phase of growth were determined by microarray analysis comparing FA19 (WT) to JF5 (*mpeR::kan*).



MpeR Activated Genes

NGO1179	1.97	hypothetical protein
NGO1270	1.95	conserved hypothetical protein, putative phage associated protein
NGO1170	1.95	conserved hypothetical protein, putative phage associated protein
NGO1151	1.63	hypothetical protein
NGO1498	1.58	hypothetical protein

MpeR Repressed Genes

NGO1951 *prfB*	2.40	peptide chain release factor 2
NGO0672	1.98	hypothetical protein
NGO0365	1.94	site-specific DNA-methyltransferase M.NgoVII
NGO1176	1.88	hypothetical protein
NGO2042	1.87	hypothetical protein
NGO1915	1.81	3-deoxy-D-manno-octulosonic-acid transferase
NGO1313	1.76	hypothetical protein
NGO1366 *mtrR*	1.74	mtrCDE transcriptional regulator, repressor
NGO1342 *dhpS*	1.67	dihydropteroate synthetase
NGO1847	1.65	hypothetical protein
NGO1771	1.63	hypothetical protein
NGO1107	1.62	hypothetical protein, putative phage associated protein
NGO1159	1.59	hypothetical protein
NGO1481	1.52	hypothetical protein
NGO0924	1.51	hypothetical protein
NGO0952	1.50	putative TonB-dependent receptor protein

Figure 6. Growth profile of strain FA19, FA19 *glnA::kan*, and FA19 *glnA::kan/glnA**. Strains FA19, FA19 *glnA::kan*, and FA19 *glnA::kan/glnA** grown in liquid broth containing Kellogg's Supplements 1 and 2 for 24 hours. The FA19 *glnA::kan*, mutant shows an extended lag phase followed by normal growth rate, but does not reach the same optical density as the wild type parental strain FA19. When the *glnA::kan* mutation is complemented with *glnA* under its native promoter's control, utilizing the pGCC3 complementation vector integrated at the *proAB* locus, the growth profile returns to near wild type like.

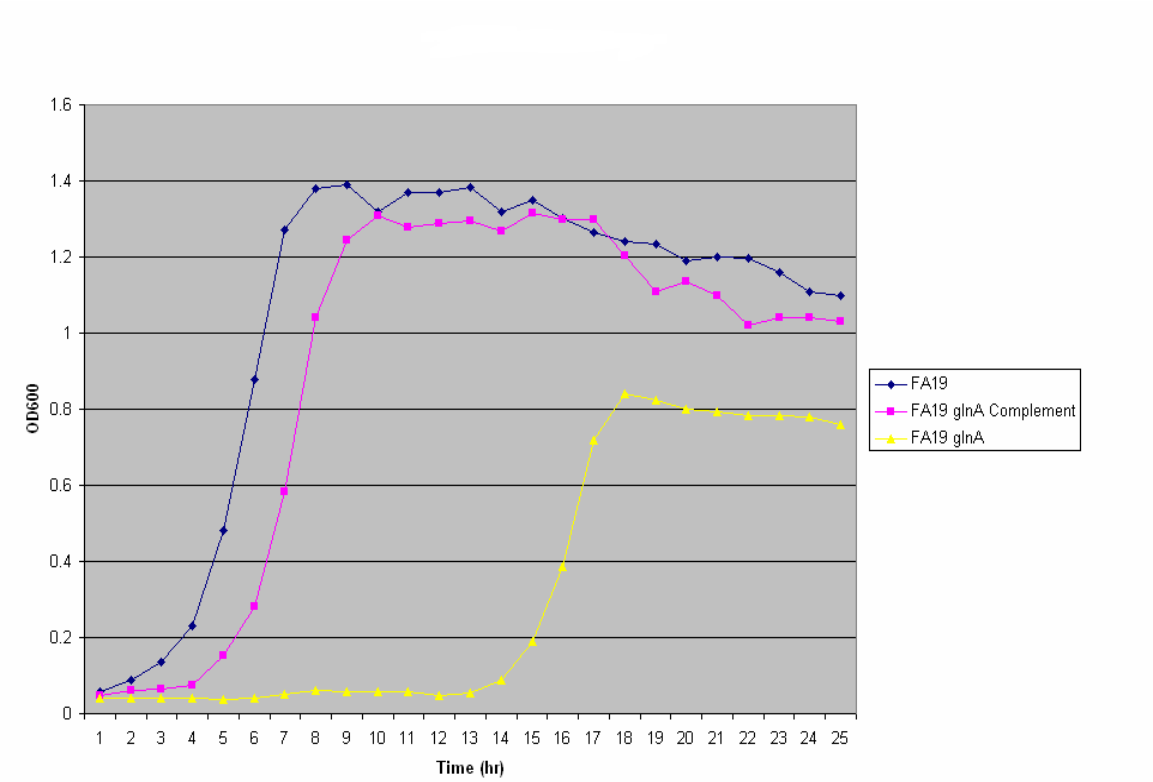

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Time (hr)
FA19
FA19 glnA Complement
FA19 glnA

Table 1. Antimicrobial susceptibility of *glnA::kan* and *glnA::kan/glnA** (pGCC3 *glnA*) compared to parental strain FA19 and Δ*mtrR* strain JF1. The pGCC3 vector carries the erm resistance cassette and the * in the table indicates resistance mediated by this cassette. FA19 1:4 indicates strain FA19 that was grown in 0.25x GCB broth in order to mimic the growth profile of the *glnA::kan* mutant strain.

	Chloramphenicol	Crystal Violet	Erythromycin	Penicillin	Polymixin B	TX-100
FA19	0.5	2.5	0.25	0.0075	62	125
JF1 (Δ*mtrR*)	1	5	1	0.015	125	1000
glnA::kan	2	5	2	0.0075	1000	125
*glnA::kan/ glnA**	0.5	2.5	4*	0.0075	62	125
FA19 1:4	0.5	ND	0.125	ND	62	62.5

Chapter 5: Discussion

The work presented here has focused on characterizing the regulation exhibited by the transcriptional regulator MtrR on genes not previously identified as being under its control. Since its original identification 37 years ago (31), the investigation of the *mtr* system's true identity and function has been an ongoing project in our and other laboratories. MtrR was originally identified in *Neisseria gonorrhoeae* as a local regulator of the genes encoding the MtrC-MtrD-MtrE efflux pump (21, 38). We now know that MtrR plays an important role in gene regulation in this strict human pathogen. This well adapted human pathogen has become a significant public health burden due to its antibiotic resistance levels. In 2007, the CDC added *N. gonorrhoeae* to its list of "superbugs" following its acquisition of clinically relevant levels of resistance to all but one class of antimicrobial agents available for treatment (3-5). The MtrC-MtrD-MtrE efflux pump performs an important role in the development of gonococcal resistance to certain antibiotics. In this respect, the regulatory action of MtrR in modulating such resistance is an important consideration.

Studies in a variety of bacterial efflux pump systems have demonstrated that mutations that abrogate the function of transcriptional repressors can occur and result in increased resistance to antimicrobial compounds exported by these pumps (16). This is also true of the *mtr* system of *N. gonorrhoeae* as disruptions that occur in the gene encoding MtrR lead to higher levels of antibiotic resistance through loss of function of MtrR and increased expression of the *mtrCDE* operon. These mutations, however, do not lead to the same level of antimicrobial resistance as those *cis*-acting mutations that occur in the intergenic region between *mtrR* and the *mtrCDE* operon (49). An important link

between gonococcal mechanisms of antimicrobial resistance and pathogenesis was identified in studies that established a connection between antimicrobial efflux pumps, their regulators, and the ability to establish infections in the female BALB/c mouse model (48). In this model, loss of MtrR has been shown to increase the fitness of the gonococcus in early stages of infection. This increased fitness is presumably due to the increased expression of the *mtrCDE* operon, whose product is able to recognize and export a number of structurally diverse antimicrobial agents including certain host derived antimicrobial compounds (such as LL-37) (44).

In addition to the study that established the importance of MtrR in a female BALB/c model (48), our data showed that MtrR could regulate genes beyond the *mtrCDE* locus, including the *farR* (29), *pilMNOPQ* and *ponA* loci (13) (Figure 1). The first target outside of the *mtr* system identified as being regulated by MtrR was the gene encoding the transcriptional regulator FarR, which acts as a repressor of the *farAB* operon (29). The FarA-FarB efflux pump system is responsible for efflux of long chain fatty acids (30) that the gonococcus is sensitive to and this system is indirectly regulated by MtrR. Other previously identified MtrR regulatory targets studied outside of the classical efflux pumps are the *pilMNOPQ* (MtrR-repressed) and the *ponA* (MtrR-activated) loci (13). These two loci are important in the ability of MtrR to modulate levels of resistance to antimicrobial compounds as the *pilMNOPQ* locus contains the *pilQ* gene, which encodes the pilus secretin (9). In addition to functioning as the pore through which the pilus extends, PilQ enables a number of antimicrobial agents to gain access to the cell (6, 51). The *ponA* gene encodes penicillin binding protein 1 (PBP-1) that, in addition to

being a target of penicillin, is an essential gene involved in the cross linking of peptide groups during peptidoglycan synthesis (40).

MtrR's regulation of genes beyond the classically defined *mtrCDE* operon, coupled with the lack of ubiquitous *mtrR* mutant strains, led us to hypothesize that MtrR may be responsible for regulating genes in the gonococcus that would make its retention beneficial at certain times during an infection, or at certain infection sites. This proposed function is in addition to regulating genes whose increased expression would confer an initial fitness advantage *in vivo*. Therefore, I chose to characterize the extent to which MtrR, and, to a lesser extent, other known regulators of the *mtrCDE* locus, control gene expression in the gonococcus in order to better understand gene regulation in this human pathogen. The results of this work were reported in Chapters 2 and 4. This work lead to the discovery that MtrR regulates a significant number of genes, either directly or indirectly, that are involved in divergent aspects of gonococcal biology including biosynthesis, transport, and stress response.

The observation that loss of MtrR leads to an initial fitness advantage *in vivo* is a benefit originally ascribed to the loss of repression of the *mtrCDE* operon and is supported by the evidence that loss of MtrR activity results in elevated resistance to antimicrobial compounds (20, 44), including host-derived antimicrobial compounds, via the action of the MtrC-MtrD-MtrE efflux pump. The data presented in Chapters 2, 3, and 4 indicates that the initial benefit conferred by loss of MtrR is perhaps more complex than previously hypothesized. In order to evaluate this new hypothesis, we began by examining the regulation of *rpoH* (Chapter 2). *rpoH* was identified in our microarray work as potentially being regulated by MtrR and regulation of this gene could help form

the basis for a link between resistance to oxidative and non-oxidative killing resistance mechanisms in the gonococcus.

rpoH encodes the alternative sigma factor (sigma 32) in both *E. coli* and *N. gonorrhoeae*. In *E. coli*, RpoH is an important mediator of the response to heat shock, general stress response, oxidative stress, and resistance to lysis by some beta-lactams (27, 39). Due to its central importance in the cellular response to various forms of stress, *rpoH* regulation is complicated. The most thoroughly studied aspect of regulation of RpoH production has been post-transcriptionally by way of a thermosensor in the mRNA, increased translation, and loss of DnaK inhibition (15, 34, 35) . However, other work has identified an *rpoH* promoter that is intricately regulated by a number of mechanisms, including recognition by sigma 70 and sigma E, as well as transcriptional regulation mediated by CRP and DnaA (12, 36). Among these mechanisms of transcriptional regulation is repression of the transcripts from the *rpoH* promoter by the transcriptional regulator CytR. Under normal conditions, CytR binding to the *rpoH* promoter region results in a three-fold reduction in transcription from the P3, P4 and P5 promoters, while over-production of CytR results in a ten-fold reduction in transcription from the P3, P4, and P5 promoters (24). In the gonococcus, *rpoH* is an essential gene whose product is required for growth under normal growth conditions as well as in response to various stressors (10, 17). RpoH responds to various forms of stress in the gonococcus including heat shock and growth during cellular contact (10, 11, 17). In the early stages of infection, the gonococcus is exposed not only to stress from cellular contact, but also elevated temperature, if the patient is febrile, as well as a massive oxidative and non-oxidative killing response from the influx of neutrophils (45). As non-oxidative and

oxidative killing mechanisms are both expressed by neutrophils, we thought it possible that MtrR may play a role in regulating genes involved in the response to oxidative killing in addition to its role in regulating genes involved in the response to non-oxidative killing (e.g., that mediated by antimicrobial peptides).

In studying the regulation of *rpoH* by MtrR, we found that MtrR repressed *rpoH* expression and that inducible expression of MtrR could lead to decreased production of RpoH, resulting in decreased resistance of gonococci to certain oxidative killing mechanisms studied, i.e. hydrogen peroxide. As indicated previously, loss of MtrR results in increased fitness in the *in vivo* BALB/c mouse model, whereas loss of a structural component of the MtrC-MtrD-MtrE efflux pump results in lower fitness in the female BALB/c mouse model. However, it seems likely that loss of MtrR also results in increased fitness in the early infection cycle due to the loss of repression of *rpoH* in addition to the loss of repression of the *mtrCDE* operon. This resistance scheme seems likely since the antigonococcal factors that are countered by RpoH are present during the early stages of infection, particularly in the phagolysosome and during the oxidative burst (7, 37, 42, 43, 46). This increased expression of *rpoH*, and corresponding increase in expression of members of the RpoH regulon that help protect against the effects of heat shock or exposure to reactive oxygen species (ROS) such as *grpE* and *clpB*, would occur in a *mtrR* mutant strain. In this manner, an *mtrR* mutant would confer further characteristics upon the gonococcus that would likely be beneficial in the early stages of infection.

The heat shock chaperone gene *hsp33* (23) was also identified as being an MtrR repressed gene. While we did not explore the regulation of this gene by MtrR in detail, a

potential MtrR binding site was identified in the putative promoter region of *hsp33* (Chapter 2). If MtrR regulates *hsp33*, either directly or indirectly, then loss of MtrR could result in increased expression of *hsp33* which, like RpoH and the RpoH regulated genes, could be part of the observed increase in fitness during the early part of an infection cycle.

The regulation of *rpoH* by MtrR represents the first evidence of MtrR linking the mechanisms of resistance to oxidative and non-oxidative killing in the gonococcus and indicates that MtrR may be acting in a capacity that regulates the general stress response systems of the gonococcus in addition to its previously identified functions (Figure 1). However, due to the lack of ubiquitous mutations in the *mtrR* gene, even though loss of MtrR or MtrR functionality confers an initial survival advantage, we hypothesized that MtrR has been maintained by the majority of gonococcal strains because it regulates, directly or indirectly, genes that are either important in later stages of infection or perhaps at different infection sites. In the studies undertaken to examine the extent of the MtrR regulon, a number of classes of genes were identified that could be relevant to this hypothesis (Chapters 2 and 4).

Among QacR/TetR transcriptional regulators, such as MtrR, the ability to regulate a number of diverse cellular processes that are important during an infection is not without precedent. For example, the CmeR regulon of *Campylobacter jejuni* consists of 28 genes, but CmeR is so vital that its loss results in a colonization deficiency in the host. This deficiency occurs even though, presumably, the CmeA-CmeB-CmeC efflux pump encoding operon would be relieved of repression, resulting in greater production of this efflux pump and greater resistance to its substrates (18). Strains of gonococci that do not

produce a functional MtrR do not exhibit this extreme result; however, after an initial fitness advantage, it has been observed that the recovery and fitness of these strains does begin to decrease after day seven in *in vivo* gonococcal infections in the female BALB/c mouse model (48). These two observations about CmeR and MtrR suggest that the retention of these transcriptional repressors could be due to effects that they have on other genes which are not initially understood when these regulators are viewed solely in the context of their originally identified regulatory targets. The key to understanding the reason for the continued retention of these types of transcriptional repressors therefore likely rests with the other genes that they regulate.

One facet of gonococcal physiology that was chosen for further study, due to its deviation from the originally identified MtrR target genes and its vital function in the gonococcus, was metabolism. When RNA was isolated from cells at the mid-log phase of growth or late-log phase of growth and gene expression was examined by microarray, we found 16 metabolism genes regulated by MtrR (Chapter 2) and 11 genes that were regulated by MtrR (Chapter 4), respectively. Genes involved in the biosynthesis of thiamine, tryptophan, and glutamine were among these genes identified. Interestingly, of these genes, only *glnE*, the glutamine adenyltransferase, was found to be significantly regulated at both phases of growth (7.04 fold at mid-log and 8.88 fold at late-log). When the genes involved in glutamine biosynthesis or acquisition were investigated more closely, it became apparent that MtrR was intricately involved in regulating the production and acquisition of this amino acid. Not only was *glnE* regulated by MtrR, but also *glnA* (glutamine synthetase), *glnM* (glutamine permease), *glnQ* (glutamine transporter ATP binding protein), and *gltS* (L-glutamate permease). Due to the number

of glutamine synthesis, regulation, and transport genes in the MtrR regulon identified by microarray analysis, this pathway was selected for further study. In particular, this work focused on *glnA* and *glnE*. It was found that MtrR can directly activate *glnE* and indirectly represses *glnA*. MtrR-mediated repression of *glnA* involves FarR, the transcriptional repressor of the *farAB* efflux pump operon (29). As is described in Chapter 3, FarR can activate *glnA*, but its binding to a target DNA sequence upstream of the *glnA* promoter was blocked or reduced in the presence of MtrR. Since MtrR represses *farR* expression (29), mutations that abrogate MtrR function or production result in elevated expression of *glnA*. The results of this study were discussed in detail in Chapter 3, but this work clearly shows that MtrR's regulatory effects are more significant than previously thought and implicate MtrR as a transcriptional regulator that is involved in cellular processes more diverse than general stress response or efflux of antimicrobial agents.

The regulation of biosynthesis genes is not without precedent among QacR/TetR family regulators, as AmtR of *Corynebacterium glutamicum*, an organism used in the industrial production of glutamine, is known to regulate a number of genes involved in the production of amino acids (1). Interestingly, *glnA* (glutamine synthetase) is among the genes identified as being repressed by AmtR. Further, AmtR was found to be involved in the transcriptional regulation of a number of glutamate and glutamine acquisition and biosynthetic pathway genes, similar to what we found with MtrR (1, 14). This introduces the possibility that MtrR, and perhaps other QacR/TetR transcriptional regulators in other bacteria, is maintained due to an involvement in the regulation of basic biosynthetic pathways. In both the AmtR and the MtrR regulons, the genes regulated are

mostly repressed, including *glnA*. In contrast to this, *glnE* expression is activated by MtrR at both mid-log and late-log phases of growth. This activation is likely to be directly mediated by MtrR's interaction with the DNA region upstream of *glnE* (Chapter 3). By DNase I protection assays, we found that MtrR could bind three sites within and flanking the *glnE* promoter. Interestingly, these sites were on the sense and anti-sense strands, but complementary sequences on the opposing strand were not protected. Additionally, we noted two interesting features in the *glnE* promoter. First, we noted the presence of an extended -10 motif that could mitigate the impact of a poor -35 element identified from the annotated sequence (25). Previous work in *N. gonorrhoeae* has observed this extended -10 motif and established its ability to direct high levels of transcription which leads to hypothesize that this may play a role in regulation of the *glnE* promoter (22). Secondly, we identified a possible alternative -35 hexamer sequence that is more consistent with the sigma-70 consensus sequence -35 hexamer in *E. coli*. This alternative -35 element results in a sub-optimal 21 nucleotide spacing between the -10 and -35 hexamers. Accordingly, we propose that binding of MtrR to this region could enhance interaction of the promoter with RNA polymerase leading to increased transcription of *glnE*. However, further work will be required to determine the exact mode of activation and to determine if all three MtrR binding sites identified are required for activation of *glnE* by MtrR.

This project has also indicated that *glnA* is non-essential, while *glnE* appears to be essential in the gonococcus (Chapter 4). This is not without precedent as in *Mycobacterium tuberculosis glnE* is essential, while *glnA* is not (2). Moreover, in *Salmonella typhimurium*, while not essential, loss of *glnE* results in serious growth

defects and significant fitness costs (50). The reason for *glnE*'s central importance in these two examples is likely due to a combination of factors that originate with the glutamine precursor molecule, glutamate.

Glutamate is an important precursor in many biosynthetic pathways such as the production of amino acids including glutamine, proline, and arginine as well as polyamines. As glutamine synthetase is synthesized in an active form, it immediately begins synthesizing glutamine from glutamate and NH_3 following its translation. If the *glnE* gene, which encodes the regulator of glutamine synthetase activity PII, is disrupted, then glutamine synthetase activity would proceed unregulated in the cell. This could result in the depletion of the glutamate pool and the over-production of glutamine to the exclusion of proline, arginine, and their subsequent products (e.g., polyamines), which are vital in cellular functions. In addition, depletion of the glutamate pool in *Salmonella typhimurium* results in a disruption of the K+ pool and a much abrogated growth rate (8, 50). Given the possible negative consequences of disregulated glutamine synthetase production and regulation as well as the importance of being able to adapt to new environments in which nutrient availability varies, it seems likely that MtrR's involvement in fine-tuning expression of *glnA* and *glnE* has contributed to its maintenance in gonococci. This involvement may also partially explain the decreasing fitness seen in the female BALB/c mouse model after the seven-day time point that has been observed in strains where the *mtrR* gene is disrupted (48).

Additional classes of genes were identified in this work that may contribute to the understanding of why MtrR is maintained in the gonococcus. Among these MtrR-activated is *recQ*, which is involved in DNA repair (26, 32, 47). In the gonococcus,

external sources of DNA damage vary widely, but can include damage from ROS generated during the acute infection phase by the host's innate immune response. DNA repair in general is of great importance throughout the gonococcus' life cycle (28) and MtrR may be involved in regulating this important aspect of gonococcal biology. RecQ is also partly responsible for the antigenic and phase-variable nature exhibited by the gonococcus's pilus (26, 32). This unique feature of the gonococcus occurs when silent copies, *pilS*, of the actively transcribed main structural gene of the pilus, *pilE*, recombine with one another and result in unique DNA sequences that ultimately lead to variation in the protein PilE. This mechanism bestows a survival advantage upon the gonococcus as the host is unable to mount an effective immune response based on recognition of previous exposure (19, 33, 41). This inability occurs because the majority of the surface-exposed pilin protein is composed of this highly variable component. As such, RecQ represents an MtrR regulated gene that would be important for both initial colonization and long term survival of the gonococcus.

The studies carried out on the MtrR regulon beyond the *mtrCDE* operon presented here, and by others, have found MtrR to be a global regulatory protein of significant importance in the gonococcus. This work has established the importance of MtrR in regulating genes involved in resistance to both oxidative and non-oxidative mechanisms, the importance of MtrR in regulating at least one key biosynthetic pathway, and produced a working hypothesis to explain the observed affects of MtrR mutants in *in vivo* mouse infection model studies. It has also provided a hypothesis for the maintenance of MtrR, even though its loss provides increased resistance to oxidative and non-oxidative killing mechanisms. While the regulation of *glnA* and *glnE* could account for both the

maintenance of MtrR and the fitness pattern seen over the course of *in vivo* studies, the identification of other genes that are regulated by MtrR in this work, but not yet studied, give us reason to believe that MtrR's maintenance is likely more complicated. Characterization of MtrR's regulation of these other genes, including *hsp33*, *dphS*, *recQ* and others, will continue to add to our understanding of what MtrR's full role is in the gonococcus, how MtrR modulates resistance to oxidative and non-oxidative killing mechanisms, how MtrR regulates biosynthetic pathways, and how MtrR affects gonococcal fitness.

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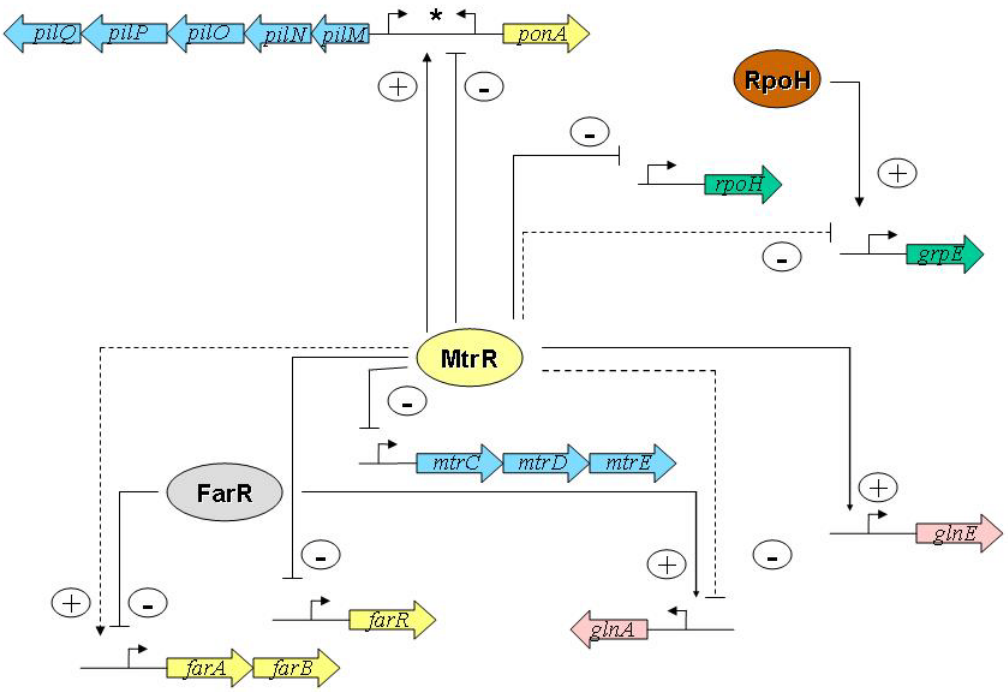
Figure 1. Schematic of genes belonging to the MtrR regulon whose regulation has been investigated in detail during this work, and previous work in our laboratory. MtrR is a global transcriptional regulator of significant importance in the gonococcus. Those genes of the currently known MtrR regulon whose regulation has been characterized in detail are summarized here. The bent arrows indicate the promoter for each gene, or operon, identified as being regulated by MtrR. The regulation of these genes may be direct (indicated by a solid line) or indirect (indicated by a dashed line), as well as positive (indicated by an arrow head and a +) or negative (indicated by a blocked line and a -). The * in the *ponA-pilMNOPQ* locus indicates that these two promoters overlap. Thus, the promoter for *pilMNOPQ* is the promoter to the right and is repressed by MtrR, while the *ponA* promoter is to the left and is activated by MtrR.


pilQ
pilP
pilO
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*
ponA
RpoH
rpoH
grpE
MtrR
mtrC
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FarR
glnE
glnA
farR
farA
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