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Characterization of MpeR and its role in high-level antimicrobial resistance in *Neisseria gonorrhoeae*

By

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By

Alexandra Maria Mercante

B.S. Microbiology, Louisiana State University, 2000

Advisor: William M. Shafer, PhD.

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

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The Gram-negative strict human pathogen *Neisseria gonorrhoeae* is the causative agent of the sexually transmitted infection gonorrhea. Worldwide, 95 million cases of gonorrhea are reported each year. Damage to reproductive organs due to gonococcal infection, particularly in females, can lead to pelvic inflammatory disease, ectopic pregnancy, and in some cases infertility. Currently only third generation cephalosporins are recommended to treat gonorrhea, yet recent reports of cephalosporin resistance poses a serious public health concern. The gonococcal MtrCDEF efflux pump exports structurally diverse hydrophobic antimicrobials, thus greatly impacting antimicrobial resistance. Several DNA-binding proteins transcriptionally regulate the expression of both the *mtrCDE* operon and *mtrF*. The global transcriptional regulator MtrR not only dampens expression of the *mtrCDE* operon but also regulates a number of genes important for both gonococcal physiology and metabolism. Upon investigation of the regulatory properties of *mtrF*, it was discovered that MpeR repressed its expression. Interestingly, the expression of *mpeR* was derepressed under iron-deplete conditions similar to those found *in vivo*. Thus, this work sought to determine the regulatory capacity of MpeR and how the availability of free iron could impact resistance to antimicrobials recognized by the MtrCDEF efflux pump. It was discovered that MpeR is a global transcriptional regulator, activating and repressing a number of genes at both the mid-log and late-log phases of growth. Among the genes that were repressed in a direct manner at the mid-log phase of growth was *mtrF*. We found that high-level antibiotic resistance to a substrate of the MtrCDE efflux pump, mediated by MtrF, could be modulated by both MpeR and the availability of free iron. At the late-log phase of growth, when *mpeR* was maximally expressed, *mtrR* was directly repressed by MpeR, resulting in increased expression of the *mtrCDE* operon in an iron-dependent manner. Additionally, MpeR can directly activate expression of *fetA* encoding the single-component TonB-dependent outer membrance receptor, FetA, important for xenosiderophore recognition. Taken together, this work establishes a novel link, mediated by MpeR, between how changes in the availability of free iron can impact resistance to antimicrobials recognized by the MtrCDE efflux pump; thus expanding our understanding of gonococcal pathogenesis.

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Dedication

This work is dedicated to my parents Herman and Regina S. Dubon for instilling in me the courage and tenacity to strive for success, to my sister Julia Isabel Dubon for being my best friend, and to my husband Jeffrey Mercante for his loving and undying support. This work is also dedicated to the loving memory of my grandmother Marina Zelaya Martinez and my uncle Arturo Dubon.

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Chapter 1: Introduction

I. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative diplococcus and the etiologic agent that causes the sexually transmitted disease gonorrhea. This obligate human pathogen belongs to the family *Neisseriaceae* that includes Gram-negative aerobic bacteria from fourteen genera. The genus *Neisseria* contains two important human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, the latter being a major cause of septicemia and meningitis. *Neisseria gonorrhoeae* is an ancient pathogen with biblical references (Leviticus 15:1–19) but was not discovered to be the causative agent of gonorrhea until 1879 by the Polish physician Albert Ludwig Sigesmund Neisser (98). *Neisseria gonorrhoeae* is a heterotrophic organism measuring 0.6 to 1.0 μm in diameter. The gonococcus is microaerophilic, grows optimally at 35-37°C with 3-10% CO_2 added to the atmosphere, and requires cysteine, iron, yeast extract, and glucose (55). A facultative aerobic intracellular pathogen, the gonococcus exhibits twitching motility and uses type IV pili to adhere to surfaces. The gonococcus exhibits the ability to be naturally competent for genetic transformation at all phases of growth (20, 69, 183). This frequent uptake of exogenous DNA (i.e. transformation) exhibited by the gonococcus results in a high rate of genetic variation. Transformation and the prevalence of nearly 100 phase variable genes all lead to extensive genome plasticity. Remarkably, the gonococcus has recently demonstrated the ability to acquire human DNA through horizontal gene transfer which is the first evidence of the transfer of genetic material from a human to a bacterial pathogen (9).

i. Disease and Epidemiology

The gonococcus primarily attaches to nonciliated columnar epithelial cells on mucosal surfaces. In males, the gonococcus infects the urethra, resulting in symptoms such as purulent discharge and dysuria. In some cases inflammation of the prostate or testicles resulting in prostatitis or orchitis (respectively) can occur. Gonococcal infections in males can also cause epididymitis, the painful swelling of the duct that connects the testicle with the vas deferens, which, if not properly treated could lead to stricture and infertility. In females the gonococcus primarily infects the endocervix, causing symptoms including vaginal discharge, dysuria, and intermenstrual bleeding. Women often suffer from asymptomic infections that can progress to pelvic inflammatory disease (PID). If left untreated, such gonococcal infections lead to scarring of the fallopian tubes resulting in ectopic pregnancy or infertility. Disseminated gonococcal infections (DGI) are caused by hematogenous dissemination of gonococci from the primary site of infection. Symptoms vary greatly but can include joint pain, fever, and skin lesions. Certain strains of *Neisseria gonorrhoeae* that result in asymptomatic gonococcal infections are often associated with DGI (86).

Both men and women can also exhibit gonococcal infections of the pharynx, rectum, and conjunctiva. Pharyngeal gonococcal infections result following orogenital contact with an infected individual and are often asymptomatic. Rectal infections can occur commonly in men who have sex with men or in women with cervical gonococcal infections. Symptoms of rectal gonorrhea include purulent discharge and rectal bleeding. In adults, ocular infections typically are the result of autoinoculation from the primary site of infection such as the genitals. In neonates, bilateral conjunctivitis can occur in an

infant that passes through the vaginal canal of an infected mother. The newborn can exhibit symptoms such as eye pain, redness, and purulent discharge. Neonatal ocular gonococcal infections can cause permanent damage to the eyes requiring prompt treatment of the infected infant. Current treatment guidelines state that third generation cephalosporins such as ceftriaxone or cefixime are the accepted treatment for uncomplicated gonococcal infections of the urethra, cervix, or rectum while pharyngeal infections and gonococcal conjunctivitis are typically treated with ceftriaxone (214).

In the United States, approximately 300,000 new gonococcal infections occur annually with the state of Georgia having the sixth highest rate of gonorrhea (103). In Atlanta alone the rate for gonorrhea is 300 cases per 100,000 population. Rates of gonorrhea are much higher among African Americans, 15-30 year-old unmarried persons, and among individuals living in the rural parts of the southeastern portion of the United States as well as inner city areas throughout the country (44). Worldwide, there are an estimated 95 million cases of gonococcal infections diagnosed each year (205). In Southeast Asia alone there are an astounding 27 million new cases of gonorrhea reported to occur each year. Developing parts of the world such as Africa and Central and South America suffer more from complications due to gonorrhea than other parts of the developed world. This high rate of complication is attributed mostly to inadequate heath care and lack of availability of antibiotics commonly used to treat gonorrhea.

II. Gonococcal Virulence Factors

i. The Pilus

The gonococcus employs several mechanisms for invading its host and causing disease, which will be discussed in the following sections. The hypervariable type IV pilus of the gonococcus serves to attach to host epithelial cells (190). Gonococcal type IV pili are surface fibrils whose expression is required for natural competence (2). The propensity for the gonococcus to exchange DNA via horizontal gene transfer is an important factor contributing to its genetic variation. Additionally, the gonococcal type IV pilus is important in the establishment of a sustained infection (101, 102). Piliated gonococci adhere to the cell surface by forming dense microcolonies (131, 142, 190). The two primary genes important for pilin expression are *pilE* and *pilS*. The structural gene *pilE* encodes for the pilin precursor protein, propilin. Posttranslational cleavage of the PilE protein at a 7 amino-acid N-terminal signal sequence results in the mature pilin monomer (133). The gonococcal *pilS* gene is found in multiple silent copies; for example, strain FA1090 contains 19 unique *pilS* copies grouped into 5 chromosomal loci (80). The gonococcus has the unique ability to transition from piliated to non-piliated cells at a rate of 10^{-2}, as well as vary the production of different pilin antigenic types (72-74). The production of variable pilin types that results from pilin antigenic variation occurs either by *pilE*/*pilS* intragenic recombination or by posttranslational modifications of the pilin protein. Pilin variation contributes to differences in how gonococci adhere to epithelial and endothelial cells and serves to aid in immune evasion (27, 146, 178, 195).

In addition to PilE and PilS, other proteins are important for the formation of a stable and functional type IV pilus (152, 213). The proteins PilD, PilC, PilQ, PilG, PilF, and PilP have been shown to be required for pilus assembly by forming a complex that spans the periplasm thus crossing over to the outer membrane. PilD functions to process propilin into mature pilin by cleaving a seven-amino-acid leader sequence and methylating the N terminus of the pilin protein (54, 147). The 110 kDa protein PilC, the major pilus adhesin required for type IV pilus biogenesis and function, plays a vital role in infection of both epithelial and endothelial cells (152, 213). The two copies of PilC found in the gonococcus, named PilC1 and PilC2 encode proteins with similar functions involved in adhesion (96). It has been shown that piliated PilC-null mutants lose the capacity to adhere to epithelial cells (143, 168). PilQ, a member of the secretin family of pore-forming proteins that is stabilized by PilW, forms a multimeric outer membrane pore through which pilus assembly occurs (64, 196). PilG serves to counteract the effects of retraction of the pilus mediated by PilT (194). The PilT protein belongs to a large family of putative nucleotide binding proteins called TrbB-like proteins and provides the energy for pilus retraction. Mutations of the highly conserved *pilT* gene result in a non-motile phenotype, therefore PilT is essential for twitching motility (211, 212, 216).

ii. Opa Proteins

The gonococcal surfaced expressed opacity (Opa) proteins are encoded by a group of highly conserved genes and serve to establish intimate contact with host epithelial cells (191). This close host cell attachment is mediated through protein-protein interactions between CEACAM (human carcinoembryonic antigen-related cellular

adhesion molecule family) or CD66 receptors and Opa proteins (47). The *opa* genes have two regions of hypervariability, HV1 and HV2, and antigenic variation of Opa proteins occurs when genetic rearrangements occur in these hypervariable sequences (186). Phase variation happens at the translational level among Opa proteins and is due to changes in the number of CTCTT pentamers in the coding repeat found near the 5' end of *opa* genes (141). Using the male volunteer experimental infection model, the importance of Opa phase variation *in vivo* was exhibited by the recovery of Opa+ gonococci when Opa- gonococci were used to infect the volunteers (93). It has been hypothesized that in women certain Opa phenotypes are better adapted for different stages of the reproductive cycle (91). Utilizing a murine-mouse model of infection, it has been demonstrated that Opa proteins do enhance persistence of gonococci in the female genital tract and that *opa* phase variation contributes to gonococcal survival as the bacteria encounter host factors of the mammalian reproductive cycle. The variability of Opa proteins due to antigenic and phase variation serves to alter gonococcal surface appearance, size, and interaction with neighboring colonies *in vitro*, as well as enhance host interactions and pathogenicity *in vivo* (48).

iii. Lipooligosaccharide (LOS)

In Gram-negative bacteria, lipopolysaccharide (LPS) is a major component of the outer membrane that contributes to the stability of the cell membrane, increases the overall net negative charge of the organism, and protects the membrane from certain types of chemical attack. Gonococcoal lipooligosaccharide (LOS) is anchored to the bacterial cell wall membrane via lipid A and differs from LPS in that it lacks the long

repeating O antigen; instead, the side chain consists of three short chains named α, β, and γ (28). The 7 gonococcal glycosyltransferases encoded by 3 chromosomal loci comprise the genes involved in LOS biosynthesis. The highly conserved gonococcal glycosyltransferase genes *lgtA*, *lgtC*, *lgtD*, and *lgtG* undergo phase variation due to slipped-strand base mispairing that occurs at homopolymeric G tracts, and as a result, gonococci can express more than one antigenically distinct LOS on their cell surface at one time (11, 217, 221). Variations in sialylation of LOS by the *lst* gene, which encodes for α-2,3-sialyltransferase, also lead to mutability among gonococcal LOS types, leading to improved protection from polymorphonuclear leukocyte (PMN) killing (124). It has been well established that phase and antigenic variation along with sialylation of gonococcal LOS plays an important role in establishment of an infection and immune evasion (11, 172, 193). Variations in gonococcal LOS can result in a less effective adaptive immune response by limiting surface exposed antigens available for antibody recognition (201). Changes in LOS structure, due to phase variation in *lgtA*, *lgtC*, or *lgtD* also modulate gonococcal susceptibility to normal human serum (NHS), greatly impacting the ability of gonococci to resist host defense factors (180). Modifications to gonococcal LOS can also occur through the addition of phosphoethanolamine (PEA) to not only the core oligosaccharide but also lipid A (13, 41, 123, 148, 215). The enzyme phosphoethanolamine transferase that is responsible for adding a positively charged PEA to the 4′position of lipid A is encoded by *lptA* (41). Recently it has been demonstrated that disruption of *lptA* expression increases gonococcal susceptibility not only to the cationic antimicrobial peptide polymixin B, but also to complement-mediated killing by

NHS thus further influencing the interplay between the gonococcus and host defense mechanisms (111).

iv. Porin

In bacteria, porins are β-barrel proteins that act as channels to transport molecules cross a cellular membrane. Gonococcal porin belongs to the Gram-negative porin superfamily and is essential for survival of the organism (50, 92, 136). PorB, a trimeric protein composed of mainly β-pleated sheets and encoded by the *porB* gene, is the major outer membrane protein of the gonococcus. Only one of two *porB* alles, *porB1a*, encoding PIA, or *porB1b*, encoding PIB, is harbored by the gonococcus. Variability among gonococcal porin occurs mainly within the surface-exposed loops, but, unlike other surface exposed gonococcal components, PorB is generally antigenically stable, which makes for a useful serotyping tool that also plays a critical role in gonococcal pathogenesis (65). Strains of the gonococcus responsible for disseminated disease containing porin PorB1A are more resistant to killing by normal human serum and invade cells more efficiently *in vitro* than gonococci harboring the PorB1B porin (200). This suggests that differences among the subtypes of gonococcal porin serve as a critical component in facilitating the invasion of eukaryotic cells. The interaction between PorB1A and Factor H (fH), the alternative pathway complement inhibitor, serves to protect the gonococcus from human complement mediated phagocytosis. Additionally, gonococcal strains containing PorB1A can disrupt the classical pathway of complement by binding to the regulatory protein C4b-binding protein (C4 bp) (158, 159). In addition to providing a conduit for the transfer of material between and a bacterial cell and the

extracellular environment, gonococcal porins can physically interact with and insert into host epithelial cells when gonococci intimately associate with these host cells during an infection (130, 132). This close association between the host and invading gonococci could be an important mediator of gonococcal pathogenicity (167). The role of porin in other capacities such as the induction or inhibition of apoptosis and antibiotic resistance demonstrate the importance of this outer membrane protein (17, 68, 140, 144).

v. Resistance to Oxidative Stress

Common oxidants found in biological systems are the reactive oxygen species (ROS), which include the superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), along with the reactive nitrogen species (RNS), which include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$). Exposure to these oxidative stresses can result in damage to bacterial DNA, proteins, and cell membranes all of which can lead to cell death (177). The host environment inhabited by the gonococcus contains a variety of oxidants that are generated as a by-product of gonococcal metabolic processes or as key factors of the innate immune response to gonococci, which is characterized by the influx of PMNs to the site of infection (82). Exudate released as a result of inflammation containing PMN's with attached and internalized gonococci is a frequent clinical manifestation of gonorrhoeal disease (182). Therefore, the gonococcus has evolved a number of mechanisms to combat various oxidative stresses encountered at the site of acute infection within its host environment.

The gonococcus employs the use of several transcriptional regulators to modulate changes in the expression of genes involved in the protection from oxidative damage.

Hydrogen peroxide is sensed by the DNA-binding protein OxyR, which belongs to the LysR family of transcriptional regulators (36, 220). Several members of the OxyR regulon were found to be upregulated in response to oxidative stress, including the gene for catalase, *katA*, which encodes the enzyme responsible for converting hydrogen peroxide to oxygen and water (128, 174, 175). In addition, numerous genes involved in the heat shock response and iron acquisition were also regulated by OxyR (187). Another gonococcal transcriptional regulator important for protection from oxidative damage, PerR, is a member of the Fur (ferric uptake regulator) family of metalloregulatory proteins whose regulon includes antioxidant enzymes such as KatA, the ferritin-like Dps protein MrgA, and the *mntABC* regulon responsible for manganese (Mn) transport which serves as a critical ROS quenching agent (83, 197). Additionally, the oxygen-dependent FNR (fumarate and nitrate reductase regulator) has been found to regulate the gonococcal periplasmic cytochrome *c* peroxidase (Ccp), which is involved in protection from hydrogen peroxide-induced killing (117, 199). In addition to OxyR, PerR, and FNR, the zinc-dependent thiol-based NmlR regulon and the iron-dependent Fur regulons all coordinate a robust response to oxidative stress (16, 66, 104). Further study of genes encompassing these regulons will lead to a greater understanding of gonococcal survival in the presence of this harsh environmental factor.

vi. Iron Acquisition

Iron is an essential nutrient for bacterial growth (26). The metal is also indispensable for mammalian survival and promotes the proper functioning of immune cells. Both prokaryotic and eukaryotic cellular activities such as oxygen transport and

storage, electron transport, and the decomposition of peroxides are dependent on the presence of iron (118, 145). Iron can also be deleterious to the host due to the fact that ferric iron (Fe^{3+}) can stimulate free radical formation via the Fenton reaction, causing damage to DNA and other cellular components. Therefore the balance of iron availability, termed nutritional immunity, is key for survival of the host as well as protection from invading pathogens, termed nutritional immunity (209). As a result, iron is complexed intracellulary with ferritin and hemoproteins and is bound extracellularly to transferrin and lactoferrin, minimizing free iron concentrations in the human host.

In order to invade and survive, pathogens such as *Neisseria gonorrhoeae* have developed several strategies to acquire iron from an iron-limiting host environment, allowing for successful proliferation *in vivo*. A common mechanism for bacterial iron acquisition is the secretion of siderophores. These low-molecular-weight high affinity chelators of iron successfully compete with transferrin and lactoferrin for the metal. Iron-bound siderophores are then transported into the bacterial periplasm via specific outer membrane receptors that are TonB-dependent and transferred into the cytoplasm via ABC transporters. Once in the bacterial cytoplasm, the Fe^{3+}-siderophore complex is reduced to Fe^{2+} to release bound iron (12, 42). The gonococcus, however, does not produce or secrete siderophores. Instead it employs the use of TonB-dependent receptors and the TonB-ExbB-ExbD energy transduction complex, similar to TonB-ExbB-ExbD in *E.coli*, to bind and remove iron directly from transferrin, lactoferrin, hemoglobin, and hemoglobin complexed with haptoglobin. It has been demonstrated that inactivation of *tonB* has negative effects on the ability of the gonococcus to acquire iron from host sources (19, 188).

Gonococcal two-component TonB-dependent receptors are unique in that they contain an accessory lipoprotein in addition to the TonB-dependent gated channel protein. The best-characterized gonococcal two-component TonB-dependent receptor is TbpAB that was discovered for its ability to singularly remove and internalize iron from transferrin (8, 23, 156, 173). The major source of iron in the bloodstream during systemic gonococcal infections is transferrin. Additionally, there are permissive levels of transferrin in serum to allow for bacterial growth, highlighting the importance of transferrin as an iron source for the pathogenic *Neisseria* (135). The subunits of the two-component receptor are co-transcribed, iron-regulated, and encoded by *tbpA* and *tbpB* (39). TbpA serves as the TonB-dependent outer membrane receptor while TbpB acts as the surface-exposed accessory lipoprotein (8, 37, 38). The importance of TbpAB during a gonococcal infection was demonstrated using the human male urethral challenge model in which expression of TbpAB was necessary for *in vivo* gonococcal colonization (40).

The gonococcus employs the use of the two-component TonB-dependent receptor LbpAB to acquire iron from lactoferrin, the principal iron source on mucosal epithelial surfaces (127, 134). The genes *lbpA* and *lbpB* are in an iron-regulated operon similar to *tbpAB* and encode for the lactoferrin-binding TonB-dependent receptor LbpA and the accessory lipoprotein LbpB (21, 25, 115). Gonococcal mutants of the LbpA receptor are unable to acquire iron from lactoferrin while mutants of the LpbB accessory protein display diminished growth, lactoferrin binding capacity, and iron uptake rates as compared to wild-type (18, 21, 24, 115, 154, 157). The acquisition of iron from lactoferrin is not required for gonococcal colonization of the urogenital mucosa. However, the use of a derivative of strain FA1090 in the human male challenge model of

infection that utilizes lactoferrin, but not transferrin, as an iron source and is still able to cause symptomatic infections suggests the importance of the acquisition of iron from lactoferrin by LbpAB for gonococcal pathogenesis (7). Interestingly the expression of both transferrin and lactoferrin receptors provides a competitive advantage to the gonococcus in a co-infection of male volunteers with a strain bearing only the transferrin receptor (7). Yet, despite the advantage of expressing both receptors and the fact that ample amounts of lactoferrin can be found both on mucosa and purulent exudates, 50% of clinical isolates do not make a lactoferrin receptor. Thus there could be a selective advantage for gonococci not expressing the lactoferrin receptor and this could be mediated by variations in both sites and stages of infection.

The third two-component TonB-dependent transporter HpuAB allows the gonococcus to acquire iron from hemoglobin and hemoglobin complexed with haptoglobin, both of which are significant sources of iron in the human host (67, 107). While the genes *hpuA* and *hpuB* that encode for HpuAB are also in an iron-regulated operon like *tbpAB* and *lbpAB*, HpuAB expression is also phase-variable due to slipped-strand base mispairing that occurs at a poly(G) repeat (33, 113, 114). Mutants of *hpuA* and *hpuB* demonstrate that both proteins are required for iron acquisition using hemoglobin or hemoglobin complexed with haptoglobin, yet the role of HpuAB during infection is not known (33, 34, 112, 113, 161).

The 76-kDa iron-repressible single-component TonB-dependent receptor FetA allows the gonococcus to acquire iron from siderophores such as aerobactin and enterobactin, both of which are synthesized by enteric bacteria (30, 189, 192, 210). The presence of FetA antibodies in patients infected with *Neisseria gonorrhoeae* indicates the

importance of FetA expression *in vivo* and its use as a potential gonococcal vaccine candidate (22). The open-reading frame downstream of *fetA*, termed *fetB*, encodes FetB which shares homology with siderophore periplasmic binding proteins (30). Additionally, *fetA* is phase variable due to slipped-strand base mispairing that occurs at a poly(A) repeat found in the promoter region, suggesting that variable expression of FetA aids not only in iron acquisition but also immune evasion (31). Other putative single-component iron transporters including TdfG and TdfH have been reported, yet their roles in neisserial iron acquisition remain to be determined (198). The single-component iron transporter TdfF, however, has been demonstrated to be required for gonococcal survival in cervical epithelial cells (75). While gonococcal iron acquisition depends heavily on TonB-dependent mechanisms, the utilization of ferric citrate and heme by the gonococcus is also TonB-independent (19). It has recently been reported that the FbpABC iron transport system also uses TonB-independent means to acquire iron from xenosiderophores (siderophores produced by organisms other than the pathogenic *Neisseria*), further expanding the repertoire of iron scavenging mechanisms of the gonococcus. Intracellular iron storage in *Neisseria gonorrhoeae* is achieved by the proteins bacterioferritin (Bfr) and ferritin (10, 32, 63). The expression of Bfr is also important for protection from oxidative stress (35). Future research on these and other intracellular iron storage mechanisms will lead to new insights concerning the fate of iron internalized by the gonococcus.

It has been determined that approximately 10% of gonococcal genes modulate in expression due to changes in iron levels, suggesting the importance of this nutritional factor for the success of gonococcal pathogenesis (53). The regulation of gene

expression in response to the availability of iron is coordinated by the Fur (ferric uptake regulator) protein. Fur was first discovered in *Salmonella typhimurium* but has been well characterized in *E. coli* (43, 56, 60, 84). This 15kDa protein forms a dimer and, with Fe(II) as a cofactor, binds to the promoter regions of genes important for iron acquisition. The 19bp consensus sequence DNA-binding region, or "Fur box", recognized by Fur was first characterized in *E.coli* and allows for determination of how Fur functions as a regulator of gene expression (45). Classically, Fur bound with Fe (II) acts a repressor, precluding the binding of RNA polymerase; however, several examples of Fur activation have been demonstrated in organisms such as *E.coli* and the *Salmonella* (52, 57-59, 79). The pathogenic *Neisseria* contain a Fur homolog that shares 50% homology with Fur of *E.coli*, suggesting that this iron-responsive protein has similar effects on the expression of genes in the gonococcus and the meningococcus (16, 100). The gonococcal Fur protein is considered to be a global regulator of gene expression due to its ability to regulate genes important not only for iron acquisition but also catabolism, recombination, adherence to host cells, and protection from oxidative stress (90, 176).

III. Mechanisms of Antibiotic Resistance in the Gonococcus

i. Disease Treatment

Treating gonorrhea during the pre-antibiotic era was unpleasant for the afflicted patient and rudimentary for the healthcare provider: physicians administered urethral irrigations with potassium permanganate and silver nitrate, while dysuria was treated with a soap and water enema and oral laudanum containing opium, which served to ease pain

and discomfort. Patients were also advised to abstain from alcohol use and sexual contact during the treatment process (150). The modern era of antibiotic use to treat gonorrhea began in 1935 when Gerhard Domagk discovered the antibiotic sulphanilamide (1). In the late 1940's, after 14 years of sulphanilamide use to treat gonorrhea, almost all (90%) of gonococcal clinical isolates were resistant to sulphonamides (99). Fortunately, in 1944 penicillin was available and first used to treat only sulphonamide-resistant cases of gonorrhea, but the drug quickly became the standard course of treatment (202). Gonococcal resistance to penicillin also arose, and in 1985 worldwide use of penicillin to treat gonorrhea ended (153, 163). Other families of antibiotics, including tetracyclines, aminoglycosides, macrolides, and fluoroquinolones have all been used to treat gonorrhea in the recent past, but resistance to all of these antibiotics has arisen in the gonococcus. Unfortunately, in 2007, the gonococcus joined the list of other "super bugs" due to its resistance to fluoroquinolone treatment. As mentioned earlier, current treatment for gonorrhea includes the use of third generation cephalosporins (i.e. ceftriaxone and cefixime), yet treatment failure using these drugs has been reported in Japan (46, 89, 214). The potential loss of third generation cephalosporins as a viable option for the treatment of gonorrhea is a pressing public health concern; therefore, understanding the mechanisms by which the gonococcus develops resistance to antibiotics is useful in helping to combat this serious sexually transmitted disease. Resistance to antimicrobial agents encountered by the gonococcus can develop in one of several ways, including entry exclusion, drug modification, target modification, or drug efflux.

ii. Entry Exclusion

The inability of an antimicrobial compound to penetrate Gram-negative bacteria due to the possession of an outer membrane provides the target microbe with a suitable first line of defense against invading antimicrobial agents and is known as entry exclusion. Some penetration of the outer membrane to obtain nutrients and compounds is necessary for gonococcal survival and occurs through the use of porins, channels, and transporters. These same conduits can be hijacked by antibiotics to serve as a way for antibacterial agents to enter into bacterial cells. In order to prevent penetration of the bacterial cell by antibiotics, mutations in porins, channels, and transporters have developed. For example, changes in porin alleles, termed *penB*, result in mutations on loop 3, leading to decreased antibiotic levels in the periplasmic space (68, 149). Interestingly, the gonococcal PilQ secretin, which forms a pore in the outer membrane, can also serve as a means by which antimicrobials penetrate the gonococcal periplasm. Alterations to *pilQ* that occur in combination with alterations to *penA*, *mtrR*, and *penB* result in increased resistance to both penicillin and tetracycline (219).

iii. Drug modification

Antibiotics that are able to cross the cell wall are still subject to bacterial defense strategies. Many bacterial species have the capacity to produce enzymes that inactivate an antibiotic, thus rendering the drug ineffective. A well characterized example is the enzyme β-latamase, first described in 1940, that catalyzes the hydrolysis of the β-lactam ring of penicillin, abrogating the ability of this drug to disrupt cell wall synthesis (3).

While most resistance to β-lactams is chromosomally mediated, strains of *Neisseria gonorrhoeae* isolated from patients in Great Britain, the United States, Africa, and southeast Asia harboring β-lactamase-producing plasmids were isolated in 1976 (51). Thus the acquisition of β-lactamase-producing plasmids played an accessory role in the development of resistance to β-lactams such as penicillin with approximately 11% of gonococcal strains harboring β-lactamase-producing plasmids (14, 184).

iv. Target modification

Antibiotics act to either kill or inhibit the growth of bacteria by targeting and disrupting a specific cellular function or process. Bacteria utilize target modification as a tool to combat the action of an antibiotic. Mutations that arise and result in subtle changes to specific cellular targets of the antibiotic can render the bacteria resistant to an antimicrobial agent. For example as mentioned earlier, β-lactams such as penicillin disrupt cell wall synthesis by covalently binding to penicillin-binding proteins (PBPs), which results in irregularities in peptidoglycan synthesis (208). However, a T to C transition mutation in *ponA*, encoding PBP1, leads to a substitution of proline for leucine at position 421 thus lowering the affinity to penicillin (162). Additionally, the *penA* gene encodes altered forms of PBP2 that contain an insertion of an additional aspartic acid residue at position 345, resulting in acylation by penicillin that is reduced by 5-10 fold (185). Moreover, a mosaic-like *penA* gene isolated from male patients with urethritis conferred reduced susceptibility to cefixime. The mosaic-like *penA* gene harbors a transpeptidase-encoding domain similar to that of *penA* genes in *Neisseria perflava* (*N. sicca*), *Neisseria cinerea, Neisseria flavescens*, and *Neisseria meningitidis*. Thus the

exchange of genetic material between *Neisseria gonorrhoeae* and other Neisserial species can occur resulting in the acquisition of mosaic-like genes, such as *penA*, that can contribute to changes in susceptibility to antibiotics used to treat gonorrhea (6, 110, 116).

The antibiotic tetracycline acts to kill a bacterial cell by inhibiting protein synthesis. Specifically, the drug inhibits the binding of amino-acyl tRNA to the mRNA-ribosome complex. In the gonococcus, tetracycline resistance can be acquired from the plasmid-mediated *tetM* determinant whose product functions by mimicking translation elongation factors thus preventing the antibiotic from binding to the ribosome or promoting release of the bound antibiotic (29, 138, 160, 169). High-level chromosomal tetracycline resistance can occur due to a Val-57-Met point mutation in the *rpsJ* gene that encodes the ribosomal protein S10, thus altering the affinity of tetracycline for the ribosome (87).

Fluoroquinolones function by disrupting DNA replication in the gonococcus. Mutations in *gyrA* and *parC*, both of which are responsible for DNA maintenance, result in high levels of resistance to the fluoroquinolone ciprofloxacin (15). Utilizing entry exclusion, drug modification, and target modification, the gonococcus has developed several mechanisms to successfully resist every antibiotic that has been used to treat gonorrhea.

v. Drug Efflux

A fourth mechanism of antibiotic resistance employed by the gonococcus is the expression of efflux pumps. Both prokaryotic and eukaryotic cells possess efflux pumps, although in eukaryotic cells efflux pumps mainly contribute to anticancer drug resistance,

first described by Juliano and Ling in 1976 with the discovery of p-glycoprotein (97). Efflux pumps are defined as energy-dependent transporters made up of proteins that are localized to the cell membrane and serve to pump out or export a variety of agents from a cell. Bacterial efflux systems consist of one or more components and have the capacity to transport several different antibiotics, leading to their association with multi-drug resistance (MDR). There are five families of efflux pumps associated with MDR and they include the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the resistance nodulation division (RND) family. All five families of efflux pumps are characterized based on the number of components they contain, the number of membrane spanning domains possessed by the transporter protein, the energy mechanism utilized for transport, and the types of agents they export. The gonococcus expresses several different efflux pumps that will be described in the following section. A summary of the organization of these pumps along the cell envelope as well as their substrates is summarized in Figure 1. Greater detail regarding neisserial efflux is provided in Chapter 5.

IV. Gonococcal Efflux Pumps

i. NorM

The NorM efflux pump of *Neisseria gonorrhoeae* shares homology with NorM of *Vibrio parahaemolyticus* and YdhE of *E.coli* and belongs to the MATE family of efflux pumps. The MATE family consists of transporters with 12 membrane spanning domains

coupling Na^+ import with drug transport (106, 137). The gonococcal NorM efflux pump recognizes cationic toxic compounds such as ethidium bromide, acriflavine hydrochloride, 2-*N*-methylellipticinium and berberine, all of which have a quaternary ammonium on an aromatic ring. Additionally, Rouquette-Loughlin *et al.* found that a point mutation upstream of the *norM* gene which causes overexpression of *norM* results in decreased susceptibility to fluoroquinolones such as ciprofloxacin which was recently used to treat gonorrhea, suggesting the importance of this efflux pump in the development of resistance to treatment options of recent clinical relevance (121, 165).

ii. MacAB

The MacAB efflux system of *Neisseria gonorrhoeae* was discovered because of its homology to the MacA-MacB efflux system of *E.coli* (105). MacA, a membrane fusion family protein, and MacB, which contains an ATP-binding domain, along with the TolC-like outer membrane protein MtrE, form an ABC transporter system that utilizes ATP hydrolysis to export macrolides such as azithromycin. Mutations in the -10 promoter region of the *macAB* operon result in increased expression of *macAB* which leads to enhanced resistance to macrolides recognized by the MacAB efflux system (166). A single-dose oral regimen with the azithromycin was once a popular treatment option, but the World Health Organization (WHO) no longer recommends the use of macrolides such as azithromycin for the treatment of gonorrhea due to worldwide reports of resistance (81, 218).

iii. FarAB

In 1983 McFarland *et al.* reported cases of resistance to the fatty acids linoleic acid, palmitic acid, and oleic acid in rectal clinical isolates of *Neisseria gonorrhoeae* obtained from men who have sex with men. This observation suggested that efflux presented a plausible mechanism of resistance to these agents that are known to have *in vitro* antigonococcal activity (129, 139). In 1999, Lee and Shafer reported that the *farAB*-encoded efflux system, which shares homology with the *E.coli emrAB*-encoded efflux system and belongs to the major facilitator superfamily (MFS) of efflux pumps, utilizes proton motive force to export long-chained fatty acids (FAs) (109, 119). Regulation of *farAB* expression is mediated by the DNA-binding protein FarR, which belongs to the MarR family of proteins and acts to repress expression of *farAB.* Other MarR family members act as repressors of efflux operons, including EmrR, which represses expression of *emrAB* efflux genes (120). Stabilization of the binding of FarR to the *farAB* promoter region is enhanced by the histone-like DNA binding protein Integration Host Factor (IHF) (108).

iv. MtrCDE

The *mtr* (multiple transferrable resistance) system was first described by Maness and Sparling in 1973. They observed strains of gonococci that were resistant to various hydrophobic antimicrobials and hypothesized that a common genetic factor such as mutational changes in cell-envelope permeability could be responsible for the phenotype (125). In 1982 it was reported that approximately 12% of patients were infected with gonococci bearing the *mtr* locus, indicating the clinical relevance of the *mtr* system (139).

In 1994, Pan and Spratt successfully cloned part of the *mtr* locus that contained a mutant allele of the *mtrR* gene, a then-putative transcriptional repressor. Additionally, they reported that the *mtr* locus shares significant homology to the *E.coli acrAB* locus that is also involved in modulating susceptibility to hydrophobic agents (151).

The MtrCDE efflux pump, encoded by the *mtrCDE* locus, belongs to the RND family of efflux pumps which utilizes proton motive force to export structurally diverse hydrophobic agents, including antibiotics such as penicillin and erythromycin, detergents such as Triton X-100, dyes including crystal violet, the spermicide nonoxynol-9, and the host-derived antimicrobial peptide LL-37 (77, 181). The 114kDa transporter protein MtrD contains 12 membrane-spanning domains and shares similarity to other cytoplasmic RND membrane proteins. The 42 kDa membrane spanning lipoprotein MtrC serves as the link between MtrD and the TolC-like outer membrane protein MtrE, from which hydrophobic agents are expelled out of the bacterial cell. Insertional mutations resulting in the loss of *mtrD*, *mtrC*, or *mtrE* expression result in hypersusceptibilty to hydrophobic agents recognized by the pump (49, 76, 77). In addition to the core components of the MtrCDE efflux pump an accessory protein to the pump, termed MtrF, is needed for high-level resistance (62). The discovery of MtrF occurred upon examination of HA-hypersusceptible clinical isolates. One strain of interest did not harbor any mutations in the *mtrCDE* operon, but it did contain a phenotypically suppressed *mtrR* missense mutation in the helix-turn-helix region of MtrR and a second mutation that mapped to a gene found downstream of *mtrR*, and thus was named *mtrF*. MtrF is a 56.1kDa putative inner membrane protein with 12 transmembrane spanning domains. Upon comparison of the MtrF amino acid sequence, homologs to hypothetical

proteins in several bacterial species including *Neisseria meningitidis* MC58, *Vibrio cholerae*, and *Staphylococcus aureus* COL were found. The only protein of known function that shares homology to MtrF is the *E. coli* AbgT transporter of the folate catabolite *p*-amino-benzoyl-glutamate (88). While the exact function of MtrF is still unknown, it has been shown that expression of *mtrF* is necessary for high-level resistance to hydrophobic agents recognized by the MtrCDE efflux pump in strains lacking a functional MtrR and displaying increased *mtrCDE* expression (204).

In addition to their role in the development of multidrug resistance in a number of pathogens such as *E.coli*, *Psuedomonas aeruginosa*, and *Vibrio cholerae*, multidrug efflux pumps, including the MtrCDE efflux pump of *Neisseria gonorrhoeae*, have been suggested to be vital mediators of both colonization and bacterial persistence within the human host (155). Utilizing a female murine mouse model for lower genital gonococcal infections, it was demonstrated that a functional MtrCDE efflux system enhances gonococcal infectivity while loss of the efflux pump proved to be disadvantageous to the survival of gonococci *in vivo* (94). This is the first report uncovering the importance of the expression of a multidrug efflux system during infection of the female genital tract by gonococci. Therefore multidrug efflux pumps should be considered a bacterial virulence factor due to their capacity to not only render a bacterium resistant to antimicrobial agents but also their crucial task in the establishment of infection (94).

V. Genetic regulation of the MtrCDE efflux pump

While MDR efflux pumps can be advantageous for bacterial colonization and pathogenesis, constitutive expression of these pumps can be deleterious to bacterial survival. Therefore, transcriptional regulation of the expression of MDR efflux genes is typically observed; both *cis*- and *trans*- acting regulatory factors are important in such gene control. Several well-characterized examples of DNA binding proteins that act to either activate or repress efflux gene expression on a local level include the *E.coli* TetR repressor of tetracycline efflux genes, the BmrR activator of *bmr*-mediated efflux in *Bacillus subtilis,* the QacR repressor of *qacAB* in *Staphylococcus aureus,* and EmrR, which acts to repress the *emrAB* efflux operon in *E.coli* (4, 71, 85, 120). Global regulatory proteins that modulate the expression of multiple genes can also serve to regulate efflux gene expression. For example, the *acrAB* efflux operon of *E.coli* is activated by MarA, Rob, and SoxS, all of which act as global transcriptional regulators (5). The gonococcus employs the use of several DNA-binding proteins to both activate and repress expression of the *mtrCDE* efflux genes.

i. MtrA

The AraC-XylS family transcriptional regulator MtrA had been shown to activate expression of the *mtrCDE* efflux operon when gonococci are exposed to sublethal concentrations of the HA TritonX-100, which is a substrate of the MtrCDE efflux pump. It has been proposed that induction of pump expression serves as an efficient defense mechanism that prepares the gonococcus to expel antibiotics or host defense factors thus

ensuring infectivity and survival (164). Utilizing a mouse infection model it has been demonstrated that a gonococcal strain bearing a mutation in *mtrA* is significantly less fit *in vivo*, indicating the importance of this DNA-binding protein for gonococcal pathogenicity (206). Unpublished observations by Zalucki and Shafer suggest that MtrA is a global regulatory protein and acts directly or indirectly to activate or repress a number of gonococcal genes in addition to the *mtrCDE* efflux operon.

ii. MtrR

Repression of the *mtrCDE* efflux system is mediated by the DNA-binding protein MtrR, which was first characterized by Pan and Spratt (151) who cloned the *mtrR* gene that is 250 base pairs immediately upstream, but divergently transcribed from the *mtrCDE* operon. Inactivation of *mtrR* results in increased resistance of gonococci to HAs recognized by the MtrCDE efflux pump (151). MtrR has a mass of 23kDa and belongs to the QacR-TetR family of transcriptional regulators and interacts with DNA by the binding of two homodimers to the promoter of the *mtrCDE* operon (77, 122). Utilizing a mouse infection model it was shown that loss of the *mtrR* gene enhances gonococcal fitness *in vivo*, suggesting that derepression of the MtrCDE efflux pump results in a strain that is better adapted to surviving conditions within the genital tract (206). Derepression of *mtrCDE* expression can also occur as a result of point mutations in the MtrR binding site, a single-base-pair deletion within a 13bp inverted repeat sequence in the *mtrR* promoter, or missense mutations in the helix-turn-helix motif of MtrR, all of which result in elevated MtrCDE pump levels of clinical significance *in vitro* and *in vivo* (78, 122, 179, 207). Additionally, mutations to the promoter region of *mtrR* coupled with

mutations in both *penA* and *penB* result in elevated MtrCDE pump levels that render the gonococcus resistant to penicillin (203). Recently it has been reported that mtr_{120}, a C-to-T transition mutation found 120bp upstream of the *mtrC* start codon, results in a consensus -10 region. This *cis*-acting mutation generates a novel *mtrCDE* promoter element that is not subject to either MtrA or MtrR regulation yet results in increased expression of the *mtrCDE* operon and thus elevated levels of resistance to antimicrobials (Ohneck *et al*. in press).

Using microarray analysis, it was found that MtrR can directly or indirectly activate or repress 69 gonococcal genes at different phases of growth including the *mtrCDE* efflux genes. For example, the gonococcal *rpoH* gene that encodes the stress response sigma factor RpoH is directly repressed by MtrR, thus linking this transcriptional repressor of a drug efflux operon to survival of gonococci in H_20_2 rich environments (61). Repression of *glnA*, encoding glutamate synthetase, an enzyme involved in amino acid biosynthesis, is mediated by the ability of MtrR to preclude binding of *glnA* activator FarR to the *glnA* promoter region. Thus, in addition to its role as a repressor of *mtrCDE* expression, MtrR has the capacity to function as a global regulator of gene expression in the gonococcus (95).

iii. MpeR

In addition to the regulatory action of the DNA binding proteins MtrA and MtrR, a third gonococcal DNA binding protein termed MpeR (Mtr protein efflux regulator) is also involved in the regulation of *mtr* efflux components. MpeR was first reported by Folster and Shafer who searched the FA1090 gonococcal genome for novel putative

transcriptional regulators that could serve to regulate the expression of *mtrF* (62). The repression of *mtrF* expression by MpeR prompted the investigation of the global impact that MpeR could have as a DNA-binding protein in the gonococcus. MpeR is 35.7kDa in size and is characterized as an AraC-family protein due to sequence similarity of its C-terminal helix-turn-helix motif to that of other AraC-like proteins (62). The first member of the AraC family to be characterized was the AraC protein of *E.coli*, which regulates the L-arabinose operon (70, 170, 171). Members of the AraC family are found in a wide range of bacterial species and can act as both activators and repressors of transcription.

The AraC family of proteins have the capacity to regulate genes with bacterial functions that include sugar catabolism, stress response, and virulence (126). Interestingly, recent microarray studies have demonstrated that expression of *mpeR* is derepressed under low iron conditions and is regulated by Fur + iron in the gonococcal strain FA19 (53, 90). This lead us to hypothesize that the expression of *mpeR* could be important for gonococcal pathogenesis *in vivo* where levels of free iron are limited.

Due to the evidence that MpeR is implicated in the regulation of the MtrCDE efflux component MtrF, and the fact that *mpeR* expression is modulated by the availability of free iron, this body of work sought to determine the role that MpeR plays in the regulation of antimicrobial efflux mechanisms occurring in an iron-dependent mechanism (Figure 2), thus leading to novel insights into gonococcal pathogenesis. As such, the following specific aims listed below will be addressed in this work.

Specific Aims:

I. Define the MpeR regulon in *Neisseria gonorrhoeae* in order to determine the global regulatory capacity of MpeR.

II. Determine how MpeR impacts antimicrobial resistance through its regulation of both *mtrF* and *mtrR*.

III. To establish a novel link, mediated by MpeR, between how changes in the availability of free iron can impact resistance to antimicrobials recognized by the MtrCDE efflux pump.

IV. Elucidate the role that MpeR plays in regulating the expression of the single-component TonB-dependent iron acquisition system, FetA.

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Figure 1: Efflux pumps possessed by *Neisseria gonorrhoeae*.

The multi-drug efflux transporters utilized by *Neisseria gonorrhoeae* to export a wide variety of antimicrobials are depicted. The MATE family pump NorM couples Na^+ import with the export of cationic toxic compounds, all of which have a quaternary ammonium on an aromatic ring. The MacAB efflux pump belongs to the ABC superfamily of multi-drug transporters and utilizes ATP hydrolysis to export macrolides such as azithromycin. The FarAB efflux pump belongs to the MF superfamily and along with the outer membrane protein MtrE utilizes proton motive force to export long-chain fatty acids. The Mef efflux pump another member of the MF superfamily also exports macrolides. The RND family MtrCDEF efflux system utilizes proton-motive force to export a number of structurally diverse hydrophobic antimicrobial agents

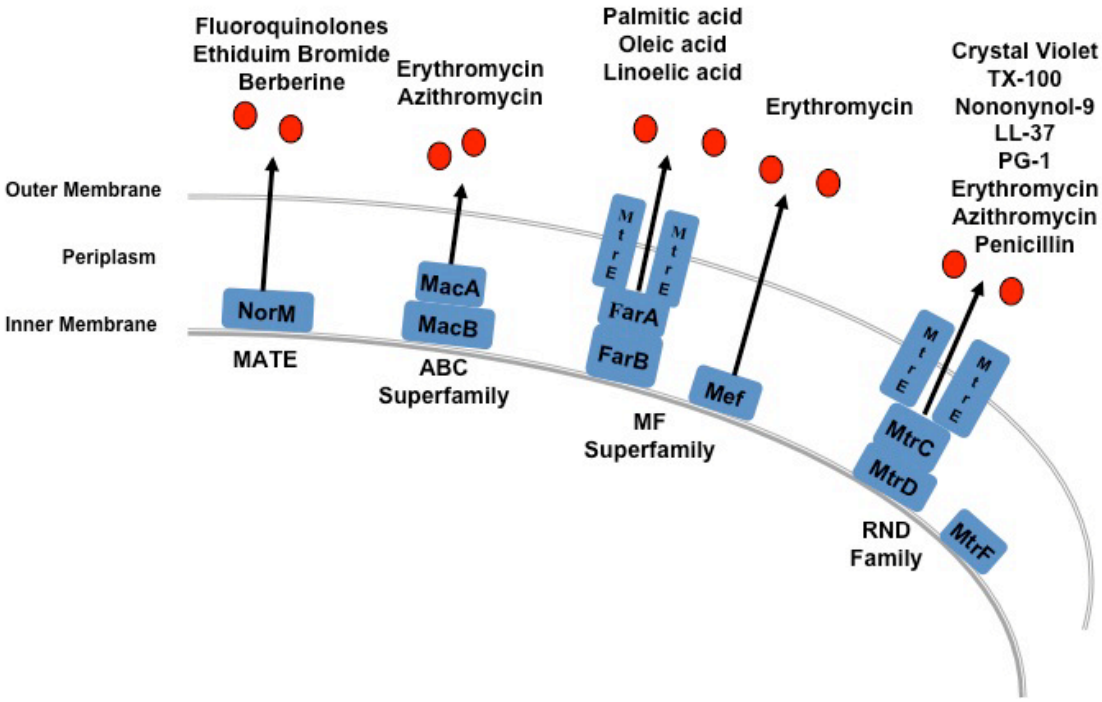
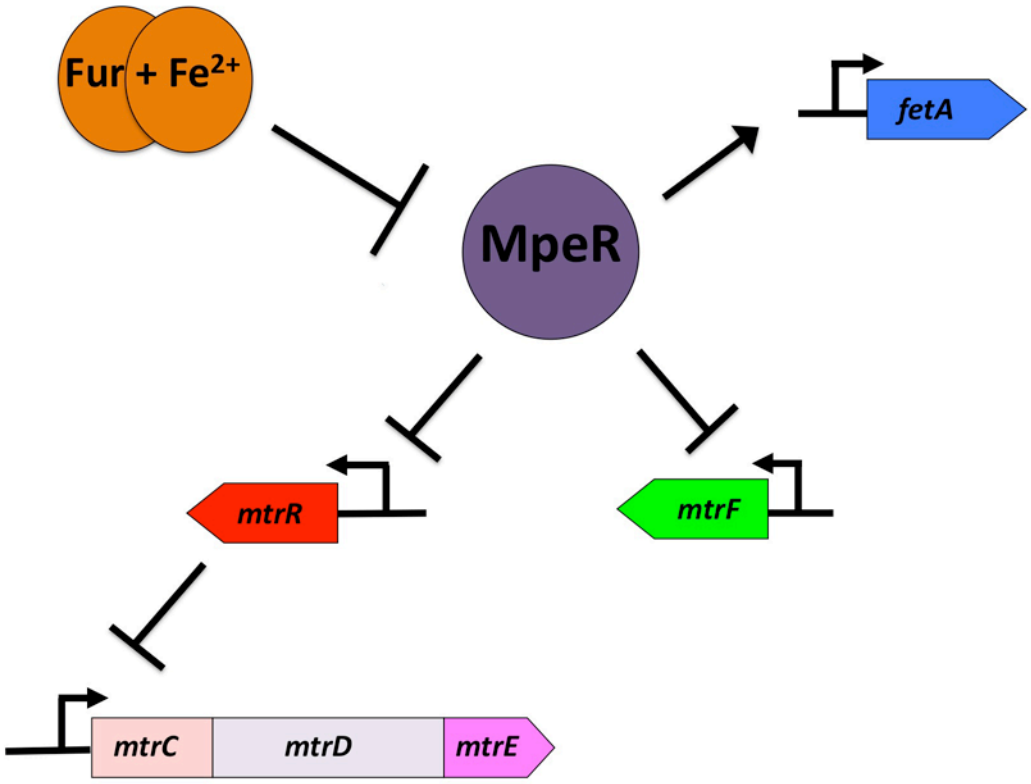

Fluoroquinolones
Ethiduim Bromide
Berberine
Erythromycin
Azithromycin
Palmitic acid
Oleic acid
Linoelic acid
Erythromycin
Crystal Violet
TX-100
Nononynol-9
LL-37
PG-1
Erythromycin
Azithromycin
Penicillin
Outer Membrane
Periplasm
Inner Membrane
NorM
MATE
MacA
MacB
ABC
Superfamily
MtrE
MtrE
FarA
FarB
Mef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

Figure 2: Model for iron-dependent gene regulation mediated by MpeR in *Neisseria gonorrhoeae*.

Under iron-replete conditions Fur+Fe^{2+} acts to repress the expression of *mpeR*. Under iron deplete conditions the repression of *mpeR* is relieved resulting in elevated levels of *mpeR* expression. In strain FA19, MpeR represses the gene *mtrF* encoding for an accessory protein of the MtrCDE efflux pump whose expression is important for high-level resistance mediated through the MtrCDE efflux pump. Therefore the repression of *mtrF* by MpeR serves to modulate high-level resistance to hydrophobic agents recognized by the MtrCDE efflux pump in an iron-dependent manner. Additionally, MpeR represses expression of the *mtrR* gene encoding for the MtrR protein, a global regulatory protein responsible for repressing expression of the *mtrCDE* operon. The iron dependent regulation of *mtrCDE* expression occurs through the repressive action on *mtrR* by MpeR. In strain FA1090 MpeR activates expression of *fetA* that encodes for the single-component TonB-dependent receptor responsible for acquiring iron through its recognition of xenosiderophores. Take together, the regulatory properties of MpeR provide a novel link between the availability of iron and antimicrobial resistance mediated through the MtrCDEF efflux system.


Fur + Fe2+
fetA
MpeR
mtrR
mtrF
mtrC
mtrD
mtrE

Chapter 2: Function and Regulation of *Neisseria gonorrhoeae* Efflux Pumps

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The export action of efflux pumps is a nearly universal mechanism used by bacteria to escape the action of toxic compounds in their environment. Antimicrobials faced by bacteria include various biocides (natural or synthetic) and classical antibiotics used in therapy of infections. Certain efflux pumps also export antimicrobials produced by their hosts and this ability likely enhances the survival of the infecting pathogen, especially during early stages of infection when mediators of innate host defense normally function to reduce the microbial load. This review is concerned with the roles of efflux pumps produced by *Neisseria gonorrhoeae* in contributing to its resistance to antimicrobials used in therapy of infections or those that participate in innate host defense. Specific emphasis is placed on the genetic organization, transcriptional regulation, and function of gonococcal efflux pumps. The major theme of this review is that in addition to their role in enhancing bacterial resistance to classical antibiotics and biocides, certain efflux pumps, such as those harbored by strict human pathogens like gonococci, can also influence in vivo fitness and survival of bacteria since they provide a mechanism to resist natural antimicrobials produced by their host.

Introduction

N. gonorrhoeae is an important, strict human pathogen that is transmitted by sexual contact and causes the disease termed gonorrhea, which can have many clinical manifestations. Historical writings suggest that the gonococcus (or GC) has been a human curse for thousands of years (66). Thus, the ancient nature of gonorrhea is suggested by warnings in the Book of Leviticus in the Old Testament that women should avoid men with discharges. Later descriptions of the disease by the second century Greek physician Galen confirms that gonorrhea has been a disease of mankind for centuries. In sharp contrast, the earliest description of disease caused by the related human pathogen *N. meningitidis* (the meningococcus or MC), which can be carried commensally in the nasopharynx of 10-30% of the population, can be found in the early 19th century writings by Vieusseaux (71). Due to the lack of earlier accounts of disease, which can have dramatic presentations in the forms of meningitis or septicemia and result in rapid death, it has been suggested that only in recent times did MC evolve into a pathogen.

As emphasized in other chapters of this volume and elsewhere (39, 50, 66), drug efflux pumps can help bacteria resist the action of clinically useful antibiotics. One can even imagine that in the pre-antibiotic era, soil microbes found efflux pumps beneficial to help them resist antibiotics produced by other microbes. At first glance, it is more difficult to understand how bacteria that principally live inside a host developed the need for efflux pumps unless one considers that the respective host also produces antimicrobials. Thus, strict human pathogens such as GC are confronted with a wide range of host-derived antimicrobials (e.g., antimicrobial peptides, toxic free fatty acids,

bile salts and progesterone, etc.) that can have potent activity against them. We now know that many of these antimicrobials can be recognized and exported by efflux pumps produced by GC (66). Moreover, there is very good evidence (see below) that production and levels of one particular pump, MtrC-MtrD-MtrE, can substantially enhance both the duration of a gonococcal infection (29) and in vivo fitness (78, 79) of GC in an experimental female mouse model of lower genital tract infection (28). This same efflux pump is also required for high level, chromosomally-mediated resistance to penicillin that can be expressed by GC (75). Accordingly, studies on efflux pumps produced by GC provide a unique opportunity to understand the role of bacterial drug efflux pumps in the overall pathogenic mechanisms of bacteria during infection and how they also contribute to resistance to antibiotics used clinically today or in the past. Thus, this review will concentrate on these issues as well as provide insights as to how GC controls expression of efflux pump genes and how such regulatory systems modulate expression of other genes involved in fitness and pathogenicity.

GC Efflux Pumps and Roles in Antimicrobial Resistance and Survival In Vivo

In contrast to other bacteria that express a wide number and type of drug efflux pumps (55), most GC strains produce only four drug efflux pumps (Figure 1). These pumps belong to the resistance-nodulation-division (RND) family (MtrC-MtrD-MtrE), the major facilitator (MF) family (FarA-FarB-MtrE), the ABC transporter family (MacA-MacB-MtrE) and the multidrug toxic compound extrusion (MATE) family (NorM); these pumps have been reviewed elsewhere (66). Additionally, some (rare) clinical isolates have been reported to harbor the *mef* gene (45), which encodes a pump that exports

macrolides. The MtrC-MtrD-MtrE efflux pump, like other RND drug efflux pumps, can export structurally diverse hydrophobic antimicrobial agents, including antibiotics (macrolides, beta-lactams, rifampin, etc.), non-ionic detergents (triton X-100 [TX-100]) (44, 77), biocides (the spermicide nonoxynol-9) and host-derived antimicrobials (e.g., certain antimicrobial peptides, progesterone, bile salts) that participate in innate host defense (22, 58, 67, 79). Export of human antimicrobial peptides, such as LL-37, by the MtrC-MtrD-MtrE pump by both GC (67) and MC (72), provides a way for these pathogens to capture them in the periplasm after they have breached the barrier imposed by the outer membrane. This is important since LL-37 has been proposed to inhibit cell wall biogenesis (69). Sub-lethal levels of LL-37 frequently bathe mucosal surfaces, including the genital tract (6) and can transiently increase LL-37 resistance by a mechanism involving a two component regulatory system that indirectly enhances transcription of *mtrCDE* (see below). Progesterone, also recognized by MtrC-MtrD-MtrE, behaves as a detergent on gonococcal membranes (48) and its importance in protecting the female lower genital tract from infection is supported by the observation that surgical removal of ovaries from mice increased their susceptibility to an experimental GC infection (29). The FarA-FarB-MtrE efflux pump recognizes long-chain, unsaturated fatty acids (e.g., oleic and palmitic acid) (38), which can have detergent-like action against GC (48). The MacA-MacB pump can export macrolides, but is often silent due to promoter mutations (62). NorM has the ability to export quaternary ammonium compounds and quinolone antibiotics (62).

Mutations that increase the level of bacterial efflux pump proteins can reduce the efficacy of antibiotic treatment regimens (39, 50, 66). As a specific example, over-

expression of the *mtrCDE* efflux pump operon due to *trans*- or *cis*-acting mutations (see below) can decrease gonococcal susceptibility to penicillin (75). Importantly, in strains that harbor other mutations that impact the structure of penicillin-binding proteins 1 (PBP1) and 2 (PBP2), or outer membrane porin proteins (13, 52, 57, 70, 75) Shafer et al., 2010) over-expression of *mtrCDE* conferred a level of penicillin resistance that resulted in clinical failure (13). Collectively, these mutations ultimately led to the removal of penicillin from the CDC-recommended treatment regimen. Importantly, genetic inactivation of the pump operon in a resistant strain resulted in a return to a level of penicillin-susceptibility well below the MIC breakpoint (75). This observation supports the notion (42) that efflux pump inhibitors (EPI) could restore previously used antibiotics that were lost due to the development of resistance. Moreover, inhibiting the action of efflux pumps that also recognize host antimicrobials may have the added benefit of making EPI-treated bacteria less fit during natural infection. Looking toward the future, the third generation cephalosporin ceftriaxone is now the mainstay antibiotic used for treating gonorrhea, but strains expressing decreased susceptibility to this important antibiotic have emerged and at least one strain has been documented to be clinically resistant to cure by ceftriaxone (M. Unemo, personal communication); hence, it will be important to follow the spread of such strains and determine the role that efflux pumps like MtrC-MtrD-MtrE play in resistance. As has been recently emphasized (10), the public health problem of GC strains resistant to antibiotics is becoming more severe and strains expressing clinical resistance to ceftriaxone would leave little therapeutic options in controlling gonorrhea. In the absence of new antibiotics that recognize novel targets, this problem will only worsen.

In the absence of classical antibiotics, does production of an efflux pump provide an advantage for gonococci during infection? Results from studies on the MtrC-MtrD-MtrE pump suggest that this is the case since its production enhanced gonococcal survival during experimental infection in the lower genital tract of female mice (29). Moreover, differential expression of the *mtrCDE* operon (see below) can modulate fitness levels in this infection model (78, 79). Thus, we proposed that the MtrC-MtrD-MtrE efflux pump is important in the ability of GC to survive in vivo when challenged by mediators of innate host defense (67, 79) This capacity to resist mediators of innate host defense may be important during human infection because GC elicit a strong TH17, pro-inflammatory response (41) characterized by enhanced production of antimicrobial peptides. Other RND-type pumps similar to MtrC-MtrD-MtrE produced by Gram-negative enteric pathogens (e.g., AcrA-AcrB-TolC) are also likely to enhance bacterial survival during infection (2) (see Chapters 9 and 12). The capacity of GC to export free long-chained fatty acids (e.g., palmitic and oleic acid) via the FarA-FarB-MtrE pump may help it survive in the rectum where concentrations of such fatty acids are elevated (48, 65), however, direct experimental evidence is lacking since FarA-FarB-MtrE was not required for in vivo survival in the mouse infection model (29). Nevertheless, results from studies on the Mtr system suggest that prior to the clinical use of antibiotics, efflux pumps served important roles for bacteria during stages of infection when they encounter host-derived antimicrobials.

Organization of GC Efflux Pump Genes

Of the four GC efflux pumps (66), three are encoded by genes organized in an operon (*mtrCDE*, *farAB* and *macAB*) while the fourth (*norM*) is encoded by a stand-alone

gene (Figure 2). The *mtr* (*m*ultiple *t*ransferable *r*esistance) system was first identified by (46) when they isolated a spontaneous mutant that exhibited increased resistance to multiple, structurally diverse antimicrobial hydrophobic compounds. It was originally thought that *mtr* regulated outer membrane permeability by overproducing a membrane protein and increasing the degree of peptidoglycan cross-linking (20). However, subsequent cloning/sequencing experiments in the 1990s (23, 53) showed that the mutation was located within a gene encoding a transcriptional repressor (MtrR) of an upstream, but transcriptionally divergent operon (*mtrCDE*) that would encode the tripartite MtrC-MtrD-MtrE efflux pump similar to other RND-type pumps of Gram-negative bacteria (9, 21, 23, 53). Like other RND efflux pumps, the three proteins that form the pump are a cytoplasmic membrane transporter (MtrD), a membrane fusion protein (MtrC) and an outer membrane channel protein (MtrE) (Figure 1). It is likely that MtrE also serves as the outer membrane channel protein for the FarA-FarB and MacA-MacB efflux pumps (Figure 1). Directly or indirectly, other proteins also participate in efflux mediated by the Mtr pump. In this respect, Veal and Shafer (76) identified an accessory protein (MtrF) which, for reasons that are not yet clear, is required when the host strain expresses high levels of antimicrobial resistance via the MtrC-MtrD-MtrE efflux system Additionally, energy supplied by the TonB-ExbB-ExbD system is needed for inducible antimicrobial resistance mediated by MtrC-MtrD-MtrE (60) that also requires the participation of a transcriptional activator termed MtrA (see below). Lipooligosaccharide (LOS) structure is also important in the function of the MtrC-MtrD-MtrE efflux pump in strains over-expressing *mtrCDE* because a deep rough LOS mutant

expressing a core oligosaccharide that was severely truncated was unable to express high level resistance to substrates of the pump (44).

Transcriptional Regulation of GC Efflux Genes

Discoveries of bacterial efflux pumps were often made prior to the availability of genome sequence databases and in these instances were facilitated by the isolation of mutants that expressed decreased susceptibility to antimicrobials (50, 66). These resistance-conferring mutations frequently mapped to a gene that encoded a DNA-binding protein that would normally dampen expression of a closely linked gene or operon encoding efflux pump proteins. Pan and Spratt (53) discovered the *mtr* locus (Figure 2) in GC strain CH95 in this way when they identified a mutation in a gene (*mtrR*) that encodes a DNA-binding protein similar to TetR and QacR (18) responsible for the Mtr phenotype described by Maness and Sparling (46). CH95 expressed high-level resistance to a panel of structurally diverse hydrophobic agents (HA), but introduction of CH95 *mtrR* coding mutation (H105Y) into a sensitive recipient only resulted in a slight (2-4 fold) increase in resistance. However, when the DNA sequence upstream of *mtrR*, which included promoters for *mtrR* and *mtrCDE* transcription, was introduced into a sensitive strain, higher levels of HA-resistance similar to the donor could be obtained in transformants. The reason for this difference was discovered by Hagman *et al.* (23) to be due to the presence of a single base pair (bp) deletion in a 13 bp inverted repeat sequence within the *mtrR* promoter of the donor strains CH95 or FA171 (Figure 3). Subsequent examination (65, 66) of CH95 and other high-level resistant GC strains showed that this single bp deletion was frequently present in a 13 bp inverted repeat and that introduction of this mutation with or without *mtrR* coding region

mutations into the sensitive strain FA19 would result in high level HA-resistance. Taken together, GC has both *cis*- and *trans*-acting elements that impact transcription of efflux pump genes and these are discussed separately below.

Cis-acting factors that regulate efflux pump genes

Point mutations, deletions, or insertions in the nucleotide sequences between *mtrR* and *mtrCDE* can provide GC with higher levels of HA-resistance than mutations within the *mtrR*-coding region (Figure 3). As mentioned above, a single bp deletion within the 13 bp inverted repeat element localized in the *mtrR* promoter can significantly enhance transcription of *mtrCDE* (23). This mutation is frequently found in GC clinical isolates that express the Mtr phenotype (40, 80); some clinical isolates have a dinucleotide insertion within this inverted repeat (81). In either case, the optimal 17 bp spacing between the -10 and -35 elements is disrupted and this significantly reduces *mtrR* transcription. This, however, cannot be the sole reason why such strains express high level HA-resistance since their level of *mtrCDE* expression is greater than strains with *mtrR* loss-of-function or null mutations. Instead, because the promoters for *mtrR* and *mtrCDE* transcription overlap and are divergent (Figure 3), it is most likely that the mutations enhance RNA polymerase interactions with the *mtrCDE* promoter.

A limited number of GC strains (e.g., MS11 (79)) have a point mutation (C→T) located 120 nucleotides upstream of the *mtrC* translational start codon (mtr_{120}), changing the sequence at this locus from TATAAC to TATAAT and thereby generating a consensus -10 element (Figure 3) (51). This new -10 element acts as a stronger promoter for *mtrCDE* transcription and results in high levels of *mtrCDE* expression and HA-resistance, as well as enhanced in vivo fitness (79). It appears not to be under the control

of the transcriptional regulators MtrR or MtrA (see below), which act on the wild type promoter. Although analysis of the genome sequence for fourteen GC strains indicates that this mutation is rare (1/14 strains), one recent clinical isolate in a collection of 113 strains studied by M. Unemo and co-workers (personal communication) in Orebro, Sweden, was found to contain this mutation. Since the mtr_{120} mutation significantly enhanced GC fitness in a murine model of lower genital tract infection (79), it may provide the bacteria with a competitive advantage in the community. More detailed molecular surveillance will be needed to determine if such strains emerge at a higher frequency.

In 1999 a number of azithromycin-resistant GC clinical isolates were obtained from patients in Kansas City, MO (32). These strains contained a 153 bp insertion in the *mtrR-mtrCDE* intervening region and expressed cross-resistance to structurally diverse HA that was mediated by the MtrC-MtrD-MtrE efflux pump. Our work (61), showed that virtually all meningococci (MC) contain a 155-159 bp insertion within this region, identified as a Correia Element (CE) (8), with some serogroup Y strains also having a tandemly linked IS*1301* sequence; more recent studies have shown a low percentage of meningococcal strains lacking the CE (11). In MC, the presence of the CE element dampened *mtrCDE* expression as a result of providing a binding site for integration host factor (IHF) and a new site for post-transcriptional processing of the *mtrC* transcript; IHF is also important in regulating the *farAB* efflux pump operon (see below). It is unclear, however, how the CE and perhaps more importantly the IHF-binding site and IHF, functions in GC since HA-resistance is elevated in strains bearing the CE sequence. Nevertheless, the presence (albeit rare) of the CE at this site in GC suggests that

horizontal gene transfer occurred between MC and GC and emphasizes the importance of recombination events in generating diversity in clinical strains of both pathogens.

The *norM* and *macAB* genes are also regulated by *cis*-acting control elements that were defined by mutations (Figure 3). The presence of point mutations in the -35 hexamer of the *norM* promoter (C→T) or in the ribosome-binding site (A→G) can enhance bacterial resistance to certain quaternary ammonium compounds as well as to norfloxacin and ciprofloxacin (59). A point mutation in the -10 hexamer of the *macAB* promoter (G→T) has been identified that increases expression of *macAB* and levels of macrolide resistance in GC (62). Unlike *cis*-acting mutations that influence *mtrCDE* expression, for both the *norM* and *macAB* systems, the aforementioned mutations are not likely by themselves to confer clinically significant levels of resistance to their antibiotic substrates. However, together with other mutations that impact antibiotic susceptibility levels, the presence of these *cis*-acting mutations could influence the efficacy of antibiotic therapy, particularly for strains with susceptibilities near the MIC breakpoint for the relevant antibiotics. Like the mtr_{120} mutation for *mtrCDE*, the point mutations found to regulate *norM* and *macAB* alter the sequence of their respective promoter elements to be closer to consensus (3, 68). Thus, it appears that point mutations within bacterial promoters can upregulate gene expression by enhancing promoter recognition by RNA polymerase outside of the control of transcriptional regulators, or, in the case of *norM*, may increase translation through improved ribosomal recognition of the *norM* transcript.

Trans-acting factors that regulate efflux pump genes

A host of DNA-binding proteins directly or indirectly control expression of genes encoding structural proteins of drug efflux pumps in gonococci (18, 66). Many of these DNA-binding proteins also have the ability to directly modulate expression levels of other genes important in gonococcal physiology and metabolism, as well as regulating genes encoding transcriptional regulators of efflux pump determinants. In this respect, the DNA-binding proteins that control *mtrCDEF and farAB* expression are interconnected (Figure 4) and specific examples are discussed in detail below.

MtrR control of gene expression

MtrR, first described by Pan & Spratt (53), performs a central role in regulating *mtrCDE* directly and *farAB* indirectly (37). It is a member of the TetR/QacR family and binds as two homodimers to the *mtrCDE* promoter region to dampen expression of this operon (Figure 5) (24, 43). Studies on clinical isolates that express elevated levels of resistance to hydrophobic agents recognized by the MtrC-MtrD-MtrE efflux pump have shown that such strains bear mutations that cause radical amino acid replacements in the helix-turn-helix (HTH) motif (residues 32-53) that significantly reduce MtrR-binding to target DNA sequences (65). Some mutations that can confer a phenotype are outside of the HTH region and they might alter dimer formation or drug interactions (65). These loss-of-function mutations can enhance *mtrCDE t*ranscription 2-3 fold and, depending on the substrate, increase resistance to antimicrobials by 4-10 fold (75, 81). It is important to emphasize that because loss-of-function mutations of *mtrR* have been observed in clinical isolates, their occurrence is relevant for considering antibiotic treatment regimens used worldwide. In addition to contributing to antibiotic resistance, loss of MtrR, leading to

increased levels of the MtrC-MtrD-MtrE efflux pump, conferred a fitness advantage in vivo in the female mouse model of lower genital tract infection (78). These results confirmed previous findings showing that bacterial strains expressing the MtrC-MtrD-MtrE efflux pump had greater and longer survival in the genital tract of BALB/c mice than isogenic strains that did not express an active pump (29).

Using classical genetic and microarray technologies (14, 15, 37), we have learned that MtrR directly or indirectly controls over 65 genes at different phases of growth. We performed a microarray analysis using RNA extracted from mid-logarithmic phase cultures of strain FA19 and an isogenic derivative bearing an *mtrR* deletion to identify MtrR-repressed and –activated genes (15). The cumulative results from these studies have lead us to conclude that MtrR is a global regulatory protein and its capacity to control genes involved in stress response (*rpoH*) (15), peptidoglycan biosynthesis (*ponA*) (14), amino acid biosynthesis (*glnA*) (31), polyamine uptake (*potF and potH*) and regulation of a regulator (*farR*) of the *farAB* efflux operon (37) may be as important as its regulation of *mtrCDE*. We have termed these genes as being “off-target”, since MtrR is acting as a *trans*-regulator to distinguish them from the adjacent *mtrCDE* genes (31).

Examples of specific off-target genes and their role in GC physiology are described below. MtrR can directly repress *rpoH*, which encodes an essential sigma factor involved in environmental stress response (19). This regulation results in expression changes of a panel of RpoH-RNA polymerase transcribed genes (15). RpoH expression is involved in the ability of GC to resist oxidative stress and loss of MtrR production enhances bacterial resistance to hydrogen peroxide (15). Thus, MtrR can modulate levels of GC susceptibility to both oxidative (e.g., H_2O_2) and nonoxidative

(e.g., antimicrobial peptides) killing systems of neutrophils. Furthermore, MtrR can regulate expression of efflux genes other than *mtrCDE* (37). It can directly repress *farR,* which encodes the transcriptional repressor of the *farAB* efflux pump operon (Figure 4). In opposing manners, MtrR and FarR can regulate expression of *glnA,* which encodes glutamine synthetase. FarR binds upstream of the *glnA* translational start and activates its expression (31). However, MtrR can bind upstream of the FarR-binding site and dampen transcription of *glnA* by reducing FarR binding to the *glnA* promoter and FarR expression. Curiously, MtrR can activate *glnE* (31), which reveals that it can differentially control genes encoding enzymes that function in the same metabolic pathway. In addition to the mentioned regulatory roles of MtrR, preliminary studies suggest that MtrR also influences the extent of biofilm formation in *N. gonorrhoeae*. However, further research is needed to determine the MtrR-regulated genes involved in this process since none of the genes known to be involved in biofilm formation, such as *norB*, *estD*, or *aniA* (12), were identified as MtrR-regulated by microarray analysis (15) .

An important evolutionary question is why did GC not dispense with MtrR since its loss increases antibiotic resistance and *in vivo* fitness? It is likely that genes activated by MtrR hold the key. In the mouse infection experiments performed by Warner *et al*. (78), loss of MtrR afforded a fitness advantage for the first five days, but this advantage waned, suggesting that possession of MtrR may be advantageous at later stages of infection. One of these MtrR-activated genes that may be important in vivo is *glnE* (15), which is of significant importance in *Salmonella typhimurium* growth and fitness *in vivo* (34), is essential in mycobacteria (54) and apparently is essential in GC as we have been unable to construct a *glnE* null mutation. GlnE encodes the enzymatic regulator of

glutamine synthetase and can activate or de-activate the enzyme. Since levels of glutamine are low at mucosal surfaces and within phagocytes, the ability to synthesize glutamine *in vivo* may be important for survival and maximal fitness. Alternatively, or in conjunction with this necessity to produce glutamine at mucosal surfaces, the importance of MtrR regulation of off-target genes may be more integral in the long-term maintenance of gonococcal infections than previously understood due to its involvement in the regulation of biosynthetic pathways. Indeed, we found (15) that the largest subpopulation of MtrR regulated genes of known function were those involved in biosynthetic pathways and it is not without precedent that TetR/QacR transcriptional regulators can play a role in regulating expression of genes involved in such pathways (56). Certainly, other examples of MtrR-activated genes exist, but this subset of the MtrR regulon may explain why *mtrR* has not been discarded over the millennia.

MtrA regulation of efflux gene expression

MtrA is a member of the AraC family of transcriptional regulators and was discovered during experiments that evaluated whether growth of the gonococci in sub-lethal level of substrates recognized by MtrC-MtrD-MtrE could enhance bacterial resistance to such antimicrobials (58). A panel of strains was tested and two urogenital isolates (strains FA19, FA889) and an invasive bloodstream isolate (strain UUI) were able to increase resistance to TX-100 when incubated overnight in sub-lethal levels of TX-100. For five other strains tested, including strain FA1090, no increase in resistance was observed (58). A database search revealed an open reading frame that was likely to encode a transcriptional activator (*mtrA*) similar to those that positively regulate the expression of other bacterial efflux operons (1, 17, 33). Comparing the sequence of *mtrA*

from both strain FA19 and FA1090 revealed the presence of an 11 bp deletion in the coding region of FA1090, which would result in the production of a non-functional protein. Subsequent sequencing of PCR products from many gonococcal strains and whole genome sequencing efforts have shown that the 11 bp deletion in FA1090 is common, present in nearly 40% of strains. Introducing a null mutation into *mtrA* in strain FA19 resulted in the loss of the induction phenotype, suggesting that MtrA induces expression of the *mtrCDE* efflux pump when grown in sub-lethal concentrations of TX-100 (58). This inducible resistance was later found to be dependent on the TonB-ExbBD system for energy (60).

MtrA is not only a positive regulator of *mtrCDE*, it also controls (directly or indirectly) other genes involved in efflux or regulation of efflux genes (e.g. *mpeR*). Importantly, loss of MtrA was found to negatively impact the in vivo fitness of gonococci in the mouse infection model (78). This could be due the loss of induction of the *mtrCDE* pump operon by MtrA when GC are in the presence of antimicrobials recognized by the efflux pump. Supporting this conclusion, two second site suppressor mutations in the *mtrR* gene were isolated that gave increased fitness of the *mtrA* mutant in the mouse model (78). This suggests that loss of the repressor (MtrR) compensates for the loss of the activator (MtrA) of the *mtrCDE* efflux pump operon. However, as MtrA is a global regulator of genes, further experiments are needed to determine the cause of the loss of fitness of MtrA mutants in the mouse model.

Recently, our group has purified MtrA as a maltose-binding protein fusion protein and found that it can bind in a specific manner upstream of the MtrR-binding site near *mtrCDE* (Figure 5). The binding of MtrA to this site is enhanced by the presence of an

efflux pump substrate (e.g., TX-100) and in this instance, endows MtrA with a greater affinity for the target DNA than that of MtrR (Zalucki *et al*., manuscript in preparation). Taken together, the earlier observations of Rouquette *et al.* (58) regarding the induction of *mtrCDE* and TX-100 resistance can now be explained by the ability of MtrA to bind upstream of *mtrCDE* with a greater affinity than the natural repressor (MtrR) and promote transcription of the efflux pump operon.

MpeR regulation of GC genes

MpeR was discovered by Folster and Shafer (16) during a search of the FA1090 genome sequence for regulators, in addition to MtrR, that might control expression of *mtrF*. As described above, MtrF is an apparent accessory protein of the MtrC-MtrD-MtrE efflux pump and is needed for high-level resistance of gonococci to antimicrobials recognized by this pump (16, 76). MpeR also negatively regulates expression of *mtrR* (Figure 4, Mercante *et al*., unpublished). Interestingly, *mpeR* was found to be under the negative control of Fur complexed with iron (26). Additionally, *mpeR* is maximally expressed at the late log phase of growth when available free iron levels begin to diminish (Mercante *et al*., unpublished). The capacity of Fur + iron to repress *mpeR* and the ability of MpeR to repress *mtrR*, likely explains why *mtrCDE* is maximally expressed late in growth when levels of free iron would be depleted. Recent collaborative work with C. Cornelissen has shown that MpeR activates expression of *fetA*, which encodes a single-component TonB-dependent receptor that allows the gonococcus to acquire iron from enterobactin-like siderophores produced by enteric bacteria (5, 25). This regulation was observed in strain FA1090, but not FA19, because MtrA is a negative regulator of *mpeR* (Figure 4) and, as mentioned above, FA1090 is a natural *mtrA* null mutant, thus

removing this regulatory control mechanism. Taken together, these findings show that MpeR plays important roles in both drug efflux and iron acquisition by gonococci, emphasizing the need to consider regulators of efflux genes in a larger context with respect to bacterial physiology and pathogenesis.

Involvement of a two component regulatory sytem in controlling mtrCDE

The MisR response regulator and MisS sensor kinase proteins form a classical two-component regulatory system (TCS) that is similar to but distinct from the PhoP/PhoQ system of *Salmonella enterica* serovar Typhimurium (47). The *misRS* operon has been extensively studied in MC through both classical genetics and microarray technology (74), and was named for its ability to influence meningococcal LOS inner core structure (73). It also controls expression of *dsbD*, which is required for the maintenance of disulfide bonds in periplasmic/envelope proteins that may become damaged by the host redox defenses (35). MisR/MisS also enhances expression of hemoglobin uptake and utilization proteins by serogroup B MC, suggesting that this TCS plays a role in iron scavenging (82). Other studies have shown that MisR/MisS is crucial for meningococcal pathogenesis. Serogroup C MC that lacked a functional *misR* gene were deficient in their ability to colonize human endothelial cells *in vitro* (27) and were avirulent in a murine intraperitoneal infection model (49).

In GC, a link between *misRS* expression and the *mtrCDE* efflux pump operon was established when growth in sub-lethal levels of the antimicrobial peptide LL-37 increased transcription of the *misR* and *misS* genes as analyzed by microarray and qRT-PCR (Shafer *et al*., unpublished observations), resulting in inducible, high-level LL-37 resistance, which required this TCS. Because LL-37 is a known substrate of the MtrC-

MtrD-MtrE efflux pump (67), we tested if MisR/MisS could regulate expression of *mtrCDE* and found that the MisR/MisS TCS indirectly controls *mtrCDE* by dampening expression of *mtrR*, the master repressor of *mtrCDE* (Kandler & Shafer, unpublished). Thus, strong de-repression of *mtrCDE* is most likely to occur when levels of MpeR, which represses *mtrR* (Figure 4), are high during iron starvation, and when the MisR/MisS TCS is activated by sub-lethal concentrations of host defense peptides. Since the human host restricts the availability of free iron, and mucosal surfaces often contain low levels of LL-37 during inflammation resulting from infection (6), it is likely that *mtrCDE* expression is higher during infection than when GC are cultivated in the laboratory in iron-rich culture media. Furthermore, just as MpeR allows GC to adjust gene expression to deal with falling iron levels (Mercante & Shafer, unpublished), the MisR/MisS TCS may provide a mechanism for GC to sense and adapt to increasing concentrations of antimicrobial peptides as they progress through the different stages of infection. MisR/MisS also modulates levels of antimicrobial peptide susceptibility in MC (30, 49, 73). Although the MtrC-MtrD-MtrE pump produced by MC can export antimicrobial peptides, it is unclear if meningococcal MisR/MisS acts to modulate levels of peptide resistance by an efflux-dependent process.

The FarR DNA-binding protein and gene expression

FarR is the local repressor of the *farAB* efflux operon (37). FarR-mediated repression of *farAB* also requires IHF, and a model for this joint repression has been published (36). Interestingly, expression of *farR* is negatively regulated by MtrR (37). Thus, MtrR can directly and indirectly regulate levels of two efflux systems in gonococci. In contrast, *farAB* is not negatively regulated by FarR in the naturally fatty-acid resistant

MC (64). Instead, meningococcal FarR appears to control expression of *nadA* (63), which encodes a surface-exposed outer membrane protein produced by 40% of capsular serogroup B strains involved in attachment to and invasion of host cells (4). NadA is currently under investigation as a vaccine candidate that could be used to help protect against capsular serogroup B MC strains, for which no vaccine is available (7).

Conclusions

We propose that for strict human pathogens, such as GC, bacterial efflux pumps perform important roles in promoting survival when they are confronted by host antimicrobials or antibiotics. Unlike other Gram-negative pathogens, GC possesses a limited number of pumps (Figure 1), yet uses these few pumps effectively to avoid the action of antimicrobials. From a clinical perspective, over-expression of the *mtrCDE*-encoded efflux pump system was a key component in the downfall of penicillin as a useful (and cheap) antibiotic in treating gonorrhea (75). We further propose that the ability of GC to transcriptionally modulate expression of efflux pump genes promotes survival and determines fitness during infection. There is clear evidence for this in an animal model system, but the frequent isolation of strains bearing mutations in regulatory genes that control efflux pump levels (especially those negative regulatory proteins) from patients suggests that the same is true for human infections (65, 66). It is important to recognize that many of these regulatory proteins are interconnected and also control other important genes involved in basic metabolism and pathogenesis. Thus, by using efflux pump gene regulators to control a larger set of genes, GC has economically tailored itself to both respond to adverse conditions (e.g., presence of antimicrobials) as well as perform vital physiological functions, both of which are important during infection. Finally, it is

essential that we further consider how host factors impact efflux pump gene expression during infection and how these changes influence survival of GC in vivo.

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FIGURE LEGENDS

Figure 1. Membrane organization of antimicrobial efflux pumps of gonococci. The proposed membrane organization and class of the known antimicrobial efflux pumps common to gonococcal strains (NorM, MacA-MacB-MtrE, FarA-FarB-MtrE and MtrC-MtrD-MtrE) and how each pump is energized are depicted in this figure. MtrE acts as an outer membrane channel protein for three pumps. MtrF is shown as an accessory protein for the MtrC-MtrD-MtrE pump.

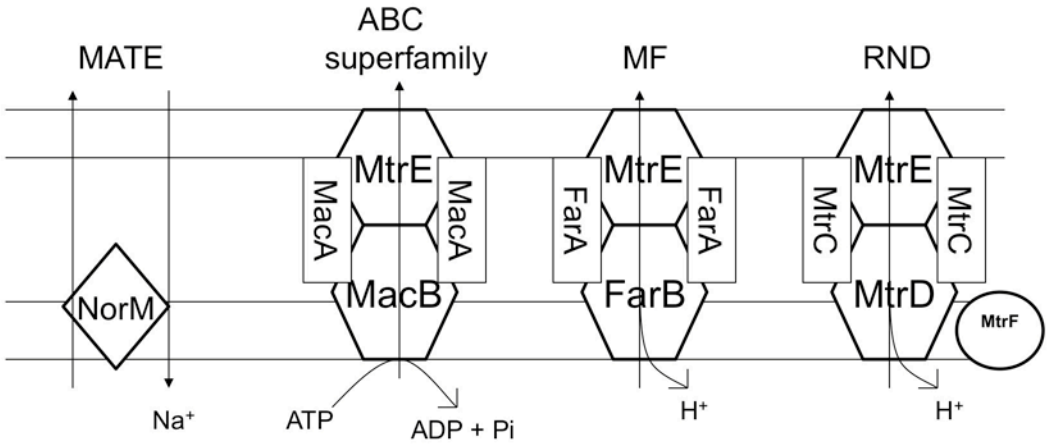
Figure 2. Genetic organization of the efflux components of gonococci. Shown are the genes that encode components of the four drug efflux pumps (MtrC-MtrD-MtrE, FarA-FarB-MtrE, MacA-MacB-MtrE and NorM) expressed by gonococci; all genes are drawn to scale. Please see text for details of genes and corresponding pumps.

Figure 3. *Cis*-acting mutations regulate efflux pump expression in gonococci. A. The *mtrR-mtrC* intergenic region. The 13 bp inverted repeat is boxed. The site of the single bp deletion described by Hagman et al (1995) is bolded. The consensus -10 element generated by the mtr_{120} mutation is shown, with the C→T mutation in bold text. B. The *norM* promoter region. Single base pair mutations resulting in increased *norM* expression are indicated by bold nucleotides. C. The *macAB* promoter region. The G→T mutation in the -10 element that results in increased *macAB* expression is bolded.

Figure 4. Schematic of the regulatory circuit mediated by the DNA-binding proteins that modulate *mtr* efflux expression. The details of the genes and regulation are discussed in the text; arrows indicate gene activation and bared lines indicate gene repression.

Figure 5. MtrR binding site within the *mtrR-mtrC* intergenic region and helix-turn-helix motif of MtrR. (A) Schematic representation of the *mtrR* and *mtrC* genes in *N. gonorrhoeae*. The double-stranded nucleotide sequence pictures the start sites (+1) of *mtrR* and *mtrCDE* (in boldface) and the -10 and -35 sequences for *mtrR* and *mtrC* (underlined and overlined, respectively). The binding site for the two MtrR homodimers (Hoffman et al., 2005) is highlighted in gray. The *cis*-acting 13-bp inverted repeat element within the *mtrR* promoter is shown and the site of the single bp deletion frequently found in clinical isolates is indicated by a box. In the diagram, bent arrows indicate the starting sites for each gene. The *mtrR* gene region encoding the helix-turn-helix (HTH) motif, characteristic of TetR/QacR transcriptional regulators, is indicated by the hatched pattern in the *mtrR* coding region. (B) Amino acid sequence alignment of the helix-turn-helix motif of MtrR involved in DNA binding is over lined. Residues involved in turns are indicated by double lines. Missense mutations frequently found in clinical isolated are indicated by an asterisk (A39T, G45D). Residues involved in DNA binding identified by crystal structures in TetR and QacR are indicated in boldface (for review see Ramos, 2005). α-helices in TetR and QacR are shaded. Highly conserved residues overlap with helix and turns.

Figure 1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MF
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ADP + Pi
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H+

Figure 2

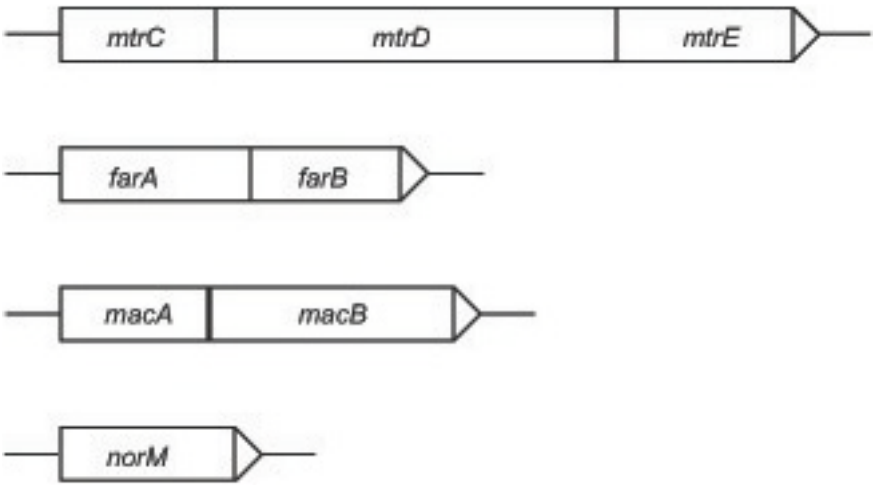
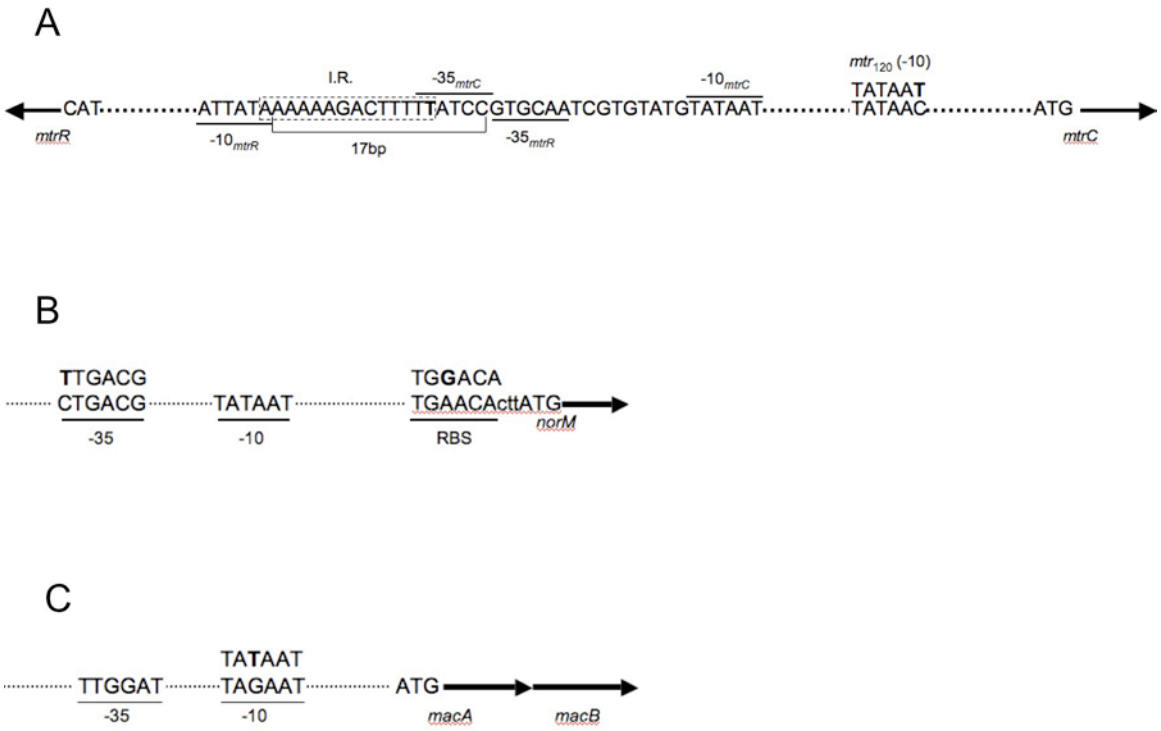


Figure 3


A
I.R.
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-10mtrC
mtr120 (-10)
TATAAT
CAT
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ATG
mtrR
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17bp
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mtrC
B
TTGACG
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TATAAT
TGGACA
TGAACActtATG
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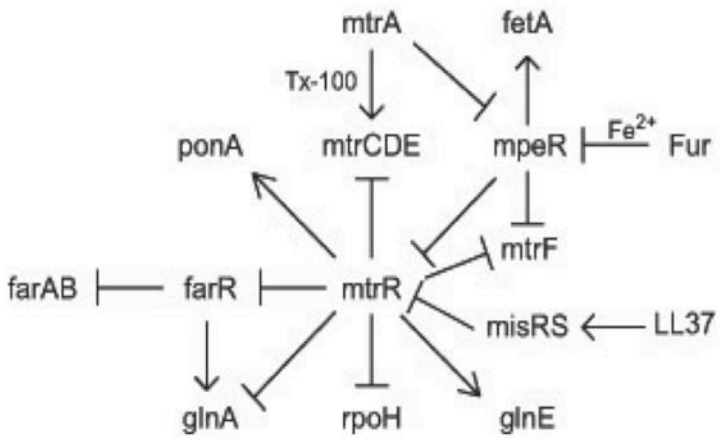
macA
macB

Figure 4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fetA
Tx-100
ponA
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LL37
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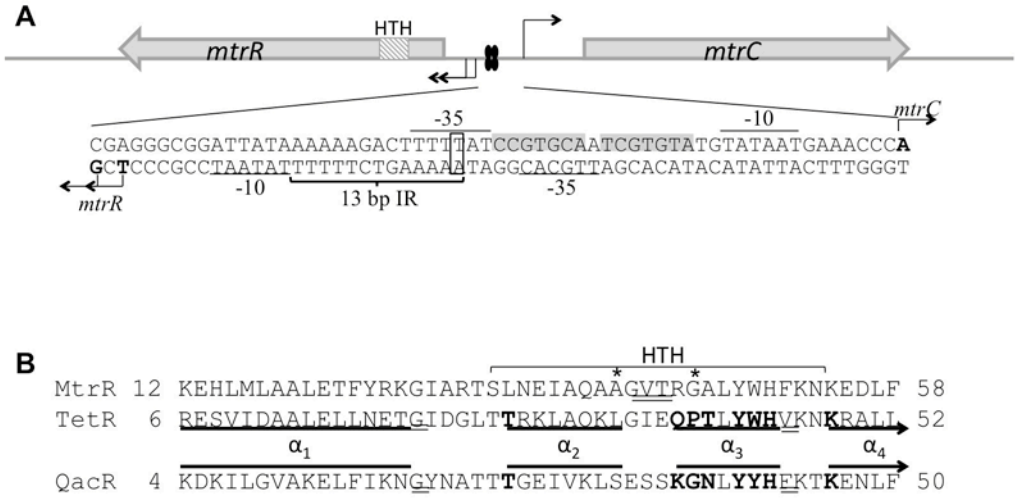
rpoH
glnE

Figure 5


A
HTH
mtrR
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-35
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mtrC
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-10
13 bp IR
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mtrR
B
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MtrR 12 KEHLMLAALETFYRKGIARTSLNEIAQAAGVTRGALYWHFKNKEDLF 58
TetR 6 RESVIDAALELLNETGIDGLTTRKLAQKLGIEQPTLYWHVKNKRALL 52
α1
α2
α3
α4
QacR 4 KDKILGVAKELFIKNGYNATTTGEIVKLSESSKGNLYYHEKTKENLF 50

Chapter 3: MpeR regulates the *mtr* locus in *Neisseria gonorrhoeae* and modulates antimicrobial resistance by an iron-responsive mechanism.

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This manuscript was written by first author Alexandra Dubon Mercante with editorial changes made by David W. Dyer and William M. Shafer.

Previous studies have shown that the MpeR transcriptional regulator produced by *Neisseria gonorrhoeae* represses expression of *mtrF*, which encodes a putative inner membrane protein (MtrF). MtrF works as an accessory protein with the MtrC-MtrD-MtrE efflux pump helping gonococci to resist high levels of diverse hydrophobic antimicrobials. Regulation of *mpeR* has been reported to occur by an iron-dependent mechanism involving Fur (Ferric uptake regulator). Collectively, these observations suggest the presence of an interconnected regulatory system in gonococci that modulates expression of efflux pump protein-encoding genes in an iron-responsive manner. Herein, we describe this connection and report that levels of gonococcal resistance to a substrate of the *mtrCDE*-encoded efflux pump can be modulated by MpeR and the availability of free iron. Using microarray analysis, we found that the *mtrR* gene, which encodes a direct repressor (MtrR) of *mtrCDE*, is an MpeR-repressed determinant in the late-logarithmic phase of growth when free iron levels would be reduced due to bacterial consumption. This repression was enhanced by conditions of iron-limitation and resulted in increased expression of the *mtrCDE* efflux pump operon. Futhermore, as judged by DNA-binding analysis, MpeR-mediated repression of *mtrR* was direct. Collectively, our results indicate that both genetic and physiologic parameters (e.g., iron availability) can influence expression of the *mtr* efflux system and modulate levels of gonococcal susceptibility to efflux pump substrates.

INTRODUCTION

Neisseria gonorrhoeae is a strict human pathogen and the causative agent of the sexually transmitted infection (STI) termed gonorrhea. Gonorrhea ranks second in the United States as the most common STI with a rate of infection reported to be 112 cases per 100,000 people in 2008 (7). Worldwide, it has been estimated that over 90 million cases of gonorrhea occur each year (35). This annual incidence of gonorrhea, the increasing prevalence of antibiotic-resistant clinical isolates (39), especially those expressing decreased susceptibility or clinical resistance to ceftriaxone (6, 18, 38), evidence that repeated gonococcal infections increase host susceptibility to infection by the Human Immunodeficiency Virus (4, 25), the lack of a protective vaccine (5) and the serious consequences of infection on the reproductive health of males and females, makes gonorrhea a major global health problem.

With respect to antibiotic resistance, the Centers for Disease Control and Prevention placed *N. gonorrhoeae* on the "Super Bugs" list in 2007 and growing concern exists about the future of antibiotic therapy in treating gonorrhea (17, 23). Accordingly, it is important to understand the genetic and physiologic processes that govern antibiotic resistance in the gonococcus. In this respect, energy-dependent efflux of multiple, structurally diverse antimicrobials by the MtrC-MtrD-MtrE (Mtr) system is an important mechanism used by gonococci to resist the bactericidal action of certain antibiotics (e.g., β-lactams and macrolides), topically applied spermicides (e.g., nonoxynol-9) and host-derived compounds that participate in innate host defense (e.g., progesterone and the antimicrobial peptide LL-37 (20, 29)). Not only does the Mtr efflux pump assist gonococci in developing clinically significant levels of antibiotic resistance, it also

provides an advantage during infection since its production is required for a sustained lower genital tract infection in a female mouse model (20). Moreover, the ability of gonococci to up-regulate its production provides a fitness benefit because certain *cis*- or *trans*-acting mutations that enhance *mtrCDE* expression can significantly increase levels of *in vivo* fitness in the mouse model of infection (36, 37).

Besides the *mtrCDE* efflux pump-encoding operon, two additional genes within the *mtr* locus are important in the ability of gonococci to export antimicrobials recognized by the Mtr efflux pump. The *mtrR* gene, which encodes a transcriptional repressor (MtrR) of *mtrCDE*, is closely linked to the *mtrF* gene, which encodes a putative inner membrane protein (MtrF); both genes are upstream of *mtrCDE* (15). MtrR, a member of the QacR/TetR family of DNA-binding proteins, dampens *mtrCDE* expression by binding within the promoter region of *mtrCDE* (15, 24). MtrR can also repress *mtrF* (12) and can positively or negatively control expression of over 65 genes outside of the *mtr* locus that are scattered throughout the chromosome (11). Although the precise function of MtrF in gonococci remains to be determined, it seems to function as an accessory protein for the Mtr efflux pump, needed for high levels of antimicrobial resistance mediated by the Mtr efflux pump (12, 34). In addition to being under the negative control of MtrR (11, 12), *mtrF* is subject to repression by MpeR, which is an AraC-like transcriptional regulator (12). The *mpeR* gene is restricted to the pathogenic *Neisseria* (9) and is negatively regulated by Fur (Ferric uptake regulator) and iron (19). Interestingly, MpeR has recently been found by Hollander *et al.* (16) to directly activate the *fetA* gene of *N. gonorrhoeae* strain FA1090; FetA is a surface exposed receptor for enterobactin-like siderophores (2). MpeR may also be linked to iron acquisition systems

in *N. meningitidis* as Fantappie *et al.* (9) reported that it can bind near the promoter for a gene (annotated as NMB1880) that encodes a FetB-like lipoprotein thought to bind siderophores in the periplasmic space.

Given the likely importance of MpeR in determining levels of gonococcal resistance to antimicrobials and a possible connection of this regulatory protein with iron acquisition and regulatory processes, we sought to determine the number and types of genes controlled by MpeR. In this context, we now report that expression of the gene encoding the major transcriptional repressor (MtrR) of the *mtrCDE* efflux pump operon is controlled by MpeR and levels of free iron.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains TOP10 (Invitrogen, Carlsbad, CA) and DH5α mcr were used in all cloning experiments. *E.coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates and incubated at 37 °C. *N.gonorrhoeae* strain FA19 was used as the primary gonococcal strain (30) and all strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, MI) containing defined supplements I and II at 37°C under 3.8% (vol/vol) CO_2 or in GCB broth with supplements I and II and sodium bicarbonate as previously described (30). Iron-deplete cultures were grown in GCB broth containing the supplements described above and 200 μM deferoxamine mesylate (Desferal). The plasmids and oligonucleotide primers used in this study are listed in Tables 1 and 2. All chemicals were purchased from Sigma Biochemical (St. Louis, MO.).

Antimicrobial susceptibility assays. The minimal inhibitory concentration (MIC) of selected antimicrobial agents including triton X-100, potassium tellurite (K_2TeO_3), and silver nitrate ($AgNO_3$) was determined using the agar dilution assay previously described (15).

RNA Isolation, microarray design, cDNA labeling and hybridization, and array analysis. Strains FA19 and JF5 (as FA19 but *mpeR*::Km (13)) were grown in 50 ml GCB broth and samples of each strain were harvested at both the mid-log (OD_{600} = 0.6) and late-log (OD_{600} = 0.8) phases of growth for RNA isolation using the hot-phenol method as previously described (9). Following DNase treatment (Qiagen DNase Kit), RNA recovery (Qiagen RNeasy Mini kit) and quantification by Nano-drop 1000 (NanoDrop Technologies), microarray analysis was performed. The microarray design, cDNA labeling and hybridization, as well as data analysis from the array were all conducted as previously described (12). Genes that showed expression differences of 1.5-fold ($P \leq 0.05$) were considered to be subject to MpeR regulation.

Inactivation of *mpeR* in strain FA140. Chromosomal DNA from strain JF5 was used as a template to PCR-amplify *mpeR*::Km with primers 5'*mpeR* and 3'*mpeR* (Table 2). The resulting PCR product was gel-purified and used to transform FA140 and transformants were selected on GCB agar with 50 μg/ml of kanamycin; a representative transformant (see strain AD9 in Table 1) was chosen for further study and the presence of an inactivated *mpeR* gene was confirmed by PCR and DNA sequence analyses.

Complementation of *mpeR*::*km*. In order to complement *mpeR*::Km in gonococcal strain JF5, primers 5'pMpeRpac and 3'GC4MpeR (Table 2) were used to PCR amplify *mpeR* and 250 bp of upstream DNA from FA19 chromosomal DNA. The resulting DNA fragment was cloned into pGCC3 (33) at the PmeI and PacI (New England Biolabs) sites to produce pGCC3-*mpeR*. Following DNA sequencing to confirm the correct orientation, this construct was transformed into gonococcal strains JF5 *mtrF-lacZ*, JF5 *mtrR-lacZ*, JF5 *mtrC-lacZ* and JF5 (Table 1) as previously described (14). Transformants in which *mpeR* recombined into the chromosome between the genes *lctP* and *aspC* were selected on GCB agar containing 1 μg/ml of Ery (erythromycin) and the presence of the inserted DNA was confirmed by PCR analysis using primers 5'mpeR and 3'mpeR. The resulting strains were named AD1 (JF5 *mtrF-lacZ*, *mpeR*$^+$), AD3 (JF5 *mtrR-lacZ*, *mpeR*$^+$), AD4 (JF5 *mtrC-lacZ*, *mpeR*$^+$), and AD5 (JF5, *mpeR*$^+$) (Table 1).

Construction of *lacZ* fusion strains in gonococci, preparation of cell extracts, and β-galactosidase assays. All translational *lacZ* fusions were constructed as previously described (32). In order to construct FA19 *mpeR-lacZ* (Table 1), the 161 bp sequence upstream of *mpeR* was PCR-amplified from strain FA19 using primers 5'pmpeR and 3'pmpeR (Table 2) and inserted into the BamHI site of pLES94. This construct was transformed into DH5α TOP10; the correct insertion and correct orientation was confirmed by DNA sequencing. The plasmid construct was then transformed into FA19, which allowed for insertion of the translational fusion between the *proAB* genes. Transformants were selected on GCB agar with 1 μg/ml of chloramphenicol (Cm). Strain

JF5 *mtrR-lacZ* was constructed by PCR amplifying *mpeR*::Km from strain JF5 using primers 5'*mpeR* and 3'*mpeR* [Table 2]. This DNA fragment was used to transform FA19 *mtrR-lacZ*. Transformants were selected on GCB agar with 50 μg/ml of Km and sequencing of a PCR-amplified product was performed to confirm disruption of *mpeR*. In order to construct strain JF1 *mtrC-lacZ*, the promoter sequence of *mtrC* (16) was cloned into pLES94 as previously described (13) and this plasmid construct was transformed into JF1. Insertion and selection of transformants was as described for construction of FA19 *mpeR-lacZ*. Strains harboring *lacZ* fusions were grown in GCB broth and 15 ml of culture were harvested by centrifugation at 5,000xg for 10 min. The supernatant was discarded and the remaining pellet was washed once with phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer (0.25 mM Tris [pH 8.0]). Cells were broken and β-galactosidase assays were performed as previously described (13, 34).

Inactivation of *terC* and complementation of the *terC* null mutant. The *terC* gene (NGO1059) was inactivated as previously described (13, 24). Briefly, the primer sets 5'A*terC* and 3'B*terC*Sma along with 5'C*terC*Sma and 3'D*terC* were used to PCR-amplify *terC* from FA19 chromosomal DNA in order to engineer a SmaI site within the gene. The products from these two PCR reactions were then used as a template to PCR-amplify the entire gene using primers 5'A*terC* and 3'D*terC*. This 1500 bp PCR product was inserted into pBAD-TOPO-T/A and transformed into *E.coli* TOP10 as described in the manufacturer's protocol (Invitrogen). The non-polar kanamycin resistance cassette, *aphA-3*, (28) was digested from pUC18K with SmaI and cloned into the engineered SmaI site of *terC*. This construct was transformed into DH5α TOP10 and, following plasmid

purification, the inactivated *terC*::Km sequence was PCR-amplified using primers 5'A*terC* and 3'D*terC*. This plasmid was used to transform FA19 and transformants harboring the disrupted gene (see strain AD6 in Table 1) were selected on GCB agar containing 50 μg/ml Km. The presence of the inactivated gene was confirmed by PCR and sequencing analysis. Complementation of *terC* in strain AD6 was done as described for *mpeR* (above) except that primers 5'p*terC*Pac and 3'GC4*terC* were used to amplify *terC* from FA19 chromosomal DNA. The resulting complemented strain was termed AD7 (Table 1).

Immunodetection of MtrR. Strain FA19 was grown in GCB broth under either iron-replete or iron-deplete conditions as described above. At the late-logarithmic phase of growth, samples were harvested by centrifugation and cell extracts were solubilized and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblotting was conducted as described previously (11). MtrR was detected using rabbit anti-MtrR antiserum (1:1,000) and goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (1:1,000) as described previously (12).

Construction and purification of maltose-binding protein (MBP)-MpeR fusion protein. MpeR was fused in frame at its N-terminus to MBP by using the pMal-c2x fusion vector (New England Biolabs, Beverly, MA) as previously described (17) utilizing primers 5'malEmpeRF and 3'malEmpeRR (Table 2). Growth of the *E. coli* transformant bearing the plasmid construct, induction of expression and purification of MBP-MpeR was performed as previously described (23).

Electrophoretic mobility shift assay (EMSA). The *mtrR* probe was PCR- amplified from FA19 chromosomal DNA using primers 5'KH9#1 and 3'cel2A (Table 2) to generate a 360 bp product and purified using the QIAquick PCR purification kit (Qiagen). The PCR product was radiolabeled and purified as previously described (17). The labeled DNA fragment (10 ng) was incubated with 15 μg of fusion protein (MBP-MpeR). Specificity of binding was shown by incubating 15 μg of MBP-MpeR and 10 ng of the 360 bp radiolabeled *mtrR* probe with increasing concentrations of cold specific competitor (non-radiolabeled *mtrR*) and with increasing concentrations of a cold non-specific competitor (non-radiolabeled *misR*) amplified using 5'*misR*-205 and 3'*misR*+104 to generate a 309 bp product. All reactions were incubated along with DNA binding buffer (10mM Tris-HCl, 200mM NaCl, 1mM DTT, 1 μg/ml of Poly dIdC) for 30 min. at room temperature. Samples were subjected to electrophoresis on a 6% (wt/vol) polyacrylamide gel at 4°C followed by autoradiography.

Primer extension of *mtrF*. In order to determine the start site for *mtrF* transcription total RNA was prepared from a mid-logarithmic culture of strain FA19 as described above. The primer 3'*mtrF-R* was radiolabeled using [γ^{32}P] and T4 polynucleotide kinase and used to reverse transcribe 10μgs of total RNA. The primers 5'*mtrF*-F and 3'*mtrF*-R were used along with FA19 chromosomal DNA as a template to amplify the *mtrF* promoter region in order to generate reference sequence products using the SequiTherm EXCEL II DNA Sequencing kit (Epicenter). Both the primer extension product and the reference sequence were subjected to electrophoresis on a 6% (w/v) sequencing gel which was dried and autoradiography was performed for visualization.

RESULTS

MpeR regulation of *mtrF* and influence of iron availability. Prior to determining the MpeR regulon in *N. gonorrhoeae* strain FA19 (see below), we confirmed that *mtrF* expression is negatively and directly controlled by MpeR (13) and that such regulation is influenced by iron availability as suggested by previous studies (9, 20). For this purpose, we constructed a complemented derivative of strain JF5 (as FA19 but *mpeR*::*km*) and termed this as strain AD1 (Table 1), which expressed *mpeR* ectopically from its own promoter when placed between the *lctP* and *aspC* genes (33). In order to monitor *mtrF* expression, we used a previously described (13) *mtrF::lacZ* translational fusion that harbors 161 bp of the DNA sequence upstream of *mtrF* (Figure 1A) that contains a promoter element for *mtrF* transcription. This promoter was assigned as such based on results from primer extension analysis that showed a transcriptional start point (data not presented) nine nucleotides upstream of a near consensus -10 hexamer sequence that was separated from a putative -35 hexamer sequence by an optimal 17 nucleotides. When the *mtrF-lacZ* fusion was introduced into strains FA19, JF5 and AD1, we found that expression of *mtrF-lacZ* was significantly higher in the *mpeR*::*km* mutant (JF5) compared to either the wild type or complemented strains (Figure 1B).

We next tested if conditions of iron depletion would impact *mpeR* expression in strain FA19 as previously reported by Ducey *et al.* (9) and later found by Jackson *et al.* (20) to involve a Fur-dependent mechanism. As is shown in Figure 2, the peak of *mpeR* expression in strain AD2 (as FA19 but *mpeR-lacZ*) grown in iron-replete GCB broth occurred at the late-logarithmic phase of growth when free iron levels would be reduced

due to consumption. Based on this result, we next monitored *mpeR-lacZ* expression during growth of strain AD2 in iron-replete vs. iron-deplete (+ desferal) conditions and harvested cells at the late-log phase of growth. As expected, the culture growing under iron-deplete conditions had a severe growth defect compared to the control (-desferal) culture, yet expressed *mpeR* at higher levels (Figure 3). This result was consistent with the conclusions reached by Ducey *et al.* (9) and Jackson *et al.* (20) that *mpeR* is an iron-repressed gene in gonococci.

Antimicrobial susceptibility of gonococci can be modulated by MpeR and iron availability. In order to learn if production of MpeR, which negatively regulates expression of *mtrF* (13), can influence levels of gonococcal resistance to antimicrobials recognized by the Mtr efflux pump system in an iron-dependent manner, we assessed the susceptibility of strain FA140 to a known substrate (e.g., triton X-100 [TX-100]) of the pump when this strain was grown under iron-replete vs. iron-deplete conditions. We used FA140 for this purpose because loss of *mtrF*, in this strain, but not in wild type strain FA19, has a phenotype (e.g., hypersusceptibility to antimicrobials such as TX-100 (36). FA140 expresses a high level of resistance to TX-100 mediated by the Mtr efflux pump due to a single bp deletion in the *mtrR* promoter that abrogates *mtrR* expression resulting in elevated expression of *mtrCDE* (35). With FA140, we found that TX-100 resistance was substantially higher (> 64-fold) in the iron-replete culture compared to the iron-deplete culture (Table 3). In order to determine if this iron-dependent, TX-100 resistance required *mpeR* and/or *mtrF*, we next examined genetic derivatives of FA140 that contained inactivated copies of these genes. Using strain WV16 (as FA140 but

mtrF::km), we confirmed that loss of *mtrF* significantly enhances gonococcal susceptibility to TX-100 (Table 3). Importantly, there was no difference in the TX-100 MIC values against the cultures grown in iron-replete vs. iron-deplete conditions. In contrast to parent strain FA140, strain AD9 (as FA140 *mpeR::km*) expressed a high level of TX-100 resistance independent of iron availability (Table 3), indicating that the iron-dependent phenotype of antimicrobial susceptibility requires MpeR.

Identification of MpeR-regulated genes associated with antimicrobial resistance. In order to identify MpeR-regulated genes besides *mtrF* that are involved in antimicrobial resistance, we used RNA from strains FA19 and JF5 to identify the MpeR regulon in wild type strain FA19. As is shown in Table 4, microarray analysis of RNA pairs prepared from mid-logarithmic and late-logarithmic phase cultures of these strains revealed a total of 67 MpeR-regulated genes with forty-six being MpeR-repressed (30 at mid- and 16 at late-log) and twenty-one activated in the presence of MpeR (16 at mid-log and 5 at late-log). The finding that *mtrF* expression was elevated in strain JF5 was consistent with earlier work ((13) and see Figure 1B) that it is an MpeR-repressed gene (Table 4A.) and served as an internal control that validated our use of the microarray system to detect MpeR-regulated genes. Interestingly, for both MpeR-repressed and activated genes, there was no overlap of regulated genes in the mid- and late-log RNA samples.

With respect to genes known or possibly involved in antimicrobial resistance, we identified, in addition to *mtrF*, two genes of interest: *mtrR* and *terC*. Expression of *mtrR*, which encodes the major transcriptional repressor (MtrR) of the *mtrCDE* operon (16) and can also negatively control *mtrF* independently of MpeR (13), was identified as an

MpeR-repressed gene (1.74X) in the late-log culture of strain FA19. In contrast, *terC* (NGO1059), which has been provisionally annotated (www.genome.ou.edu) as encoding a membrane protein (TerC) involved in tellurium resistance (3), was found to be MpeR-activated (2.17X), but only in the mid-log phase of growth. While much is known about *mtrR*, little information is available about *terC* in gonococci but it is of interest, however, because it contains a polynucleotide repeat (A-8) in its coding sequence, making it a candidate for being a phase-variable gene. Since analysis of the FA1090 genome sequence (www.genome.ou.edu) and our own sequencing of the *terC* gene in FA19 (data not presented) showed that *terC* would be in the "phase-on" sequence in these strains, we asked if it was involved in resistance to tellurium as well as antimicrobials recognized by the Mtr efflux pump. For this purpose, a null mutation in *terC* was constructed in strain FA19; this mutant strain was named AD6. Strain AD6 was then complemented with the wild type *terC* gene from FA19, which was cloned into pGCC3 and expressed from its own promoter between *lctP* and *aspC* genes in the chromosome; this complemented strain was termed AD7. Using these strains, we found that loss of *terC* increased gonococcal susceptibility to potassium tellurite (Table 5), but not other tested antimicrobials (e.g., silver nitrate and TX-100) including one (TX-100) recognized by the Mtr pump; this change in tellurium susceptibility endowed by the null mutation could be reversed by complementation. Consistent with *terC* being an MpeR-activated gene, loss of *mpeR* in strain JF5 also resulted in hypersusceptibility of gonococci to potassium tellurite, but this was reversed by complementation when a wild type copy of *mpeR* was expressed ectopically (see strain AD5 in Table 5). In that the *terC* mutant did not show

hypersusceptibility to a substrate of the Mtr pump (e.g., TX-100), we concentrated on MpeR control of *mtrR* and a target of MtrR regulation (*mtrCDE*).

MpeR regulation of *mtrR and mtrCDE*. The observation that *mtrR* expression was repressed by MpeR in the late-log phase culture of strain FA19 (Table 4) suggested that MpeR could indirectly regulate the *mtrCDE* efflux pump operon by modulating levels of its main repressor (MtrR) and that such regulation would be iron-dependent. To test this hypothesis, we examined *mtrR* and *mtrC* expression in MpeR-positive and MpeR–negative strains grown under iron-replete and iron–deplete conditions. Using strains FA19 *mtrR-lacZ*, JF5 *mtrR-lacZ* and the *mpeR* complemented strain with the *mtrR-lacZ* fusion (AD3), we found that *mtrR* expression was decreased in the MpeR-positive strains compared to MpeR-negative strain JF5, confirming that it is an MpeR-repressible gene (Figure 4). Based on this result, we next tested if levels of MtrR would differ in wild type strain FA19 when it was grown under iron-replete vs. iron-deplete conditions. Using SDS-PAGE and Western blot analysis of whole cell lysates that employed anti-MtrR antiserum we found (Figure 5) that the level of MtrR in strain FA19 was increased when grown under iron-replete vs. iron-deplete conditions.

Our finding that levels of MtrR in gonococci can change due to the presence of MpeR and levels of iron suggested that MtrR control of the *mtrCDE* efflux pump operon is regulated by MpeR and iron availability. As a further test of this model, we monitored expression of an *mtrC-lacZ* fusion in FA19, JF5 and the *mpeR*-complemented strain termed AD4 (Table 1). We found that loss of *mpeR* in strain JF5 decreased *mtrC-lacZ* expression by 19.23 % (P = 0.002), which was reversed by complementation when the wild type *mpeR* gene was expressed ectopically in strain AD4 (Figure 6A); this small but

significant difference in *mtrC-lacZ* expression may explain why the *mtrCDE* genes were not identified in the microarray studies as being MpeR-regulated. Additionally, we observed that iron-deplete conditions (+desferal) enhanced *mtrC-lacZ* expression in strain FA19 (Figure 6B). Since MtrR is a direct repressor of *mtrCDE* (16, 26) and its expression can be controlled by levels of iron and MpeR, we hypothesized that expression of *mtrC-lacZ* would not be impacted by the availability of iron in an MtrR-negative strain. In order to test this possibility, we used strains FA19 and JF1 (as FA19 but Δ*mtrR*) containing an *mtrC-lacZ* translational fusion and found that although the level of *mtrC-lacZ* expression was higher in the JF1 background, unlike that of strain FA19, it was not influenced by iron-limitation (Figure 6B).

Binding of MpeR to *mtrR* target DNA. In order to determine if MpeR-mediated repression of *mtrR* was direct, we used EMSA to detect specific binding of an MBP-MpeR fusion protein to a target 360 bp DNA sequence that contained 186 bp of the upstream region containing the *mtrR* promoter (15, 16) and part (174 bp) of the *mtrR* coding sequence. Using a competitive EMSA, we found that incubation of the target DNA with 15 μg of the MBP-MpeR fusion protein resulted in the appearance of two shifted complexes. Of these two complexes only the slower migrating species (shown by the arrow in Figure 7) gave evidence of being a specific complex as it was reduced in intensity by the presence of specific, unlabeled probe but not a nonspecific probe (*misR*) of similar length.

DISCUSSION

Based on the data presented herein, we propose the existence of a regulatory pathway in gonococci that controls expression of the *mtr* efflux pump locus. In this

model (Figure 8), expression of *mtrF* is repressed by MpeR in an iron-responsive process shown by others to involve Fur + iron mediated repression of *mpeR* (9, 20). We propose that MpeR also indirectly enhances *mtrCDE* expression due to its ability to directly repress *mtrR*, which encodes the main repressor of this operon (29), especially when free iron levels are decreased. Taken together, we suggest that since MtrR is the direct repressor of *mtrCDE* (16), levels of free iron would modulate expression of this operon by controlling levels of MpeR and, in turn, MtrR. We cannot presently rule out, however, that MpeR-binding to the *mtrR-mtrCDE* intervening sequence might additionally activate *mtrCDE* expression directly and additional DNA-binding studies are required to resolve this issue.

How might our observations impact the biology and antimicrobial resistance potential of the gonococcus during infection on the genital mucosal surface especially when it is confronted with antimicrobials recognized by the Mtr efflux pump? For instance, in an infection where the gonococcus is confronted with an iron-restricted environment, the negative influence of Fur on *mpeR* expression would be diminished. We hypothesize that iron-restriction would increase *mpeR* expression and the increased level of MpeR would dampen transcription of *mtrR.* As a consequence of this control process, levels of the Mtr efflux pump would be increased, resulting in decreased susceptibility to antimicrobials recognized by the pump. In contrast to the iron-restricted situation, women infected with gonococci can develop serious complications and often present with pelvic inflammatory disease within days of menses onset when iron levels would be elevated due to blood flow. In this scenario, *mpeR* expression would be reduced because the repressive action of Fur would be favored and levels of MtrR would be higher than in an

iron-restricted environment and *mtrCDE* expression and antimicrobial resistance mediated by the pump, could as a consequence, be lowered. We do not yet know if conditions of iron restriction are responsible for the remarkable differences in the number and types of genes that were observed by microarray analysis to be regulated by MpeR during mid- vs. late-log phases of growth (Table 4). Despite this uncertainty, our data emphasizes that transcriptional regulators such as MpeR can exhibit very different regulons depending on growth phase. Therefore transcriptional profiling studies, particularly those involving investigations on antimicrobial resistance, should take this into account.

In addition to its importance in regulating *mtr*-associated efflux genes in gonococci, MpeR has been recently found (17) to directly activate transcription of *fetA* in strain FA1090 under iron-replete conditions. It is important to note that strain FA1090, employed by Hollander *et al.* is unrelated to the strain (FA19) employed in this investigation and differences in how *mpeR* expression is regulated in these two strains may account for why we did not detect *fetA* as an MpeR-regulated gene in our array studies. Despite this uncertainty, FetA is of interest as it is the outer membrane transporter of a TonB-dependent receptor complex employed by gonococci to obtain iron bound to enterobactin-like siderophores produced by other bacteria and is also produced by *N. meningitidis* (2). FetA is immunogenic as evidenced by the presence of anti-FetA antibodies in convalescent serum from patients recovering from meningococcal disease and these antibodies cross-react against gonococcal strains (1). Moreover, monoclonal antibodies against FetA are bactericidal against meningococci. In conclusion, although MpeR was first identified based on its ability to control levels of gonococcal resistance to

antimicrobials (13), it may have even greater significance for controlling genes of the pathogenic Neisseria that encode proteins involved in iron utilization and perhaps other systems that are important in virulence expressed by gonococci and meningococci.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Relevant genotype[a]	Source or reference
Neisseria gonorrhoeae		
FA19	Wild type	P.F. Sparling
JF1	Δ*mtrR* in FA19	(13)
JF4	Inactivation of *mtrF* with the insertion of *aphA-3* in FA19 (Km^r)	(13)
JF5	Inactivation of *mpeR* with the insertion of *aphA-3* in FA19 (Km^r)	(13)
FA140	Mutation in promoter region of *mtrR*, missense mutation at codon 45 (Gly-45 to Asp-45) of *mtrR*	P.F. Sparling
WV16	Inactivation of *mtrF* with insertion of *aphA-3* in FA140 (Km^r)	(36)
FA19 *mtrF-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* in FA19 (Cm^r)	(13)
JF5 *mtrF-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* in JF5 (Cm^r), (Km^r)	(13)
AD1	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* (Cm^r), *mpeR*$^+$(Ery^r) in JF5 (Km^r)	This study
AD2	Chromosomal insertion of a translational fusion of l*acZ* to the promoter region of *mpeR* in FA19 (Cm^r)	This study
FA19 *mtrR-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* in FA19 (Cm^r)	(12)
JF5 *mtrR-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* in JF5 (Cm^r), (Km^r)	This study
AD3	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* (Cm^r), *mpeR*$^+$(Ery^r) in JF5 (Km^r)	This study
FA19 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in FA19 (Cm^r)	(13)
JF1 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in JF1 (Cm^r)	This study
JF5 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in JF5 (Cm^r), (Km^r)	(13)
AD4	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* (Cm^r), *mpeR*$^+$(Ery^r) in JF5 (Km^r)	This study
AD5	Chromosomal complementation of *mpeR* (Ery^r) in strain JF5 (Km^r)	This study
AD6	Inactivation of *terC* with the insertion of *aphA-3* in FA19 (Km^r)	This study
AD7	Chromosomal complementation of *terC* (Ery^r) in strain AD6 (Km^r)	This study
AD8	Chromosomal complementation of *mpeR* (Ery^r) in strain AD7 (Km^r)	This study
AD9	Inactivation of *mpeR* with the insertion of *aphA-3* in FA140 (Km^r)	This study
Escherichia coli		
DH5α	[F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*]	Invitrogen
TOP10	(F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*)ϕ80 *lacZ*ΔM15 Δl*acX74 recA1 deoR araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r *endAI nupG*)	Invitrogen
Plasmids		
pMAL-c2X	Vector for expression of protein fusions to maltose-binding protein	New England Biolabs
pLES94	pUC18-derived cloning vector for fusion of gonococcal genes to a promoterless *lacZ* and chromosomal insertion between *proA* and *proB* (Ap^r)	(32)
pGCC3	NICS vector for cloning *mpeR* and *terC* under the control of their own promoters and chromosomal complementation between *lctP* and *aspC*	(33)

[a]Cm^r, chloramphenicol resistance, Ery^r, erythromycin resistance, Km^r, kanamycin resistance, Ap^r, ampicillin resistance

Table 2. Oligonucleotides used in this study

Oligonucleotide used	Sequence (5'→3')
5'pMpeRpac……..	GGTTAATTAAGCAAACAACCTGCAGAAACC
3'GC4MpeR2…….	GGTTTAAACTCAGCACTTTTTCACATCCGA
5'AterC……..	GCAACACATAGGGAGACGCTTT
3'BterCSma……	CATAATCATGTCCTTCCCGGGAAC
5'CterCSma……	GTTCCCGGGAAGGACATGATTATG
3'DterC……..	GCTTTCGCGTATGCCCATCATAC
5'pterCPac……..	GGTTAATTAATCTCGCCGAAGGGGAGGA
3'GC4terC……..	GGTTTAAACTTCTTCGGGCAATTTGGTGAT
5'mpeR……..	ATGAACACCGCCGCCATCT
3'mpeR……..	GCACTTTTTCACACTCGAAGG
5'malEmpeR-F……..	CACTGGGGATCCATGAATACCGCCGCCATCT
3'malEmpeR-R……..	CACTGGCTGCAGTCAGCACTTTTTCACATCCGA
5'mtrRJK1……..	CATCAGGTGTTCTTTGGTTTTCAA
3'mtrC-promR……	CATCGCCTTAGAAGCATAAAAAGC
5'misR-205…….	CACCGCTGCTGCCCGAACTGCTC
3'misR+104……	AGCAGGGCATCGTCATCTACGAG
5'pmtrF-F……..	TTAATTTCCCCTATCATCGCA
3'pmtrF R……..	TAAATTTTTGAATTTAACATGAAG
5′pmpeR……	TAGGATCCGCAAACAACCTGAAGAAACC
3′pmpeR……	ATGGATCCCGGTTCATGATTGGATAGGAAC

TABLE 3: Effect of iron and *mpeR* on high-level TX-100 resistance due to *mtrR* mutations

Strain	Minimum Inhibitory Concentration (μg of TX-100/mL)[a,b]
FA140 +Fe	>16,000
FA140 -Fe	375
WV16 +Fe	125
WV16 -Fe	125
AD9 +Fe	>16,000
AD9 -Fe	>16,000

[a]MICs of TX-100 (tritonX-100).
[b]All values represent average results from three independent experiments

TABLE 4: MpeR regulon in *Neisseria gonorrhoeae*

TABLE 4A: MpeR-regulated genes at mid-log

Gene	Common name	Change (fold)	Functional classification
MpeR repressed			
NGO0018	NGO0018	1.53	unknown
NGO0205	*lolA*	1.60	putative outer membrane lipoprotein carrier protein
NGO0373	NGO0373	1.75	putative ABC transporter permease
NGO0393	NGO0393	1.52	putative TetR-family transcriptional regulator
NGO0678	NGO0678	1.59	unknown
NGO0679	*leuC*	1.51	isopropylmalate isomerase large subunit
NGO0754	*mobA*	1.56	putative molybdoprotein guanine dinucleotide biosynthesis protein
NGO0795	*bfrB*	2.24	bacterioferritin B
NGO0863	NGO0863	1.69	putative oxidoreductase
NGO0891	NGO0891	1.95	unknown
NGO0916	*sucB*	1.65	dihydrolipoamide acetyltransferase
NGO1046	*clpB*	1.66	putative ClpB protein
NGO1273	NGO1273	1.51	unknown
NGO1368	***mtrF***	**2.22**	**Mtr efflux pump protein component**
NGO1416	*nqrD*	1.80	NADH-ubiquinone reductase
NGO1418	*nqrF*	1.70	Na(+)-translocating NADH-quinone reductase subunit F
NGO1422	*grpE*	1.71	Putative heat shock protein
NGO1428	NGO1428	1.53	unknown
NGO1494	*potF*	1.71	spermidine/putrescine ABC transporter
NGO1600	*glnA*	1.95	glutamine synthetase
NGO1665	*ilvE*	1.62	branched-chain amino acid aminotransferase
NGO1685	NGO1685	1.68	unknown
NGO1749	*nuoC*	1.52	NADH dehydrogenase subunit C
NGO1765	pglA	1.51	putative glycosyltransferase
NGO1770	*prlC*	1.54	oligopeptidase A
NGO1780	NGO1780	1.67	unknown
NGO1809	*valS*	1.50	valyl-tRNA synthetase
NGO2013	*glnQ*	1.82	putative ABC transporter, ATP-binding protein
NGO2014	*cjaA*	2.15	putative ABC transporter, periplasmic binding protein
NGO2094	*groES*	1.84	co-chaperonin GroES
MpeR activated			
NGO0221	NGO0221	1.57	putative deoxyribonucleotide triphosphate pyrophosphatase
NG00527	NGO0527	1.58	unknown
NGO0567	NGO0567	1.69	putative hydrolase
NGO0606	NGO0606	1.58	putative sodium-dependent transport protein
NGO0694	NGO0694	1.62	unknown
NGO0820	*mesJ*	1.75	putative cell cycle protein
NGO0876	NGO0876	1.90	unknown
NGO0958	*rph*	1.62	putative ribonuclease PH
NGO1058	*surE*	1.64	stationary phase survival protein
NGO1059	**NGO1059**	**2.17**	**putative tellurium resistance gene**
NGO1079	NGO1079	1.69	putative oxidoreductase
NGO1406	*gcvT*	1.55	aminomethyltransferase
NGO1488	NGO1488	1.53	unknown
NGO1810	NGO1810	1.62	unknown
NGO2132	NGO2132	1.58	unknown
NGO2167	NGO2167	1.51	unknown

TABLE 4: MpeR regulon in *Neisseria gonorrhoeae*

TABLE 4B: MpeR-regulated genes at late-log

Gene	Common name	Change (fold)	Functional classification
MpeR repressed			
NGO0365	*dcmG*	1.94	site-specific DNA-methyltransferase
NGO0672	NGO0672	1.98	unknown
NGO0924	NGO0924	1.51	unknown
NGO0952	NGO0952	1.51	putative TonB-dependent receptor protein
NGO1107	NGO1107	1.62	unknown
NGO1159	NGO1159	1.59	unknown
NGO1176	NGO1176	1.88	unknown
NGO1313	NGO1313	1.76	unknown
NGO1342	*dhpS*	1.67	dihydropteroate synthase
NGO1366	***mtrR***	**1.74**	***mtrCDE* transcriptional regulator, repressor**
NGO1481	*bioC*	1.52	biotin synthesis protein
NGO1771	NGO1771	1.63	unknown
NGO1847	NGO1847	1.65	unknown
NGO1915	*kdtA*	1.81	3-deoxy-D-manno-octulosonic-acid transferase
NGO1951	*prfB*	2.40	peptide chain release factor 2
NGO2042	*pilS*	1.87	pilin silent gene cassette
MpeR activated			
NGO1151	NGO1151	1.63	unknown
NGO1170	NGO1170	1.95	unknown
NGO1179	NGO1179	1.97	unknown
NGO1270	NGO1270	1.95	unknown
NGO1498	NGO1498	1.58	unknown

TABLE 5: Effect of *terC* and *mpeR* on susceptibility of gonococci to potassium tellurite

Strain	Minimum Inhibitory Concentration (μg/mL)[a,b]		
	K_2TeO_3	$AgNO_3$	TX-100
FA19	1.0	20	100
AD6	0.05	20	100
AD7	0.5	20	100
JF5	0.05	20	100
AD5	1	20	100

[a]MICs of K_2TeO_3 (potassium tellurite), $AgNO_3$ (silver nitrate), and TX-100 (tritonX-100).
[b]All values represent average results from three independent experiments

FIGURE LEGENDS

Figure 1. Regulation of *mtrF* by MpeR. (A). Primer extension analysis identifying the transcriptional start site of *mtrF*. The sequence reaction is depicted and the predicted -10 and -35 promoter elements are underlined while the start of transcription is marked by an asterisk. Primer extension products were generated from RNA samples harvested from strain FA19 at the mid-log phase of growth and under iron replete conditions. **(B).** The specific activity or nanomoles of o-nitrophenyl-β-D-galactopyranoside hyrdrolyzed per mg of protein is shown for measuring *mtrF* expression in strains FA19 *mtrF-lacZ*, JF5 *mtrF-lacZ*, and AD1 (JF5 *mtrF-lacZ*, *mpeR*$^{+}$ [Table 1]). Samples were harvested from gonococci at the mid-log phase of growth. The above experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. The difference in expression between FA19 vs. JF5 and JF5 vs. AD1 were significant ($p = 0.001$).

Figure 2. Maximal expression of *mpeR*. The specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hyrdrolyzed per mg of protein) is shown for measuring *mpeR* expression in strain AD2 (FA19 *mpeR-lacZ* [Table 1]) at different phases of growth under iron replete conditions. The above experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation and all differences were significant ($p < 0.05$).

Figure 3. **Expression of *mpeR* under iron replete and deplete conditions.** The specific activity was as described above and is shown for measurements of *mpeR*

expression in strain AD2 (FA19 *mpeR-lacZ* [Table 1]) grown under either iron replete (+Fe) or iron deplete (-Fe) conditions. Samples were harvested from gonococci at the late-log phase of growth. The above experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. The difference between cultures grown under iron-replete vs. iron–deplete conditions was significant (*P*-value = 0.007).

Figure 4. Regulation of *mtrR* by MpeR. *mtrR* expression in strains FA19 *mtrR-lacZ*, JF5 *mtrR-lacZ*, and AD3 (JF5 *mtrR-lacZ,mpeR*$^+$ [Table 1]) was measured using *lacZ* fusions. Samples were harvested from gonococci at the late-log phase of growth. The above experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. All differences were significant ($p \leq 0.001$).

Figure 5. Iron modulates levels of MtrR. (A). Wild-type strain FA19 was grown under iron-replete and -deplete conditions. Samples from each growth condition were harvested at the late-log phase of growth, solubilized, and separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **(B).** Following transfer to nitrocellulose, the blot was probed with anti-MtrR antiserum.

Figure 6. Regulation of *mtrCDE* expression is modulated by *mpeR* expression and levels of free iron but not *mtrR* expression. (A). *mtrCDE* expression in strains FA19 *mtrC-lacZ*, JF5 *mtrC-lacZ*, and AD4 (JF5 *mtrC-lacZ*, *mpeR*$^+$ [Table 1]) was measured.

The difference in *mtrCDE* expression between strains FA19 and JF5 was significant (p= 0.002) as was that between strains JF5 and AD4 (p= 0.0001). **(B).** *mtrCDE* expression in strains FA19 *mtrC-lacZ* and JF1 *mtrC-lacZ* [Table 1] grown under either iron replete or iron deplete conditions was determined. The difference in expression between FA19 +Fe and FA19 -Fe was significant (p = 0.0023) while that between JF1 +Fe and JF1 –Fe was not significant (p = 0.3132). The above experiments were done in triplicate and are representative examples of three independent experiments. Error bars represent one standard deviation.

Figure 7. MpeR binds specifically to the upstream region of *mtrR*. The 363bp *mtrR* upstream region was radiolabeled and 10ng of this DNA was incubated with 15μg of MBP-MpeR alone. In order to demonstrate that binding of MBP-MpeR to *mtrR* was specific, increasing concentrations (2x, 5x, 10x) of cold specific competitor (*mtrR*) or increasing concentrations (2x, 5x) of cold non-specific competitor (*misR*) were added. An arrow marks specific binding while an asterisk marks non-specific binding.

Figure 8. Model demonstrating the regulatory properties of MpeR that impact the high-level resistance mediated by the MtrCDE efflux system in an iron-dependent manner. (A). Under iron-replete conditions Fur complexed with iron represses the expression of *mpeR*. **(B).** Under iron-deplete conditions mpeR is derepressed and acts to repress the expression of both *mtrF* and *mtrR* in strain FA19. In strain FA1090 MpeR activates the expression of *fetA*.

Figure 1

A.

TCAAGTTTTCGGATTACCGCCTTTATGAGAATAACGATGTGGGCATTTTCCGT

TTTAATCTATTACGGTTATATACATATACGATTATTTTAGTTTGCTTACAAAAC

ACTTCATGTTAAATTCAAAAATTTAATGCACTCAATATATTTTTTTAAGGAGA

AGCAG

-35

-10

*

B.

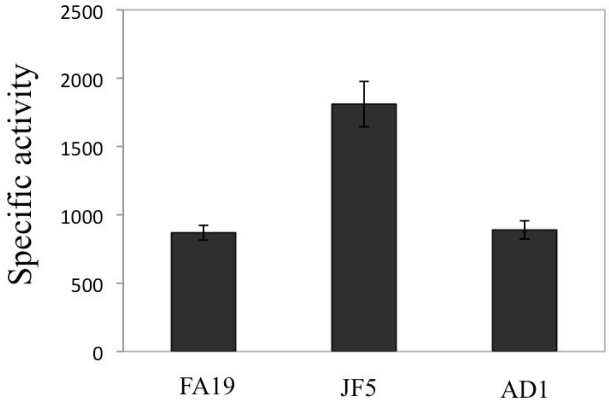
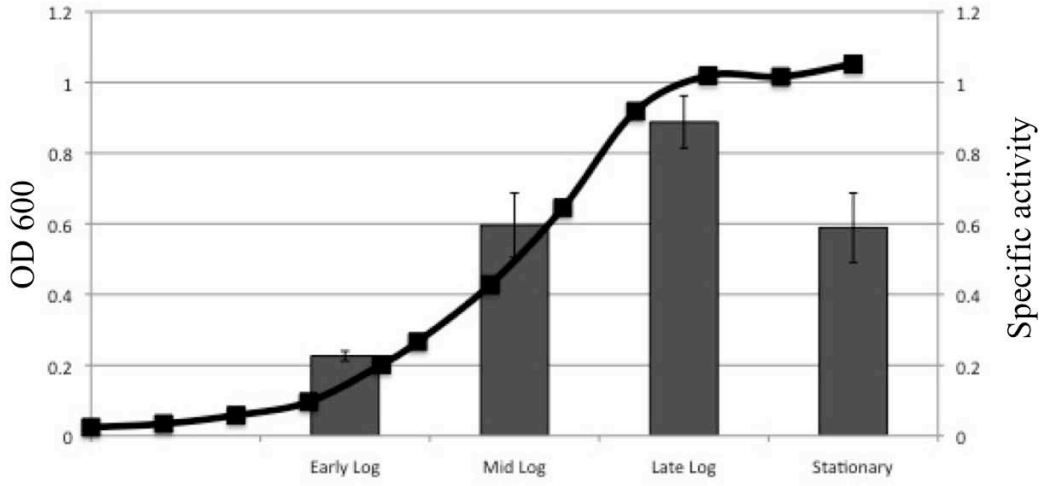


Figure 2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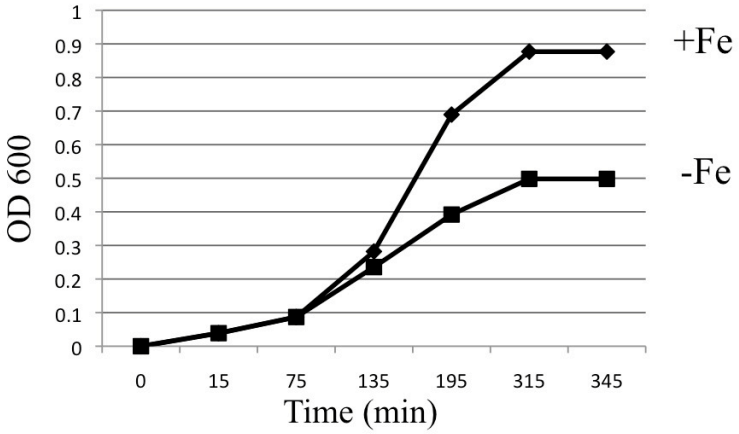
0.6
0.4
0.2
0
OD 600
Specific activity
Early Log
Mid Log
Late Log
Stationary

Figure 3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0.8
0.7
0.6
0.5
0.4
0.3
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0.1
0
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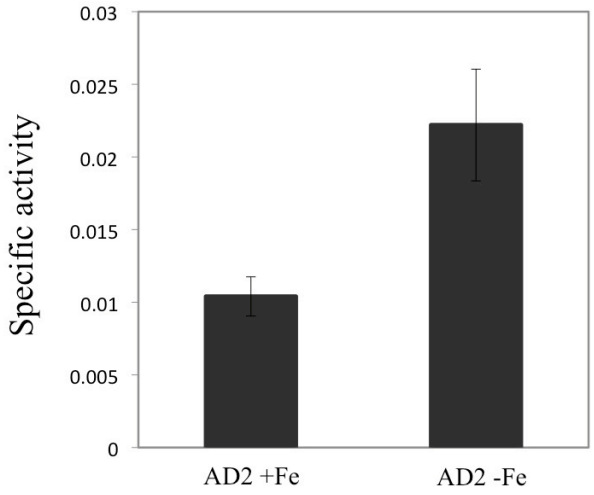
15
75
135
195
315
345
Time (min)
+Fe
-Fe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0.01
0.005
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Specific activity
AD2 +Fe
AD2 -Fe

Figure 4

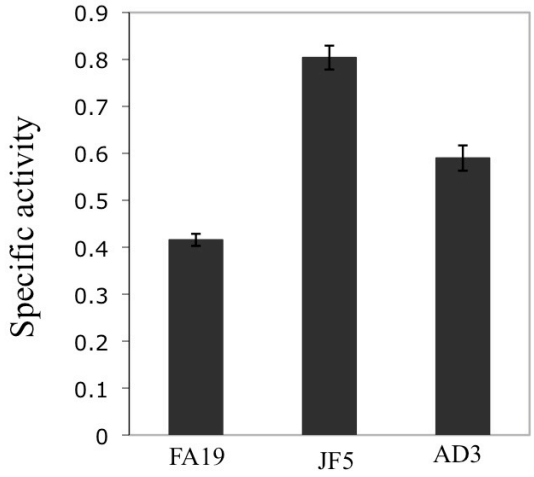


Figure 5

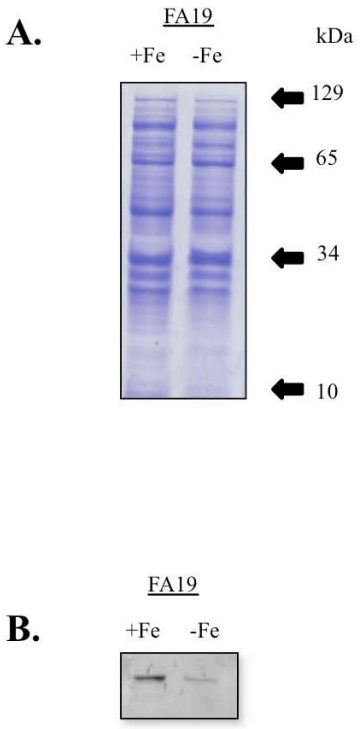


Figure 6

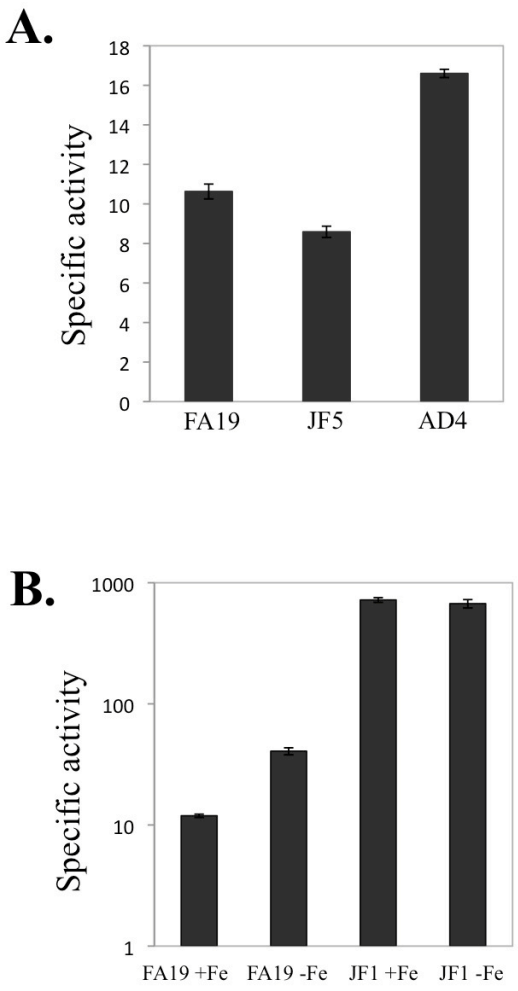

A.
Specific activity
18
16
14
12
10
8
6
4
2
0
FA19
JF5
AD4
B.
Specific activity
1000
100
10
1
FA19 +Fe
FA19 -Fe
JF1 +Fe
JF1 -Fe

Figure 7

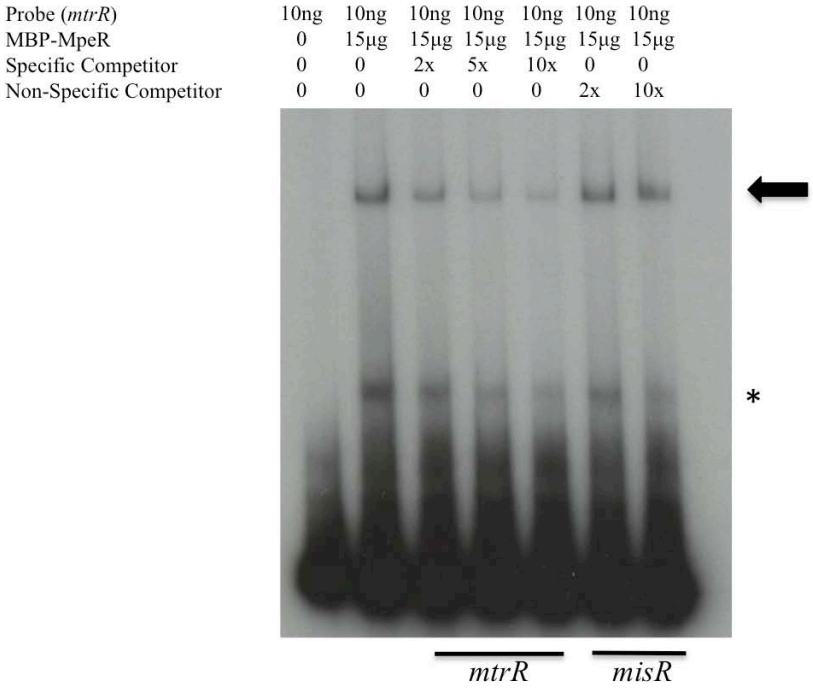
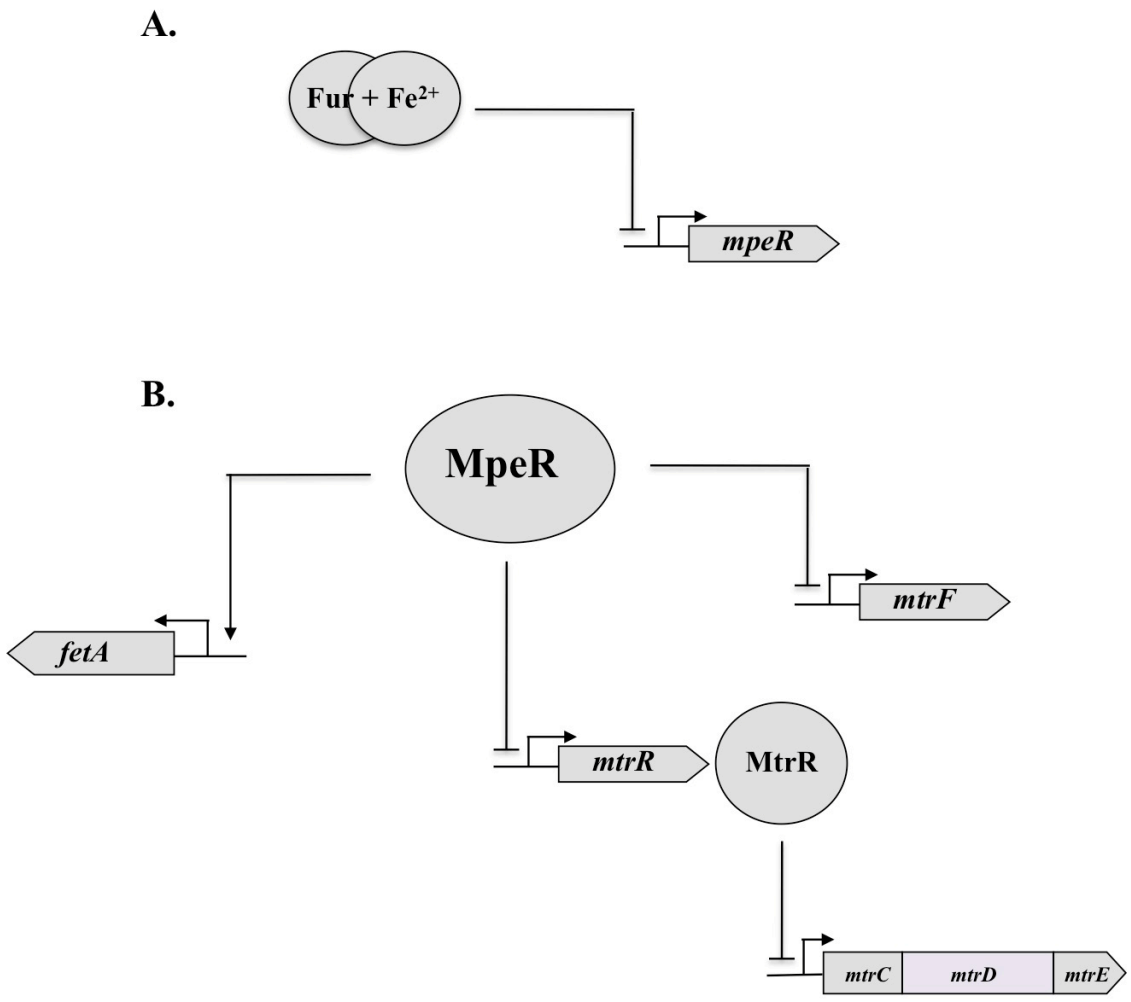

Probe (*mtrR*) 10ng 10ng 10ng 10ng 10ng 10ng 10ng
MBP-MpeR 0 15µg 15µg 15µg 15µg 15µg 15µg
Specific Competitor 0 0 2x 5x 10x 0 0
Non-Specific Competitor 0 0 0 0 0 2x 10x
*
mtrR
misR

Figure 8



Chapter 4: The iron-repressed, AraC-like regulator, MpeR, activates expression of *fetA* in *Neisseria gonorrhoeae*

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This manuscript was written by first author Aimee Hollander and Cynthia Nau Cornelissen with editorial changes made by Alexandra Dubon Mercante and William M. Shafer. Alexandra Dubon Mercante wrote the section Specific binding of MpeR to DNA upstream of *fetA* and conducted the electrophoretic mobility shift assay (figure 3) along with primer extension analysis (figure 5).

Neisseria gonorrhoeae is an obligate human pathogen that causes the common sexually- transmitted infection, gonorrhea. Gonococcal infections cause significant morbidity, particularly among women, as the organism ascends to the upper reproductive tract, resulting in pelvic inflammatory disease, ectopic pregnancy and infertility. In the last few years, antibiotic resistance rates have risen dramatically, leading to severe restriction of treatment options for gonococcal disease. Gonococcal infections do not elicit protective immunity nor is there an effective vaccine to prevent the disease. Thus, further understanding of the expression, function and regulation of surface antigens could lead to better treatment and prevention modalities in the future. In the current study, we determined that an iron-repressed regulator, MpeR, interacted specifically with the DNA sequence upstream of *fetA* and activated FetA expression. Interestingly, MpeR was previously shown to regulate the expression of gonococcal antimicrobial efflux systems. We confirmed that the outer membrane transporter, FetA, allows gonococcal strain FA1090 to utilize the xenosiderophore, ferric-enterobactin, as an iron source. However, we further demonstrated that FetA has an extended range of substrates that encompasses other catecholate xenosiderophores, including ferric-salmochelin and the dimers and trimers of dihydroxybenzoylserine. We demonstrated that *fetA* is encoded as part of an iron-repressed, MpeR-activated operon, which putatively encodes other iron transport proteins. This is the first study to describe a regulatory linkage between antimicrobial efflux and iron transport in *N. gonorrhoeae*. The

regulatory nidus that links these systems, MpeR, is exclusively expressed by pathogenic *Neisseriae* and is therefore expected to be an important virulence factor.

INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen that primarily infects the urogenital or anorectal mucosa following intimate contact. *N. gonorrhoeae* is the etiological agent of gonorrhea, which is the second most commonly-reported, notifiable infectious disease in the United States. In 2009, the Centers for Disease Control and Prevention reported a total of 301,174 cases of gonorrhea in the United States (14); however, this is thought to be a conservative estimate due to underreporting. In men, a gonococcal infection is characterized by acute urethritis with symptoms that include purulent discharge and dysuria. It is estimated that up to 80% of women infected with *N. gonorrhoeae* are asymptomatic or present with very minor symptoms (45). Women with symptomatic disease experience cervicitis and vaginal discharge. When left untreated, due to the asymptomatic nature of the infection in women, the bacteria can ascend to the upper female genital tract. This ascending infection can result in pelvic inflammatory disease, which may lead to ectopic pregnancy or infertility (47, 61). The Centers for Disease Control currently only recommends extended-spectrum cephalosporins for treatment due to increased antimicrobial resistance to all previously-recommended therapies (15, 33, 39). Unfortunately, resistance to this class of antimicrobial agent has already emerged (14). *N. gonorrhoeae* infections do not elicit protective immunity and there is evidence that gonococcal infections increase the spread of HIV (18, 44). Since

gonococcal disease poses a significant public health challenge, it is important to understand the pathogenesis of *N. gonorrhoeae* in order to identify new therapies.

Iron is an essential nutrient for most microorganisms including the *Neisseriae* (10). Many microorganisms acquire iron from the human host by synthesizing and secreting siderophores. Siderophores are low-molecular weight iron-chelating molecules that scavenge iron from the environment or host iron binding proteins. *N. gonorrhoeae* does not synthesize siderophores but instead obtains iron directly from human iron binding proteins including transferrin, lactoferrin, and hemoglobin in a receptor-mediated mechanism (7, 17, 19, 37). Expression of either the transferrin or lactoferrin receptor by *N. gonorrhoeae* is necessary to establish infection in human male volunteers (2, 20). Gonococci can also hijack siderophores produced by other bacteria, which is known as xenosiderophore utilization. It has been previously demonstrated that strains of gonococci can obtain iron from the xenosiderophores enterobactin, aerobactin, and salmochelin, which are all synthesized by enteric bacteria (12, 62, 65).

Iron acquisition is tightly regulated since excess iron can promote Haber-Weiss-Fenton chemistry, creating highly reactive, toxic hydroxyl radicals within the cell (30). In many bacteria including *N. gonorrhoeae*, the ferric uptake regulator (Fur) acts as a transcriptional regulator for iron acquisition genes. Under iron-replete (+Fe) conditions, a dimer of Fur binds to its co-repressor, ferrous iron, and assumes a DNA binding conformation. The Fur-Fe^{2+} complex binds to a specific DNA sequence called the Fur box found in the promoter regions of iron regulated genes (4). Once Fur is bound to the Fur box it blocks gene transcription. As intracellular iron stores become depleted, apo-Fur dissociates from the Fur box allowing RNA polymerase to bind to the promoter and

transcribe the Fur regulated gene (22). Gonococcal Fur not only regulates iron acquisition genes but also impacts the expression of a broad range of genes including those that encode Opa proteins, NADH dehydrogenase, sodium pumps and other transcriptional regulators (34). The regulation of genes involved in iron acquisition, adhesion and metabolism establishes Fur as a global regulator (34, 58).

Fur may not be the sole transcriptional regulator of iron acquisition systems in *N. gonorrhoeae*. AraC-like regulators operate as both positive and negative regulators of iron acquisition systems in other microorganisms including *Pseudomonas aeruginosa, Yersinia pestis*, and *Bordetella pertussis* (5, 23, 32, 53). In these microorganisms, the AraC-like regulator functions as a transcriptional regulator of siderophore biosynthesis and acquisition genes. The mechanism of AraC-like regulation of siderophore genes involves the cognate siderophore functioning as a co-inducer. The AraC-like regulator is under the transcriptional control of Fur and therefore AraC-like regulation occurs under iron deplete conditions. The gonococcal genome encodes multiple AraC-like regulators and it has been recently demonstrated that one of these regulators, MpeR (25) is Fur regulated (34).

In this study, we demonstrate that MpeR, an iron-regulated AraC-like regulator is required for up-regulated expression of the outer membrane transporter, FetA, in gonococcal strain FA1090. We also establish that *fetA* is part of an iron regulated operon that encodes a periplasmic binding protein and components of a putative ABC transport system; however, only *fetA* but not the downstream genes require MpeR for detectable expression. Furthermore, we determined that gonococcal strain FA1090 acquires iron from enterobactin, enterobactin derivatives and salmochelin S2 in a FetA- and TonB-

dependent manner. Expression of MpeR was necessary to achieve maximal growth on these siderophores, but none of the utilizable iron sources appear to act as a co-inducer for MpeR-dependent activation of *fetA*.

MATERIALS AND METHODS

Bacterial growth conditions. Gonococcal strains were maintained on GC medium base (GCB; Difco) containing Kellogg's supplement 1 (35) and 12 μM ferric nitrate. For mutant selection, strains were grown on media supplemented with 50 μg/ml kanamycin or 1μg/ml erythromycin. For iron-depleted growth conditions, gonococci were grown in a defined medium that was treated with Chelex 100 (BioRad) (CDM) (66). For iron-replete conditions, gonococcal strains were grown in CDM for one mass doubling before the addition 12 μM ferric nitrate. For growth in the presence of siderophores, gonococci were grown in liquid CDM for one mass doubling, and then ferric siderophores were added at a concentration of 10 μM. All liquid cultures were grown at 37°C with 5% CO_2. For large scale preparation of gonococcal membrane proteins, GCB broth containing Kellogg's supplement 1 was inoculated with plate-grown, non-piliated gonococci as described previously (19). For iron-deplete conditions, the iron chelator Desferal (deferroxamine mesylate; Sigma Aldrich) was added to a final concentration of 50 μM; for iron-replete conditions, 12 μM ferric nitrate was added.

Construction of gonococcal mutants. All strains and plasmids used in this study are listed in Table 1. Gonococcal strain FA1090 has been described previously (9). To create

gonococcal strain MCV304, the *mpeR* gene was inactivated by the insertion of a kanamycin resistance cassette (*aphA-3*) as previously described (25). All oligonucleotide primers used in this study are described in Table 2. Primers 5'mpeR and 3'mpeR were used to PCR amplify chromosomal DNA from FA19 *mpeR*::*aphA-3* strain (25). The amplicon was then used to transform gonococcal strain FA1090. To select for allelic exchange, FA1090 transformants were plated on GCB agar containing kanamycin. Strain FA6959 was previously constructed (12) by insertional inactivation of the FA1090 *fetA* gene with a polar Ω cassette (54). For the construction of the complemented strain, *mpeR*^{C} (MCV305), the *mpeR* coding sequence from gonococcal strain FA19 was amplified using primers 5′ pMpeR pac and 3′ GC4 MpeR. The resulting amplicon contains the *mpeR* gene and 250 bp upstream of the *mpeR* start codon. The amplicon was inserted between the PacI and PmeI sites of pGCC3 (59). The resulting plasmid, pGCC3-*mpeR*, was then digested with ClaI and the fragment containing *mpeR*, *lctP*, *aspC*, and the erythromycin resistance cassette was purified and used to transform MCV304. Transformants were selected on GCB agar supplemented with erythromycin. The resulting complemented strain, MCV305, contains the original *mpeR* mutation and an ectopically-inserted copy of the wild-type *mpeR* gene, preceded by 250 base pairs of upstream regulatory signals. The *fetA*, *mpeR* double mutant strain, MCV306, was constructed by transforming FA6959 with the *mpeR*::*aphA-3* amplicon as described in the construction of MCV304. GCB agar supplemented with kanamycin was used for selection.

Isolation of cell fractions containing membrane proteins. As described previously (19), gonococci were pelleted after large scale growth under iron-replete or iron-deplete conditions. Gonococcal cells were then resuspended in 10mM HEPES [4-(2-hydroxyethyl)-1-piper azine-ethanesulfonic acid] and passed through a French pressure cell once at 20,000 lb/in^2. Intact gonococcal cells were removed by centrifugation at 8,500 x *g* and membrane proteins were pelleted by centrifugation at 140,000 x *g* for 1h. Total membrane protein fractions were resuspended in 10mM HEPES and protein concentrations were determined by bicinchoninic acid assay (Pierce).

Separation of total membrane protein fractions and mass spectrometry analysis. Membrane protein fractions, isolated as described above, were solubilized and proteins were separated on a 7.5% polyacrylamide gel. Proteins were visualized by Coomassie blue staining. One band of interest was extracted from the stained gel and submitted to the VCU Mass Spectrometry Resource Center for identification. The sample was digested overnight with trypsin and the resulting peptides were extracted. The peptides were analyzed on a LC-MS system that consisted of a Thermo Electron Deca XP Plus mass spectometer with a nanospray ion source interfaced with a reversed-phase capillary column.

Immunodetection of FetA and TbpA. For detection of FetA in total membrane protein preparations, aliquots containing 20µg of protein were resolved using SDS-PAGE. For detection of FetA in whole cell lysates, gonococcal strains were grown in the presence of iron-containing catechols for 6 hours; every 2 hours, aliquots were removed and

standardized to culture density. Cells were pelleted and lysed with Laemmli solubilizing buffer (36) and stored at –20°C. Before use, 5% β-mercaptoethanol was added to all preparations prior to heating at 95°C for 3 minutes. After SDS-PAGE (19), proteins were electroblotted to nitrocellulose membranes in 20 mM Tris base, 150 mM glycine, and 20% methanol (64) within a submerged transfer apparatus (BioRad). For detection of FetA, membranes were blocked with 5% skim milk in low-salt TBS (LS-TBS). FetA blots were then probed with a FetA-specific monoclonal antibody (13) and washed with LS-TBS, followed by a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (BioRad). Blots were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

For detection of TbpA in whole cell lysates, proteins were separated by SDS-PAGE as described above, followed by electroblotting to nitrocellulose. Membranes were blocked with 5% bovine serum albumin (Roche) in high-salt Tris-buffered saline (TBS) plus 0.05% Tween 20 (Sigma). TbpA blots were then probed with a polyclonal, TbpA-specific antiserum (19) and washed with high-salt TBS plus 0.05% Tween, followed by a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (BioRad). Blots were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

RNA isolation. Gonococcal strains were grown in CDM under iron-deplete and iron-replete conditions as described above in bacterial growth conditions. After one mass doubling, 12 μM $FeNO_3$ (iron replete), or no additional iron (iron-deplete) was added and the cultures were grown for an additional 2 h. Total RNA was isolated from cultures

using the RNeasy mini kit as directed by the manufacturer (Qiagen). Purified RNA was treated twice with RNase-free DNase as directed by the manufacturer (Qiagen). SUPERase-In (Ambion) was added before storage at 80°C.

Qualitative reverse transcriptase PCR (RT-PCR). Portions of *16S rRNA*, *fetA*, *mpeR*, and intergenic regions between the *fet* genes were amplified using the Thermoscript RT-PCR system (Invitrogen) as described previously (29). A portion of this reaction was used as template for PCR amplification with Platinum *Taq* Polymerase (Invitogen) according to the manufacturer's protocol. Oligonucleotide sequences of the primers used in this analysis are listed in Table 2. After an initial denaturation step at 94°C for 3 min, DNA was amplified for 30 cycles. Each cycle consisted of 1 min at 94°C, 30 s at 60°C and 1.5 min at 72°C, followed by a final extension step of 10 min at 72°C. To detect any DNA contamination of RNA preparations, parallel RT-PCR reactions were conducted in the absence of reverse transcriptase. Amplicons resulting from the RT-PCR reactions were detected by ethidium bromide staining of agarose gels.

Real time RT-PCR. cDNA was generated by reverse transcription of 100 ng of total RNA using the Accuscript High Fidelity 1st strand cDNA synthesis Kit (Stratagene) according to the manufacturer's protocol. Synthesized cDNA was used as PCR template. The SensiMix SYBR No-ROX Kit (Bioline) and CFX96 Real Time System (BioRad) was employed for the real time RT-PCR reactions. Oligonucleotide sequences of the primers used in this analysis are listed in Table 2. The polar W insertion in *fetA* was

located between the binding sites for the primers used to detect *fetA* expression; the *aphA-3* cassette was located downstream of the primer binding sites for *mpeR* expression analysis. The cDNA/SensiMix mixture was initially heated to 94°C for 10 min and subjected to 40 cycles conducted under the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Transcription of *rmpM* was employed as an internal control. For each experimental condition, *fetA, mpeR,* and *ng2091* transcripts were normalized to *rmpM* levels. The relative C_T method (41) was employed to compare normalized expression levels under different conditions. Three biological replicates were analyzed, each conducted in triplicate.

Identification of *fetB* transcriptional start site by primer extension analysis. In order to identify the *fetB* transcriptional start site, RNA was harvested from FA1090 grown under iron-replete or iron-deplete conditions as described above. The reverse primer, oVCU676 (Table 2), was radiolabeled with [γ- ^{32}P] using T4 polynucleotide kinase; 5μg of RNA was incubated with the radiolabeled primer and reverse transcriptase to generate the primer extension product. In order to generate reference sequence products, plasmid pVCU366 (Table 1) was sequenced using the reverse primer, oVCU676, and the SequiTherm EXCEL II DNA Sequencing kit (Epicenter) as described previously (57). The sequencing template plasmid, pVCU366, was generated by amplification of wild-type FA1090 genomic DNA with the primers oVCU498 and oVCU499 (Table 2). The resulting amplicon contained the *fetB* promoter region and was cloned into pCR 2.1 (Invitrogen). Both the primer extension product and the reference sequence were subjected to electrophoresis on a 6% acrylamide sequencing gel, which was dried and subjected to autoradiography for visualization.

Electrophoretic mobility shift assay (EMSA). MpeR was fused in-frame at its amino-terminus to the maltose-binding protein (MBP) using the pMal-c2x fusion vector (New England Biolabs). For this purpose, the *mpeR* coding region was PCR-amplified from FA19 chromosomal DNA, isolated as described (43) using primers 5′ malEmpeRF and 3′malEmpeRR (Table 2). The resulting amplicon was purified using the QIAquick PCR purification kit (Qiagen). Both the vector and PCR product were digested with PstI and BamHI (New England Biolabs) and ligated using T4 DNA Ligase (New England Biolabs). *E. coli* transformants harboring the construct were selected on LB agar (Difco) plates containing 100μg/ml of ampicillin. Both strands of the cloned insert were sequenced to ensure fidelity of the PCR amplification reaction and in-frame fusion with *malE*. Growth of the *E. coli* transformant bearing the plasmid construct, induction of expression and purification of MBP-MpeR was performed as described previously for an MBP-MtrR fusion (38).

For the EMSA studies, the 500 bp, intergenic region immediately upstream of the FetA start codon was PCR-amplified from FA1090 chromosomal DNA using primers 5′fetAup and 3′fetAup (Table 2). This upstream region was further divided into two smaller products by PCR amplification. The 5′fetAup and 3′fetAupint primers (Table 2) resulted in *fetA*1. The 5'fetAupint and 3'fetAup primers (Table 2) were used to amplify *fetA*2 region. These three PCR products were purified using the QIAquick PCR purification kit (Qiagen) and end-labeled with [γ- ^{32}P] (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). The radiolabeled PCR products were purified by excising the DNA from nondenaturing polyacrylamide gels, and recovered by crush-soak elution overnight at 37°C into 750 μl of PB buffer from the QIAquick PCR

purification kit (Qiagen). The radiolabeled DNA/buffer mixture was centrifuged at 15,800 x g for 10min and the resulting supernatant was removed and added to a QIAquick PCR purification kit (Qiagen) column, which was then washed with 750 µl PE buffer. The DNA was eluted in 100 µl of nuclease-free water (Ambion). Five nanograms of each radiolabeled probe were incubated with 10 ng of MBP-MpeR for 30 minutes at room temperature.

The specificity of the MpeR-*fetA* promoter interaction was evaluated by adding either specific or non-specific, unlabeled competitor DNA to the binding reactions. The specific, unlabeled competitor DNA was generated by PCR amplification of the *fetA*1 promoter region as described above. The *fetA*2 sequence was also amplified as described above and used as an unlabeled competitor. The non-specific competitor DNA was generated by PCR amplification of a portion of the *rnpB* gene using the primers rnpB1F and rnpB1R (Table 2). All binding reactions were incubated in DNA binding buffer (20mM Tris-HCl, 200mM NaCl, 1mM DTT, 1µg/ml Poly dIdC) for 30 min. at room temperature. All samples were subjected to electrophoresis on a 5% (wt/vol) polyacrylamide gel at 4°C. After electrophoresis, the gel was dried onto Whatman filter paper and exposed to X-ray film for autoradiography.

Preparation of ferric-siderophores. The siderophores used in this study were purchased from EMC Microcollections (Tübingen, Germany). Siderophores were resuspended to a final concentration of 1mg/ml in sterile deionized water and ferrated to 80% saturation using $FeCl_3$ (62). In some experiments, the siderophores were purchased pre-ferrated and the lyophilized ferric-siderophores were dissolved in methanol (enterobactin) or water prior to final dilution in water. The results of the siderophore utilization assays were the

same regardless of whether the siderophores were purchased in the ferrated state or ferrated immediately before use. The following is a list of the siderophores tested for growth support of gonococcal strain FA1090: ornibactin, aerobactin, ferrichrysin, ferrirubin, coprogen, neocoprogen, enterobactin, dihydroxybenzoylserine (DHBS) monomer, DHBS dimer, DHBS trimer, salmochelin S4 and salmochelin S2.

Xenosiderophore utilization assays. Plate bioassays to evaluate xenosiderophore utilization were performed using CDM plates supplemented with 2.5 μM apo-bovine transferrin to chelate excess iron. As described previously (62) strains were inoculated onto plates using a sterile Dacron swab (Puritan). A sterile Pasteur pipette was used to bore a well into the agar. Subsequently, 10μl of the diluted ferric-siderophore solution (100 mg/ml) was added to each well. Ten microliters of apo-bovine transferrin at a concentration of 10mg/ml was used as a negative control and 10μl of ferric citrate (10 μM) was used as a positive control. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours and then evaluated for ferric-siderophore dependent growth.

Statistical analysis. Statistical significance of xenosiderophore-dependent growth data was determined by using a two-tailed, unpaired Student's *t* test. *P* values for specific comparisons are reported in the figure legend. For real time RT-PCR data (Table 3), the relative C_T method (41) was utilized to calculate the fold change for each comparison. The range for each value is shown in parentheses. The average fold change values were calculated from three independently conducted RT-PCR reactions and are representative of results generated from three independent RNA preparations (biological replicates).

RESULTS

The *mpeR* mutant displays differential protein expression under iron-deplete conditions. The *mpeR* gene was originally identified by Folster *et al.* as encoding an AraC-like regulator that plays a role in the coordinate expression of hydrophobic agent efflux pumps (25). We began investigating whether MpeR plays other regulatory roles in gonococci due to its proximity to a gene that encodes an uncharacterized TonB-dependent transporter, TdfF (29). We initially determined that *mpeR* was iron regulated, which was recently confirmed by Jackson *et al.* via microarray, FURTA assays, and the presence of a Fur box in its promoter (34). We hypothesized that since MpeR is iron regulated, it could also regulate iron acquisition systems in gonococci, particularly the one putatively encoded by the *tdfF* locus. To test this hypothesis, the wild type (FA1090) and *mpeR* mutant (MCV304) were grown under iron-deplete (-Fe) and iron-replete (+Fe) conditions and total membrane protein fractions were isolated. Using SDS-PAGE analysis, we determined that an 80 kDa protein was expressed by the wild-type strain only under iron-deplete conditions, but this protein was not detectable in the *mpeR* mutant strain (Fig. 1A). The 80 kDa band was excised from the gel and analyzed by mass spectrometry, which unambiguously identified the protein as FetA. Western blot analysis of total membrane proteins isolated from the wild type (FA1090), *mpeR* mutant (MCV304), *fetA* mutant (FA6959), and *mpeR*^{C} (MCV305) grown under iron-deplete (-) and iron-replete (+) conditions were used to confirm the mass spectrometry results. The wild-type and complemented strains grown under iron-deplete conditions expressed detectable levels of FetA, whereas neither the *fetA* mutant nor the *mpeR* mutant expressed

this protein (Fig. 1B). From this analysis, we concluded that the iron-regulated AraC-like regulator, MpeR, controlled the expression of FetA in gonococcal strain FA1090.

***fetA* transcription requires MpeR expression and iron-deplete growth conditions.**

fetA, previously called *frpB*, encodes a TonB-dependent outer membrane transporter (FetA) that is immunogenic, and subject to Fur regulation (6, 12, 51, 63). FetA was renamed by Carson *et al.* when they discovered that this transporter was necessary for efficient ferric-enterobactin transport (12). To determine whether MpeR transcriptionally regulated *fetA*, end-point relative RT-PCR was utilized to detect *fetA* and *mpeR* transcripts. RNA was isolated from gonococcal strains grown under iron-deplete (-) and iron-replete (+) conditions. Transcripts from *fetA* and *mpeR* were detected in the wild-type strain preferentially under iron-deplete conditions (Fig. 2), consistent with previous published studies (13, 34). The *fetA* transcript was not detected in the *mpeR* mutant (MCV304). In the *mpeR*^{C} strain (MCV305), both *mpeR* and *fetA* transcripts were detected (Fig. 2) indicating that the MpeR effect on *fetA* expression was restored by complementation. Expression of *mpeR* in the *mpeR*^{C} strain (MCV305) was iron regulated (Fig. 2), consistent with the presence of iron-sensitive regulatory signals in the sequence upstream of the MpeR start codon, which were included in the complementation construct. Overall, these data allow us to conclude that MpeR is necessary for *fetA* expression under iron-deplete conditions.

Real time RT-PCR was employed to confirm the end-point RT-PCR results and to quantify the level of gene expression. All C_T values were normalized to *rmpM* gene expression and then fold change was determined using the relative C_T method (41). The *rmpM* gene encodes an outer membrane protein that is constitutively expressed under all

growth conditions tested in this study. In Table 3, the average fold change is shown, representing the comparison between expression under iron-deplete and iron-replete conditions or expression by wild-type and mutant under iron deplete conditions. As expected, the fold change in expression under iron deplete vs. iron replete conditions was large for both *fetA* and *mpeR* (97-fold for *fetA* and 198-fold for *mpeR*; Table 3). When the wild type and its isogenic *fetA* mutant were compared, there was 194-fold more *fetA* expression in the wild type. *mpeR* expression was unaffected in the *fetA* mutant. When the wild type and the *mpeR* mutant were compared, there was a 117-fold change in *fetA* expression (Table 3), which is consistent with the inability to detect *fetA* transcripts in the *mpeR* mutant as observed by qualitative RT-PCR (Fig. 2). As shown in Table 3, the restoration of the *mpeR* gene in the *mpeR*^{C} strain resulted in *mpeR* expression levels that approached that of the wild-type (1.9 fold difference between wild-type and the *mpeR*^{C} strain). Commensurate with the return of *mpeR* expression, we detected an increase in FetA expression in the *mpeR*^{C} strain relative to the *mpeR* mutant (Table 3), again supporting the relative RT-PCR data (Fig. 2). Together, the expression data support the previous observations that both *fetA* and *mpeR* are iron regulated (6, 34) and furthermore demonstrate that *mpeR* expression was not impacted by the *fetA* mutation. Results from the relative and real-time expression studies establish that when *mpeR* was absent, *fetA* expression decreased and when *mpeR* expression was restored by complementation with a wild-type copy, *fetA* levels returned to near wild-type levels under iron-deplete conditions. Cumulatively, these data indicate that MpeR is necessary for *fetA* transcription under iron-deplete conditions.

Specific binding of MpeR to DNA upstream of *fetA*. In order to determine whether MpeR-dependent *fetA* regulation occurred as a result of direct interaction, we used EMSA to test whether MpeR bound to target DNA sequences upstream of the FetA start codon. Results from preliminary EMSA experiments using increasing amounts of MBP-MpeR (1-10 µg) incubated with the radiolabeled *fetA* probe showed shifting of a DNA fragment of 500 bp in length , whereas MBP alone showed no shift (data not shown). This upstream region was further divided into two smaller DNA fragments of 250 bp in length (Fig. 3A), both of which were tested for MpeR interaction by EMSA. The probe fragment corresponding to the most upstream portion of the intergenic region (*fetA*1, Fig. 3A) was shifted by addition of MpeR protein (Fig. 3B), while MBP alone did not shift the *fetA1* fragment (data not shown). The probe fragment corresponding to the sequence immediately preceding the *fetA* start codon (*fetA*2; Fig. 3B) was not shifted in the presence of MpeR (data not shown). In order to determine whether MpeR binding upstream of *fetA* was specific, a competitive EMSA experiment was performed in which increasing amounts (2-20X) of excess unlabeled specific (*fetA*) or non-specific (*rnpB*) DNA fragments were added to the binding reaction. The results demonstrate that the specific competitor, but not the non-specific competitor, reduced binding of MpeR to the labeled *fetA* probe (Fig. 3B). The unlabeled *fetA*2 sequence modestly competed with the binding of MpeR to the *fetA*1 probe, suggesting that MpeR may bind preferentially to *fetA*1 sequence and with a lower affinity to the *fetA*2 sequence. Cumulatively, these results allow us to conclude that binding of MpeR to the DNA sequence upstream of *fetA* is specific and that its capacity to activate *fetA* expression is likely by a direct mechanism.

***fetA* is part of an iron responsive operon that is differentially affected by MpeR expression.** The DNA region downstream of *fetA* encodes putative xenosiderophore acquisition genes (Fig. 4A). The genes downstream of *fetA* include: *fetB*, which encodes a putative periplasmic binding protein; *ng2091* and *ng2090*, which encode two predicted permease proteins; and *ng2088*, encoding an ATP binding protein. The latter three genes are therefore predicted to form an ABC transport complex. *ng2089* encodes an uncharacterized protein. Most siderophore acquisition systems are co-transcribed and all genes involved in the system are coordinately regulated. Therefore, end point RT-PCR was utilized to determine if *fetA* and the downstream genes were co-transcribed and also activated by MpeR. Primers were designed to amplify the intergenic regions between each gene in the hypothetical operon (Fig. 4A). RNA was isolated from gonococcal strains grown under iron-deplete (-) and iron-replete (+) conditions. In the wild-type strain (FA1090) all amplicons were detected under iron-deplete conditions (Fig. 4B) indicating that *fetA* and downstream genes are part of an iron-regulated operon. Interestingly, the *fetA* mutant, which contains a polar W (54) insertion in the *fetA* gene, maintained the ability to express all of the downstream putative genes (Fig. 4B). Similar results were observed in the *mpeR* mutant, in which genes *fetB*-NG2088 were co-transcribed under iron-deplete conditions (Fig. 4B). A *fetA mpeR* double mutant strain was also employed to investigate co-transcription of the ABC transport genes. Again, the downstream putative transport genes, in the absence of both *fetA* and *mpeR* were all transcribed under iron-deplete conditions (Fig. 4B). Finally, when the *mpeR* mutant was complemented with the wild-type *mpeR* gene, *fetA* and the downstream co-transcripts were detected under iron-deplete conditions, similar to the wild-type strain. Based on

these RT-PCR results, we propose that the wild-type strain produces two different transcripts under iron-deplete conditions (Fig. 4A). One transcript encodes *fetA-ng2088* whereas the other transcript encodes *fetB-ng2088*, expression of which is iron regulated but not qualitatively affected by MpeR (Fig. 4A). The *fetA* transcriptional start site was previously mapped (13) and is identified in Figure 3. To confirm the presence of a second transcriptional start site upstream of *fetB*, we employed primer extension analysis. As shown in Figure 5, we identified a transcriptional start site downstream of several possible -10 sequences, within the previously identified Fur binding site (34). The transcript starting with *fetB* was modestly iron regulated and the initiation site was located 72 nucleotides upstream of the FetB start codon.

To establish whether *mpeR* or *fetA* interruption quantitatively impacted expression of the downstream ABC transport genes, real-time RT-PCR was employed. *ng2091* is the first gene transcribed downstream of the gene encoding the putative periplasmic binding protein (*fetB*) (Fig. 4A). The same RNA samples isolated for qualitative RT-PCR (Fig. 4B) were used for real-time RT-PCR analysis. All C_T values were normalized to *rmpM* gene expression. In Table 3, the average fold change is shown, representing the comparison between expression under iron-deplete and iron-replete conditions or expression by wild-type and mutant under iron deplete conditions. As shown in Table 3, *ng2091* gene expression was iron repressed; 16.3 fold more transcript was detected under iron deplete conditions. When the wild-type and mutant strains were compared, we observed that *ng2091* gene expression levels were 23-fold higher in the wild type relative to the *fetA* mutant. Similarly, *ng2091* expression was 22.3-fold higher in the wild type relative to the *mpeR* mutant. The wild-type strain expressed 11.5-fold more *ng2091*

transcript than the *fetA mpeR* double mutant (Table 3). The *mpeR*^{C} strain expressed more *ng2091* transcript than did the *mpeR* mutant, as expected. Cumulatively, these results indicate that *fetA* is part of an iron-regulated operon encoding a putative periplasmic binding protein and ABC transport system. The quantitative expression studies suggest that the ABC transport system is not as tightly iron-regulated as is either *fetA* or *mpeR*, which is consistent with the transcriptional start site mapping data presented in Figure 5. Furthermore, the real-time RT-PCR data indicate that expression of *ng2091* is activated by MpeR, but that the extent of this activation is not as great as was detected for *fetA*.

Xenosiderophore-iron acquisition by gonococcal strain FA1090. *N. gonorrhoeae* does not produce siderophores; however, the pathogen is capable of utilizing siderophores synthesized by other microorganisms (12, 62, 65). In gonococcal strain FA1090, Carson *et al.* demonstrated that FetA functions as a receptor for the xenosiderophore enterobactin (12). Enterobactin is a cyclic catecholate siderophore composed of three 2,3-dihydroxybenzoylserine (DHBS) subunits and was first characterized in *E. coli* and *Salmonella* (49, 52). Enterobactin derivatives including the DHBS monomer (D1), the DHBS dimer (D2), and the DHBS trimer (D3) have also been identified as siderophores secreted by *E. coli* (31, 48). Carson *et al.* determined that enterobactin is utilized by gonococcal strain FA1090 in a FetA- and TonB-dependent manner (12). We tested whether strain FA1090 could also utilize other xenosiderophores in a similar manner. Since MpeR activates *fetA* expression, MpeR was predicted to affect catecholate utilization in gonococcal strain FA1090 as well.

Plate bioassays were employed to measure siderophore-dependent growth of gonococcal strain FA1090. We tested a variety of siderophores including: ornibactin,

aerobactin, ferrichrysin, ferrirubin, coprogen, neocoprogen, enterobactin, DHBS and salmochelin for growth support. We found that ferrichrysin, ferrirubin, coprogen and neocoprogen did not support growth at all. Ornibactin and aerobactin supported growth but only in a TonB-independent fashion (data not shown). Thus we focused on the catecholate-type siderophores including: enterobactin, DHBS monomer (D1), DHBS dimer (D2), DHBS trimer (D3), salmochelin S4 (a cyclic, diglucosylated form of enterobactin), and the linear derivative of salmochelin (S2) (46). We evaluated xenosiderophore utilization in the wild type, the *tonB* mutant, the *fetA* mutant, the *mpeR* mutant, the *mpeR*^{C} strain, and the double *fetA mpeR* mutant. Ferric citrate was used as a positive control and bovine transferrin was used as a negative control since gonococci cannot utilize iron bound to non-human transferrin (3, 37). Figure 6 shows the average growth zone in millimeters detected around each iron source with standard deviation reflecting the variability within four or five independently conducted experiments, each of which was performed in triplicate. As previously shown by Carson *et al.* (12), gonococcal strain FA1090 grew in the presence of enterobactin (Fig. 6). Enterobactin-dependent growth by the *fetA* and *tonB* mutants was reduced to just above background levels (dotted line, Fig. 6). Enterobactin-dependent growth by the *mpeR* mutant was also decreased relative to the wild-type strain, although growth inhibition was not as great as was seen with the *fetA* and *tonB* mutant strains. Enterobactin-dependent growth was restored in the *mpeR*^{C} strain to near wild-type levels, consistent with the recovery of MpeR regulatory function in this strain. The double *fetA mpeR* mutant was severely restricted in enterobactin-dependent growth. These findings are consistent with those of

Carson *et al.* (12), but further extend our observation that MpeR activates FetA expression, and leads to an enhanced ability to utilize the xenosiderophore enterobactin.

As shown in Fig. 6, our analysis additionally demonstrates that gonococcal strain FA1090 can employ the enterobactin derivatives D2 and D3 in a FetA- and TonB-dependent manner. As was detected with enterobactin, use of these enterobactin derivatives was also maximized when MpeR was expressed. The *mpeR* mutant was capable of less growth around D2 and D3 (Fig. 6) relative to the wild-type strain and complementation of the *mpeR* mutation led to an increase in D2- and D3-dependent growth. Interestingly, there was also a significant decrease in salmochelin S2-dependent growth by both the *fetA* mutant and *tonB* mutant, indicating that FA1090 utilized salmochelin S2 in a FetA-and TonB-dependent manner as well. These results demonstrate that gonococcal strain FA1090 is capable of employing enterobactin, the DHBS dimer (D2) and trimer (D3) derivatives and salmochelin S2 as iron sources. FetA, TonB and MpeR are critical for this process, consistent with the ability of MpeR to induce expression of FetA. This is the first demonstration that gonococcal strain FA1090 utilizes DHBS dimers and trimers and salmochelin S2 as iron sources and that this uptake pathway depends upon expression of FetA, TonB, and MpeR activation. While salmochelin S4 was not utilized by any FA1090 variants tested in this analysis, the DHBS monomer was employed by all strains, both mutant and wildtype. These results imply that FA1090 does not have the capacity to internalize salmochelin S4 but can import DHBS in a TonB- and FetA-independent pathway.

Xenosiderophores that support growth do not serve as co-inducers for MpeR-dependent *fetA* activation. AraC-like transcriptional regulators are distinguished by a

C-terminal helix-turn-helix motif, which is responsible for DNA binding. These regulators also contain an N-terminal binding site to which activator molecules bind conferring specificity on regulation (26). MpeR, an AraC-like regulator, enhances *fetA* transcription (Figs. 2 and 4) and MpeR binds to the region upstream of the *fetA* gene (Fig. 3). Gonococcal strain FA1090 can utilize enterobactin, DHBS, and salmochelin S2 as iron sources (Fig. 6). Given these findings, we tested whether these xenosiderophores could serve as co-inducers for MpeR-dependent FetA activation. Western blot analysis was utilized to investigate FetA expression when gonococci were grown in the presence of ferrated catecholates. The wild-type strain was grown in the presence of the ferrated forms of enterobactin, DHBS monomer (D1), DHBS dimer (D2), DHBS trimer (D3), salmochelin S4, and salmochelin (S2). FA1090 grown in the presence of all tested siderophores except salmochelin S4 exhibited FetA expression levels similar to that detected under iron-replete conditions (Fig. 7A). The wild-type strain, grown in the presence of S4, exhibited similar FetA expression levels to those expressed when grown in iron-deplete conditions (Fig. 7A). To determine whether the decrease in FetA expression was siderophore specific or due to iron status, we analyzed TbpA expression from the same cultures. TbpA is a Fur regulated, outer membrane transporter of iron from human transferrin (19). TbpA expression mirrored that of FetA as a function of growth on xenosiderophores (Fig. 7A). As shown in Fig. 6, salmochelin S4 did not support the growth of FA1090. Thus the increase in FetA expression in the presence of salmochelin S4 is due to iron stress rather than S4 serving as a co-inducer for MpeR in the activation of FetA. Furthermore, the other siderophores and derivatives that supported growth resulted in high internal iron pools and consequently resulted in repression of FetA. We

repeated this experiment, replacing the ferrated siderophores with the iron-free forms during gonococcal growth (Fig. 7B). As seen with the ferrated-xenosiderophores, there was no evidence of siderophore-dependent induction as all strains expressed FetA at levels similar to those detected in iron depleted growth conditions. Cumulatively, these results suggest that FetA expression is sensitive to the iron status of the cell and that the presence of the xenosiderophores, either in ferrated or iron-free form, did not further influence FetA expression.

DISCUSSION

MpeR was first identified by Folster and Shafer as a homolog of other AraC-like regulators (25). MpeR was originally described as a transcriptional regulator of the *mtrF* gene, which encodes a protein that modulates antimicrobial efflux pump activity in gonococcal strain FA19. In the present study, we determined that in contrast to its repressive action on *mtrF*, MpeR activates *fetA* transcription under iron-deplete conditions. While MpeR clearly plays a role, other regulators or co-factors may also be involved in controlling *fetA* expression. Importantly, this is the first example of an AraC-like regulator that is involved in the regulation of an outer membrane xenosiderophore transporter in *N. gonorrhoeae*. Thus, the transcriptional regulatory activities of MpeR impact at least two important properties needed for survival of gonococci during infection: efflux of host-derived antimicrobials by the Mtr system (25) and xenosiderophore-iron acquisition via FetA. In the context of the present work, we suggest that MpeR regulation of *fetA* may aid in gonococcal immune evasion. Anti-FetA antibodies are present in sera from patients convalescing from meningococcal disease and these antibodies are cross-reactive against gonococcal strains (1, 8). In addition, monoclonal antibodies against FetA are bactericidal in the presence of human complement (51). Therefore, continuous, unregulated FetA expression during the entirety of an infection is expected to elicit a host response that would inhibit the bacteria from thriving *in vivo*. Thus *fetA* expression is expected to be tightly controlled.

In the current study, we determined that enterobactin and DHBS dimers (D2) and trimers (D3) were utilized by strain FA1090 in a FetA- and TonB-dependent manner.

MpeR-dependent activation of FetA enhanced the ability of FA1090 to employ these xenosiderophores as sole iron sources. While the ability of *N. gonorrhoeae* to utilize enterobactin had been previously recognized (12), this is the first demonstration of gonococcal use of DHBS dimers (D2) and trimers (D3) as iron sources. Like enterobactin, these enterobactin derivatives were employed by wild-type FA1090 in a FetA-, TonB-, and MpeR-dependent mechanism. This is somewhat surprising since these siderophores are internalized via distinct TonB-dependent transporters in other Gram-negative bacteria (27, 67) Moreover, strain FA1090 can also employ the xenosiderophore salmochelin S2 in the same FetA-dependent pathway. The broad specificity of FetA for all four xenosiderophores is remarkable, as their import into *E. coli* is facilitated by four distinct transporters, including FepA, Cir, FiuA and IroN (67). Carson *et al.* (12) noted that the sequence of FetA from gonococcal strain FA1090 retained those residues known to be important for enterobactin binding to FepA (16); however, the spacing between the conserved residues was distinct. In addition, the binding affinity of FetA for enterobactin was found to be much weaker (12) than that described for *E. coli* FepA (11). Thus we propose that FetA has evolved as a gonococcal transporter capable of importing a broad spectrum of catecholate-type xenosiderophores, perhaps at the expense of high affinity interactions with any single siderophore.

Salmochelin is known as a "stealth siderophore" and is derived by glucosylation of enterobactin via the products of the *iroA* locus (46). Virulent pathogens, including uropathogenic *E. coli*, *Salmonella enterica*, and *Shigella dysenteriae* harbor the *iroA* locus, sometimes on pathogencity islands (50). Modification of the enterobactin molecule by addition of two glucose molecules leads to increased hydrophilicity and to the ability

of salmochelin to evade the host's innate immune response. In the human host, enterobactin is sequestered and made ineffectual by the innate immunity protein, siderocalin (also known as lipocalin 2), whereas salmochelin is not (24, 28). Thus, salmochelin production by pathogens allows for efficient iron acquisition in the presence of siderocalin (56), which is found in lymphocytes. Salmochelin S4 is a cyclic form of the siderophore and salmochelin S2 is the linear derivative of S4; both forms coordinate iron and can be employed as "stealth siderophores". Gonococcal strain FA1090 did not utilize iron from salmochelin S4 but did obtain iron from S2. As microbial producers of enterobactin, DHBS and salmochelin inhabit the same niche as the gonococcus (21, 55), it seems likely that the ability to hijack these siderophores, in the presence of neutrophil-derived siderocalin, enhances the survival of *N. gonorrhoeae* in vivo.

Interestingly, our laboratory has observed differences in xenosiderophore utilization among gonococcal strains. Strain FA19 utilizes enterobactin, D1, and S2 in a TonB- and FetA-independent mechanism that requires expression of the FbpABC system (62). The mechanism by which these siderophores cross the outer membrane was not precisely defined, but we proposed that entry may be via non-specific diffusion through abundant porin proteins (62). In the current study, we demonstrated that FA1090 utilizes D1 in a TonB- and FetA-independent mechanism. However, FA1090 utilizes enterobactin, D2, D3, and salmochelin S2 in a FetA- and TonB-dependent manner. Our hypothesis for the differences in xenosiderophore utilization phenotypes between strains is that there are two different pathways by which iron from xenosiderophores can be transported into the gonococcus. One pathway is TonB- and FetA-dependent while the other pathway is TonB-independent and requires the FbpABC system. In gonococcal

strain FA1090, the *fetA* and *tonB* mutants were capable of significantly less growth with enterobactin, D2 D3, and S2 relative to the wild-type strain; however, both mutants exhibit xenosiderophore-dependent growth above background (dotted line in Fig. 5). Growth was diminished but not abolished in the *tonB* and *fetA* mutant strains; therefore we hypothesize that the FbpABC-dependent, Ton-independent pathway is also employed by these mutants in the FA1090 background. In further support of this hypothesis, Carson *et al*. demonstrated that both *fetA* and *fetB* mutants of FA1090 exhibited a decrease in growth in the presence of enterobactin but growth was not abolished (12). Similar results were also observed with the *tonB* mutant in the previous study (12). Thus, some iron was transported into the *fet* and *tonB* mutants in an energy- and *fet* operon-independent pathway. The difference in xenosiderophore-dependent growth between FA19 and FA1090 is likely due to differential use of these two distinct pathways. Gonococcal strain FA19 seems to be limited to use of the TonB-independent pathway that employs the FbpABC system for iron transit through the periplasm and cytoplasmic membrane.

In *N. gonorrhoeae*, the *fetA* gene is the only TonB-dependent transporter that is also encoded with a periplasmic binding protein and a complete set of ABC transport genes (*fetB-ng2088*). Data presented in the current study suggest that the putative periplasmic binding protein and ABC transport system are co-transcribed with *fetA*, but also independent of FetA and MpeR. Expression of the shorter transcript encoding *fetB-ng2088* was detected only in gonococci grown in iron-deplete conditions. A Fur box upstream of *fetB* was identified by FURTA and EMSA assays (34), consistent with our finding of a separate iron-regulated transcript including the *fetB-ng2088* genes. The

FetA-independent but iron regulated transcription of the ABC transport genes suggests that the ABC transport system could be utilized by other TonB-dependent transporters, in addition to FetA.

Enterobactin, D2, D3, and salmochelin S2 were acquired by gonococcal strain FA1090, which led us to consider the possibility that one or all of these xenosiderophores could serve as a co-inducer of MpeR for the activation of *fetA* expression. However, contrary to our hypothesis, FetA expression was not altered by the presence of any of the siderophores tested, regardless of their iron status. We are currently entertaining three possible mechanisms to explain these results. First, MpeR may not require a co-inducing molecule in order to activate FetA expression. Second, because MpeR is involved in modulating the expression of proteins involved in antimicrobial efflux, the co-inducing agent for MpeR-dependent regulation could be related to efflux. In this context, since the Mtr system is necessary for efflux of host-derived antimicrobials, MpeR might sense an efflux substrate as a signal of location within the host. And third, other catechols might serve as co-inducers with MpeR. Given the broad specificity of FetA, it is possible that an as yet unrecognized catecholate molecule could serve as an inducer and also provide iron in a FetA-dependent manner.

The *mpeR* gene has only been identified in the genomes of the pathogenic *Neisseria* and is absent from the genomes of commensal *Neisseria* (42, 60). The MpeR proteins are highly conserved, sharing 97-100% sequence identity among the pathogenic *Neisseria* species (data not shown). This conserved, pathogen-specific regulator controls the expression of FetA, which is a pan-*Neisseria* transporter (42). In *Staphylococcus aureus*, different community-acquired strains vary in their virulence. The difference

amongst strains in pathogenic potential is linked to increased expression of core genome-encoded virulence genes (40). It was hypothesized by Li *et al.* (40) that global regulators of virulence genes are responsible for variable virulence amongst strains. Similarly, commensal *Neisseria* have an extensive repertoire of virulence alleles that are also expressed by pathogenic *Neisseria* and other bacterial genera (42). Thus differential regulation of these alleles, rather than their presence or absence, may contribute to increased virulence in the pathogenic *Neisseria*. Perhaps MpeR-dependent activation of *fetA* and other potential transporters enhances virulence in pathogenic *Neisseriae* relative to the commensals.

In conclusion, in this study we demonstrated that MpeR, a pathogen-specific regulator in *N. gonorrhoeae* enhances expression of the siderophore receptor FetA under iron-deplete conditions. A second, internal transcriptional start site was identified upstream of *fetB*, positioned near several potential -10 promoter elements, overlapped by the Fur-binding site. Additional studies will be required, however, to fully define the mechanisms that control *fetB* transcription and whether other regulatory proteins are involved. We also determined that enterobactin, D2, D3 as well as salmochelin S2 were utilized in a FetA- and TonB-dependent mechanism in strain FA1090. Expression of MpeR enhanced the ability of strain FA1090 to utilize enterobactin and salmochelin, consistent with MpeR-dependent activation of FetA. None of the catecholates that supported growth in this study appeared to act as a co-inducer for MpeR-dependent activation of *fetA*. To our knowledge, this study represents the first description of a pathogen-specific regulator in *N. gonorrhoeae* that activates expression of a pan-*Neisseria* TonB-dependent transporter. In addition, the current study is the first to link

regulation of iron transport and antimicrobial efflux systems through the action of a pathogenic *Neisseria*-specific regulator.

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TABLE 1. Strains and plasmids used in this study.

Strains and plasmids	Genotype [a] and/or relevant characteristics	Reference or Source
Strains		
FA1090	Wild type (Δ*lbpA*, HpuAB off)	(9)
FA6959	FA1090 *fetA*::Ω (Str^r Spc^r)	(12)
MCV656	FA1090 *tonB*::Ω (Str^r Spc^r)	(29)
MCV304	FA1090 *mpeR*::*aphA-3* (Str^r Spc^r Km^r)	This study
MCV305	$mpeR^C$(Str^r Spc^r Km^r Erm^r)	This study
MCV306	*fetA*::Ω, *mpeR*::*aphA-3* (Str^r $Spcr^r$ km^r)	This study
Plasmids		
pGCC3-mpeR	pGCC3 containing the full-length *mpeR* gene and 250 bp of upstream sequence	This study
pVCU366	pCR 2.1 containing 800bp of *fetA* through *fetB* amplified by oVCU498 and oVCU499	This study

[a.] Str^r, streptomycin resistance; Spc^r, spectinomycin resistance; Kan^r, kanamycin resistance Erm^r, erythromycin resistance

TABLE 2. Oligonucleotides used in this study.

Oligonucleotide	Amplicon	Sequence (5′-3′)
5′mpeR	*mpeR*::*aphA-3*	ATGAACACCGCCGCCATCT
3′mpeR	*mpeR*::*aphA-3*	GCACTTTTTCACATCCGAAGG
5′pMpeR pac	$mpeR^{C}$ construct	GGTTAATTAACGAAACAACCTGCAGAAACC
3′ GC4 MpeR	$mpeR^{C}$ construct	GGTTTAAACTCAGCACTTTTTCACATCCGA
malEmpeRF	*mpeR* coding region	CACTGGGGATCCATGAATACCGCCGCCATCT
malEmpeRR	*mpeR* coding region	CACTGGCTGCAGTCAGCACTTTTTCACATCCGA
5′fetAup	500bp upstream *fetA* probe	GCCCGAACGGTTCGGACAAATT
3′fetAup	500bp upstream *fetA* probe	TTGTTCGTCCTTTTGAGTGT
3′fetAupint	*fetA*1 upstream probe	TCACTTGGTGCTTCAGCACC
5′fetAupint	*fetA*2 upstream probe	GGTGCTGAAGCACCAAGTGA
rnpB1F	*rnpB*	CGGGACGGGCAGACAGTCGC
rnpB1R	*rnpB*	GGACAGGCGGTAAGCCGGGTTC
oVCU 484	*mpeR* RT-PCR Fwd	GCGTTTCCCACCGAAATCCACAAT
oVCU 485	*mpeR* RT-PCR Rev	AGCGTAATAATCGGGCGGAGAGTT
oVCU 486	*fetA* RT-PCR Fwd	AAAGATTACGAAGCCGGCAAAGGC
oVCU487	*fetA* RT-PCR Rev	TTCAACAGGGTTTGTTCGGCAAGG
oVCU498	*fetA*-*fetB* Fwd	CCAACGCTGCACCAATACCCTGC
oVCU499	*fetA*-*fetB* Rev	CCTTCAGCTTGTCGGCTTCCGCCTG
oVCU500	*fetB*-NG2091 Fwd	CAGGCGGAAGCCGACAAGCTGAACG
oVCU501	*fetB*-NG2091 Rev	GCACAGGCTGACGGCAAACAATACC
oVCU513	NG2091-NG2090 Fwd	GGTATTGTTTGCCGTCAGCCTGTCG
oVCU516	NG2091-NG2090 Rev	GCTCGCTCGGGACGGTATTGAATC
oVCU515	NG2090-NG2089 Fwd	GACTTTGTCTTGCACCTGCGCCTG
oVCU518	NG2090-NG2089 Rev	CAGTTCGCGGACGATGACGAACTG
oVCU517	NG2089-NG2088 Fwd	CTGTGCGGACATTCCTGCCCGAC
oVCU553	NG2089-NG2088 Rev	TGCTTTGGCAGAACACCATCGCAA
oVCU110	*16S* rRNA Fwd	TATCGGAACGTACCGGGTAGC
oVCU111	*16S* rRNA Rev	GTATTACCGCGGCTGCTGGCA
oVCU529	*fetA* qRT-PCR Fwd	ATCCAACACCAATTTGGCGTA
oVCU530	*fetA* qRT-PCR Rev	CATCGGCGGAATAGCGTTT
oVCU461	*mpeR* qRT-PCR Fwd	TCTACCGCCAGTACCAAACC
oVCU462	*mpeR* qRT-PCR Rev	GGCTGAAATTGTGGATTTCG
oVCU457	*rmpM* qRT-PCR Fwd	GGAGCAGGCTCCTCAATATG
oVCU458	*rmpM* qRT-PCR Rev	TAAAGTCGGTATGGCCTTCG
oVCU576	NG2091 qRT-PCR Fwd	GTGAGCGTGAATTTGGGTTT
oVCU577	NG2091 qRT-PCR Rev	TATTGCCGACCGTTACAATG
oVCU676	*fetB* primer extension	CAG GGT CGG AAT TTT GCG GCG AAC AC

TABLE 3. Effects on gene expression measured by real time RT-PCR.

Comparison	Normalized target gene expression ratio		
	fetA	*mpeR*	*ng2091*
WT-Fe / WT+Fe	97 (103-83)[1]	198 (301-106)	16.3 (17-16)
WT-Fe / FA6959-Fe	194 (209-167)	2.2 (3.1-.6)	23 (28-16)
WT-Fe / MCV304-Fe	117 (185-33)	62 (123-46)	22.3 (24.22)
WT-Fe / MCV305-Fe	9.6 (15-5)	1.9 (2.4-1.3)	7.1 (9.7-5)
WT-Fe / MCV306-Fe	108 (154-70)	37 (45-26)	11.5 (16-9)

[1] Average fold change for each comparison is shown, with the range detected among three independently-conducted real time RT-PCR assays shown in parentheses.

FIGURE LEGENDS

FIGURE 1. FetA expression is regulated by MpeR. (A) SDS-PAGE analysis of protein expression. Total membrane proteins were isolated from WT (FA1090) and *mpeR* mutant (MCV304) strains grown under iron-deplete (-) and iron-replete (+) conditions for 4 hours. Proteins were separated on a 7.5% acrylamide gel. The arrow on the left indicates the band that was excised and identified as FetA by mass spectrometry analysis. The position of molecular weight markers is indicated on the right. (B) Western blot analysis of FetA expression. The WT (FA1090), *mpeR* mutant (MCV304), *fetA* mutant (FA6959) and the complemented *mpeR*^{C} strain (MCV305) were grown under iron-deplete (-) and iron-replete (+) conditions. Total membrane proteins from each strain were isolated and standardized before being separated by SDS-PAGE and then transferred to nitrocellulose. Blots were probed with an anti-FetA monoclonal antibody.

FIGURE 2. MpeR activates *fetA* transcription under iron-deplete conditions. The WT (FA1090), *fetA* mutant (FA6959), *mpeR* mutant (MCV304), and the complemented *mpeR*^{C} strain (MCV305) were grown under iron-deplete (-) and iron-replete (+) conditions. RNA samples isolated from each gonococcal strain were analyzed for expression of *fetA* and *mpeR* by RT-PCR. *16S rRNA* (*16S*) was used as a positive control because it was constitutively expressed under all growth conditions. Expression of *16S rRNA* in the absence of reverse transcriptase, (*16S* (-)) was used as a negative control.

FIGURE 3. MpeR binds upstream of *fetA* in a specific manner. (A) Sequence of the 500-base pair, intergenic region immediately upstream of *fetA*. The sequence highlighted in teal is contained within the *fetA*1 probe employed for the EMSA shown in panel B. The sequence highlighted in yellow is contained within the *fetA*2 competitor DNA. The sequence highlighted in blue is contained in both *fet*A1 and *fetA*2 ampicons. The Fur-binding site (34) is highlighted in gray. The previously-mapped (14) promoter elements (underlined) and transcriptional start site (asterisk) are identified. The start codon for FetA is shown in red. (B) 5 nanograms of the 250-base pair, labeled *fetA*1 probe (lane 1) was incubated with 10 µg of MBP-MpeR in the absence of unlabeled competitor (lane 2) or in the presence of 2, 10 or 20X excess unlabeled competitor DNA. Lanes 3-5 contain reactions including increasing concentrations of the specific competitor (*fetA*1); lanes 6-8 contain reactions including increasing concentrations of *fetA*2; and lanes 9-11 contain reactions including increasing concentrations of the non-specific competitor, *rnpB*.

FIGURE 4. FetA is encoded as part of a multi-gene operon. (A) Genetic locus including *fetA* and downstream genes. Genes are depicted by boxes. Hatched regions 5′ of *fetA* and *fetB* indicate approximate locations of Fur boxes (34). Below the chromosomal locus are small dark arrows indicating primer locations. Numbered black bars denote the amplicons generated from each primer set. Long, dark gray arrows indicate length and start positions of two proposed transcripts. (B) RT-PCR analysis of the *fet* operon. RNA was isolated from the indicated gonococcal strains which were grown under iron deplete (-) and iron replete (+) conditions. Amplicon numbers correspond to the diagram in panel A. *16S rRNA* (*16S*) was used as a positive control

because it is constitutively expressed under all conditions test. Expression of *16S rRNA* in the absence of reverse transcriptase, (*16S* (-)) was used as a negative control.

FIGURE 5. Identification of the *fetB* transcriptional start site. (A) Sequence of the intergenic region immediately upstream of *fetB*. The ATG at the end of the sequence represents the FetB start codon. Several overlapping, potential -10 promoter elements are underlined. The Fur-binding site (34) is highlighted in gray. The transcriptional start site identified in this analysis is identified by the asterisk. (B) Primer extension products generated from RNA samples harvested from wild-type gonococcal strain FA1090 grown under iron replete (+Fe) and iron deplete (-Fe) conditions. Equivalency of the amount of RNA template in each sample was confirmed by ethidium bromide staining of RNA separated on an agarose gel. For comparison, the sequencing reaction using the same primer as was used for the primer extension reaction, is shown on the left. The T residue highlighted by the asterisk marks the point of transcript initiation on the non-coding strand.

FIGURE 6. Xenosiderophore utilization by gonococcal strain FA1090. CDM plates were supplemented with apo-bovine transferrin and wells within the plates were inoculated with the following siderophores: ENT: enterobactin; D1: dihydroxybenzoylserine (DHBS); D2: the dimer form of DHBS; D3: the trimer form of DHBS; S2: the linear derivative of salmochelin; and S4: the cyclized form of salmochelin. Ferric citrate (+) was used as the positive control and apo-bovine transferrin (-) was used as the negative control as indicated along the x-axis of the graph.

Each bar indicates the average growth in millimeters around each siderophore source; the average and standard deviations were determined from seven independent experiments, each conducted in triplicate. Bars represent average growth zone for the following strains: wild type FA1090 (black bars), *fetA* mutant strain FA6959 (checkered bars), *tonB* mutant strain MCV656 (gray bars), *mpeR* mutant strain (white bars), and *mpeR*^{C} complement strains (striped bars). The horizontal dotted line indicates the diameter of the well containing each iron source. Pairwise comparisons between the wild-type and mutant strains resulted in the following *P*-values: * $\leq$ 0.001; # = 0.0124; ^ = 0.0197.

FIGURE 7. FetA expression is not induced by the presence of xenosiderophores. (A) WT (FA1090) was grown in CDM with the indicated ferrated-xenosiderophores (final concentration of 10 mM) as the sole iron source. The following ferric-xenosiderophores were tested: ENT: enterobactin; D1: dihydroxybenzoylserine (DHBS); D2: the dimer form of DHBS; D3: the trimer form of DHBS; S2: the linear derivative of salmochelin; and S4: the cyclized form of salmochelin. As controls the WT strain was grown in the absence of iron (-) or with ferric nitrate (+) but without the addition of siderophores. Aliquots collected at 2, 4 and 6 hours (indicated above the blots) were lysed and subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose. Blots were probed with anti-FetA (top) or anti-TbpA antibodies (bottom). (B) As in panel A, except the WT (FA1090) was grown in CDM with the indicated xenosiderophores in the iron-free or apo form.

Figure 1

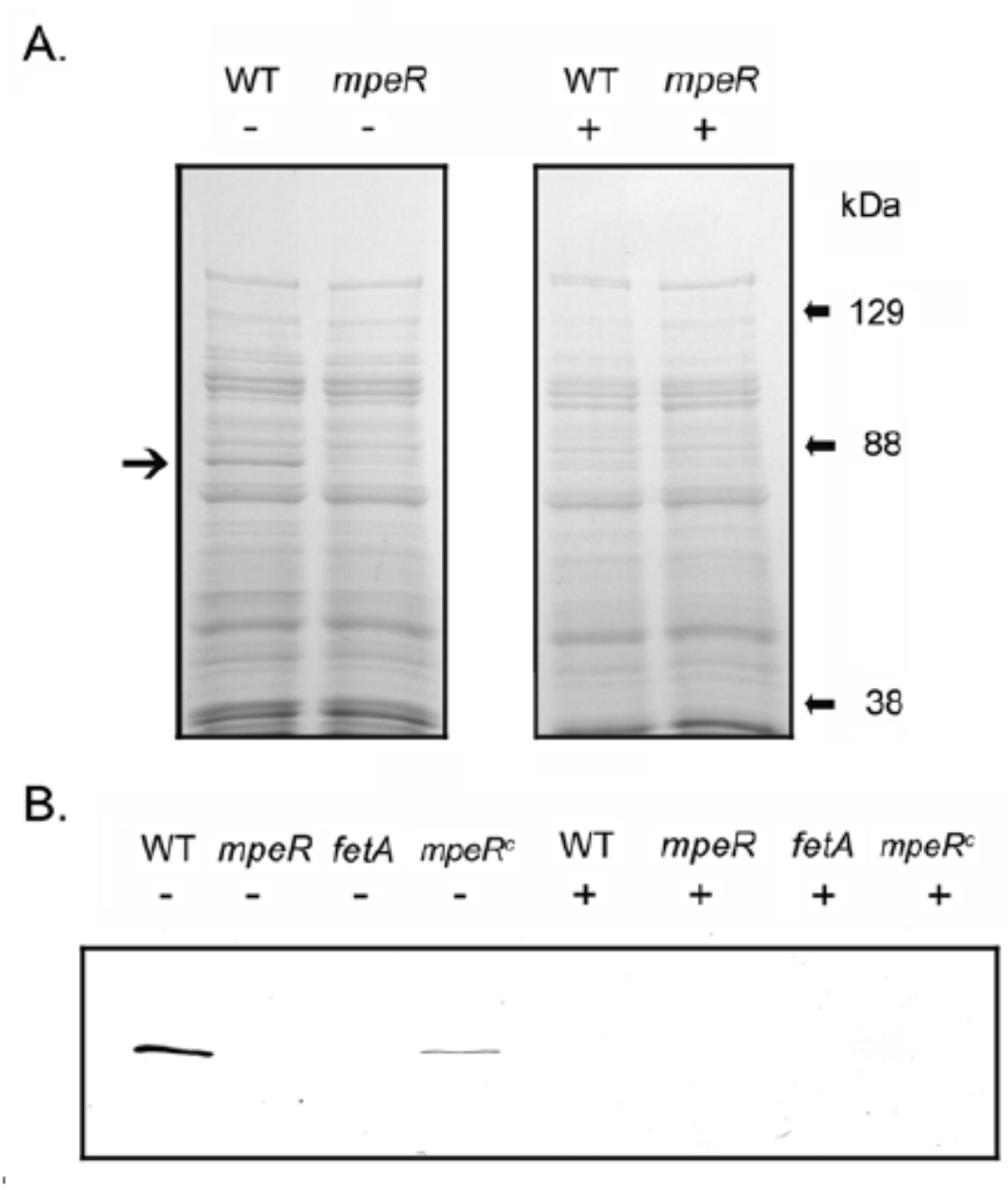
A.
WT
mpeR
WT
mpeR
-
-
+
+
kDa
129
88
38
B.
WT
mpeR
fetA
mpeRc
WT
mpeR
fetA
mpeRc
-
-
-
-
+
+
+
+

Figure 2

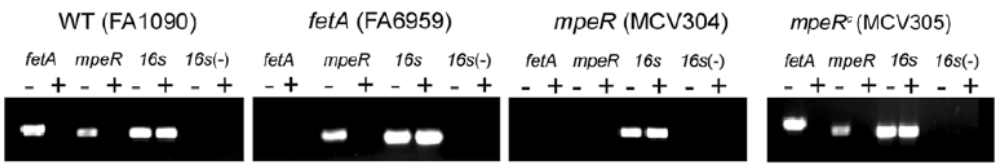
WT (FA1090)
fetA (FA6959)
mpeR (MCV304)
mpeR^c (MCV305)
fetA mpeR 16s 16s(-)
- + - + - + - +

Figure 3

A.

CTCCGATAAATGGTTTGAAAACAATCATCTGCCCGAACGGTTCG
GACAAATTGAAGTGGAAACCGGGCTGCCGCCTAACGGCCTGCCC
GTACAAGTTGGCAAAATTAGGGTGTGTGCGGGAAAATTCAAGTG
AGGCGGAAAAAAATTTATTTCCGCCGTTTTTTATAGTGGACTAA
ATTTAAACCGGTACGGCGTTGCCTCGCCTTAGCTCAAAGAGAAC
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ATCTGTACTGCCTGCGGCTTCGCCGCCTTGTCCTGATTTTTGTT
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GCTTCGCAAATAAAAACGATAATCAGCTTTACACAACCCCCCCC
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B.

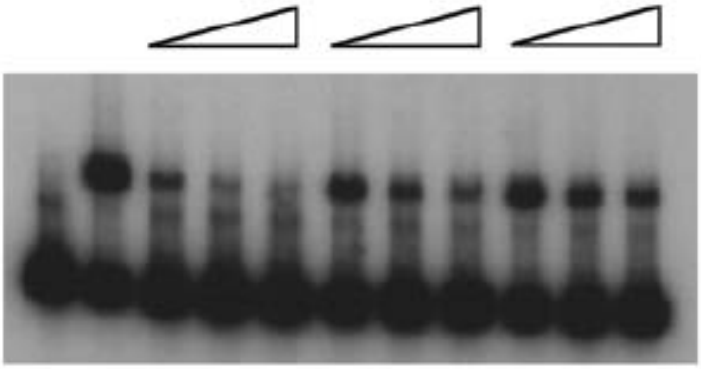


Figure 4

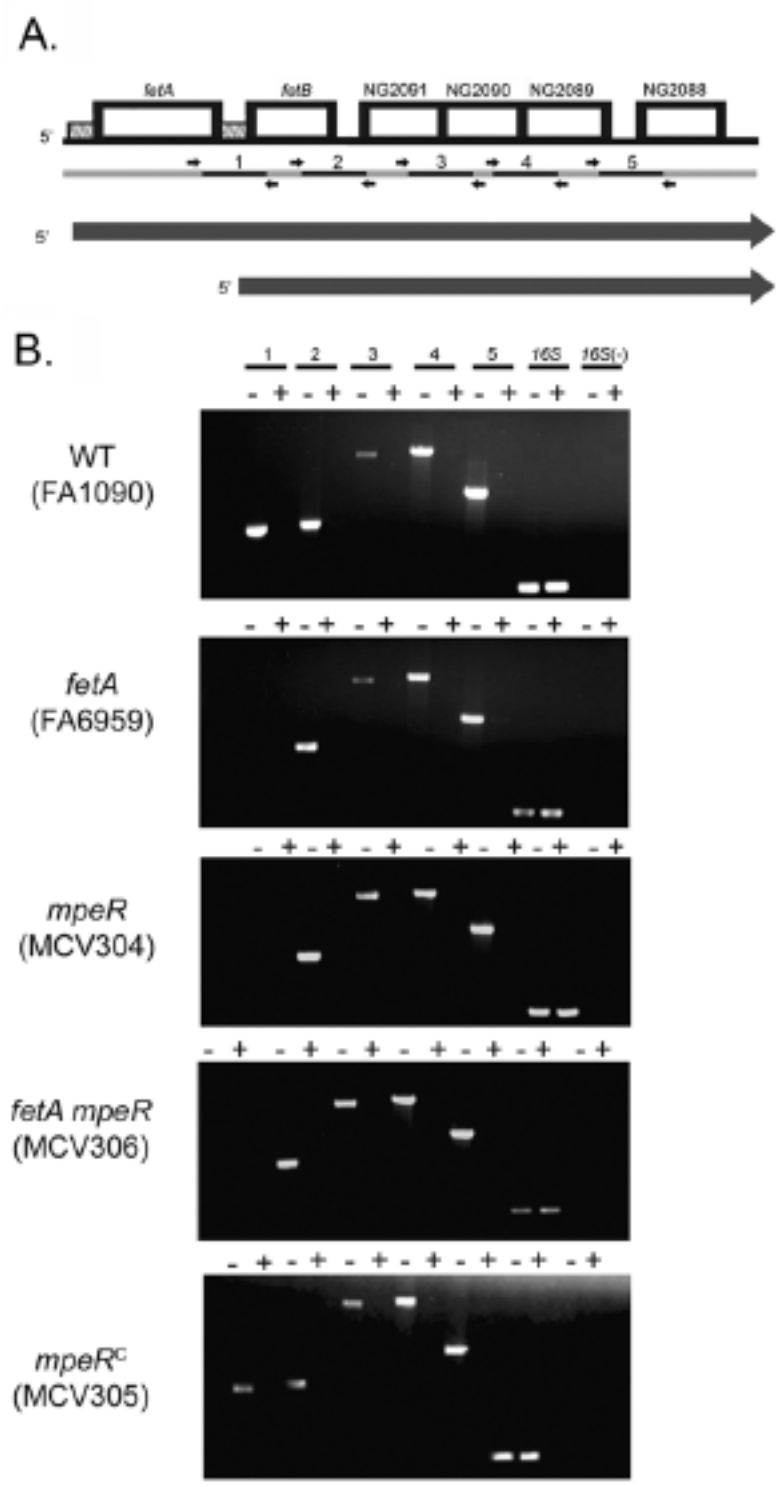
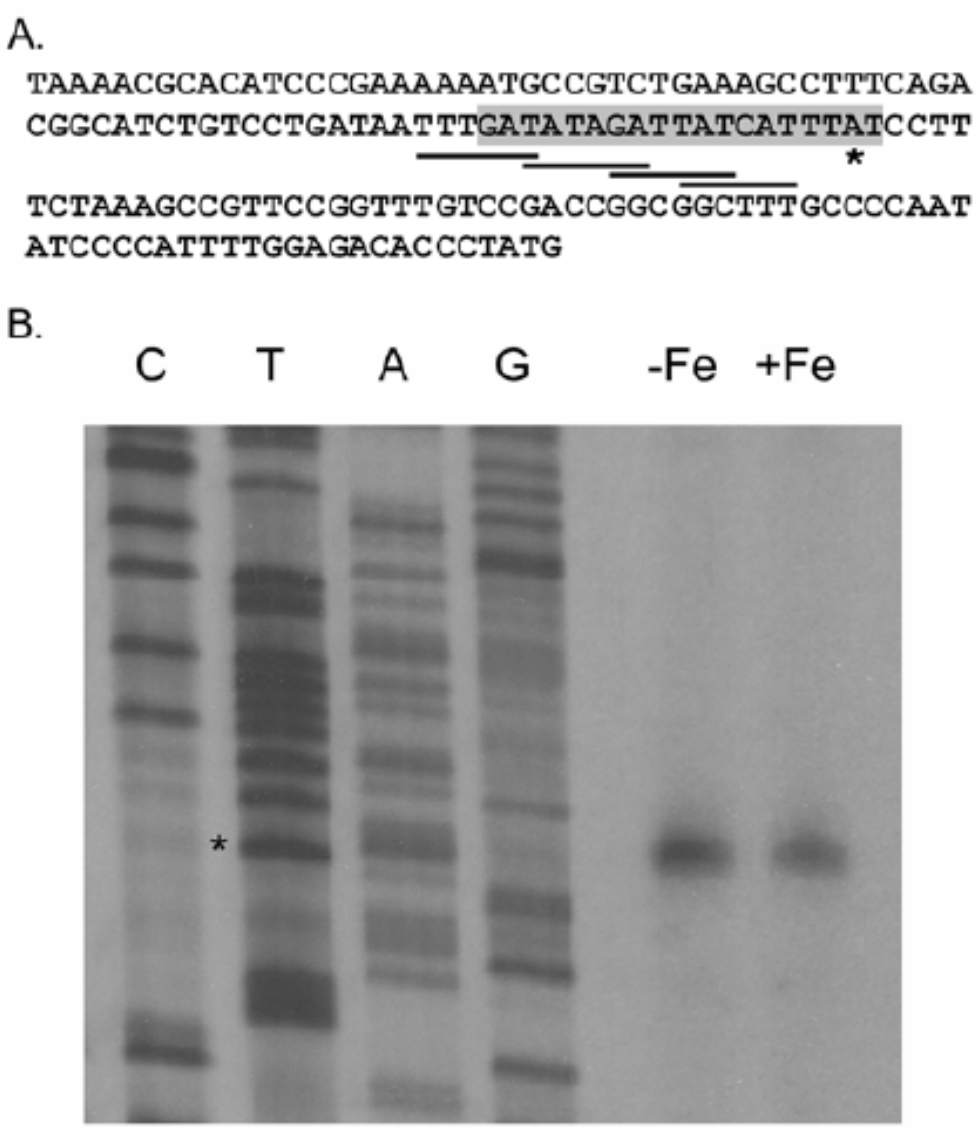

A.
fetA
fetB
NG2091
NG2090
NG2089
NG2088
5'
1
2
3
4
5
5'
5'
B.
1
2
3
4
5
16S
16S(-)
WT
(FA1090)
fetA
(FA6959)
mpeR
(MCV304)
fetA mpeR
(MCV306)
mpeR^C
(MCV305)

Figure 5


A.
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*
TCTAAAGCCGTTCCGGTTTGTCCGACCGGCGGCTTTGCCCCAAT
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B.
C
T
A
G
-Fe
+Fe
*

Figure 6

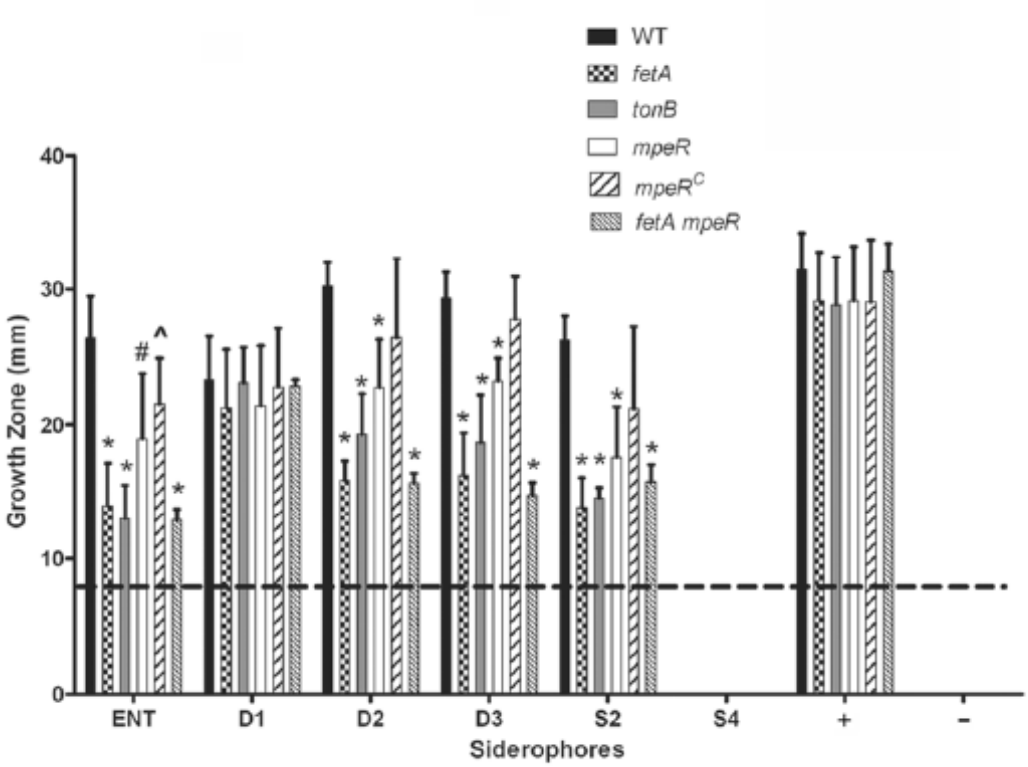
WT
fetA
tonB
mpeR
mpeR^C
fetA mpeR
Growth Zone (mm)
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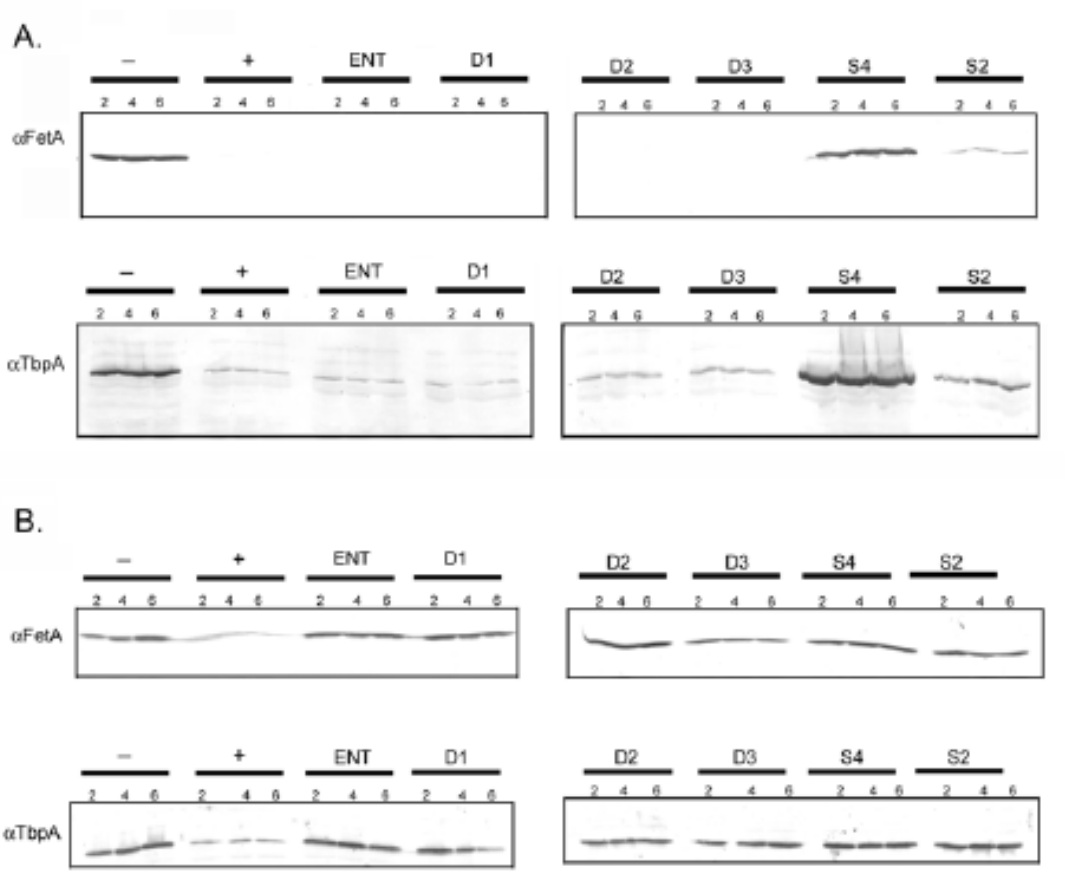
Figure 7



Chapter 5: Summary and Final Discussion

The ancient organism *Neisseria gonorrhoeae* has proven to be a successful human pathogen partly because it has evolved to employ a number of diverse defense mechanisms to survive within its human host. One useful survival strategy is the expression of multi-drug efflux pumps that serve to protect the gonococcus from the deleterious effects of a number of antimicrobial agents. It has been established that the expression of multi-drug efflux pumps also aids in both bacterial colonization and persistence within the human host (22). Thus, it is important to consider multi-drug efflux pumps as a bacterial virulence factor that can be influenced by a number of host-mediated factors. While multi-drug efflux pumps serve important roles, their expression can be costly in terms of cellular energy and is tightly regulated. Therefore, elucidating the transcriptional regulation of efflux genes can give us a more comprehensive understanding of gonococcal pathogenesis. This body of work sought to uncover the role of the AraC family transcriptional regulator, MpeR, in regulating expression of genes in the *mtr* efflux pump locus and many other genes important for the pathogenesis of *Neisseria gonorrhoeae*.

Expression of the *mtrCDE* operon, which encodes the MtrCDE efflux pump, is repressed by the TetR family transcriptional regulator MtrR (19, 25). Transcriptional activation of the pump operon is mediated, in an inducible manner, by the AraC transcriptional activator MtrA (19, 25). The discovery by Veal and Shafer that the MtrCDE accessory protein MtrF is important for high-level resistance prompted questions about the regulation of this efflux component (27). Due to the close proximity

of *mtrR* and *mtrF* on the gonococcal genome it was hypothesized by Folster and Shafer (10) that MtrR could serve to regulate the expression of *mtrF*, yet DNA binding studies could not prove that MtrR was regulating the expression of *mtrF* in a direct manner. Thus, the gonococcal genome was searched for additional transcriptional regulatory proteins, and subsequent β-galactosidase assays revealed that the AraC-like protein MpeR could serve to repress the expression of *mtrF* (10). According to microarray studies conducted by Ducey *et al*., the expression of *mpeR* is derepressed under iron-deplete conditions similar to those found in the human host (6). These initial observations lead us to hypothesize that the changes in free iron levels encountered by the gonococcus within the human host could serve to influence resistance to antimicrobials recognized by the MtrCDE efflux pump in an MpeR dependent manner.

Results in Chapter 3 confirming that MpeR represses the expression of *mtrF* in a direct manner and previous data demonstrating that *mtrF* is important for resistance of gonococci to hydrophobic agents when MtrCDE pump levels are high lead us to utilize the strain FA140 in order to determine if both MpeR and the availability of free iron could impact changes in antimicrobial resistance. Strain FA140 contains a base pair deletion in the *mtrR* promoter region that results in sub-optimal binding of RNA polymerase to the *mtrR* promoter, thereby greatly increasing *mtrCDE* transcription. This strain also contains mutations in the MtrR helix-turn-helix domain that abrogate the capacity of MtrR to bind DNA (27).

Interestingly, a significant decrease in the high-level resistance of FA140 to Triton X-100, a hydrophobic agent exported by the MtrCDE efflux pump, was due to the iron-dependent repression of *mtrF* by MpeR (Chapter 3). This observation suggests that

iron stress may render the gonococus unable to support high MtrCDE pump levels. While it is clear that MtrF is important for high-level resistance, its mechanism of action remains to be determined. Two hypotheses for the function of MtrF proposed by Veal and Shafer (27) are that it could act to stabilize the MtrCDE efflux pump or that MtrF could serve to shuttle hydrophobic agents to the MtrCDE efflux pump. These hypotheses may explain why *mtrF* would be repressed by MpeR under low iron conditions.

It was also important to determine whether MpeR regulates the expression of other genes of interest so as to test if MpeR, like MtrR (9), acts as a global regulator of gonococcal gene expression. Thus, microarray analysis was conducted at both the mid-log and late-log phases of growth to compare changes in gene expression between wild-type strain FA19 and strain FA19 bearing a disruption in *mpeR*. We found that MpeR both activates and represses multiple and distinct gonococcal genes at different phases of growth (Chapter 3). Typically AraC-like regulators function as transcriptional activators. However, the AraC regulator CelD in *E.coli* functions as a transcriptional repressor of the of the *celABCF* operon, involved in carbon metabolism (20). Moreover, the AraC protein YbtA of *Yersinia pestis* functions as both a repressor and an activator of transcription at different promoters (8). Interestingly, there was no overlap between the genes that were regulated by MpeR at the mid-log phase of growth and those regulated by MpeR at the late-log phase of growth. This lack of overlap may represent an efficient means by which the gonococcus utilizes a single transcriptional regulator to modulate changes in a number of different genes due to a host-mediated factor (ie. free iron availability) at different phases of growth.

Included in the microarray data among the genes that were regulated by MpeR is *mtrR*, shown to be repressed by MpeR at the late-log phase of growth when *mpeR* is maximally expressed (Chapter 3). Aside from repressing the expression of the *mtrCDE* operon (16, 19), the TetR family transcriptional regulator MtrR is a global regulator of gene expression (9). Among the genes that MtrR regulates are *rpoH*, which encodes the alternative sigma factor (sigma 32) found in both *E. coli* and *N. gonorrhoeae* to be important for not only the heat shock response but also resistance to oxidative stress (9, 14, 23). Additionally, MtrR plays an important role in the regulation of genes involved in the biosynthesis of thymine, tryptophan, and glutamine, all of which are important for gonococcal metabolism (9). Specifically it was shown by Johnson *et al.* that MtrR indirectly represses *glnA* (glutamine synthetase) by inhibiting the binding of FarR, the transcriptional regulator of the FarAB efflux pump, which activates *glnA* expression by binding upstream of the *glnA* promoter region (13, 15).

The finding that MpeR represses *mtrR* expression in a direct manner at the late-log phase of growth when free iron levels are limited and *mpeR* is maximally expressed (Chapter 3) led us to hypothesize that both MtrR protein levels and *mtrCDE* expression could be modulated in an iron-dependent manner. Of significant importance is the implication of the work presented in Chapter 3 that provides a novel link between free iron availability in the host and regulation of gonococcal antimicrobial resistance, through the regulatory capacity of MpeR, thus impacting the global regulator MtrR. Importantly, such control of *mtrR* expression also impacted expression of the *mtrCDE* operon. Accordingly, it is plausible that other genes regulated by MtrR could also be impacted by iron availability. Therefore, the interplay of host mediated cues (such as iron

availability and antimicrobial defenses) and the mechanisms used by the gonococcus to detect and adapt to these signals merits further study.

Utilizing a female BALB/c mouse model of lower genital tract infection, it has been demonstrated that loss of MtrR increases fitness of the gonococcus (28). This survival advantage, along with the increased antibiotic resistance observed in *mtrR* mutants, calls into question why evolution has not selected against MtrR. This concept has been a topic of great interest in the investigation of the regulatory role of MtrR in gonococcal pathogenesis. Along with the regulation of metabolic genes by MtrR, the effects of iron-dependent regulation of *mtrR* expression through MpeR may help explain the maintenance of MtrR in the gonococcus. One hypothesis could be that, because the gonococcus has evolved in an iron-restricted host environment, repression of *mtrR* due to host-mediated iron levels is one mechanism by which the gonococcus can ensure fitness levels in response to its environment.

In addition to regulating the expression of genes important for Mtr-mediated resistance, findings presented in Chapter 4 demonstrate that MpeR also serves to directly activate the gene *fetA*, encoding the single-component TonB-dependent receptor, FetA. Specifically, FetA has the capacity to recognize and utilize iron bound by xenosiderophores, low molecular weight iron scavengers produced by other bacteria (4). While several examples exist of AraC-like proteins regulating the expression of genes important for siderophore production (2, 8, 18) this work provides the first example of the regulation of a xenosiderophore receptor by an AraC-like protein in *Neisseria gonorrhoeae*.

It has been reported that anti-FetA antibodies present in patients recovering from meningococcal disease are cross-reactive against gonococcal strains (1, 3) and that monoclonal antibodies against FetA, along with human complement, are bactericidal (21). Because of this, constitutive *fetA* expression would elicit a host response that could be detrimental to the survival of the gonococcus *in vivo*, therefore; activation of *fetA* by MpeR under iron deplete conditions may help limit FetA expression to aid the gonococcus in evasion of the host immune response.

Importantly, the activation of *fetA* by MpeR under iron deplete conditions enhances FetA recognition of salmochelin S2, enterobactin, and the dimer and trimer forms of dihydroxybenzoylserine (5). These findings demonstrate that FetA has the capacity to recognize a broad spectrum of xenosiderophores produced by organisms that share the same environmental niche as the gonococcus (7, 24). Taken together, the body of work in Chapter 4 is the first report illustrating the activation of an iron acquisition system by a transcriptional regulator that also acts to mediate expression of an antimicrobial efflux system. It is important to note, however, that *fetA* was not one of the genes regulated by MpeR according to the microarray data presented in Chapter 3. This is because the microarray examined changes in gene expression in the FA19 strain background while the discovery that MpeR activates *fetA* expression in an iron-dependent manner occurred in strain FA1090. Interestingly *mpeR* was sequenced and found to be present only in pathogenic strains of *Neisseria* (11, 17, 26). Thus, it would be informative to investigate which genes MpeR regulates in other pathogenic strains aside from FA19 and FA1090 in order to uncover novel regulatory connections, mediated by MpeR, that could serve to enhance gonococcal pathogenesis.

As a whole, this body of work has lead to new insights into the regulatory capacity of MpeR in *Neisseria gonorrhoeae*. Importantly, through MpeR, a link has been established that seems to allow the gonococcus to sense a host-mediated factor (the availability of free iron) and translate that to changes in *mtrCDE* expression through the regulation of *mtrR*. To date, this is the first report demonstrating the regulation of MtrR, a global regulatory protein of great importance for antibiotic resistance, proper metabolic function, resistance to oxidative stress, and gonococcal fitness. The iron-dependent regulation of MpeR also leads to changes in high-level resistance to hydrophobic agents recognized by the MtrCDE efflux pump through the repression of *mtrF*.

At first glance, the repression of both *mtrF* and *mtrR* by the same regulator was perplexing to us, given that the former is necessary for high levels of resistance and the latter is necessary to dampen pump expression. However, when we considered that the stresses of hypoferric conditions and antimicrobials are likely dynamic during infection, it seemed plausible that the gonococcus might use MpeR to fine tune efflux activity in response to fluctuating iron availability sensed by Fur. Therefore, this trans-acting factor may provide a means to translate a host-mediated signal into changes in the expression of a number of genes important for antibiotic resistance, metabolism, resistance to oxidative stress, and fitness for specific survival needs at different sites of infection. Moreover, the direct MpeR-dependent activation of the gene encoding the iron-acquisition receptor FetA provides the first evidence of a connection between the acquisition of iron and the regulation of efflux components and enhances the interconnectivity between these two processes, which are both important factors for gonococcal survival within the host.

An important factor to consider, based on the findings presented herein is that the resistance of gonococci to antimicrobial agents may be drastically different *in vivo* where free iron levels are low. Typically in the laboratory, bacterial cultures are grown in media and on agar plates with an ample supply of iron. Therefore, it may be more accurate to determine minimal inhibitory concentrations of antimicrobials both under iron replete and iron deplete conditions to get a better assessment of bacterial resistance or sensitivity to such agents.

Microarray studies conducted by Jackson *et al.* report that MpeR itself is among a number of transcriptional regulators that are either activated or repressed by the ferric uptake regulator, Fur, in *Neisseria gonorrhoeae* (12). The hypothesis behind this regulation is that Fur, through its regulation of a number of secondary regulators, is able to control a number of genes that serve varied capacities. Thus, changes in free iron levels found within the host have a more significant impact on the gonococcus than was previously thought. Therefore, continued studies on the regulatory capacity of MpeR in response to iron levels will lead to further insights into the pathogenic potential of *N. gonorrhoeae*.

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