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Elizabeth A. Ohneck Date

A Novel Mechanism of Enhanced Efflux Pump Expression in *Neisseria gonorrhoeae*: Implications for Antimicrobial Resistance and Cell Physiology

By

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Doctor of Philosophy

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By

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B.S. Biology, University of Dayton, 2008

Advisor: William M. Shafer, Ph.D.

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

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Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea, a significant global public health concern as it is estimated that over 106 million cases occur each year worldwide. Infection control is complicated by the lack of a vaccine against *N. gonorrhoeae* and widespread antibiotic resistance mediated by multiple mechanisms. The *N. gonorrhoeae* MtrCDE multidrug efflux pump confers resistance to a diverse array of antimicrobial agents. Expression of this pump is tightly regulated by a transcriptional repressor, MtrR, and an inducible transcriptional activator, MtrA. The *mtrR* gene lies 250 base pairs upstream and is transcribed divergently from the *mtrCDE* operon. Isolates of *N. gonorrhoeae* expressing clinically significant levels of resistance to MtrCDE substrate antimicrobials often contain mutations in *mtrR* or in the *mtrR-mtrCDE* intergenic region. Recently, a C-to-T transition mutation was identified 120 base pairs upstream of the *mtrC* start codon and shown to be sufficient to confer high-level resistance to MtrCDE substrates. This work was designed to characterize the mechanism by which this mutation, termed mtr_{120}, affects MtrCDE-mediated resistance. It was determined that the mtr_{120} mutation generates a second, highly active promoter for *mtrCDE* transcription, resulting in increased MtrCDE production and correspondingly high-level resistance. This novel promoter was shown to act outside of MtrR or MtrA regulation, thereby offering a unique opportunity to study the physiological effects of efflux pump overexpression on gonococcal cells. This work also demonstrates that global transcriptional changes occur in gonococci when *mtrCDE* is overexpressed by the mtr_{120} promoter, including the downregulation of a previously uncharacterized transcriptional regulatory protein, GepR, that appears to be involved in regulation of genes important for antimicrobial resistance, including the *mtrCDE* operon. Disruption of GepR was shown to cause hypersusceptibility to MtrCDE substrates and clinically relevant antibiotics. In summary, this research characterizes a novel mechanism of high-level efflux-mediated antimicrobial resistance with global physiological implications in the gonococcus, and identifies a new transcriptional regulator important for clinically significant levels of antibiotic resistance, which may offer a unique target in the development of novel drugs to combat *N. gonorrhoeae*.

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Chapter 1: Introduction

I. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative, aerobic diplococcus and the causative agent of the sexually transmitted infection, gonorrhea. *N. gonorrhoeae* is one of two important human pathogens belonging to the genus *Neisseria*, which is comprised of many species that are commensals and pathogens of humans and animals. The other human pathogen, *Neisseria meningitidis*, is a major cause of meningitis and septicemia. While the earliest references to meningococcal disease date back to the 19th century writings of Vieusseaux, gonorrhea is an ancient disease, with descriptions found as far back as the Old Testament in the Book of Leviticus (61). Gonococcal disease was termed "gonorrhea" by the second century physician Galen from the Greek words "gonos" (seed) and "rhia" (flow), in reference to the hallmark discharge observed in male infection; the causative agent, however was not identified until 1879 by the Polish physician Albert Ludwig Seigesmund Neisser (130, 180). Thus, *N. gonorrhoeae* has been evolving with mankind for thousands of years, allowing ample time for the gonococcus to develop into an efficient human pathogen.

Several factors contribute to the success of *N. gonorrhoeae* as a pathogen. The gonococcus is naturally competent for genetic transformation, allowing frequent uptake of exogenous DNA and thus a high rate of genetic variation by horizontal gene transfer (HGT). Transformation is most efficient from other *Neisseria* spp. based on the presence of a 10 base pair specific DNA uptake sequence (74). However, there are examples of HGT from other organisms, most remarkably of which is the presence of a fragment of the human nuclear element L1 gene recently identified in approximately 11% of *N.*

gonorrhoeae strains, which also serves as the first example of HGT between humans and a bacterial pathogen (5). In addition, the gonococcus possesses many phase variable genes, which in conjunction with frequent transformation, allows extensive genome plasticity, facilitating adaptation to environmental changes in the host and evasion of the host immune response. *N. gonorrhoeae* also demonstrates the ability to form biofilms, both *in vitro* and *in vivo*, which may contribute to the development of asymptomatic infection in women and provide increased resistance to antimicrobials (99, 234). Furthermore, *N. gonorrhoeae* has been shown to survive and replicate within neutrophils (262) and possesses a number of mechanisms to resist oxidative stress, antimicrobial agents, and the development of lasting immunity in the host. Together, these traits make *N. gonorrhoeae* a highly effective human pathogen.

II. Gonococcal Epidemiology, Disease and Treatment

A. Epidemiology

Gonorrhea is the second most prevalent bacterial sexually transmitted infection, with an estimated 106 million cases occurring each year worldwide, and 600,000 cases in the United States alone (238, 267). In the U.S., rates of gonococcal infection are highest among 15 - 24 year old persons, individuals living in the southern and midwest portions of the United States, and the African American population (238). In addition, the incidence of gonorrhea is higher in women than in men, and this discrepancy may be larger than is evident due to the higher occurrence of asymptomatic infection in women (59, 238). Worldwide, the rate of gonococcal infection is highest in Sub-Saharan Africa, southeast Asia, Latin America, and the Caribbean (267). Developing countries struggle

with a high rate of complicated gonococcal infection due to inaccessibility of adequate healthcare and effective antibiotic treatments.

B. Disease

Gonorrhea is primarily an uncomplicated infection of the lower urogenital tract. In males, urethral infection causes painful or difficult urination, known as dysuria, and purulent discharge, a hallmark of male gonococcal infection, as well as inflammation of the prostate or testicles in some cases (112). In women, infection primarily occurs in the endocervix and results in dysuria, vaginal discharge, and intermenstrual bleeding (112). If left untreated, serious complications can arise in both genders. In men, epididymitis, a swelling of the duct that connects the testicles with the vas deferens, can result in stricture of the duct and lead to sterility (112). Women infected with *N. gonorrhoeae* are often asymptomatic, putting them at increased risk for complications (112). Untreated infection in females can progress to pelvic inflammatory disease (PID) and scarring of the fallopian tubes, resulting in infertility or ectopic pregnancies (112). Prolonged infection also increases the likelihood of disseminated gonococcal infection (DGI), which occurs when the gonococcus crosses the epithelium and enters the blood stream (131). Although rare, symptoms of DGI can be quite severe and include fever, joint pain, dermatitis, and endocarditis (131). Additionally, gonococcal infection increases the risk for HIV acquisition and transmission, likely due to induction of inflammation and recruitment of immune cells stimulated by gonococcal infection (82).

N. gonorrhoeae may also infect other sites, including the rectum, pharynx, and conjuctiva (112). Rectal infections are common in men who have sex with men and women with cervical infections, and are characterized by rectal bleeding and purulent

discharge (112). Infection of the pharynx occurs upon orogenital contact with an infected person and is often asymptomatic (112). Conjunctival infection may cause symptoms including eye pain, redness, and purulent discharge (112). In adults, ocular infection is often due to autoinoculation from the primary site of infection. Neonatal ocular infection may occur in infants born to mothers with gonorrhea, and requires prompt treatment to prevent permanent damage to the eyes (270).

C. Treatment

The gonococcus exhibits high rates of phase and antigenic variation, resulting in failure to induce a protective immune response in the host, making reinfection quite common. These same traits have complicated the development of a vaccine against *N. gonorrhoeae*. Thus, antibiotic therapy is the primary means of treatment and control of infection within the community. Unfortunately, the gonococcus has effectively and rapidly developed resistance to all antibiotics introduced during the course of modern chemotherapy.

Early treatments for gonorrhea were crude and unpleasant for the patient, including urethral irrigation with silver nitrate or potassium permanganate preparations (186). The discovery of sulphonamide in 1935 offered a far more pleasant option for treatment; however, by the 1940s, sulphonamide resistant strains of *N. gonorrhoeae* were widespread (62, 70). Penicillin, introduced in 1944, allowed efficient treatment of sulphonamide-resistant strains and quickly became the standard course of treatment (258). Penicillin resistance first emerged in the late 1950s, and by 1987, penicillin was no longer recommended for use against gonococcal infection (44, 45). Numerous other classes of antibiotics have been used to treat gonococcal infection, including

tetracyclines, aminoglycosides, macrolides and fluoroquinolones, but *N. gonorrhoeae* has developed mechanisms of resistance against all. In 2007, *N. gonorrhoeae* was classified as a "superbug" by the CDC due to the widespread prevalence of multidrug resistant strains (47). The last remaining class of antibiotics available for single-dose therapy is the third-generation cephalosporins. However, in 2011 a strain resistant to third-generation cephalosporins was isolated from a female sex worker in Japan who failed cefixime therapy (183). The subsequent emergence of similar strains in multiple countries prompted a change in the CDC-recommended treatment for gonococcal infection to combination therapy involving a single intramuscular dose of ceftriaxone and a single oral dose of azithromycin or doxycycline twice daily for 7 days (48).

D. Models of gonococcal infection

With no new antibiotics currently in development, understanding mechanisms of virulence and antibiotic resistance utilized by the gonococcus is critical to facilitate the discovery of new therapies and ways to overcome resistance. Because *N. gonorrhoeae* is a human-specific pathogen, however, in-depth study of pathogenesis *in vivo* can be difficult. A human male volunteer urethral infection model has been used extensively in research and provided insight into multiple characteristics of gonococci during infection, but due to ethical implications and other considerations, types of studies using this model are limited (51). Chimpanzees, our closest primate relatives, may be experimentally infected with *N. gonorrhoeae,* but more distantly related primates are not readily colonized, and the expense of primate models, along with other considerations, limit use of this model (11). Recently, a female BALB/c mouse model of lower urogenital tract infection has been developed, and has been an excellent tool in better understanding

gonococcal virulence and antimicrobial resistance *in vivo* (121, 122). In addition, several *ex vivo* cell and organ culture systems have been developed and can lend further insight into gonococcal pathogenesis (72). Information gained from research with these models in combination with *in vitro* laboratory studies has offered insight into virulence factors important in gonococcal pathogenesis (discussed in Section III) and mechanisms by which gonococci develop resistance to antimicrobials (discussed in Section IV).

III. Gonococcal Virulence Factors

A. Pili

Gonococci express type IV pili, surface fibrils composed of multiple repeating subunits of an approximately 19kDa pilin protein. Expression of pilin and the type IV pili biogenesis machinery is required for the natural competence exhibited by *N. gonorrhoeae*; loss-of-function mutations in any of several pili-related genes disrupt the characteristic efficient, sequence-specific transformation of the gonococcus (1). Additionally, type IV pili serve as an important virulence factor, as pili have been shown to play a critical role in the initial attachment of gonococci to host epithelial cells and are essential for establishing infection *in vivo* (110, 241). Gonococcal pili are subject to both phase and antigenic variation, which contributes to immune evasion and facilitates gonococcal adaptation to different environments within the human host.

The structural pilin gene, *pilE*, is comprised of three distinct regions: a 5' conserved region encoding the first 45 – 50 amino acids which is invariant among all pilin genes analyzed to date and is thus likely crucial for assembly or function of pili; a middle semivariable region that contains single amino acid substitutions among pilin

variants; and a 3’ hypervariable region that carries insertions and deletions as well as single amino acid changes and constitutes the immunodominant portion of intact pili (223, 232). The semivariable and hypervariable regions are interspersed with short conserved segments, which may function in maintenance of the structural and functional integrity of assembled pili (101, 232). Expression of *pilE* results in production of the pilin precursor, prepilin, which carries a 7 amino acid N-terminal signal sequence that is posttranslationally cleaved to achieve a mature pilin monomer (168). Gonococcal strains may contain one or two *pilE* genes; in strains containing two *pilE* loci, only one is required for full pilin expression (167, 168, 221).

A number of genes are required for biogenesis and function of gonococcal pili. The prepilin peptidase, which cleaves the prepilin product of *pilE* to mature pilin, is encoded by *pilD* (71). Assembled pilin fibers emerge from the cell through an outer membrane pore formed by multimers of the PilQ protein; these PilQ pores are stabilized by the proteins PilW and PilP (40, 67). This extrusion is powered by a hexameric ATPase, PilF, with the aid of an inner membrane protein, PilG (116). Several other genes, including *pilM*, *pilN*, and *pilO*, which lie in an operon with the *pilP* and *pilQ* genes, are involved in the formation of the inner membrane subcomplex involved in pilus biogenesis (92). An additional NTPase, PilT, is involved in the disassembly and retraction of pili, and is required for both the twitching motility and natural competence demonstrated by *N. gonorrhoeae* (269).

The protein PilC, which is localized to the pilus tip and may also reside in the outer membrane, is necessary for both pilus biogenesis and natural transformation competence (199, 214, 215). Most strains of *N. gonorrhoeae* express two forms of the

110 kDa protein, designated PilC1 and PilC2 (123, 125). These proteins are structurally and functionally similar, although not identical, and expression of the *pilC* genes is subject to high frequency phase variation (123, 125). PilC serves as the major pilus adhesion molecule, and is therefore critical for attachment to host cells (213, 215). It was originally thought that pilus-mediated attachment to epithelial cells occurred through interaction of PilC with the CD46 receptor (129); newer evidence, however, suggests this interaction occurs through an alternative receptor (134).

Gonococcal pili are subjected to both antigenic and phase variation. The gonococcal genome contains multiple silent pilin gene copies, termed *pilS*, which lack a promoter, ribosomal binding site, and the 5' conserved region found in *pilE* (101, 102). Each *pilS* locus may contain one to several *pilS* copies, with the total number of loci and copies varying by strain (102, 108). High frequency antigenic variation of pilin occurs via recombination of *pilE* with one or more *pilS* copies, with approximately 4×10^{-3} recombination events occurring per cell generation (55, 101). The antigenic properties of pili may also vary due to changes in posttranslational modifications. For example, Ser^{63} of PilE may be glycosylated by an O-linked 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) with a terminal α-anomeric galactopyranose (αGal) (111, 190, 206). The gene encoding the galactosyltransferase responsible for formation of the 1,3-glycosidic linkage of the αGal residue to DATDH, *pgtA*, exists as two mutually exclusive alleles, one containing a phase-variable poly-G tract (*pgtA1*), and one that is not phase variable due to an A and a C substitution within this tract (*pgtA2*) (15). Strains carrying the *pgtA1* gene have the ability to express pilin of two antigenically different glycosylation types: when the *pgtA1* gene is in phase, the pilin protein will be glycosylated with the complete

disaccharide; however, when the *pgtA1* gene is out of phase, the αGal will be absent. Furthermore, pilin residues Ser^{68} and Ser^{156} may be modified by the addition of either phosphoethanolamine or phosphocholine, depending on the primary pilin structure, the glycosylation state of Ser^{63}, and the stoichiometric concentration of the PilV protein, although the mechanisms of these variations are unclear (2, 111).

Phase variation of gonococcal pili is evidenced by the fact that *N. gonorrhoeae* can switch between a piliated and non-piliated state at frequencies of approximately 10^{-2} per cfu per generation (138, 281). This high frequency on/off switching is mediated by a number of mechanisms. One such mechanism involves gross DNA arrangements within the pilin expression locus, often with deletion of 5'-terminal and promoter sequences, resulting in a pilus+ to pilus- phase change (243). These pilus- variants do not express pilin and do not revert to the pilus+ phenotype, except in the rare event of restoration of the 5' *pilE* sequences (243). A second mechanism involves frameshift mutations in the *pilC* gene, which contains a 5' poly-G nucleotide tract; frameshift mutations within this tract result in on/off switching of PilC expression by moving the start codon in or out of frame (123). Strains expressing PilC have a tract of 13 G nucleotides, while strains failing to express PilC were found to contain 11 or 12 (123). Cells that do not express PilC are pilus-, but still express the pilin protein and are able to revert to the pilus+ phenotype; thus, lack of PilC likely prevents assembly of pilin into functional pilus fibers, and restoration of the *pilC* reading frame results in expression of PilC, allowing pilus assembly (123).

Finally, the mechanism of *pilE/pilS* recombination leading to antigenic variation as described above can also result in phase variation if *pilE* recombines with an aberrant

or “unorthodox” *pilS* locus. For example, recombination with a *pilS* copy that contains a frameshift or point mutation creating a nonsense mutation can result in a truncated, immunologically undetectable pilin (19). Subsequent recombination with a *pilS* locus encoding a segment that corrects for the nonsense mutation results in expression of full length pilin and reversion to the pilus+ phenotype (19). Recombination of *pilE* with a *pilS* locus carrying joint *pil* gene copies can result in a *pilE* locus with a tandem arrangement of a complete pilin gene and a partial pilin gene under the control of the same *pilE* promoter; when these tandemly arranged copies are in the same translational frame, oversized pilin molecules are produced, which cannot be assembled into pili (161). Such variants are thus pilus-, but may revert by a deletion event in the *pilE* locus that removes one of the pilin gene copies (161).

Several studies provide evidence that gonococci undergo pilin antigenic variation *in vivo*. Pili of gonococci isolated from different anatomical sites have different antigenic properties (251). Gonococcal isolates from sexual partners have been found to differ in pilus type, and isolates from a male urethra after experimental challenge with gonococci of a known pilus type were found to express pili that were both antigenically and genetically distinct from that of the parent strain used for infection (245, 277). Additionally, after cloning, sequencing, and mapping the repertoire of pilin genes in *N. gonorrhoeae* strain FA1090, Hamrick *et al.* found that upon infection of 3 male volunteers with the same variant, multiple *pilS* sequences could be found in the *pilE* locus of recovered isolates, and isolates differed broadly in pilin type (108). Taken together, these studies strongly suggest a selective pressure for pilin antigenic variation in human infection.

Multiple roles for the high frequency antigenic variation of pili have been suggested. Because patients with gonorrhea have both mucosal and serum antibodies to most major gonococcal surface proteins but may be cured and reinfected multiple times without showing signs of immunity, and because it has been shown that anti-pilus antibodies are not widely cross-reactive, one possible role of pilin antigenic variation is immune evasion (33, 181, 224, 250). The maintenance of a large repertoire of possible pilin compositions and the ability to frequently alter its pilin sequence during infection would allow the gonococcus to prevent the host from mounting an efficient antibody-mediated immune response, as well as inhibit the development of a protective memory response, thereby permitting reinfection and facilitating the pathogen's spread within the population. Variation in pilin posttranslational modifications may also contribute to immune evasion. For example, in strains carrying the phase variable *pgtA1* allele, a switch to the "off" phase results in a lack of the αGal residue at Ser^{63}, which may facilitate gonococcal survival in the blood through the avoidance of recognition by anti-αGal IgG, an antibody present in high levels in human sera (93). Alternatively, pili play a critical role in initial attachment of gonococci to human epithelial cells, and even minor amino acid variations in the pilin sequence have been shown to significantly alter bacterial binding properties and tissue tropism (124). Thus, pilin antigenic variation may facilitate gonococcal spread from the initial site of infection throughout the genitourinary tract or to other sites during disseminated gonococcal infection by increasing the affinity of gonococci for specific cell types and preventing recognition by the host immune system.

Non-piliated gonococci have been isolated from patients with gonorrhea, including patients with disseminated gonococcal infection (DGI), suggesting that pilus+ to pilus- phase variation occurs *in vivo* (182, 244). Pilus- gonococci must therefore have some advantage during infection, although the specific benefit has yet to be determined. One might hypothesize that pilus phase variation further contributes to immune evasion, as switching off pilin expression would remove a surface antigen recognized by host immune factors, which in turn might facilitate colonization or persistence at certain infection sites. Alternatively, as pili are critical mediators of attachment of gonococci to host cells, dissemination of gonococci between sexual partners or to distal infection sights may require silencing of pilin expression to decrease gonococcal affinity for host cells, allowing spread from the primary infection site.

Variation of pilin glycosylation has also been implicated in facilitating dissemination of gonococci, as there is a strong correlation of the presence of the phase-variable *pgtA1* allele with more invasive gonococcal disease, including PID (93). It has been suggested that expression of *pgtA* leading to glycosylation of pilin with the complete αGal-1,3-DATDH disaccharide aides colonization of the lower urogenital tract because the presence of αGal triggers anti-αGal antibodies of the IgA and secretory IgA isotypes, the primary antibody isotypes at mucosal surfaces (107). These IgA antibodies act as "blocking antibodies" by downregulating complement-mediated killing of gonococci (107). However, in strains carrying the phase-variable *pgtA1* allele, the ability to turn off *pgtA* expression and prevent the addition of αGal to the DATDH residue at Ser^{63} may facilitate migration of gonococci from primary sites of urogenital infection through the epithelium, sub-epithelium, and endothelium to cause DGI, or movement of

gonococci up the mucosa of the female genital tract, resulting in PID, as well as protect gonococci from recognition by the more bacteriocidal anti-αGal IgG antibodies which are present at higher concentrations in distal infection sites (93).

In summary, the gonococcal pilus serves as an important virulence factor, due to both its critical role in initial attachment to host epithelial cells, and its potential roles in immune evasion and gonococcal dissemination. The high frequency antigenic variation of the pilin protein and phase variation of pilus production and posttranslational modifications provide a large repertoire of piliation types that increase gonococcal versatility and adaptability to specific environmental niches within the human host.

B. Opa

The opacity (Opa) proteins, previously known as outer membrane protein II (P.II), are a family of heat-modifiable proteins ranging in molecular weight from 24 – 30 kDa expressed on the surface of the gonococcal cell (242). Opa proteins play a role in the formation of an intimate interaction between the host cell and the gonococcus, and stimulate phagocytosis or cellular invasion of host cells by gonococci (63). Some Opa proteins mediate uptake by binding heparan sulfate proteoglycans on the surface of host cells; most Opa types, however, mediate host cell attachment via protein-protein interactions with CEACAMs, also referred to as CD66, receptors on the host cell surface (63). Opa proteins are encoded by a group of highly conserved genes, with approximately 80% identity in the DNA sequences. The 5’ end of the structural genes contains multiple but variable repeats of the nucleotide pentamer CTCTT and encodes a hydrophobic peptide signal sequence, while the 3’ end contains a Neisserial DNA uptake sequence predicted to also serve as the transcriptional terminator (20). The Opa genes differ

primarily in two short regions, designated hypervariable regions 1 (HV1) and 2 (HV2); while variable in specific sequence, these domains preferentially encode hydrophilic amino acids, suggesting that the HV regions comprise the surface-exposed portion of the Opa protein (235). Each strain of *N. gonorrhoeae* may have up to 12 *opa* gene copies, and variants may express from none to three Opa protein types on the cell surface (94, 242). In general, Opa expression influences the surface appearance, size, interaction, and organization of gonococci within colonies *in vitro*, and multiple studies suggest that variations in Opa protein expression also confer different antigenic and pathogenic phenotypes on the gonococcus *in vivo* (64).

Opa proteins are subject to both phase and antigenic variation. Each *opa* gene copy has its own promoter, and all are constitutively transcribed (235). Phase variation of expression of each Opa protein occurs independently at the translational level due to changes in the number of CTCTT pentamers in the coding repeat found at the 5' end of the *opa* gene, which encodes the hydrophobic core of the Opa leader peptide (175, 235). The coding repeat may contain from 7 to 57 pentamer units; the number of units present determines whether the start codon will be in phase to allow production of a full-length, functional Opa protein, or out of phase, resulting in a severely truncated peptide (20, 235). Antigenic variation in Opa proteins is due to the presence of multiple distinct gene copies with variable antigenic sequences dispersed through the genome that are expressed independently (235). The hypervariable regions exist as discrete sequences that may be present within a gene in multiple combinations. The existence of multiple unique HV sequences and differential combination of HV1 and HV2 segments, along with the presence of multiple *opa* gene copies in the chromosome, allows the gonococcus to

maintain a repertoire of antigenically distinct Opa proteins and the potential for greater cell surface variation.

The importance of Opa expression *in vivo* is demonstrated by the recovery of primarily Opa+ variants from cervical, urethral and rectal infections (246). Strong evidence for Opa phase variation *in vivo* also exists in that predominantly Opa+ gonococci are recovered from the urethras of male volunteers experimentally inoculated with Opa- organisms (120). Antigenic variation occurs during infection as well, as demonstrated by the recovery of variants expressing multiple different Opa+ phenotypes from individual patients, and the finding that the specific sets of anti-Opa antibodies in the sera of sexual partners infected with the same strain differs (246, 277). It is important to note, however, that Opa- gonococci are preferentially isolated from the fallopian tubes and from infected females during menses, as well as during DGI, indicating that that the Opa- phenotype also has a significant role in gonococcal pathogenesis *in vivo* (68, 117, 182).

As previously mentioned, Opa proteins interact with CD66 receptors on the surface of host cells. Individual CD66 receptors are differentially expressed by epithelial and endothelial cells of different tissue types, and variant Opa proteins preferentially bind only specific CD66 receptors (97, 198, 248). Thus, by varying the Opa proteins expressed, the gonococcus can alter its tissue tropism and adapt to the environment at a specific infection site. Multiple studies support this conclusion by demonstrating that only certain Opa variants can invade a specific cell type (80, 139, 156). The importance of Opa expression in host cell attachment may also explain why Opa- variants are preferentially isolated from DGI, as a lack of Opa proteins on the gonococcal surface

would decrease bacterial affinity for host cells, thereby facilitating spread of gonococci by preventing attachment to host cells. Similarly, such decreased attachment could aid in transmission to a new host. Hence, both phase and antigenic variation of Opa proteins appear to contribute to the pathogenicity of *N. gonorrhoeae* by mediating interactions between gonococci and human cells.

Controversial evidence exists regarding the role of Opa variation in gonococcal interaction with neutrophils. It was previously determined that Opa binding to CD66a on host polymorphonuclear leukocytes (PMNs) triggers bacterial uptake and stimulates an enhanced respiratory burst (97, 136). Newer evidence, however, suggests that Opa- gonococci can also be taken up efficiently by PMNs and can actually inhibit the oxidative burst; the same study also found that stimulation of the oxidative response by Opa+ organisms was minimal (56). Regardless of uptake mechanism, it has been shown that gonococci can survive inside PMNs and that intracellular killing of gonococci by phagocytes involves primarily non-oxidative means (57, 202). Therefore, enhanced uptake by PMNs, whether through expression of Opa proteins that bind CD66a or by phase variation to an Opa- state, may provide an advantage in cases where survival within PMNs is favored, such as evasion of host immune factors, dissemination to distal infection sites, or transmission to a new host in the PMN-rich exudate of infected individuals.

In addition to determining tissue tropism, Opa variation may aid in immune evasion. Antibodies to Opa proteins isolated from sera of infected patients show little to no cross-reactivity, reacting to only a single Opa type (277). Thus, by changing the Opa proteins expressed, gonococci might be better able to avoid the host humoral immune

response. Phase variation to an Opa- phenotype may also contribute to evasion of an antibody-mediated response, as removal of the Opa antigen altogether from the gonococcal cell surface would prevent recognition by any anti-Opa antibodies developed in an infected individual; the isolation of primarily Opa- variants from DGI supports such a hypothesis, as bacteria would encounter high levels of antibodies in the serum during transport in the blood (182). Additionally, Opa+ gonococci may be more sensitive to serine proteases than Opa- gonococci, which would confer an advantage on Opa- gonococci when serine protease levels are high (25, 117). As the levels of several proteases fluctuate during the mammalian reproductive cycle, this difference in sensitivity might contribute to the selection for Opa- variants observed during menses, while lower levels of certain proteases and a corresponding fluctuation of protease inhibitors may protect Opa+ gonococci during other stages of the reproductive cycle, resulting in the cyclic recovery of Opa+ versus Opa- variants recently observed by Cole *et al.* in the mouse model of infection (52, 133).

In summary, the ability to vary the number and antigenic type of Opa proteins expressed on the bacterial cell surface is an important factor in gonococcal pathogenesis, as variation of Opa proteins appears to mediate tissue tropism, dissemination, and transmission of *N. gonorrhoeae*, as well as contribute to efficient immune evasion.

C. Lipooligosaccharide

LOS mediates several aspects of gonococcal disease. It stimulates bactericidal antibodies found in normal human serum, and contributes to gonococcal invasiveness and immune evasion (8, 256). Additionally, LOS affects attachment to host cells, in concert with pili and Opa proteins, and is responsible for much of the tissue damage that occurs

during gonococcal infection (98). The LOS of *N. gonorrhoeae* is composed of a branched oligosaccharide structure anchored to the bacterial cell membrane via lipid A (35). Unlike the lipopolysaccharide of other Gram-negative bacteria, gonococcal LOS lacks the long repeating O antigen; rather, the O side chain consists of three short chains, referred to as α, β, and γ (76). The α chain is highly variable, and variants differ in both length and carbohydrate composition of this chain (96, 274). Gonococci may express more than one antigenically distinct LOS on the cell surface at one time (9). At least six different LOS serotypes have been identified, and LOS antigenic variation is predicted to occur at a frequency of approximately $10^{-2} – 10^{-3}$ (7, 9, 218).

Antigenic variation of LOS is intimately linked to phase variation, as changes in the expression of the glycosyltransferases involved in LOS biosynthesis determine which carbohydrate moieties will be added to the O side chain of the LOS molecule. In the gonococcus, 7 glycosyltransferase genes are encoded by three chromosomal loci; the genetic structure of these loci, and the genes they encode, appear to be highly conserved in most strains of *N. gonorrhoeae* (284). The *lgt-1* locus comprises 5 genes, *lgtA – E*, involved in synthesis of the α chain of LOS (284). The *lgt-2* locus encodes *lgtF*, also involved in α chain synthesis, and *rfaK*, necessary for synthesis of the γ chain (284). The *lgt-3* locus contains only *lgtG*, which encodes a glucosyltransferase needed for β chain synthesis; *ltgE* is also required for β chain completion (76, 284). The proteins encoded by the *lgt* genes are highly conserved in sequence and function; however, four of these genes, *lgtA*, *lgtC*, *lgtD*, and *lgtG* have homopolymeric G tracts, which result in phase variation of their expression by slipped-strand mispairing (76, 274, 284). The repertoire of phase-on genes therefore determines which carbohydrate modifications will be made

to LOS. However, because LOS synthesis occurs in a stepwise fashion, the addition of variable carbohydrate moieties is also influenced by the structure of the growing LOS molecule. For example, when both *lgtA* and *lgtC* are phase-on, LgtC cannot transfer galactose to the primary glucose of the oligosaccharide chain, because LgtC competes poorly with LgtA, and addition of N-acetylglucosamine by LgtA blocks the substrate recognized by LgtC (96). When *lgtA* is phase-off, however, LgtC is able to add galactose to the primary glucose, resulting in variation of the second residue of the α chain (96). Thus, phase variation of the glycosyltransferases involved in LOS synthesis, in conjunction with the structure of the LOS molecule itself, results in the expression of antigenically distinct molecules on the gonococcal cell surface.

Gonococcal LOS is also subject to varying degrees of sialylation by the outer membrane α-2,3-sialyltransferase, encoded by the *lst* gene (159). Sialylation involves the transfer of N-acetylneuraminic acid moiety from cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA) to the terminal sugar residues of the LOS O side chains (256). The *lst* gene is constitutively transcribed, is not subject to antigenic or phase variation, and is transcribed at relatively constant levels between strains (188). Therefore, other, currently undetermined factors must regulate the variability of LOS sialylation. Phase variation of the *lgt* genes, for example, may result in the absence or presence of suitable sialylation sites on the LOS α chain (256). Additionally, point mutations in the *lst* gene that do not affect overall enzymatic function may lead to differences in the intrinsic specific activity of the Lst enzymes among different gonococcal populations; mutations with this potential have been previously identified (188). Finally, as yet

unknown Neisserial factors may regulate Lst activity, perhaps through binding to the Lst enzyme or to sites on the LOS molecule (188).

As mentioned previously, gonococci can express multiple LOS types at once (9). The relatively independent events of *lgt* phase variation of LOS sialylation provide an expanded repertoire of LOS types, as sialyl groups and specific sugar residues can be grouped in multiple combinations. Competition between Lgt enzymes, such as that seen for LgtA and LgtC, could result in two different LOS types if both are expressed, with that created by the poorer competitor in lower concentration on the cell surface. Additionally, some LOS molecules may be transported to the cell surface before all modifications are made, resulting in the expression of both a fully modified and a partially modified LOS variant (9, 76). While the total number of different LOS types that may be produced by *N. gonorrhoeae* is extensive, the repertoire of any one strain is limited and is a stable attribute of that specific strain; in other words, there is little variation in the number and quantity of each LOS type produced by different populations of a single strain (9, 157, 217).

Evidence exists that LOS phase and antigenic variation, as well as sialylation, occur both in tissue culture and *in vivo*, and may be necessary for the establishment of gonococcal infection and disease progression (10, 219, 256). For example, experimental infection of male volunteers with a low molecular weight, nonsialylated LOS variant results in recovery of large numbers of this variant early in infection; however, a subsequent shift to recovery of variants with multiple higher molecular weight, sialylated LOS types is seen as disease progresses to inflammation and dysuria, and eventually urethritis and discharge (219). Together with the fact that variants with sialylated LOS

have been shown to be more invasive than those whose LOS is not sialylated, these findings suggest that nonsialylated variants establish infection, while sialylated variants are responsible for disease symptoms (256). Thus, variation of LOS plays an important role in the establishment and progression of *N. gonorrhoeae* infection *in vivo*.

Both structural LOS variation and sialylation also contribute to immune evasion. Frequent alteration of the carbohydrate composition of the O side chain prevents the development of an effective antibody response by differing the surface-exposed antigens available for antibody binding (256). Certain LOS types may even be unrecognizable to the host adaptive immune response, as antibodies to these LOS epitopes are not found in the sera of infected patients (216). In contrast, sialylation does not appear to affect antibody binding, but does cause a nonfunctional deposition of complement on the gonococcus, thereby inhibiting complement-mediated killing (256). Sialylation also prevents adherence to, and therefore killing by, PMNs (132). Thus, structural LOS phase variation and sialylation work cooperatively to evade multiple aspects of the host immune response, contributing to persistence and pathogenicity of *N. gonorrhoeae* during infection.

N. gonorrhoeae also uses LOS to exploit host factors for immune evasion. CMP-NANA, which provides the substrate for sialylation of LOS, is a compound readily available in concentrations sufficient for sialylation in human serum, professional phagocytes, and genital secretions (256). Use of CMP-NANA is therefore an efficient mechanism of protection from immune responses, as the gonococcus is not required to synthesize its own substrate for sialylation, and the host is unlikely to mount an antibody response to a compound found so abundantly in his or her own tissues. Additionally,

some LOS epitopes closely resemble epitopes of human blood group antigens, as shown by the cross-reactivity of monoclonal antibodies to human blood group antigens with certain LOS variants (158). This molecular mimicry further allows evasion of the adaptive response, as the host will not produce antibodies to his or her blood group antigens, and so will not produce antibodies that recognize such LOS types. These mechanisms highlight the close evolutionary relationship between the gonococcus and its human host, as well as the successful adaptations made by the gonococcus in order to evade the immune response and persist within the host.

Interaction of host dendritic cells (DCs) with invading microorganisms is an important step in mounting an effective immune response, as activation of DC receptors can induce phagocytosis and guide cytokine production, thereby directing the immune response. While the lipid A portion of gonococcal LOS is recognized by the receptor TLR4, the carbohydrate moieties serve as ligands for various sugar-dependent receptors, the specificity of which is determined by the carbohydrate variant present (259). Interaction with the C-type lectin receptor DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) promotes internalization of the microbe; however, only specific LOS variations have been found to interact with DC-SIGN, and LOS variation might thus serve as a means of avoiding phagocytosis and the subsequent DC-mediated response (280). Alternatively, interaction of gonococci with DC-SIGN results in upregulation of interleukin-10 (IL-10) production, an anti-inflammatory cytokine that downregulates the production of cytokines that promote the Th1 response, which is the response most protective against *N. gonorrhoeae* (259). Several pathogens known to establish chronic infections, including *Mycobacterium tuberculosis* and *Helicobacter*

pylori, have been shown to interact with DC-SIGN to stimulate IL-10 production (18, 91). Together, this evidence suggests that variation to an LOS type than can interact with DC-SIGN can inhibit an effective immune response against *N. gonorrhoeae* and may aid the gonococcus in establishing a latent or asymptomatic infection. Additionally, certain LOS variants have been found to interact with the C-type lectin DC receptor macrophage galactose-type lectin (MGL); activation of MGL shifts the immune response to Th2, which is less effective in the elimination of gonococci (259). It therefore appears that *N. gonorrhoeae* can selectively alter DC activation, cytokine production, and the polarization of the immune response by varying the LOS carbohydrate moieties to interact with specific DC cell receptors.

D. Porin

Neisserial porins are the most abundant gonococcal outer membrane proteins (126). These trimeric β-barrel channels are essential for gonococcal survival, as they are involved in ion and nutrient exchange with the environment (126). Gonococci possess two porin genes, *porA* and *porB*; only *porB* encodes a functional porin protein, however, as *porA* is a pseudogene. *porB* exists as two alleles, of which a gonococcal strain can contain only one: *porB1a*, which encodes PIA, and *porB1b*, encoding PIB (87). These variants differ in size and orientation in the outer membrane (126). PorB demonstrates some antigenic variability in the surface-exposed loops, but is more antigenically stable than other gonococcal cell surface components, making PorB a useful tool in gonococcal serotyping (87).

Porins facilitate gonococcal interaction with host cells by physically interacting with and inserting into host epithelial cell membranes (164, 166). In addition, porins have

a number of effects on host immune cells, including activation of B cells and the inhibition of neutrophil actin polymerization, degranulation, expression of opsonin receptors, phagocytosis, and host cell apoptosis (22, 266). Gonococci expressing PIA are largely responsible for disseminated infections, likely due to the enhanced invasiveness and increased serum resistance conferred by this allele due to its ability to effectively bind C4bp and Factor H, which inhibits the classical and alternative complement pathways, respectively (38, 200, 257). PIB may also bind C4bp, thereby conferring serum resistance on some gonococcal strains expressing this allele (200). PIB expression, however, is generally associated with localized infections, and this variant confers elevated levels of resistance to antibiotics such as rifampicin and tetracycline in comparison to PIA, possibly due to a difference in charge imparted on the cell membrane by these two variants (34, 37, 39, 247).

E. Resistance to Oxidative Stress

Within the human host, *N. gonorrhoeae* encounters multiple sources of oxidative stress, including products of its own respiration, oxidizing agents produced by commensal microorganisms, and interactions with host innate immune cells, particularly PMNs, which are recruited in large numbers to sites of gonococcal infection (222). Oxidizing agents frequently encountered by the gonococcus include reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (HO·), superoxide anions (O_2·), and reactive nitrogen species such as peroxynitrite (ONOO-) and nitric oxide (NO·) (254). Exposure to oxidizing agents can lead to damage of DNA and proteins and ultimately lead to cell death. Thus, the gonococcus has developed a number of mechanisms to resist the action of oxidizing agents.

Exposure to H_2O_2 results in a number of transcriptional changes in the gonococcus, many of which are regulated by the peroxide-sensitive transcriptional regulators OxyR and PerR (236). OxyR-regulated genes include *katA*, which encodes catalase, *gor*, which encodes a glutathione oxidoreductase, and *prx*, which encodes peroxiredoxin (222). The catalase enzyme converts H_2O_2 to water and contributes significantly to the ability of gonococci to survive in the presence of H_2O_2 and PMNs. Gor maintains a pool of reduced glutathione, which acts as a hydrogen donor to restore oxidized molecules and is considered the first line of defense against oxidative stress. The Prx enzyme catalyzes reduction of alkyl hydroperoxide. The Fur family manganese-dependent regulator PerR regulates expression of the MntABC transport system, which transports manganese into the cell as an ROS quenching agent. Mutants in MntABC display slower growth rates and increased sensitivity to $O_2\cdot$ and H_2O_2. PerR also regulates expression of *ccpR*, which encodes a periplasmic cytochrome c peroxidase that neutralizes H_2O_2 to water. CcpR and KatA are functionally redundant, as mutation in either gene alone results in only a modest increase in H_2O_2 sensitivity, but a double mutant is significantly more sensitive compared to wild type and single mutant strains (254). Other enzymes involved in resistance to oxygen radicals possessed by *N. gonorrhoeae* include a superoxide dismutase (SodB), thioredoxin, glutaredoxin, and a fused methionine sulfoxide reductase (MsrA/B) (222).

Defense against RNS is of particular concern during anaerobic growth. Gonococcal defenses against RNS include AniA, a nitrite reductase, and NorB, a nitric oxide reductase (222). These enzymes neutralize RNS to nitrous oxide and are necessary for anaerobic growth. Transcription of *aniA* is regulated by the fumarate and nitrate

reductase regulator (FNR), which is also a regulator of *ccpR* and an important regulator of nitrate- and nitrite-induced genes in other bacteria (149). In addition, cytochrome c ≠, CycP, acts as a buffer against NO· by binding this radical, preventing the formation of peroxynitrite from reaction of NO· with O_2· until NorB has accumulated to sufficient levels to neutralize NO· (255). More recently, a MerR-family transcriptional regulator, termed NmlR, was identified that regulates a zinc-dependent alcohol dehydrogenase, AdhC, and other genes potentially involved in resistance to RNS (222).

F. Iron acquisition

Iron is an essential nutrient for both bacterial and eukaryotic cells, involved in oxygen transport, electron transport, and other important cellular activities (150). In a mammalian host, over-abundance of free iron can be deleterious, leading to an increase in free radical formation via the Fenton reaction, which can result in damage to DNA and other cellular components. Thus, the concentration of free iron is controlled by complexing iron with ferritin and hemoproteins within mammalian cells and transferrin and lactoferrin extracellularly (150). As such, the human host is an iron-limited environment for bacterial pathogens such as the gonococcus.

Many bacteria secrete low molecular weight, high-affinity iron chelating proteins known as siderophores that compete with transferrin and lactoferrin for iron and are transported into the bacterial cell (13, 58). The gonococcus, however, instead uses several TonB-dependent receptors and the TonB-ExbB-ExbD energy transduction complex to bind and remove iron directly from host proteins (21, 237). The two-component TonB-dependent receptor TbpAB removes and internalizes iron from transferrin, which is the major source of iron in the bloodstream encountered by gonococci during DGI (193).

Additionally, TbpAB was shown to be required for successful colonization in the male urethral infection model (54). LbpAB, a second two-component TonB-dependent receptor, is used to acquire iron from lactoferrin, which serves as the primary iron source on mucosal epithelial surfaces (169). LbpAB is not required for colonization of the urogentical mucosa, but expression of LbpAB in a strain with a nonfunctional TbpAB receptor allows colonization and symptomatic infection, and strains expressing both receptors demonstrate a competitive advantage over strains expressing only one during co-infection in male volunteers (4).

The HpuAB two component receptor is required for acquisition of iron from hemoglobin and hemoglobin complexed with haptoglobin (146). The *hpuAB* operon is phase variable due to slipped-strand mispairing in a poly(G) repeat (147). The role of HpuAB in infection is still unknown. Another iron-regulated and phase variable TonB-dependent receptor is the single component receptor FetA, which acquires iron from siderophores produced by other bacteria (41, 42). Interestingly, antibodies to FetA can be isolated from patients with gonococcal infection, making FetA a potential vaccine candidate (23). *N. gonorrhoeae* possesses a number of other TonB-dependent and independent receptors, endowing this organism with a large repertoire of methods to scavenge iron from the human host and other bacteria (207).

Expression of genes involved in iron acquisition is modulated by the global transcriptional regulator ferric uptake regulator (Fur) (77). Genes regulated by Fur, including the TonB-dependent receptors, contain a 19bp DNA consensus sequence termed the “Fur box,” which is recognized and bound by Fur complexed with Fe(II) (77, 115). While classically a repressor, Fur has been shown to activate a number of genes and

is considered a global regulator due to its ability to regulate genes involved in functions other than iron acquisition, including catabolism and oxidative stress resistance (115, 220).

IV. Mechanisms of Antimicrobial Resistance in the Gonococcus

Within the human host, pathogens such as *N. gonorrhoeae* are constantly subjected to toxic compounds, including antimicrobial agents produced by commensal flora, host immune compounds, and antibiotics during treatment. Thus, the gonococcus has developed a number of ways to protect itself from these antimicrobial compounds. In general, *N. gonorrhoeae* utilizes four methods of neutralizing antimicrobials: modification or destruction, exclusion from the cell interior, modification of the target, and active efflux from the cell.

A. Drug modification or destruction

Bacteria may produce a number of enzymes to modify or destroy antimicrobial agents that successfully enter the cell. A classic, well-characterized example is the nearly ubiquitous β-lactamase enzymes, which cleave the characteristic ring of β-lactam antibiotics such as penicillin (3). Penicillin disrupts cell wall synthesis by molecular mimicry of the D-Ala-D-Ala motif in penicillin binding proteins (PBPs) that form the peptidoglycan layer (265). The lactam ring binds the active site of PBPs and prevents cross-linking of *N*-acetylglucosamine and *N*-acetylmuramic acid, thereby preventing formation of the peptidoglycan layer (265). β-lactamases recognize and hydrolyze the lactam ring, rendering the antibiotic inactive (60).

Strains of *N. gonorrhoeae* producing β-lactamase were first identified in the 1970s (12, 192). This TEM-1-type β-lactamase is carried on a 5.1 kb or 7.1 kb R plasmid containing a Tn2-transposable element that harbors the *bla* gene, encoding the β-lactamase (75, 203, 230). It is thought that this plasmid was obtained from a horizontal gene transfer event from *Haemophilus parainfluenzae* (75, 203). While not all strains of *N. gonorrhoeae* produce β-lactamase, 50 - 80% of isolates in certain geographical areas such as Latin America and the Western Pacific may carry an R plasmid producing β-lactamase. Widespread β-lactamase producing strains render treatment with penicillin unreliable to ineffective.

B. Entry Exclusion

The cell membrane of Gram-negative bacteria provides a barrier to entry of unwanted compounds into the cell. However, exchange of ions, nutrients and metabolic waste products requires access between the cell interior and the external environment, and thus the cell envelope contains a number of pores, channels, and transporters to allow entry of compounds necessary to the cell's survival. Certain antimicrobial agents may take advantage of these entry points to gain access to targets within the cell interior. Mutations in the proteins that make up these channels, porins, and transporters may block entry of antimicrobials; this process is known as entry exclusion.

Penicillin and tetracycline diffuse into the periplasmic space through porins. As previously mentioned, gonococci may express one of two porin alleles, PorB1A (PIA) or PorB1B (PIB) (87). Strains that express PorB1B are less sensitive to penicillin and tetracycline than those expressing PorB1A (95, 184, 231). Additionally, amino acid substitutions in loop 3 of PorB1B may increase resistance to penicillin and tetracycline

(95, 184). The best studied of these mutations, collectively termed *penB*, are a glycine to lysine change at position 120, or glycine to aspartic acid and alanine to aspartic acid substitutions at positions 120 and 121, respectively (184). Interestingly, the effects of the *penB* mutation are only observable in strains bearing mutations in *mtrR* that over-express the MtrCDE multidrug efflux pump (discussed in Section V. D.), and mutations in *penB* cannot be selected without the presence of mutations in *mtrR* (231).

Similarly, mutations may occur in PilQ, a doughnut-shaped structure in the outer membrane composed of 12 subunits used as a channel through which the pilus is secreted (66, 67). PilQ also appears to allow entry of antimicrobial agents, as null or missense mutations in PilQ have been shown to increase resistance of gonococci to a number of structurally diverse antimicrobials (49, 282). A glutamic acid to lysine change at position 1996, termed *penC*, prevents mutlimerization of PilQ, which has been shown to confer increased resistance to penicillin (282). Like *penB* mutations, however, the *penC* mutation is not sufficient to confer significant levels of penicillin resistance on its own; rather, it lends a further increase in resistance in strains carrying both the *mtrR* and *penB* mutations (282).

C. Target Modification

In general, antibiotics have specific targets to which they bind to inhibit cellular processes essential for bacterial growth or survival. Mutants in target proteins that modify the antibiotic binding site or sterically hinder antibiotic binding can confer resistance to these toxic compounds. Importantly, these mutations must disrupt the antibiotic binding site without drastically affecting function of the target protein, or growth or fitness of the bacterium may suffer. Mutations that effectively confer resistance without a significant

growth or fitness cost may persist in a bacterial population in the absence of antibiotic pressure. Resistance mutations that significantly impact the activity of the target protein and slow growth or diminish general fitness may be lost from the population in the absence of antibiotic selection, or may lead to the development of compensatory mutations that restore growth rate and fitness (discussed in Chapter 2).

Mutations in the PBPs of *N. gonorrhoeae* have been shown to contribute to penicillin resistance. The *penA* gene, which encodes PBP2, may contain an addition of an aspartic acid residue at position 345, which is just downstream of the penicillin acylation site, and 4 to 8 substitutions in the carboxyl terminal region of the protein, resulting in a slower acylation rate by penicillin (30). This *penA* variant arose from the acquisition of segments of *penA* from commensal *Neisseria* species with PBP2s demonstrating decreased acylation rates by penicillin (29, 233). The mosaic *penA* allele decreases susceptibility of gonococci harboring this mutation (231). Additionally, the *ponA* gene, which encodes PBP1, may contain a T to C transition mutation that results in the substitution of proline for leucine at position 421, lowering the affinity for penicillin and decreasing the acylation rate (208). Neither the *penA* nor *ponA* mutations are sufficient to confer clinically significant levels of penicillin resistance alone, and require the presence of *penB* and *mtrR* mutations to impart an altered resistance phenotype on gonococcal cells (78, 208).

Macrolide antibiotics, such as erythromycin, inhibit protein synthesis by binding to the 23S rRNA of the 50S subunit of the ribosome causing the release of incomplete peptides. Bacteria can resist macrolides by methylation of an adenosine in the peptidyl transferase domain of the 23S rRNA, which interferes with macrolide binding to the

target site. Methylation is carried out by enzymes known as methyltransferases, encoded by a set of genes termed *erm* genes (162). Some clinical isolates of *N. gonorrhoeae* demonstrating decreased susceptibility to erythromycin or azithromycin have been shown to contain *ermB*, *ermC*, or *ermF* genes (205). These methyltransferases are important in achieving clinically significant levels of resistance to macrolide antibiotics (205). Interestingly, *ermF* is part of a conjugal element and may be transferred from gonococci to other gonococci, meningococci, and *Enterococcus faecalis* (205).

Two mechanisms mediate tetracycline resistance in gonococci. First, presence of a conjugal plasmid bearing the *tetM* gene results in high-level tetracycline resistance (204). TetM mimics protein elongation factors and binds to ribosomes, resulting in release of bound antibiotic (36). Alternatively, chromosomally-mediated high-level tetracycline resistance is due to mutations in *rpsJ,* which results in amino acid substitutions at position 57 in ribosomal protein S10 (113). Position 57 lies near the tetracycline binding site, and substitution of the valine at this position for a large or uncharged amino acid such as leucine, glutamine or methionine alters the rRNA structure, thereby reducing affinity for tetracycline (113). Effectivity of the *rpsJ* mutation requires the presence of *mtrR* and *penB* mutations (113).

Quinolone antibiotics target bacterial topoisomerases, which are critical in DNA replication and bacterial chromosome segregation. Mutations in topoisomerases can prevent recognition of quinolone antibiotics and confer resistance. The gonococcal *gyrA* gene encodes the A subunit of DNA gyrase, and mutations generating amino acid substitutions at positions 91 and 95 (commonly Ser91Phe and Asp95Asn) confer resistance to ciprofloxacin (16, 69). Mutations in the *parC* gene, which encodes

topoisomerase IV, also confer resistance to ciprofloxacin, commonly due to an Asp86Asn mutation in ParC (16, 69). A number of *N. gonorrhoeae* isolates resistant to ciprofloxacin have been shown to contain both the *gyrA* and *parC* mutations, signifying these mutations are important in clinically significant quinolone resistance (252).

Rifampin, or rifampicin, is a semisynthetic derivative of the rifamycin class of antibiotics, which exert bactericidal activity by inhibition of RNA polymerase (86). Rifampin binds to the β subunit of RNA polymerase and sterically hinders the initial chain formation of RNA synthesis (173). In the United States, the only uses of rifampin approved by the Food and Drug administration are the treatment of *Mycobacterium tuberculosis* and prophylactic therapy for individuals exposed to patients with *Neisseria meningitidis*, such as household members and medical caretakers (46, 86). However, due to the rapid spread of resistance to antibiotics commonly used to treat bacterial infections, rifampin has been examined for its efficacy against treatment of resistant nonmycobacterial pathogens. Several studies from the 1980s demonstrated successful treatment of gonococcal infection in both men and women using rifampin or rifampin-erythromycin dual therapy (17, 26, 187). Unfortunately, treatment with rifampicin often results in the rapid emergence of resistance (86). Indeed, although a highly effective prophylactic against *N. meningitidis*, rifampin-resistant strains have emerged that have caused systemic infection, failure of prophylaxis, and localized outbreaks (46, 273). This resistance is due to a variety of mutations in *rpoB*, which encodes the RNA polymerase β-subunit, particularly involving amino acid substitutions of the histidine at position 35 in the *N. meningitidis* RpoB (43). High frequency of spontaneous rifampin resistance in combination with the natural competence and propensity for transformation exhibited by

Neisseria spp. limit the applicability of this antibiotic in treatment of gonococcal infection, as well as cause concern for the continued use of rifampin as a successful prophylactic for meningococcal infection.

D. Drug Efflux

Active efflux of antimicrobials from the cell interior decreases cellular concentration of these compounds and limits their ability to access and act upon their targets. Such removal of toxic substances is carried out by efflux pumps, which are energy-dependent transporters found in all kingdoms of life that localize to the cell membrane and export a wide variety of agents from cells. Efflux pumps are classified into five major families based primarily on the structure and number of membrane spanning domains of the transporter component and the energy source used to drive export: the ATP-binding cassette (ABC) superfamily, the mutlidrug and toxic compound extrusion (MATE) family, the major facilitator (MFS) superfamily, the small multidrug resistance (SMR) superfamily, and the resistance-nodulation-division (RND) family (148). In general, efflux transporters involved in multidrug resistance have large binding pockets with low-level specificity, allowing recognition of a broad array of structurally diverse agents (148).

The first efflux pump involved in multidrug resistance to be identified was actually a eukaryotic efflux pump, P-glycoprotein (Pgp), an ABC superfamily transporter found in a tumor cell line demonstrating anticancer drug resistance (127, 128). The first bacterial efflux pumps involved in antimicrobial resistance to be characterized were the *E. coli* Tet pump, a plasmid-encoded MFS pump that recognizes tetracycline and related antibiotics, and the *E. coli* AcrAB system, which has become the canonical example of

RND family multidrug efflux pumps in Gram-negative bacteria (14, 145, 155). In fact, efflux of antimicrobial compounds from Gram-negative bacteria poses an additional level of complexity, in that substances must be transported through the periplasm, either from the cell interior or from capture in the inner membrane. For this reason, efflux systems in Gram-negative bacteria are often tripartite, composed of the inner membrane transporter, an outer membrane channel, and a periplasmic fusion protein that serves to connect the inner and outer membrane components (148, 178).

The gonococcus expresses four characterized efflux pumps, a limited number compared to other Gram-negative bacterial pathogens such as *E. coli*, which expresses at least 37 different pumps, and *P. aeruginosa*, which possess approximately 65 (103, 144, 179, 211, 212, 239). These pumps are described in detail in Section V.

E. Interplay of resistance mechanisms

Often, multiple mutations affecting several resistance mechanisms are required for clinically significant levels of resistance to an antimicrobial. As mentioned above, gonococcal high-level tetracycline resistance requires the *penB*, *rpsJ*, and *mtrR* mutations, which involves alteration of PBP2, ribosomal protein S10, and expression of a multidrug efflux pump, respectively (113). The same is true for chromosomally-mediated penicillin resistance, which requires the cumulative effects of mutations in *penA*, *penB*, *mtrR*, and *ponA* (78, 208). In the face of increasing resistance to last-line antibiotics for gonococcal treatment, it is important to understand how the interplay of resistance mechanisms affects resistance to third-generation cephalosporins. For example, clinically significant resistance to ceftriaxone requires both mosaic PBPs, resulting from *penA* and *penB* mutations, and over-expression of the MtrCDE efflux pump (27). The mechanism

of interplay between resistance determinants is currently not well understood, but is an important factor to consider in developing ways to overcome resistance.

V. Efflux pumps in *Neisseria gonorrhoeae*

A. NorM

The NorM efflux pump of *N. gonorrhoeae* belongs to the MATE family of efflux pumps (211). The first MATE family pumps to be identified were YdhE of *Escherichia coli* and NorM of *Vibrio parahaemolyticus*, the latter being the first characterized Na+-coupled multidrug efflux pump (170, 171). Subsequently, MATE family pumps have been reported in all three kingdoms of life and are known to be present in almost all prokaryotes and eukaryotes (31, 185). These pumps have 12 putative transmembrane domains, recognize structurally diverse cationic toxic compounds, and rely on an electrochemical cation gradient across the cell membrane for drug transport (140). Most MATE family pumps are Na+/drug antiporters (140); however, some, such as AbeM of *Acinetobacter baumannii* and PmpM of *Pseudomonas aeruginosa*, rely on H+ as the transport coupling cation (109, 240). While MATE family transporters have been characterized in numerous pathogens, including *V. paraphaemolyticus* (NorM, VmrA), *E. coli* (YdhE), and *Haemophilus influenza* (HmrM), there is little evidence that these pumps contribute significantly to virulence (50, 170, 171). In mammals, MATE transporters have been shown to be involved in excretion of metabolic waste products and xenobiotics, and in plants, these pumps act to detoxify metals and secondary metabolites (such as phenols) by contributing to vesicular storage or extrusion (172).

Thus, MATE family pumps may contribute more to overall cell physiology than to resistance specifically.

The NorM efflux pump of *N. gonorrhoeae* is homologous to NorM of *Vibrio parahaemolyticus* and YdhE of *E. coli* (211). The gonococcal NorM is an Na+/drug antiporter that recognizes structurally diverse cationic toxic compounds including dyes such as ethidium bromide and crystal violet, quaternary ammonium compounds such as berberine and benzalkonium, and fluoroquinolone antibiotics such as norfloxacin and ciprofloxacin, which was a preferred antibiotic for treatment of gonococcal infections until widespread fluoroquinolone resistance developed and fluoroquinolones were removed from the CDC recommended treatment guidelines in 2007 (47, 211). In strain FA19, wild-type NorM confers only low-level resistance to substrate antimicrobials; however, mutations have been identified in the upstream regulatory region of the *norM* gene that increase resistance to NorM substrates (211). Thus, it has been suggested that NorM contributes to clinically significant levels of resistance, particularly to ciprofloxacin, in strains with other mutations that raise the MIC near levels that result in treatment failure (153).

B. MacAB

The gonococcal MacAB efflux pump is a member of the ABC transporter family (212). ABC proteins are characterized by the presence of a highly conserved 215 amino acid nucleotide binding domain, including the unique ABC superfamily signature sequence LSGGQ, and use phosphate bond hydrolysis to drive a particular physiological process (272). ABC transporters are composed of an ABC protein associated with a hydrophobic transmembrane domain, either as two distinct peptides or one polypeptide,

and it is the transmembrane domain that determines the specificity of the transporter substrates (141, 276). ABC transporters have been identified in all three kingdoms of life and are involved in both the import and export of a wide variety of molecules, including small solutes, polysaccharides, lipids, and peptides (276). Thus, the ability to transport drugs may be an adaptation from an original role of these transporters in removal of toxic metabolites or environmental biocides, as well as excretion of and immunity to antimicrobial compounds produced by the cell itself, such as the Bcr transporter of *Bacillus licheniformis*, which confers resistance to the bacitracin this organism produces (194, 276).

In Gram-positive bacteria, ABC family transporters are most often associated with single drug resistance, such as the MsrA transporter of *Staphylococcus epidermidis*, which confers resistance to erythromycin (141, 209, 276). The first multidrug resistance ABC transporter identified was LmrA of *Lactococcus lactis*, which recognizes and exports a broad range of drugs, including aminoglycosides, macrolides, quinolones, and tetracyclines (28, 195). It was originally thought that Gram-negative bacteria did not use ABC transporters in drug efflux, and relied on secondary transporters for antimicrobial resistance (276). However, in 2001, Kobayashi *et al.* demonstrated that the ABC-type transporter MacB of *E. coli*, in conjunction with the MFP MacA and the OMP TolC, confers resistance to macrolide antibiotics, thereby providing the first example of a Gram-negative ABC drug efflux transporter (137). It is important to note, however, that the effects of MacAB were studied in an AcrAB-deficient strain, as AcrAB is known to be largely responsible for macrolide resistance in *E. coli* (137, 283).

MacAB of *N. gonorrhoeae* is homologous to *E. coli* MacAB, with MacB belonging to the ABC transporter family and MacA being an HlyD-type MFP (212). Rouquette-Loughlin *et al.* showed that the *macA* and *macB* genes were located together in an operon, and that gonococcal MacAB can confer resistance to erythromycin and azithromycin (212). Importantly, the effects of MacAB were not apparent in strains with an intact MtrCDE efflux pump because, like AcrAB in E. coli, the MtrCDE pump contributes strongly to macrolide resistance in gonococci and masks resistance due to MacAB (212). However, MacAB was shown to confer resistance in strains with a nonfunctional MtrCDE pump or strains known to have an *mtr*-independent mechanism of macrolide resistance (212). Thus, it is possible that MacAB may contribute to increased macrolide resistance in strains with MICs higher than those conferred by the MtrCDE efflux pump, particularly in the event of mutations that result in increased *macAB* expression.

C. FarAB

The FarAB efflux pump of *N. gonorrhoeae* is a major facilitator superfamily (MFS) transporter (144). MFS transporters exist in all living organisms, have 12 or 14 transmembrane domains, and can be divided into 5 functional groups: drug resistance, uptake of Krebs cycle intermediates, phosphate ester/phosphate antiport, oligosaccharide uptake, and sugar uptake (83, 191). Most MFS transporters are substrate specific; however, several families of MDR transporters have been identified, and members of the MFS comprise the largest family of multidrug efflux pumps (83). MFS transporters rely on proton motive force for energy, and the MDR efflux pumps are H+/drug antiporters (83, 191). In Gram-positive bacteria, MFS transporters have been shown to confer

clinically significant levels of antimicrobial resistance. For example, deletion of Bmr in *B. subtilis* renders these mutants more sensitive to rhodamine G6 (177), while NorA of *S. aureus* has been shown to confer resistance to toxic cationic compounds, including quinolone antibiotics (275). In Gram-negative bacteria, however, resistance provided by MFS efflux pumps is often overshadowed by the action of MDR pumps of the RND family, as the tripartite nature of these pumps allows efflux across both membranes (83). Some exceptions have been identified, such as EmrAB from *E. coli*, which confers clinically relevant resistance to antibacterial uncoupling agents (151). It is likely that the presence of the MFP, EmrA, in conjunction with the transporter, EmrB, facilitates transport across both membranes by allowing interaction with an OMP, resulting in a higher, more physiologically significant level of resistance to EmrAB substrates (83).

FarAB was identified in a gonococcal genome search to determine the mechanism of fatty acid resistance in clinical isolates that did not show resistance to other antimicrobial hydrophobic agents that are substrates of the MtrCDE efflux pump (144). FarAB is homologous to EmrAB of *E. coli* and its counterpart in *Vibrio cholerae*, VceAB, and, like these pumps, is composed of a MFS transporter, FarB, and a MFP, FarA (53, 144, 151). Additionally, Lee and Shafer showed that FarAB utilizes the OMP MtrE, thereby allowing transport of substrates across both membranes (144). Unlike EmrAB and VceAB, however, which confer resistance to uncoupling agents, FarAB mediates resistance to long-chained fatty acids (144).

The *farA* and *farB* genes comprise an operon that is repressed by a MarR-family protein, FarR (142, 144). MarR proteins function by binding to DNA as dimers through a conserved winged helix-turn-helix motif, often via recognition of direct or inverted repeat

sequences, and control a variety of physiological functions, including response to oxidative stress, virulence factor production, and catabolism of aromatic compounds (268). Many regulators of efflux pump operons belong to the MarR family, including EmrR and MarR of *E. coli*, which regulate *emrAB* and *marRAB* expression, respectively, and MexR of *P. aeruginosa*, which represses expression of the *mexA-mexB-oprM* operon (152, 197, 225). FarR was identified in a search of the meningococcal genome for MarR-like proteins, and subsequently shown by Lee *et al.* to directly repress expression of *farAB* by binding to the *farAB* promoter (142). Additionally, FarR was shown to be autoregulatory, in that it represses its own expression by binding its own promoter (142). Expression of *farR* is also repressed by MtrR; thus, MtrR serves as an indirect activator for *farAB* (142).

Repression of *farAB* by FarR is dependent upon Integration Host Factor (IHF), a histone-like DNA binding protein present in all Gram-negative bacteria (89, 106, 143). IHF is composed of two subunits, ihfA and ihfB, and binds DNA as a heterodimer in a sequence-specific manner (81, 89). IHF plays an accessory role in several cellular processes, including DNA replication and recombination and gene regulation, and can positively or negatively influence transcription by facilitating binding of RNA polymerase or transcriptional regulatory proteins (79, 88, 114, 176). In the context of the *far* system, IHF binds specifically to the -36 to -47 region upstream of *farAB* and bends DNA to stabilize binding of FarR (143). Binding of IHF alone significantly represses *farAB* transcription, while FarR does not significantly repress *farAB* expression in the absence of IHF; full repression of *farAB* requires the binding of both regulatory proteins (143).

D. MtrCDE

The MtrCDE efflux pump of *N. gonorrhoeae* is a member of the resistance/nodulation/division (RND) permease superfamily, a ubiquitous superfamily of proton motive force-dependent permeases represented in all major kingdoms of life (105, 191, 253). RND family transporters are characterized by 12 α-helical transmembrane domains, with two large hydrophilic extracyoplasmic loop domains between transmembrane helices 1 and 2, and 7 and 8 (253). The RND superfamily is divided into 7 subfamilies, based on substrate specificity; MtrCDE belongs to the hydrophobe/amphiphile efflux-1 (HAE1) subfamily, which consists primarily of transporters found in Gram-negative bacteria that recognize drugs and hydrophobic compounds (253). The prototype RND efflux systems in Gram-negative bacteria are the AcrAB-TolC efflux system of *E. coli*, and the MexAB-OprM efflux system of *P. aeruginosa,* both of which are contribute to multidrug resistance in these organisms (reviewed in (24, 191)).

The first evidence for the *mtr* efflux system was the observation of strong correlations between resistance levels of gonococci to a range of hydrophobic antimicrobial agents by Maness and Sparling in 1973 (160). It was also observed that a single mutational event could lead to multiple drug sensitivity with respect to these antimicrobials, as well as reversion to multiple drug resistance (160). Because spontaneous mutations rarely affect more than one genetic locus, it was predicted that the multidrug resistance was due to a single gene, the product of which likely affects cell envelope permeability, a hypothesis supported by the finding that resistant mutants were less permeable to crystal violet than sensitive strains (100, 160). The *mtr* locus was not

characterized, however, until 1994, when Pan and Spratt identified and cloned the *mtrR* gene and a partial ORF on the opposite strand, which was termed *mtrC* (189). This locus was found to be homologous to the *envCD* locus of *E. coli*, known to confer resistance to antibiotics, dyes, and detergents (135, 189). It was subsequently found that the *mtr* locus consists of the *mtrR* gene, encoding a transcriptional regulatory protein, and a divergently transcribed operon of three genes, *mtrC*, *mtrD*, and *mtrE*, the products of which form a tripartite efflux pump belonging to the RND family (65, 103, 105).

The MtrCDE efflux pump recognizes and exports a diverse array of hydrophobic antimicrobial compounds, including antibiotics, such as erythromycin, azithromycin, and penicillin; dyes, such as crystal violet; detergents, including Triton X-100 (TX-100) and the sperimicide nonoxonyl-9; and host antimicrobial compounds, such as fatty acids, bile salts, progesterone, and the antimicrobial peptide LL-37 (103, 227, 260). MtrC is a 44 kDa membrane fusion protein that connects the inner and outer membrane components of the pump and is homologous to AcrA of *E. coli* and MexA of *P. aeruginosa* (103, 249). The RND transporter, MtrD, is a 114 kDa inner membrane protein with 12 transmembrane segments, homologous to AcrB of *E. coli* and MexB of *P. aeruginosa* (105). MtrD monomers associate to form trimers that recognize substrates in the outer leaflet of the inner membrane and use proton motive force to actively pump substrates out of the gonococcal cell (Fig. 1A) (118, 174). The outer membrane channel is composed of a trimer of MtrE, a 48.3 kDa lipoprotein homologous to OprM of *P. aeruginosa* (65, 118). In an assembled pump, 6 molecules of MtrC bind to connect the MtrD and MtrE trimers, making the stoichiometry of an assembled pump 3:6:3 (Fig. 1A) (118). In the absence of substrate, the MtrCDE channel is closed; substrate binding to MtrD induces a

conformational change that is transmitted through MtrC to open the MtrE channel for drug extrusion (119). Inactivation of any individual gene in the operon causes hypersusceptibility to pump substrates, indicating that all three components are required to form a functional pump and confer antimicrobial resistance (65, 103, 105). An additional accessory protein, termed MtrF, has also been identified; although its function is currently unknown, it has been shown to be involved in facilitating constitutive high-level MtrCDE-mediated resistance, and is required for high-level inducible resistance in the presence of sublethal levels of pump substrates (84, 261).

The *mtrCDE* operon is repressed by MtrR, a TetR family transcriptional regulator (103, 104, 189). TetR family regulators are classified by a conserved N-terminal helix-turn-helix (HTH) DNA binding domain and bind as homodimers to palindromic operator sites (201). Most TetR family proteins act as repressors and are located upstream and divergently transcribed from the gene or operons they regulate (201). However, a growing body of evidence suggests that members of this family may also act as activators and serve as global regulators within a bacterial cell. For example, LuxR, the master regulator of the quorum-sensing response in *Vibrio harveyi*, has been shown to directly activate and repress a number of genes in the quorum-sensing regulon (196). MtrR itself has been shown to be a global regulator in the gonococcus, acting as both a repressor and an activator to regulate a variety of systems, including stress response, metabolism, and antibiotic resistance (85).

The *mtrR* gene is 250 base pairs upstream and is transcribed divergently from the *mtrCDE* operon (Fig. 1B) (189). Like other TetR family members, MtrR binds as a homodimer to an inverted repeat within the *mtrCDE* promoter (154). Mutations in MtrR

that disrupt domains important for binding can relieve repression of *mtrCDE* and result in increased MtrCDE-substrate resistance. For example, mutations within the HTH DNA binding domain abrogate binding of MtrR to its target site; one particularly well-characterized example is a glycine to aspartic acid change at amino acid 45 (103). Additionally, mutations may occur in a more central domain of MtrR, such as a histidine to tyrosine change at amino acid 105, that are predicted to prevent MtrR repression by interfering with multimerization of MtrR (228). MtrR has also been shown to repress the gene encoding MtrF, which lies just downstream and is transcribed divergently from *mtrR*, although this regulation appears to be indirect (Fig. 1B) (84).

The *mtrCDE* operon is subject to positive regulation by MtrA, an AraC family regulatory protein (210). AraC family proteins are primarily transcriptional activators, although some also demonstrate activity as repressors, and regulate diverse functions including sugar catabolism, stress response, and virulence (90, 163). These regulators are characterized by a 100 amino acid region of homology, most often at the C-terminal domain, that forms two HTH DNA binding domains, and may bind DNA as monomers or dimers (90, 163). The N-terminal domain may function in multimerization or response to ligands that affect the regulator activity of these proteins (73). The canonical regulator for which this family is named is AraC of *E. coli*, which activates *araBAD*, *araFGH*, and *araE*, involved in transport and catabolism of L-arabinose, as well as represses its own expression (90).

The gonococcal MtrA was identified in a search for transcriptional activators upon the observation that *mtrCDE* transcription and MtrCDE-mediated resistance were enhanced when gonococci were grown in the presence of sublethal levels of TX-100 and

nonoxonyl-9 (210). MtrA binds to a site in the *mtrR-mtrCDE* intergenic region approximately 20 - 30 base pairs upstream of the MtrR binding site; these sterically close sites result in competition between MtrR-mediated repression and MtrA-mediated activation of pump operon transcription (Fig. 1B) (278). In the absence of ligands, MtrA demonstrates weak binding affinity to the *mtrCDE* promoter region, and MtrR binding is favored (278). In the presence of inducing antimicrobials, however, affinity of MtrA for its binding site is increased, and binding of MtrA is favored over MtrR (278). Interestingly, many gonococcal isolates contain an 11bp deletion in the *mtrA* gene that results in the production of a truncated, non-functional protein; these strains are not inducible to increased MtrCDE-mediated resistance in the presence of sublethal concentrations of pump substrates (210).

An additional AraC family regulatory protein, MpeR, also acts at the *mtr* locus to regulate *mtrCDE* expression in an iron-responsive manner. Expression of *mpeR* is repressed by Fur (ferric uptake regulator) when levels of free iron are high (115). MpeR, in turn, directly represses expression of *mtrR* (165). Thus, under iron replete conditions, repression of *mpeR* by Fur allows sufficient expression of *mtrR* to keep levels of MtrCDE low via MtrR repression of the *mtrCDE* operon (165). In contrast, under iron deplete conditions, increased production of MpeR results in decreased expression of *mtrR*, relieving repression of *mtrCDE,* thereby allowing increased MtrCDE production and enhanced MtrCDE-mediated resistance (165). MpeR has also been shown to repress *mtrF* when levels of free iron are high; thus, MpeR contributes an important regulator role to high-level and inducible MtrCDE-mediated resistance (84).

VI. Role of *cis*-acting Mutations in Regulation of Antimicrobial Resistance

Genes encoding efflux systems are often controlled by complex regulatory circuits. Constitutive high-level expression of efflux pumps poses a potential metabolic stress for cells; thus, expression of efflux genes is often damped by the action of transcriptional repressors and induced when efflux-mediated resistance is most beneficial by relief of repression or activation by other transcriptional regulators (178). Antimicrobial stress can lead to the accumulation of mutations that enhance efflux-mediated resistance by increasing pump production. Such mutations often occur in transcriptional repressors of pump gene expression, such as those described previously for MtrR, and act to relieve repression by disrupting the ability of the repressor to bind its target site. However, *cis*-acting mutations may also occur that increase pump expression by affecting pump gene promoter elements. Examples of such *cis*-acting mutations have been identified for several gonococcal efflux pumps.

Under wild-type conditions, *norM* is expressed at low levels and confers only low-level resistance to its substrate antibiotics. However, Rouquette-Laughlin *et al.* identified two mutations upstream of the *norM* coding region that increase resistance to NorM substrate antibiotics (211). The first is a C-to-T transition mutation in the -35 promoter element that confers intermediate-level resistance to NorM substrates. This mutation changes the -35 element sequence from the wild-type CTGACG to TTGACG (Fig. 2A). As the sequence generated by this base pair change results in a -35 sequence closer to consensus (TTGACA), it is likely that this mutation increases expression of *norM* by enhancing the ability of RNA polymerase to recognize and bind the *norM* promoter, resulting in increased production of NorM and therefore increased resistance

(32). The second mutation is an A-to-G change in the ribosome binding site (RBS) of the *norM* transcript that, when present in conjunction with the -35 element mutation, results in high-level resistance to NorM substrates. This mutation alters the RBS from the wild-type TGAACA to TGGACA (Fig. 2A). Like the -35 mutation, the RBS mutation generates a sequence closer to consensus (AGGAGG), which would improve recognition of the *norM* transcript by the ribosome, leading to enhanced translation and increased NorM production (229).

The *macAB* promoter was found to contain a -10 element with the near-consensus sequence TAGAAT (Fig. 2B) (32, 212). The low levels of *macAB* expression detected by Rouquette-Loughlin *et al.* from this promoter sequence suggested that the nonconsensus G dampens *macAB* expression (212). It was found that replacing the G with the consensus T nucleotide resulted in 33.3- and 8.3-fold increased resistance to azithromycin and erythromycin, respectively, in a strain with a non-functional MtrCDE pump (212). It is likely that the G-to-T change, like the -35 mutation in the *norM* promoter, enhances the ability of RNA polymerase to recognize the *macAB* promoter, thereby increasing transcription of *macAB*, resulting in higher pump levels and increased resistance to macrolide antibiotics recognized by the MacAB pump.

Expression of *mtrCDE* may be impacted by *cis*-acting mutations that occur in the *mtrR-mtrCDE* intergenic region. The *mtrR* promoter contains a 13 base pair inverted repeat element that is important for *mtrR* transcription (Fig. 2C). The deletion of a single A-T base pair within the *mtrC*-proximal portion of this repeat results in high-level *mtrCDE* expression and correspondingly high levels of MtrCDE-mediated resistance (103). This mutation is frequently found in isolates of *N. gnorrhoeae* demonstrating

clinically significant levels of resistance to MtrCDE substrates (226, 271, 279). High-level resistance in strains bearing this mutation likely results from two distinct mechanisms. First, deletion of base pair from the inverted repeat element decreases the spacing between the -10 and -35 elements of the *mtrR* promoter from an optimal 17 base pairs to a sub-optimal 16 base pairs, which is sufficient to abrogate *mtrR* transcription, as strains containing this deletion have been shown to be negative for MtrR production (Fig. 2C) (32, 104). Additionally, because the *mtrR* and *mtrCDE* promoters overlap, competition for RNA polymerase recognition between the two promoters is reduced, leading to increased transcription from the *mtrCDE* promoter (Fig. 2C) (104, 264). Thus, levels of MtrCDE production and antimicrobial resistance are higher than in strains containing a mutation in the structural region of MtrR (103, 264).

Recently, a second *cis*-acting mutation in the *mtr* locus was identified in *N. gonorrhoeae* strain MS11, originally isolated from a patient with an uncomplicated cervical infection and since used extensively in gonococcal research (241). This mutation, a C-to-T base transition mutation 120 base pairs upstream of the *mtrC* start codon subsequently termed mtr_{120}, was shown to increase transcription of *mtrCDE* and confer levels of MtrCDE-mediated antimicrobial resistance similar to those of the single base pair deletion mutation described above by an MtrR-independent mechanism (Fig. 2C) (264). Characterization of this mutation and its mechanism of action are a major focus of this work (Chapter 3).

VII. Effects of efflux pump expression on gonococcal fitness

Mutations that confer antibiotic resistance often also confer a growth defect or fitness disadvantage on bacterial cells (6). In regard to efflux pumps, it has been hypothesized that overexpression of these large cellular machines may be metabolically taxing for the cell, necessitating the complex regulatory circuits that often control transcription of efflux pump genes (178). A number of mutations have been identified in *N. gonorrhoeae*, however, that actually enhance gonococcal fitness (reviewed in Chapter 2). Importantly, *mtr* mutants that demonstrate increased expression of MtrCDE are often recovered from patients during outbreak investigations, and many of these mutations have been shown to enhance gonococcal survival in a female BALB/c mouse model of lower genital tract infection (263, 264).

Amino acid substitutions in MtrR that abrogate its ability to bind and repress *mtrCDE* transcription are found frequently in clinical isolates of *N. gonorrhoeae*, and these mutants, as well as laboratory mutants in which *mtrR* has been deleted or knocked out demonstrate a significant survival advantage over a wild type isogenic parent during experimental murine infection (263, 264). In contrast, mutants with a non-functional MtrCDE pump show a severe fitness disadvantage compared to wild type gonococci in similar experiments (263). Importantly, mutants deficient in MtrA, which lose the ability to induce *mtrCDE* expression in the presence of sublethal concentrations of pump substrates, also show a severe fitness disadvantage over wild type gonococci (263). In fact, during a study by Warner *et al.*, some MtrA-deficient mutants developed compensatory mutations in *mtrR* during the course of infection that led to a non-functional MtrR protein, thereby allowing increased expression of *mtrCDE* (263). Furthermore, a strain carrying the single base pair deletion in the *mtrR* promoter, which

produces greater levels of MtrCDE than an MtrR structural mutant, not only shows a greater fitness advantage over wild type gonococci than do MtrR mutants during murine infection, but actually has a fitness advantage over the MtrR mutants themselves (264). Thus, it appears that overexpression of the MtrCDE efflux pump is actually advantageous to gonococcal survival *in vivo*.

Interestingly, the mtr_{120} mutation conferred a fitness advantage similar to that of the single base pair deletion in the female mouse model of infection (264). Additionally, as enhanced *mtrCDE* transcription mediated by mtr_{120} was independent of MtrR, this mutation offered a unique opportunity to examine the physiological effects of efflux pump overproduction on the gonococcal cell, without the disruption of the pump operon repressor. As such, the following specific aims were addressed in this work:

1. To characterize the mtr_{120} mutation and determine its mechanism of action
2. To examine the global cellular response of gonococci to overproduction of MtrCDE

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Figure legends

Figure 1: The MtrCDE efflux pump of *Neisseria gonorrhoeae*. (A) The MtrCDE efflux pump is a tripartite pump composed of a trimer of the inner membrane RND family transporter MtrD and a trimer of the outer membrane protein MtrE, connected by six monomers of the periplasmic fusion protein MtrC. MtrD recongizes substrates in the outer leaflet of the inner membrane and uses proton motive force to actively pump substrates out of the cell through the channel formed by MtrE. **(B)** The genes encoding the MtrCDE efflux pump lie in an operon. Expression of *mtrCDE* is regulated by the transcriptional repressor, MtrR, and the inducible transcriptional activator, MtrA. The *mtrR* gene lies 250 base pairs upstream of the *mtrCDE* operon, and its expression is repressed by the transcriptional regulatory protein MpeR in response to levels of free iron. The *mtrF* gene, which encodes an accessory protein of unknown function, lies 2 open reading frames upstream of *mtrR* and is subject to repression by both MtrR and MpeR.

Figure 2: *cis*-acting mutations that enhance efflux pump gene expression in *Neisseria gonorrhoeae.* (A) The *norM* promoter. A nonconsensus -35 element and nonconsensus RBS result in low levels of constitutive expression. A C-to-T base pair substitution in the -35 element and an A-to-G substitution in the RBS bring the sequences of these elements closer to consensus and result in increased *norM* expression and resistance to NorM substrate antimicrobials. **(B)** The *macAB* promoter. A nonconsensus -10 element results in low levels of constitutive expression. A G-to-T substitution in the -10 element brings this sequence closer to consensus, resulting in increased transcription

of *macAB* and increased resistance to MacAB substrate antibiotics. **(C)** The *mtrR*-*mtrCDE* intergenic region. The -10 and -35 elements of the *mtrR* and *mtrCDE* promoters are indicated. The -10 and -35 elements of the *mtrR* promoter are separated by an optimal 17 base pairs, the sequence of which contains an inverted repeat important for *mtrR* transcription. The deletion of an A-T base pair in the inverted repeat, indicated in bold type, decreases this spacing, abrogating *mtrR* transcription and improving access for RNA polymerase to the *mtrCDE* promoter, resulting in increased *mtrCDE* expression and enhanced resistance. The C-to-T transition mutation (mtr_{120}) upstream of the *mtrC* start codon that is the subject of this work is indicated in bold type. This mutation generates a consensus -10 element and results in high-level expression of *mtrCDE* and correspondingly high levels of resistance.

FIGURE 1

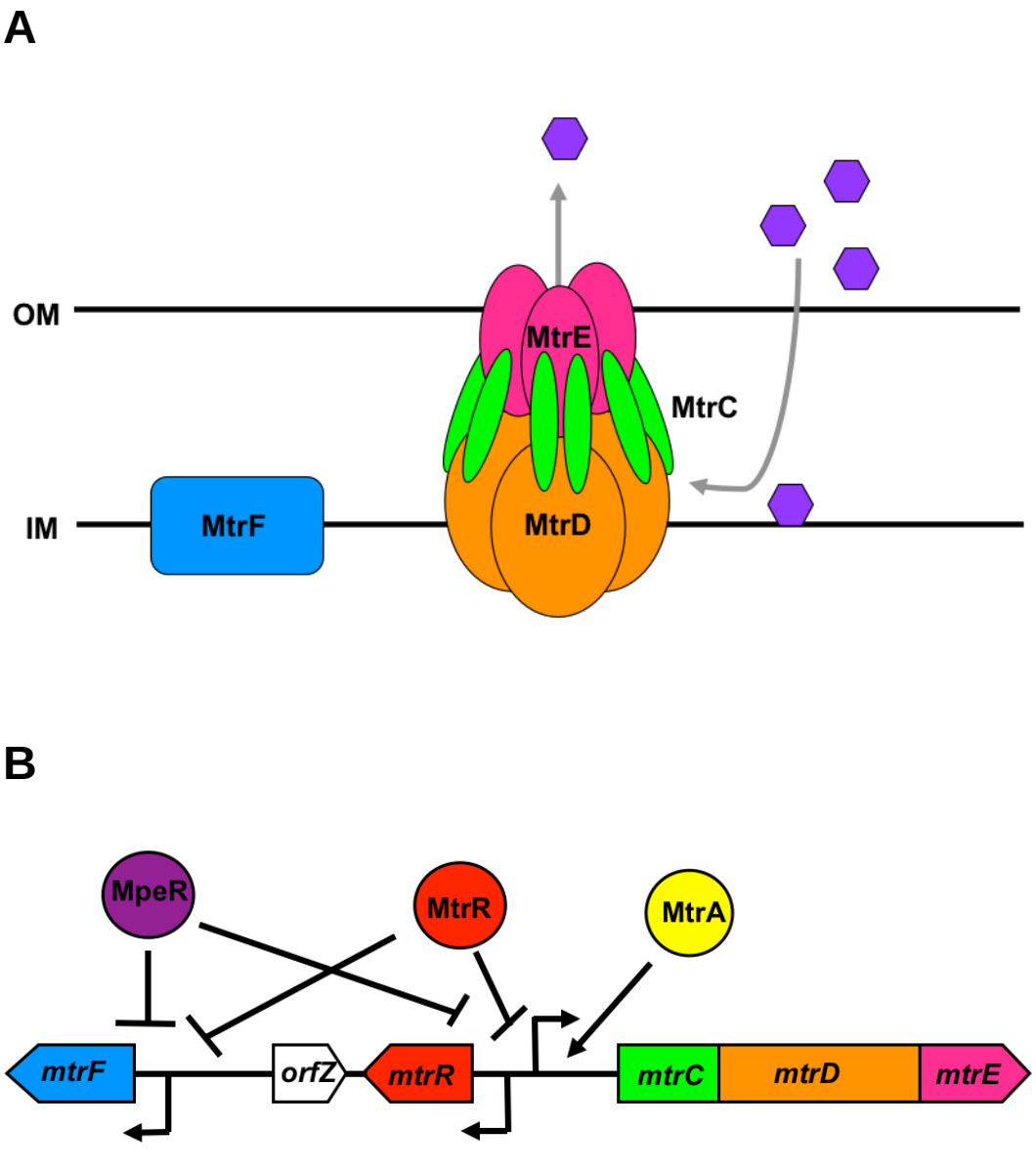
A
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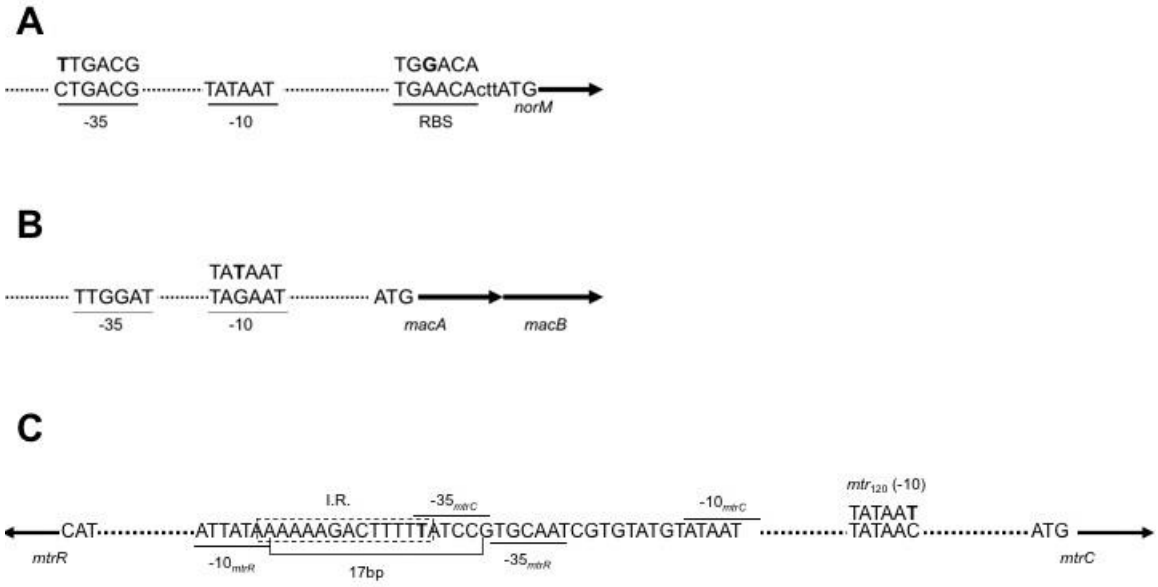
FIGURE 2


A
TTGACG
CTGACG
TATAAT
TGGACA
TGAACActtATG
norM
-35
-10
RBS
B
TATAAT
TTGGAT
TAGAAT
ATG
macA
macB
-35
-10
C
I.R.
-35mtrC
-10mtrC
mtr120 (-10)
TATAAT
CAT
ATTATAAAAAAGACTTTTTATCCGTGCAATCGTGTATGTATAAT
TATAAC
ATG
mtrR
mtrC
-10mtrR
17bp
-35mtrR

Chapter 2: Clinically Relevant Antibiotic Resistance Mechanisms Can Enhance the *in vivo* Fitness of *Neisseria gonorrhoeae*

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1. Introduction

In 2007 the Centers for Disease Control and Prevention placed *Neisseria gonorrhoeae* on the infamous "Super Bugs" list to highlight the high prevalence of strains resistant to relatively inexpensive antibiotics, such as penicillin, tetracycline and fluoroquinolones, previously used in therapy to treat gonorrhea (Shafer *et al.*, 2010). This event was significant because the gonococcus, a strict human pathogen, causes > 95 million infections worldwide each year and since the mid-1940s mankind has relied on effective antibiotic therapy to treat infections and stop local spread of disease. Today, such therapy is threatened by antibiotic resistance. Specifically, the third generation cephalosporins, especially ceftriaxone, may be losing their effectiveness since some (albeit still rare) isolates in the Far East, most recently Japan, and Europe have displayed clinical resistance to currently used levels of ceftriaxone, and treatment failures have been reported (Ohnishi *et al*., 2011; Unemo *et al*., 2010). Concern has been raised that the spectrum of resistance expressed by some gonococcal strains may make standard antibiotic treatment for gonorrhea ineffective in the not too distant future (Dionne-Odom *et al*., 2011). Without new, effective antibiotics or novel combination therapies of existing antibiotics, the reproductive health of the world's sexually active population may be placed at risk due to such antibiotic resistant gonococci.

An important question regarding antibiotic resistance is whether a particular resistance mechanism has a fitness cost for the bacterium (Andersson & Levin, 1999; Andersson & Hughes, 2010), especially in the community where it competes with its antibiotic sensitive brethren. A fitness cost is typically experimentally measured as a deleterious change in bacterial growth rate in laboratory media or survival in

experimental infection in the absence of antibiotic pressure. Fitness costs (or benefits) are best viewed during co-cultivation of isogenic strains that differ only in the resistance mechanism under study. For certain antibiotic resistance mechanisms, a significant fitness cost can be incurred. This general observation led to the idea that removal of the selective pressure imposed by the antibiotic in question would favor sensitive strains to predominate in the community and allow for the return of the antibiotic in question to treat the infection in question. By and large, this has proven not be the case (Andersson and Hughes, 2010). There are many reasons for this, including the unintentional selective pressure exerted by the widespread availability and use of antibiotics to treat bacterial infections in general, over-the-counter antimicrobials that confer selective pressure and provide cross-resistance (or decreased susceptibility) to the antibiotic in question, and host-derived antimicrobials that select for the particular resistance determinant. In addition to antimicrobial pressures, it has been repeatedly documented that compensatory, second site mutations can develop that reverse fitness costs while maintaining resistance (Schrag *et al*., 1997; Marcusson *et al*., 2009; Andersson and Hughes, 2010).

More recently, a new view has been taken regarding antibiotic resistance and fitness: some resistance systems actually provide the resistant strain with a fitness advantage over wild type strains or can reverse a fitness burden imposed by a separate mutation that also participates in resistance to a particular antibiotic. Evidence for enhanced fitness of bacterial pathogens, in laboratory media or in experimental infection models, due to mutations or gene acquisition events that increase resistance to antibiotics has been provided for *Campylobacter jejuni* (Luo *et al*., 2005) and *Neisseria*

gonorrhoeae (Warner *et al*., 2007, 2008). The idea that an antibiotic resistance mechanism could have negligible and even beneficial effects on fitness could help to explain, in part, why resistant strains persist in the community long after the antibiotic has been removed from the treatment regimen. For instance, gonococci expressing resistance to penicillin, tetracycline and/or fluoroquinolones have persisted in the community despite the removal of these antibiotics from the recommended gonorrhea treatment regimen for several years. Against this background, we herein review data and provide models as to how two mechanisms of antibiotic resistance expressed by *N. gonorrhoeae* can enhance fitness *in vivo*. The *in vivo* system employed in these studies is a female mouse model of lower genital tract infection that recapitulates many features of infection in human females, most notably the development of inflammation that occurs during cervicitis (Jerse, 1999; Packiam *et al*., 2010; Song *et al*., 2008). The two resistance mechanisms discussed below are multi-drug efflux by the MtrC-MtrD-MtrE pump (Hagman *et al*., 1995; Jerse *et al*., 2003) and quinolone resistance that develops due to point mutations in *gyrA* and *parC*. We discuss concepts regarding the evolution of antibiotic resistance expressed by gonococci in the context of how these resistance mechanisms may have endowed this strict human pathogen with a fitness advantage during infection.

2. Antimicrobial Efflux and Gonococcal Fitness

The MtrC-MtrD-MtrE efflux pump of *N. gonorrhoeae* is a resistance-nodulation-division (RND) efflux pump family member that recognizes a diverse array of hydrophobic antimicrobial agents and exports these toxic compounds out of the

gonococcal cell (Hagman *et al*., 1995). The *mtrCDE* operon is composed of three structural genes that encode the core proteins of the efflux pump: *mtrC*, which encodes a periplasmic membrane fusion protein; *mtrD*, encoding an energy-dependent inner membrane transporter; and *mtrE*, which encodes a TolC-like outer membrane channel protein (Delahay *et al*., 1997; Hagman *et al*., 1995; Hagman *et al*., 1997). In addition to these core efflux proteins, an accessory protein termed MtrF is required for high-level resistance to substrates of the pump and its gene (*mtrF*) is also located within the *mtr* locus (Figure 1) (Veal & Shafer, 2003).

Transcription of the *mtrCDE* operon is negatively regulated by the TetR family transcriptional regulator, MtrR, which represses *mtrCDE* expression by the binding of two homodimers to pseudo-direct repeats within the *mtrCDE* promoter (Hoffman *et al*., 2005; Lucas *et al*., 1997). The *mtrR* gene is located 250 base pairs upstream of and is transcribed divergently from *mtrCDE* (Pan & Spratt, 1994). Additionally, transcription of *mtrCDE* may be induced in the presence of sub-lethal concentrations of nonionic, membrane-acting detergents through the action of an AraC/XylS family transcriptional activator, MtrA (Rouquette *et al*., 1999). Expression of *mtrF* is negatively regulated by both MtrR and the AraC family regulator MpeR (Folster and Shafer, 2005), as well as the availability of free iron (Mercante *et al*., manuscript in preparation).

The MtrC-MtrD-MtrE efflux pump mediates resistance to structurally diverse hydrophobic antimicrobial agents, including ß-lactam antibiotics such as penicillin, macrolide antibiotics including erythromycin and azithromycin, dyes such as crystal violet, and detergents such as Triton X-100 and nonoxynol-9 (Hagman *et al*., 1995; Rouquette *et al*., 1999). Additionally, MtrC-MtrD-MtrE confers resistance to host

antimicrobial compounds, including fatty acids, bile salts, progesterone, and the antimicrobial peptide LL-37 (Jerse *et al*., 2003; Morse *et al*., 1982; Shafer *et al*., 1995; Shafer *et al*., 1998). MtrC-MtrD-MtrE efflux pump-deficient mutants are highly attenuated in a female BALB/c mouse model of lower genital tract infection, even in the absence of pump substrate antibiotic treatment (Jerse *et al*., 2003). This attenuation is likely due to an increased susceptibility to host antimicrobial compounds, highlighting the importance of the *mtr* system in establishing gonococcal infection.

The production of efflux pumps is an energy-expensive process, and it might be hypothesized that high levels of MtrC-MtrD-MtrE production could stress the gonococcus, resulting in slower or defective growth, thereby conferring a fitness cost on strains with increased *mtrCDE* expression. In this respect, Eisenstein and Sparling noted that a mutant strain displaying the Mtr phenotype, now known to be due to a single base pair deletion in the inverted repeat in the *mtrR* promoter (Figure 1) that results in high-level antibiotic resistance through increased transcription of *mtrCDE* (Hagman & Shafer, 1995), had a slower growth rate *in vitro* (Eisenstein & Sparling, 1978). However, this same mutation confers a fitness advantage during competitive infection against wild-type strain FA19 in the female mouse model of infection in the absence of antibiotics (Warner *et al*., 2008) and is frequently found in clinical isolates (Shafer *et al*., 1995; Zarantonelli *et al*., 1999), particularly from men who have sex with men (Shafer *et al*., 1995; Xia *et al*., 2000). Additional mutations in the *mtrR* coding region and the *mtrR* promoter have been identified in clinical isolates that increase resistance to MtrC-MtrD-MtrE pump substrates as well as confer a survival advantage in the female mouse infection model (Table 1) (summarized in Warner *et al*., 2008). Mutations in the *mtrR* coding region,

particularly those resulting in radical amino acid changes in the MtrR helix-turn-helix DNA binding domain, lead to low or intermediate levels of antimicrobial resistance that corresponds to a low to intermediate survival advantage during competitive infection in female mice (Warner *et al*., 2008). The single nucleotide deletion in the inverted repeat of the *mtrR* promoter and a recently identified mutation 120 base pairs upstream of the *mtrC* start codon (mtr_{120}) confer high-level resistance to pump substrates as well as a greater fitness advantage *in vivo* (Warner *et al*., 2008). These changes in fitness require an active efflux pump, as the effects were reversed in the regulatory mutant strains when the efflux pump system was genetically inactivated. Thus, it appears that the level of antibiotic resistance due to increased *mtrCDE* expression corresponds positively to the strength of the fitness advantage observed *in vivo*.

Induction of *mtrCDE* expression by the activator MtrA is also important for gonococcal survival *in vivo*. Strains carrying a disrupted *mtrA* gene display a significant fitness disadvantage during competitive infection with wild-type strain FA19 in the female mouse model of infection (Warner *et al*., 2007). MtrA induction of *mtrCDE* expression occurs in the presence of nonionic detergents such as Triton X-100 (Rouquette *et al*., 1999). The presence of host antimicrobial factors that are pump substrates, such as fatty acids or CRAMP-38, the mouse homologue of the human cathelicidin LL-37, may have a similar effect. The decreased fitness of the *mtrA* mutants *in vivo* would therefore be attributed to failure of the gonococcus to respond to host defense factors due to inability to upregulate expression of the pump. Interestingly, in a study by Warner *et al*., 2007, some *mtrA*-deficient strains developed mutations in the *mtrR* gene ($mtrR_{1\text{-}53}$ and $mtrR_{E202G}$) after inoculation into mice in the absence of antibiotics; these strains were

recovered in high numbers and displayed increased antibiotic resistance as well as a fitness advantage during subsequent competitive infection against wild-type FA19 (Table 1). The development of compensatory mutations to overcome the cost of *mtrA* disruption highlights the importance of the MtrC-MtrD-MtrE efflux pump to gonococcal fitness *in vivo*.

The importance of the MtrC-MtrD-MtrE efflux pump *in vivo*, even in the absence of antibiotic treatment, suggests that this pump originally evolved as a mechanism to aid the gonococcus in escaping host defense mechanisms, rather than in response to the introduction of antibiotics to treat gonococcal infection. Increasing antibiotic use and the availability of the over-the-counter spermicide nonoxynol-9 may then have selected for pump mutants, such as those containing *mtrR* mutations frequently isolated from patients with gonococcal infection. These strains are not only able to resist antibiotic treatment, but also better able to resist host antimicrobial compounds, giving them a survival advantage *in vivo* and in the community (Xia *et al*., 2000). Thus, increased production of the MtrC-MtrD-MtrE efflux pump represents a mechanism of antibiotic resistance that imparts a fitness advantage upon the gonococcus, rather than a fitness cost. It is important to note that homologues of both the pump and its regulatory proteins exist in other Gram-negative bacteria. For example, the AcrA-AcrB-TolC efflux system of *Salmonella enterica* enhances the capacity of this pathogen to cause experimental infection in chickens (Webber *et al*., 2009). Lessons learned with the gonococcus regarding drug efflux and fitness may therefore have broader implications for how bacterial pathogens escape both classical antibiotics and host defense compounds.

3. Quinolone Resistance and Gonococcal Fitness

The limited use of quinolones in the treatment of bacterial infections began after the 1962 discovery of nalidixic acid as a product of chloroquine synthesis. Subsequent development of fluoroquinolone derivatives amassed broad-spectrum appeal due to their effective targeting of many Gram-positive and Gram-negative pathogens (Emmerson, 2003). Continued development of this class of antibiotics was fueled by the concurrent progression of bacterial resistance to penicillin and tetracycline, including *N. gonorrhoeae* (Covino *et al*., 1990). By 1993, fluoroquinolones were recommended by the CDC as the first-line treatment option for uncomplicated gonococcal infections; however, within 10 years, over 80% of gonococcal isolates in the western Pacific region were ciprofloxacin resistant (Cip^R) (Tapsall, 2005; Trees *et al*., 2001). The eventual spread of quinolone resistant *N. gonorrhoeae* (QRNG) in the United States led to the removal of fluoroquinolones from the list of recommended first-line antibiotics for treatment of gonorrhea and related conditions by the CDC in 2007 (CDC, 2007).

Quinolones induce bacterial cell death by inhibiting the activity of the bacterial type IIA DNA topoisomerases DNA gyrase and topoisomerase IV (Emmerson, 2003; Hooper, 1999). These enzymes are responsible for managing the topological state of genomic DNA and are necessary for resolving regions of topological stress that occur during critical cell processes such as DNA replication and the regulation of gene expression. DNA gyrase and topoisomerase IV are heterotetramers that bind to DNA and generate a double-stranded break in one region of the bound DNA duplex, which results as a complex referred to as the G-segment. A second region of distant DNA duplex, referred to as the T-segment, passes through the G-segment and the cleaved substrate

held in the G-segment is subsequently relegated to complete a single round of topological adjustment (Bates *et al*., 2011; Chen and Lo 2003; Morais Cabral *et al*., 1997). Quinolones specifically target the G-segment of the enzyme-DNA complex. Presently, there is no universally accepted mechanism of how quinolones kill bacteria; however, mounting evidence suggests that two quinolone molecules stabilize the cleaved DNA duplex, resulting in the accumulation of lethal lesions within the genome of the cell (Laponogov *et al*., 2009).

Quinolone resistance in *N. gonorrhoeae* is due to point mutations in the quinolone resistance determining region (QRDR) of the A subunits of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) (Tanaka *et al*., 2000; Trees *et al*., 2001). Belland and colleagues were the first to delineate the genetic basis of quinolone resistance in *N. gonorrhoeae* in 1994. By analyzing ciprofloxacin resistant (Cip^R) mutants selected *in vitro*, these investigators showed Cip^R in *N. gonorrhoeae* is a two-step process in which intermediate-level ciprofloxacin resistance (Cip^I) occurs via point mutations in *gyrA* that encode amino acid substitutions at positions Ser91 and Asp95. Cip^I *gyrA* mutants then become Cip^R when point mutations occur in *parC* (Belland *et al*., 1994). This sequence of events is consistent with data from numerous molecular epidemiologic studies (Kam *et al*., 2003; Morris *et al*., 2009; Starnino *et al*., 2010; Tanaka, 1992; Trees *et al*., 2001; Vereshchagin *et al*., 2004). Analyses of clinical isolates have also provided insights into the nature of mutations directly associated with fluoroquinolone resistance in *N. gonorrhoeae*. Commonly isolated substitutions in the Ser91 position of the GyrA subunit include amino acids with bulky side chains (phenylalanine and tyrosine) and the hydrophobic leucine, while arginine is the most common substitution at position Asp95

(Kam *et al*., 2003; Morris *et al*., 2009; Ruiz *et al*., 2001; Starnino *et al*., 2010; Tanaka *et al*., 2000; Trees *et al*., 2001; Vereshchagin *et al*., 2004; Vernel-Paulillac *et al*., 2009). Double point mutations in *gyrA* that result in these amino acid substitutions are sufficient and also largely responsible for sterically hindering the intercalation of quinolone molecules (Xiong *et al*., 2011). The location specificity of *parC* mutations that lead to high-level Cip^R, appears to be less stringent than mutations in *gyrA*, with alterations at position 91 (the most common), 86, 87, or 88 identified among Cip^R isolates (Dewi *et al*., 2004; Morris *et al*., 2009; Tanaka *et al*., 2000; Trees *et al*., 2001;) (Figure 2).

The impact of quinolone resistance mutations on microbial fitness has been studied in several bacterial species. Topoisomerase mutations often are associated with an *in vitro* fitness cost, although not all *gyrA* mutations or combinations of *gyrA* mutations or *gyrA, parC* mutations result in decreased growth *in vitro* (Bagel *et al*., 1999; Marcusson *et al*., 2009, Pope *et al*., 2008; Luo *et al*., 2005). Interestingly, in 2005 Zhang and colleagues showed *gyrA* mutations confer a fitness benefit to *C. jejuni in vivo* using a chicken intestinal colonization model (Luo *et al*., 2005). Based on this report and the wide prevalence of QRNG strains, we hypothesized that fluoroquinolone resistance mutations in *N. gonorrhoeae* may be accompanied by a transmission or survival advantage. To address the possibility that QRNG may be more fit *in vivo*, we constructed Cip^I and Cip^R mutants in *N. gonorrhoeae* strain FA19 that carry the commonly isolated *gyrA* (Ser91Phe and Asp95Asn) or *gyrA* (Ser91Phe and Asp95Asn) and *parC* (Asp86Asn) mutations, respectively and measured their fitness relative to the Cip^S parent strain in the murine genital tract infection model. No *in vitro* fitness cost was associated with acquisition of the $gyrA_{91/95}$ mutations based on comparing the growth rates of the

gyrA$_{91/95}$ mutant and the Cip^{S} wild-type strain, although a slight reduction (3-fold) in the recovery of the mutant was observed when co-cultured with the Cip^{S} wild-type strain (Table 2). Interestingly, however, the Cip^{I} *gyrA*$_{91/95}$ mutant exhibited a clear fitness advantage *in vivo* as evidenced by high competitive indices (CIs) over time and the isolation of only Cip^{I} bacteria from some mice on days 5 and 7 post-inoculation. In contrast, the Cip^{R} *gyrA*$_{91/95}$, *parC*$_{86}$ mutant grew significantly more slowly *in vitro* and exhibited reduced fitness *in vivo* relative to the wild-type Cip^{S} strain (Table 2) (Kunz *et al*., submitted).

As discussed, it is well established that *mtr* locus mutations increase gonococcal fitness in the mouse model, and we therefore wondered whether the fitness benefit conferred by *gyrA*$_{91/95}$ mutations would enhance the fitness advantage afforded by increased efflux of host substrates through the MtrC-MtrD-MtrE active efflux pump. Our alternative hypothesis was that increasing numbers of resistance mutations would impair growth to such an extent as to abrogate the fitness benefits associated with either resistance mutation. To test this hypothesis, we constructed *gyrA*$_{91/95}$ and *gyrA*$_{91/95}$, *parC*$_{86}$ mutants in an *mtr* mutant of strain FA19 that carries a commonly isolated *mtrR* promoter mutation (the single base pair deletion in the *mtrR* promoter termed hereafter as *mtrR*$_{-56}$). The *gyrA*$_{91/95}$, *mtrR*$_{-56}$ mutant (Cip^{I}) showed no fitness difference compared to the *mtrR*$_{-56}$ mutant parent strain *in vitro*, but significantly out-competed the *mtrR*$_{-56}$ mutant during experimental murine infection. In contrast, the highly Cip^{R} *gyrA*$_{91/95}$, *parC*$_{86,}$ *mtrR*$_{-56}$ mutant was severely attenuated both *in vitro* and *in vivo* relative to the *mtrR*$_{-56}$ mutant, with only *mtrR*$_{-56}$ mutant gonococci recovered from a majority of mice 5 days after inoculation (Table 2) (Kunz *et al*., submitted).

From these studies we conclude that the *gyrA*$_{91/95}$ mutation confers a fitness benefit to *N. gonorrhoeae* that is independent of the MtrC-MtrD-MtrE efflux pump system, but that an additional *parC*$_{86}$ mutation results in a net fitness cost. These data are intriguing and may help to explain the frequent isolation of Cip^{R} gonococci that also carry *mtrR* promoter or *mtrR* structural gene mutations, which has been interpreted by others as evidence that active efflux through the MtrC-MtrD-MtrE pump is another mechanism of fluoroquinolone resistance in *N. gonorrhoeae* (Dewi *et al*., 2004; Vereshchagin *et al*., 2004). The fact that we found no difference in the Cip MICs of the *gyrA*$_{91/95}$ versus *gyrA*$_{91/95}$*, mtrR*$_{-56}$ mutants or of *gyrA*$_{91/95}$, *parC*$_{86}$ versus *gyrA*$_{91/95}$*, parC*$_{86,}$ *mtrR*$_{-56}$ mutants (Kunz *et al*., submitted), is strong genetic evidence that *mtr* mutations do not contribute to Cip^{R} in *N. gonorrhoeae*. Instead, the prevalence of Cip^{R} *mtr* strains may reflect increased microbial fitness conferred by these mutations.

It is important to remember that while mutations in both *gyrA* and *parC* led to reduced fitness in the mouse model, compensatory mutations may occur in nature that restore fitness while maintaining high-level Cip^{R}. There is much evidence that fitness compensation can occur in bacteria without loss of antibiotic resistance (Balsalobre *et al*., 2011; Bjorkholm *et al*., 2001; Bjorkman *et al*., 1998; Giraud *et al*., 1999; Komp Lindgren *et al*., 2005; Marcussen *et al*., 2009; Nagaev *et al*., 2001). In support of this possibility for QRNG, we have observed that while Cip^{R} gonococci were outcompeted by Cip^{S} (wild-type) or Cip^{I} bacteria in a majority of mice tested, only Cip^{R} gonococci were recovered from some mice (10-17%) as infection progressed in each of several experiments (Figure 3). To further investigate this observation, we analyzed Cip^{R} bacteria isolated on day 5 in pure culture from a mouse inoculated with a mixture of Cip^{I} (*gyrA*$_{91/95}$*, mtrR*$_{-56}$) and Cip^{R}

(*gyrA*$_{91/95}$, *parC*$_{86}$, *mtrR*$_{-56}$) mutants. Interestingly, these Cip^{R} bacteria grew better than the Cip^{I} and Cip^{R} strains used to inoculate the mouse and the Cip^{S} *mtr* parent of the Cip^{I} and Cip^{R} strains. Unlike either of these strains, the *in vivo*-selected Cip^{R} mutant had a wild type *mtr* locus and a *gyrA* allele that was predicted to encode a leucine instead of phenylalanine residue at position 91 (Leu91) (Kunz *et al*., submitted). We conclude that one or more compensatory mutations occurred during infection that allowed highly Cip^{R} gonococci to out-compete Cip^{I} bacteria *in vivo*.

The basis for the reported *in vivo* fitness benefit shown by *gyrA* mutants in *N. gonorrhoeae* or *C. jejuni* (Luo *et al*., 2005) is not known. As topoisomerase mutations are accompanied by alterations in supercoiling (Bagel *et al*., 1999; Luo *et al*., 2005), changes in the expression of genes important for colonization, growth on mucosal surfaces, or evasion of host defenses are one possible explanation (Luo *et al*., 2005; Zhang *et al*., 2006). It is also possible that the *in vivo* fitness benefit exhibited by *gyrA*$_{91/95}$ mutants in *N. gonorrhoeae* is due to secondary mutations that were selected to compensate for alterations in GyrA as proposed by Marcusson *et al.* to explain the increased fitness of *gyrA* mutants of *E. coli* in a urinary tract infection model (Marcusson *et al*., 2009). The *E. coli* mutants tested in this study showed various degrees of fitness costs *in vitro*, however, and thus it is reasonable to assume that one or more compensatory mutation would be needed to promote fitness *in vivo*. In contrast, while *gyrA* mutations are associated with increased *in vivo* fitness in *C. jejuni* (Luo *et al*., 2005) and *N. gonorrhoeae* (Kunz *et al*., submitted), these mutations do not confer a significant growth cost *in vitro*; therefore, secondary mutations that restore growth may not be required for full fitness *in vivo*. Additionally, while not definitive evidence that *gyrA* mutations alone are responsible for

the fitness we observe in the mouse model, we recently demonstrated that *gyrA*$_{91/95}$ mutations are accompanied by a pronounced fitness benefit in two other *N. gonorrhoeae* strains, and that this benefit was detected within one day of infection (Jonathan A. D'Ambrozio & Ann E. Jerse, unpublished observation).

Identification of the mechanism by which *gyrA* mutations enhance gonococcal fitness during experimental murine infection is important as it may reveal new and interesting facets of gonococcal pathogenesis. Additionally, our data suggest Cip^{I} strains may serve as a reservoir for Cip^{R} in *N. gonorrhoeae* since a single step mutation in *parC* is all that is then required for high-level resistance. We postulate the following scenario by which this may occur. First, low levels of antibiotic pressure due to fluoroquinolone treatment for other infections or self-medication, selects for Cip^{I} strains. Cip^{I} strains are then maintained within sexual networks, or even flourish, due to the fitness benefit conferred by the *gyrA*$_{91/95}$ mutations. Highly Cip^{R} strains would not flourish, possibly due to the more severe growth defect construed by mutation in *parC*$_{86}$ or the possibility that the *parC*$_{86}$ mutation or the combination of the *parC*$_{86}$, *gyrA*$_{91/95}$ mutations may have a negative impact on the expression of genes important for survival *in vivo*. However, some Cip^{R} gonococci will be selected due to compensatory mutations that restore fitness while maintaining high level Cip^{R}. Continued study of the frequency and nature of compensatory mutations that allow maintenance of high level Cip^{R} is important for understanding the spread of QRNG.

4. Conclusion

Antibiotic resistance expressed by many of the bacterial pathogens that infect humans represents one of the most important public health challenges for clinical medicine in the 21st century. During the early years of the antibiotic era of medicine (circa. 1945-1950) it became clear to physicians that antibiotic treatment failures were frequently the result of the infecting bacteria being resistant to the antibiotic being used; indeed, penicillinase-producing strains of *Staphylococcus aureus* were recognized and became wide-spread soon after penicillin was introduced as a therapeutic agent in 1943 (Bud, 2007). As the antibiotic era progressed and more antibiotics became available, disturbing reports of treatment failures became more prevalent. Fortunately, researchers trained in microbial physiology and bacterial genetics undertook studies to learn the mechanisms used by bacteria to resist a given antibiotic. These early investigators soon learned that while an antibiotic resistant strain had an advantage over a susceptible strain in the presence of the antibiotic in question, the resistance mechanism frequently came at a cost in the absence of the antibiotic. Thus, in the absence of the selective pressure brought by the antibiotic, the resistant strain frequently grew slower *in vitro* and in model systems of infection (cell culture or animals). However, for some resistance mechanisms, there was little if any cost when compared to a sensitive, but otherwise isogenic strain. The resulting dogma from this work was that antibiotic resistance in the absence of selective pressures could be costly for bacteria. In this case, removing the selective pressure would result in the evolution of more susceptible strains that would have an advantage in the community. By and large, this has not been the case (Anderson & Hughes, 2010).

Less clear, however, was whether in the absence of selective pressure, a resistant strain would have a fitness benefit during an infection over a sensitive counterpart. In this respect, the report of Luo *et al*. (2005) dealing with the increased fitness of a ciprofloxacin resistant strain of *C. jejuni* over a sensitive parent strain *in vivo* was a "game-changer" for antibiotic resistance researchers. Briefly, it forced us to consider the rather scary possibility that a mechanism of antibiotic resistance can actually enhance the ability of a pathogen to survive in the community. This possibility has a number of important implications for our understanding of bacterial pathogenesis and bacterial infections that should be considered. First, are there "antibiotic substitutes" *in vivo* that the resistance mechanism recognizes, allowing the resistant strain to out-compete the sensitive strain? Might these "host antibiotics" provide the selective pressure in the community? This is certainly likely for the fitness benefit imparted to those *N. gonorrhoeae* strains that over-expresses the Mtr efflux pump system. This pump, along with similar pumps produced by other Gram-negatives (Shafer *et al*., 2010), recognizes host antimicrobials (e.g., antimicrobial peptides) in addition to antibiotics such as beta-lactams and macrolides. In this context, efflux pump inhibitors (Lomovskaya & Bostian, 2006) may have clinical use as they would increase bacterial susceptibility to classical antibiotics as well as host antimicrobials. A second issue that requires further investigation is whether a resistance mechanism has secondary effects on the physiology of the resistant strain that results in an advantage during infection. This hypothesis may help to explain why $gyrA_{91/95}$ mutations can enhance the fitness of Cip^I strains of *N. gonorrhoeae*. Hopefully, ongoing transcriptional profiling studies that compare isogenic Cip^I and Cip^S strains will provide insights that will help us to understand fitness

differences. A third point merits consideration: stable mutations that decrease bacterial susceptibility to a given antibiotic, but not to an extent that it pushes them across the MIC breakpoint, may be more advantageous for the bacteria than previously thought. In this respect, as emphasized throughout this text, mutations in *mtrR* or *gyrA* provide gonococci with a fitness advantage *in vivo*, but do not push them across the MIC breakpoint for either beta-lactams or quinolones, respectively. Importantly, both are necessary for clinically significant levels of resistance imparted by other mutations. Accordingly, strains bearing *mtrR* and/or *gyrA* mutations may not only be more fit during infection, but also more likely to subsequently develop clinical resistance to beta-lactams and quinolones than fully sensitive strains. This issue is of greater urgency now because the gonococcal strain that caused a ceftriaxone-resistant infection in Japan is an *mtrR* mutant (Ohnishi *et al.*, 2011) even though the mutation by itself has little impact on the level of beta-lactam resistance (Veal & Shafer, 2003). Finally, if a resistance mutation enhances fitness and is stably maintained in a bacterial pathogen for years, it may be yet another reason why antibiotic re-cycling after extended absence from the treatment regimen may not be a viable option to combat the emergence and spread of antibiotic resistant bacteria.

We have used *N. gonorrhoeae* as a model human pathogen for studies on how bacterial fitness can be impacted by mechanisms of antibiotic resistance. Having been intimately associated with humans for thousands of years, it is of no surprise that the gonococcus has evolved novel ways to evade or resist the multitude of toxic agents that it encounters during infection. The continued emergence of strains expressing decreased susceptibility or even clinical resistance to frontline antibiotics used today (e.g., ceftriaxone) in therapy emphasizes the remarkable adaptive ability of this pathogen. The

examples provided herein with the gonococcus emphasize that mechanisms of antibiotic resistance can enhance bacterial virulence, as defined by increased *in vivo* fitness. Understanding the processes that lead to increased fitness of the gonococcus (or any other pathogen) due to antibiotic resistance may result in novel strategies that could be used to inhibit bacterial replication *in vivo* directly or indirectly by enhancing the efficacy of the defensive systems of the host that operate locally.

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TABLE 1: Fitness of *mtr* regulatory mutants in mice compared to wild-type. CI: competitive index. Ratio of mutant to wild type CFU (vaginal isolates) divided by mutant to wild type CFU (inoculum).

Genotype	**CI at day 3**
Single bp deletion in *mtrR* promoter	1000
mtr_{120} point mutation	1000
A39T change in DNA binding domain of MtrR	100
mtrA::Km^R	0.005
mtrA::Km^R $mtrR_{1\text{-}53}$	100
mtrA::Km^R $mtrR_{E202G}$	10
mpeR::Km^R	1

Table 2: Fitness of FQ-R mutations in mice compared to wild-type or *mtr* mutant *Gc.* CI: competitive index. Ratio of mutant to wild type CFU (vaginal isolates) divided by mutant to wild type CFU (inoculum).

Genotype	CI at day 3
gyrA$_{91/95}$	5-fold increase; 30-fold increase on day 5 compared to Cip^S wild-type strain
gyrA$_{91/95}$, *parC*$_{86}$	2-fold decrease compared to Cip^S wild-type strain
gyrA$_{91/95}$, *mtr*$_{-56}$	40-fold increase compared to Cip^S *mtr*$_{-56}$ mutant parent strain
gyrA$_{91/95}$, *parC*$_{86}$, *mtr*$_{-56}$	50-fold decrease compared to Cip^S *mtr*$_{-56}$ mutant parent strain

Figure legends

Figure 1. Organization of the *mtr* locus of *N. gonorrhoeae.* Bent arrows mark the *mtrR*, *mtrF*, and *mtrCDE* promoters. *mtrR* and *mtrCDE* are divergently transcribed on opposite strands. Circles represent the transcriptional regulatory proteins MtrR and MtrA. The box represents the location of the expanded sequence. The *mtrR* and *mtrCDE* promoter elements are indicated in the expanded sequence; the dashed box marks the inverted repeat element of the *mtrR* promoter.

Figure 2. Evolution of quinolone resistance in *N. gonorrhoeae.* Quinolone resistance in *N. gonorrhoeae* is a two-step process, beginning with point mutations in the QRDR of *gyrA*, which increase resistance to intermediate levels. Positions 91 and 95 are most often altered, with Ser91Phe and Asn95Asn the most common substitutions. Other substitutions have also been reported. High-level resistance occurs upon mutation of the QRDR of *parC* mutation. *parC* mutations carried by Cip^R gonococci most often cause amino acid substations at position 91, 86, 87 or 88 (Kam *et al*., 2003; Ghanem *et al*., 2005; Morris *et al*., 2009; Ruiz 2001; Starnino *et al*., 2010; Tanaka *et al*., 2000; Trees *et al*., 2001; Vereshchagin *et al*., 2004; Vernel-Paulillac 2009).

Figure 3. The fitness disadvantage of Cip^R gonococci can be overcome by selection for compensatory mutations. Vaginal inoculation of estradiol-treated BALB/c mice with populations of Cip^S or Cip^I gonococci (white) mixed with similar numbers of Cip^R gonococci (black) results in the recovery of a higher proportion of Cip^S or Cip^I CFU, with some mice clearing the Cip^R bacteria. However, in 10-17% of mice tested, high numbers

of only Cip^R CFU were recovered as infection progressed, most likely due to compensatory mutations (Kunz *et al*, submitted).

FIGURE 1

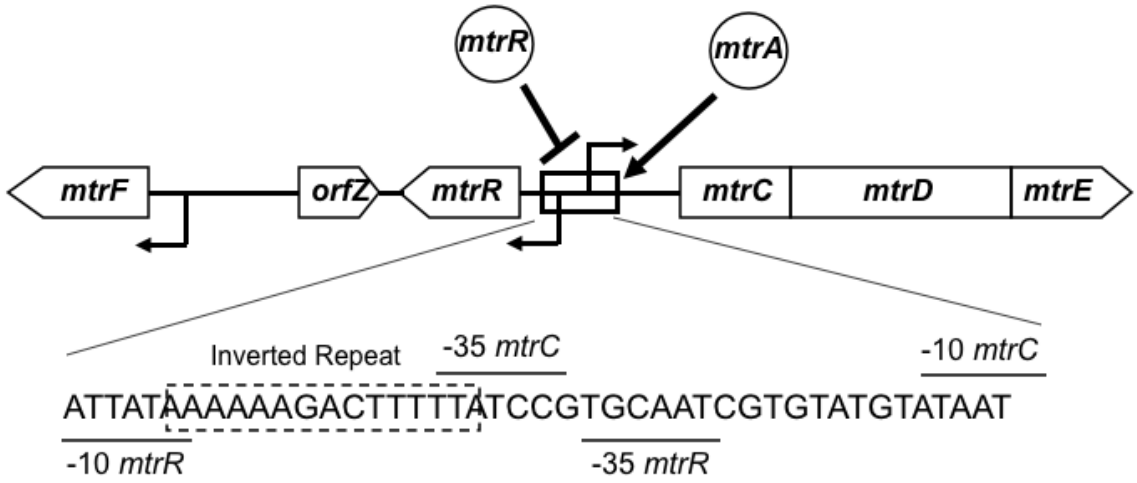

mtrR
mtrA
mtrF
orfZ
mtrR
mtrC
mtrD
mtrE
Inverted Repeat
-35 mtrC
-10 mtrC
ATTATAAAAAAGACTTTTTATCCGTGCAATCGTGTATGTATAAT
-10 mtrR
-35 mtrR

FIGURE 2

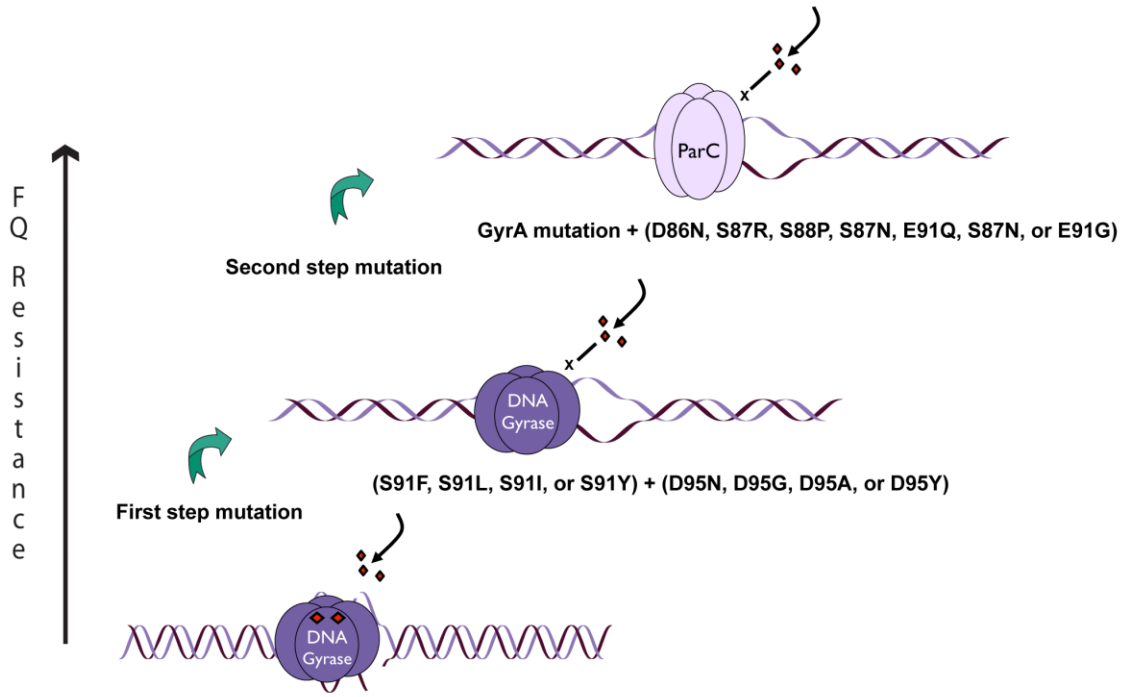
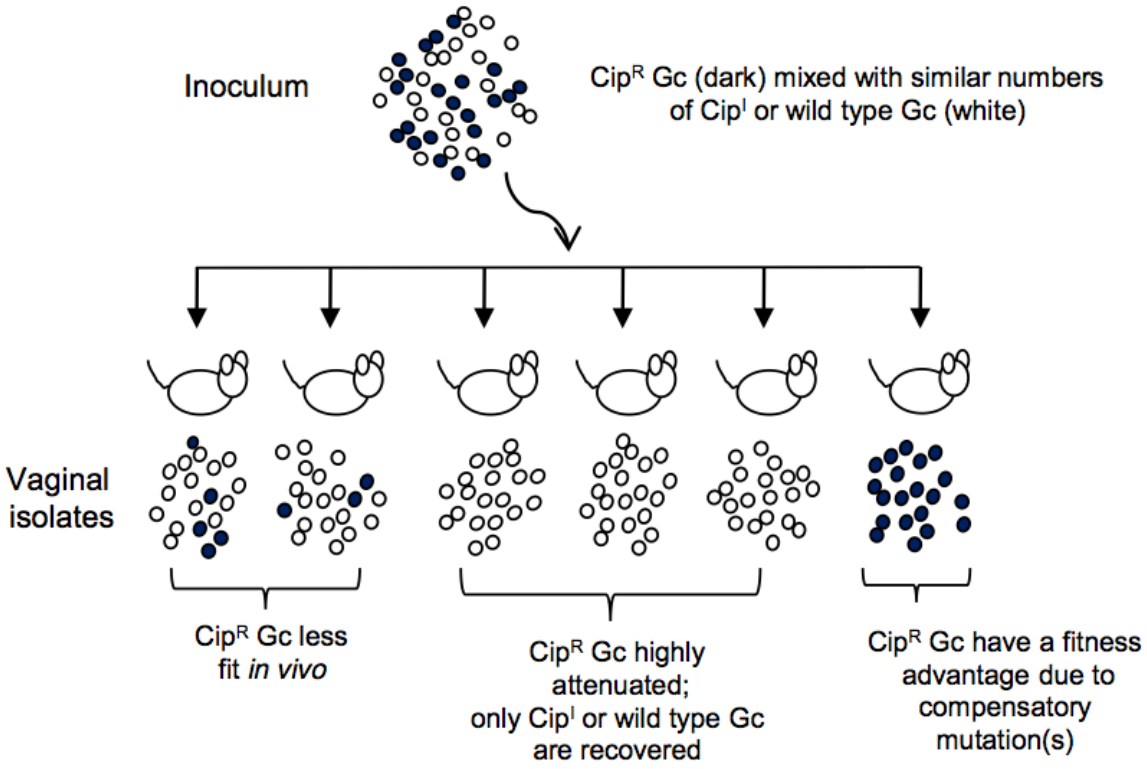

FQ Resistance
ParC
GyrA mutation + (D86N, S87R, S88P, S87N, E91Q, S87N, or E91G)
Second step mutation
DNA Gyrase
(S91F, S91L, S91I, or S91Y) + (D95N, D95G, D95A, or D95Y)
First step mutation
DNA Gyrase

FIGURE 3


Inoculum
Cip^R Gc (dark) mixed with similar numbers of Cip^I or wild type Gc (white)
Vaginal isolates
Cip^R Gc less fit *in vivo*
Cip^R Gc highly attenuated; only Cip^I or wild type Gc are recovered
Cip^R Gc have a fitness advantage due to compensatory mutation(s)

Chapter 3: A novel mechanism of high-level, broad-spectrum antibiotic resistance caused by a single base pair change in *Neisseria gonorrhoeae*

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ABSTRACT

The MtrC-MtrD-MtrE multidrug efflux pump of *Neisseria gonorrhoeae* confers resistance to a diverse array of antimicrobial agents by transporting these toxic compounds out of the gonococcus. Frequently in goncoccal strains, expression of the *mtrCDE* operon is differentially regulated by both a repressor, MtrR, and an activator, MtrA. The *mtrR* gene lies 250 base pairs upstream and is transcribed divergently from the *mtrCDE* operon. Previous research has shown that mutations in the *mtrR* coding region and in the *mtrR-mtrCDE* intergenic region increase levels of gonococcal antibiotic resistance and *in vivo* fitness. Recently, a C to T transition mutation 120 base pairs upstream of the *mtrC* start codon, termed mtr_{120}, was identified in strain MS11 and shown to be sufficient to confer high levels of antimicrobial resistance when introduced into strain FA19. Here, we report that this mutation results in a consensus -10 element, and that its presence generates a novel promoter for *mtrCDE* transcription. This newly generated promoter was found to be stronger than the wild type promoter and does not appear to be subject to MtrR repression or MtrA activation. Although rare, the mtr_{120} mutation was identified in an additional clinical isolate during sequence analysis of antibiotic resistant strains cultured from patients with gonococcal infection. We propose that *cis*-acting mutations can develop in gonococci that significantly alter the regulation of the *mtrCDE* operon and result in increased resistance to antimicrobials.

IMPORTANCE

Gonorrhea is the second most prevalent bacterial sexually transmitted infection and a worldwide public health concern. As there is currently no vaccine against *N. gonorrhoeae*, appropriate diagnostics and subsequent antibiotic therapy remains the

primary means of infection control. However, the effectiveness of antibiotic treatment is constantly challenged by emergence of resistant strains, mandating a thorough understanding of resistance mechanisms to aid the development of new antimicrobial therapies and genetic methods for antimicrobial resistance testing. This study was undertaken to characterize a novel mechanism of antibiotic resistance regulation in *N. gonorrhoeae*. Here, we show that a single base pair mutation generates a second, stronger promoter for *mtrCDE* transcription that acts independently of the known efflux system regulators and results in high-level antimicrobial resistance.

INTRODUCTION

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection, gonorrhea, is a Gram-negative diplococcus and a strict human pathogen. Clinical isolates of *N. gonorrhoeae* frequently exhibit high levels of antimicrobial resistance mediated by multiple mechanisms, including active efflux of antimicrobials by four known efflux pumps (1-5). The MtrC-MtrD-MtrE efflux pump is a well-characterized system that recognizes and exports a wide variety of antimicrobial agents, including macrolide and β-lactam antibiotics, detergents, and host antimicrobial factors (1, 6). Transcription of the *mtrCDE* operon is differentially regulated by a repressor, MtrR, and an activator, MtrA (7-9). The *mtrR* gene is located 250 base pairs upstream of and is transcribed divergently from *mtrCDE* (3). MtrR represses expression of *mtrCDE* via binding of two homodimers to pseudo-direct repeats within the *mtrCDE* promoter (10); the MtrR helix-turn-helix (HTH) DNA-binding motif resides between residues 32 – 53 (7, 11).

A variety of mutations in *mtrR* and in the *mtrR-mtrCDE* intergenic region have been identified in antibiotic resistant gonococcal strains recovered from outbreak investigations (12). Strain MS11, originally isolated in the 1960s from a patient with an uncomplicated cervical infection (13) and since used extensively by many researchers, exhibits higher levels of intrinsic *in vitro* resistance to MtrC-MtrD-MtrE substrates than other laboratory strains (12). Sequence analysis of the MS11 *mtr* locus revealed that MS11 is a natural *mtr* mutant, containing an alanine to threonine substitution at position 39 in the MtrR DNA binding domain, as well as a novel C to T transition mutation located 120 base pairs upstream of the *mtrC* start codon (mtr_{120}) (12). Introduction of the mtr_{120} mutation into laboratory strain FA19 yielded one of the highest reported levels of

MtrC-MtrD-MtrE-based antimicrobial resistance (12). Additionally, this mutation increased resistance to the host-derived antimicrobial compounds progesterone and CRAMP-38, the murine homologue of the human cathelicidin LL-37, suggesting that the mtr_{120} mutation facilitates resistance to host defense mechanisms (12). In agreement with this hypothesis, the mtr_{120} mutation increased *in vivo* fitness in a female mouse model of lower genital tract infection by nearly three logs compared to wild-type strain FA19 during competitive infection in the absence of antibiotic treatment (12).

Here, we demonstrate that the mechanism of mtr_{120}-based antimicrobial resistance is the generation of a consensus -10 element (14) that acts as a second, stronger promoter for *mtrCDE* transcription, resulting in substantially increased pump expression and enhanced resistance to antimicrobials. This promoter appears to function independent of MtrR and MtrA regulation. Additionally, we report that, while rare compared to other mutations that enhance *mtrCDE* expression, the mtr_{120} mutation was found in an additional multi-drug resistant strain that has been included in the 2008 WHO *Neisseria gonorrhoeae* reference strain panel (15).

RESULTS

Analysis of the mtr_{120} locus in MS11 revealed that this C to T transition creates the consensus sequence for a -10 element (TATAA**T**) (Fig. 1) (14). A near-consensus -35 element sequence (TTGAGA) was located upstream of the potential -10 element; however, this putative -35 hexamer was separated from the potential -10 element by 24 base pairs rather than the optimal 17 base pairs (14). To determine if this -10 element could act to promote transcription, primer extension of *mtrC* was performed using total

RNA from FA19 and DW120 cultures at mid-log phase (Fig. 2). As expected, primer extension of RNA from FA19 yielded a single *mtrC* transcript, which mapped to the previously identified transcription start site (Fig. 1) (1). The presence of the mtr_{120} mutation, however, resulted in a second, shorter *mtrC* transcript, that was more intense than the wild-type *mtrC* transcript, suggesting a higher concentration of the shorter transcript. Importantly, the start site for this second transcript mapped to a site 7 base pairs downstream of the -10 consensus sequence generated by mtr_{120}, a reasonable distance to suggest that this -10 sequence could act to promote expression of this transcript.

To rule out the possibility that the mutant transcript was a result of differential mRNA processing due to the mtr_{120} mutation, β-galactosidase assays were carried out with FA19 containing a promoterless *lacZ* gene translationally fused to either the entire *mtrC* promoter region ($mtrC_F$-*lacZ*) or a truncated region lacking the wild-type *mtrC* promoter ($mtrC_T$-*lacZ*) (Fig. 3A) (16). The mtr_{120} $mtrC_F$-*lacZ* fusion showed significantly higher β-galactosidase activity than the wild-type $mtrC_F$-*lacZ* fusion. As expected, the wild-type $mtrC_T$-*lacZ* fusion showed no β-galactosidase activity. However, the mtr_{120} $mtrC_T$-*lacZ* fusion showed levels of β-galactosidase activity significantly higher than that of wild-type $mtrC_F$-*lacZ* fusion, demonstrating that the -10 element generated by mtr_{120} mutation is sufficient for transcription in the absence of the wild-type promoter and is stronger than the wild-type promoter, in agreement with the more intense band seen in the primer extension from mtr_{120}. As further verification, we prepared *mtrC-lacZ* translational fusions containing a mutated -10 sequence (TATAAT to TGTCAC) of the wild-type *mtrCDE* promoter ($mtrC_{mut}$-*lacZ*). We observed that *mtrC-lacZ* expression was

abrogated when the wild-type promoter was mutated and the sequence at position 120 was wild-type (Fig. 3B). However, *mtrC-lacZ* expression remained high when the mtr_{120} mutation was present. Taken together, we propose that the mtr_{120} mutation defines a new and highly active promoter for *mtrCDE* transcription.

To characterize the resistance phenotype of the mtr_{120} mutation and determine if MtrR or MtrA, the known regulators of *mtrCDE* wild-type promoter, affect resistance levels conferred by this mutation, MICs were determined for antimicrobials against strains FA19, DW120, and *mtrR* or *mtrA* knock-out derivatives of each strain (genotypes described in Table 1; results shown in Table 2). In agreement with the increased mRNA levels detected in the primer extension experiment and high levels of β-galactosidase activity from the *mtrC-lacZ* fusions carrying the mtr_{120} mutation, the presence of mtr_{120} resulted in increased resistance to the MtrC-MtrD-MtrE pump substrates erythromycin (Erm), rifampin (Rif), crystal violet (CV), and Triton X-100 (Tx-100). In contrast, the presence of mtr_{120} did not affect resistance to the non-pump substrate kanamycin (Km) in strains isogenic for *mtrR* or *mtrA*; increased kanamycin resistance in *mtrR* and *mtrA* knock-out strains is due to the presence of the *aphA-3* cassette within these genes, and differences in resistance between strains with *mtrR* disruption and *mtrA* disruption may be attributed to differences in promoter strength for these genes. Importantly, the absence of MtrR and MtrA did not affect resistance levels to pump substrates in strains with mtr_{120}, suggesting that, unlike the wild-type promoter (17), the mtr_{120} promoter is not subject to MtrR or MtrA regulation.

To verify that the observed increased antimicrobial resistance was due to increased levels of MtrC-MtrD-MtrE pump production, Western blot analysis was

conducted to determine the effect of the *mtr*$_{120}$ mutation on MtrE production. In agreement with the results of the MIC assays, strains bearing the *mtr*$_{120}$ mutation produced much greater amounts of MtrE than strains with a wild-type sequence at this locus (Fig. 4). Additionally, the absence of MtrR and MtrA did not appear to affect MtrE levels in the presence of *mtr*$_{120}$, further suggesting that these regulators do not act on the promoter generated by this mutation.

The *mtr*$_{120}$ mutation was originally identified in strain MS11 (12), which is a commonly utilized laboratory strain that has been used in gonococcal research for many years. Thus, to determine if the *mtr*$_{120}$ mutation is present in strains isolated during recent clinical infection, as well as to compare its frequency to other *mtr* locus mutations in a clinical setting, the *mtrR* gene and the *mtrR-mtrC* intergenic region of 113 clinical isolates and 8 WHO reference strains were selected and sequenced based on their MIC to azithromycin. The azithromycin MIC range for the sequenced *N. gonorrhoeae* isolates, including the eight 2008 WHO reference strains (15), was 0.125 to 8 µg/ml, with 76% of strains found to be resistant to azithromycin by EUCAST standards (MIC >0.5 µg/mL). Among these strains, only one, the 2008 WHO reference strain WHO L (15), was found to contain the *mtr*$_{120}$ mutation. This strain was also found to contain the previously described G45D mutation in the HTH DNA-binding domain of MtrR (11). In contrast to the low frequency of the *mtr*$_{120}$ mutation, the previously defined single nucleotide (A) deletion in the 13 bp inverted repeat located between the -10 and -35 sequences of the *mtrR* promoter was found in 86 isolates (71%), of which 8 (7%) also had the G45D amino acid alteration in the coding region of *mtrR* (7). Moreover, five additional mutations in the promoter region of *mtrR* were found in a total of 12 isolates. Alteration

of G45 (G45D [n=11] and G45S [n=3]) in the *mtrR* coding region alone was present in 14 isolates (12%). Other frequently occurring amino acid alterations found in the coding region of *mtrR* were: A86T found in 109 isolates (90%), Y105H in 25 isolates (21%), D79N in nine isolates (7%), A39T in seven isolates (6%), and L99G/H in three isolates (2%) (Table 3).

DISCUSSION

The mtr_{120} mutation is novel in its mechanism of providing antimicrobial resistance in that it creates an entirely new promoter for *mtrCDE* transcription and acts independently of the MtrR and MtrA transcriptional regulatory proteins. To our knowledge, this is the first report of such a mechanism of efflux pump regulation. Two precedents exist in *N. gonorrhoeae*, however, for upregulation of efflux pumps through *cis*-acting point mutations at existing promoters. First, expression of *norM*, a gene encoding a multidrug and toxic compound extrusion family exporter that contributes to quaternary ammonium compound, norfloxacin, and ciprofloxacin resistance, is upregulated by point mutations in the -35 hexamer (C to T) of the *norM* promoter or in the ribosome binding site (A to G) (5). Second, a point mutation in the -10 hexamer (G to T) of the promoter for *macAB*, which encodes an ABC transporter family efflux system that contributes to macrolide resistance, results in increased expression of this efflux pump (4). Like the mtr_{120} mutation, these point mutations bring their respective promoter elements closer to the consensus sequences (TTGACA for the -35; AGGAGG for the ribosome binding site), thereby enhancing recognition by RNA polymerase or, in the case of *norM*, the ribosome (14, 18). The mtr_{120} mutation is novel, however, in that there is no

expression from the wild type sequence at this locus, and the consensus -10 element generated by the *mtr*$_{120}$ mutation acts as a second, independent promoter for *mtrCDE* transcription.

The *mtr*$_{120}$ mutation appears to offer the gonococcus a relatively simple and convenient mechanism of antibiotic resistance. The change of this single base pair significantly increases *mtrCDE* expression and confers high-level antimicrobial resistance without disrupting other components of the efflux system, including the regulators MtrR and MtrA. Both MtrR and MtrA are global regulators in *N. gonorrhoeae*, controlling a multitude of genes outside of the *mtrCDE* operon, many of which are important for virulence and *in vivo* fitness (19, 20). Thus, the ability to upregulate *mtrCDE* without affecting the regulation of other genes needed for infection and survival would be a highly efficient and minimally disruptive mechanism of developing antimicrobial resistance. It is therefore somewhat surprising that this mutation is so rare, especially when compared to the frequency of *mtrR* promoter and coding region mutations in the clinical isolates examined in this study.

The reason for the rarity of the *mtr*$_{120}$ mutation is thus a matter of speculation. The production of efflux pumps is an energy-expensive process, and it is therefore possible that the high levels of MtrC-MtrD-MtrE production stimulated by this mutation may stress the gonococcus, resulting in slower or defective growth. In this respect, Eisenstein and Sparling noted that a single base pair deletion in the inverted repeat in the *mtrR* promoter, a mutation which also confers high-level antibiotic resistance through increased transcription of *mtrCDE*, results in a slower growth rate *in vitro* (7, 21). However, unlike *mtr*$_{120}$, this mutation was recovered with high frequency in the strains

sequenced in this study, and with the more recent finding that MtrR acts as a global regulator in the gonococcus, it is possible that the observed growth defect was at least in part due to lack of *mtrR* expression. Additionally, we have noticed no difference in the growth kinetics *in vitro* between strains carrying mtr_{120} and strains wild-type at this site (data not shown), and Warner *et al.* found that strain DW120 has a fitness advantage *in vivo* over FA19 in a female mouse model of lower genital tract infection (12).

Another possibility to explain the rarity of the mtr_{120} mutation is that the mutational event required for this change occurs infrequently compared to other mutations, particularly the deletion in the inverted repeat. A specific nucleotide change at a single base pair locus is required to generate the mtr_{120} phenotype. However, one of any five base pairs may be deleted in the inverted repeat to cause high-level resistance. Thus, it may be that the mtr_{120} mutation is simply less likely to occur, accounting for its scarcity in the isolates sequenced. Further analysis of the mtr_{120} mutation and its overall effects will be required to elucidate the reason for this mutation's relative infrequency.

It is important to note that the mtr_{120} mutation was originally identified in strain MS11 (12). This strain has been used extensively in the laboratory for studies on neisserial pili (22-25), Opa proteins (26, 27), *in vitro* cell infection (28, 29), antimicrobial resistance (30), and *in vivo* pathogenesis in male volunteers (31-34). The possession of this rare mutation, however, makes MS11 uncommon compared to other gonococcal strains, enhancing its resistance not only to antibiotics, but also to host antimicrobial compounds that are MtrC-MtrD-MtrE substrates, such as the antimicrobial peptide LL-37 (6). MS11 has been found to be more infectious than another commonly studied *N. gonorrhoeae* strain, FA1090, in experimental infection in male volunteers (35), and it is

likely that increased resistance to host antimicrobial compounds due to the *mtr*$_{120}$ mutation plays an important role in this increased infectivity. It is therefore important to consider this mutation when interpreting findings from previous studies using strain MS11, particularly those involving antimicrobial resistance, pathogenesis and human volunteer studies in which virulence factors important in evasion of innate defenses were assessed (35).

It is also important to note that the WHO reference strain WHO L carries the *mtr*$_{120}$ mutation. Although its resistance levels to erythromycin and azithromycin were found to be slightly higher than reference strains with the single nucleotide deletion in the 13 bp inverted repeat of the *mtrR* promoter, WHO L does not contain this deletion and its resistance was thus considered to be attributed to the G45D mutation in the HTH domain of MtrR (15). However, as mutations in the HTH domain of MtrR generally confer only low levels of antimicrobial resistance, it is far more likely that the higher level of azithromycin resistance of WHO L is due to the presence of *mtr*$_{120}$, which is an important factor to consider in its use as a reference strain.

This study demonstrates the significant impact single base pair mutations may have on gene expression and the development of antimicrobial resistance, and characterizes a novel *cis*-regulatory mechanism for efflux pump expression. Sequence analysis of the promoter regions of other efflux pumps, both in *Neisseria* and in other pathogenic organisms, will determine if this mechanism is widely used among pathogens or is unique to *N. gonorrhoeae* and *mtrCDE*. Additionally, the *mtr*$_{120}$ mutation provides a unique opportunity for study of the physiological consequences of efflux pump over-expression on bacterial cells. This single point mutation in a non-coding region allows

over-production of the MtrC-MtrD-MtrE efflux pump without disruption or altered expression of local or global regulatory proteins. Thus, the direct phenotypic consequences of high-level efflux pump production can be examined without the introduction of confounding effects on cell physiology due to altered regulation of other genes, a challenge which has been difficult to overcome. Further study of the mtr_{120} mutation will help advance our understanding of antimicrobial resistance mechanisms as well as elucidate the physiological consequences of efflux pump over-expression on bacterial cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. Gonococci were routinely grown on GCB agar (Difco Laboratories, Detroit, MI) containing defined supplements I and II (36) at 37°C under 4% CO_2, or in GCB broth (Difco Laboratories, Detroit, MI) containing defined supplements I and II and 0.048% (v/v) sodium bicarbonate with shaking at 37°C. *E. coli* DH5α was routinely grown on LB agar or in LB broth (Difco Laboratories, Detroit, MI).

The *N. gonorrhoeae* strains used in this study are described in Table 1. Oligonucleotide primers are listed in Table 4. Strains DW120, KH9, and CR1 were previously described (1, 8, 12). Strain EO1 was constructed by transformation of DW120 with the *mtrR*::Km^R gene from KH9 chromosomal DNA by PCR amplification using primers KH9#10B, which anneals 10 base pairs downstream of the *mtrR* translational start, and CEL1, which anneals 120 base pairs downstream of the *mtrR* translational stop (1). Strain EO2 was constructed by transformation of DW120 with the *mtrA*::Km^R gene

from CR1 PCR amplified using primers C6, which anneals 255 base pairs upstream of the *mtrA* translational stop, and C7, which anneals 232 base pairs downstream of the *mtrA* translational start. PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc, Valencia, CA). Purified products were transformed into FA19 and transformants were selected on GC agar supplemented with 50 μg/mL kanamycin. Transformations were performed as previously described (37). All transformants were confirmed by PCR.

Primer extension of *mtrC*. Total RNA was prepared from gonococci by the method of Baker and Yanofsky (38). Primer extension analysis of *mtrC* was performed using the SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Briefly, 50 μg of total RNA was reverse transcribed using the ^{32}P-5′-end-labeled oligonucleotide primer PEmtrC181, which anneals to the first seven codons of *mtrC* (Table 4). To determine transcriptional start sites, primer extension products were electrophoresed on a 6% sequencing gel alongside reference sequencing reaction products derived from the PEmtrC181 primer using an *mtrCDE* promoter region PCR product amplified using primers mtrC_F and mtrC_R as template. Sequencing was performed using the SequiTherm EXCEL II DNA Sequencing kit (Epicenter Biotechnologies, Madison, WI) following the manufacturer's instructions. The dried gel was exposed to Kodak XAR film overnight at -70°C and developed using a Kodak X-OMAT 1000A film processor.

Construction of *mtrC-lacZ* fusions. Translational *lacZ* fusions were constructed as previously described (16). Briefly, the *mtrC* promoter region from FA19 or DW120 was amplified using primers that introduce a BamH1 restriction site at the end of the PCR

products; primer sequences are listed in Table 4. For all fusions, mtrC_7, which anneals to the first six codons of *mtrC*, was used as the reverse primer. Forward primer mtrC_3 was used to amplify the *mtrC* promoter sequence beginning 239 base pairs upstream of the *mtrC* start codon, encompassing all *mtrC* promoter elements, to make fusions wild-type $mtrC_F$-*lacZ* and mtr_{120} $mtrC_F$-*lacZ*. Forward primer mtrC_4 was used to PCR amplify a region beginning 157 base pairs upstream of the *mtrC* start codon from FA19 or DW120, excluding the previously identified *mtrC* promoter and transcription start site (1), to make fusions wild-type $mtrC_T$-*lacZ* and mtr_{120} $mtrC_T$-*lacZ.* Forward primer PmtrCmut was used to PCR amplify a region beginning 254 base pairs upstream of the *mtrC* start codon from FA19 or DW120 and mutate the -10 of the wild-type *mtrC* promoter from TATAAT to TGTCAC. PCR products were digested with BamH1 and the resulting DNA fragments were inserted into the BamH1 site of pLES94 (16). Recombinant plasmids were transformed into *E. coli* DH5α. Transformants were selected on LB agar containing 100 μg/mL ampicillin. Correct insertion and orientation were confirmed by PCR analysis and DNA sequencing. The plasmids were transformed into FA19 to allow insertion into the chromosomal *proAB* locus. Transformants were selected on GCB agar containing 1 μg/mL chloramphenicol.

Preparation of cell extracts and β-galactosidase assays. Strains containing translational *mtrC-lacZ* fusions were grown overnight on GCB agar plates containing 1 μg/mL chloramphenicol. Cells were scraped, washed once with phosphate buffered saline (pH 7.4), and resuspended in lysis buffer (24 mM Na_2HPO_4, 16 mM NaH_2PO_4, 4 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$). Cells were lysed by repeated freeze-thaw cycles and cell

debris was removed by centrifugation at 9,300×g for 10 min at 4°C. β-galactosidase assays were performed as previously described (39).

Minimum inhibitory concentration assays. The MICs of erythromycin, rifampin, crystal violet, Triton X-100, and kanamycin were determined by two-fold agar dilution assay (36). Strains were grown on GCB agar and resuspended in GCB broth to OD600 of 0.1, and 5 μL of these suspensions were inoculated onto GCB agar plates containing two-fold serial dilutions of antibiotics. Plates were incubated overnight at 37°C under 4% CO_2. Differences in MIC values greater than twofold were considered significant.

Western blot analysis of MtrE expression. Whole cell lysates from late log cultures (approximately 10^8 cells per sample) were run on a 10% SDS-PAGE gel and transferred to nitrocellulose. The membrane was probed with 1:10,000 dilution of rabbit polyclonal antibodies against amino acids 110 – 120 of MtrE (RQGSLSGGNVS) (20). Detection was performed with 1:10,000 dilution of goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) exposed to BCIP and NBT (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

Detection of the mtr_{120} mutation in clinical isolates. All examined clinical *N. gonorrhoeae* isolates (n=113) were obtained at the National Reference Laboratory for Pathogenic Neisseria, Örebro University Hospital, Sweden from 2002 through 2009. Isolates were cultured from patients exposed to infection in many countries worldwide, and were included based on having an azithromycin MIC of ≥0.38 μg/ml. Furthermore, the 2008 WHO *N. gonorrhoeae* reference strains (n=8) were included for examination

and quality control in all assays (15). All isolates were species confirmed by sugar utilization test and/or Phadebact GC Monoclonal Test (Boule Diagnostics AB, Huddinge, Sweden), and preserved as previously described (40).

The MIC (µg/ml) of azithromycin was determined using the Etest method (AB bioMérieux, Solna, Sweden) as previously described (41). The breakpoints used for susceptibility (S), intermediate susceptibility (I) and resistance (R) were according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org); for azithromycin, S≤0.25 µg/ml and R>0.5 µg/ml.

N. gonorrhoeae DNA was isolated in the NorDiag Bullet instrument (Nordiag ASA Company, Oslo, Norway) using the BUGS'n BEADS STI-*fast* kit (Nordiag ASA Company), according to the manufacturer's instructions. To identify putative mutations that cause enhanced expression of the MtrC-MtrD-MtrE efflux pump, the *mtrR-mtrC* intergenic region was amplified in a LightCycler 1.2 real-time PCR system (Roche Molecular Biochemicals, Mannheim, Germany) using the primers mtrC_F, which anneals 11 base pairs upstream of the *mtrR* start codon and 249 base pairs upstream of the *mtrC* start codon (17), and mtrC_R, which anneals 24 nucleotides downstream of the *mtrC* translational start (1). Additionally, the promoter and coding region of *mtrR* was amplified using primers MTR1 and MTR2 (42) as previously described (43). All PCR amplified products were purified prior to sequencing using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Both DNA strands of amplicons were sequenced using the same primers as in the PCR amplification described above using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Genetic Analyzer 3120 (Applied Biosystems)

according to the manufacturer's instructions. Multiple-sequence alignments of nucleotide sequences and amino acid sequences were performed using the BioEdit (Version 5.0.9) software.

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TABLE 1: Strains used in this study.

Strain	Relevant Genotype	Reference
FA19	Wild-type	Sarubbi *et al.*, 1974
DW120	FA19*mtr*$_{120}$	Warner *et al.*, 2008
KH9	FA19 *mtrR*::Km^{R}	Hagman *et al.*, 1995
CR1	FA19 *mtrA*::Km^{R}	Rouquette *et al.*, 1999
EO1	FA19*mtr*$_{120}$ *mtrR*::Km^{R}	This study
EO2	FA19*mtr*$_{120}$ *mtrA*::Km^{R}	This study
RD1	*mtrE*::Km^{R}	Delahay *et al.*, 1997

TABLE 2: Sensitivity to substrates of the MtrC-MtrD-MtrE efflux system

		Minimum Inhibitory Concentration (μg/mL)				
Strain	**Genotype**	**Erm**	**Rif**	**CV**	**Tx-100**	**Km**
FA19	Wild-type	0.25	0.06	0.6	125	30
KH9	FA19 *mtrR*::Km^R	1	0.12	1.25	250	480
CR1	FA19 *mtrA*::Km^R	0.25	0.06	0.6	125	240
DW120	FA19mtr_{120}	2	0.25	2.5	>16,000	30
EO1	FA19mtr_{120} *mtrR*::Km^R	2	0.25	2.5	>16,000	480
EO2	FA19mtr_{120} *mtrA*::Km^R	2	0.25	2.5	>16,000	240

TABLE 3: The MIC (µg/mL) of azithromycin and frequency of recovery of mutations in the promoter region of *mtrR*, the coding region of *mtrR* and the mtr_{120} mutation in *N. gonorrhoeae* clinical isolates from 2002 to 2009 (n=113) and the 2008 WHO *N. gonorrhoeae* reference strains (n=8).

Azithromycin MIC (No. isolates)	*mtrR* promoter mutation						Nonsynonymous mutation in *mtrR* coding region						
	ΔA*	a	b	c	d	e	G45D/G45S	A86T	Y105H	D79N	A39T	L99G/L99H	mtr_{120}
0.125(2)	-	-	-	-	-	-	-	1	-	-	-	-	-
0.25(4)	4	-	-	-	-	-	2	4	-	-	-	-	-
0.38 (14)	7	3	-	-	-	-	2	9	2	5	2	2	-
0.5 (9)	4	2	-	-	-	-	1	7	2	2	1	1	1
0.75 (43)	34	-	1	1	2	-	10	41	12	1	2	-	-
1 (30)	26	1	-	-	-	-	3	29	3	1	2	-	-
1.5 (8)	7	-	-	-	-	-	1	8	1	-	-	-	-
2 (4)	2	-	-	-	-	1	1	4	1	-	-	-	-
4 (2)	2	-	-	-	-	-	-	2	-	-	-	-	-
6 (4)	-	-	1	-	-	-	3	3	3	-	-	-	-
8 (1)	-	-	-	-	-	-	1	1	1	-	-	-	-
Total 121	86	6	2	1	2	1	24	109	25	9	7	3	1

*Deletion of A in 13 bp inverted repeat in the *mtrR* promoter.

[a]Transversion from C to A 19 nucleotides upstream of where the A deletion occurs.

[b]Insertion of one T 10 nucleotides downstream of where the A deletion occurs.

[c]Deletion of one G 34 nucleotides upstream of where the A deletion occurs.

[d]Deletion of one T 21 nucleotides upstream of where the A deletion occurs.

[e]Transversion from A to C in the inverted repeat 3 nucleotides upstream of where the A deletion occurs.

TABLE 4: Oligonucleotide primers used in this study.

Name	Sequence	Purpose
KH9#10B	5'-CCAAAACCGAAGCCTTGAAAACCAA-3'	*mtrR*::Km^R amplification
CEL1	5'-GACAATGTTCATGCGATGATAGG-3'	*mtrR*::Km^R amplification
C6	5'-CGACATTCCATTCGTCTTCCGG-3'	*mtrA*::Km^R amplification
C7	5'-GCCACGACGGAAAATGCGGAG-3'	*mtrA*::Km^R amplification
PEmtrC181	5'-CCTTAGAAGCATAAAAAGCCAT-3'	Primer extension of *mtrC*
mtrC_3	5'-AGTCGGATCCGGTTTGACGAGGG CGGAT-3'	Full *mtrC-lacZ* fusion
mtrC_4	5'-AGTCGGATCCAATTGAGACTGCATCT CAACT-3'	Truncated *mtrC-lacZ* fusion
PmtrCmut	5'-AGTGGATCCGTTTCGGGTCGGTTTGA CGAGGGCGGATTATAAAAAAGACTTTTT ATCCGTGCAATCGTGTATGTAGCACGAA ACCCA-3'	Wild-type *mtrC* promoter -10 mutation for *lacZ* fusion
mtrC_7	5'-AGTCGGATCCGAAGCATAAAAAGCC-3'	Reverse *mtrC* promoter primer
mtrC_F	5'-CGTTTCGGGTCGGTTTGACG-3'	*mtrR-mtrC* intergenic region amplification
mtrC_R	5'-CATCGCCTTAGAAGCATAAAAAGCC-3'	*mtrR-mtrC* intergenic region amplification
MTR1	5'-AACAGGCATTCTTATTTCAG-3'	*mtrR* amplification
MTR2	5'-TTAGAAGAATGCTTTGTGTC-3'	*mtrR* amplification

Figure legends

Figure 1. The *mtr* locus in *Neisseria gonorrhoeae*. A. Organization of the *mtr* locus. Bent arrows mark the *mtrR* and *mtrCDE* promoters (P). *mtrR* and *mtrCDE* are divergently transcribed on opposite strands. The location of the mtr_{120} mutation and *mtrC-lacZ* fusion start sites are indicated. The hatched box represents the location of the expanded sequence. **B.** Sequence of the *mtrR-mtrCDE* intergenic region. The previously characterized *mtrR* and *mtrCDE* promoter elements, the consensus -10 sequence generated by the mtr_{120} mutation, and the putative -35 element for the mtr_{120} promoter are indicated in the expanded sequence. The transcriptional start site from the previously characterized *mtrCDE* promoter is marked by single cross; the transcription start point from the mtr_{120} promoter is marked by a double cross.

Figure 2. Primer extension analysis of *mtrC* from FA19 and DW120. Primer extension products were generated using an *mtrC*-specific oligonucleotide (Table 4) hybridized to 50 μg of total RNA harvested from each strain. The DNA sequence was produced using the same oligonucleotide and is complementary to the mRNA. The wild type and mutant sequences at the mtr_{120} locus are expanded, with the mutated nucleotide in bold.

Figure 3. Expression of β-galactosidase from the mtr_{120} locus. The β-galactosidase activities per mg total protein in cell extracts of FA19 containing translational *mtrC-lacZ* fusions are shown. Assays were performed in triplicate. Error bars represent 1 standard deviation. Asterisks correspond to a *p* value (Student's t test) <0.01. **A.** The mtr_{120} locus

is sufficient for *mtrC-lacZ* expression in the absence of the wild-type promoter. **B.** The mtr_{120} locus promotes *mtrC-lacZ* expression when the wild-type *mtrC* promoter is inactivated.

Figure 4. Expression of MtrE by wild-type and mtr_{120} strains. Western blot analysis of whole cell lysates from late-log phase cultures was conducted using polyclonal rabbit MtrE-specific antibodies followed by goat α-rabbit IgG-alkaline phosphatase. Strain RD1 (44) contains a Km^R insertion in *mtrE* and was used as a negative control. The total protein from all strains was equally loaded, as assessed by Coomassie blue stain on a separate SDS PAGE gel (data not shown).

FIGURE 1

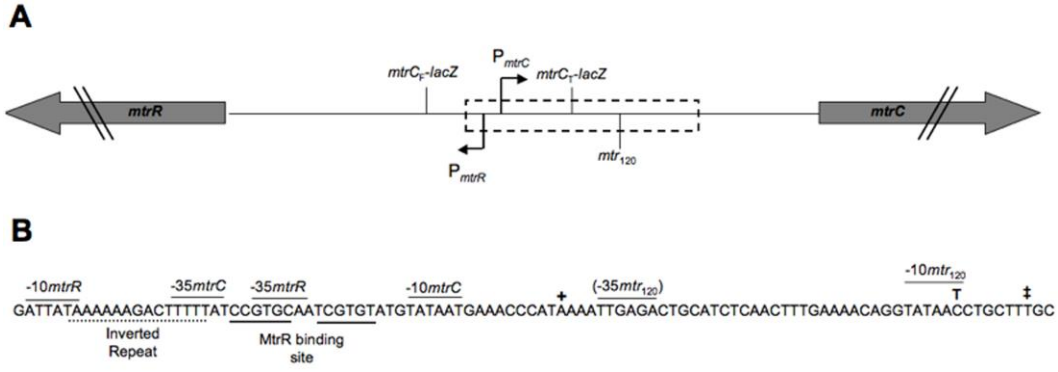

A
mtrR
mtrC
mtrC_F-lacZ
P_mtrC
mtrC_T-lacZ
mtr_120
P_mtrR
B
-10mtrR
-35mtrC
-35mtrR
-10mtrC
(-35mtr_120)
-10mtr_120
T
‡
GATTATAAAAAAGACTTTTTATCCGTGCAATCGTGTATGTATAATGAAACCCATAAAATTGAGACTGCATCTCAACTTTGAAAACAGGTATAACCTGCTTTGC
Inverted Repeat
MtrR binding site

FIGURE 2

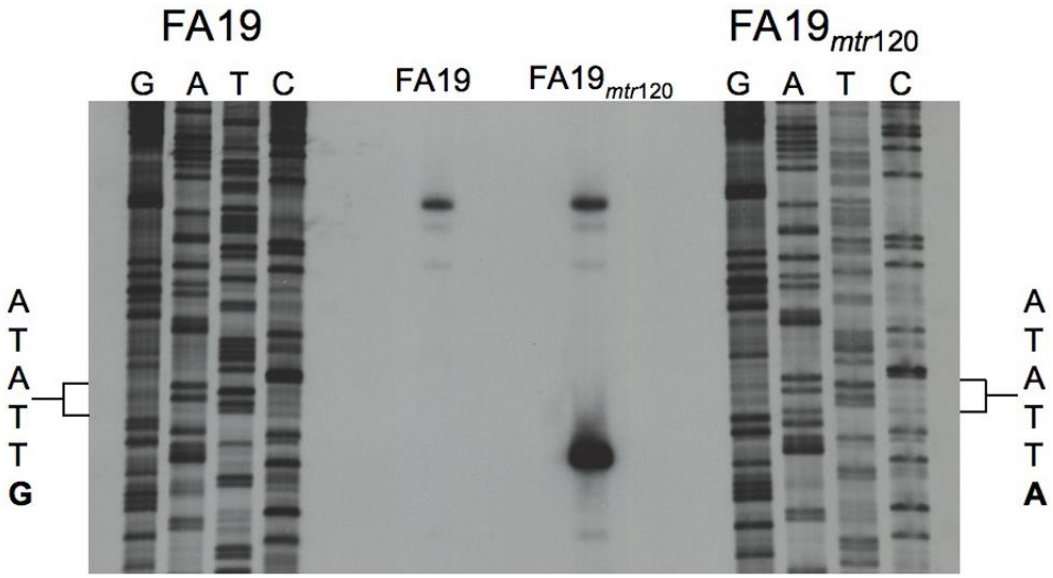


FIGURE 3

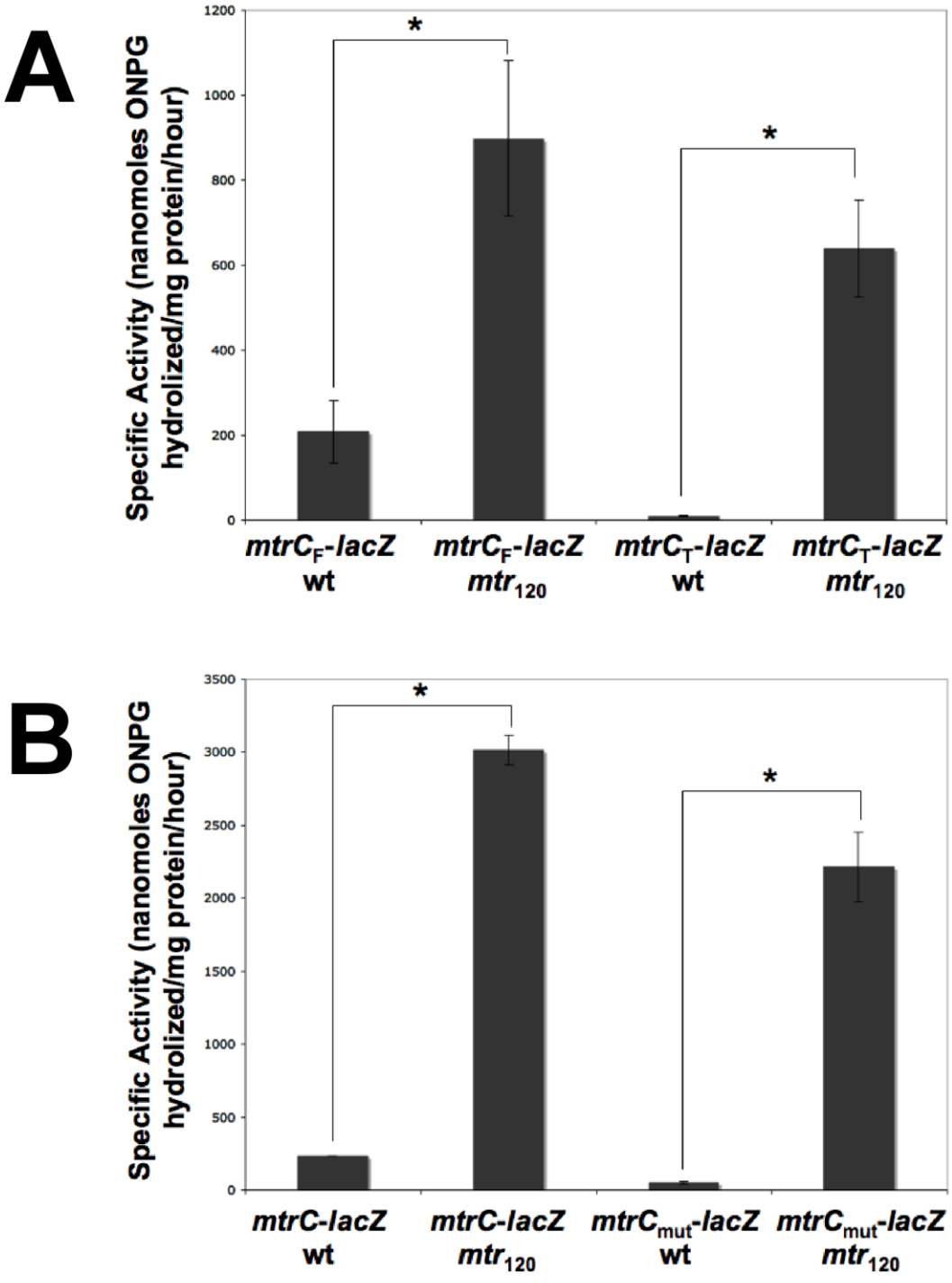
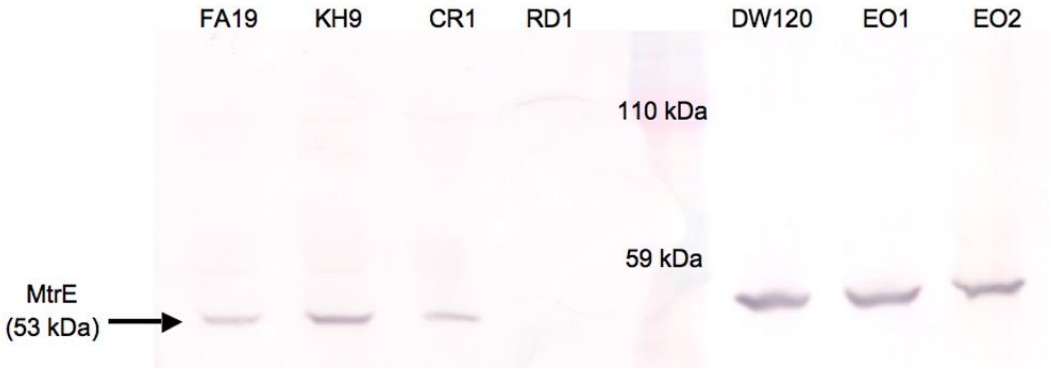

A
Specific Activity (nanomoles ONPG hydrolized/mg protein/hour)
1200
1000
800
600
400
200
0
*
*
mtrC_F-lacZ wt
mtrC_F-lacZ mtr_120
mtrC_T-lacZ wt
mtrC_T-lacZ mtr_120
B
Specific Activity (nanomoles ONPG hydrolized/mg protein/hour)
3500
3000
2500
2000
1500
1000
500
0
*
*
mtrC-lacZ wt
mtrC-lacZ mtr_120
mtrC_mut-lacZ wt
mtrC_mut-lacZ mtr_120

FIGURE 4



Chapter 4: Identification of a regulatory protein (GepR) in *Neisseria gonorrhoeae* required for antimicrobial resistance

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ABSTRACT

The MtrCDE multidrug efflux pump of *Neisseria gonorrhoeae*, which confers resistance to a diverse array of hydrophobic antimicrobial agents, is tightly regulated by a transcriptional repressor, MtrR, and an inducible transcriptional activator, MtrA. The *mtrR* gene lies 250 base pairs upstream and is transcribed divergently from the *mtrCDE* operon. Isolates of *N. gonorrhoeae* demonstrating clinically significant levels of resistance to MtrCDE substrate antimicrobials often have mutations within the *mtrR* gene or the *mtrR-mtrCDE* intergenic region that result in over-production of the MtrCDE efflux pump. One such mutation is a C-to-T transition mutation 120 base pairs upstream of the *mtrC* start codon, termed mtr_{120}, that generates a second highly active promoter for *mtrCDE* transcription. As the mtr_{120} promoter acts outside of MtrR and MtrA regulation, this mutation offers a unique opportunity to examine the cellular effects of efflux pump overexpression on the gonococci without deletion or mutation of *mtrR* and the corresponding perturbation in expression of other MtrR target genes. A transcriptome comparison between a strain bearing the mtr_{120} mutation and its isogenic wild-type parent revealed global transcriptional changes occur in the gonococcus when MtrCDE is produced at high levels. Most importantly, this transcriptional profiling revealed a gene positioned downstream of *mtrCDE* that encodes a transcriptional regulatory protein in the GntR/FadR family required for antimicrobial resistance mediated by the MtrCDE efflux pump. Herein, we report that loss of this putative regulatory protein, termed GepR (Gonococcal efflux pump gene Regulator), can substantially decrease expression of the *mtrCDE* operon. Importantly, loss of GepR can also reverse resistance of gonococci, including strains from patients who failed antibiotic therapy, to antimicrobial substrates

of the MtrCDE efflux. We propose that GepR may serve as a novel target for drug development to combat the increasing problem of antibiotic resistance expressed by gonococci.

IMPORTANCE

Gonorrhea poses a significant global public health concern, as it is the second most prevalent sexually transmitted bacterial infection. The lack of a vaccine and widespread antibiotic resistance expressed by geographically disperse strains complicate control of infection. The recent emergence of *N. gonorrhoeae* strains resistant to third-generation cephalosporins presents a significant threat to the effectiveness of antibiotic therapy. A thorough understanding of resistance mechanisms is imperative in facilitating the development of new drugs and the discovery of methods to overcome resistance. Bacterial multidrug efflux pumps play a significant role in antimicrobial resistance in numerous pathogens; thus, study of the action of these pumps and the physiological consequences of their expression could reveal novel drug targets. This study was undertaken to examine the cellular response of *N. gonorrhoeae* to efflux pump overexpression. Here, we show that high-level production of MtrCDE affects the expression of a novel gene encoding a putative transcriptional regulatory protein required for expression of resistance to β-lactam and macrolide antibiotics. We found that loss of this regulatory protein can reverse antibiotic resistance expressed by clinical isolates, suggesting that it may be a novel target for drug therapy.

INTRODUCTION

Neisseria gonorrhoeae is a Gram-negative diplococcus and a strict human pathogen that causes the sexually transmitted infection gonorrhea. *N. gonorrhoeae* presents a significant global health burden, as it is estimated that over 106 million cases occur annually worldwide, with an estimated 600,000 cases occurring in the United States (3, 52). Unfortunately, *N. gonorrhoeae* has efficiently and often rapidly developed resistance to nearly every antibiotic introduced through the history of modern chemotherapy to treat infection. Recently, due to emerging resistance to third-generation cephalosporins, the CDC-recommended therapy for gonococcal infection was changed to dual therapy with ceftriaxone and azithromycin or doxycycline (8). With no new antibiotics currently in development, it is essential that a better understanding of antimicrobial resistance mechanisms utilized by the gonococcus is obtained so that new strategies can be developed to overcome resistance and facilitate drug development.

Clinically relevant levels of antimicrobial resistance in *N. gonorrhoeae* are mediated by a number of mechanisms, including active efflux of antimicrobial compounds, particularly by the resistance-nodulation-division (RND) family efflux pump, MtrCDE (13, 29). MtrCDE is a well-characterized efflux system that recognizes and exports a wide variety of structurally diverse antimicrobial agents, including macrolide and β-lactam antibiotics, detergents such as the spermicide nonoxonyl-9, dyes such as crystal violet, cationic antimicrobial peptides such as polymyxin B and the human cathelicidin LL-37, and certain host-derived compounds including progesterone and bile salts that posses potent anti-gonococcal activity (13, 38). MtrCDE is a tripartite pump,

consisting of an RND family transporter, MtrD, an outer membrane channel, MtrE, and a periplasmic fusion protein, MtrC (9, 13, 15).

Transcription of the *mtrCDE* operon encoding these components is subject to a complex regulatory circuit. A TetR-like transcriptional regulatory protein, MtrR, represses transcription of the operon by binding as two homodimers to two psuedodirect repeats within the *mtrCDE* promoter (14). In the presence of sublethal concentrations of pump substrates, transcription of *mtrCDE* may be induced by an AraC family activator, MtrA, which binds upstream of the *mtrCDE* promoter and in the presence of certain pump substrates has a greater affinity for this region of DNA than MtrR (32, 53).

The *mtrR* gene lies 250 bp upstream and is transcribed divergently from *mtrCDE* (Fig. 1A) (13, 29). Antimicrobial resistant clinical isolates of *N. gonorrhoeae* often contain mutations within MtrR, particularly in the helix-turn-helix (HTH) DNA-binding domain, or mutations within the intergenic region between *mtrR* and *mtrCDE* (51). Mutations in the HTH DNA-binding domain of MtrR generally confer low levels of resistance, while *cis*-acting mutations in the intergenic region may result in high-level resistance to pump substrates (51). One such *cis*-acting mutation, which is frequently found in clinical isolates, is the deletion of a single A-T base pair in an inverted repeat element within the *mtrR* promoter (Fig. 1B) (13). This mutation decreases the spacing between the -10 and -35 elements of the *mtrR* promoter, thereby abrogating transcription of *mtrR* and allowing improved access for RNA polymerase to the overlapping *mtrCDE* promoter (13). Recently, another important *cis*-acting mutation was identified in strain MS11, which was originally isolated in the 1960s from a patient with an uncomplicated cervical infection and since used extensively by researchers (51). This mutation, a C-to-

T transition mutation 120 base pairs upstream of the *mtrC* start codon, termed mtr_{120}, was shown to generate a second, highly active promoter for *mtrCDE* transcription outside of the influence of MtrR and MtrA (Fig. 1B) (27, 51). Both of these *cis*-acting mutations result in high levels of MtrCDE production and correspondingly high levels of antimicrobial resistance (13, 27, 51).

Often, mutations that enhance antibiotic resistance also result in a fitness cost for the bacterium, observed in the laboratory as slowed *in vitro* growth rate or poor survival during experimental infection compared to antibiotic-sensitive isogenic strains (1). In regard to efflux pumps, it has been hypothesized that over-production of these large cellular machines would be metabolically draining on a bacterium and thus confer a growth or fitness cost (25). In fact, gonococci that overexpress MtrCDE have been shown to be more fit in a female BALB/c mouse model of lower genital tract infection than their wild type counterparts, and the frequent recovery of *mtr* mutants from patients suggests that MtrCDE overexpression is likely advantageous to gonococci (49, 51). Nevertheless, it could be expected that large-scale production of a tripartite pump and insertion of a large number of these pumps in the bacterial cell wall would perturb cell homeostasis and require metabolic or structural compensation by the bacterium for the observed enhanced levels of *in vivo* fitness. In order to examine the consequences of overexpression of a drug efflux pump on cell homeostasis, it is necessary to have a system in which the pump is overexpressed in the absence of contributions made by transcriptional regulators. This caveat is of importance because DNA-binding proteins that control efflux pump gene expression can have global regulatory action and modulate genes outside to the efflux pump gene locus. For example, MtrR has been shown to regulate nearly 70 genes within

the gonococcus (11). Thus, overexpression of an efflux pump by deletion or disruption a regulator would complicate differentiation between which of the observed changes are due to overexpression of the pump and which are due to perturbations of other systems controlled by the transcriptional regulator.

The *mtr*$_{120}$ mutation provides such a unique opportunity to study the physiological effects of MtrCDE overexpression without the disruption of the pump repressor and global transcriptional regulator, MtrR. Here, we show that increased MtrCDE production stimulates global transcriptional changes in the gonococcus. In addition, we have identified a novel transcriptional regulatory protein, which we have termed GepR (Gonococcal efflux pump gene Regulator), that appears to be involved in the regulation of antimicrobial resistance in *N. gonorrhoeae*.

RESULTS AND DISCUSSION

Identification of gonococcal genes involved in antimicrobial resistance and regulated by over-production of the MtrCDE efflux pump.

To determine whether global gene expression in the gonococcus is affected when the MtrCDE efflux pump is overexpressed, RNA-Seq was conducted to compare the transcriptomes of wild-type strain FA19 and FA19 carrying the *mtr*$_{120}$ mutation (Table 1). Analysis of the RNA-Seq results revealed changes in the expression of a number of genes distributed throughout the genome and representing a variety of functional classes when MtrCDE is overexpressed (Table 2). For the purposes of this study, we chose to focus on genes that were differentially expressed between the wild-type strain and the mutant strain with a change in expression ≥2-fold (along with a Bonferroni-corrected p-value

≤0.05) and were likely involved in the resistance of gonococci to antimicrobials. As expected, expression of *mtrC*, *mtrD*, and *mtrE* was highly upregulated in FA19*mtr*$_{120}$, which served as an internal control for the results of the transcriptome comparison.

Our analysis of the transcriptional profiling data revealed two genes possibly involved in gonococcal resistance to antimicrobials. The first, based on annotation of the FA1090 genome sequence (www.genome.ou.edu), is *ccpR*, encoding a cytochrome C peroxidase implicated in hydrogen peroxide resistance (45). We found that the expression of *ccpR* was enhanced in FA19 *mtr*$_{120}$ compared to wild-type strain FA19. The second gene of interest was downregulated in FA19*mtr*$_{120}$ and is positioned three open reading frames downstream of the *mtrCDE* operon (Fig. 1A). This gene (NGO1360; GenBank AAW90008.1) was of particular interest as it encodes a 239 amino acid member of the FadR/GntR family of regulators, classified by a highly conserved N-terminal DNA binding domain and a variable C-terminal domain involved in ligand binding (16, 30). GntR family regulators have been shown to be involved in the regulation of efflux pump genes and other genes involved in antimicrobial resistance expressed by other bacteria (22, 26, 44). Since further studies (see below) revealed that the product of NGO1360 performs a role in modulating expression levels of gonococcal resistance to antimicrobials recognized by the MtrCDE efflux pump, we provisionally termed this gene *gepR* (*g*onococcal *e*fflux *p*ump gene *R*egulator). Importantly, expression of the two genes encoding hypothetical proteins located between *gepR* and *mtrE* was not affected by the presence of the *mtr*$_{120}$ mutation as determined by analysis of the RNA-Seq results.

We examined the homology of GepR to other relevant members of the GntR family using the Protein Basic Local Alignment Search Tool (BLASTP). Alignment of

GepR with GntR, the gluconate operon repressor of *Bacillus subtilis* (GenBank BAA21579.1), for which the GntR family is named, revealed 48% homology over 46 amino acids in the N-terminal domain. Alignment of GepR with FadR, the fatty acid metabolism transcriptional regulator of *Escherichia coli* (GenBank CAA30881.1), showed an N-terminal region of homology with 30% homology over 51 amino acids, and a second region of 25% homology over 52 amino acids toward the C-terminus. We also determined the homology of GepR to other GntR-family regulators known to be involved in regulation of antimicrobial resistance. SP1714 (GenBank ABJ55088.1), a repressor of 2 ABC transporters involved in LL-37, licomycin, and gramicidin resistance in *Streptococcus pneumoniae*, showed 23% similarity over 79 amino acids in the N-terminal domain (22). CrgR (GenBank AAK34584), which is involved in regulation of cathelicidin resistance in *Streptococcus pyogenes*, showed 28% similarity over 76 amino acids at the N-terminus (26). NorG (UniProtKB/Swiss-Prot Q7A875.1), a *Staphylococcus aureus* GntR/FadR family activator of the NorB efflux pump, which mediates quinolone resistance, and repressor of the AbcA efflux pump, involved in β-lactam resistance, showed a region of 30% similarity over 64 amino acids at the N-terminus, and a region of 30% similarity over 37 amino acids toward the C-terminus (44). Thus, GepR possesses an N-terminal domain homologous to other members of the GntR transcriptional regulator family, and a C-terminal domain similar to members of the FadR subfamily, including GntR/FadR regulators known to be involved in antimicrobial resistance.

Antimicrobial susceptibility profile of gonococcal mutants lacking *ccpR* or *gepR*.

To determine whether *ccpR* or *gepR* contribute to antimicrobial resistance expressed by gonococci, particularly that mediated by the MtrCDE efflux pump system,

we constructed null mutations of these genes in wild-type strain FA19 and its genetic derivatives that over-produce the MtrCDE efflux pump due to a single base pair deletion in the *mtrR* promoter (strain KH15; see Table 1) or possession of the mtr_{120} promoter (strain FA19mtr_{120}). We tested these strains, along with a genetic derivative of strain FA19 lacking a functional MtrCDE efflux pump due to insertional inactivation of *mtrC* (strain KH12; see Table 1), for differences in susceptibility to the MtrCDE-substrate antimicrobials erythromycin (Erm), crystal violet (CV), Triton-X 100 (Tx-100), and polymyxin B (PxB). Additionally, as *ccpR* encodes a cytochrome C peroxidase, we suspected that it may help gonococci resist the antimicrobial action of peroxides (45). We therefore also tested strains FA19, FA19mtr_{120}, KH15, and their *ccpR* null mutants for differences in susceptibility to hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (tBuOOH). Although loss of *ccpR* did not influence levels of gonococcal susceptibility to antimicrobials recognized by the MtrCDE efflux pump (Table 3), its loss did have a modest, albeit reproducible, impact on gonococcal susceptibility to H_2O_2 and tBuOOH (Table 4). In this respect, both FA19mtr_{120} and KH15 showed a modest increase in resistance to H_2O_2 compared to FA19, and this resistance returned to wild-type levels upon disruption of *ccpR*. In contrast to hydrogen peroxide, both FA19mtr_{120} and KH15 showed a significant increase in resistance to tBuOOH, but this resistance was not significantly impacted by disruption of *ccpR*. That we observed only modest changes in resistance to peroxides is, in fact, not surprising, as the gonococcus has a number of mechanisms to neutralize toxic oxygen radicals, and it has been previously shown that knockout of *ccpR* alone does not significantly impact oxidative stress resistance (2, 45).

These results do suggest, however, that MtrCDE overexpression in general affects levels of peroxide resistance, as similar results are observed in both FA19*mtr*$_{120}$ and KH15.

In contrast to *ccpR* mutants, mutants of strains FA19 or KH15 lacking *gepR* displayed hypersusceptibility to antimicrobials, notably those recognized by the MtrCDE efflux pump (Table 3). Interestingly, loss of *gepR* did not impact antimicrobial susceptibility in the FA19*mtr*$_{120}$ genetic background. These results suggest that GepR is necessary for MtrCDE-based resistance when the wild-type *mtrCDE* promoter drives pump gene expression, but does not affect resistance when expression is mediated by the *mtr*$_{120}$ promoter. In order to evaluate the clinical significance of this finding, we inactivated *gepR* in clinical isolates obtained from patients who failed antibiotic (cefixime or ceftriaxone) therapy; these isolates also express resistance to azithromycin and penicillin and possess the well-characterized single base pair deletion mutation in the *mtrR* promoter (data not presented) known to result in elevated levels of *mtrCDE* expression. As is shown in Table 5, *gepR* null mutants of these clinical isolates displayed a significant increase in susceptibility to azithromycin and penicillin and were 2- to 4-fold more susceptible to cefixime and ceftriaxione compared to their respective parent strain. Importantly, with respect to azithromycin and penicillin, the presence of the *gepR* null mutation in these clinical isolates resulted in MIC values that would be well below the MIC breakpoint for both antibiotics (1 - 2 μg/ml).

GepR regulates levels of the MtrCDE efflux pump.

Our finding that loss of *gepR* could significantly reduce the resistance of gonococci to antimicrobials recognized by the MtrCDE efflux pump, and the similarity of GepR to members of the GntR/FadR family of regulators, suggested that GepR acts as a

constitutive and positive regulator the *mtrCDE* efflux pump operon. In order to test this possibility, we evaluated levels of the MtrE protein, which serves as the outer membrane protein channel for the pump, in strains FA19, FA19*mtr*$_{120}$, KH15, and their respective *gepR::kan* mutants. As is shown in Figure 2, we found that in both the FA19 and KH15 backgrounds, MtrE could not be detected in the *gepR* mutants. However, consistent with the results from antimicrobial susceptibility testing, loss of *gepR* did not seem to impact levels of MtrE in strain FA19*mtr*$_{120.}$ Since the *mtr*$_{120}$ promoter is present in < 1% of strains expressing high level antimicrobial resistance mediated by the MtrCDE efflux pump, we hypothesize that inactivation of GepR by inhibitors could reverse antibiotic resistance (27).

GepR has regulatory action outside of the *mtr* locus.

In order to learn if GepR can regulate gonococcal genes outside of the *mtr* locus and thus potentially act as a global gene regulator, we examined expression levels of *ccpR* in strains FA19, FA19*mtr*$_{120}$, and their respective *gepR*::*kan* mutants using a *ccpR-lacZ* reporter fusion. We selected *ccpR* since its expression, like *mtrCDE*, was elevated in FA19*mtr*$_{120}$ while *gepR* expression was decreased in this strain (Table 2). In agreement with the results of the RNA-Seq analysis, expression of *ccpR* was significantly upregulated in strain FA19*mtr*$_{120}$ compared to wild-type strain FA19 (Fig. 3). Interestingly, we found that loss of GepR resulted in decreased expression of *ccpR* in both the FA19 and FA19*mtr*$_{120}$ backgrounds, suggesting that GepR may serve as a positive regulator of *ccpR* expression, and the capacity of GepR to regulate gonococcal genes is not restricted to the *mtr* locus (Fig. 3). However, it is also likely that expression

of *ccpR* is subject to GepR-independent regulation, as possession of the mtr_{120} promoter results in elevated *ccpR* expression but decreased expression of *gepR* (Table 2).

Our effort to determine the global consequences of overexpression of the *mtrCDE* efflux pump operon in *N. gonorrhoeae,* independent of loss of known transcriptional regulators MtrR and MtrA, using the mtr_{120} promoter identified a previously undescribed gene (*gepR*) positioned just downstream of the operon that seems to be needed for this pathogen to express antimicrobial resistance. As GepR is a member of the GntR/FadR family of regulators, we hypothesize that it serves as a positive and constitutive regulator of the *mtrCDE* operon. Although its mechanism of control of *mtrCDE* expression, the nature of other target genes subject to its control, and the mechanism by which *gepR* expression is decreased when the mtr_{120} promoter is preferentially used to transcribe *mtrCDE* are currently unknown, it is important to note that to our knowledge GepR is the first transcriptional regulator in bacteria to be associated with expression of constitutive antimicrobial resistance. Efforts are now underway to define the DNA-binding activity of GepR using target DNA sequences within the *mtr* locus and upstream of *ccpR* so as to determine the mechanisms by which it controls genes involved in gonococcal resistance to antimicrobials.

It is noteworthy that *gepR* null mutants of clinical isolates of gonococci obtained from patients who failed antibiotic therapy were hypersusceptible to azithromycin and penicillin. These clinical isolates were obtained from patients who failed treatment by cefixime or ceftriaxione and were cross-resistant to penicillin and/or azithromycin. With respect to penicillin, this relatively inexpensive antibiotic was highly effective for treating gonorrhea for over forty years (7). However, the development of chromosomal mutations,

notably the single bp deletion in the *mtrR* promoter and mutations in *penA*, which encodes penicillin-binding protein 2, resulted in the emergence of strains expressing clinically relevant levels of penicillin resistance (42, 47, 48). Earlier work by Veal *et al.* showed the importance of the MtrCDE pump in chromosomally-determined penicillin resistance since loss of this pump due to inactivation of the *mtrD* gene, which encodes the cytoplasmic membrane transporter protein, in a penicillin-resistant isolate increased penicillin susceptibility to a level below the MIC breakpoint (10, 15, 48). Thus, based on our past studies and those reported herein, we propose that therapeutics that interrupt expression of the *mtrCDE* operon, in the absence of production of a β-lactamase, could permit the return of penicillin as an effective antibiotic to treat gonorrhea. In addition to restoring the use of penicillin as an effective antibiotic, a GepR inhibitor could also render gonococci susceptible to mediators of innate host defense, since the MtrCDE pump recognizes antimicrobial peptides and other host-derived antimicrobials (progesterone and bile salts). This hypothesis is supported by the finding of Jerse *et al.* that mutants of strain FA19 lacking *mtrD* or *mtrE* were unable to cause a sustained infection in the lower genital tract of female mice (18). The global public health problem of antibiotic resistance expressed by gonococci (and other pathogens) requires new strategies to effectively treat infections. Further research on how transcriptional regulators such as GepR modulate efflux pump gene expression should provide important information that could lead to the development of new antimicrobials that combat antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Gonococci were routinely grown on GCB agar (Difco Laboratories, Detroit, MI) containing defined supplements I and II (36) at 37°C under 4% CO2 or in GCB broth (Difco Laboratories, Detroit, MI) containing defined supplements I and II and 0.048% (v/v) sodium bicarbonate with shaking at 37°C. *E. coli* DH5α was routinely grown on LB agar or in LB broth (Difco Laboratories, Detroit, MI). The *N. gonorrhoeae* strains used in this study are described in Table 1. In some experiments, clinical isolates obtained from pateints who failed treatment with cefixime or ceftriaxone were also used; these strains are described in Table 5. The oligonucleotide primers used are listed in Table 6. Strains FA19, FA19mtr_{120}, KH15, and KH12 were previously described (13, 14, 27, 35). Strain FA19 *ccpR::km* was kindly provided by E.-H. Lee (Emory University, Atlanta, GA, USA). Strains FA19mtr_{120} *ccpR*::*km* and KH15 *cppR*::*km* were constructed by PCR amplification of *ccpR* containing the *aphA*-3 cassette from FA19 *ccpR*::*km* chromosomal DNA using primers ccp1 and ccp4 (Table 6) and transformation of the product into strains FA19mtr_{120} and KH15, with selection on GC agar supplemented with 50 μg/mL kanamycin. To construct strains with insertional inactivation of *gepR*, oligonucleotides gntR1 and gntR2 (Table 6) were used to amplify a 778 bp region from FA19 genomic DNA. The resulting product was cloned in pBAD-TOPO (TOPO TA cloning kit, Invitrogen), then digested with StuI, a unique restriction site located at the 5' end of the insert. The *aphA*-3 cassette was prepared from pUC18K and cloned into the StuI site described above. A recombinant plasmid was purified from a kanamycin-resistant transformant of *E.coli* DH5-α and used to transform strains FA19, KH15 and FA19mtr_{120} for resistance to kanamycin (50 μg/ml) as described

in (12). Genomic DNA for transformation experiments or PCR was prepared as described in (23).

Preparation of samples for RNA-Seq analysis. Strains FA19 and FA19*mtr*$_{120}$ were grown in liquid culture in triplicate to late-log phase as described above. Total RNA was harvested from cultures using the Ambion RiboPure Bacteria kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. DNA was removed by treatment with Ambion TURBO DNA-*free* according to manufacturer's instructions. rRNA was depleted using two rounds of Ambion MICROBExpress according to manufacturer's instructions. cDNA libraries were prepared from RNA using the Invitrogen SuperScript Double-stranded cDNA Synthesis kit (Life Technologies, Grand Island, NY) and random hexameric primers according to manufacturer's instructions. cDNA libraries were amplified by 15 cycles of PCR for the addition of adaptors with TruSeq indexes using the Illumina TruSeq Sample Preparation kit according to manufacturer's instructions. After KAPA quantitation and dilution, the 6 libraries were clustered in a single lane and sequenced on an Illumina HiSeq 2000 instrument with 100 base pair paired end (PE) reads.

Identification of differentially expressed genes. The raw reads were trimmed by removing adapter sequences and ambiguous nucleotides. Reads with quality scores less than 20 and length below 30 base pairs were all trimmed. The resulting high-quality reads from each of the 3 replicate samples were mapped onto the *N. gonorrhoeae* FA1090 reference genome (GenBank accession: AE004969) using CLC Genomics Workbench software. For the reference mapping, at least 95% of the bases were required to align to the reference genome and a maximum of 2 mismatches were allowed. The total number

of reads mapped for each transcript was determined and then normalized to detect the reads per kilobase per million reads (RPKM) measure. The proportions-based test was used to identify the differentially expressed genes between the wild-type FA19 strain (control) and the mutant FA19*mtr*$_{120}$ strain with a Bonferroni-corrected p-value ≤0.05. The proportions-based test method allows an estimation of differential expression based on single measurements of read counts for the two treatment conditions. After quantile normalization of the RPKM values, fold changes were calculated. This analysis was performed using the RNA-Seq module and the expression analysis module in CLC Genomics Workbench. Transcripts with fold change values ≥2 and total read number larger than 5, along with a Bonferroni-corrected p-value ≤0.05, were included in the analysis as differentially expressed genes.

Antimicrobial susceptibility testing. The minimal inhibitory concentration (MIC) of Erm, CV, PxB, and TX-100 were determined by 2-fold agar dilution assay (36). Strains were grown in liquid culture to late-log phase, the optical density at 600nm was adjusted to 0.05, and 5 µL samples of suspensions were spotted onto GCB agar containing 2-fold serial dilutions of antibiotics. Plates were incubated at 37°C under 4% (v/v) CO_2. Differences in MIC values greater than 2-fold were considered significant. To test gonococcal susceptibility to peroxides, strains were grown in liquid culture to late-log phase, and the optical density at 600nm was then adjusted to 0.005. 100 µL of suspensions was added to 100 µL GCB supplemented with 2-fold dilutions of H_2O_2 or tBuOOH in 96 well plates. Plates were incubated at 37°C for 45 minutes. 5 µL from each well was spotted onto GC agar plates and plates were incubated at 37°C under 4% CO_2

overnight. The results are reported as the minimal bactericidal concentration and represent average values from at least three determinations.

Detection of MtrE. MtrE was detected in whole-cell lysates of gonococci by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western immunoblotting using rabbit polyclonal antibodies against MtrE as previously described (49).

Construction of *lacZ* fusions and analysis of expression. Translational *lacZ* fusions were constructed as previously described (40). Briefly, the region upstream of *ccpR* was amplified using primers that introduce a BamH1 restriction site at the end of the amplified region; primer sequences are listed in Table 6. PCR products were digested with BamH1, and resulting DNA fragments were introduced into the Bam H1 site of pLES94 (40). Recombinant plasmids were transformed into *E. coli* DH5α and transformants were selected on LB agar supplemented with 100 µg/mL ampicillin. Correct insertion and orientation were confirmed by PCR analysis and DNA sequencing. Plasmids were transformed into *N. gonorrhoeae* to allow insertion into the chromosomal *proAB* locus. Transformants were selected on GCB supplemented with 1 µg/mL chloramphenicol.

Strains containing translational *ccpR-lacZ* fusions were grown in liquid culture to late-log phase. Three 1 mL samples were taken from each culture and cells were pelleted by centrifugation. Cells were washed once with phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer (24 mM Na_2HPO_4, 16 mM NaH_2PO4, 4 mM KCl, 0.4 mM $MgSO_4 \oplus 7H_2O$). Cells were lysed by repeated freeze-thaw cycles and cell debris was removed by centrifugation. β-galactosidase assays were performed as previously described (41).

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TABLE 1: Strains used in this study

Strain	Relevant Genotype	Reference
FA19	Wild type	Sarubbi *et al* 1974
FA19*mtr*$_{120}$	C to T 120bp upstream of *mtrC* start	Ohneck *et al* 2011
KH15	ΔT in IR of *mtrR* promoter	Hagman and Shafer 1995
KH12	*mtrC::Km*	Hagman *et al* 1995
FA19 *ccpR*::*km*	*aphA*-3 insertional inactivation of *ccpR*	This study
FA19*mtr*$_{120}$ *ccpR*::*km*	*aphA*-3 insertional inactivation of *ccpR*	This study
KH15 *ccpR*::*km*	*aphA*-3 insertional inactivation of *ccpR*	This study
FA19 *gepR*::*km*	*aphA*-3 insertional inactivation of *gepR*	This study
FA19*mtr*$_{120}$ *gepR*::*km*	*aphA*-3 insertional inactivation of *gepR*	This study
KH15 *gepR*::*km*	*aphA*-3 insertional inactivation of *gepR*	This study
FA19 *ccpR-lacZ*	translational *ccpR-lacZ* fusion inserted at non-essential *proAB* site in chromosome	This study
FA19*mtr*$_{120}$ *ccpR-lacZ*	translational *ccpR-lacZ* fusion inserted at non-essential *proAB* site in chromosome	This study
FA19 *gepR::km ccpR-lacZ*	translational *ccpR-lacZ* fusion inserted at non-essential *proAB* site in chromosome	This study
FA19*mtr*$_{120}$ *gepR*::*km ccpR-lacZ*	translational *ccpR-lacZ* fusion inserted at non-essential *proAB* site in chromosome	This study

TABLE 2: Transcriptional response of gonococci to over-expression of the MtrCDE efflux pump

Genes up-regulated in the presence of *mtr*$_{120}$			
Gene	Common Name	Fold Change	Functional Classification
NGO1363	*mtrE*	4.44	Mtr efflux pump protein component: outer membrane channel protein
NGO1364	*mtrD*	5.63	Mtr efflux pump protein component: RND family transporter
NGO1365	*mtrC*	5.42	Mtr efflux pump protein component: periplasmic fusion protein
NGO1769	*ccpR*	2.52	cytochrome c peroxidase
Genes down-regulated in the presence of *mtr*$_{120}$			
Gene	Common Name	Fold Change	Functional Classification
NGO0218	NGO0218	-15.02	hypothetical
NGO0585	NGO0585	-2.02	hypothetical integral membrane protein
NGO0593	*clpP*	-2.03	ATP-dependent Clp protease subunit
NGO0618	NGO0618	-2.14	hypothetical
NGO0650	NGO0650	-2.05	ATP-dependent RNA helicase
NGO1058	*surE*	-2.14	stationary phase survival protein
NGO1246	*sohB*	-2.72	periplasmic peptidase, S49 family
NGO1248	NGO1248	-5.23	hypothetical
NGO1360	NGO1360	-3.56	FadR/GntR family transcriptional regulator (gepR)
NGO1368	*mtrF*	-2.67	Mtr efflux pump accessory protein
NGO1481	NGO1481	-4.59	Putative SAM-dependent methyltransferase
NGO1857	*secE*	-4.83	protein translocase channel subunit
NGO1917	*terC*	-3.06	transmembrane transporter, tellurium resistance

TABLE 3: Sensitivity of gonococci to antimicrobial substrates of the MtrCDE efflux system

	MIC (μg/mL)			
Strain	Erm	CV	TX-100	PxB
FA19	0.25	0.31	125	100
KH12	0.03	0.16	15.6	25
FA19 *ccpR*::*km*	0.25	0.31	125	100
FA19 *gepR*::*km*	0.03	0.16	15.6	25
FA19mtr_{120}	2	1.25	>16,000	400
FA19mtr_{120} *ccpR*::*km*	2	1.25	>16,000	400
FA19mtr_{120} *gepR*::*km*	2	1.25	>16,000	400
KH15	2	1.25	>16,000	800
KH15 *ccpR*::*km*	2	1.25	>16,000	800
KH15 *gepR*::*km*	0.03	0.16	15.6	25

TABLE 4: Sensitivity of gonococci to oxidative stress-inducing compounds

	MBC (%)	
Strain	H_2O_2	tBuOOH
FA19	0.002	0.004
FA19 *ccpR*::*km*	0.002	0.004
FA19mtr_{120}	0.004	0.016
FA19mtr_{120} *ccpR*::*km*	0.002	0.008
KH15	0.004	0.016
KH15 *ccpR*::*km*	0.002	0.016

TABLE 5: Sensitivity of patient isolates to clinically relevant MtrCDE substrates.

	MIC µg/mL			
Strain	Penicillin G	Cefixime	Ceftriaxone	Azithromycin
H041[a]	4	8	4	1
H041 *gepR::km*	0.5	4	1	0.047
S180[b]	0.75	4	2	0.5
S180 *gepR::km*	0.094	1.5	0.5	0.016
246/-10[c]	2	0.19	0.094	0.5
246/-10 *gepR::km*	0.25	0.047	0.012	0.032
272/-10[d]	3	0.38	0.125	0.25
272/-10 *gepR::km*	0.38	0.094	0.047	0.032
3694/-10[e]	4	0.25	0.125	0.38
3694/-10 *gepR::km*	0.19	0.064	0.023	0.023

[a] First clinical isolate demonstrating high-level resistance to ceftriaxone (28)
[b] Clinical isolate from Spain demonstrating high-level resistance to ceftriaxone (6)
[c] Isolate from patient that failed cefixime therapy
[d] Isolate from patient that failed cefixime therapy
[e] Isolate from patient that failed cefixime therapy

TABLE 6: Oligonucleotide primers used in this study

Name	Sequence	Purpose
ccp1	5’-CGAAAGCAAACAGGAAAGCCTA TGG-3’	*ccpR*::km amplification
ccp4	5’-CCTTGGACCAGACCGAATTTCTG-3’	*ccpR*::km amplification
gntR1	5’-GTGATGAAACTGGTAAGGC-3’	construction of *gepR::km*
gntR2	5’-CCTCCCAATCCTGCACGC-3’	construction of *gepR::km*
pLESccp_F	5’-AGTCGGATCCTGTCGGAAAATAAA ATGCCG-3’	*ccpR-lacZ* fusion
pLESccp_R	5’-AGTCGGATCCAAAGACATCGTGT ATTCCTTATG-3’	*ccpR-lacZ* fusion

Figure legends

Figure 1. The *mtr* locus of *Neisseria gonorrhoeae*. A. Genetic organization of the *mtr* locus. *mtrR* and *mtrCDE* are divergently transcribed on opposite strands. *gepR* lies 3 open reading frames downstream of *mtrCDE.* Bent arrows denote promoters. Shaded arrows represent open reading frames encoding hypothetical proteins. **B.** Sequence of the *mtrR-mtrCDE* intergenic region. The -10 and -35 elements of the *mtrR* and *mtrCDE* promoters and the -10 element generated by the mtr_{120} mutation are indicated. The -10 and -35 elements of the *mtrR* promoter are separated by an optimal 17 base pairs as noted. The hatched box outlines the inverted repeat element important for *mtrR* transcription. The nucleotide positions of the single base pair deletion and the mtr_{120} mutation are indicated by bold letters.

Figure 2. GepR affects production of MtrE. Western blot analysis of whole cell lysates separated by SDS-PAGE was conducted using polyclonal rabbit MtrE-specific antibodies followed by goat α-rabbit alkaline phosphatase. Lanes: (1) FA19, (2) FA19 *gepR::km*, (3) KH15, (4) KH15 *gepR::km*, (5) FA19mtr_{120}, (6) FA19mtr_{120} *gepR::km*. A companion SDS-PAGE gel was stained with Coomassie Brilliant Blue and confirmed equal loading of protein in each lane (data not presented).

Figure 3. GepR affects expression from the *ccpR* promoter. The β-galactosidase activities from whole cell lysates of late-log phase cultures of strains containing translational *ccpR-lacZ* fusions are shown. Assays were performed in triplicate. Error

bars represent 1 standard deviation. Asterisks correspond to a *p* value (Student's t test) <0.05.

FIGURE 1

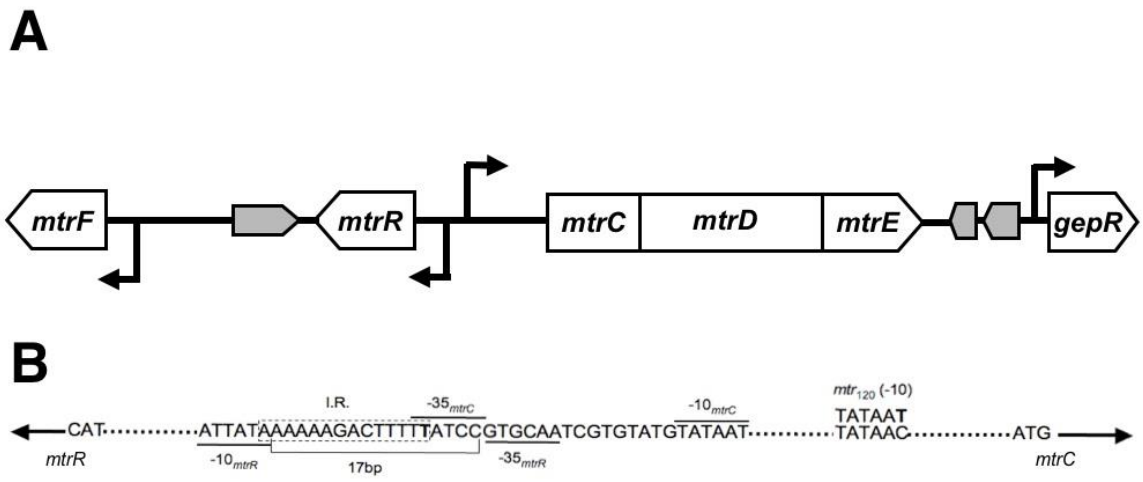

A
mtrF
mtrR
mtrC
mtrD
mtrE
gepR
B
I.R.
-35mtrC
-10mtrC
mtr120 (-10)
TATAAT
CAT
ATTATAAAAAAGACTTTTTATCCGTGCAATCGTGTATGTATAAT
TATAAC
ATG
mtrR
-10mtrR
17bp
-35mtrR
mtrC

FIGURE 2

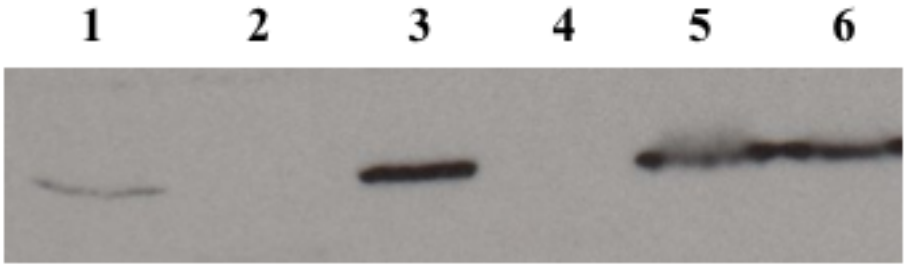
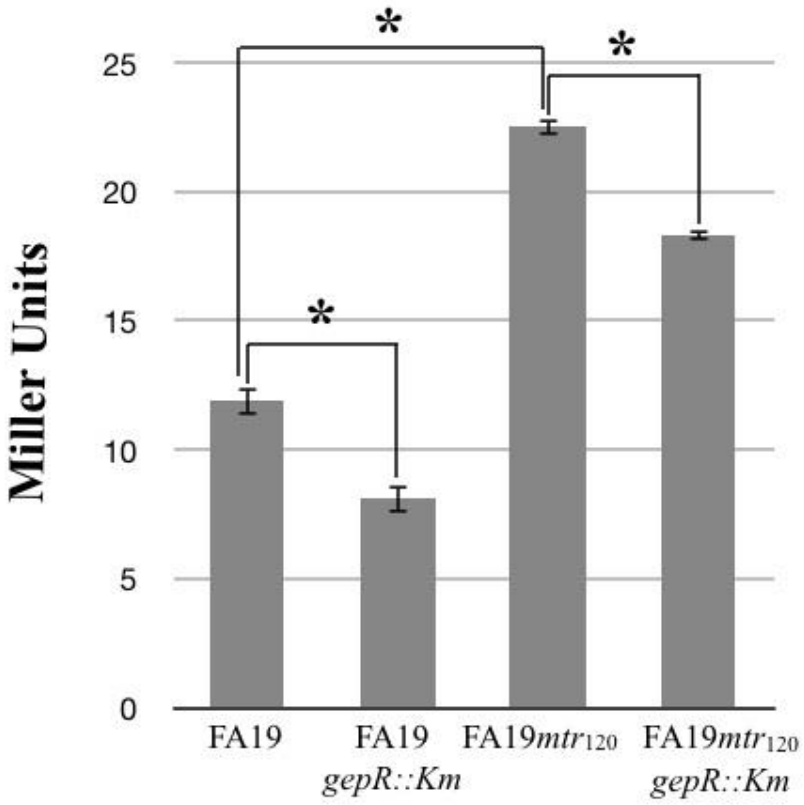


FIGURE 3


Miller Units
25
20
15
10
5
0
*
*
*
FA19
FA19 gepR::Km
FA19mtr120
FA19mtr120 gepR::Km

Chapter 5: Discussion

Neisseria gonorrhoeae has been evolving within the human population for thousands of years, allowing this bacterium ample opportunity to develop into an efficient pathogen. A number of factors contribute to the success of the gonococcus as a human pathogen, including multiple mechanisms to evade the host immune response and resist antibiotic therapy. One important mechanism is the expression of several efflux pumps which recognize and export from the gonococcal cell both antimicrobial compounds naturally present in the host at the site of infection and upon induction of the host immune response, and antibiotics introduced to treat infection (6, 10, 15, 16). In particular, the MtrCDE multidrug efflux pump protects the gonococcus from a diverse array of antimicrobial agents and contributes to clinically significant levels of resistance to antibiotics commonly used in infection therapy (6).

Often, mutations that confer antimicrobial resistance come at a cost, as these mutations can involve the increased expression of enzymes or protein complexes, resulting in metabolic stress, or the alteration of certain cellular factors important for growth or replication to a suboptimal composition or structure, resulting in slower or less efficient function and subsequently slower growth (1). Theoretically, it is possible that the over-expression of efflux pumps would confer such a fitness cost, as high-level production of these large, multicomponent cellular machines could be metabolically draining for a bacterial cell. As such, efflux pump genes are often subject to complex regulatory circuits, and the *mtrCDE* operon is no exception. Expression of this operon is subject to repression by the TetR-family transcriptional regulator, MtrR, and inducible activation by the AraC-family transcriptional regulator MtrA (7, 14). In addition,

expression of MtrR is controlled by another transcriptional regulator, MpeR, which regulates expression of *mtrR*, and so indirectly expression of *mtrCDE,* in response to iron levels in the environment (13).

However, over-expression of *mtrCDE* is actually advantageous to the gonococcus, as mutants that demonstrate increased levels of MtrCDE production are frequently isolated from patients with gonorrhea and out-compete an isogenic wild-type strain in a female BALB/c mouse model of lower genital tract infection (21, 22). These mutants often have mutations in MtrR that disrupt its ability to repress the *mtrCDE* operon, frequently due to amino acid substitutions in the helix-turn-helix DNA binding domain (12, 22). Such mutations lead to a modest increase in MtrCDE production and a corresponding intermediate level of resistance to MtrCDE substrates (22). Other strains fail to produce MtrR at all due to the deletion of a single base pair within an inverted repeat element in the MtrR promoter (6). The dual effects of this *cis*-acting mutation, namely, abrogating *mtrR* transcription and improving accessibility to the overlapping *mtrCDE* promoter for RNA polymerase, result in very high levels of pump production and high-level resistance to antimicrobials recognized by MtrCDE (6).

This work characterizes the mechanism of action of a second *cis*-acting mutation that results in high-level MtrCDE-mediated resistance. Originally identified by Warner *et al*. in a comparison of clinically relevant *mtr* locus mutations, this C-to-T transition mutation 120 base pairs upstream of the *mtrC* start codon, termed mtr_{120}, generates a consensus -10 promoter element (22). We have demonstrated that this promoter element is sufficient for transcription at levels higher than those from the wild-type promoter, resulting in high levels of MtrCDE production and correspondingly high levels of

resistance to MtrCDE substrates. Other examples exist in the gonococcus of single base pair changes that act in *cis* to affect efflux gene expression; in general, these mutations change a regulatory sequence closer to consensus sequence for the given element. For example, expression of *norM*, which encodes a gonococcal efflux pump involved in resistance to cationic toxic compounds, can be enhanced by base pair changes in the -35 promoter element and the ribosome binding site that generate sequences closer to consensus (3, 15, 18). Similarly, a base pair change in the -10 promoter element for the operon encoding MacAB, an efflux pump that recognizes macrolide antibiotics, increases expression of the operon by generating a consensus -10 sequence (3, 16). However, to our knowledge, the mtr_{120} mutation is the first example of a single base pair change that creates an entirely new promoter for efflux operon transcription.

The extreme transcriptional sensitivity of the mtr_{120} locus is intriguing, especially in comparison to the -10 element mutation in the *macAB* promoter. A consensus -10 element reads TATAAT, from which both the wild-type *macAB* -10 element and the wild-type mtr_{120} locus differ by only a single base pair, reading TAGAAT and TATAAC, respectively (3, 16, 22). However, while detectable transcription occurs from the *macAB* promoter containing the nonconsensus -10 element, no detectable transcription occurs from the wild-type mtr_{120} locus (16). Substitution of a T for the G in the -10 element of *macAB* to generate the consensus sequence results in a moderate increase in transcription, whereas substitution of a T for the C to generate the mtr_{120} promoter results in high levels of transcription of *mtrCDE* (16). The difference in sensitivity of these -10 element loci is uncertain, but may involve the relative importance of individual base pair positions in recognition of the -10 element by RNA polymerase, or the action of other regulatory

elements or proteins in the upstream region of the *macAB* and *mtrCDE* operons. For example, there is a recognizable, although nonconsensus, -35 element located the optimal 17 base pairs upstream of the -10 element in the *macAB* promoter, which might contribute to the low but detectable levels of transcription from the *macAB* promoter when the -10 element sequence is nonconsensus (16). In contrast, the mtr_{120} -10 element is not preceded by a recognizable -35 element, which might contribute to the lack of transcription from this locus when the sequence is the nonconsensus wild-type. However, in general, the -35 element does not contribute significantly to transcription from gonococcal gene promoters, and high-level expression from the mtr_{120} promoter occurs despite the absence of a -35 element. Thus, other factors must contribute to the differing strength of the *macAB* and mtr_{120} promoters upon mutation to consensus sequence.

The mtr_{120} mutation was originally identified in strain MS11, which was isolated from a patient with an uncomplicated cervical infection in the 1960s and has since been used extensively in gonococcal research (19, 22). As this mutation has been identified in only 1 of the 14 laboratory strains with genomes available through the Broad Institute, and is isolated infrequently from patients, the possession of the mtr_{120} mutation is an important characteristic to consider when analyzing results of studies using strain MS11. Because the mtr_{120} mutation confers high-level resistance to both antibiotics and host antimicrobial compounds and confers a fitness advantage in a mouse model of infection, it is particularly important to consider this mutation in those studies regarding antimicrobial resistance and fitness or survival in host models, including the female BALB/c model of lower genital tract infection and the male volunteer urethral infection model (22). However, because the mtr_{120} mutation does occur in strains isolated from

patients, results of studies using strain MS11 may provide insight into the role and importance of this mutation during infection.

Strains of *N. gonorrhoeae* isolated from patients with gonococcal infection often contain mutations in *mtrR* or the *mtrR-mtrCDE* intergenic region, suggesting that increased production of MtrCDE is beneficial during human infection (17). In addition, strains with *mtr* locus mutations show a fitness advantage over wild-type isogenic parent strains during competitive infection in female BALB/c mice (21, 22). Surprisingly, however, the mtr_{120} mutation is found very infrequently compared to other *mtr* locus mutants. In a panel of 121 isolates sequenced for this study, nearly all isolates contained mutations in the *mtrR* coding region, while the mtr_{120} mutation was found in only 1. Such a discrepancy in frequency might suggest that while moderately increased levels of pump production are beneficial, high-level overexpression is somewhat disadvantageous. However, the single base pair deletion in the inverted repeat of the *mtrR* promoter, which results in similar levels of MtrCDE production as the mtr_{120} mutation, was isolated in 86 of the 121 strains sequenced in this study. Additionally, strains with the single base pair deletion or mtr_{120} show a greater competitive advantage than strains with mutations in the *mtrR* structural region during infection in female mice (22). Also, in the male volunteer urethral infection model, strain MS11 is approximately 100-fold more infection than strain FA1090, which contains a wild-type *mtrCDE* promoter but does not demonstrate inducible resistance due to a mutation in *mtrA* (8). Thus, the reason for the infrequency of the mtr_{120} mutation in clinical isolates remains unknown. One possibility is that the mtr_{120} mutation is disadvantageous at certain sites of infection within the human host, or that strains bearing this mutation are less transmissible than other *mtr* mutants. Importantly,

strains with the single base pair deletion do not produce MtrR, whereas the *mtr*$_{120}$ mutation does not disrupt expression or structure of MtrR (7). As MtrR is a global regulator in the gonococcus, it could be that absence of this transcriptional regulator is advantageous during certain stages of infection or transmission (5). Further study of the circumstances that give rise to and select for the *mtr*$_{120}$ mutation may help elucidate subtle effects of different mechanisms of *mtrCDE* overexpression and the consequences of the mechanism of overexpression on gonococcal fitness, infectivity, and transmissibility.

Despite the fact that *mtr* mutants that produce increased amounts of MtrCDE demonstrate a fitness advantage *in vivo*, it is probable that the gonococcus must compensate for the overexpression of *mtrCDE* in some manner, as a larger amount of cellular resources for transcription, translation, and insertion of proteins into the cell envelope will be diverted for the production of the MtrCDE pump. The *mtr*$_{120}$ mutation offers an important and unique opportunity to examine these effects. Previous studies of the effects of *mtrCDE* overexpression on the gonococcal cell would have required deletion or mutation of MtrR to relieve repression of the pump operon. As previously mentioned, however, MtrR is a global regulator within in the gonococcus, regulating nearly 70 genes involved in functions as diverse as metabolism, stress response, and antimicrobial resistance (5). Thus, disrupting MtrR would complicate the differentiation of cellular responses due to overexpression of the pump and changes due to the disruption of other genetic systems regulated by MtrR. Because the *mtr*$_{120}$ promoter allows high-level *mtrCDE* expression without the disruption of any transcriptional activators known to regulate the *mtrCDE* locus, the specific cellular responses to MtrCDE over-production

can be determined. In this study, we used RNA-Seq to compare the transcriptomes of wild-type strain FA19 and FA19 bearing the *mtr*$_{120}$ mutation and confirmed that global transcriptional changes do occur within the gonococcus in response to *mtrCDE* overexpression. Transcription of a number of genes from different functional categories was affected by the presence of *mtr*$_{120}$. Interestingly, most of the differentially expressed genes were downregulated in the *mtr*$_{120}$ mutant, and many of these genes encode membrane proteins. Decreased expression of other membrane proteins might help compensate for the diversion of metabolic resources to increased MtrCDE production and changes in the cell envelope structure or composition that would result from insertion of a large number of MtrCDE efflux pumps.

By the statistical tests employed in our study, the only gene significantly upregulated in the FA19*mtr*$_{120}$ mutant other than *mtrC*, *mtrD*, and *mtrE*, was *ccpR*, which encodes a cytochrome c peroxidase that helps protect the gonococcus from oxidative stress by neutralizing hydrogen peroxide to water (20). Despite this seemingly important role in gonococcal physiology, insertional inactivation of *ccpR* did not have a detrimental effect on resistance to peroxides or MtrCDE substrates. Additionally, the FA19*mtr*$_{120}$ mutant was only slightly more resistant to peroxides than wild-type strain FA19, despite expressing higher levels of *ccpR*. These findings are not surprising, as the gonococcus has many mechanisms for neutralizing reactive oxygen species (ROS) and other sources of oxidative stress (2, 20). The action of *ccpR* is redundant to that of catalase, encoded by the *katA* gene in the gonococcus, as a strain with a knock-out in either gene demonstrates only a small increase in sensitivity to ROS, but a strain in which both genes are disrupted is hypersusceptible to ROS (20). However, it is thought that exposure to low

concentrations of some antibiotics can stimulate the production of ROS (9); induction of *ccpR* would help neutralize such a response. It is also interesting to note that a strain that overexpresses *mtrCDE* due to the single base pair deletion demonstrates the same slight elevation in peroxide resistance as FA19*mtr*$_{120}$, which might suggest that increased resistance to oxidative stress may be important when *mtrCDE* is highly expressed. Alternatively, insertion of a large number of efflux pumps into the cell membrane could trigger general envelope response systems, which could in turn indirectly result in the upregulation of *ccpR* (4). Further analysis of the interplay between efflux pump overexpression and upregulation of *ccpR* may help elucidate connections between antimicrobial resistance and responses to oxidative and envelope stress.

The down-regulation of NGO1360, a GntR/FadR family transcriptional regulator which we have termed GepR, in FA19*mtr*$_{120}$ led to the identification of another protein involved in regulation of MtrCDE-mediated resistance. Insertional inactivation of *gepR* results in hypersensitivity to MtrCDE substrate antimicrobials, apparently by abolishing production of the MtrCDE efflux pump, as MtrE is undetectable by Western blot analysis in *gepR* null mutants. These findings suggest that GepR is required for constitutive MtrCDE-mediated resistance. In the presence of *mtr*$_{120}$, however, resistance and MtrE production are unaffected by the absence of *gepR*, suggesting that, as for MtrR and MtrA, the *mtr*$_{120}$ promoter acts outside of GepR regulation. Additionally, expression of *ccpR* is diminished in strains lacking *gepR*. Together, these results indicate that GepR may be a global regulator of response to antimicrobial stress or toxic compound resistance, as it may potentially regulate genes involved in antimicrobial efflux and oxidative stress response.

The mechanism of downregulation of *gepR* in the mtr_{120} mutant is unclear. An initial suspicion was that the high level of activity from the mtr_{120} promoter results in a localized transcriptional sink, diverting the transcriptional machinery away from the nearby *gepR* promoter. However, it would then be expected that other genes near the *mtrCDE* locus would also be downregulated, but neither the two open reading frames between the *mtrCDE* operon and the *gepR* gene nor *mtrR* were determined to be differentially expressed by RNA-Seq analysis. A second hypothesis is the existence of a feedback loop involved in monitoring *mtrCDE* expression or efflux activity that regulates expression of *gepR*, either through GepR itself, or through an alternative transcriptional regulator. As GepR is required for constitutive MtrE production, sufficiently high levels of MtrCDE production may transmit a signal that reduces *gepR* expression to in turn reduce further production of MtrCDE, preventing accumulation of MtrCDE to levels that would be stressful to the cell. The high-level *mtrCDE* expression from the mtr_{120} promoter might be sufficient to signal such downregulation. To this end, it would be useful to examine *gepR* expression levels in other mutants that overexpress *mtrCDE*, as well as a strain that does not express the pump operon, to determine how varying levels of MtrCDE production affect expression of *gepR*.

Interestingly, knock out of *gepR* in multidrug resistant strains isolated from patients who failed cefixime or ceftriaxone therapy significantly increased susceptibility of these isolates to azithromycin and penicillin, as well as increased susceptibility to cefixime and ceftriaxone. Thus, GepR could serve as a novel target for drug development, as inactivation of this protein would increase susceptibility to both antibiotics and naturally occurring antimicrobial compounds. A GepR inhibitor might

serve as a more attractive compound for development than efflux pump inhibitors, which are a current focus of therapeutic development. The development of effective efflux pump inhibitors is complicated by the fact that many multidrug efflux transporters have multiple binding pockets, and an inhibitor must therefore block each pocket to effectively disrupt resistance to all substrates of the pump (11). Additionally, an ideal broad-spectrum efflux pump inhibitor would block efflux pumps belonging to multiple transporter families, to avoid the need for the simultaneous administration of multiple efflux pump inhibitors (11). If GepR is necessary for transcription of *mtrCDE*, inhibition of GepR would block MtrCDE production altogether, thereby increasing sensitivity to all MtrCDE substrate antimicrobials. Also, MtrE serves as the outer membrane channel component for the FarAB and MacAB efflux systems; thus, because GepR is necessary for production of MtrE, inhibition of GepR could effectively block multiple gonococcal efflux pumps (10). Further study of the of the GepR regulon, the regulatory characteristics of GepR, and the mechanism by which GepR regulates expression of *mtrCDE* will reveal the importance of this protein in gonococcal antimicrobial resistance and hopefully facilitate the development of novel drug therapies to treat gonorrhea or overcome resistance to allow re-introduction of previously used antibiotics.

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