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Emerging Infectious Diseases Program

Uniformed Services University

Title of Dissertation: "The role of transcriptional regulators of the gonococcal MtrC-MtrD-MtrE efflux pump in pathogen fitness."

Douglas Michael Warner, 2007

Thesis directed by: Ann E. Jerse, Ph.D. Associate Professor, Department of Microbiology

Abstract

Antibiotic resistance has been a health burden for humankind since the inception of antibiotic therapy. Generally, antibiotic resistance levies a fitness toll on the bacterium; however, previous work showed that the MtrC-MtrD-MtrE antibiotic efflux pump of the pathogen *Neisseria gonorrhoeae* is important for bacterial colonization of the murine lower genital tract in the absence of antibiotic substrates. The *mtrCDE* operon is under the control of MtrR, a repressor protein which, when mutated, increases the level of resistance to MtrC-MtrD-MtrE substrates. Results here show that mutation of *mtrR* confers an in vivo fitness advantage to *N. gonorrhoeae* via derepression of the *mtrCDE* operon. Conversely, when MtrA, the transcriptional activator of *mtrCDE,* is mutated MtrA-deficient strains are attenuated in vivo. Mutations in *mtrR* are found in clinical

isolates, and here I show that the most common natural mutations display a gradient of antibiotic resistance. These different levels of in vitro resistance are mimicked by in vivo fitness increases. Additionally, we report that laboratory strain MS11 is a natural *mtr* mutant. One of the two *mtr* mutations in strain MS11 has not been previously described in *Neisseria*, and here we show that the mutation increases the half-life of the *mtrC* transcript. Modification of RNA half-life has not been described as a mechanism of antibiotic resistance for this family of bacterial efflux pumps. By performing in vivo fitness experiments in β-estradiol-treated female mice that were not treated with antibiotic substrates of the MtrC-MtrD-MtrE efflux pump, we propose that the advantage conveyed by *mtrCDE* derepression is due to a pump substrate present in the genital tract. Therefore, we established the levels of gonococcal resistance to antimicrobial peptides that we propose are the chief innate substrates that select for increased expression of the *mtrCDE* operon in this model. This work is a member of a growing group of examples of antibiotic resistance mechanisms that confer a fitness advantage even in the absence of antibiotics.

Title Page

"The role of transcriptional regulators of the gonococcal MtrC-MtrD-MtrE efflux pump in pathogen fitness."

By

Douglas Michael Warner

Dissertation submitted to the Faculty of the Department of Emerging Infectious Diseases Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements of Doctor of Philosophy 2007

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I would not have completed this work without the guidance of Ann Jerse. Her dedication, knowledge, and most of all patience made this work possible. The help and encouragement of Bill Shafer was also indispensible.

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The list of friends is too long to write, but I will always remember the camaraderie I shared with my fellow students.

And of course, Katie, for being my rock and having more faith in me than I had in myself.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Neisseria gonorrhoeae: Disease Manifestations and Control

The *Neisseria* genus was first described in 1879 by Albert Neisser (100), and *N. gonorrhoeae* was first cultured by Leistikow and Loffler in 1882 (163). Bacteria in the family *Neisseriaciae* are classically described as Gram-negative, oxidase-positive diplococci (Fig. 1). The pathogenic species of *Neisseria* are human-specific colonizers that cause meningitis and septicemia (*N. meningitidis*) or gonorrhea (*N. gonorrhoeae*). There are many differences between the two pathogenic *Neisseria* species, including different modes of transmission, different disease states, and the presence of a polysaccharide capsule for *N. meningitidis*. Additionally, while vaccines are available against some serogroups of *N. meningitidis*, there is currently no vaccine available for *N. gonorrhoeae* (163).

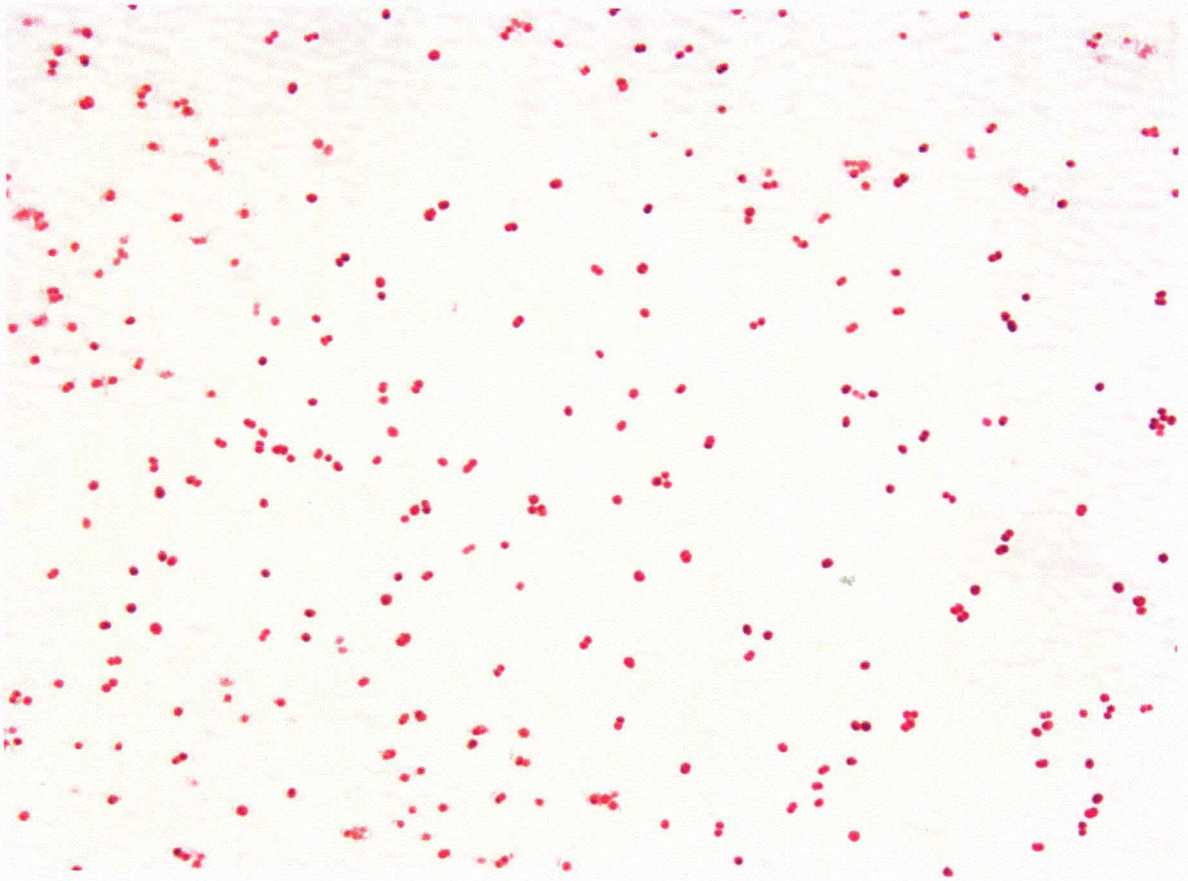
The disease state of gonorrhea was first named by Galen in the second century A.D. from the Latin word which means "flow of seed" (76). The disease was also described by such early physicians as Hippocrates and Celsus (76). Currently, gonorrhea is second only to chlamydial infection as the most commonly reported bacterial infection to the CDC, with more than 300,000 cases reported every year (203). This estimate is probably low and may represent only half of the actual number of infections due to non-reporting and asymptomatic infections (27). The disease gonorrhea can manifest in a variety of ways, the most common of which is a localized mucosal infection of the pharynx, rectum, or urogenital tract. Bacterial infection with *N. gonorrhoeae* is

characterized by a high influx of polymorphonuclear lymphocytes and is accompanied by a purulent exudate (163). In both males and females this localized colonization can exist in either a symptomatic or an asymptomatic state. In 10-20% of cases, infection can ascend in the genital tract to cause epididymitis and prostatitis in men, and salpingitis, cervicitis, and pelvic inflammatory disease in women (76). In both sexes ascension can lead to sterility, and in females ascending infection has a high co-incidence with ectopic pregnancy, which leads to health concerns for both mother and child. In 0.5-3.0% of cases, *N. gonorrhoeae* can cause disseminated gonococcal infection (DGI), which manifests as arthritis and/or dermatitis (74, 76). Recent studies have shown that gonorrhea infection can be an important risk factor in HIV infection, due to a possible increase in access of the virus to the lymphoid system through damage to the epithelial mucosa from gonococcal infection. (31).

The enormous public health impact of gonococcal infection has led to a series of studies intended to identify vaccine candidates. Vaccine targets tested include porin, pili, transferrin receptor, lactoferrin receptor, Opa proteins, and reduction modifiable protein (Rmp) (reviewed in (32)). Porin has shown the most promise, as convalescent sera from patients showed better bactericidal activity to *N. gonorrhoeae* when anti-porin antibodies were present (68, 145). Unfortunately, the porin molecule is stably expressed but shows antigenic variability between strains, and vaccination methodologies for production of protective IgA antibodies at the mucosal site of infection are currently unavailable. As such, antibiotic treatment for the pathogen *N. gonorrhoeae* is pivotal to public health and disease control.

Figure 1. The gonococcus. A microscope slide viewed under 100X, which shows a Gram-stain of heat-fixed *N. gonorrhoeae*. The pink color is characteristic of a Gram-negative bacterium, which retains the counterstain employed in the preparation of a Gram-strain. This field also shows the characteristic diplococcal appearance of the bacterium.

Figure 1. The gonococcus



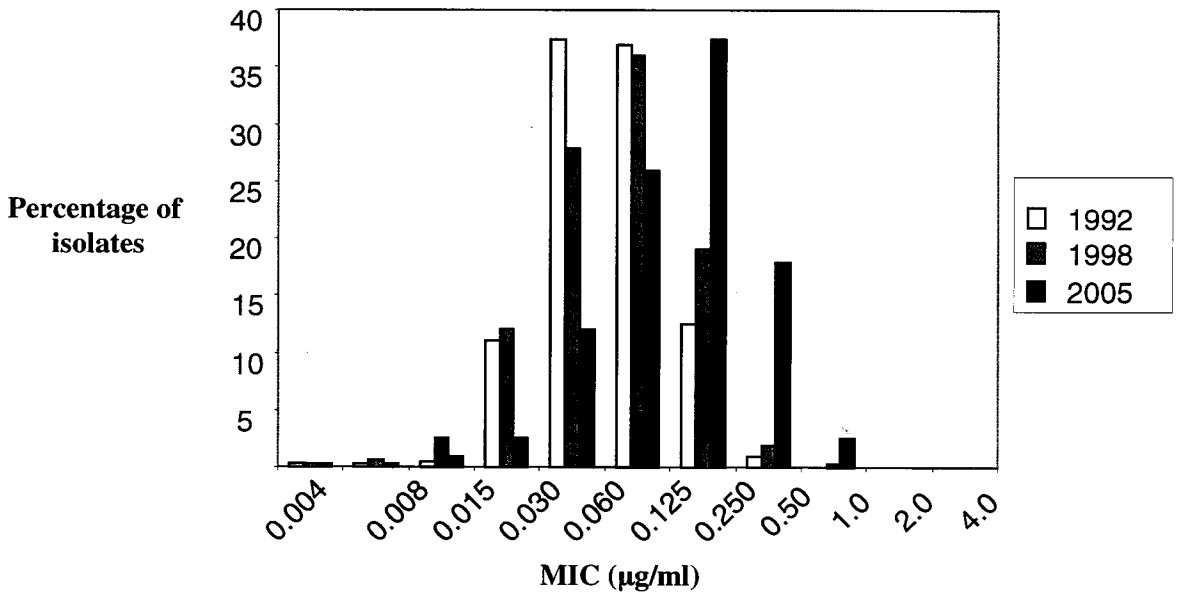
Antibiotic Use and Resistance

Therapy for gonococcal infection has changed greatly since the 19th century, when gonococcal infection was treated with local genital irrigation with silver nitrate or potassium permanganate (89). Upon the discovery of sulfonamides in the 1930s, antibiotic therapy became the most frequently used method of disease control. By the end of 1944, however, resistance to sulfonamides had increased so drastically that a new therapy was required. Penicillin had been discovered and used to treat countless wound infections during WWII, and initial testing showed *N. gonorrhoeae* to be extremely sensitive [minimum inhibitory concentration (MIC) = 0.003-0.03 units/ml] (1unit = 0.6 μg). However, by 1957 there were increasing reports in Europe and the United States of treatment failure with penicillin. MICs of resistant strains were found to be 0.01-0.2 units/ml. By the 1970s, resistant strains from the United States could be found with susceptibility levels at 3.5-5.0 units/ml. *N. gonorrhoeae* developed chromosomal penicillin resistance during the 1950s and 1960s, but in the 1970s plasmids that encoded high levels of resistance began to be reported and characterized (reviewed by (179)). At the same time, other antibiotics were being developed and employed, which selected for the evolution of resistance by the pathogen to tetracycline, erythromycin, and chloramphenicol. The issue of the spread of antibiotic resistant infections became so important, that the Centers for Disease Control created the Gonococcal Isolate Surveillance Project (GISP) in 1986 to monitor susceptibility levels of clinical isolates throughout the United States (56).

Reports from GISP have been effective at detailing the emergence of antibiotic resistance, and their findings have chronicled the quick development of antibiotic resistance over a twenty year period since the creation of GISP. Initial reports from GISP detailed an initial decrease in penicillin resistance. GISP reports show increases in resistance to a variety of antibiotics, such as macrolides as shown in Figure 2. However, in 2001, 20% of isolates collected in the United States were resistant to penicillin, tetracycline, or both (1). At that time, the first line treatments for gonorrhea were fluoroquinolones or third-generation cephalosporins, with spectinomycin advised for patients with allergies or who are pregnant. Since 2001, conditions have changed such that spectinomycin has been discontinued for US distribution (2) and fluoroquinolone resistance has increased to an extent these antibiotics are contra-indicated (28). The only current recommended treatments for gonorrhea infection are cefiximine and ceftriaxone; however, resistance to these agents are increasing as well (2).

Figure 2. Trends in gonococcal macrolide resistance. Data collected by the Gonococcal Isolate Surveillance Program (GISP) is presented here to show the increase in gonococcal resistance to macrolide antibiotics in the United States over a thirteen year period. The trend seen here with macrolides is likely due to increases in the use of erythromycin and azithromycin as therapeutics for uncomplicated genital tract infections as gonococcal penicillin resistance became widespread. Significantly, the MtrC-MtrD-MtrE efflux pump acts to expel macrolide antibiotics. This figure was modified from the GISP report of 2005 (148).

Figure 2. Trends in gonococcal macrolide resistance



Gonococcal Mechanisms of Antibiotic Resistance

The gonococcus employs all four general mechanisms of antibiotic resistance described for Gram-negative bacteria. Examples of these various mechanisms of antibiotic resistance will be summarized using the gonococcus as the model organism. The information presented in this section is summarized in Table 1.

One of the first mechanisms of antibiotic resistance described in gonococci is the production of enzymes to disrupt the action of an antibiotic agent, as exemplified by the β-lactamases. β-lactamase producing plasmids were isolated and characterized from *N. gonorrhoeae* in the 1970s and are hypothesized to have been obtained by horizontal transfer from the bacterium *Haemophilus influenzae* (25, 155). β-lactamase acts to cleave the lactam ring of penicillin, which renders the molecule inactive. To date, β-lactamase is the most potent mechanism of penicillin resistance in *N. gonorrhoeae*, and it increases the MIC of strains 16-fold (13, 95, 155).

Another mechanism that the gonococcus utilizes to resist penicillin is the modification of the antibiotic target protein, as illustrated with the *pen* or *pon* mutations. *pen* and *pon* mutations lead to subtle changes in two of the three gonococcal protein targets of penicillin, which render the antibiotic less effective (12). These chromosomal penicillin resistance mutations increase resistance 4-fold (76). Other noteworthy examples of *N. gonorrhoeae* modifying the targets of a drug are the *gyrA* and *parC* mutations, which alter the bacterial DNA binding proteins gyrase and topoisomerase,

respectively (36). Such mutations in these DNA binding proteins make quinolone antibiotics less effective (15).

A third commonly described mechanism of bacterial antibiotic resistance is a decrease in cell membrane permeability which curtails the amount of harmful substances that can penetrate the cell. Two examples of this mechanism of resistance in *N. gonorrhoeae*, are the *penB* (35) and *pilQ/penC* (213) mutations. These two mutations cause subtle changes to the porin or pilus secretin proteins which allow periplasmic or cytoplasmic entry for antibiotics. Thus, small changes in these portals lead to decreases in entry for certain antibiotics, including penicillin.

The last mechanism of antibiotic resistance utilized by Gram-negative bacteria is to expel the antibiotic or antimicrobial agent across the inner and outer membranes of the bacteria, which keeps periplasmic or cytoplasmic concentrations of the substance at a low level. This mechanism, known as efflux, will be discussed more completely in the next section.

Table 1. Gonococcal mechanisms of antibiotic resistance

Mechanism of Resistance	Mutation	Specific Mechanism	Drugs Effected	Reference
Alteration of membrane permeability	*penB*	Reduces porin permeability by mutating the porin PIB allele	Pen[a], Tet[b]	(35)
	penC	Mutates the protein PilQ which decreases antibiotic uptake	Pen, Tet	(213)
Modification of the enzyme/protein target	*penA*	Reduces the binding of PBP2 to Pen.	Pen, Cef[c]	(106)
	ponA	Reduces the binding PBP1 to Pen.	Cef, Pen	(156)
	parC	Alters the topoisomerase IV targeted by FQ.	FQ[d]	(36)
	gyrA	Alters the DNA gyrase targeted by FQ.	FQ	(36)
Creation of an enzyme to counteract/neutralize the antibiotic	N/A	Plasmid-derived enzyme, which breaks down the β-lactam ring of Pen.	Pen	(18)
Expulsion of the antibiotic	*mtrR*	Derepresses the MtrC-MtrD-MtrE efflux pump.	Erm[e], Az[f], Pen, Cef	(65)

[a] Penicillin
[b] Tetracycline
[c] Ceftriaxone
[d] Fluoroquinolone
[e] Erythromycin
[f] Azithromycin

Antibiotic Efflux

The molecular mechanism of transmembrane transport has been heavily researched for all kingdoms of life (146). Transmembrane transporters are characterized as a complex of interacting proteins that utilize energy from ATP hydrolysis, proton-motive force, or anti-porter systems (reviewed in (94)). The mechanism of efflux was initially characterized as a mechanism of transmembrane transport. It was not until the late 1970s when the P-glycoprotein efflux pump was found in cancer cells that were resistant to chemotherapies (88) that such transporters were thought to facilitate protection from chemotherapeutics. The over-expression of these P-glycoprotein efflux pumps in eukaryotic cancer cells led to increased resistance to chemotherapy. Shortly thereafter, the Tet efflux pump was described as a bacterial antibiotic resistance determinant (99, 119). Antichemotherapeutic efflux pumps have also been described for other pathogenic organisms such as *Candida albicans* (CaMDR1) (124).

Bacterial antibiotic efflux pumps are classified according to structural and functional homology. There are five families of bacterial efflux-pump proteins: the ATP-binding cassette superfamily (ABC), the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion family (MATE), the small multidrug resistance family (SMR), and the resistance-nodulation-division family (RND).

The ABC family of efflux pumps is related to the superfamily of ATP-driven membrane transport proteins. These pumps generally exist as homodimers that span a single membrane and use the energy from the hydrolysis of an ATP molecule to export

such substrates as sugars, ions, drugs, and proteins (45). The two most commonly described examples of ABC pumps are the LmrA pump of *Lactococcus lactis* (22) and the MacAB pump of *Eschericia coli* (92). Recently, a MacAB homolog was characterized in *N. gonorrhoeae*, which is the first example of a gonococcal ABC family transporter. MacAB acts to efflux macrolide antibiotics; however, it exists in a nearly non-expressed state due to a naturally mutated promoter sequence (161).

Members of the MFS family of bacterial efflux pumps are identified by 12-14 transmembrane spanning regions within the large protein that makes up the pump. Generally, these pumps exist as a large monomer embedded in the inner membrane of bacteria and utilize proton motive force as an energy source (reviewed in (164)). These pumps are more common in Gram-positive bacteria due to their single membrane spanning property. However, there are examples of these pumps in Gram-negative bacteria in which the protein monomers pair with the outer membrane portion of other pumps such as the EmrAB-TolC multidrug efflux pump of *E. coli* (102).

The MATE family of efflux pumps is similar in size, structure and function to the MFS efflux pump family; however, the two families share no sequence homology. An example of this efflux family is the NorM pump of *Vibrio hemolyticus* (125). Recently a homolog of NorM was characterized in *N. gonorrhoeae*, which acts to efflux substances with an aromatic ring, such as ethidium bromide and berberine (158).

The SMR family of efflux pumps is also an efflux pump system that acts using the proton motive force across the cytoplasmic membrane. These proteins are generally one third the size of other bacterial efflux pumps, have fewer transmembrane spanning regions, and recognize fewer substrates (reviewed in (143)) than other bacterial efflux

pumps. Examples of this family are the Smr protein of *Staphylococcus aureus* (58), and the EmrE pump of *E. coli* (169).

The final efflux pump family is the RND family, which is the most commonly described pump in Gram-negative bacteria due to the fact that it generally consists of a three protein complex that acts to span both bacterial membranes and the periplasm. RND-type efflux pumps are made up of an inner membrane-spanning transport protein, an outer membrane channel, and a membrane fusion protein. The outer membrane transport protein trimerizes to form a complex that utilizes the proton motive force of the bacterial inner membrane to transport substrates to the periplasmic portion of the pump (reviewed in (94)). Recent crystallization of AcrB of *E. coli* and other studies have shown that the inner membrane portion of the RND efflux pumps can recognize and efflux substrates in both the cytoplasm and periplasm of the bacterium (128). Mutation of the periplasmic loops of the *P. aeruginosa* inner membrane pump component MexB, has shown that substrates are recognized and effluxed from the periplasmic space and/or the inner membrane (188).

The outermost component of the RND efflux pump is a periplasm-spanning outer membrane pore, which expels pump substrates from both the cytoplasm and periplasm of the bacterium. The protein homo-trimerizes to create a long channel made up of α-helices. Crystal structure studies with the *E. coli* efflux protein TolC have found that the pore-forming complex spans the periplasm and docks with the β-barrel of the inner membrane transporter to form the pump (93). Studies with mini-cells have shown that the pore exists in the "closed" configuration until efflux occurs (7). As the pore-forming portion is the only efflux protein that spans the entire periplasm, this portion of the pump

pairs with the inner membrane pump component to facilitate efflux of a variety of substrates, as is seen with TolC of *E. coli* (21) and OprM of *P. aeruginosa* (reviewed in (147)).

The membrane fusion protein of RND pumps is localized to the periplasm and acts to stabilize the interaction of the inner membrane and pore-forming portions of the pumps (207). Both outer membrane homologues TolC and OprM have lipoprotein motifs encoded in their periplasmic domains (reviewed in (94)), which suggests that these proteins may bind directly to the inner membrane. However, other studies have shown an association between the membrane fusion protein and the inner membrane (3, 70, 207, 212), which shows the role for the membrane-fusion protein as a stabilizer of the three protein RND pump complex.

Possible roles for RND-type efflux pumps in pathogenicity have appeared in the literature within the last decade, with demonstration of structural pump mutants displaying attenuated phenotypes. Stone and Miller (181) first described that *tolC* mutants of *Salmonella enterica* type *Enteritidis* were avirulent in mice. This finding was followed by Hirakata et al. showing that the *P. aeruginosa* efflux pump MexAB-OprM is important to host cell invasion and infection of rats and mice (71). The *Campylobacter jejuni* RND-pump CmeABC was shown to be important in the colonization of chickens (101). Further investigations into the roles these pumps play in pathogenicity are detailed in Chapter 4.

Neisserial Efflux Pump Systems

The first neisserial efflux pump was described by Guymon et al. in 1975 (61), before the mechanism of efflux had been characterized. This seminal paper described strains with a "multiple-transformable resistance" (*mtr*) phenotype, which was genetically transferable and conveyed increased resistance to erythromycin, dyes, and hydrophobic agents. That same year, a paper by Sarubbi et al. (166), characterized a mutation termed *env*, which displayed decreased resistance to erythromycin, dyes, and hydrophobic agents (HA). When the *env* mutation was transformed into the previously described *mtr* strains, the resistance phenotype was lost. In 1984, Shafer et al., described the 52 kDa protein Env-10 as the outer-membrane protein responsible for the loss of the resistance phenotype seen in the Sarubbi study (175).

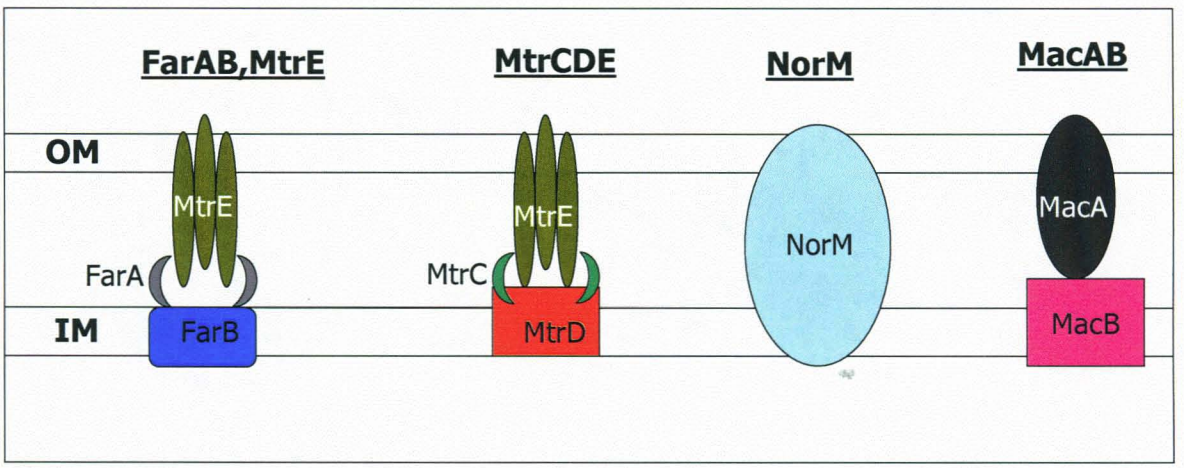
In the early 1990s, the final pieces of the *mtr* puzzle came together with the description of the *mtrR* transcriptional repressor by Pan and Spratt (140) and the subsequent cloning and characterization of the *mtrCDE* operon that is under its control by Hagman et al. (64). Mutations in the *mtrR* gene have since been characterized as a means of increasing transcription of the efflux operon and lowering susceptibility to pump substrates (65). Additionally, the presence of *mtrR* mutations in clinical isolates was noted with early estimates that up to 15% of clinical isolates displayed the *mtr* phenotype that is associated with *mtrR* mutations (140, 210).

The MtrC-MtrD-MtrE efflux pump was fully characterized through a series of papers from William Shafer's laboratory, which identified: 1) the MtrD protein to have

homology with RND-type efflux pumps (63), 2) the MtrE protein as a pore-forming outer membrane pump component (37), and 3) various loss of function mutations within the *mtrCDE* operon (196). Further, two studies from Shafer's group have detailed the existence of another *mtr* gene, *mtrF*, which is located downstream from the *mtrR* gene (195). *mtrF* encodes an inner membrane protein that plays a role in higher levels of resistance to MtrC-MtrD-MtrE substrates and is under the transcriptional control of MtrR (48). Further work by the Shafer lab has identified other efflux pumps in the *N. gonorrhoeae* genome, including the FarAB-MtrE pump (97), the NorM pump (158), and the MacAB pump (161). The known gonococcal efflux pumps and their substrates are summarized in Figure 3.

Figure 3. Gonococcal efflux pumps. The four known gonococcal efflux pumps are shown. The FarA-FarB pump pairs with the MtrE protein to form a functional transporter, which acts to expel long chain fatty acids (97). The MtrC-MtrD-MtrE efflux pump was the first gonococcal efflux pump described and has the widest range of pump substrates, which include bile salts, fatty acids, macrolide antibiotics, penicillin (64), hormones (81), and antimicrobial peptides (176). The NorM efflux pump recognizes cationic compounds including norfloxacin, ciprofloxacin, and ethidium bromide (158). Finally, the MacA-MacB efflux pump is expressed at very low levels in *N. gonorrhoeae;* however, experimental alteration of the promoter sequence showed that the complex expels macrolide antibiotics (161).

Figure 3. Gonococcal efflux pumps



Negative Regulation of the mtrCDE Operon

Most RND-type efflux operons are under the negative control of repressor proteins, many of which are encoded directly upstream and divergently from the start of the pump operon that they control. The presence of these repressors suggests that there is a need to down-regulate the expression of these efflux pumps, and some models suggest that these repressors slow or stop transcription of efflux pumps at times when the bacterium does not need the pump (reviewed in (153)). Indeed, some local RND type pump repressors are directly affected by the substrates that are effluxed by the pumps they control. Binding of pump substrates causes the repressors to dissociate from DNA when the cytoplasmic concentration of a pump substrate necessitates efflux (141).

The *mtrCDE* operon is under the negative transcriptional control of the repressor protein MtrR. As noted previously, one of the early descriptions of the *mtr* phenotype was an increase in resistance to certain hydrophobic agents. Work by Pan and Spratt (140) identified the *mtrR* repressor gene, and further work by Hagman et al. (65) showed that the de-repressed phenotype caused increased antimicrobial resistance due to an increase in transcription of the *mtrCDE* operon. Most recently, DNA microarray and other analyses have demonstrated that the *ponA* and *pilMNOPQ* genes are also under the control of the MtrR protein (47).

The MtrR protein is a member of the TetR family of repressors, a well-described family that includes the *S. aureus* repressor TetR, as well as the *E. coli* homologue MarR

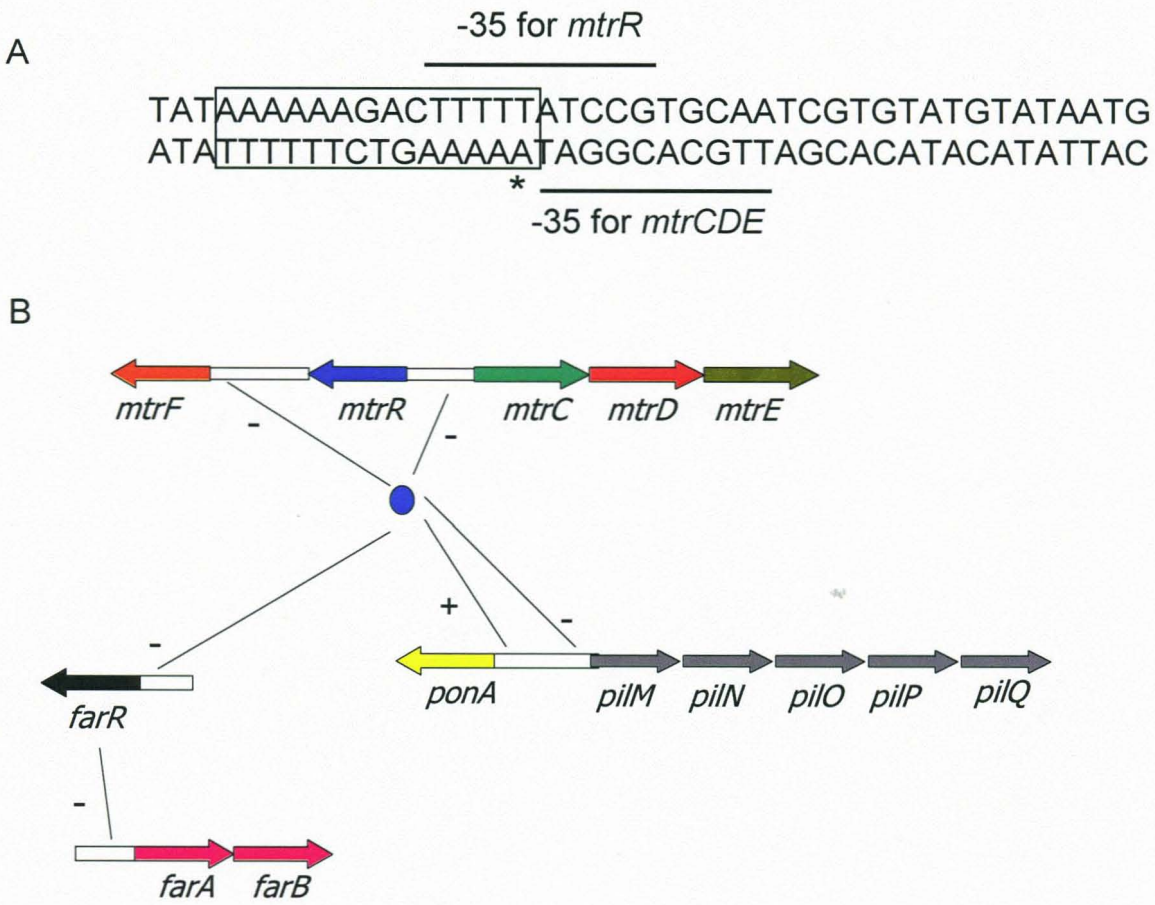
(153). TetR-type repressors act by homo-dimerizing and binding inverted repeat regions of DNA operators. This family of repressors contains a helix-turn-helix (HTH) DNA binding motif near the N-terminal end of the protein (reviewed in (153)). Studies to characterize the repression dynamics of MtrR began with Lucas et al. in 1997, who used electrophoretic mobility shift assays and primer extension analysis to illustrate that the MtrR protein binds the DNA sequence between the *mtrR* and *mtrC* genes (103). The binding region was finely mapped to the area noted in Figure 4. These works were followed by a study that used molecular physics to show the binding of MtrR to itself and to its DNA target (73). This work redefined the way MtrR was thought to bind to itself, and showed that the protein homodimerizes then binds another MtrR homodimer to form a dimer of dimers. Similar binding dynamics are also seen in the QacR repressor of *S. aureus* which controls the expression of the QacA efflux pump (59). There are several similarities between MtrR and QacR, as QacR is a similarly sized 188 amino acid repressor protein that is also encoded upstream and divergently from the efflux pump QacA. Similarly, QacR also binds a pseudo-palindromic repeat that overlaps the transcriptional start of the pump operon. (59, 142)

Besides the findings summarized above, numerous studies of antibiotic-resistant clinical isolates of *N. gonorrhoeae* have increased our understanding of the *mtrR* gene. A single base pair change in a region upstream of the *mtrR* gene was found to alter the susceptibility of the bacterium to hydrophobic agents (104). This region was further described as the transcriptional start for both the *mtrR* gene and the *mtrCDE* operon, and the single base pair change both stopped transcription of the *mtrR* gene and increased transcription of the *mtrCDE* operon. This single base pair change is the most commonly

described mutation that yields the *mtr* phenotype (65, 113, 115, 118, 194, 210, 211). Other mutations found in the intergenic region between *mtrR* and *mtrCDE* include insertions that disrupt MtrR binding (85, 118). The most notable of such insertions is a Correia element found in *N. meningitidis,* which also increases transcription of the *mtrCDE* operon (160). In addition to promoter mutations, mutations within the *mtrR* gene itself have been described in clinical isolates. Most *mtrR* mutations are found in the region that encodes the HTH motif within the first 130 amino acids of the protein, with the most notable being the A39T (38), G45D (174, 184), and H105T (113, 140) mutations. In all of these cases, it can be hypothesized that DNA-binding by the mutated MtrR proteins is disrupted. While there are many studies of MtrR regulation and the effects of *mtr* mutations, there have been no studies of the effect of *mtrCDE* derepression on bacterial survival or fitness to date.

Figure 4. The MtrR regulon. (A) The intergenic region located between *mtrR* and *mtrCDE* is shown. The boxed region denotes the MtrR binding site (103). This region contains the mutation found in strain KH15 and the majority of *mtr* clinical isolates (*). This region also contains the overlapping -35 promoter sites of the *mtrCDE* and *mtrR* operons (denoted by bars) (65, 103). (B) The MtrR repressor negatively regulates the transcription of *mtrF* (48), *mtrCDE* (65), *pilMNOPQ* (47), and *farR* (96). Additionally, MtrR acts as an activator of the *ponA* gene by an unknown mechanism (47). By repressing expression of *farR*, the MtrR protein is also an indirect activator of the *farAB* operon (96).

Figure 4. The MtrR regulon



Positive Regulation of the mtrCDE Operon

In many cases, RND-type efflux pump operons are under the control of positive transcriptional regulators or activators. Activators allow the organism to up-regulate the transcription of a needed efflux pump when conditions are present that favor the pump (43). Efflux pumps are often under the control of both local and global activators (67). However, there are no transcriptional activator homologs found within 2 kb of the *mtrCDE* locus, so this discussion will center on global activators of efflux pumps. The best described examples of global efflux activators are the three activators that control the AcrAB-TolC efflux pump of *E. coli*, namely, MarA, SoxS, and Rob. MarA and SoxS are small DNA-binding proteins of the XylS/AraC family of transcriptional activators, and control over 60 chromosomal genes (11) MarA is part of the *mar* regulon, which was originally described for its ability to increase antibiotic resistance (62). SoxS was initially described as part of a response regulon to oxidative stress (6). Rob is a larger protein that responds to increasing concentrations of fatty acids or bile salts by binding them, and then binding to efflux pump operons to induce expression of AcrAB (157). A DNA binding site for these activators was found upstream of the *tolC* gene, which is induced by these different activators, but is not located near the *acrAB* operon (9). These findings and the discovery of XylS/AraC-type activators that controlled efflux pump expression in other Gram-negative pathogens (reviewed in (51)) prompted the examination of the gonococcal genome for similar activator homologs. The transcriptional activator MtrA was discovered using reverse genetics to screen for AraC-

type regulators that affect gonococcal levels of resistance to substrates of the MtrC-MtrD-MtrE pump. An *mtrA* mutant has similar levels of resistance to the wild type parent strain under normal culture conditions. However, when the *mtrA*-mutated bacteria are grown in a sub-lethal level of pump substrate there is no change in resistance levels. This effect is in stark contrast to the wild type strain where these growth conditions increase transcription of the *mtrCDE* operon, which causes a ten-fold increase in resistance compared to a non-activated strain (162).

The *mtrA* gene exists in its wild type state in only half of the gonococcal strains described. Strains FA19 and MS11 have a functional *mtrA* gene, however the *mtrA* of strain FA1090 has an 11-bp deletion early in the gene, which renders the activator useless. Indeed, strain FA1090 displays no increase in resistance to MtrC-MtrD-MtrE substrates when grown on sublethal levels of substrate (162). Little else is known about the *mtrA* gene, though data presented in chapter two, in addition to ongoing work in other laboratories, will provide insight into the effects of *mtrA* on downstream gene regulation and pathogenicity. The studies described in chapter two are the first in which the role of this activator in pathogenesis is shown.

In vivo Models of Gonococcal Infection

Studies of pathogenic bacteria are best completed with the use of an in vivo model that emulates the host niche of the pathogen. An in vivo model of infection allows investigators to examine the: 1) changes a microbe goes through while colonizing the host, 2) contribution of specific genes to pathogenicity and bacterial fitness, 3) immune

response of the host to the infection, 4) effectiveness of potential therapeutics, and 5) efficacy of potential vaccine candidates.

Though microbiologists utilized primate models of gonococcal urethritis in the 1970s to examine the disease state and immunological response to the gonococcus (105), this model is expensive and difficult to use. Other animal models of gonococcal infection include endocarditis in dogs and rabbits (41), infection of the anterior chamber of the rabbit eye (123), and subcutaneous implantation of a chamber containing *N. gonorrhoeae* in rabbits, guinea pigs, hamsters, mice, and rats (10). While these models have provided data concerning bacterial growth and host response, the site of infection and method of inoculation greatly diverge from the natural process of gonococcal infection.

Use of human volunteers as test subjects has yielded useful data concerning the interactions of gonococci with the male urethra. Gonococcal phenotypic variation was observed during experimental urethritis in human male subjects, with extensive variation of both pilin and opacity proteins observed after inoculation (79, 172). Genes such as RecA (32), IgA1 protease (82), and iron uptake receptors (8, 33) were mutated and tested to assess contributions to pathogenicity. The only mutants that were attenuated in this model of infection were those lacking a transferrin receptor protein for iron acquisition (33). Also, vaccine candidates pilin and porin were tested in human male volunteers. Tests with pilin vaccines showed an immune response but no protection (23, 84). Results with porin vaccines were similar, with development of an immune response, but no protection from infection (75). Of note, no efflux pump mutants have been tested in this infection model.

While the male urethritis model has many advantages, it does not address gonococcal urogenital infections in females. Human females cannot be recruited as volunteers for experimental infection due to the increased risk of complications detailed earlier in this chapter. Instead, in vitro cell culture systems have been employed using primary uterine (190) and cervical cells (29), and immortalized cervical (46) and ovarian cell lines (127). Fallopian tube organ cultures have also been used to investigate gonococcal infection (116, 199). Though such studies are useful, they can only evaluate gonococcal interactions with a single cell type and cannot fully account for all of the extraneous factors that exist in a living system.

The use of female mice as a surrogate host for *N. gonorrhoeae* infection has been described in various publications since 1949 (123). Braude et al., reported differences in recovery of the gonococcus from female mice that coincided with estrous phase (24), and Vedros and Kenyon (197) reported the use of mice with imperforate vaginas as a model for infection in 1978. Kita et al. reported the gonococcal infection of female ddY mice inoculated in the appropriate stage of estrous (late proestrus /early estrus). However, the inoculation step in this study occurred only after the vagina was disinfected and pH increased to allow for better bacterial survival, and only short-term colonization was achieved (91). Taylor-Robinson reported the use of estradiol in germ-free mice to promote long term colonization of *N. gonorrhoeae* in female mice (186). These publications contributed to the development of the current female mouse model of genital tract infection established in 1999 (78). This model, called the 17-β-estradiol-treated mouse model, relies on the implantation of a 17-β-estradiol pellet in the shoulder of female mice to stabilize the estrous cycle in an estrus-like phase. The animals are also

treated with antibiotics to suppress the overgrowth of normal vaginal flora that occurs under the influence of estradiol. In this model, piliation is unnecessary for colonization (78) and Opa protein expression is selected for during infection in the majority of mice (177). Experiments in 2002 illustrated that the gonococcal transferrin receptor, which allows *N. gonorrhoeae* to obtain iron from human transferrin, was not necessary for vaginal colonization of mice (80). This result is in contrast to experiments performed with the male urethritis model (33), and is most likely due to differences in host iron sources. Further studies have detailed the attenuation of gonococci mutated in sialyltransferase (204), lactate acquisition (44), and a gonococcal OmpA homolog predicted to be important in defenses against macrophages during murine infection (173).

Like any animal model, there are drawbacks to the use of the estradiol-treated mouse model of gonococcal infection, which include differences in host iron sequestration factors (168), complement factors (131), and gonococcal colonization receptors (55, 83). However, *N. gonorrhoeae* is a human-specific pathogen, and therefore, host restrictions are to be expected with any non-primate animal model. While there are host differences between mice and humans, similarities include such key factors as low levels of oxygen and iron, a pH that is similar to that of the human cervix, the presence of innate immune effectors such as complement and PMNs, and the presence of some natural flora, the presence of hormones and hormonally-regulated factors. In summary, the murine model of lower genital tract infection is a relatively inexpensive in vivo model that enables investigation of previously unexplored areas of gonococcal research.

Previous Work with Neisserial Efflux and Pathogenicity

The specific aims in the next section were formulated based on the principles of efflux pumps detailed above, and the report from this laboratory that illustrated a role for the MtrC-MtrD-MtrE pump in the colonization of the murine lower genital tract (81). In brief, mice inoculated with mutants of strain FA19 in the *mtrD* and *mtrE* genes showed significantly lower bacterial loads compared to mice inoculated with the wild type parent strain. In contrast, a *farB* mutant, which is unable to produce the FarA-FarB-MtrE efflux pump, was not attenuated in this model. These findings were further tested by performing competitive infections with the wild type strain and each of the three mutants, *mtrD*, *mtrE*, and *farB*. The competitive infection data confirmed the single strain infection data by showing severe attenuation for strains mutated in the *mtrCDE* pump. The fact that the *farB* mutant was not attenuated in vivo is evidence that the effects seen with the *mtrE* mutant were directly related to its role in the MtrC-MtrD-MtrE efflux pump complex rather than the FarA-FarB-MtrE pump. These studies were performed in mice that were not treated with antibiotics that are substrates of the MtrC-MtrD-MtrE efflux pump. Therefore, additional studies were performed to determine the role of the ovarian hormone progesterone, which was found to be a substrate of the MtrC-MtrD-MtrE (81). Competitive infections in mice that were ovarectomized or left intact showed that the attenuation of MtrC-MtrD-MtrE mutants was delayed, but still present in mice that were unable to produce ovarian progesterone. The delayed attenuation of the *mtrE* mutant in ovarectomized mice led to the conclusions that both hormonally-independent

and -dependent factors may challenge MtrC-MtrD-MtrE-deficient gonococci. It is likely that in vivo levels of progesterone are too low in the mouse to be the factor that is directly challenging MtrE-deficient gonococci.

This study prompted further investigation into the in vivo roles of transcriptional regulators of the MtrC-MtrD-MtrE pump. Additionally, the effect of naturally occurring *mtr* mutations on pathogen fitness was of interest. The previous study also raised the question of what the in vivo selective factor was that makes the MtrC-MtrD-MtrE pump important to the pathogen. Recent findings that the antimicrobial peptide LL-37 is a substrate of the gonococcal efflux pump MtrC-MtrD-MtrE (176) led us to investigate the role of the mouse homolog, CRAMP, in challenging MtrC-MtrD-MtrE–deficient gonococci.

Specific Aims

The work presented herein tests the hypothesis that transcriptional regulators of the *mtrCDE* operon play important roles in the pathogenicity of *N. gonorrhoeae*. Specifically, I hypothesize that derepression of the efflux pump due to *mtr* mutations, both naturally occurring and laboratory-created, confers fitness advantages to *N. gonorrhoeae* in the murine model of lower genital tract infection. I theorize that derepression of the *mtrCDE* efflux pump operon via mutation of the transcriptional activator MtrR will lead to an increase in the fitness of *mtrR* mutant compared to the parental strain. Conversely, mutation of the activator protein MtrA will cause a decrease in fitness for *mtrA* mutants. Therefore, the first specific aim is to investigate the role of

the *mtrCDE* transcriptional regulators in gonococcal fitness using the murine model of female genital tract infection.

My second aim is to determine if naturally isolated *mtr* mutants display different levels of resistance to macrolide antibiotics and other MtrC-MtrD-MtrE pump substrates. An examination of the literature suggests that certain *mtr* mutations are found more often than others in clinical isolates. I also hypothesize that greater levels of *mtrCDE* derepression will lead to greater pathogen fitness. The results of these investigations are presented in chapters two and three. Additionally, I performed an analysis of clinical isolates from Baltimore, which is presented as an appendix following chapter four.

Chapter 2: Regulation of the MtrC-MtrD-MtrE Efflux Pump System Modulates the in vivo Fitness of *Neisseria gonorrhoeae*

Douglas M. Warner, Jason P. Folster, William M. Shafer, and Ann E. Jerse

Abstract

The *Neisseria gonorrhoeae* MtrC-MtrD-MtrE multidrug resistance efflux pump expels macrolide antibiotics, penicillin, and antimicrobial effectors of the innate defense. Mutation of the *mtrR* locus, which encodes a transcriptional repressor of the *mtrCDE* operon, increases gonococcal resistance to these agents. Here we report that an *mtrR* mutant is more fit than wild type bacteria in a mouse infection model. Consistent with derepression of the *mtrCDE* operon as the primary reason for the fitness benefit, an *mtrR,mtrE* double mutant conferred no increased survival phenotype over an *mtrE* mutant. Gonococcal mutants deficient in MtrA, an activator of the *mtrCDE* operon, exhibited significantly reduced fitness in vivo, and *mtrA* mutants with spontaneous compensatory *mtrR* mutations were selected during infection. Our results confirm the importance of the MtrC-MtrD-MtrE efflux system during experimental gonococcal genital tract infection and also illustrate an antibiotic resistance mechanism that is accompanied by a fitness benefit rather than a fitness cost.

Note: All of the experimentation in this study was performed by Douglas Warner except for the construction of the complemented *mtrR* mutant JF1(*mtrR'*), which was

performed by Dr. Jason Folster. At the time of this writing, this manuscript is in press in *The Journal of Infectious Diseases.*

Introduction

Gonorrhea is the second most frequently reported infection to the Centers for Disease Control and Prevention (77) and an estimated 67 million cases occur annually worldwide (53). Concern over the high prevalence of gonorrhea is intensified by the frequency of ascended infections and serious complications in women (76) and the fact that gonorrhea is a co-factor in the spread of human immunodeficiency virus (31). Antibiotic treatment of infected individuals is an important public health tool in controlling the spread of gonorrhea; the effectiveness of antibiotics against *Neisseria gonorrhoeae*, however, is continually challenged by the relatively rapid emergence of resistant strains (185) (203). It is therefore imperative to fully understand how antibiotic resistance mechanisms relate to fitness in this pathogen.

Bacterial resistance to antibiotics can occur by modification of the target, breakdown of the antibiotic, reduced permeability, and increased expression of multidrug resistance (MDR) efflux pumps via mutation in pump repressor genes (67). Derepression of MDR efflux pumps can have enormous clinical consequences since elevated expression of these systems can increase resistance to multiple antibiotics that differ in target specificity (132). The gonococcus expresses four efflux pump systems, namely MtrC-Mtr-D-MtrE (64), FarA-FarB-MtrE (97), NorM (158), and MacA-MacB (161). The MtrC-Mtr-D-MtrE efflux pump system belongs to the resistance/nodulation/division (RND) family of pumps and bears significant homology to the *Pseudomonas aeruginosa* MexA-MexB-OprM system and the AcrA-AcrB-TolC system in *Escherichia coli* (reviewed in (132)). There are numerous reports of clinical isolates of *N. gonorrhoeae*

that harbor mutations in *mtrR*, the repressor gene of the *mtrCDE* operon, or its promoter region, which is located immediately upstream and divergently transcribed from the *mtrCDE* operon (65, 103). These isolates exhibit increased resistance to penicillin (137, 194), erythromycin, and azithromycin (184, 205, 210). Of these, only azithromycin is still used to treat gonorrhea (203), although the emergence of azithromycin resistant strains, many of which carry an *mtrR* mutation, is a growing concern (118, 211).

It is widely theorized that MDR efflux pumps evolved before the use of antibiotics to counteract antimicrobials in their natural environment. In support of this hypothesis, we previously found that *mtrCDE* operon mutants of *N. gonorrhoeae* were highly attenuated in the female mouse model of genital tract infection (81). The reason the MtrC-MtrD-MtrE efflux pump system confers increased gonococcal survival in vivo is not yet known. One hypothesis is that this system protects against natural immune effectors at the infection site, from which one might predict that mutations in the *mtrR* locus might benefit the bacterium for reasons that are independent of protection against antibiotics. This hypothesis must be carefully tested because MtrR directly or indirectly regulates other genes that may play a role in pathogenesis, including *mtrF* (48), the *farAB* operon (48, 96), *pilM*, and *ponA* (47). Additionally, the importance of the activator protein MtrA, which belongs to the AraC-type family of activators, must be considered. Expression of *mtrA*, which is unlinked to the *mtrCDE* locus, is induced by sub-lethal levels of pump substrates and causes increased expression of the *mtrCDE* operon (162). Recent transcriptional profiling studies (Shafer et al., unpublished observations) indicate the MtrA is involved in regulating other gonococcal genes, the expression of which may be important in gonococcal adaptation or virulence. Therefore, it is important to

distinguish what effects, if any, occur due to a lack of MtrA-regulated gene expression in an infection model.

Here we assessed the in vivo fitness-cost balance that accompanies increased antibiotic resistance in *N. gonorrhoeae* via mutation of the *mtrR* locus. We also measured the in vivo fitness of an *mtrA* mutant to determine if MtrA contributes to gonococcal adaptation in vivo.

Methods

Strains and Culture Conditions: Bacterial strains and primers used are described in tables 2 and 3. All mutants and recombinant strains were resistant to streptomycin (Sm) and constructed in strain FA19Sm^R, a spontaneous Sm^R mutant of wild type strain FA19. The cloning strategy used to genetically mark strains and construct mutants is outlined in figure 5. The Neisseria chromosomal integration system (NCIS) vectors pGCC3 and pGCC5 (121) (provided H.S. Seifert, Northwestern University) were used to express the wild type *mtrR* or *mtrA* gene from a nonessential site in mutants JF1mtrR' and DW5, respectively. A wild type copy of the *mtrR* gene and promoter region from strain FA19 was PCR-amplified with primers 5'mtrR and 3'mtrR; the *mtrA* gene and its promoter region was amplified with primers mtrAfor and mtrArev. The *mtrR* locus in mutants 9AR and 11AR was amplified and sequenced with primers C-Xho and R-Xho. PCR and nucleotide sequence analysis were used to confirm all plasmid constructs, mutants, and recombinant strains. *N. gonorrhoeae* was cultured on supplemented GC agar plates (Difco) as described (81). Antibiotic concentrations were 20 μg/ml chloramphenicol

(Cm), 50 μg/ml kanamycin (Km), 200 μg/ml ampicillin (Amp) for *E. coli*, and 0.5 μg/ml Cm, 50 μg/ml Km, 100 μg /ml Sm and 0.5 μg/ml erythromycin (Em) for *N. gonorrhoeae*.

In vitro Growth Kinetics: Gonococci were cultured in 125 ml of supplemented GC broth at 37° C with aeration as described (204). The starting optical density at 600 nm (OD_{600}) was 0.07-0.08. Growth was measured by change in OD_{600} over time; for *in vitro* competition experiments, mixed broth cultures were incubated and the growth of each strain was followed by quantitative culture on GC agar with the appropriate antibiotic selection.

Experimental Murine Infection: Female BALB/c mice (6-8 weeks old, National Cancer Institute) were treated with 17-β-estradiol and antibiotics to increase susceptibility to *N. gonorrhoeae* and reduce the overgrowth of commensal flora that occurs under the influence of estrogen (81). None of the antibiotics used are substrates of the MtrC-MtrD-MtrE pump and the sensitivity of wild type and MtrC-MtrD-MtrE-deficient bacteria to these antibiotics was shown to be equivalent previously (81). For competitive infections, similar numbers of each strain or mutant to be tested were inoculated into mice (n = 5-8 per mixture) [total dose: 10^6 colony forming units (CFU)] as described (81). Nonpiliated variants were used to minimize the potential transfer of selectable markers during infection by natural transformation. The number of CFU of each strain within the inocula and vaginal mucus was determined by quantitative culture on GC agar with Sm (total number) and GC with Sm plus Km, Cm, or Em. Data were expressed as competitive indices $[(\text{mutant titer})/(\text{wt titer})]_{\text{output}}$ / $[(\text{mutant titer})/(\text{wt titer})]_{\text{inoculum}}$ as described (204).

The limit of detection (4 CFU per 100 µl vaginal swab suspension) was used for samples from which no colonies of *N. gonorrhoeae* were isolated. For infectious dose studies, mice were inoculated with 20 µl of PBS containing 10-10,000 CFU of KH15 or wild type FA19 bacteria (6-8 mice per dose). Vaginal mucus was cultured for *N. gonorrhoeae* on days 2, 4, 6, and 8 post-inoculation. Mice with positive cultures for 6-8 days were considered infected (limit of detection: 1 CFU per 100 µl vaginal swab suspension). The ID_{50}, ID_{80}, and 95% confidence intervals were established by Probit analysis (201) for each strain. Animal experiments were conducted in the laboratory animal facility at Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol approved by the University's Institutional Animal Care and Use Committee.

Minimal Inhibitory Concentrations (MIC): The MIC of Triton X 100 (TX-100), Em, azithromycin (Az), and Km was determined by agar dilution (81).

Western Blot Analysis: Bacteria that were cultured on GC agar for 22-24 hrs were suspended in PBS, sonicated (3 min, 30 second blasts), and centrifuged at 5,000 rpm to remove unbroken cells. Total membranes were isolated by centrifugation at 100,000 x *g* for 2 hr. Membrane proteins were fractionated on SDS-polyacrylamide gels under denaturing conditions and probed with rabbit polyclonal antibodies generated against peptide RQGSLSGGNVS, which corresponds to amino acids 110-120 of the 467 amino acid MtrE protein. Peptide synthesis, KHL conjugation, immunizations, and affinity purification was by Bethyl Laboratories (Montgomery, TX).

Results

Increased in vivo Fitness of an *mtrR* Mutant: To address the hypothesis that derepression of the *mtrCDE*-encoded efflux pump yields a strain that is better adapted to the genital tract, we compared the fitness of mutant JF1, which contains an internal *mtrR* deletion, to that of wild type bacteria in the murine genital tract infection model. A Cm^R derivative of $FA19Sm^R$, the parent strain of JF1, was used as the wild type strain to facilitate accurate determination of the relative number of mutant and wild type bacteria in vaginal mucus. JF1 and $FA19Cm^R$ bacteria showed no difference in growth kinetics or recovery when cultured together in broth (in vitro competition assay). The presence of the *cat* cassette in $FA19Cm^R$ also did not alter the in vitro growth kinetics of the wild type strain (Fig. 6A, inset). In contrast, following inoculation of mice with similar numbers of JF1 and $FA19Cm^R$ gonococci, JF1 bacteria exhibited a significant fitness advantage over the wild type strain as evidenced by a 100- to 10,000-fold increase in the competitive index (CI) within 1 day post-inoculation. This advantage was maintained throughout the course of infection in three separate experiments and wild type bacteria were no longer detected by day 3 in a majority of mice (Fig. 6B, open circles). Expression of *mtrR* from an ectopic site resulted in loss of the in vivo fitness advantage (Fig. 6C), despite the fact that resistance to TX-100, a laboratory substrate of the MtrC-MtrD-MtrE pump (Table 4) and the production of MtrE (Fig. 7) were only partially restored in the complemented mutant JF1(*mtrR'*). The simplest explanation for the incomplete restoration of these in vitro phenotypes is the possibility that *mtrR* expression from a site distant from the

mtrCDE locus may not be the same as that which occurs in its native site. However, considering the fact that MtrR acts on other loci besides *mtrCDE* (47, 48, 96), these results also raised the possibility that targets of MtrR other than the MtrC-MtrD-MtrE pump may contribute to the fitness advantage demonstrated by the *mtrR* mutant in vivo.

We therefore next compared the fitness of a mutant unable to produce both MtrR and a functional MtrC-MtrD-MtE pump with that of a mutant that only lacked the MtrC-MtrD-MtrE system. We predicted that if genes other than *mtrCDE* were responsible for the observed fitness advantage of the *mtrR* mutant, then a mutant unable to produce both MtrR and a functional MtrC-MtrD-MtE pump would still have a competitive advantage over gonococci that were only deficient in the MtrC-MtrD-MtrE system. Competitive infections were performed between an *mtrR,mtrE* double mutant (strain DW4) and strain DW3K, which carries a Km^R marker and the same disrupted *mtrE*::Cm locus found in DW4 bacteria. DW4 and DW3K bacteria had identical in vitro growth dynamics compared to their parent strains and each other (data not shown), and no fitness difference was observed in vivo as evidenced by CI values that remained consistently around 1.0 (Fig. 6D). We conclude that derepression of the *mtrCDE*-encoded efflux pump system via loss of MtrR was responsible for a distinct fitness advantage in vivo.

To examine if the fitness advantage conferred by *mtrCDE* derepression was associated with increased infectivity we performed infectious dose studies with wild type FA19Sm^R bacteria and mutant KH15, which carries a single bp deletion in the 13-bp inverted repeat within the *mtrR* promoter that is contained within the MtrR binding site upstream of the *mtrCDE* operon (103). This deletion is frequently found in clinical isolates and confers greater resistance to hydrophobic agents and higher levels of

mtrCDE transcription than that conferred by mutations in the *mtrR*-coding sequence (64, 103, 211). KH15 bacteria reproducibly infected more mice at each dose, with approximately 2 x 10^3 wild type CFU versus 3 x 10^2 KH15 CFU required to infect 50% of the mice, and 3 x 10^5 wild type CFU versus 1 x 10^4 KH15 CFU required to infect 80% of mice. Confidence intervals overlapped, however, and therefore, these differences were not considered significant. No spontaneous *mtrR* mutants were detected in mice inoculated with wild type bacteria, and in vitro growth dynamics of FA19Sm^R and KH15 bacteria appeared identical (data not shown).

***mtrA* Mutants are Attenuated in vivo**: We also compared the relative fitness of wild type gonococci and the *mtrA* mutant JF3, which is incapable of inducing expression of *mtrCDE* through the action of the MtrA activator protein (162). JF3 bacteria showed no fitness difference compared to wild type FA19Sm^R gonococci in vitro (Fig. 8A). However, recovery of JF3 bacteria from mice was decreased 10-1,000-fold compared to wild type gonococci within two days after inoculation (Fig. 8B). Recovery was restored to wild type levels by genetic complementation (Fig. 8C). As with MtrR, MtrA affects the transcription of other gonococcal genes (Shafer *et al.*, unpublished observations) and therefore, we created an *mtrA,mtrE* double mutant (DW6) for comparison with mutant DW3, which carries only an *mtrE* mutation. No fitness advantage was detected in competitive infections with mutants DW6 and DW3 (Fig. 8D). We thus conclude that the *mtrA* mutant was attenuated in the murine genital tract primarily due to its incapacity to induce expression of *mtrCDE* above wild type levels.

Interestingly, despite clearance of *mtrA::aphA3* mutant bacteria in a majority of mice co-inoculated with wild type and MtrA-deficient bacteria (Fig. 8B, open symbols), in one experiment we recovered high numbers of Km^R colonies from two mice 4 days after inoculation, which is the time point that coincided with loss of the wild type strain. Consistent with an *mtrR* phenotype, the Km^R isolates grew on GC agar with 0.5 μg/ml Em. PCR analysis revealed that the *mtrA* locus remained disrupted. A 1-bp insertion in the *mtrR* gene was identified at nucleotide 160 in the *mtrR* structural gene in one of the in vivo-derived mutants (strain AR11). This mutation is predicted to cause a frameshift that would encode a truncated 52 amino acid protein in which the DNA-binding site would be disrupted (103). The other mutant, AR9, contained a 1-bp change, which would result in a glycine instead of glutamic acid at amino acid 202, which is near the C-terminal end of the 210 amino acid MtrR protein. We do not yet know how mutations in this region impact MtrR activity; MtrR represses *mtrCDE* transcription through two homodimers (73) and it is conceivable that the E202G substitution may impact the dimerization process.

Replacement of the wild type *mtrR* gene in strain $FA19Sm^R$ with the $mtrR_{1\text{-}52}$ and $mtrR_{E202G}$ mutant alleles by allelic exchange to create mutants DW9 and DW11 confirmed linkage of the Em^R and TX-100 resistance phenotypes to these mutated genes (Table 4). Mutant DW11 ($mtrR_{1\text{-}52}$) exhibited resistance levels that were similar to that of the *mtrR* deletion mutant JF1 and 3-fold greater than that of wild type gonococci, and as predicted, this frame-shift mutation conferred a strong fitness advantage in vivo (Figure 9A). Mutant DW9 ($mtrR_{E202G}$) demonstrated an intermediate level of resistance to these agents; however, DW9 gonococci exhibited only slightly elevated fitness compared to

FA19Cm^R bacteria in vivo, with mean CI values of 5.1 (day 2), 1.8 (day 4), and 2.2 (day 6) (Fig. 9B). The *mtrR*$_{E202G}$ allele was originally identified as a secondary mutation in an *mtrA* mutant, which appeared to compensate for the lack of MtrA since the recovery of these mutant gonococci was similar to that of the wild type strain (Fig. 9B). Expression of the *mtrR*$_{E202G}$ allele in a clean *mtrA* mutant background also resulted in a fitness phenotype that was equal or slightly elevated compared to the wild type strain (Fig. 9C), which confirms the compensatory nature of the *mtrR*$_{E202}$ mutation when in a MtrA-deficient background.

Discussion

In general, acquisition of antibiotic resistance is accompanied by a biological cost, which is usually measured as a reduced in vitro growth rate of resistant bacteria (98). More recently, the impact of antibiotic resistance on microbial fitness *in vivo* has been examined. Specifically, a fitness loss accompanied antibiotic resistance due to mutation of the target gene in animal models of *Staphylococcus aureus* (129), *Salmonella enterica* serovar Typhimurium (20, 111), and *Streptococcus pneumoniae* (189) infection. An exception to these studies is the reported colonization advantage displayed by some fluoroquinolone-resistant *Campylobacter jejuni* strains due to mutation of *gyrB* (108). Here we report that the acquisition of antibiotic resistance in *N. gonorrhoeae* via derepression of an MDR-encoding pump operon is accompanied by fitness benefits in vivo, which for an organism with no outside reservoir like *N. gonorrhoeae*, is the only relevant setting for measuring fitness cost. Our use of the mouse model to study the

consequence of *mtrR* mutation in *N. gonorrhoeae* is supported by physiological and immunological similarities between the lower genital tracts of women and female mice (44, 80, 204). Overexpression of the *mtrCDE* operon during infection may increase gonococcal evasion of natural immune effectors such as bile salts (63), progesterone (81), the human antimicrobial peptide LL-37 (176), and in the case of experimental murine infection, the cathelicidin-related protein (CRAMP). CRAMP, the murine homologue of LL-37, is expressed by epithelial cells and is present in the granules of polymorphonuclear leukocytes (178), and is known to be important in the immune response to bacterial infections (136).

The importance of MtrC-MtrD-MtrE system and its regulators in vivo is further supported by the unexpected selection for compensatory *mtrR* mutations in a genetically engineered *mtrA* mutant during murine infection. The *Campylobacter jejuni* CmeA-CmeB-CmeC (101) and the *Pseudomonas aeruginosa* MexA-MexB-OprM (71) efflux systems also play a role in pathogenesis, although the *P. aeruginosa* MexA-MexB-OprM and MexA-MexB-OprJ efflux systems may reduce or increase fitness depending on the infection model used (86, 165); these latter results might be predicted based on the diverse niches inhabited by this pathogen and the role of these efflux systems in both extrusion of antibiotics and secretion of virulence factors and quorum-sensing signals.

Both MtrA and MtrR up- and down-regulate other gonococcal genes, including *mtrF*, *ponA*, *farR*, and *pilM* (47, 48, 96) (and W.M. Shafer et al., un-published). Comparisons of mutants deficient in an active MtrC-MtrD-MtrE efflux system versus both the MtrC-MtrD-MtrE pump and either of these two regulators showed modulation of the *mtrCDE* operon was the primary reason for the increased and reduced fitness of *mtrR*

and *mtrA* mutants in vivo, respectively. This conclusion should be qualified, however, by limitations in the competitive infection assay. Because MtrC-MtrD-MtrE-deficient bacteria are significantly attenuated in the murine model (81), the overall bacterial load in mice inoculated with mixtures of bacteria unable to express the pump were two to three logs lower than that recovered from mice infected with MtrC-MtrD-MtrE-proficient bacteria. The sensitivity of the CI value is reduced with lower colonization by both strains, and thus leaves open the possibility that other MtrR- and MtrA-regulated factors(s) may also contribute to pathogenicity. Studies similar to those reported herein using mutants in these regulated genes should clarify this issue.

Since mutations in *mtrR* provide a fitness advantage for gonococci in the genital tract infection model employed herein, why are such strains not more frequently isolated, especially from the lower genital tract of females? It is not known if MtrR-deficient strains would demonstrate an altered fitness phenotype in other body sites colonized by *N. gonorrhoeae*, although there is evidence that *mtrR* mutant isolates may have a selective advantage during rectal infections (126, 205) where it is postulated that resistance to long chain fatty acids in fecal lipids may, in certain instances, be *mtr*-dependent. It is also possible that there are other genes regulated by MtrA or MtrR, which are important for gonococcal survival but do not play a role in the female genital tract or are host-restricted. The time period studied here might also be insufficient for measuring the effects of *mtrR* mutation over the course of a prolonged infection. These effects could be as simple as the *mtrR* mutant having a minor growth disadvantage that we did not detect under standard culture conditions, but which was reported to accompany overexpression of other resistance pumps (5). We propose a more complex explanation

based on the existence of both positive (MtrA) and negative (MtrR) regulators, which have other targets in addition to the *mtrCDE* operon. At early stages of infection, such as in the 5-7 day time period used in our experiments, the increased resistance of gonococci to local antimicrobials afforded by the pump can be achieved by derepression (loss of MtrR) or by activation (MtrA-dependent). Based on our competitive infection data, it seems likely that at least in vivo, induced levels of the pump are not as high as that which occurs when *mtrR* is mutated. At later stages of infection, other antimicrobial pressures may be operative and resistance mechanisms distinct from the pump may be more critical. Such later stage resistance mechanisms may be optimally expressed in strains that possess an active MtrR protein; additionally, other bacterial functions that are encoded by MtrR-regulated genes outside of the *mtrCDE* locus may also be important during prolonged infections. Continued delineation of the MtrR and MtrA regulons and their importance in vivo should reveal important insights into the host and bacterial factors that have shaped the evolution of this highly successful and well-adapted pathogen.

Figure 5. Plasmid and strain construction. The 5.5-kb *ClaI* fragment of plasmid pGCC5 (121) shown at the top of the figure was used to create strain FA19Cm^R as described (177). Strain FA19 Km^R was constructed by replacing the *cat* marker of strain FA19Cm^R with the *aphA-3* cassette from plasmid pGCCKm, the construction of which is shown in the left side of the figure. To construct pGCCKm, a 2.3-kb PCR fragment from pGCC5 was amplified using primers F1-lctP (44) and icatB (177) and ligated it into the *MscI* site of pBR322 to create pBR-GCC (Amp^R, Cm^R). An 850-bp *SmaI* fragment containing a nonpolar *aphA-3* cassette from pUC18K (122) was then inserted into the *MscI* site within the *cat* gene of pBR-GCC to create pGCCKm. Mutants DW3 (*mtrE*::*cat*) and DW4 (*mtrE, mtrR*) were constructed by introducing the *mtrE*::*cat* fragment into strains FA19Sm^R and JF1*mtrR*', respectively on a 2.4-kb *BamHI, EcoRV* fragment from pCRmtrE::Cm. The construction of plasmid pCRmtrE::Cm is shown on the right side of the figure. Briefly, a 760-bp PCR product containing the *cat* gene from pGCC5 was amplified using primers icatB and icatC (177) and ligated into the *BsrBI* site within the *mtrE* gene cloned into pCR-Blunt (pCR-mtrE). Klenow fragment was used to fill in *BsrBI* ends prior to ligation. The source of the *mtrE* gene in plasmid pCR-mtrE was a 1.7-kb PCR fragment amplified from strain FA19 using primers mtrE-for and mtrE-rev. Mutant DW6 (*mtrA,mtrE*) was constructed by transforming mutant DW3 with a PCR-amplified *mtrA::aphA3* gene from strain JF3 obtained with primers mtrAfor and mtrArev. To create an *mtrE* mutant in a Km^R background (strain DW3K), FA19Km^R was transformed with the *mtrE::Cm* PCR fragment from strain DW4 obtained with primers mtrE-for and mtrE-rev. *E. coli* strain TOP-10 (Invitrogen) was used for all cloning experiments and PCR fragments were introduced into *N. gonorrhoeae* by transformation (60) or electroporation (42).

Figure 5. Plasmid and strain construction

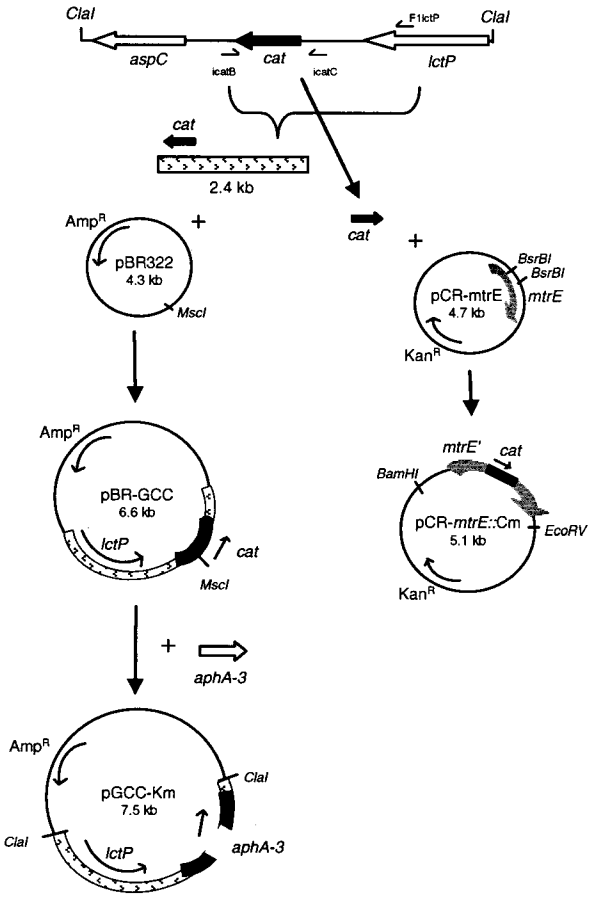


Figure 6. Effect of *mtrR* mutation on in vitro and in vivo fitness. (A) in vitro competition assays for FA19Cm^R versus *mtrR* mutant JF1 (solid diamond) and FA19Cm^R versus FA19Sm^R (open square). Bacteria were cultured together in GC broth. Data are expressed as competitive indices (CIs) as defined in the Methods. The inset shows total growth over time as measured by OD_{600}. Competitive infection of female mice was used to compare the in vivo fitness of (B) FA19Cm^R versus JF1, (C) FA19Cm^R versus JF1mtrR', and (D) DW3K versus DW4 bacteria. Symbols correspond to CI values from individual mice within an experiment; open circles represent cultures from which no wild type (B and C) or DW3K (D) bacteria were recovered. Bars indicate the geometric mean. The dashed line denotes a CI of 1.0 (no fitness difference). Asterisks in panels C and D denote a significant difference in the distribution of CIs compared to those obtained from mice inoculated with FA19Cm^R and JF1 gonococci (panel B) (Students unpaired t test, $p < 0.05$).

Figure 6. Effect of mtrR mutation on in vitro and in vivo fitness

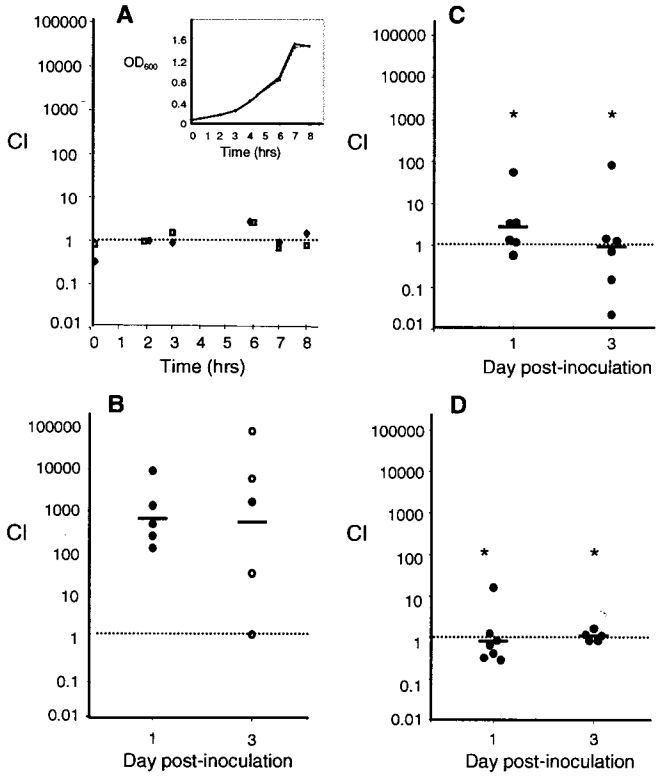


Figure 7. MtrE expression by wild type and mutant strains used in this study. Immunoblot of total membrane proteins incubated with polyclonal MtrE-specific rabbit antibodies followed by anti-rabbit IgG-horse radish peroxidase. There is increased expression of MtrE in *mtrR* mutant JF1 (lane 2) compared to wild type FA19Sm^R gonococci (lane 1) and partial restoration of expression to wild type levels by genetic complementation of the mutation (lane 3). Lanes 4 and 5 show no expression of MtrE by strains DW3 and DW4, which carry *mtrE::cat* mutations. All lanes were equally loaded as assessed by Coomassie blue stain. Detection was by chemiluminescence (Amersham).

Figure 7. MtrE expression by wild type and mutant strains used in this study

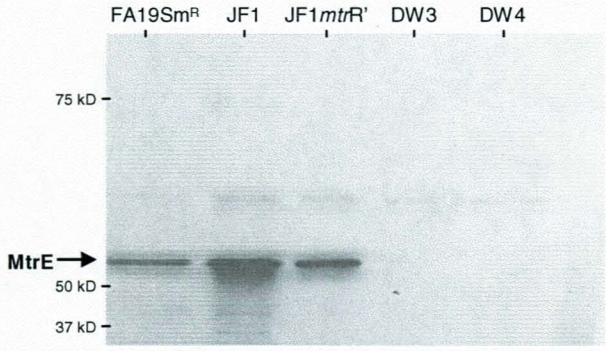


Figure 8. Effect of *mtrA* mutation on fitness in vitro and in vivo. (A) in vitro fitness of FA19Sm^R versus *mtrA* mutant JF3 bacteria when cultured together in GC broth (as described in the Fig. 6 legend). The inset shows total growth over time as measured by OD_{600}. Competitive infection of female mice was used to assess the in vivo fitness of (B) FA19Sm^R versus JF3, (C) FA19Sm^R versus DW5, and (D) DW3 versus DW6 bacteria. Symbols correspond to CI values from individual mice within an experiment; open circles in panel B represent cultures from which no MtrA-deficient bacteria were recovered. The geometric mean is indicated by a bar. The dashed line denotes a CI of 1.0 (no fitness difference). Asterisks in panels C and D denote a significant difference in the distribution of CIs when compared to those obtained from mice inoculated with FA19Cm^R and JF3 gonococci (panel B) (Students unpaired t test, $p < 0.05$). † indicates cultures from which spontaneous *mtrR,mtrA* double mutants AR9 and AR11 were isolated.

Figure 8. Effect of *mtrA* mutation on fitness in vitro and in vivo

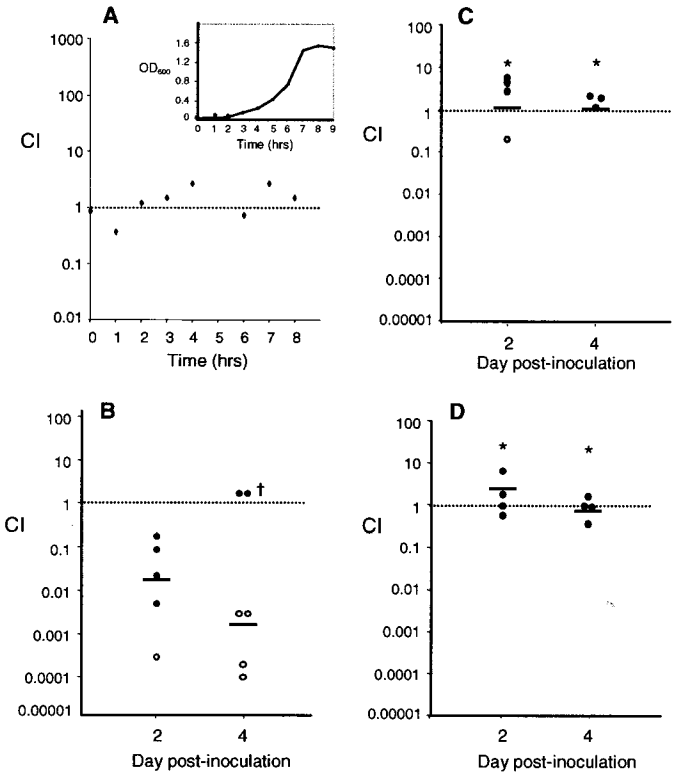


Figure 9. In vivo fitness of wild type or *mtrA* mutant bacteria carrying mouse-derived *mtrR* mutations. Competitive infection of female mice was used to assess the *in vivo* fitness of FA19Cm^R versus *mtrR* mutants (A) DW11 ($mtrR_{1\text{-}52}$) and (B) DW9 ($mtrR_{E202G}$). (C) FA19Sm^R versus mutant DW9A, which is an *mtrA* mutant that also carries the $mtrR_{E202G}$ mutation. As a control, another group of mice was infected with *mtrA* mutant JF3 and FA19Sm^R in parallel, and unlike mutant DW9A, a dramatic loss in the recovery of JF3 bacteria occurred over time (data not shown), similar to that shown in Fig. 8B. Symbols correspond to CI values from individual mice within an experiment; open circles represent cultures from which no wild type (A and B) bacteria were recovered. Bars indicate the geometric mean. The dashed line denotes a CI of 1.0 (no fitness difference). All strains showed equal fitness when co-cultured in broth cultures (data not shown)

Figure 9. In vivo fitness of wild type or *mtrA* mutant bacteria carrying mouse-derived *mtrR* mutations

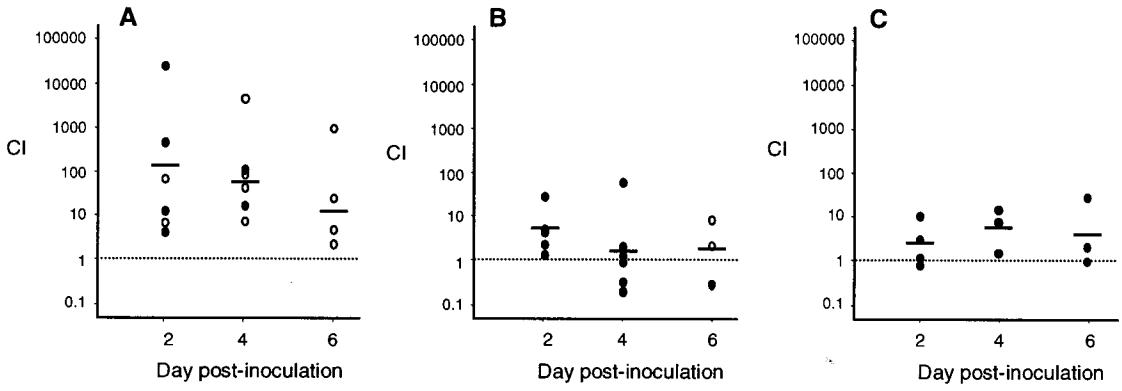


Table 2. *Neisseria gonorrhoeae* strains used in this study

Strain	Relevant Genotype	Reference
FA19Sm^R	spontaneous Sm^R mutant of wild type FA19	(81)
	parent strain of all strains used in this study	
FA19Cm^R	*cat* gene inserted between *aspC* and *lctP* locus	this study
FA19Km^R	*aphA3* gene inserted between *aspC* and *lctP*	this study
JF1	$mtrR_{\Delta 55\text{-}633}$	(48)
KH15	*mtrR* with1-bp missense promoter mutation	(64)
JF1mtrR'	JF1 complemented with *mtrR*	this study
DW3	*mtrE*::*cat* mutation	this study
DW4	$mtrR_{\Delta 55\text{-}633}$, *mtrE*::*cat*	this study
DW3K	*mtrE*::*cat* mutation	this study
JF3	*mtrA*::*aphA3* mutation	(162)
DW5	JF3 complemented with *mtrA*	this study
DW6	*mtrA*::*aphA3*,*mtrE*::*cat*	this study
AR9[a]	*mtrA*::*aphA3*, $mtrR_{E202G}$	this study
AR11[a]	*mtrA*::*aphA3*, $mtrR_{1\text{-}52}$	this study
DW9	$mtrR_{E202G}$[b]	this study
DW11	$mtrR_{1\text{-}52}$[b]	this study
DW9A[c]	$mtrR_{E202G}$,*mtrA*::*aphA3*	this study

[a]Mouse-derived JF3 derivative.

[b]Mouse-derived mutation transformed into wild type FA19Sm^R background.

[c]Strain DW9 transformed with the *mtrA::aphA3* cassette.

Table 3. Oligonucleotide primers used in this study

Primer	Primer sequence
Afor	5' ATGTACGTAGGATGCGCCTTACCCA 3'
Arev	5'GCAATGCATTGAAATGACGCGGTAC 3'
5'mtrR	5'GGTTAATTAACGCCTTAGAAGCATAAAAAGC 3'
3'mtrR	5'GGGTTTAAACTTATTTCCGGCGCAGGCAG 3'
mtrE-for	5'CCTGTATATTGCCGCTGGTG 3'
mtrE-rev	5'TGCCGTCTGAAACCTTATCG 3'
C-Xho	5'ATACTCGAGATCCGCTTTGGCAAGCGTTG 3'
R-Xho	5'CCAGCTCGAGTTGTGTCATTTTGATGCCG 3'

Table 4. Strain susceptibility to substrates of the MtrC-MtrD-MtrE efflux pump system

	Minimum Inhibitory Concentration (μg/ml)				
	Em	**Az**	**Km[a]**	**TX-100**	
Strain				**Uninduced**	**Induced[b]**
FA19Sm^R	0.5	0.06	31	62	200
FA19Cm^R	0.5	0.06	31	62	200
FA19Km^R	0.5	0.06	100	62	200
JF1	4.0	0.125	31	500	750
JF1mtrR'	NA[c]	NA[c]	31	250	
DW3K	<0.05	0.03	100	31	
DW3	<0.05	0.03	31	31	
JF3	0.5	0.06	31	62	62
DW5	0.5	0.06	31	62	200
DW6	<0.05	0.03	31	31	
9AR[d]	1.0	0.125	31	125	
11AR[d]	4.0	0.125	31	500	
DW9	2.0	0.125	31	125	
DW11	4.0	0.125	31	500	
DW9A	1.0	0.125	31	125	

[a]Km is not a substrate of the MtrC-MtrD-MtrE system and was used as a control.

[b]Increased expression of MtrC-MtrD-MtrE was induced by growth on GC agar with subinhibitory concentrations of Triton X-100 (50 μg/ml) (162).

[c]N/A, not applicable due to presence of an *ermC* cassette in strain JFmtrR'

[d]Original mouse-derived strain.

Chapter 3: Clinically Isolated Mutations in the MtrR Repressor of the MtrC-MtrD-MtrE Efflux Pump Confer Different Levels of Antibiotic Resistance and in vivo Fitness to *Neisseria gonorrhoeae*

Abstract

The *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump is a critical factor for both antibiotic resistance and protection from antimicrobial peptides. Derepression via mutation of *mtrR*, a gene that encodes a transcriptional repressor of the pump, results in increased expression of the *mtrCDE* operon and a subsequent increase in resistance to erythromycin, azithromycin, and penicillin. Here we examined naturally occurring *mtrR* mutants, including one found in the well-characterized laboratory strain MS11 for differences in the level of efflux pump derepression. We found that mutations upstream of the *mtrR* gene, including the MtrR binding region and a novel mutation located 131-bp upstream of the *mtrR* gene showed the highest levels of antibiotic resistance and *mtrCDE* transcription. Mutations located in the MtrR structural gene had an intermediate or low resistance phenotype. The gradient of resistance was paralleled by a gradient of increased fitness in the murine model of female genital tract infection. The resistance of these natural mutants to the murine antimicrobial peptide CRAMP also showed a gradient, which in general corresponded to the differences seen in vivo. Additionally, we report the presence of two mutations in the *mtr* locus of strain MS11, one of which, mtr_{-131} is a novel mutation in the 5' untranslated region of the *mtrCDE* transcript. We showed that the -131 mutation increases the half-life of *mtrC* RNA. These studies

establish a hierarchy of mutations with regard to regulation of pump efflux, and suggest that selection for more de-repressed mutants may occur in mixed infections.

Note: All of the experimentation in this study was performed by Douglas Warner. At the time of this writing, this manuscript is in preparation for submission to Antimicrobial Agents and Chemotherapy.

Introduction

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, is the second most commonly reported bacterial infection in the United States (77). While disease mortality is low, complications in women can lead to pelvic inflammatory disease, sterility, and ectopic pregnancy (14). Since no vaccine currently exists for *N. gonorrhoeae*, antibiotic therapy is a major component of infection control. The use of antibiotics against gonorrhea began with the introduction of sulfonamides in the 1930s (179). Soon thereafter, reports of *N. gonorrhoeae* strains with decreased susceptibility to sulfonamides began to arise. These findings prompted the use of penicillin for treatment of gonococcal infection. However, reports of penicillin resistant strains surfaced within five years of its introduction as a therapeutic (reviewed in (179)). The rapid emergence of antibiotic resistance in *N. gonorrhoeae* has been seen with other drugs, including tetracycline, ampicillin, and most recently fluoroquinolones (185). Thus

the ability of *N. gonorrhoeae* to quickly develop resistance to antibiotic treatments has been an obstacle to disease control as long as chemotherapeutics have been employed.

Mechanisms of antibiotic resistance include mutation of the antibiotic target, enzymatic breakdown of the antibiotic, decreased permeability of the bacterial membrane, and both the presence and derepression of transmembrane efflux (reviewed in (4). The MtrC-MtrD-MtrE efflux pump system is one of four known efflux pumps of *N. gonorrhoeae* (97, 158, 161). The MtrC-MtrD-MtrE efflux pump expels macrolide antibiotics and is implicated in high level penicillin resistance (194). Substrates of the MtrC-MtrD-MtrE pump also include the hydrophobic agents Triton X-100, ethidium bromide, progesterone, bile salts, and antimicrobial peptides (37, 64). We previously examined the role of the MtrC-MtrD-MtrE pump in vivo using a female mouse model of lower genital tract infection, and found that mutants in the MtrC-MtrD-MtrE pump were highly attenuated. In contrast, mutants in the FarAB efflux pump were not attenuated compared to the wild type strain (81). The reason for the observed attenuation is not known. Of note, the human cathelicidin LL-37 is a substrate of the MtrC-MtrD-MtrE pump (176), and has been localized to a variety of mucosal surfaces including the urogenital tract (30).

The MtrC-MtrD-MtrE pump is a member of the resistance-nodulation-division (RND) family of efflux pumps, and like other homologous pumps, is under the tight control of both a repressor, MtrR (65) and an activator MtrA (162). The *mtrR* gene is located 250-bp upstream and is divergently transcribed from the *mtrCDE* operon. The MtrR repressor is a TetR type repressor that binds to an inverted repeat in the intergenic region between *mtrCDE* and *mtrR* via a four alpha-helix domain located near the N-

terminus of the protein (73). In its bound form, the MtrR repressor is a four protein dimer of a dimer, much like the similar transcription factor QacR (170).

Strains that bear mutations in *mtrR* have been recovered in a variety of *N. gonorrhoeae* outbreak investigations. The most common mutation is a single base pair deletion located in the inverted repeat that acts as the shared but divergent promoter regions of *mtrR* and *mtrCDE* (34, 38, 104, 130, 184, 210, 211). Other commonly described mutations are located in the structural gene of *mtrR*, and include a G45D mutation (38, 174, 184, 198, 210) and an A39T mutation (38), which are found in the proposed DNA-binding domain of MtrR. All of these mutations confer decreased susceptibility to antibiotics. There are two reports that compared the resistance differences between a promoter mutation and an inactivated *mtrR* gene (65, 174). To date, there are no further studies that detail the differences in derepression conveyed by the most common *mtrR* mutations found among clinical isolates. Additionally, none of these naturally occurring mutations has been tested for in vivo fitness. More recently, we reported that derepression of the *mtrCDE* operon via mutation of the *mtrR*-encoded transcriptional repressor increased fitness in the lower genital tract of female mice (Warner et al., 2007). The reason for these in vivo findings is currently unknown, but is likely due to a role for the efflux pump in the evasion of innate immune defenses (81, 191).

Here we compared five different *mtrR* mutations that were isolated clinically or from experimentally infected mice to determine if they confer differential levels of *mtrCDE* operon derepression as measured by levels of RNA transcript, protein production, antibiotic resistance, and in vivo fitness. Additionally we report that the

frequently used laboratory strain MS11 is a natural *mtr* mutant, which harbors a mutation that heretofore has not been described.

Methods

Bacterial Strains and Culture Conditions: *Neisseria gonorrhoeae* strain FA19 Sm^R is a spontaneous streptomycin resistant mutant of strain FA19 (*porB1A*, serum resistant) and was described previously (81). Strain FA19 Cm^R and *mtr* mutants JF1, DW9, DW11, and KH15 were described previously (Warner et al., 2007). A series of additional mutants in the *mtrR* gene or promoter region were constructed in strain FA19Sm^R and are described in Table 5. All *mtr* transformants were isolated on GC agar supplemented with 0.5 μg/ml erythromycin (Em), a phenotype conveyed by derepression of the *mtrCDE* operon. Mutant *mtrR* loci were PCR-amplified from the clinical isolates LG5 or LG7 (Garvin et al., in preparation), or strain MS11 with primers R-Xho and C-Xho as described (Warner et al., 2007), purified by agarose gel fractionation, and transformed into strain FA19Sm^R. The *cat* gene of pGCC-5 (provided by H.S. Seifert, Northwestern University) was used to mark strains FA19 Sm^R, DW9, DW39, and DW45 in an untranscribed chromosomal region between the *lctP* and *aspC* genes as described previously (177), which yielded strains FA19Cm^R DW9Cm^R, DW39Cm^R, and DW45Cm^R. *mtrA* mutant strain DW131A was created by PCR amplification of the mutant *mtrA::aphA3* allele from strain JF3 using primers mtrAfor and mtrArev (Warner et al., 2007). Strains DW131, FAMS, and A39T were transformed with the resultant 1900-bp product and transformants were selected on GC agar supplemented with

kanamycin (Km). To construct mutant JF1$_{-131}$, which contains both the *ΔmtrR* and *mtr*$_{-131}$ mutations, the PCR-amplified *mtrR* locus from strain JF1 was transformed into strain DW131. All transformations were performed by the method of Gunn & Stein (60). The nucleotide sequences of the *mtr* regions of strains DW9, DW11, DW39, DW45, MS11, DW131, and FA19$_{MS11mtr}$ were determined by PCR amplification and subsequent sequencing with primers C-Xho and R-Xho. All sequencing was performed using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems), by the Biomedical Instrumentation Center of the Uniformed Services University of the Health Sciences (USUHS). All bacterial strains were cultured on supplemented GC agar (Difco) as described in (204). All antibiotics were from Sigma and were used to supplement selective media at the following concentrations: Cm at 0.6 μg/ml, Em at 0.5 μg/ml, and Km at 50 μg/ml.

Determination of Minimum Inhibitory Concentration (MIC): The MIC of Triton X-100, Em, azithromycin (Az), and Km against wild type parent strain FA19 Sm^R and the *mtrR* mutants was established using standard two-fold agar dilution assays (Warner et al., 2007).

Resistance to Antimicrobial Peptides and Progesterone: Sensitivity to a single concentration of water-soluble progesterone (35 μg/ml) (Sigma) was tested in vitro using a plating efficiency assay as described (81). Susceptibilities to antimicrobial peptides LL-37 and cathelicidin related antimicrobial peptide (CRAMP) were determined by incubating 10^6 CFU of each strain suspended in PBS with CRAMP or LL-37 in a total

volume of 100 µl and at final concentrations of 4 µg/ml and 8 µg/ml, respectively. These concentrations correspond to a slightly lower MIC value than that of wild type FA19. The mixtures were incubated in a 37° C CO_2 incubator for 45 min before serial dilution and culture on GC agar. All assays were performed in triplicate and repeated at least once to test reproducibility. Peptides LL-37 and CRAMP were synthesized and purified at the Microchemical Facility of Emory University.

Western Blot Analysis: Outer membrane proteins were fractionated on a 12% SDS/PAGE gel, transferred to PolyVinylidene DiFluoride membranes, and incubated with rabbit polyclonal antibodies against MtrE as described previously (Warner et al., 2007). Detection was performed with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Bethyl), followed by chemiluminescence with the ECL reagent (GE Healthcare). Densitometry was performed using the Image J program.

Real Time PCR: Bacterial strains were grown to early log phase (OD_{600} = 0.2-0.4) in supplemented GC broth with aeration. RNA-later reagent (Ambion) was added to a 300 µl aliquot and samples were stored at –80° C. Cells were lysed and RNA was prepared as recommended in the Qiagen RNAeasy kit (Qiagen) followed by treatment with Turbo DNAse (Ambion). A 100 ng sample of RNA from each strain was then reverse-transcribed using the Superscript III RT Kit (Invitrogen). Briefly, 10 µl of RNA were mixed with 150 ng of random primers, 10 mM dNTPs, 10 mM dithiothreonol, synthesis buffer, and the Superscript III enzyme. The mixture was incubated at room temperature for 10 min and then 50°C for one hour before deactivation of the enzyme for 10 min at

95°C. Real time PCR was carried out in 96 well plates with a Sybr-Green reaction mix (Applied Biosystems). Relative levels of transcript for 16S rRNA, *rmp, mtrC, mtrR*, and *mtrA* were determined by utilizing the relative method of comparison for each of the mutants to the wild type strain. All primers used are described in Table 6. All transcripts were assayed in triplicate with a no reverse transcriptase control. Dissociation curves were used to confirm the specificity of each reaction and two biological samples were tested to confirm results. The threshold for significance expressed as fold differences was established by examining the fold change between strains with respect to the constitutively expressed gene *rmp*. All delta Ct values were normalized to 16S rRNA.

RNA half-life Determination: The half-lives for *mtrC* and *rmp* mRNA from strains FA19, JF1, and DW131 were determined using the quantitative method of RT-PCR. Liquid broth cultures were grown to early log phase (OD= 0.2), treated with rifampicin (200 μg/ml), and sampled immediately afterwards (T_0) and at 2, 4, 8, 12, and 20 minutes post-rifampicin treatment. Samples were suspended in RNA-later reagent (Ambion) and frozen at -80° C before RNA was extracted with the RNAeasy Kit (Qiagen). RNA was quantified by spectophotometric analysis, and 100 ng per sample were converted to cDNA. cDNA was then used with the primers rmp-forRT, rmp-revRT, mtrC-forRT, and mtrC-revRT (Table 6) in a Sybr-Green Real-Time PCR reaction plate as detailed above. Each reaction plate also contained dilutions of FA19 genomic DNA to establish a standard curve for both primer sets. Ct values were converted to actual RNA values using the standard curve, and an RNA degradation curve was established for both genes in all three bacterial strains. These degradation curves were used to calculate the amount

of time for a given strain to degrade one half of the starting amount of mRNA. The experiment was performed twice on different biological samples.

Experimental Murine Infection: Female BALB/c mice were treated with 17-β-estradiol and antibiotics to promote long term infection as previously described (78). The competitive infection technique was used to assess fitness in vivo as described (Warner et al., 2007), in which a defined mixture of two strains were co-inoculated into a mouse, with one strain marked with a Cm^R cassette. Bacterial suspensions were prepared as described (204), adjusted to an OD_{600} of 0.07 (~$1x10^6$ CFU/10 µl), and then mixed to obtain a 1:1 ratio for each pair of strains. Twenty µl of the mixture were inoculated intravaginally into mice (n = 5 mice per mixture) and vaginal mucus was cultured every other day. The titer of each strain within the inoculum and vaginal mucus was determined by quantitative culture of equal volumes on GC agar with Sm (total CFU) and with Sm and Cm (wild type or *mtrR* mutant CFU, depending on the experiment). Competitive indices were calculated according to Unsworth et al. (192) in which a ratio of (*mtrR* mutant / wild type)$_{output}$ / (*mtrR* / wild type)$_{input}$ was calculated. A CI >1 was considered a fitness advantage for the *mtrR* mutant. In cases in which one strain was not recovered, the limit of detection (4 CFU/100 µl) was used to calculate CIs. All strains were also tested in in vitro competition assays as described previously (Warner et al., 2007). For infections in which Cm^R wild type strains were tested, randomly selected colonies were screened on GC media supplemented with Em (0.5 µg/ml) to confirm the correct ratio of *mtrR* mutants to wild type bacteria. Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association

for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

Results

Construction of a Series of *mtr* Locus Mutations in Strain FA19Sm^R: Investigations of clinical outbreaks have shown that gonococcal isolates with increased resistance to Em, Az, and penicillin frequently harbor naturally occurring mutations in the *mtrR* gene or its upstream region. Here we created a series of mutants that represent the majority of natural promoter and structural gene mutants that have been reported in the *mtr* loci of clinical isolates (Fig. 10). The A39T and G45D mutations, which are in the theorized DNA-binding domain of MtrR, were amplified from a set of clinical isolates from Baltimore (117), (Garvin et al., in preparation). Two other mutants were constructed, using mutant *mtrR* loci that carried structural gene mutations, which were isolated from experimentally infected mice (Warner et al., 2007). One is an *mtrR* frameshift mutation, while the other mutation is an E202G amino acid change near the C-terminus of the MtrR protein. These two structural MtrR mutations were PCR amplified and introduced into the FA19Sm^R background to create transformants DW11 and DW9, respectively. Mutant strain KH15 is a transformant of strain FA19Sm^R that contains a thymidine deletion on the end of the 13-bp inverted repeat denoted in Fig 10. This inverted repeat is the MtrR binding region and also encompasses the -35 promoter region for both *mtrR* and *mtrCDE* (103).

Based on the observation in our laboratory that the commonly used laboratory strain MS11 exhibits higher levels of in vitro resistance to TX-100, Em, and progesterone compared to strain FA19Sm^R, we hypothesized that strain MS11 may contain one or more *mtr* locus mutations. Sequence analysis of the *mtr* locus of strain MS11 revealed the A39T mutation in the MtrR DNA binding domain and an adenine to guanine transition located 131-bp upstream of the *mtrR* start codon and 119-bp upstream of the *mtrC* start codon (Fig. 10). This mutation has not been described previously among clinical isolates, however we have isolated mtr_{-131} in vitro when measuring the rate of spontaneous Em resistance in FA19 (data not shown).

Natural *mtr* Mutations Confer Differential Levels of Antibiotic Resistance: While all mutants used in this study were isolated on agar containing Em at 0.5 μg/ml, higher levels of resistance have been described for the *mtr* mutant strain KH15, which displays increased levels of antimicrobial resistance compared to an *mtrR* deletion strain (65, 174). As a first step towards further phenotypic characterization of these naturally occurring mutations, we determined the MICs for three substrates of the MtrC-MtrD-MtrE efflux pump (TX-100, Em, and Az) against our collection of *mtr* locus mutants. Km was used as a non-substrate control. Based on the MIC for Em, we defined a stepwise gradient of resistance, with three levels of resistance clearly illustrated (Table 7). Mutant strains DW9, JF1, and DW45 showed only a slight increase in Em resistance, which was a 2-fold increase in the MIC compared to the parent strain. A 2-fold increase in the MIC of Az was also exhibited by these strains; resistance to TX-100 was 2-fold (DW9) and 4-fold higher (JF1, DW45) than that of the wild type strain. The next level of resistance

(intermediate) was defined as a 4-fold increase in the MIC of Em, and was exhibited by the MtrR truncation mutant DW11 and the MtrR structural gene mutant DW39. DW39 displayed a higher level of resistance to Az and TX-100 (4-fold and 8-fold respectively), compared to DW11 (4-fold with TX-100). The third group of mutants, KH15 and DW131, displayed the highest levels of resistance to Em (16-fold increase). Interestingly, strains KH15 and DW131 both carry mutations outside of the MtrR structural gene. The mutation in strain KH15 is a -T mutation located 79-bp upstream of the *mtrR* start codon, which is one of the most commonly described mutations isolated in clinics. The -131 mutation in strain DW131 is novel and not in a region known to be involved in interactions between MtrR and *mtrCDE*. Mutants KH15 and DW131 exhibited levels of resistance to Az and TX-100 that were also notably higher than the other strains, with increases in MIC of 8-fold and 256-fold, respectively. Interestingly, complete transformation of the MS11 *mtr* locus (A39T and mtr_{-131}) yielded a lower MIC than transformation of the -131 mutation alone (Table 7).

Analysis of the *mtr* Locus in Strain MS11 To further characterize the *mtrR* locus of strain MS11, we PCR amplified the *mtr* region of strain MS11 or the spontaneous FA19 mutant that carries the -131 mutation that we had previously isolated. The primers used (R-Xho and C-Xho) annealed 48-bp after the translational stop site of *mtrR* and 391-bp interior of the *mtrC* gene, respectively. The resultant 1335-bp products were transformed into strain FA19Sm^R and followed by selection on Em (0.5 μg/ml). Genetic transfer of the two different mutations together or of the -131 mutation individually created strains $FA19_{MS11mtr}$ and DW131, respectively. Results from these two strains displayed a linkage

between the MS11 *mtr* locus and resistance to Em and TX-100 (Table 7). To confirm the role of the MtrC-MtrD-MtrE efflux pump in the resistance phenotype we introduced an *mtrE* mutation from plasmid pCR-mtrE (Warner et al., 2007) into strain MS11, and tested the resultant strain ($DW3_{MS11}$) for levels of resistance compared to the *mtrE* mutant in strain FA19 (DW3). Disruption of the *mtrE* gene in strain MS11 conveyed a decrease in resistance equal to that of the strain DW3.

The location of the -131 mutation was puzzling as it is too far upstream to alter MtrR binding or transcription of the *mtrR* gene. Therefore, we tested whether the -131 mutation was affected by the action of MtrR or MtrA, which are the repressor and inducer of the MtrC-MtrD-MtrE pump, respectively. The -131 mutation was transformed into *mtrR* and *mtrA* mutant strains, and the resultant double mutants were tested for sensitivity to Em and TX-100. Mutants DW131A (mtr_{-131}, *mtrA::aphA3*) and $JF1_{-131}$ (mtr_{-131}, *ΔmtrR*) showed no change in the MIC of TX-100 or Em compared to the wild type strain (Table 7). From these MIC values, we concluded that the basis of the increased resistance conveyed by the -131 mutation is MtrR- and MtrA-independent. Further sequence analysis determined that the *mtrA* gene in strain MS11 is identical to that of strain FA19, this finding confirmed that the increased resistance seen with MS11 is not due to any differences in the promoter or structural gene of the activator MtrA.

Effect of *mtr* Locus Mutations on *mtrCDE* Expression and mRNA half-life We next determined if the observed functional differences were consistent with differences in MtrE protein expression. MtrE-specific antibodies were used to visualize the amount of MtrE in outer membranes isolated from each of the *mtrR* mutants and the wild type strain

(Fig. 11). Increased band intensity was seen for all *mtrR* mutant strains compared to wild type bacteria, although a gradient in expression was not easily discernable by this method. We therefore used RT-PCR to examine the effect of the mutations on the levels of *mtrC, mtrR*, and *mtrA* transcript from each of the mutants (Fig. 12). As reported previously (65), no *mtrR* mRNA was detectable in the deletion strain (JF1) or strain KH15 (data not shown). None of the differences in antibiotic resistance conferred by the other mutations tested could be attributed to differences in expression of the transcriptional activator MtrA or repressor MtrR, because there was no discernable differences among the *mtrR* mutants and the wild type strain with respect to levels of *mtrR* or *mtrA* mRNA. Differences in the level of *mtrC* transcript, in contrast, were consistent with differences in antibiotic resistance, with strains DW39, DW131, $FA19_{MS11mtr}$, and KH15 expressing 3- to 6-fold greater amounts of *mtrC* mRNA than the wild type strain. These strains showed intermediate (DW39) to high (DW131, $FA19_{MS11mtr}$, and KH15) levels of resistance to Em and Az (Table 7). Mutants (DW9, JF1, and DW45) showed 1.5- to 2-fold greater amounts of *mtrC* expression. These results are consistent with the lower increases in antibiotic resistance demonstrated by these strains. Mutant DW11 had an intermediate MIC for Em, but lower MIC for Az and TX-100, and gave *mtrC* expression levels similar to the lower expression mutants. Taken together, these results suggest that subtle differences at the mRNA level can alter the functional measures of the phenotype.

Interestingly, the transcriptional profile of DW131 showed no difference in the level of *mtrR* or *mtrA* compared to the wild type strain. These results are consistent with the mutation increasing levels of resistance in an MtrA- and MtrR- independent manner.

The -131 mutation is located within the 5' untranslated region of the *mtrC* RNA. The detection of high levels of *mtrC* transcript in this strain in the absence of changes in the expression of known regulators of the *mtrCDE* operon suggests there may be a difference in *mtrC* mRNA transcript stability. A similar mutation in the 5' untranslated region of an efflux pump gene was described for the NorB efflux pump of *Staphylococcus aureus*, which led to an increase in *norB* mRNA half-life (49). Therefore, we tested whether there was a difference in the *mtrC* mRNA half-life between mutant DW131 and the parent strain FA19. We found a two-fold difference in *mtrC* RNA half life in strain DW131 when compared to either strain FA19 or the *mtrR* deletion strain JF1 (Figure 13), while there were no differences detected in the half-life of mRNA for the constitutively expressed gene *rmp*. These results suggest the -131 mutation alters the structure of the *mtrC* transcript or its interaction with either RNA degradation factors or the ribosome to allow for a longer mRNA half-life.

In vivo Fitness Levels Correspond to Resistance Levels: Previously we reported that the *mtrR* deletion mutant JF1 was more fit than the wild type strain in the lower genital tract of female mice (Warner et al., 2007). Here we evaluated the in vivo fitness of mutants that represent high (KH15 and DW131), intermediate (DW39), and low (DW9) ends of the antibiotic resistance gradient. Competitive infections were performed in which similar numbers of a Cm^R marked derivative of wild type strain FA19 and each of the *mtr* mutant strains were inoculated intravaginally into BALB/c mice. Co-culture of each mutant with strain FA19Cm^R in GCB (in vitro competition assays) showed that there were no growth or survival advantages in vitro (Fig. 14A). In contrast, all four

mutants demonstrated a fitness advantage within one day post-inoculation of mice, as defined by CI values greater than one. The mere presence of fitness advantages was no surprise, as this was observed previously with the *mtrR* deletion mutant JF1 (Warner et al., 2007). These findings are novel, however, because these naturally occurring mutations, which are less disruptive to the *mtrR* locus, conveyed a more potent advantage over the wild type strain. A gradient in fitness was also observed that parallels the differences in antibiotic resistance. Strain DW9, a mutant that showed a 2-fold increase in resistance to Em, TX-100 and Az over the wild type strain displayed mean CIs of 5 and 12 on days 1 and 3, respectively with 1 of 5 mice clearing the wild type strain by day 3 (Fig. 14B). Competitive infections with mutant DW39, which showed an intermediate level of resistance to Em and Az, and FA19Cm^R resulted in CIs of 100 and 150 on days 1 and 3, respectively, with 1 of 4 mice clearing the wild type strain by day 3 (Fig. 14C). Importantly, the intergenic mutations found in mutants KH15 and DW131, which confer the highest increases in MIC (16-fold for Az and Em, and >250-fold for TX-100) were markedly more fit than the wild type strain. Mean CI values for mutant KH15 were 195 and 509 on days 1 and 3, with 2 of 4 mice clearing the wild type strain by day 3 and 3 of 4 mice by day 5 (Fig. 14D). Similarly, strain DW131 displayed mean CI values of 48 and 455 on days 1 and 3, with no wild type strain recovered from 2 of 5 mice by day 3, and from 0 of 2 mice by day 5 (Fig. 14E). In summary, these experiments show that the levels of *mtrCDE* derepression as measured by antibiotic resistance are mirrored by fitness advantages in vivo.

Gradients of Resistance to Antimicrobial Peptides and Progesterone: While there are various potential explanations for the in vivo fitness advantage conveyed by increased production of the MtrC-MtrD-MtrE efflux pump, we chose to test whether the fitness differences seen in the mouse model were reflected in susceptibility of the mutants to progesterone and the human cathelicidin LL-37 and the murine cathelicidin CRAMP, based on its homology to LL-37 and the possibility that it may challenge *N. gonorrhoeae* during murine infection. We also tested progesterone, which like LL-37, was previously determined to be a substrate of the MtrC-MtrD-MtrE efflux pump. All the mutants except DW9 were significantly more resistant to progesterone compared to the wild type strain as measured by plating efficiency (Fig. 15A). Statistical analysis also revealed that the resistance of mutant DW39 (intermediate), was significantly higher than that of mutants at the lower end of the Em resistance gradient (DW9, JF1), but significantly lower than the highly Em resistant mutants, DW131 and KH15.

Differences were also found in the sensitivity of the *mtr* mutants to the cathelicidins. All *mtr* mutants, with the exception of DW9, displayed a significant increase in resistance to both LL-37 and CRAMP compared to the wild type strain. Further analysis revealed that intermediate and low level Em resistant strains were significantly more susceptible to both LL-37 and CRAMP when compared to the susceptibility of high level Em resistant mutants KH15 and DW131 (Fig 16A and 16B). A gradient of resistance was further confirmed by the demonstration that intermediate level Em resistant strain DW39 was a significantly more resistant to CRAMP and LL-37 compared to lower level Em resistant mutants DW11 and DW9. Curiously, strain JF1 was not significantly different from strain DW39 in levels of resistance to the LL-37 (Fig.

16A) or CRAMP (Fig. 16B), and this result was reproducible. Further characterization of the derepressed phenotype of strain MS11 showed that MS11 also has a high level of resistance to LL-37 and CRAMP, which was negated with the disruption of the *mtrE* gene (Fig. 16 D and E). As expected, transformation of the MS11 *mtr* region into strain FA19 significantly increased the resistance to CRAMP (Fig. 16E).

Competition Between *mtr* Mutants: A more sensitive way to establish gradients of fitness is to perform competitive infections between two *mtr* mutant strains. We chose to compete the commonly described and highly resistant promoter mutant KH15 against structural mutants DW39, DW45, and DW9. We chose these strains because strains DW39 and DW45 contain the two most common MtrR structural gene mutations found in clinical isolates and strain DW9 provides a clearly "low" resistance phenotype. To facilitate the testing of these mixtures, we marked strains DW39, DW45, and DW9 with a Cm^R gene as described in Methods. Mixtures of strain KH15 and each of the Cm^R-marked *mtrR* mutants were inoculated into mice and infection was followed over 7 days. Our hypothesis was that the KH15 mutant would out-compete the other three mutants, but in a step-wise manner with strain DW9Cm^R displaying the most disadvantaged phenotype, followed by DW45Cm^R and lastly DW39Cm^R. To that end, all CI calculations were performed with KH15 as the numerator, as described in the legend for Fig. 17.

There were no differences in the growth and recovery of each mutant strain versus KH15 bacteria in vitro (Fig. 17A). Mutants DW9Cm^R and DW45Cm^R were greatly out-competed by mutant KH15, with CI values around 1000 by day 2. Additionally, 3 of 4

mice cleared DW9Cm^R (Fig. 17B) and 2 of 4 mice cleared DW45Cm^R (Fig. 17C) by day 4. Mutant DW39Cm^R was less fit than strain KH15, however CI values were markedly lower than with the other two mutants with a mean value of 12 by day 2 (Fig. 17D). These studies confirm that the promoter mutation provides a greater fitness advantage to *N. gonorrhoeae* than the MtrR structural mutations in vivo, and illustrates how this difference may lead to a selection for the more resistant mutant in a mixed *N. gonorrhoeae* infection, which can occur in humans (109, 112).

Discussion

Since the discovery of the MtrR repressor and the impact of mutations on antibiotic resistance in *N. gonorrhoeae*, investigations of antibiotic resistance outbreaks include this locus among others in screens for basic resistance determinants. As such, several *mtrR* mutations are repeatedly isolated. Structural mutations include truncations of the *mtrR* gene (85) or amino acid substitutions within the theoretical DNA binding domain of the MtrR protein (38, 69, 107, 113, 118, 174, 182, 184). Two key promoter mutations have also been described; one is the insertion of a Correia element in the MtrR binding site (85, 118), and the other an insertion or deletion of one or two thymidines within the pseudo-inverted repeat of the MtrR binding site (34, 38, 104, 130, 184, 210, 211). The +/-T promoter mutations in particular are frequently isolated and known to cause a greater derepression state of the *mtrCDE* operon than structural *mtrR* mutants (65, 174). The goal of the study presented here was to compare the effect of three commonly isolated *mtr* locus mutations and four *mtrR* structural gene mutations for antibiotic resistance and in vivo fitness. We also studied a novel mutation, mtr_{-131}, which we discovered in the laboratory strain MS11. Based on MIC determination, we found that the intergenic mutations conferred the highest level of resistance to antibiotic substrates of the pump and substrates that may challenge *N. gonorrhoeae* in vivo. Surprisingly, however, we found that mutations within the *mtrR* structural gene did not fall into one category, but in fact conferred significant differences in susceptibility. Among the structural gene mutations, the A39T mutation consistently stood out as having

higher in vitro resistances compared to the other three *mtrR* structural mutations, and also competed favorably in vivo with the most fit mutant, KH15. In contrast, the E202G mutation displayed lower levels of in vitro resistance and in vivo fitness. While the crystal structure for MtrR is not yet available, the importance of the A39T mutation, which is located very close to the less resistant G45D mutation, should prompt investigation into the structural requirements for the DNA binding site of MtrR.

Analysis of *mtrC* transcript levels provided further evidence of a differential level of *mtrCDE* expression, and in general the levels of *mtrC* transcript mimicked that of the MICs. Our results confirm the previously reported high levels of *mtrC* transcription and the absence of an *mtrR* transcript in strain KH15 (65). Our examination of strains DW39, DW45, DW9, and DW131 provides new insights into the mechanisms of derepression of the *mtrCDE* operon. From our results we predict that the alanine residue at position 39 in the MtrR protein is more important to DNA binding than the glycine residue located six residues away. We also hypothesize that the glutamic at position 202 plays a minor role in the ability of the repressor to regulate expression of *mtrCDE*, the specifics of which could include the ability of the MtrR proteins to dimerize. Alternatively, this mutation may alter the ability of the MtrR protein to recognize MtrC-MtrD-MtrE substrates at the C-terminal end of the repressor, which has been seen with other TetR repressors (reviewed in (153)), including QacR (170, 171).

Demonstration of fitness advantages for de-repressed *mtr* mutants poses the question of why a strain maintains *mtrR* genes. To this end, we determined the nucleotide sequence of the *mtr* region of strain MS11. Results presented here are the first to characterize strain MS11 as a natural *mtr* mutant, and furthermore, the mtr_{-131} mutation

found in MS11 is novel. Of note, repeated attempts to transform the FA19 *mtr* locus into strain MS11 were unsuccessful, which suggests an essential role for the *mtr* mutations when in strain MS11. Strain MS11 was originally isolated from the cervix of an uncomplicated genital tract infection (183). Interestingly, MS11 has a lower ID_{50} compared to strain FA1090 in the male volunteer urethritis model (167) and in female mice (78). It is not known whether this increased infectivity is due to the presence of the *mtr* mutations, since we previously showed that an *mtr* mutation did not significantly alter the infectivity of strain FA19 in mice (Warner et al., 2007). However, the results shown here contribute to further understanding and characterization of strain MS11.

The -131 promoter mutation yields one of the highest levels of *mtr*-based resistance. While the -131 mutation has not yet been reported in a clinical isolate, it is important to note that in other efflux pump systems, such as the *nor* system in *Staphylococcus aureus*, similar mutations in the 5' untranslated region yielded derepressed operon transcription (49). Here we present genetic evidence and RT-PCR results to show that the -131 mutation is independent of both MtrR and MtrA. Additionally, we showed that the -131 mutation conferred an increase in *mtrC* mRNA half-life, which is a novel mechanism by which increased resistance to MtrC-MtrD-MtrE substrates can occur. The -131 mutation suggests a role for RNA stability and the possibility of post-transcriptional regulation in the control of *mtrCDE* expression. Additional investigation is required to establish why such a mutation has not been described in clinical samples to date.

An additional objective of this study was the investigation of how these mutations may affect fitness. We previously reported that the MtrC-MtrD-MtrE pump is critical for

experimental murine genital tract infection, while the FarAB pump is not (81), and that mutant JF1 (*ΔmtrR*) exhibits increased in vivo fitness when compared to wild type parent strains (Warner et al., 2007). Results here show that naturally occurring *mtr* mutations demonstrate a gradient of fitness advantage in vivo. This fitness gradient is more clearly displayed with strain KH15, which out competes all other *mtr* mutants, except for a strain bearing the A39T mutation, which competed more favorably with mutant KH15. Such a hierarchy in fitness could allow selection for more efficient pathogens, such as KH15, in cases of mixed infection. Additionally, the increased fitness afforded to *mtr* mutants could allow for the accumulation of other mutations that may balance the potentially detrimental effect that the derepression has on other genes. Such accumulation of mutations could lead to a more persistent strain, which also has a higher degree of antibiotic resistance.

In summary, these findings add to the growing body of literature that support a role for certain RND-type pumps in Gram-negative pathogens beyond the antibiotic resistance phenotype for which they were discovered (19, 101, 134, 181). We propose that the MtrC-MtrD-MtrE efflux pump evolved to protect the pathogen from innate immune effectors such as antimicrobial peptides. Increased resistance to CRAMP may explain the basis for the observed fitness advantage in mice. The mutants also showed increased resistance to progesterone, however it seems less likely that resistance to progesterone is responsible for our in vivo observations, because progesterone concentrations in the lower genital tract are likely to be 100-fold lower than that tested here (81). Our demonstration that *mtr* mutants are more fit in vivo suggests that wild type levels of MtrR-repression are anti-pathogenic and are destined to be selected against

with further use of antibiotic therapy. Conversely, it is important to note that this infection model is a surrogate for the lower genital tract infection of females. Other body sites may not confer such an advantage to derepressed mutants. This caveat suggests a role for the MtrR repressor in nature and a reason for the existence of naturally repressed strains. Finally, we note here that the gradient in resistance to azithromycin is equally important as the fitness gradient detected in vivo, since azithromycin is increasingly utilized as a therapy for non-complicated urethritis (2).

Figure 10. Location of *mtrR* locus mutations used in this study. A schematic of the *mtrR* gene and the upstream intergenic region between *mtrR* and *mtrC* is shown. The α-helix encoded region of MtrR used for DNA binding is indicated by the square hatched pattern. This region is the location of the mutations found in strains DW39, DW45, DW11, and MS11. The E202G mutation harbored in strain DW9 is located at the C-terminal end of the MtrR protein, which is hypothesized to be involved in the dimerization of MtrR to itself. The cross hatched locus in the intragenic region has been mapped as the transcriptional start for both *mtrR* and *mtrC*, as well as the region bound by MtrR, and is the region where the mutation in strain KH15 is found, which was described previously (12). The mtr_{-131} mutation occurs further upstream of the DNA binding region and is present in strains MS11 and DW131. This mutation has not been described previously, and the G to A change is shown in the detailed DNA sequence. The transcriptional start sites for *mtrR* and *mtrC* are denoted by the arrows (→).

Figure 10. Location of mtrR locus mutations used in this study

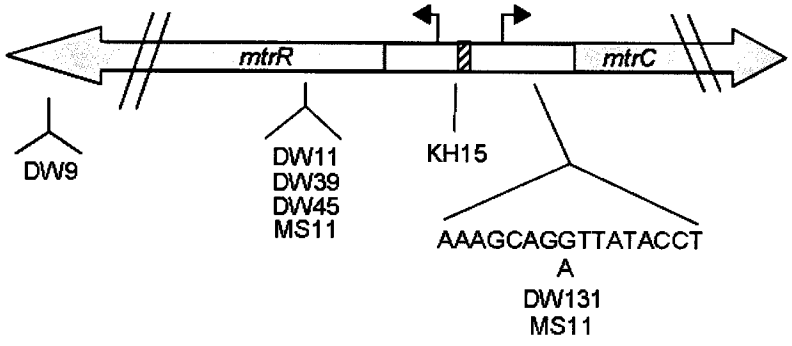


Figure 11. Expression of MtrE by wild type and mtrR mutant bacteria. Outer membrane proteins were separated by SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The 53-kDa MtrE protein was detected by a rabbit MtrE-specific polyclonal antibody described in the Materials and Methods. With the exception of mtrE mutant DW3, protein samples were loaded in ascending order of the levels of antibiotic resistance. The 32-kDa porin protein is constitutively expressed in N. gonorrhoeae and was detected by staining the PVDF membrane with amido black to show equal loading of the samples.

Figure 11. Expression of MtrE by wild type and mtrR *mutant bacteria*

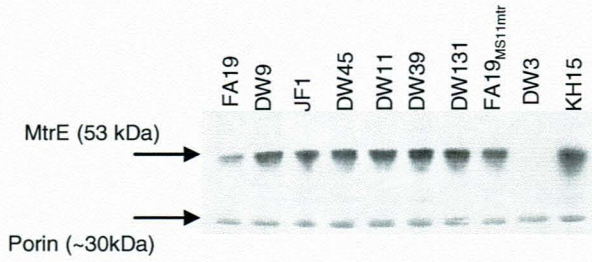


Figure 12. Transcriptional analysis of *mtrA*, *mtrR*, *mtrC*, and *rmp*. Quantitative RT-PCR was used to assess the fold difference in levels of mRNA compared to the wild type strain FA19. One representative experiment of the three biological replicates tested is shown. There were no significant differences between levels of *rmp* or *mtrA* mRNA. *mtrR* levels were highly down-regulated or non-existent in strains JF1 and KH15 as has been described previously (64, 174), and these values have been omitted to preserve the scale of the figure. Numbers in parentheses denote the fold increase in *mtrC* levels compared to wild type strain FA19. Levels of *mtrC* expression were compared using a students t-test, to evaluate differences between strains DW39, DW131, $FA19_{MS11mtr}$, and KH15, which represent isolates with higher expression of *mtrC*, and strain JF1, which represents an isolate with a lower increase in both MIC and *mtrC* mRNA level.

**Denotes $p<0.001$

Figure 12. Transcriptional analysis of mtrA, mtrR, mtrC, *and* rmp

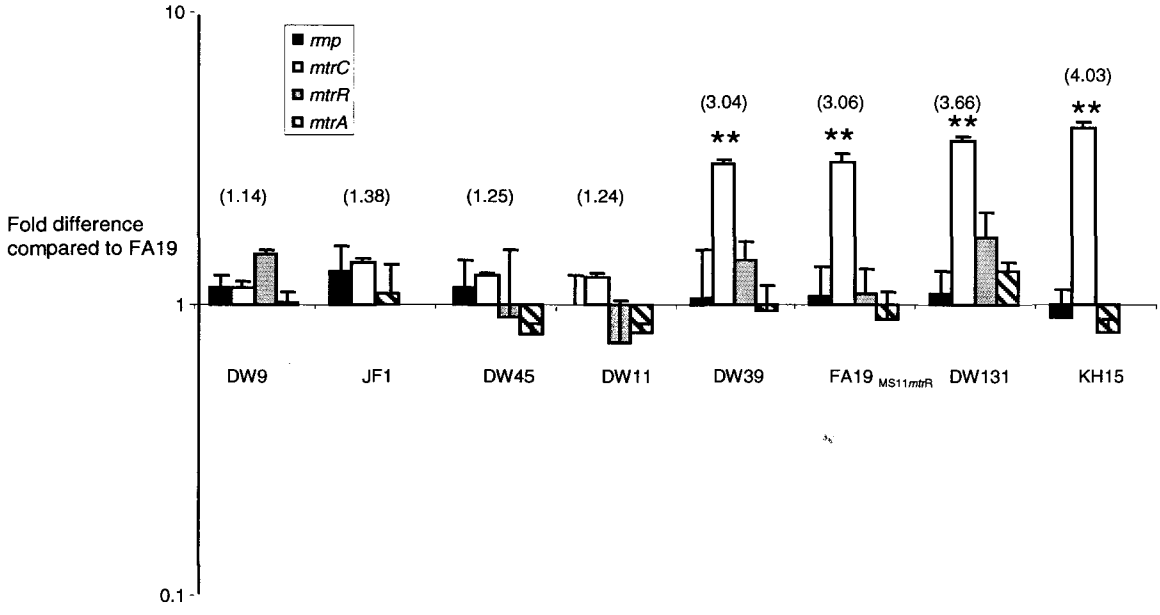


Figure 13. RNA degradation in strain DW131. (A) Equal quantities of RNA from strain FA19 (top) and DW131 (bottom) were reverse transcribed, and used in a PCR reaction to determine if there was a difference in degradation of mtrC message. Samples were taken pre (T0) and post rifampicin treatment (+Rif), and duplicate cultures were grown without the addition of rifampicin (-Rif). (B and C) RNA samples were also reverse-transcribed and used in a qRT-PCR protocol to quantify amounts of *mtrC* and *rmp* RNA from strains FA19, JF1, and DW131. These values were used to plot the degradation curves shown, which were used to calculate the RNA half life for (B) *mtrC* and (C) *rmp* in the three strains tested. Calculated RNA half life values for *rmp* were 2.3, 2.4, and 2.1 minutes for strains FA19, JF1, and DW131, respectively. Calculated RNA half life values for *mtrC* were 3.0, 3.1, and 4.2 for strains FA19, JF1, and DW131, respectively. These results are the average of two experiments.

Figure 13. RNA degradation in strain DW131

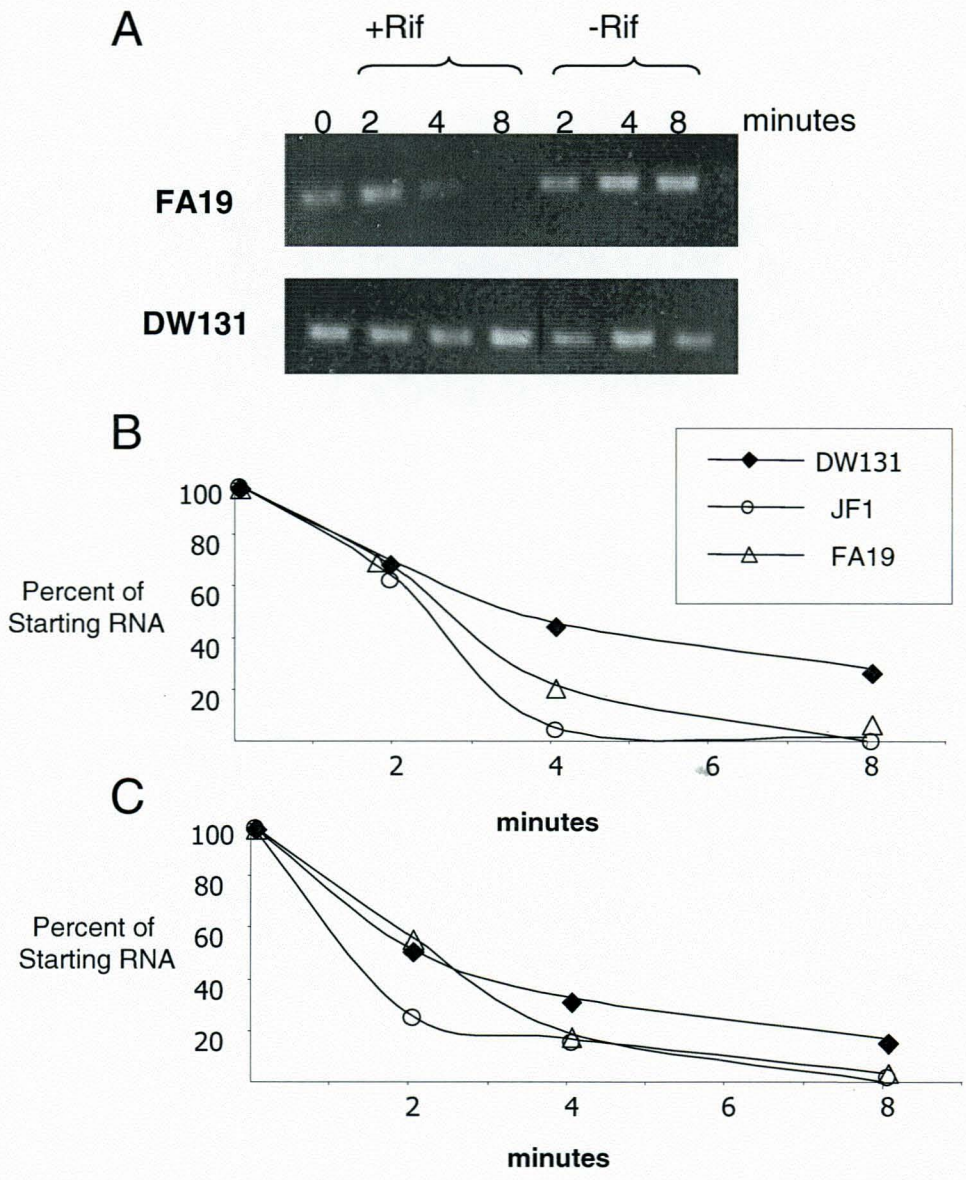


Figure 14. Differential in vivo fitness of mtrR mutants compared to the wild type strain. GC broth cultures and female mice were intravaginally inoculated with defined ratios of FA19 CmR and mtr mutant bacteria. The ratio of strains in each inoculum was used in the competitive index (CI) equation defined in Materials and Methods. (A) Mixed suspensions were cultured in GC broth (in vitro competition); recovery over the course of growth is expressed as CI. In vivo competition assays between wild type strain FA19CmR and strains (B) DW9, (C) DW39, (D) KH15 and (E) DW131 show different degrees of fitness as measured by CI. Each circle in panels B-E represents the CI from each individual mouse; open circles represent cultures from which no CmR wild type bacteria were recovered. The bars represent the geometric mean of the data, and the dotted line delineates a CI value of 1.0. In cases where a strain was no longer recovered, the limit of detection (4 CFU/100 μl of vaginal wash) was used to calculate the competitive index.

Figure 14. Differential in vivo fitness of mtrR mutants compared to the wild type strain

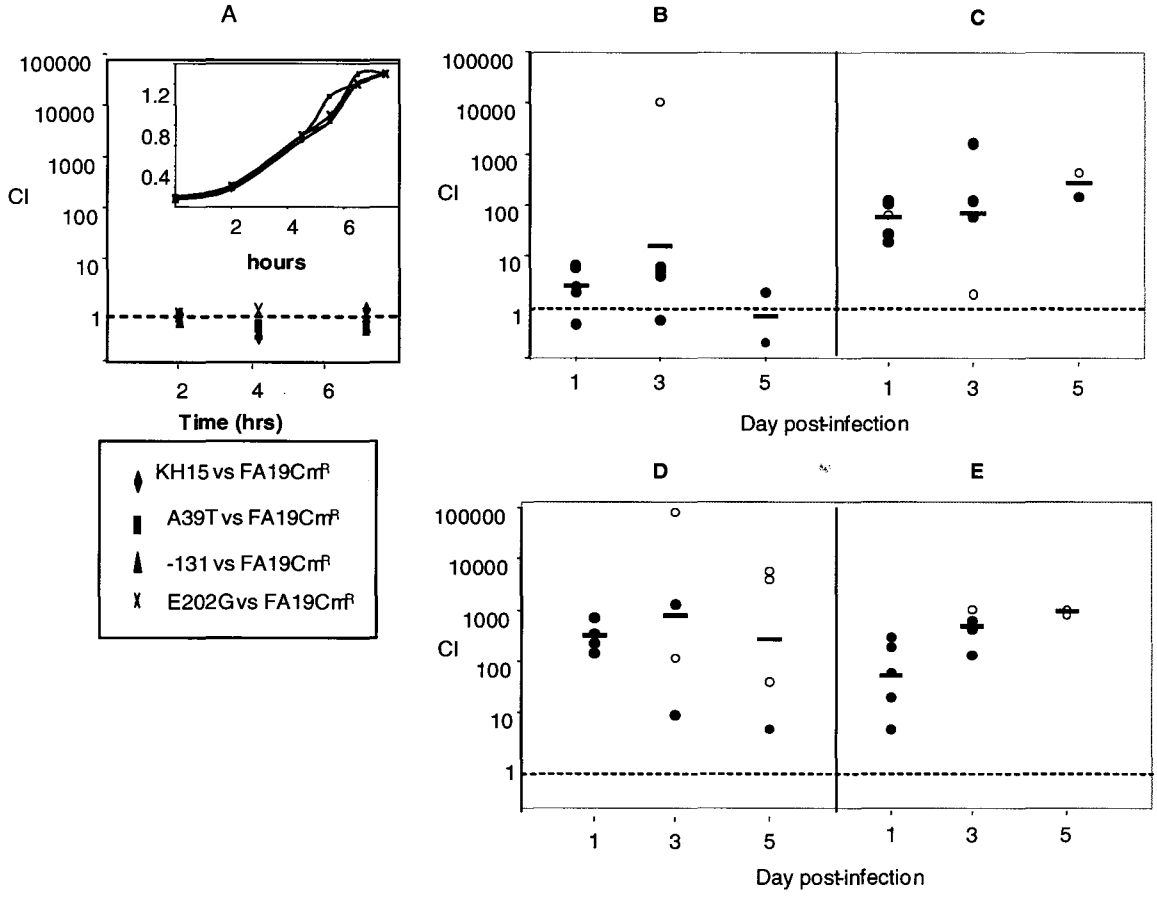


Figure 15. Progesterone resistance. Suspensions of wild type and mutant gonococci were quantitatively cultured on (A) GC agar plates supplemented with progesterone (35 μg/ml), or (B) GC agar without progesterone. The results of one of three experiments performed in triplicate are shown. Error bars represent the standard deviation of the data. Differences in bacterial recovery of mutant to wild type gonococci on progesterone were analyzed with a student's t-test; p-values comparing different mutants are denoted..

Figure 15. Progesterone resistance

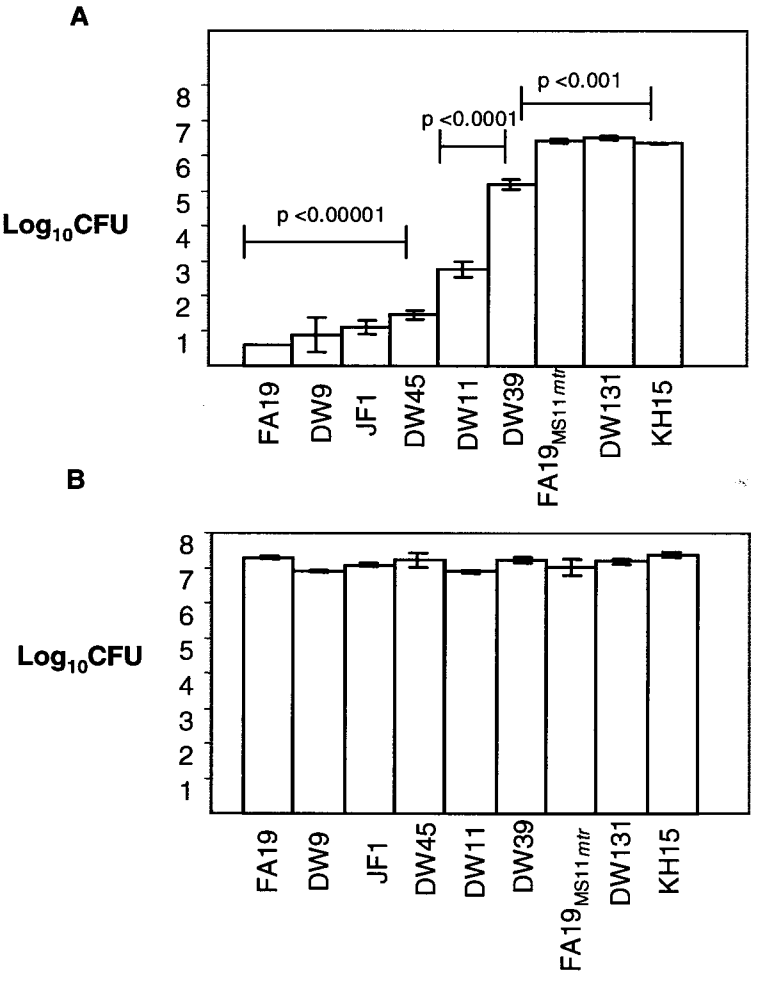


Figure 16. Resistance to antimicrobial peptides. Bacteria were incubated with LL-37, CRAMP, or PBS for 45 minutes before being quantitatively cultured on GC agar. Concentrations of LL-37 and CRAMP were 8 μg/ml and 4 μg/ml, respectively. Log_{10}CFU of bacteria recovered after incubation is shown on the y-axis. Results shown are representative of one of three experiments performed in triplicate. Panels A-C show strain FA19 and *mtr* mutants incubated in (A) LL-37, (B) CRAMP, or (C) PBS. Error bars represent the standard error of the mean. For panels A and B, p-values from t-test comparisons between the indicated data sets are shown. An asterix (*) denotes a p-value of less than 0.001 when compared to strain DW39. Panels D-F show the growth of strains MS11, $DW3_{MS11}$, FA19, and $FA19_{MS11}$ after incubation in (D) LL-37, (E) CRAMP, and (F) PBS. Double asterisk (**) denote a p-value >0.001, when comparing MS11 and $DW3_{MS11}$. In panel E, the p-value is shown for the comparison of strains FA19 and $FA19_{MS11mtr}$

Figure 16. Resistance to antimicrobial peptides

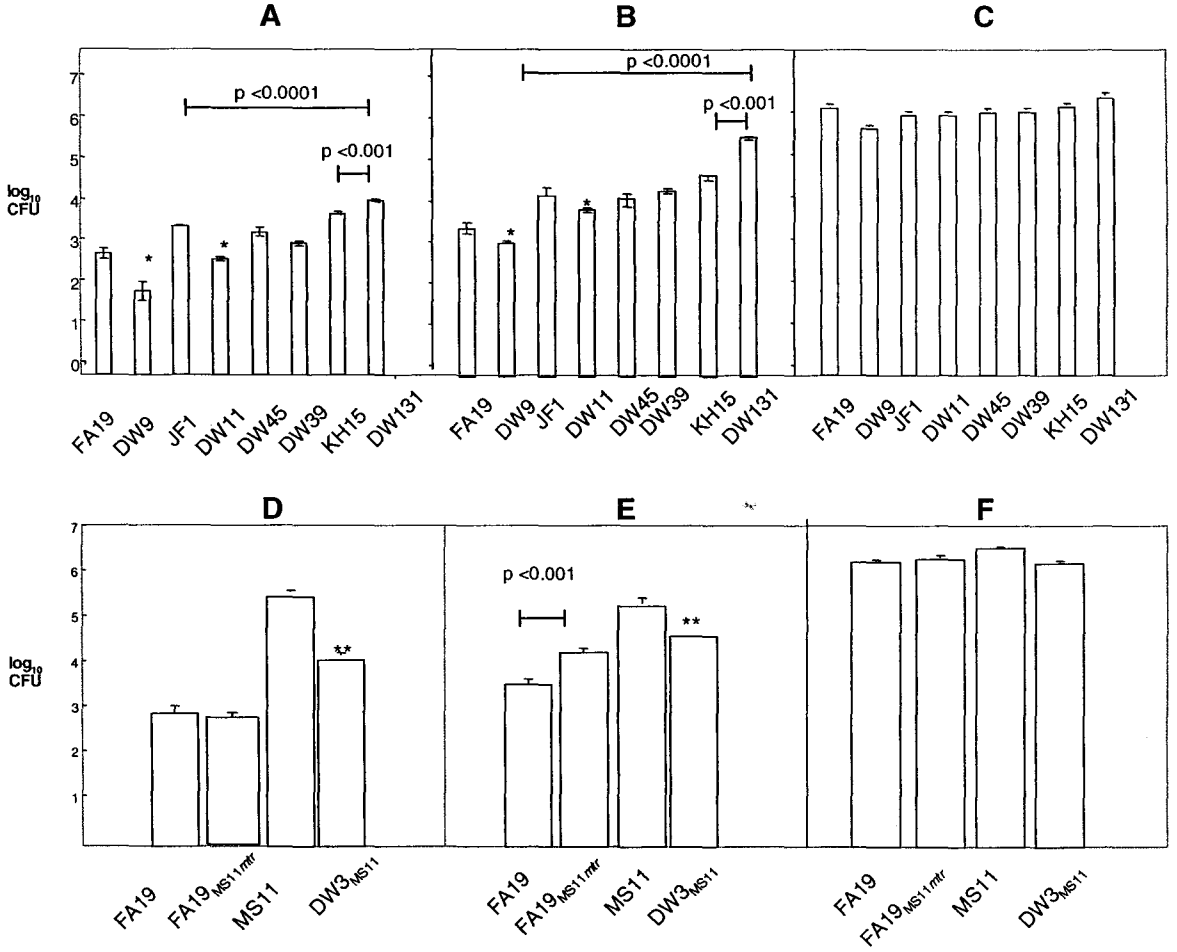


Figure 17. In vivo competition of *mtrR* mutants. Female mice were intravaginally inoculated with defined ratios of strain KH15 and different Cm^R marked *mtr* mutants. The ratio of strains in the inoculum was used in the competitive index (CI) equation defined below. (A) Mixed suspensions were inoculated into GC broth and the recovery over the course of growth is expressed as CI. The inset shows the optical density of the liquid cultures over time. In vivo competition between strains KH15 and (B) DW9Cm^R (C) DW45Cm^R and (D) DW39Cm^R are shown. In panels B-D, each circle represents the CI value for each individual mouse, and open circles signify mice from which only strain KH15 was recovered, and closed circles represent mice from which both strains were recovered. The CI of 1.0 is denoted by a dashed line, while the geometric mean of each distribution is represented by a bar. In cases where a strain was no longer recovered, the limit of detection (4 CFU/100 μl of vaginal wash) was used to calculate the competitive index. The CI equation for these experiments was defined as:

$(KH15/mtrRCm^R)_{output}/(KH15/mtrRCm^R)_{input}$.

Figure 17. In vivo competition of mtrR mutants

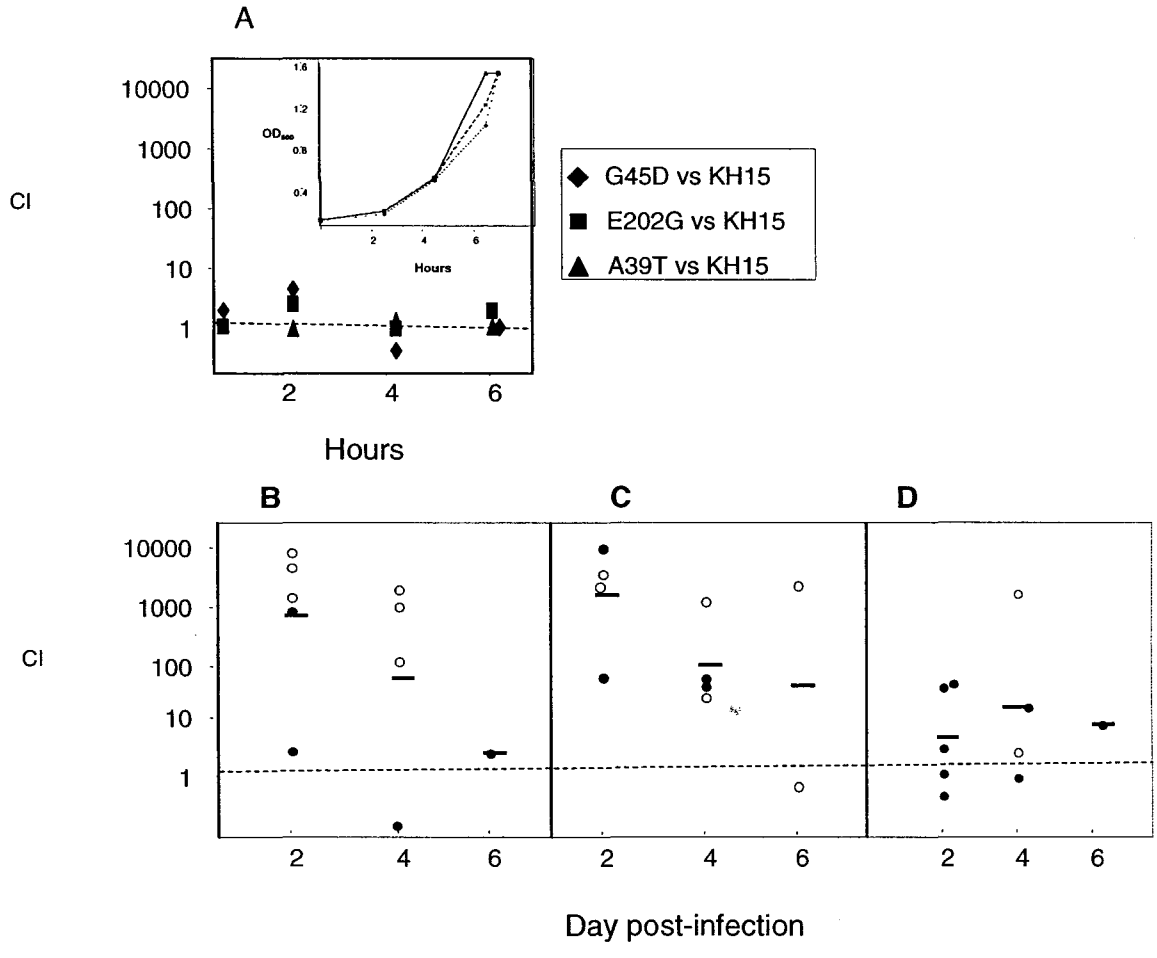


Table 5. Bacterial strains used in this study

Strain[a]	Relevant Genotype	Reference
FA19Sm^R	Parent strain	(81)
DW9	*mtrR*$_{E202G}$	Warner et al., 2007
DW11	*mtrR* $_{1-52}$	Warner et al., 2007
JF1	Δ*mtrR*	(48)
DW39	*mtrR*$_{A39T}$ (DNA-binding region)	This study
DW45	*mtrR*$_{G45D}$ (DNA-binding region)	This study
KH15	-T at MtrR binding site (*mtr*$_{-79}$)	(64)
MS11	Wild type strain, *mtr*$_{-131T>G}$, *mtrR*$_{A39T}$	(183)
DW131	FA19Sm^R *mtr*$_{-131T>G}$	This study
FA19$_{MS11mtr}$	FA19Sm^R *mtr*$_{-131T>G}$, *mtrR*$_{A39T}$	This study
JF3	*mtrA::aphA3*	(159)
DW131A	FA19Sm^R *mtr*$_{-131T>G}$, *mtrA::aphA3*	This study
DW39A	FA19Sm^R *mtrR*$_{A39T}$, *mtrA::aphA3*	This study
JF1$_{-131}$	*mtrR*$_{-131T>G}$, Δ*mtrR*	This study
DW3	*mtrE::cat*	Warner et al., 2007
DW3$_{MS11}$	*mtr*$_{-131T>G}$, *mtrR*$_{A39T}$ *mtrE::cat*	This study
FA19Cm^R	*cat* gene inserted between *aspC* and *lctP* locus	Warner et al., 2007
DW39Cm^R	*cat* gene inserted between *aspC* and *lctP* locus, *mtrR*$_{A39T}$	This study
DW45Cm^R	*cat* gene inserted between *aspC* and *lctP* locus, *mtrR*$_{G45D}$	This study
DW9Cm^R	*cat* gene inserted between *aspC* and *lctP* locus, *mtrR*$_{E202G}$	This study

[a]All strains are in the FA19Sm^R background, with the exception of strains MS11 and DW3$_{MS11}$.

Table 6. Oligonucleotide primers used for RT-PCR

Primer	Sequence (5'-3')
16S-for	GCGTGGGTAGCAAACAGGAT
16S-rev	CGCGTTAGCTACGCTACCAAG
Rmp-forRT	CAAACAACCTGGTCAGCAAC
Rmp-revRT	TTCGGCTTGACAAACTTGAG
mtrC-forRT	CCGCTTTAACGCTTGCTTCG
mtrC-revRT	CGTTACAAACCGCTGGTTTC
mtrA-forRT	GTGGTTTCAATGCTGCAACT
mtrA-revRT	AGGATAAGCACCAGCAGGAC
mtrR-forRT	AAAATTACCGCCGTTTTGAC
mtrR-revRT	CCAACGTCGATTTGATGAAG

Table 7. Sensitivity to TX-100 and antibiotic substrates of the MtrC-MtrD-MtrE efflux pump system

		Minimum Inhibitory Concentration (μg/ml)			
Strain	**Genotype**	**Em**	**Az**	**TX-100**	**Km**
FA19 Sm^R	wild type	0.5	0.125	62	30
DW9	*mtrR*$_{E202G}$	1	0.25	125	30
JF1	Δ*mtrR*	1	0.25	250	30
DW45	*mtrR*$_{G45D}$	1	0.25	250	30
DW11	*mtrR* $_{1-52}$	2	0.25	250	30
DW39	*mtrR*$_{A39T}$	2	0.5	500	30
FA19 $_{MS11mtr}$	*mtrR*$_{-131T>G}$, *mtrR*$_{A39T}$	8	0.5	8000	30
DW131	*mtrR*$_{-131T>G}$	8	1	>16000	30
KH15	*mtrR*$_{-79}$	8	1	>16000	30
MS11	*mtrR*$_{-131T>G}$, *mtrR*$_{A39T}$	8	1	>16000	30
DW3$_{MS11}$	*mtrR*$_{-131T>G}$, *mtrR*$_{A39T}$, *mtrE*::Cm	<0.12	ND	<31	30
DW3	*mtrE*::Cm	<0.12	ND	<31	30
DW131A	*mtrR*$_{-131T>G}$, *mtrA*::*aph3*	8	1	>16000	60
JF1$_{-131}$	*mtrR*$_{-131T>G}$, *mtrR*$_{A39T}$, Δ*mtrR*	8	1	>16000	30

ND, not determined

Chapter 4: Discussion

Summary of Findings

The findings presented in the previous two chapters provide evidence as to the importance of the two regulators of the MtrC-MtrD-MtrE efflux pump of *N. gonorrhoeae* in a surrogate model of female lower genital tract infection. Chapter two clearly shows an in vivo fitness advantage for an isogenic *mtrR* deletion strain and a fitness decrease for an isogenic *mtrA* mutant strain. These findings were verified with genetically complemented strains to confirm that the results were specifically due to mutation of the *mtrR* and *mtrA* loci. Further, we present evidence that the fitness phenotypes exhibited by both mutants were chiefly due to transcriptional effects on the *mtrCDE* operon and not on other loci controlled by the MtrA activator or MtrR repressor. Finally, we also showed that the commonly isolated *mtr* strain KH15 has the same infectious dose as the wild type strain, which indicates that this derepressed strain has greater fitness but equal infectivity compared to the wild type strain in this model of infection. Isolation of spontaneous *mtrR* mutants from infected mice in chapter two generated data to support the hypothesis tested in chapter three, which is that naturally occurring *mtr* mutants may display differences in both in vitro and in vivo phenotypes. This hypothesis may provide an explanation for the greater prevalence of some *mtr* mutants in clinical outbreak investigations.

isolated from experimentally infected mice, we characterized a gradient of derepression at the level of transcription, antimicrobial resistance, and in vivo fitness. Additionally, we showed similar differences in resistance to two potential in vivo MtrC-MtrD-MtrE substrates, LL-37 and CRAMP. These findings are the first to examine differences afforded by *mtr* locus mutations at the phenotypic and transcriptional level. When paired with the in vivo competition findings, these data may provide a reason for the increased isolation of *mtr* promoter mutants as opposed to *mtrR* structural mutants in outbreak investigations. Further, this work is the first to demonstrate that laboratory strain MS11 is a naturally derepressed *mtr* mutant and that one of the two *mtr* mutations it carries, mtr_{-131}, has never before been described. Additional molecular and genetic characterization of the mtr_{-131} mutation led to the identification of the mechanism of resistance, an increase in *mtrC* mRNA half-life. This is the first reported example of an RND-type efflux operon that has increased mRNA half-life due to a mutation in the 5' untranslated region of the first gene in the operon.

Significance of Findings

The findings displayed herein represent a significant addition to the fields of bacterial pathogenesis and antibiotic resistance. To date this is the first investigation of the roles of *Neisseria* transcriptional repressors or activators in pathogen fitness in an in vivo model of infection. These findings contribute to a growing list of publications that have suggested fitness advantages linked to derepression of homologous RND pumps in other organisms. Specifically, there is a study with *P. aeruginosa*, which showed that

spontaneous MexAB-overexpressing mutants were recovered from mice inoculated with wild type bacteria (86). Also, several studies detail the presence of repressor mutations in clinical isolates of *P. aeruginosa* (72, 214), *E. coli* (114, 200), *S. typhimurium* (134), and *C. jejuni* (150). Likewise, the loss of in vivo fitness demonstrated with *mtrA* mutants adds MtrA to a growing list of bacterial transcriptional activators that are crucial to pathogen fitness. A *marA* efflux pump activator mutant of *S. typhimurium* was shown to be less likely to colonize the spleens and caeca of infected chickens (154), and a triple mutant in all the activators of the *mar* antibiotic resistance operon in *E. coli* was attenuated in a murine model of pyelonephritis (26). Of note, it is known that approximately 50% of *N. gonorrhoeae* strains have an inactive *mtrA* gene (162), and we hypothesize that adaptive mutations in those strains may have circumvented the need for MtrA-based activation.

The importance of these findings with relation to the originally described phenotype of antimicrobial resistance cannot be overstated. The work shown here is critical to understanding the innate value of antibiotic efflux to the bacterial cell. Sequence analysis and mutational characterization have allowed the field to conclude that efflux systems predate the use of antibiotics (reviewed in (132)). Studies in several bacteria demonstrated that antibiotic resistance mechanisms levied a fitness-cost as manifested by a fitness decrease or metabolic disability in the absence of antibiotics (reviewed in (98)). As stated previously, the work presented here goes against this initial dogma, and proves that certain mechanisms of antibiotic resistance confer a fitness increase even in the absence of antibiotic substrates. These conclusions lead to the

following discussion of what role the MtrC-MtrD-MtrE efflux pump is playing in this infection model to yield such a marked advantage when transcriptionally derepressed.

In vivo Function of the MtrC-MtrD-MtrE Efflux Pump

Questions frequently raised by the above findings, are what the functions are of the pump in vivo and why derepression helps in pathogen fitness. Work by others has shown that antibiotic efflux pumps can act as more than just a molecular transporter of antibiotics. For example, the *P. aeruginosa* RND pump MexAB-OprM effluxes antibiotics, but also transports quorum sensing molecules to facilitate the intramolecular communication between bacteria in vivo, which effects the genetic regulation of pathogenic factors (reviewed in (90)). Thus derepression of an RND-pump could lead to an increase in quorum sensing signals. This possibility has been used to explain why derepressed *E. coli* mutants grow to a lower cell density (206), and derepressed *P. aeruginosa* mutants show decreased virulence in the *C. elegans* model of infection (165). Other roles for RND efflux pumps have been characterized that possibly contribute to increased pathogen fitness. For example, work with *E. coli* showed the TolC portion of the *E. coli* RND pump can help facilitate secretion of the effectors haemolysin (193) and colicin V (54). Also, the *P. aeruginosa* homolog of TolC is necessary for secretion of the RTX toxin (19). These additional roles of efflux pumps in other bacteria should be considered for MtrC-MtrD-MtrE as *Neisseria* has a *luxS* gene, which encodes the synthetase of the quorum sensing signal molecule (202), and also has secreted effectors (202). A caveat to studying certain putative functions by the competitive infection

technique is that any function that can be complemented in trans, could negate the fitness differences between the wild type and mutant strains. This limitation would allow for an *mtr* mutant to rescue a wild type strain with increased secretion of pathogenic factors or quorum sensing molecules.

One should also consider the evidence that transcription of other operons is affected by the derepressed phenotype of *mtr* mutant strains. As noted earlier, a recent publication showed that the MtrR protein can act as a repressor and an activator (47), (Fig. 4). The ability to detect the impact of MtrR on other target genes is challenged in competitive infections by the fact that a lower overall bacterial load is displayed by all strains with a disrupted *mtrE* gene in the murine model (81). Although disruption of the *mtrE* gene is an effective way to test for the role of the transcriptional regulators, MtrR and MtrA on other loci, any small differences in gonococcal fitness due to other genes controlled by the regulators would be hard to assess in the experiments performed in chapter two. Further tests should be performed to determine the degree to which other genes controlled by MtrR and MtrA are important to in vivo fitness. This is not to say that secretion or quorum sensing is not occurring via the MtrC-MtrD-MtrE efflux pump; however, such possible mechanisms may not have been detectable with our experimental approach.

Another reason a derepressed mutant may be outcompeting the wild type strain is that increases in the amount of pump protein complexes in the outer membrane may change the profile of the cell membrane, which could disrupt complement or antibody binding and yield a strain that is less affected by the innate or adaptive immune response. Strain FA19 is the most complement resistant strain in the laboratory due to its porin

type, so strain MS11 was utilized to study the effects of overexpression of *mtrCDE* on gonococcal susceptibility to complement-mediated killing. Strain MS11, a natural *mtr* mutant, showed no difference in complement sensitivity compared to an MS11 *mtrE* mutant (D. Warner, unpublished observation), which suggests that complement deposition does not play a role in the above findings.

The last and most obvious role the MtrC-MtrD-MtrE pump may play in vivo, is that of an expeller of host-derived substrates present on the mucosal surfaces. It was mentioned in chapter one that this pump has the widest range of substrates of all the efflux pumps in *Neisseria*. In fact, hydrophobic agents in general, such as TX-100, are pumped out by the MtrC-MtrD-MtrE complex, and one of the hydrophobic agents found at the mucosal epithelium of the genital tract is antimicrobial peptides (30). There are two families of mammalian antimicrobial peptides, cathelicidins and defensins. Cathelicidins are expressed by neutrophils, myeloid bone marrow cells, and the epithelial cells of the gastrointestinal tract, lungs, and genito-urinary tract (30) (and reviewed in (209)). While some organisms produce a great variety of cathelicidins, humans have only one, termed hCAP-18, which is encoded by the gene CAMP (39). The large protein hCAP-18 is extracellularly cleaved, yielding a cathelin and a C-terminal amphipathic antimicrobial peptide called LL-37 (178). The domain of the LL-37 peptide that has antibacterial activity is a large α-helical region that binds to the negatively charged groups on the outer membrane of a bacterium to accumulate and eventually form a pore (138). CRAMP, the murine equivalent to LL-37 shows 52% identity to the whole peptide, and 80% identity to the active portion of the molecule (52).

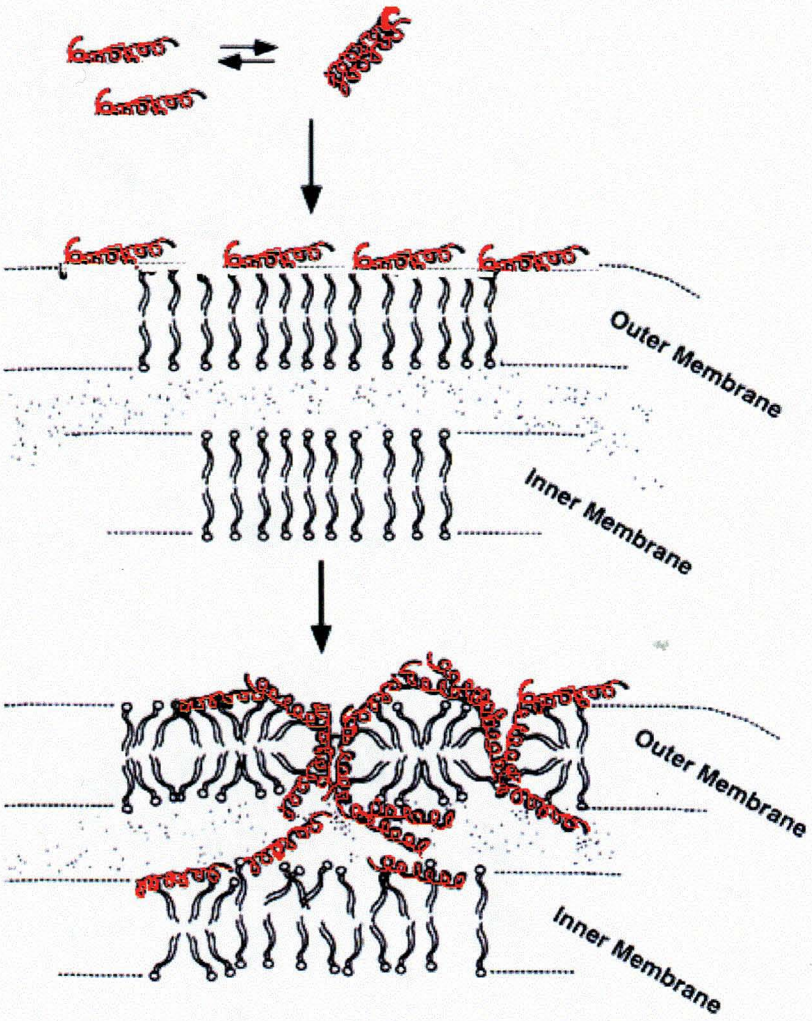
Studies with LL-37 have proven it to be a substrate of the MtrC-MtrD-MtrE efflux pump (176, 191), and here we present genetic evidence that CRAMP is also a substrate of the MtrC-MtrD-MtrE pump based on increased levels of resistance to CRAMP in strains that have higher expression levels of the *mtrCDE* operon. We propose that these antimicrobial peptides challenge the gonococcus in vivo, and that *N. gonorrhoeae* has evolved to counteract this challenge. Bacteria in general have been shown to induce eukaryotic expression of LL-37 and CRAMP (209). However, live *N. gonorrhoeae* causes a down-regulation of LL-37 in in vitro cell culture (16). Additionally, findings show that infection of CRAMP-deficient mice with *N. meningitides* leads to an increased meningococcal colonization compared to wild type mice (17).

The mechanism of action of antimicrobial peptides must be considered with discussion of this innate immune factor. As mentioned above and shown in Figure 18, the mechanism of activity of an antimicrobial peptide on Gram-negative bacteria is to aggregate along the negatively-charged outer membrane and eventually form pores that disrupt the membrane integrity of the periplasm and cytoplasm. In this model, the MtrC-MtrD-MtrE efflux pump would recognize the antimicrobial peptide in the periplasm and expel it before significant damage could occur. Additionally, the source of the antimicrobial peptides in the host must be considered. Unpublished work by Hong Wu in the Jerse lab has shown that neutrophils extracted from a CRAMP-/- mouse have less activity against an *mtrE* mutant than neutrophils derived from a wild type mouse, demonstrating that neutrophil-derived CRAMP does play a role in the innate immune response to gonococci, and that MtrC-MtrD-MtrE acts as a countermeasure. Importantly,

it is known that epithelial cells are also a source of cathelicidins, which we propose to be the origin of the CRAMP in our studies based on the selection for derepressed mutants in the murine genital tract before a poly-morphnucleocite influx is observed. Competitive infections in CRAMP-deficient mice would allow for the testing of the degree to which CRAMP is the reason for the above results. Additionally, while there is much evidence that antimicrobial peptides are acting as the efflux pump substrate responsible for our findings, it is still possible that other substrates may be present, or that the MtrC-MtrD-MtrE pump performs additional functions in vivo.

Figure 18. Model of amphipathic antimicrobial peptide activity on Gram-negative pathogens. The proposed model for how the antibacterial portions of cathelicidin antimicrobial peptides (CAMPs) cause harm to bacterial cells. Initial deposition of the CAMPs on the outer bacterial membrane leads to the insertion of the hydrophobic α-helix-rich region of the peptide into the lipid bilayer. Eventually a pore is formed, which allows entry of CAMPs to the periplasm, activating the pore-forming process at the inner membrane of the bacterium. RND-type pumps are hypothesized to deter this process by expelling the CAMPs from the periplasmic space. This hypothesis is further validated by the findings that RND-pumps recognize substrates in the periplasm. The above figure was modified from (138).

Figure 18. Model of amphipathic antimicrobial peptide activity on Gram-negative pathogens



Novel Mutations

The study of a bacterial gene or protein can be aided immensely by the occurrence of naturally occurring spontaneous mutations. The discovery of the *mtr* mutation present in strain KH15 led to the characterization of that genetic locus as the binding site of MtrR and the transcriptional start site of both *mtrCDE* and *mtrR*. Therefore, it is important to further discuss the significance of the two new *mtr* mutations discovered in this work.

We theorize that the E202G mutation in MtrR affects the homo-dimerization of the MtrR protein in such a way that fewer stable MtrR complexes can be formed. However, an alternate hypothesis is suggested by the structure-function studies of the *S. aureus* repressor homolog QacR. The crystal structures of the QacR repressor bound to six different substrates has been solved (171) and shows that QacR physically binds to the substrates that are effluxed by QacA. This and other bacterial efflux pump repressors that bind substrates (reviewed in (67, 141, 153)), leads to an alternative theory in which the E202G mutation plays a role in the hypothetical recognition and binding of MtrR to an MtrC-MtrD-MtrE efflux pump substrate. In the case of QacR, there are six α-helices in a domain that is buried in the homoquatramer. Drug binding leads to a coil to helix transition, which removes charged groups Y92 and Y93 from the hydrophobic core of the protein. The tyrosines act as a "drug surrogate" and binding of a larger aromatic ring, as is characteristic of the aromatic substrates effluxed by the pump QacA, causes a slight rotational shift in the DNA-binding region of the QacR protein. This shift causes a loss

of DNA binding and consequently repression. In fact QacR uses four glutamate residues (E) located at the C-terminal end of the protein to electrostatically neutralize cationic drugs and allow this structural shift to occur (171). The E202 residue of MtrR exists in a homologous region of the repressor protein and it is tempting to theorize that the substitution of a glycine for the glutamic acid residue may change the binding dynamics of a recognition cleft of the repressor. The unique location and phenotype of the E202G mutation further presents a need for the solution of the crystal structure for MtrR and investigation as to whether MtrR directly interacts with pump substrates.

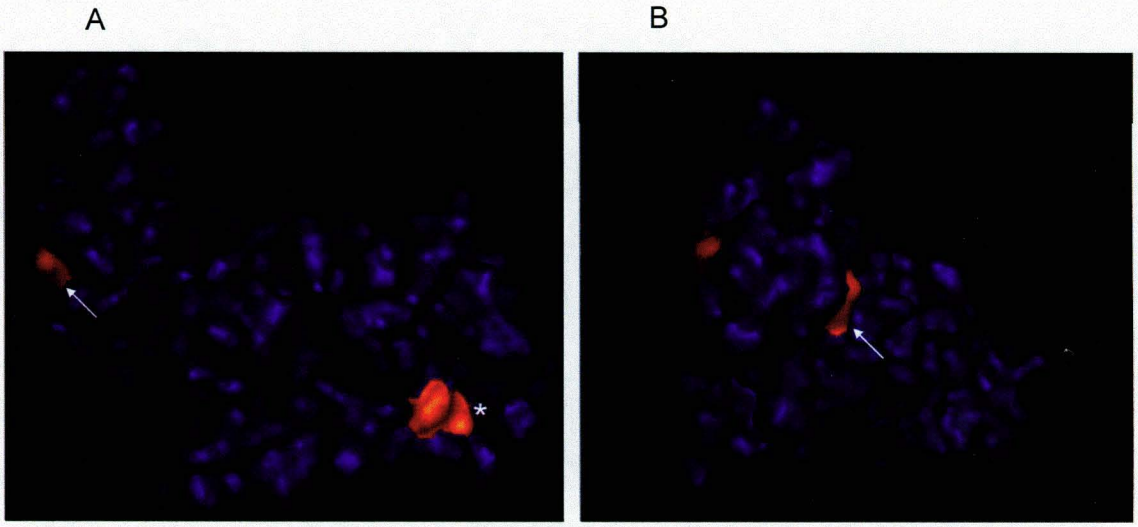
Computer modeling predicted the protein secondary structure of MtrR shown in Figure 19. The hypothetical structure predicts that the E202 residue may be buried like the tyrosine residue "drug surrogates" of QacR. Alternatively, this pocket in the protein may facilitate dimerization of the MtrR complex. Additional examination of this computer model shows that the A39 and G45 residues of the MtrR DNA binding domain are predicted to be on opposite sides of an alpha-helical structure. This distal positioning of these two amino acids may explain the resistance and fitness differences conveyed by the A39T and G45D mutations as detailed in chapter three.

The characterization of the *mtr*$_{-131}$ mutation, shown in chapter three is the first report of an *mtr* mutation that is independent of the MtrR repressor. The in vitro resistance phenotype of a -131 mutation is one of the highest levels of resistance observed and results show a similarly high level of in vivo fitness. Curiously, there have been no publications detailing such a mutation in clinical isolates. Further examination of the primers used to characterize *mtr* mutants from these clinical outbreak investigations showed that all studies which sequenced the *mtr* locus did include the -131

location. However, only a fraction of clinical outbreak investigations characterize the *mtr* phenotype by sequence analysis; most isolates with the *mtr* phenotype are identified by specific MICs. Our interest in the -131 mutation is amplified by the presence of this mutation in laboratory strain MS11, which we show contains both the -131 mutation and an A39T mutation in the MtrR protein. Strain MS11 is a cervical isolate from an uncomplicated infection (183). Numerous attempts to transform an *mtr* locus from strain FA19 into MS11 were unsuccessful, which may be due to a need for MS11 to have a mutated MtrR protein or longer RNA half-life for *mtrCDE.* Although no such findings have been reported, there are interesting experiments to perform regarding the presence of the A39T mutation in the MtrR protein of strain MS11, and the effect on the ability of the repressor to control factors in strain MS11. Such a need for derepression may be due to compensatory mutations present in strain MS11, which somehow require a naturally derepressed strain. These observations prompt further studies with both strain MS11 and mutant strain DW131 to uncover additional properties of strain MS11 and the repressor MtrR.

Figure 19. Hypothetical secondary structure of MtrR A hypothetical model of the secondary structure of the repressor MtrR was created with the 3D-Jigsaw program from www.bmm.icnet.uk/servers/3djigsaw. (A) The face of the C-terminal domain of the repressor with the E202 residue is denoted (asterix) and buried in a hypothetical binding pocket. The A39 residue is denoted by an arrow. (B) A rotation of the model shows the N-terminal face of the protein and the A39 residue is once again denoted by an arrow. The other highlighted residue is the amino acid G45. The localization of the two residues on opposite sides of a DNA binding domain may explain the differences in levels of derepression of *mtrCDE* conveyed by mutation of these amino acids.

Figure 19. Hypothetical secondary structure of MtrR



Transcription Factors and a Role for Therapeutics

The conclusions made herein lead to ideas concerning therapeutics. Obviously, treatment with antibiotics that are efflux pump substrates could enrich for natural mutant strains that are more pathogenic in certain niches. Conversely, these findings also suggest that certain in vivo niches may have already enriched for strains that are more pathogenic and resistant to antibiotic therapy. While these conclusions are frightening, the findings can also shed light on how to better deal with antibiotic resistance while simultaneously addressing pathogen fitness. Several studies have proven the principle that efflux pumps can be blocked by small peptides (reviewed in (139)). The intent of those studies was to find substances that could be co-administered with efflux pump antibiotics, to increase bacterial susceptibility to these antibiotics. My findings would lend more credence to such a pursuit, with efflux blockers also acting to decrease the bacterial fitness of pathogens in certain environments.

The same principles of pump blocking can be applied to the development of antibodies to bind and neutralize the efflux activity of pumps. Seminal studies were done with the over-expressed eukaryotic efflux pump Pgp of cancer cells, in which monoclonal antibodies that bound to the pump caused decreased resistance to anti-cancer drugs (66, 120, 135). There are no known studies on this therapeutic approach for bacterial efflux pumps, but attempts at blocking the MtrC-MtrD-MtrE efflux pump of *N. gonorrhoeae* were made by our laboratory. The MtrE-specific antibodies used in chapters two and

three were initially designed to bind regions hypothesized to be on the outer membrane-exposed domains of the MtrE protein. Attempts to characterize the binding of the antibodies to native MtrE protein showed no binding, possibly because the outer membrane-exposed region of the MtrE protein may be buried and inaccessible to antibodies. The high degree of conservation of the MtrE protein in different strains supports this theory. Alternatively, only antibodies that recognize a conformational epitope, which is dependent on the trimerization of MtrE, may be effective. The antibodies we generated were raised against linear peptides and thus are unlikely to be conformation-specific.

A further therapeutic option that my work supports is one that would change the transcription of *mtrCDE* to favor the host, such as increasing repression or nullifying activation. Evidence presented here illustrates that a transcriptionally repressed or inactivated *mtrCDE* operon would be both more susceptible to antibiotics and less pathogenic. The paradigm for transcriptional control of non-essential genes involves the bacteria utilizing response regulator systems to sense and respond to different stimuli in the environment (reviewed in (43)). As such, with further studies of factors that influence the repression and activation of the *mtrCDE* operon, designs of therapeutics that can cause the bacterium to down-regulate the transcription of *mtrCDE* may hold promise as an additional treatment for gonococcal infection. The opposite effect, one of activation or derepression, should also be considered, so that agents that come into contact with the bacteria do not increase the transcription of *mtrCDE*. Unfortunately, this second effect has already been shown to occur with the spermicidal agent Nonoxynol-9, which acts to increase transcription of *mtrA* and subsequently *mtrCDE* (162).

Evolution of Bacterial Efflux Pumps

It is of interest that only bacterial RND-type efflux pumps thus far have been shown to play roles in pathogenicity. RND-type pumps are the only known bacterial efflux pumps that recognize and respond to substrates in the periplasm (132). It is easy to imagine how this periplasmic action of an RND-pump would allow it to decrease the damage to the inner membrane of a bacteria being challenged by cathelicidins. Also, with rare exceptions RND pumps are generally chromosomally encoded and not acquired by horizontal exchange. RND pumps thus have evolved with bacteria to such an extent that their substrate range may include substrates that aid the bacteria in niche survival (133, 144). However, there is a report of plasmid-encoded pumps among environmental *Pseudomonas* species (40), which increases the likelihood of horizontal transfer of these efflux operons.

The presence of chromosomally-encoded efflux pumps has been under intense study over the past decade with the publication of many bacterial genomes. Some publications envision a "biological warfare" between plants and bacteria that long predates the interaction of bacteria with humans, which necessitated the evolution of these pumps. This theory is consistent with the fact that the largest numbers of predicted efflux pump systems are found in soil-based pathogens, such as *Pseudomonas*, which have heavy contact with plants (reviewed in (144)). Meanwhile, sequences of plants such as *Arabidopsis thaliana* show many more hypothetical efflux pumps in their genomes than are found in animal sequences (141). These genomic comparisons point to bacterial-

plant interactions as forming the evolutionary starting point for the necessity of efflux pumps. The retention of these pumps as bacteria evolved from plant to mammalian pathogens is explained with the hypothesis that the ingestion of vegetation and its subsequent harmful peptides and toxins led to the evolution of the efflux pumps to allow for bacterial success in the intestinal niche (141). These theories have led to the examination of plant antimicrobials with one, berberine, shown to be effluxed by bacterial efflux pumps, and showing considerably potency when pumps are mutated or blocked (187). Berberine is an ammonium ring-containing antimicrobial synthesized by the *Berberis* plant. The *Berberis* plant pairs berberine secretion with the secretion of an efflux pump inhibitor, which gives the combination potent activity against bacteria (180). Significantly, the *Berberis* plant has no known bacterial pathogens that prey upon it, likely due to this strong antimicrobial effect. This is an example of nature pairing efflux pump inhibitors with antimicrobials that are effluxed.

Bacteria that are human pathogens have evolved to survive in various diverse niches in the body. Studies on bile resistance have generated evidence that the evolution of efflux pumps parallels the niche that a bacterium colonizes. Gut pathogens must counteract the low pH levels encountered within the digestive tract, and work with the AcrAB pump or *E. coli* (110), the CmeABC pump of *C. jejuni* (101), the TolC protein of *V. cholerae* (19), and the AcrAB pump of *S. typhimurium* (149) shows that RND-type efflux pumps are crucial to the survival of gut pathogens in the presence of physiological levels of bile salts. Also, the FarAB pump of *N. gonorrhoeae* exports fatty acids likely to be encountered in rectal infection (126).

The growing body of evidence that RND-type efflux pumps have evolved with their pathogens to assist in the colonization of harsh biological environments calls into question the number of efflux pumps that have not yet been discovered. The above molecules were initially discovered and characterized because of their antibiotic resistance phenotype, yet more efflux pumps may exist that serve to protect the pathogen from only biological substrates. Predicted multi-drug efflux pumps generally account for 10% of all transport proteins found in a bacterial genome (141). While the characterization of many of these hypothetical efflux systems has not yet occurred, future works will likely find efflux pumps, both new and old, to be critical for fitness in many other environmental niches and infection sites.

Appendix: Phenotypic Characterization of *Neisseria gonorrhoeae* Strains that Express Frequently Recovered Variable Region (VR) P1.A Porin Types

Lotisha E. Garvin, Margaret C. Bash, Freyja Lynn, Christine Keys, Douglas M. Warner, Sanjay Ram, William M. Shafer, and Ann E. Jerse

Note: The following document summarizes the portion of work that was performed by Douglas Warner in the above manuscript. VR-typing was performed by members of Margaret Bash's laboratory at the Center for Biologics Evaluation and Research, FDA. PFGE cluster analysis and MIC determinations were performed by Lotisha Garvin with assistance from Ms. Christine Keys at the Center for Food Safety and Applied Nutrition, FDA. At the time of this writing this manuscript is in preparation.

Abstract

The porin protein of *Neisseria gonorrhoeae* is an antigenically variable protein that is essential for bacterial survival. Previous work with clinical isolates showed that certain variable region (VR) types of the porin protein are associated with more commonly isolated *N. gonorrhoeae* strains. Here we used pulsed-field gel electrophoresis (PFGE) to examine the genetic relatedness of clinical isolates with commonly isolated VR-types. Isolates were tested for several important gonococcal pathogenesis factors, including the *mtr* phenotype, to determine whether the presence of these factors correlated with the more persistent porin types. Data presented here showed

that six isolates had the A39T or G45D *mtrR* mutations including all isolates of a more persistent VR-type that belonged to the same PFGE cluster.

Introduction

In 1986, the Centers for Disease Control established the Gonococcal Isolate Surveillance Program (GISP), for the purpose of gathering clinical isolates of *Neisseria gonorrhoeae* from across the United States and analyzing the isolates for levels of antibiotic resistance (56). The use of molecular methods, such as VR-typing, is helpful in monitoring the emergence of antibiotic resistant strains. Porin, which is stably expressed but has variability in surface-expressed loops, is the basis for VR-typing in *N. gonorrhoeae* (50, 208). Porin comprises 60% of the protein content on the outer membrane of *Neisseria* and forms a homotrimer that allows entry and exit of a wide range of substrates (reviewed in (87)). Due to its transport function, certain mutations in porin lower the susceptibility of *N. gonorrhoeae* to penicillin (137). Some porin types also play an important role in immune evasion due to binding of regulatory factors in both the classical and alternative complement cascades (151, 152). Gonococcal porin exists in two mutually exclusive forms, porB.1A (PIA) and porB.1B (PIB), which are characterized based on antibody binding and sequence differences (57).

Here we characterized a set of strains collected in Baltimore, MD, which were previously tested for variations in PIA. Two particular PIA VR-types were consistently found in the isolates collected over a ten year time span (117). The basis for this finding may be that certain porin VR-types are markers of successful clones. Alternatively, certain porin VR-types may convey fitness advantages to *N. gonorrhoeae*. As part of this

analysis, we examined the "multiple transferable resistance" *mtr* phenotype, which is a porin-independent phenotype that might confer a fitness advantage to the more successful clones. The *mtr* phenotype is characterized by the increase of gonococcal macrolide resistance via transcriptional derepression of the bacterial RND-efflux pump operon *mtrCDE.* Phenotypically, these strains exhibit increased levels of resistance to MtrC-MtrD-MtrE efflux pump substrates Triton X-100 (TX-100), erythromycin (Em), and azithromycin (Az) (140). A series of publications that detailed the presence of naturally occurring *mtr* mutants in clinical isolates suggested a fitness benefit or selective factor present in nature that allows for natural *mtr* mutants to flourish (34, 113, 118, 126, 130, 184, 198, 205, 210, 211). Consistent with these findings, previous work from this lab showed that the *mtr* phenotype conveys a fitness increase in the murine model of lower genital tract infection (Warner et al., 2007). Therefore, here we wanted to determine if there was a correlation between persistent porin types and the *mtr* phenotype. These isolates were also analyzed for genetic relatedness as assessed by pulse-field gel electrophoresis (PFGE). Here we present the results of the *mtr* analysis portion of this work.

Methods and Results

The *mtr* Phenotype Was Identified in Six Clinical Isolates. All strains used in this study were initially analyzed based on their porin gene polymorphisms using a set of DNA probes designed to hybridize to sequences that encode five of the eight variable surface exposed loops of PIA (VR-types) (117). A VR-type is displayed as a series of

five numbers that correspond to the oligonucleotide probe that hybridized to that respective loop. Strains of VR-type 1;2;1;1;1 and 1;1;1;1,4;1 were the most represented among the 63 PIA strains isolated over a four year period in Baltimore (117). Twenty-five of the sixty-three strains from that study were further tested by this lab for genetic relatedness using PFGE, with 85% similarity used to define a cluster. The twenty-five isolates exhibited eight different PFGE patterns. Importantly, ten strains of the 1;2;1;1;1 porin type segregated into four unrelated clusters and seven strains from the 1;1;1;1,4;1 porin type segregated into two different clusters. These results are consistent with the hypothesis that genetically unrelated strains may express highly related porin sequences and that more persistent porin variants may therefore confer an advantage. However, to further examine the possibility that a porin-independent factor may confer an advantage to certain clones, we analyzed the isolates for the *mtr* phenotype. Laboratory strains FA19 and FA1090, which both contain wild type *mtr* loci were used as controls for all assays performed.

The resistance levels of all twenty-five clinical isolates to Em, Az and TX-100 were determined by MIC as described previously (81). Strain FA19 was included for comparison, and results are shown in Table 8. Strains LG4, LG5, LG6, LG7, and LG22 displayed increased levels of resistance to Em (>0.5 μg/ml), Az (≥0.5 μg/ml), and TX-100 (≥1000 μg/ml), compared to strains in the same genetic cluster, which suggested the presence of *mtr* mutations in these strains. To further investigate the basis for resistance to Em, Az, and TX-100, gonococcal genomic DNA was isolated from strains of interest, and the *mtr* locus was PCR amplified as described (Warner et al., 2007). PCR products

were analyzed by agarose gel electrophoresis and purified using a Qiaquick spin column (Qiagen).

After PCR amplification and gel purification, ~1 μg of the resultant *mtr* amplicons were used to transform wild type strain FA19 as described (60). Transformants were isolated on GC media supplemented with Em at a concentration of 0.5 μg/ml. This concentration of Em is sufficient to isolate strains of FA19 that contain a mutation in the *mtrR* repressor or its upstream region. Control experiments were performed with wild type *mtr* loci isolated from strain FA19 and no transformants were isolated. All transformations that did not confer a resistant phenotype were repeated to ensure proper classification. DNA from strains LG2, 4, 5, 6, 7, and 22 all produced transformants that grew on GC agar with Em. *mtr* loci from the original LG strains were then sequenced to determine the nature of the mutation(s).

Assessment of the Clonality of the Naturally-occurring *mtrR* Mutations. Sequence analysis of the *mtr* locus was performed by using 100-300 ng of the previously described *mtr* PCR amplicons in conjunction with 10 pmoles of either primer C-Xho or R-Xho and the BigDye (ABI) sequence reaction mix as described previously (Warner et al., 2007). The resultant sequencing reactions were purified using a gel filtration cartridge (Edge Biosystems) and analyzed at the USUHS Bioinstrumentation Center.

Sequence analysis of strains LG2 and LG7 showed the presence of a G>A substitution at bp 115 of the *mtrR* gene, which is predicted to cause an Ala39>Tyr substitution in the MtrR protein. Strains LG4, LG5, LG6, and LG22 harbor a G>A substitution at bp 134 of *mtrR*, which causes a Gly45>Asp substitution in the MtrR

protein. These MtrR mutations are located in the predicted DNA-binding α-helix motif of MtrR and have been described in other clinical isolates of *N. gonorrhoeae* (38, 174, 184). The significance of these mutations was further established by determining that the A39T mutation conferred a higher level of resistance to Em, Az and TX-100 when compared to strains with the G45D mutation in an FA19 background (See chapter 3).

Interestingly, comparison of the sequence data and the PFGE analysis showed that LG strains 4-6 all bore the same *mtrR* mutation (G45D) and were in PFGE cluster E. Additionally, LG strains 2 and 7 were in different PFGE clusters and had the same *mtrR* mutation (A39T). Strain LG22 harbored a G45D mutation, but was clonally dissimilar to other strains with the same mutation. The differences in MIC and *mtrR* mutation type did not appear to correlate to PFGE cluster or porin type, with exception of the A39T mutants in cluster E.

Discussion

Based on the PFGE and VR-types we can conclude that the clonality of one group of isolates did correlate with both *mtr* phenotype and VR-type. From this it is possible that cluster E isolates, which express one of the most persistent porin VR-types, may have had additional fitness advantages due to the presence of a G45D *mtrR* mutation. However, since this phenomenon occurred in only one of the seven clusters of the more persistent PIA type, *mtr* is likely not the reason for the repeated isolation of this VR-type. Conversely, MIC analysis of penicillin and tetracycline revealed that VR-type 2;1;3;3;3 had resistance levels which indicated the presence of β-lactamase producing plasmids (data not shown). Further testing for β-lactamase activity and plasmid characterization

revealed that these plasmids were present in this transiently isolated VR-type. These findings suggest that either β-lactamase activity or the β-lactamase plasmid, may confer a fitness disadvantage in the absence of the antibiotic. Of note, penicillin was no longer the recommended therapy for gonorrhea at the time these samples were isolated.

The above conclusions point out the need for additional characterization of the fitness-costs of other *N. gonorrhoeae* mechanisms of antibiotic resistance. While the *mtr* phenotype increases gonococcal fitness in competitive infections with the wild type strain (Warner et al., 2007), little else is known about the impact other antibiotic resistance determinants have on pathogen fitness. Any in vivo gonococcal fitness effects produced by antibiotic resistance determinants can be tested in the laboratory setting by performing competitive infections with a wild type strain and strains containing one or more antibiotic resistance factors. The determination of gonococcal fitness effects produced by different antibiotic resistance mechanisms could help determine the appropriateness of certain chemotherapeutics.

Table 8. The *mtr* phenotype is clonal for some but not all LG strains

VR Type	Strain	PFGE cluster	Minimal Inhibitory Concentration (μg/ml)					
			Tet	**Amp**	**PenG**	**Em**	**Az**	**TX-100**
N/A	FA19	N/A	2	0.125	1.0	0.125	0.25	50
1;2;1;1;1	LG11	B	2	0.25	0.5	0.125	0.125	50
	LG13	B	2	0.25	2.0	0.125	0.125	1000
	LG15	B	2	0.125	0.25	0.125	0.125	1000
	LG7[a]	B	2	>0.5	1.0	>0.5	0.5	>1000
	LG22[b]	A2	2	0.25	0.25	>0.5	0.5	1000
1;1;1;1,4;1	LG4[b]	E	2	>0.5	1.0	>0.5	0.5	1000
	LG5[b]	E	2	>0.5	1.0	>0.5	0.5	>1000
	LG6[b]	E	2	>0.5	1.0	>0.5	0.5	>1000
	LG21	E	2	0.5	0.5	>0.5	0.5	1000
2;4;3;3;3	LG2[a]	F	4	>0.5	>4.0	>0.5	0.25	1000
	LG8	F	2	>0.5	>4.0	0.25	0.25	1000
	LG10	F	2	>0.5	>4.0	0.125	0.25	50
	LG17	F	2	>0.5	>4.0	0.25	0.25	1000
	LG9	F	2	>0.5	>4.0	0.25	0.25	1000
	LG16	F	2	>0.5	>4.0	0.25	0.25	1000
	LG18	F	2	>0.5	>4.0	0.25	0.25	1000

[a]A39T mutant

[b]G45D mutant

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