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Journal Title: Molecular Microbiology
Volume: Volume 70, Number 2
Publisher: Wiley: 12 months | 2008-10, Pages 462-478
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1111/j.1365-2958.2008.06424.x
Permanent URL: http://pid.emory.edu/ark:/25593/fj0rk


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Accessed April 21, 2020 2:19 PM EDT
Clinically Relevant Mutations that Cause Derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE Efflux Pump System Confer Different Levels of Antimicrobial Resistance and *In Vivo* Fitness

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**Summary**

The MtrC-MtrD-MtrE efflux pump system confers resistance to macrolide antibiotics and antimicrobial substances of the host innate defense. Clinical isolates with increased resistance to erythromycin and azithromycin frequently harbor mutations in the *mtrR* structural gene, which encodes a repressor of the *mtrCDE* operon, or the *mtrR* promoter region. The MtrC-MtrD-MtrE system is important for gonococcal survival in the murine genital tract, and derepression of the *mtrCDE* operon via deletion of *mtrR* confers increased fitness *in vivo*. Here we compared isogenic strains with naturally occurring *mtrR* locus mutations for differences in *mtrCDE* expression and pump-related phenotypes. Mutations upstream of *mtrC*, including those within the MtrR binding region and a novel mutation that increases *mtrC* RNA stability conferred the highest levels of derepression as measured by *mtrCDE* transcription and resistance to antibiotics, progesterone, and antimicrobial peptides. In contrast, mutations within the *mtrR* coding sequence conferred low to intermediate levels of derepression. *In vivo*, the *mtr* mutants were more fit than the wild type strain, the degree to which paralleled *in vitro* resistance gradients. These studies establish a hierarchy of *mtrR* locus mutations with regard to regulation of pump efflux, and suggest selection for more derepressed mutants may occur during mixed infections.

**Keywords**

*Neisseria gonorrhoeae*; active efflux; MtrC-MtrD-MtrE efflux pump; antibiotic resistance; *in vivo* fitness; antimicrobial peptides; genital tract

**Introduction**

Gonorrhea is the second most commonly reported infection in the United States (Jajosky *et al.*, 2006) and it occurs at a high incidence in the developing world with an estimated 62
million annual cases worldwide (Gerbase et al., 1998). Neisseria gonorrhoeae is primarily a mucosal pathogen of the lower urogenital tract. The main site of infection is the cervix and the urethra in men. The female urethra is also often infected and rectal and pharyngeal infections are common in both genders. Lower genital tract infections are usually uncomplicated and often asymptomatic, particularly in women. Ascension to the upper reproductive tract results in more serious disease, including epididymitis, endometritis, salpingitis, and pelvic inflammatory disease (PID). Since no vaccine currently exists for N. gonorrhoeae, antibiotic therapy is a primary measure of infection control. The emergence of antibiotic resistant strains, however, continually challenges the effectiveness of antibiotics as a control strategy as exemplified by the recent removal of fluoroquinolones from the recommended therapy for gonorrhea (CDC, 2004).

A thorough understanding of gonococcal antibiotic resistance mechanisms is critical for the development of new and effective antimicrobial agents. Mechanisms of antibiotic resistance in N. gonorrhoeae include mutation of the antibiotic target, enzymatic breakdown of the antibiotic, decreased permeability of the bacterial membrane, the presence of active efflux systems, and derepression of transmembrane efflux through mutation of pump repressor genes [reviewed in (Alekshun et al., 2007)]. The MtrC-MtrD-MtrE efflux pump system is one of four known efflux pumps of N. gonorrhoeae (Hagman et al., 1995a, Lee et al., 1999, Pan et al., 1994, Rouquette-Loughlin et al., 2003, Rouquette-Loughlin et al., 2005). The MtrC-MtrD-MtrE efflux pump exports macrolide antibiotics and is implicated in high level penicillin resistance (Veal et al., 2002). We previously reported that MtrC-MtrD-MtrE-deficient mutants were highly attenuated in a female mouse model of lower genital tract infection. In contrast, mutants in the FarA-FarB-MtrE efflux pump, which are hypersusceptible to long chain fatty acids were not attenuated in this model (Jerse et al., 2003). These results are consistent with the hypothesis that the MtrC-MtrD-MtrE efflux pump system, which predates the clinical use of antibiotics, protects the gonococcus from antimicrobial substances that may be encountered during genital tract infection. Host substrates that may challenge the gonococcus include bile salts (Delahay et al., 1997, Hagman et al., 1995a), progesterone (Jerse et al., 2003), and LL37, a host-derived antimicrobial peptide found in the human genital tract (Shafer et al., 1998).

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 (Hagman et al., 1995b) and an activator MtrA (Rouquette et al., 1999). The mtrR gene is located 250-bp upstream and is divergently transcribed from the mtrCDE operon (Pan et al., 1994). The 24.5 kDa MtrR protein is a TetR-type repressor and negatively controls mtrCDE expression by the binding of two MtrR homodimers to pseudo-direct repeats within the mtrCDE promoter (Hoffmann et al., 2005); this DNA-binding action is similar to the QacR repressor that controls efflux pump gene expression in Staphylococcus aureus (Schumacher et al., 2002). A helix-turn-helix (HTH) DNA binding motif exists between residues 32-53 and missense mutations that cause radical amino acid replacements at residue 39 (A39T) or 45 (G45D) can enhance gonococcal resistance to hydrophobic antimicrobials presumably because they abrogate MtrR binding to the target DNA upstream of mtrCDE (Shafer et al., 1995, Hagman et al., 1995b). Other mutations that cause radical amino acid replacements in the center of coding sequence (H105Y) or the C-terminal domain can also impact MtrR function, possibly by altering MtrR multimer formation.

Strains that bear mtr mutations have been recovered in a variety of outbreak investigations. The most common mutation is a single base pair deletion located in the inverted repeat that is between the -10 and -35 hexamers of the mtrR promoter (Cousin et al., 2004, Dewi et al., 2004, Lucas et al., 1995, Ng et al., 2002, Tanaka et al., 2006, Xia et al., 2000, Zarantonelli et...
et al., 1999, Zarantonelli et al., 2001) Other commonly described mutations are located in the structural gene of mtrR, and include a G45D mutation (Shafer et al., 1995, Tanaka et al., 2006, Dewi et al., 2004, Vereshchagin et al., 2004, Zarantonelli et al., 1999) and an A39T mutation (Dewi et al., 2004). While all of these mutations confer increased resistance to hydrophobic antimicrobials, differences exist with respect to the level of resistance afforded by the given mutation (Hagman et al., 1995a, Shafer et al., 1995). Thus, the missense mutations in the mtrR coding sequence typically result in a low to intermediate level of resistance while the promoter mutation affords high levels of resistance. Hagman and Shafer (Hagman et al., 1995b) proposed that the promoter mutation abrogates mtrR transcription. In the promoter mutant mtrCDE transcription is elevated because MtrR is absent and RNA polymerase is better able to interact with the mtrCDE promoter that partially overlaps with the mtrR promoter at the -35 region (Lucas et al., 1997).

While the molecular mechanisms by which the mtrCDE efflux pump-encoding operon is regulated at the level of transcription are well characterized, little information is available regarding the biologic significance of the most common mtrR mutations found among clinical isolates. Additionally, while an mtrR deletion mutant demonstrated increased fitness in the lower genital tract of female mice as predicted by the demonstrated importance of the MtrC-MtrD-MtrE pump in vivo (Warner et al., 2007), the impact of naturally occurring mtr mutations on in vivo fitness has not been tested. Accordingly, here we compared five different mtr mutations found in clinical isolates or vaginal isolates from experimentally infected mice for differences in mtrCDE operon derepression as measured by levels of RNA transcript, protein production, and resistance to erythromycin (Em), azithromycin (Az), and the non-ionic detergent Triton X-100 (TX-100), all of which are substrates for the MtrC-MtrD-MtrE efflux pump. We also evaluated the consequence of each mutation on resistance to progesterone and the murine cathelicidin-related antimicrobial peptide (CRAMP-38), and on gonococcal fitness during experimental murine genital tract infection. Additionally, we report that the frequently used laboratory strain MS11 is a natural mtr mutant that harbors two mtr locus mutations, including a novel mutation that results in increased mtrCDE transcript stability. As with the mutated mtr loci amplified from clinical isolates, we show here that the mtr locus of strain MS11 confers increased resistance to CRAMP-38 and other substrates of the MtrC-MtrD-MtrE efflux pump system, including the human cathelicidin LL-37.

Results

Construction of a series of mtr locus mutations in strain FA19SmR

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 in strain FA19, which represent the majority of natural promoter and structural gene mutants that have been reported in the mtr loci of clinical isolates (Table 1). The FA19 strain used was a spontaneous streptomycin resistant mutant, which is a phenotype that is required for mouse infection studies. A schematic that shows the position of each mutation within the mtr locus is shown in Figure 1. Mutant strain KH15 is a transformant of strain FA19SmR that contains a T deletion on the end of the 13-bp inverted repeat within the mtrR promoter that is adjacent the MtrR-binding region that encompasses the mtrCDE promoter, and was previously described (Lucas et al., 1997). The A39T and G45D mutations, which are in the HTH DNA-binding domain of MtrR (Hoffmann et al., 2005, Lucas et al., 1997, Pan et al., 1994), were amplified from the urogenital isolates LG7 and LG5, respectively (McKnew et al., 2003, Garvin et al., 2008) and transformed into FA19SmR to create strains DW39 and DW45, respectively. We also included mutant DW9 in our survey, which has a one base pair change that results in a glycine residue instead of glutamic acid (E202G) near the C-
terminus of the MtrR protein. The E202G mutation was originally identified in a spontaneous EmR mutant that was isolated from experimentally infected mice (Warner et al., 2007). Mutant JF1 carries an internal deletion within mtrR (Folster et al., 2005) and was used for comparisons with mutants that carry naturally occurring mtr mutations.

We also included the commonly used laboratory strain MS11 in our analysis. Strain MS11 has been used in numerous pathogenesis studies including experiments with male volunteers (Ramsey et al., 1995, Schmidt et al., 2001, Schneider et al., 1991, Swanson et al., 1987b). Based on the observation in our laboratory that MS11 bacteria exhibit higher levels of in vitro resistance to TX-100, Em, and progesterone than strain FA19, we hypothesized that the MS11 strain may contain one or more mtr locus mutations. Sequence analysis of the MS11 mtr locus revealed the A39T mutation in the MtrR DNA binding motif and an adenine to guanine transition located between the mtrR and mtrC start codons (Fig. 1); this single base pair mutation is in the noncoding region of the mtrC transcript as it is positioned 120-bp upstream of the mtrC start codon and 42-bp downstream of the transcriptional start. This mutation (mtr120) has not been described previously among clinical isolates; however, we have isolated the mtr120 mutation when measuring the rate of spontaneous Em resistance in strain FA19 in vitro (data not shown). Here we moved the mtr120 mutation into strain FA19 to create mutant DW120. The capacity of each of these mutations to modulate levels of mtrCDE expression, resistance to antimicrobial substances and in vivo fitness is described below.

Natural mtr mutations confer differential levels of antibiotic resistance

Hagman and Shafer (1995) previously reported that the mtr mutant strain KH15 displays increased levels of antimicrobial resistance compared to an mtrR deletion mutant (Hagman et al., 1995a, Shafer et al., 1995). 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-efflux pump substrate control. Based on the MIC for Em, we observed a stepwise gradient of resistance that could be defined by three classes of resistant strains (Table 2). Compared to drug-susceptible wild type strain FA19, FA19-based mutant strains DW9 (E202G change in MtrR), JF1 (mtrR deletion), and DW45 (G45D change in MtrR) showed only a slight (two-fold), but reproducible increase in Em resistance. 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 bacteria. The next level of resistance (intermediate) was defined as a 4-fold increase in the MIC of Em, and was exhibited by mutant DW39 (A39T change in MtrR). The third group of mutants, KH15 and DW120, displayed the highest levels of resistance to Em (16-fold increase). Interestingly, strains KH15 and DW120 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 but within the mtrR promoter, and is one of the most commonly described mutations isolated in clinics. The mtr120 mutation in strain DW120, which is carried by laboratory strain MS11, is novel because it is positioned 131-bp downstream from the MtrR-binding site (Lucas et al., 1997), and is predicted to be within the mtrCDE transcript. Mutants KH15 and DW120 also exhibited levels of resistance to Az and TX-100 that were notably higher than the other strains, with increases in MIC of 8-fold and 256-fold, respectively. We tried to construct a mutant that carried both the mtrR.79 mutation in KH15 and the mtr120 mutation by transforming strain DW120 with the appropriate PCR fragment from KH15 and selecting on media with 16 μg/ml Em. No transformants were isolated. This result that suggests each of these mutations confers the highest level of MtrC-MtrD-MtrE-mediated Em resistance that can be achieved.

Mol Microbiol. Author manuscript; available in PMC 2009 October 1.
Analysis of the mtr locus in strain MS11

To further characterize the mtr locus of strain MS11, we moved the entire mutated mtr sequence of strain MS11 into strain FA19SmR. This was accomplished by transformation of a PCR product that was generated using primers that anneal 48-bp after the translational stop site of mtrR and 391-bp within the mtrC gene as described in the Experimental Procedures. The resultant 1355-bp PCR product carried both the A39T and mtr120 mutations. The resistance phenotypes of a representative transformant (FA19MS11mtr) established the genetic linkage between the MS11 mtr locus and resistance to Em, Az, and TX-100 (Table 2). Mutant DW120, which carries the mtr120 mutation in the FA19SmR background as described above, showed increased resistance to Em at a level that was comparable to that exhibited by strain FA19MS11mtr, which carries both mutations and a modest (two-fold) increase in Az and TX-100 resistance. To confirm the role of the MtrC-MtrD-MtrE efflux pump in the increased resistance phenotype exhibited by strain MS11, we introduced an mtrE mutation from plasmid pCR-mtrE (Warner et al., 2007) into strain MS11, and compared the resistance phenotypes of the resultant strain (DW3MS11) and an mtrE mutant of strain FA19 (DW3). Disruption of the mtrE gene in strain MS11, which encodes the outer membrane protein channel of the pump (Delahay et al., 1997) conferred levels of Em and TX-100 resistance that were equal to that of the FA19 mtrE mutant DW3 (Table 2).

Effect of mtr Locus Mutations on mtrCDE Expression and mRNA half-life

To verify that the mtr mutations described above impacted levels of the MtrC-MtrD-MtrE efflux pump, we next determined whether differences exist in the amount of the MtrE protein produced by our test strains. We detected increased levels of MtrE for all mtr mutant strains compared to wild type strain FA19 by immunoblot analysis of outer membrane proteins, and in general, the results reflected the gradient (Fig. 2). DW9 and JF1 gonococci had the lowest increase in band intensity (a 1.8- and 1.94-fold difference relative to the wild type strain), and both strains are at the low end of the gradient as defined by Em MIC. Strains DW45 and DW11 are classified as low and intermediate strains, respectively, and had 2.0-fold increases in MtrE compared to wild type gonococci. Mutants DW39 (intermediate) and DW120 and KH15 (both high) had band intensities that were 2.12- to 2.15-fold greater than the wild type strain. The one strain for which MtrE levels did not correspond well to the Em MIC was FA19MS11mtr, which had a 1.82-fold increase in band intensity, and was thus similar to that of mutants in the low end of the MIC gradient.

We also 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. 3). As reported previously (Hagman et al., 1995b), no mtrR mRNA was detectable in the deletion strain (JF1) or strain KH15 (data not shown). Except for levels of the mtrR transcript produced by strains JF1 and KH15, no significant differences in the expression of the transcriptional activator MtrA or repressor MtrR were detected among mtr mutant and the wild type bacteria. Differences in the level of mtrC transcript, in contrast, were consistent with the observed differences in antibiotic resistance. Mutants DW39, DW120, FA19MS11mtr, and KH15 expressed 3- to 6-fold greater amounts of mtrC mRNA than the wild type bacteria. These strains showed intermediate (DW39) to high (DW120, FA19MS11mtr, and KH15) levels of resistance to Em and Az (Table 2). Mutants DW9, JF1, and DW45 showed 1.1-to 1.4-fold greater amounts of mtrC expression, which is consistent with the lower increases in antibiotic resistance exhibited by these strains. Taken together, these results confirm that the derepressed phenotypes of the mutants are due to increased transcription of the mtrCDE operon and not to changes in the level of mtrA or mtrR transcription. These results also suggest that subtle differences at the mRNA level can alter the functional measures of the phenotype.
We also found an interesting but subtle difference between the presence of the \textit{mtrR}A39T and \textit{mtrR}120 mutations versus the \textit{mtrR}120 mutation alone. As shown above, \textit{FA19\textsubscript{MS11mtr}} gonococci, which carry both mutations, produced less MtrE than expected based on the Em MIC for this strain (Fig. 2). Strain \textit{FA19\textsubscript{MS11mtr}}, also does not fit squarely into the “high” end of the gradient when Az and TX-100 MICs were evaluated (Table 2). However, the levels of \textit{mtrC} transcripts were the same for \textit{FA19\textsubscript{MS11mtr}} and \textit{DW120} bacteria, the latter of which carry only the \textit{mtrR}120 mutation (Fig. 3). At present we can not readily explain this difference, but we hypothesize there may be regulatory factors that influence transcription or translation of \textit{mtrE} independently of \textit{mtrCD} and these are factors are influenced by the presence of one versus two of these mutations.

The location of the \textit{mtrR}120 mutation was of interest because it was positioned downstream of the MtrR binding site (Hoffmann \textit{et al.}, 2005, Lucas \textit{et al.}, 1997). We therefore sought to understand how this mutation increases gonococcal resistance to antimicrobial substances. The transcriptional profile of \textit{DW120} showed no difference in the level of \textit{mtrR} or \textit{mtrA} compared to the wild type strain, which is consistent with the mutation increasing levels of resistance in an MtrA- and MtrR- independent manner. To further test whether the \textit{mtrR}120 mutation requires MtrR (repressor of \textit{mtrCDE}) or MtrA (activator of \textit{mtrCDE}) for its activity, we transformed the \textit{mtrR}120 mutation into strains JF1 and JF3, which are \textit{mtrR} and \textit{mtrA} mutants of strain \textit{FA19SmA3}, respectively. The resultant double mutants \textit{DW120A} (\textit{mtrR}120, \textit{mtrA}\textasteriskalign{\textit{aphA}3}) and JF1\textsubscript{mtr120} (\textit{mtrR}120, \textit{AmtrR}) were tested for sensitivity to Em, Az, and TX-100. \textit{DW120A} and JF1\textsubscript{mtr120} bacteria showed no difference in the MIC of TX-100 or Em compared to \textit{DW120} (Table 2). From these results, we conclude that the basis of the increased resistance conveyed by the \textit{mtrR}120 mutation is MtrR- and MtrA-independent. Moreover, the \textit{mtrA} locus in strain MS11 is identical to that of strain FA19 based on nucleotide sequence analysis (data not shown); this finding further confirms that the resistance phenotype exhibited by wild type MS11 bacteria is not due to differences in the \textit{mtrA} promoter or structural gene.

Although the \textit{mtrR}120 mutation acted independently of MtrR and MtrA, it does impact \textit{mtrCDE} transcription (Fig. 3) and causes increased production of MtrE protein (Fig. 2). The \textit{mtrR}120 mutation is located downstream of the MtrR-binding site and the \textit{mtrCDE} promoter and is within the 5′ untranslated region of the \textit{mtrC} RNA. The position of this mutation coupled with the detection of high levels of \textit{mtrC} transcript in this strain in the absence of changes in the expression of known regulators of the \textit{mtrCDE} operon suggested that the mutation impacts \textit{mtrC} mRNA transcript stability. Indeed, a similar mutation in the 5′ untranslated region of an efflux pump gene was described for the NorB efflux pump of \textit{Staphylococcus aureus}, which led to an increase in \textit{norA} mRNA half-life (Fournier \textit{et al.}, 2001). Therefore, we tested whether there was a difference in the \textit{mtrC} mRNA half-life between mutant \textit{DW120} and the parent strain \textit{FA19}. We found a rapid decay in the \textit{mtrC} transcript in wild type bacteria but not in \textit{DW120} bacteria compared to that of the constitutively expressed gene \textit{rmp} (Fig. 4). This result suggests the \textit{mtrR}120 mutation alters the structure of the \textit{mtrC} transcript or its interaction with either RNA degradation factors or the ribosome to allow for a longer mRNA half-life.

**Gradients of resistance to antimicrobial peptides and progesterone**

Loss of MtrR production due to an \textit{mtrR} null mutation is known to increase both levels of the \textit{mtrCDE} transcript and the \textit{in vivo} fitness of gonococci in a murine vaginal infection model (Warner \textit{et al.}, 2007). While there are various potential explanations for the \textit{in vivo} fitness advantage conveyed by increased production of the MtrC-MtrD-MtrE efflux pump, we chose to examine resistance levels to progesterone and CRAMP-38, the murine homologue of the human cathelicidin LL-37 (Nizet \textit{et al.}, 2001) as two host-derived substrates that may challenge \textit{N. gonorrhoeae} \textit{in vivo}. We found mutants DW39, DW120,
and KH15 to be significantly more resistant to progesterone than the wild type strain, based on the average percentage of gonococci recovered on GC agar with progesterone versus GC agar alone from three independent experiments (Fig. 5). Consistent with the intragenic mutations conferring a higher level of antimicrobial resistance (Table 2), the percent of DW120 and KH15 bacteria recovered on agar with progesterone was consistently higher than that of DW39 bacteria, although not statistically significant, most likely due to variability in the assay. Similarly, a statistically insignificant but reproducibly higher percentage of FA19SM11mtr bacteria was recovered on agar with progesterone compared to wild type FA19 bacteria; this observation is consistent with the mtr locus of MS11 conferring increased resistance to this substrate and with the lower production of MtrE in this strain compared to strain DW120 as shown above.

Differences were also found in the sensitivity of the mtr mutants to the cathelicidin CRAMP-38, which is known to possess anti-gonococcal activity in vivo. All mtr mutant bacteria, with the exception of strain DW9, were consistently more resistant to CRAMP-38 compared to wild type FA19SmR gonococci (Fig. 6A). Consistent with the gradient of resistance as defined by Em resistance, the highly Em resistant mutants KH15 and DW120 were more resistant to CRAMP-38 when compared to intermediate and low level Em resistant strains. We also found that wild type strain MS11 was more resistant to both the human cathelicidin LL-37 and CRAMP-38 than strain FA19 (Fig. 6B). Consistent with the mtr locus of strain MS11 conferring increased resistance to these antimicrobial peptides, transformation of the MS11 mtr region into strain FA19 markedly increased the resistance of FA19 to CRAMP- and LL-37, and resistance to the peptides in strain MS11 was negated by the disruption of the MS11 mtrE gene.

**In vivo fitness levels correspond to antimicrobial resistance levels**

We recently reported that the mtrR deletion mutant JF1 was more fit than the wild type parent bacteria in the lower genital tract of female mice (Warner et al., 2007). JF1 bacteria exhibit a low resistance phenotype in all assays tested here. To better define the resistance gradient as it relates to in vivo fitness, here we tested the fitness of mutants that represent high (KH15 and DW120), intermediate (DW39), and low (DW9) classes of antibiotic resistant strains relative to the wild type parent strain. Competitive infections were performed in which similar numbers of a CmR-marked derivative of wild type strain FA19 and each of the mtr mutant strains were inoculated intravaginally into BALB/c mice. All four mutants demonstrated a fitness advantage over the strain FA19CmR within one day post-inoculation, as defined by CI values greater than 1.0 (Fig. 7B-E). Co-culture of each mutant with strain FA19CmR in GC broth (in vitro competition assays) showed that there were no growth or survival advantages in vitro (Fig. 7A). The mere presence of fitness advantages was not unexpected 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, also conferred an advantage over the wild type strain. We also observed a fitness gradient that paralleled the differences in antibiotic resistance. Strain DW9 (E202G change in MtrR), a mutant that showed a 2-fold increase in resistance to Em, TX-100 and Az, displayed mean CIs of 5 and 12 on days 1 and 3, respectively (Fig. 7B). Mutant DW39 (A39T change in MtrR) bacteria, which showed an intermediate level of resistance to Em and Az displayed a stronger advantage over FA19CmR gonococci, with CIs of 100 and 150 on days 1 and 3, respectively (Fig. 7C). Importantly, the intergenic mutations in strains KH15 and DW120, which confer the highest increases in MIC (16-fold for Az and Em, and >250-fold for TX-100), conferred the strongest fitness advantages. Mean CI values for mutant KH15 bacteria were 195 and 509 on days 1 and 3 (Fig. 7D), with 2 of 4 mice clearing FA19CmR bacteria by day 3, and 3 of 4 mice by day 5 (open circles, Fig. 7D). Similarly, strain DW120 displayed mean CI
values of 48 and 455 on days 1 and 3 (Fig. 7E), with DW120 bacteria but no FA19CmR gonococci recovered from 1 of 4 mice on day 3, and from 0 of 2 mice on day 5 (open circles, Fig. 7E). In summary, these experiments show that the levels of mtrCDE derepression as measured by antibiotic resistance are mirrored by fitness advantages in vivo.

A more sensitive method of detecting a fitness gradient is to perform competitive infections between two mtr mutant strains. Thus, we compared the commonly described and highly resistant promoter mutation found in strain KH15 against the structural gene mutations carried by strains DW39, DW45, and DW9. Strains DW39 and DW45 contain HTH mutations that are common in clinical isolates. Strain DW9, which was isolated as a spontaneous mutant from experimentally infected mice contains a single missense mutation at the 3′-end of the mtrR coding sequence and exhibits a clearly “low” resistance phenotype. To facilitate the testing of these mixtures, we marked strains DW39, DW45, and DW9 with a CmR gene as described in the Experimental Procedures. Mixtures of KH15 and each of the CmR-marked mtrR mutant bacteria were inoculated into mice and the relative recovery of each CmR mutant relative to KH15 bacteria was followed over 6 days. Our hypothesis was that the KH15 mutant would out-compete the other three mutants, but in a step-wise manner with DW9CmR bacteria displaying the most disadvantaged phenotype, followed by DW45CmR and lastly DW39CmR gonococci.

There were no differences in the growth and recovery of each mutant strain versus KH15 bacteria in vitro (Fig. 8A). Mutants DW9CmR and DW45CmR were greatly out-competed by KH15 gonococci, with CI values around 1000 on day 2 (Fig. 8A and 8B). Additionally, high numbers of KH15, but no DW9CmR bacteria were recovered from 3 of 5 mice (day 2) and 3 of 4 mice (day 4) inoculated with a mixture of KH15 and DW9CmR bacteria (open circles, Fig. 8B). Similarly, DW45CmR bacteria were not recovered from 2 of 4 mice on days 2 and 4, and only KH15 was recovered from the two mice that were culture positive on day 6 (open circles, Fig. 8C). Mutant DW39CmR was also less fit than KH15 gonococci; however, as predicted, CI values were markedly lower than that calculated for the other two mutants with a mean value of 12 by day 2 (Fig. 8D). These results confirm our conclusion that the commonly isolated –T promoter mutation carried by mutant KH15 provides a greater in vivo fitness advantage to N. gonorrhoeae than MtrR structural mutations. These data are also consistent with the A39T mutation that is carried by strain DW39 as having an intermediate fitness advantage as predicted from the MIC data.

Discussion

Since the discovery of the MtrR repressor by Pan & Spratt (1994) and the realization that it controls mtrCDE expression in N. gonorrhoeae, several studies have examined the impact of mtr mutations on gene expression and antibiotic resistance endowed by the MtrC-MtrD-MtrE efflux pump. Presently, investigations of antibiotic resistance outbreaks now include this locus among others in screens for basic resistance determinants. Curiously, in such studies certain mtr mutations are repeatedly isolated. These mutations include missense mutations that cause radical amino acid changes (e.g., A39T or G45D) in the MtrR DNA-binding domain or mutations that impact the C-terminal region of MtrR [e.g., H105Y and the E202G mutation that we isolated from experimentally infected mice (Warner et al., 2007)] (Dewi et al., 2004, Herida et al., 2004, Lundback et al., 2006, Mavroidi et al., 2001, McLean et al., 2004, Shafer et al., 1995, Sutrisna et al., 2006, Tanaka et al., 2006). In general, these coding sequence mutations elevate gonococcal resistance to structurally diverse hydrophobic antimicrobials by 2- to 4-fold. In contrast, promoter mutations are also frequently isolated in the absence of coding sequence mutations, and some enhance antimicrobial resistance by ≥ 10 fold. These promoter mutations consist of a single bp deletion or a dinucleotide insertion in a 13 bp inverted repeat sequence located within the mtrR promoter. These promoter
mutations change the spacing between the -10 and -35 hexamers of the \textit{mtrR} promoter from 17 to 16 or 19 nucleotides and these changes are sufficient to abrogate \textit{mtrR} transcription (Hagman \textit{et al.}, 1995b, Zarantonelli \textit{et al.}, 1999, Zarantonelli \textit{et al.}, 2001). Moreover, because the divergent \textit{mtrR} and \textit{mtrCDE} promoters partially overlap at the -35 regions, the \textit{mtrCDE} promoter is better recognized by RNA polymerase. The +/-T promoter mutations in particular are frequently isolated and known to cause a greater derepression state of the \textit{mtrCDE} operon than structural \textit{mtrR} mutants (Hagman \textit{et al.}, 1995b, Shafer \textit{et al.}, 1995).

The goal of the study presented here was to compare the effect of commonly isolated \textit{mtrR} locus mutations and a novel mutation, \textit{mtr}$_{120}$, which we discovered in the laboratory strain MS11 for levels of antibiotic resistance and \textit{in vivo} fitness. We also tested the relative susceptibility of the mutants to host factors that might challenge the gonococcus during infection. We found that the intergenic mutations conferred the highest level of resistance to antibiotic substrates of the pump and to progesterone and CRAMP-38, and that in general, \textit{in vitro} resistance phenotypes were predictive of \textit{in vivo} fitness advantages. Interestingly, mutations within the \textit{mtrR} structural gene did not fall into one category, but in fact conferred different levels of susceptibility. Among the structural gene mutations, the A39T mutation consistently stood out as having higher \textit{in vitro} resistances compared to the other three \textit{mtrR} structural gene mutations. Gonococci that expressed the A39T mutation also competed favorably \textit{in vivo} with the most fit derepressed mutant, KH15. In contrast, the E202G mutation conferred the lowest levels of \textit{in vitro} resistance and \textit{in vivo} fitness. While the crystal structure for MtrR has not yet been determined, it is relevant to note that the A39T mutation is within the first helical domain of the predicted HTH while the G45D mutation is positioned in the second helical domain. It may be that the former mutation has a more significant impact on MtrR-binding to its target site upstream of \textit{mtrC}. We also confirmed that the \textit{mtrR} deletion mutant, JF1, is less derepressed for \textit{in vitro} phenotypes than the promoter mutations as reported by Hagman and Shafer (Hagman \textit{et al.}, 1995b). The \textit{mtrR} deletion in JF1 was defined in Hagman \textit{et al.} (Hagman \textit{et al.}, 1995a) and Folster \textit{et al.} (Folster \textit{et al.}, 2007a), and represents a >90% deletion of the \textit{mtrR} coding sequence. It is important to note that mutant JF1 has a wild type \textit{mtrR} promoter that will more effectively compete with the \textit{mtrCDE} promoter for RNA polymerase binding than the promoter in mutant KH15 to cause a lower level of \textit{mtrCDE} transcription than that observed for KH15 (Hagman \textit{et al.}, 1995b).

Analysis of \textit{mtrC} transcript levels provided further evidence of a differential level of \textit{mtrCDE} expression, and in general the levels of \textit{mtrC} transcript mimicked that of the MIC values. Our results confirm the previously reported high levels of \textit{mtrC} transcription and the absence of an \textit{mtrR} transcript in strain KH15 (Hagman \textit{et al.}, 1995b), however, examination of strains DW39, DW45, DW9, and DW120 provides new insights into the mechanisms of derepression of the \textit{mtrCDE} operon. Thus, as suggested above, our results 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 acid residue at position 202 plays a minor role in the ability of the repressor to regulate \textit{mtrCDE} expression, 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 (Ramos \textit{et al.}, 2005)), including QacR (Schumacher \textit{et al.}, 2001, Schumacher \textit{et al.}, 2002).

The \textit{mtr}$_{120}$ mutation yields one of the highest reported levels of \textit{mtr}-based resistance. While the \textit{mtr}$_{120}$ mutation has not yet been detected among clinical isolates apart from strain MS11, it is important to note that in other efflux pump systems, such as the \textit{nor} system in \textit{Staphylococcus aureus}, similar mutations in the 5' untranslated region yielded derepressed
operon transcription (Fournier et al., 2001). Here we present genetic evidence and RT-PCR results to show that the \( mtr_{120} \) mutation is independent of both MtrR and MtrA. Additionally, we showed that the \( mtr_{120} \) 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 \( mtr_{120} \) 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.

The impact of the \( mtr_{120} \) mutation on expression of the \( mtrCDE \)-encoded efflux pump and levels of resistance and in vivo fitness is of particular interest because it was identified in strain MS11, which has been used extensively in gonococcal research. In particular, MS11 has served as a test strain for understanding mechanisms of pilus variation (Haas et al., 1987, Jonsson et al., 1994, Koomey et al., 1991, Seifert et al., 1988, Swanson et al., 1987a) and transformation (Aas et al., 2002, Chaussee et al., 1998, Hamilton et al., 2005), the kinetics of phase and antigenic variation of surface molecules during experimental urethral infection of male volunteers (Schneider et al., 1991, Swanson et al., 1988, Swanson et al., 1987), and the host response to experimental urethral infection (Ramsey et al., 1995, Schmidt et al., 2001). Strain MS11 was originally isolated from the cervix of an uncomplicated genital tract infection (Swanson et al., 1988). It now is evident that this strain over-expresses the MtrC-MtrD-MtrE efflux pump system. How derepression of this efflux pump system impacts the biologic activity of strain MS11 is of considerable interest. Interestingly, MS11 bacteria are more infectious than strain FA1090 bacteria based on the ID\(_{50}\) of these strains in the male volunteer urethritis model (Cohen et al., 1994, Schmidt et al., 2001). 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 infectious dose of strain FA19 for mice (Warner et al., 2007). However, the results shown here contribute to further understanding and characterization of strain MS11 and emphasize the need to examine how the \( mtr_{120} \) mutation impacts the ability of this strain to cause disease. We note here that repeated attempts to transform the FA19 \( mtr \) locus into strain MS11 were unsuccessful, which suggests the \( mtr_{120} \) mutation (and possibly the A39T mutation) may have been selected in this laboratory strain to compensate for a separate mutation that negatively affects its fitness in vitro or in vivo.

An additional objective of this study was the investigation of how these \( mtr \) mutations affect fitness. We previously reported that the MtrC-MtrD-MtrE pump is critical for experimental murine genital tract infection (Jerse et al., 2003) and that mutant JF1 (\( \Delta mtrR \)) 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. Strain KH15, which bears a common promoter mutation, strongly out-competed all other \( mtr \) mutants tested by 1,000-fold except for gonococci that bear the A39T mutation, which showed only a 12-fold reduction in fitness compared to KH15 bacteria. Such a hierarchy in fitness could allow selection for more efficient pathogens in cases of mixed infections with more than one \( N. gonorrhoeae \) strain, which occur frequently (Lynn et al., 2005). It seems reasonable to also hypothesize that the increased fitness afforded to \( mtr \) mutants could allow for the accumulation of other mutations that compensate for detrimental effects that may occur due to changes in the expression of \( mtrCDE \) and other MtrR-regulated genes (Folster et al., 2007b). Modulation of \( mtrCDE \) expression appears to be the main reason the \( mtrR \) mutant JF1 was more fit in the mouse model based on competitive infection experiments between an \( mtrR, mtrE \) double mutant and an \( mtrE \) mutant (Warner et al., 2007). However, MtrR can transcriptionally activate and repress over 70 genes, directly
or indirectly, and some of these gene products may be of importance (Folster et al., unpublished).

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 (Rouquette-Loughlin et al., 2003, Bina et al., 2001, Nishino et al., 2006, Stone et al., 1995). 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-38 may explain in part the basis for the observed fitness advantage in mice. The mutants also showed increased resistance to progesterone (Jerse et al., 2003); 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. It is also unlikely that exposure to high levels of estradiol used in the mouse model gives mtrR locus mutants an advantage, since estradiol does not kill N. gonorrhoeae, even when tested at levels that are greater than 1000-fold higher than normal serum levels and 100-fold higher than serum levels in mice treated with 17-β-estradiol slow-release pellets. Estradiol is also not a substrate of the MtrC-MtrD-MtrE efflux pump system (Jerse et al., 2003). Our demonstration that mtrR locus 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, and 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 azithromycin resistance is equally important as the fitness gradient detected in vivo, since azithromycin is increasingly utilized as a therapy for non-complicated urethritis (McNabb et al., 2007).

**Experimental Procedures**

**Bacterial Strains and Culture Conditions**

*Neisseria gonorrhoeae* strain FA19 SmR is a spontaneous streptomycin resistant mutant of strain FA19 (porB1A, serum resistant) and was described previously (Jerse et al., 2003). Strain FA19 CmR and mtrR mutants JF1, DW9, 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 FA19SmR and are described in Table 1. Mutant mtr sequences were PCR-amplified from strains LG5 or LG7 (Garvin et al., 2008), or strain MS11 with primers R-Xho and C-Xho as described (Warner et al., 2007), and used to transform strain FA19SmR following gel purification. Transformants were isolated on GC agar supplemented with Em (0.5 μg/ml). pGCC-5 (provided by H.S. Seifert, Northwestern University) was used to mark strains FA19 SmR, DW9, DW39, and DW45 by insertion of a cat gene into an untranscribed region between the lctP and aspC chromosomal genes as described (Simms et al., 2006). The resultant strains were FA19CmR DW9CmR, DW39CmR, and DW45CmR, respectively. mtrA mutant DW120A was created by PCR amplification of the mutant mtrA::aphA3 allele from strain JF3 using primers mtrAfor and mtrArev (Warner et al., 2007). The resultant 1900-bp product was transformed into strains DW120, and transformants were selected on GC agar supplemented with kanamycin (Km). To construct mutant JF1.120, which contains both a ΔmtrR and the mtr120 mutation, the PCR-amplified mtr locus from strain JF1 was used to transform strain DW120. All transformations were performed as described (Gunn et al., 1996). The nucleotide sequences of the mtr regions of strains DW9, DW39, DW45, MS11, DW120, and FA19MS11mtr 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 bacteria were cultured on supplemented GC agar (Difco) as described (Wu et al., 2006). All antibiotics were from Sigma and were used to supplement selective media at the following concentrations: Cm, 0.6 μg/ml; Em, 0.5 μg/ml; and Km, 50 μg/ml.

Resistance to MtrC-MtrD-MtrE pump substrates

The minimum inhibitory concentration (MIC) of Triton X-100, Em, Az, and Km against wild type parent strain FA19 SmR and the mtr mutants was established using a standard two-fold agar dilution assay (Warner et al., 2007). Sensitivity to a single concentration of water-soluble progesterone (35 μg/ml) (Sigma) was tested using a plating efficiency assay as described (Jerse et al., 2003). Susceptibility of FA19SmR and mtr mutant bacteria in this background to CRAMP-38 was determined by incubating 10⁶ CFU of each strain suspended in PBS alone or with PBS containing CRAMP-38 (4 μg/ml) in a total volume of 100 μl. The mixtures were incubated at 37°C in a CO₂ incubator for 45 min before serial dilution and culture on GC agar. For studies with FA19SmR versus wild type MS11 bacteria and the recombinant strains FA19MS11mtr and DW3MS11, 10⁴ CFU were exposed to 16 μg/ml of CRAMP-38 or LL-37 in minimal essential media (total volume 80 μl). GCB (40μl) was added after 55 min incubation at 37°C, and the suspensions were quantitatively cultured on GC agar. The number of CFU recovered from wells with peptide was divided by the number of CFU recovered from wells without peptide and the results were multiplied by 100. All assays were performed in triplicate and repeated at least once to test reproducibility. Peptides LL-37 and CRAMP-38 were synthesized and purified at the Microchemical Facility of Emory University as described (Shafer et al., 1998).

Western Blot Analysis

Outer membrane proteins were fractionated on a 12% SDS/PAGE gel, transferred to polyvinylidene difluoride (PVDF) 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.

Measurement of Transcript Levels

We used real time quantitative reverse transcriptase PCR (qRT-PCR) to measure levels of specific transcripts produced by gonococcal strains. Gonococcal strains were grown to early log phase (OD₆₀₀ = 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). RNA (100 ng) from each sample were 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 dithiothreitol, 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). All primers are described in Table 3. Relative levels of transcript for 16S rRNA, rmp, mtrC, mtrR, and mtrA were determined by the relative method of comparison for each of the mutants to the wild type strain. 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 DW120 were determined by qRT-PCR. Broth cultures were grown to early log phase (OD= 0.2), treated with rifampicin (200 μg/ml), and sampled at times 0, 2, 4, 8, 12, and 20 minutes post-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 spectrophotometric 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 3) 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. cDNA was also used in a standard PCR reaction using Taq DNA polymerase, followed by separation and visualization on an agarose gel, to visualize the decay of *rmp* and *mtrC* message from strains FA19 and DW120. The experiment was performed twice using different biological samples.

**Experimental Murine Infection**

Female BALB/c mice were treated with 17-β-estradiol and antibiotics to promote long term infection (Jerse, 1999). 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<sup>R</sup> cassette. Bacterial suspensions were prepared as described (Wu et al., 2006), adjusted to an OD<sub>600</sub> of 0.07 (~1 × 10<sup>6</sup> CFU/10 μl), and 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 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 as described (Unsworth et al., 2000) in which a ratio of (*mtrR* mutant / wild type)<sub>output</sub> / (*mtrR* / wild type)<sub>input</sub> was calculated. A CI >1.0 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 tested in *in vitro* competition assays as described (Warner et al., 2007). For infections in which Cm<sup>R</sup> wild type bacteria were tested, randomly selected colonies were screened on GC media 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.

**Acknowledgments**

This work was supported by the National Institutes of Health grants RO1-AI42053 and STI-TM-CRC grant U19 AI31496 (A.E.J.), and RO1-AI062755 (W.M.S.). W.M.S. was supported in-part by a Senior Research Career Scientist Award from the VA Medical Research Service. We thank Afrin Begum for technical assistance and Lotisha Garvin for technical assistance and help with preparation of the manuscript.
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Figure 1. Location of mtr locus mutations used in this study

A schematic of the *N. gonorrhoeae* mtr locus is shown. The α-helix-encoded region (HTH) of MtrR used for DNA binding is indicated by the hatched pattern. This region is the location of the mutations found in strains DW39, DW45, 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 mtrR and mtrCDE transcriptional start sites are indicated by the arrows and are within the intergenic region that contains the MtR binding site and the 13 bp inverted repeat, which is the region where the mutation in strain KH15 is found (Hagman *et al.*, 1995b). The mtr120 mutation occurs further upstream of the DNA binding region and is present in strains MS11 and DW120. This mutation has not been described previously, and the G to A change is shown in the detailed DNA sequence.
Figure 2. Expression of MtrE by wild type and mtr 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. The intensity of the MtrE band relative to strain FA19 and after normalization to porin is reported for each strain below the blot. Strain DW11, not described here, carries a frame-shift mutation in the mtrR gene, that is predicted to cause a 52 kD truncated MtrR protein (Warner et al., 2007).
Figure 3. 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 FA19 bacteria. 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 described previously (Hagman et al., 1995b, Shafer et al., 1995); 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 FA19 bacteria. Levels of mtrC expression were compared using a students t-test to evaluate differences between DW39, DW120, FA19 MS11mtr, and KH15 gonococci, which exhibit high levels of mtrC expression, and JF1 bacteria, which demonstrate a low-level increase in MIC and mtrC mRNA. ** Denotes p<0.001. The increase in mtrR transcription shown for strain DW120 was 1.7-fold greater than that of strain FA19, but not statistically significant when the averages from three experiments were compared.
Figure 4. Assessment of RNA decay in strain DW120
(A) Equal quantities of RNA from strain FA19 (top) and DW120 (bottom) were reverse transcribed and used in a PCR reaction to determine if there was a difference in the degradation rate of mtrC message. Samples were taken pre (T0) and post rifampicin treatment (+Rif); a second set of cultures to which no rifampicin was added (-Rif) were grown in parallel. (B) RNA samples were also reverse-transcribed and used in a qRT-PCR protocol to quantify amounts of mtrC and rmp RNA from strains FA19 and DW120. These values were used to calculate the RNA half life for mtrC and rmp. The experiment was performed twice and the results were similar.
Figure 5. Progesterone resistance
Suspensions of wild type and mutant gonococci were quantitatively cultured on GC agar plates supplemented with progesterone (35 μg/ml) or without progesterone and incubated overnight. Results are expressed as 100 × (# CFU on GC agar with progesterone divided by # CFU on GC alone). The average % recovery calculated from three independent experiments is shown; bars represent the standard error. Asterisks indicate a significant difference between DW39 (p < 0.05) or DW120 and KH15 (p < 0.01) (unpaired t test). The p value for FA19 MS11mtr versus wild type FA19 was 0.068.
Figure 6. Resistance to antimicrobial peptides

Bacteria were incubated with LL-37, CRAMP, or no peptides and then quantitatively cultured on GC agar. Results are expressed as the percentage of bacteria recovered from wells without peptide, and the graphs shown are representative of one of two or three experiments. Panels A shows the percent recovery of strain FA19 and mtr mutants incubated in CRAMP-38 (4 μg/ml); panel B shows the percent recovery of FA19SmR, FA19MS11mtr, MS11, and DW3MS11 after incubation in LL-37 or CRAMP-38 (16 μg/ml).
Figure 7. Differential *in vivo* fitness of *mtr* mutants compared to the wild type strain

Defined ratios of FA19 CmR and *mtr* mutant bacteria were inoculated into GC broth or estradiol-treated mice to assess the relative fitness of each strain under *in vitro* and *in vivo* conditions. The ratio of strains in each inoculum was used in the competitive index (CI) equation as defined in Experimental Procedures. (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) DW120 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 8. *In vivo* competition of *mtr* mutants

Defined ratios of mutant KH15 bacteria and CmR-marked *mtr* mutants were inoculated into GC broth or estradiol-treated mice to compare the relative fitness under *in vitro* and *in vivo* conditions. The ratio of strains in the inoculum was used in the competitive index (CI) equation defined below. (A) Mixed suspensions were cultured in GC broth (*in vitro* competition); 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) DW9CmR (C) DW45CmR and (D) DW39CmR 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 was defined as: (KH15/*mtrRCmR*<sub>output</sub>)/(KH15/*mtrRCmR*<sub>input</sub>), where *mtrRCmR* corresponds to strains DW9CmR, DW45CmR, or DW39CmR.
Table 1

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<td>DW39CmR</td>
<td>mtrR(_{A39T}) and cat gene inserted between aspC and lctP loci</td>
<td>This study</td>
</tr>
<tr>
<td>DW45CmR</td>
<td>mtrR(_{G45D}) and cat gene inserted between aspC and lctP loci</td>
<td>This study</td>
</tr>
<tr>
<td>DW9CmR</td>
<td>mtrR(_{E202G}) and cat gene inserted between aspC and lctP loci</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^1\) All recombinant strains were constructed strain FA19Sm\(^R\) with the exception of DW3MS11, which was made in strain MS11.
Table 2
Sensitivity to TX-100 and antibiotic substrates of the MtrC-MtrD-MtrE efflux system

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Em (μg/ml)</th>
<th>Az (μg/ml)</th>
<th>TX-100 (μg/ml)</th>
<th>Km (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA19 SmR</td>
<td>wild type</td>
<td>0.5</td>
<td>0.125</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>DW9</td>
<td>FA19 mtrR&lt;sub&gt;E202G&lt;/sub&gt;</td>
<td>1</td>
<td>0.25</td>
<td>125</td>
<td>30</td>
</tr>
<tr>
<td>JF1</td>
<td>FA19 ΔmtrR</td>
<td>1</td>
<td>0.25</td>
<td>250</td>
<td>30</td>
</tr>
<tr>
<td>DW45</td>
<td>FA19 mtrR&lt;sub&gt;G45D&lt;/sub&gt;</td>
<td>1</td>
<td>0.25</td>
<td>250</td>
<td>30</td>
</tr>
<tr>
<td>DW39</td>
<td>FA19 mtrR&lt;sub&gt;A39T&lt;/sub&gt;</td>
<td>2</td>
<td>0.5</td>
<td>500</td>
<td>30</td>
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<tr>
<td>FA19&lt;sub&gt;MS1&lt;sub&gt;1&lt;/sub&gt;&lt;/sub&gt;</td>
<td>FA19 mtr&lt;sub&gt;120&lt;/sub&gt;, mtrR&lt;sub&gt;A39T&lt;/sub&gt;</td>
<td>8</td>
<td>0.5</td>
<td>8000</td>
<td>30</td>
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<tr>
<td>DW120</td>
<td>FA19 mtr&lt;sub&gt;120&lt;/sub&gt;</td>
<td>8</td>
<td>1</td>
<td>&gt;16000</td>
<td>30</td>
</tr>
<tr>
<td>KH15</td>
<td>FA19 mtrR&lt;sub&gt;79&lt;/sub&gt;</td>
<td>8</td>
<td>1</td>
<td>&gt;16000</td>
<td>30</td>
</tr>
<tr>
<td>MS11</td>
<td>wild type; natural mtr&lt;sub&gt;120&lt;/sub&gt;, mtrR&lt;sub&gt;A39T&lt;/sub&gt; mutant</td>
<td>8</td>
<td>1</td>
<td>&gt;16000</td>
<td>30</td>
</tr>
<tr>
<td>DW3&lt;sub&gt;MS11&lt;/sub&gt;</td>
<td>MS11 mtr&lt;sub&gt;120&lt;/sub&gt;, mtrR&lt;sub&gt;A39T&lt;/sub&gt;, mtrE&lt;sub&gt;Cm&lt;/sub&gt;</td>
<td>0.04</td>
<td>ND</td>
<td>0.24</td>
<td>30</td>
</tr>
<tr>
<td>DW3</td>
<td>FA19 mtrE&lt;sub&gt;Cm&lt;/sub&gt;</td>
<td>0.04</td>
<td>ND</td>
<td>0.24</td>
<td>30</td>
</tr>
<tr>
<td>DW120A</td>
<td>FA19 mtr&lt;sub&gt;120&lt;/sub&gt;, mtrA&lt;sub&gt;CphA-3&lt;/sub&gt;</td>
<td>8</td>
<td>1</td>
<td>&gt;16000</td>
<td>60</td>
</tr>
<tr>
<td>JF1&lt;sub&gt;mtr&lt;sub&gt;20&lt;/sub&gt;&lt;/sub&gt;</td>
<td>FA19 mtr&lt;sub&gt;130&lt;/sub&gt;, mtrR&lt;sub&gt;A39T&lt;/sub&gt;, ΔmtrR</td>
<td>8</td>
<td>1</td>
<td>&gt;16000</td>
<td>30</td>
</tr>
</tbody>
</table>

ND, not determined
Table 3
Oligonucleotide primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>16S-for</td>
<td>GCGTGGGTAGCAAACAGGAT</td>
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<tr>
<td>16S-rev</td>
<td>CGCGTTAGCTACGCTACCAAG</td>
</tr>
<tr>
<td>Rmp-forRT</td>
<td>CAAACAACCTGGTCAGCAAC</td>
</tr>
<tr>
<td>Rmp-revRT</td>
<td>TCGGCTTACAAACTTGGAG</td>
</tr>
<tr>
<td>mtrC-forRT</td>
<td>CCGCTTTACCCGCTTTGCTTC</td>
</tr>
<tr>
<td>mtrC-revRT</td>
<td>CGTTACAAACCGCTTTGTTTC</td>
</tr>
<tr>
<td>mtrA-forRT</td>
<td>GTGTTTTCAATGCTGCAACT</td>
</tr>
<tr>
<td>mtrA-revRT</td>
<td>AGGATAAGCACCAGCAGGAC</td>
</tr>
<tr>
<td>mtrR-forRT</td>
<td>AAAATTACCCGTTTTGAC</td>
</tr>
<tr>
<td>mtrR-revRT</td>
<td>CCAACGTCGATTTGATGAAG</td>
</tr>
</tbody>
</table>