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Clinically Relevant Antibiotic Resistance Mechanisms Can Enhance the *In Vivo* Fitness of *Neisseria gonorrhoeae*

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1. Introduction

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

An important question regarding antibiotic resistance is whether a particular resistance mechanism has a fitness cost for the bacterium (Andersson & Levin, 1999; Andersson & Hughes, 2010), especially in the community where it competes with its antibiotic sensitive brethren. A fitness cost is typically experimentally measured as a deleterious change in bacterial growth rate in laboratory media or survival in experimental infection in the absence of antibiotic pressure. Fitness costs (or benefits) are best viewed during co-cultivation of isogenic strains that differ only in the resistance mechanism under study. For certain antibiotic resistance mechanisms, a significant fitness cost can be incurred. This general observation led to the idea that removal of the selective pressure imposed by the

antibiotic in question would favor sensitive strains to predominate in the community and allow for the return of the antibiotic in question to treat the infection in question. By and large, this has proven not be the case (Andersson and Hughes, 2010). There are many reasons for this, including the unintentional selective pressure exerted by the widespread availability and use of antibiotics to treat bacterial infections in general, over-the-counter antimicrobials that confer selective pressure and provide cross-resistance (or decreased susceptibility) to the antibiotic in question, and host-derived antimicrobials that select for the particular resistance determinant. In addition to antimicrobial pressures, it has been repeatedly documented that compensatory, second site mutations can develop that reverse fitness costs while maintaining resistance (Schrag et al., 1997; Marcusson et al., 2009; Andersson and Hughes, 2010).

More recently, a new view has been taken regarding antibiotic resistance and fitness: some resistance systems actually provide the resistant strain with a fitness advantage over wild-type strains or can reverse a fitness burden imposed by a separate mutation that also participates in resistance to a particular antibiotic. Evidence for enhanced fitness of bacterial pathogens, in laboratory media or in experimental infection models, due to mutations or gene acquisition events that increase resistance to antibiotics has been provided for *Campylobacter jejuni* (Luo et al., 2005) and *Neisseria gonorrhoeae* (Warner et al., 2007, 2008). The idea that an antibiotic resistance mechanism could have negligible and even beneficial effects on fitness could help to explain, in part, why resistant strains persist in the community long after the antibiotic has been removed from the treatment regimen. For instance, gonococci expressing resistance to penicillin, tetracycline and/or fluoroquinolones have persisted in the community despite the removal of these antibiotics from the recommended gonorrhea treatment regimen for several years. Against this background, we herein review data and provide models as to how two mechanisms of antibiotic resistance expressed by *N. gonorrhoeae* can enhance fitness *in vivo*. The *in vivo* system employed in these studies is a female mouse model of lower genital tract infection that recapitulates many features of infection in human females, most notably the development of inflammation that occurs during cervicitis (Jerse, 1999; Packiam et al., 2010; Song et al., 2008). The two resistance mechanisms discussed below are multi-drug efflux by the MtrC-MtrD-MtrE pump (Hagman et al., 1995; Jerse et al., 2003) and quinolone resistance that develops due to point mutations in *gyrA* and *parC*. We discuss concepts regarding the evolution of antibiotic resistance expressed by gonococci in the context of how these resistance mechanisms may have endowed this strict human pathogen with a fitness advantage during infection.

2. Antimicrobial efflux and gonococcal fitness

The MtrC-MtrD-MtrE efflux pump of *N. gonorrhoeae* is a resistance-nodulation-division (RND) efflux pump family member that recognizes a diverse array of hydrophobic antimicrobial agents and exports these toxic compounds out of the gonococcal cell (Hagman et al., 1995). The *mtrCDE* operon is composed of three structural genes that encode the core proteins of the efflux pump: *mtrC*, which encodes a periplasmic membrane fusion protein; *mtrD*, encoding an energy-dependent inner membrane transporter; and *mtrE*, which encodes a TolC-like outer membrane channel protein (Delahay et al., 1997; Hagman et al., 1995; Hagman et al., 1997). In addition to these core efflux proteins, an accessory protein

termed MtrF is required for high-level resistance to substrates of the pump and its gene (*mtrF*) is also located within the *mtr* locus (Figure 1) (Veal & Shafer, 2003).

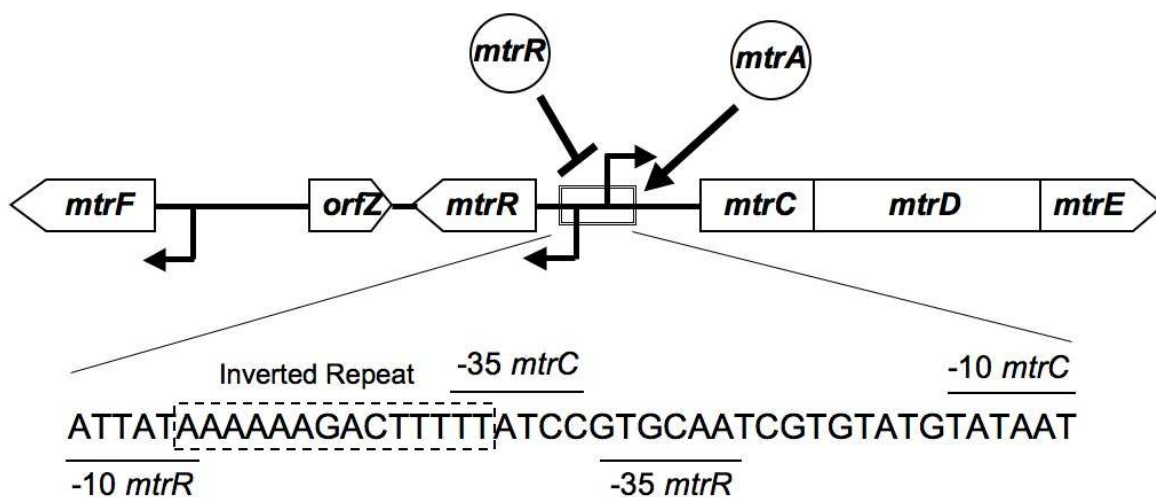


Fig. 1. **Organization of the *mtr* locus of *N. gonorrhoeae*.** Bent arrows mark the *mtrR*, *mtrF*, and *mtrCDE* promoters. *mtrR* and *mtrCDE* are divergently transcribed on opposite strands. Circles represent the transcriptional regulatory proteins MtrR and MtrA. The box represents the location of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

Transcription of the *mtrCDE* operon is negatively regulated by the TetR family transcriptional regulator, MtrR, which represses *mtrCDE* expression by the binding of two homodimers to pseudo-direct repeats within the *mtrCDE* promoter (Hoffman et al., 2005; Lucas et al., 1997). The *mtrR* gene is located 250 base pairs upstream of and is transcribed divergently from *mtrCDE* (Pan & Spratt, 1994). Additionally, transcription of *mtrCDE* may be induced in the presence of sub-lethal concentrations of nonionic, membrane-acting detergents through the action of an AraC/XylS family transcriptional activator, MtrA (Rouquette et al., 1999). Expression of *mtrF* is negatively regulated by both MtrR and the AraC family regulator MpeR (Folster and Shafer, 2005), as well as the availability of free iron (Mercante et al., 2012).

The MtrC-MtrD-MtrE efflux pump mediates resistance to structurally diverse hydrophobic antimicrobial agents, including β -lactam antibiotics such as penicillin, macrolide antibiotics including erythromycin and azithromycin, dyes such as crystal violet, and detergents such as Triton X-100 and nonoxynol-9 (Hagman et al., 1995; Rouquette et al., 1999). Additionally, MtrC-MtrD-MtrE confers resistance to host antimicrobial compounds, including fatty acids, bile salts, progesterone, and the antimicrobial peptide LL-37 (Jerse et al., 2003; Morse et al., 1982; Shafer et al., 1995; Shafer et al., 1998). MtrC-MtrD-MtrE efflux pump-deficient mutants are highly attenuated in a female BALB/c mouse model of lower genital tract infection, even in the absence of pump substrate antibiotic treatment (Jerse et al., 2003). This attenuation is likely due to an increased susceptibility to host antimicrobial compounds, highlighting the importance of the *mtr* system in establishing gonococcal infection.

The production of efflux pumps is an energy-expensive process, and it might be hypothesized that high levels of MtrC-MtrD-MtrE production could stress the gonococcus,

resulting in slower or defective growth, thereby conferring a fitness cost on strains with increased *mtrCDE* expression. In this respect, Eisenstein and Sparling noted that a mutant strain displaying the Mtr phenotype, now known to be due to a single base pair deletion in the inverted repeat in the *mtrR* promoter (Figure 1) that results in high-level antibiotic resistance through increased transcription of *mtrCDE* (Hagman & Shafer, 1995), had a slower growth rate *in vitro* (Eisenstein & Sparling, 1978). However, this same mutation confers a fitness advantage during competitive infection against wild-type strain FA19 in the female mouse model of infection in the absence of antibiotics (Warner et al., 2008) and is frequently found in clinical isolates (Shafer et al., 1995; Zarantonelli et al., 1999), particularly from men who have sex with men (Shafer et al., 1995; Xia et al., 2000). Additional mutations in the *mtrR* coding region and the *mtrR* promoter have been identified in clinical isolates that increase resistance to MtrC-MtrD-MtrE pump substrates as well as confer a survival advantage in the female mouse infection model (Table 1) (summarized in Warner et al., 2008). Mutations in the *mtrR* coding region, particularly those resulting in radical amino acid changes in the MtrR helix-turn-helix DNA binding domain, lead to low or intermediate levels of antimicrobial resistance that corresponds to a low to intermediate survival advantage during competitive infection in female mice (Warner et al., 2008). The single nucleotide deletion in the inverted repeat of the *mtrR* promoter and a recently identified mutation 120 base pairs upstream of the *mtrC* start codon (*mtr*₁₂₀) confer high-level resistance to pump substrates as well as a greater fitness advantage *in vivo* (Warner et al., 2008). These changes in fitness require an active efflux pump, as the effects were reversed in the regulatory mutant strains when the efflux pump system was genetically inactivated. Thus, it appears that the level of antibiotic resistance due to increased *mtrCDE* expression corresponds positively to the strength of the fitness advantage observed *in vivo*.

Genotype	CI at day 3
Single bp deletion in <i>mtrR</i> promoter	1000
<i>mtr</i> ₁₂₀ point mutation	1000
A39T change in DNA binding domain of MtrR	100
<i>mtrA</i> ::Km ^R	0.005
<i>mtrA</i> ::Km ^R <i>mtrR</i> ₁₋₅₃	100
<i>mtrA</i> ::Km ^R <i>mtrR</i> _{E202G}	10
<i>mpeR</i> ::Km ^R	1

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

Induction of *mtrCDE* expression by the activator MtrA is also important for gonococcal survival *in vivo*. Strains carrying a disrupted *mtrA* gene display a significant fitness disadvantage during competitive infection with wild-type strain FA19 in the female mouse model of infection (Warner et al., 2007). MtrA induction of *mtrCDE* expression occurs in the presence of nonionic detergents such as Triton X-100 (Rouquette et al., 1999). The presence of host antimicrobial factors that are pump substrates, such as fatty acids or CRAMP-38, the mouse homologue of the human cathelicidin LL-37, may have a similar effect. The decreased fitness of the *mtrA* mutants *in vivo* would therefore be attributed to failure of the gonococcus to respond to host defense factors due to inability to upregulate expression of the pump.

Interestingly, in a study by Warner et al., 2007, some *mtrA*-deficient strains developed mutations in the *mtrR* gene (*mtrR*₁₋₅₃ and *mtrR*_{E202G}) after inoculation into mice in the absence of antibiotics; these strains were recovered in high numbers and displayed increased antibiotic resistance as well as a fitness advantage during subsequent competitive infection against wild-type FA19 (Table 1). The development of compensatory mutations to overcome the cost of *mtrA* disruption highlights the importance of the MtrC-MtrD-MtrE efflux pump to gonococcal fitness *in vivo*.

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

3. Quinolone resistance and gonococcal fitness

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

Quinolones induce bacterial cell death by inhibiting the activity of the bacterial type IIA DNA topoisomerases DNA gyrase and topoisomerase IV (Emmerson, 2003; Hooper 1999). These enzymes are responsible for managing the topological state of genomic DNA and are necessary for resolving regions of topological stress that occur during critical cell processes such as DNA replication and the regulation of gene expression. DNA gyrase and topoisomerase IV are heterotetramers that bind to DNA and generate a double-stranded

break in one region of the bound DNA duplex, which results results in a complex referred to as the G-segment. A second region of distant DNA duplex, referred to as the T-segment, passes through the G-segment and the cleaved substrate held in the G-segment is subsequently religated to complete a single round of topological adjustment (Bates et al., 2011; Chen and Lo 2003; Morais Cabral et al., 1997). Quinolones specifically target the G-segment of the enzyme-DNA complex. Presently, there is no universally accepted mechanism of how quinolones kill bacteria; however, mounting evidence suggests that two quinolone molecules stabilize the cleaved DNA duplex, resulting in the accumulation of lethal lesions within the genome of the cell (Laponogov et al., 2009).

Quinolone resistance in *N. gonorrhoeae* is due to point mutations in the quinolone resistance determining region (QRDR) of the A subunits of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) (Tanaka et al., 2000; Trees et al., 2001). Belland and colleagues were the first to delineate the genetic basis of quinolone resistance in *N. gonorrhoeae* in 1994. By analyzing ciprofloxacin resistant (Cip^R) mutants selected *in vitro*, these investigators showed Cip^R in *N. gonorrhoeae* is a two step process in which intermediate level ciprofloxacin resistance (Cip^I) occurs via point mutations in *gyrA* that encode amino acid substitutions at positions Ser91 and Asp95. Cip^I *gyrA* mutants then become Cip^R when point mutations occur in *parC* (Belland et al., 1994). This sequence of events is consistent with data from numerous molecular epidemiologic studies (Kam et al., 2003; Morris et al., 2009; Starnino et al., 2010; Tanaka, 1992; Trees et al., 2001; Vereshchagin et al., 2004). Analyses of clinical isolates have also provided insights into the nature of mutations directly associated with fluoroquinolone resistance in *N. gonorrhoeae*. Commonly isolated substitutions in the Ser91 position of the GyrA subunit include amino acids with bulky side chains (phenylalanine and tyrosine) and the hydrophobic leucine, while arginine is the most common substitution at position Asp95 (Kam et al., 2003; Morris et al., 2009; Ruiz et al., 2001; Starnino et al., 2010; Tanaka et al., 2000; Trees et al., 2001; Vereshchagin et al., 2004; Vernel-Paulillac et al., 2009). Double point mutations in *gyrA* that result in these amino acid substitutions are sufficient and also largely responsible for sterically hindering the intercalation of quinolone molecules (Xiong et al., 2011). The location specificity of *parC* mutations that lead to high level Cip^R appears to be less stringent than mutations in *gyrA*, with alterations at position 91 (the most common), 86, 87, or 88 identified among Cip^R isolates (Dewi et al., 2004; Morris et al., 2009; Tanaka et al., 2000; Trees et al., 2001) (Figure 2).

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

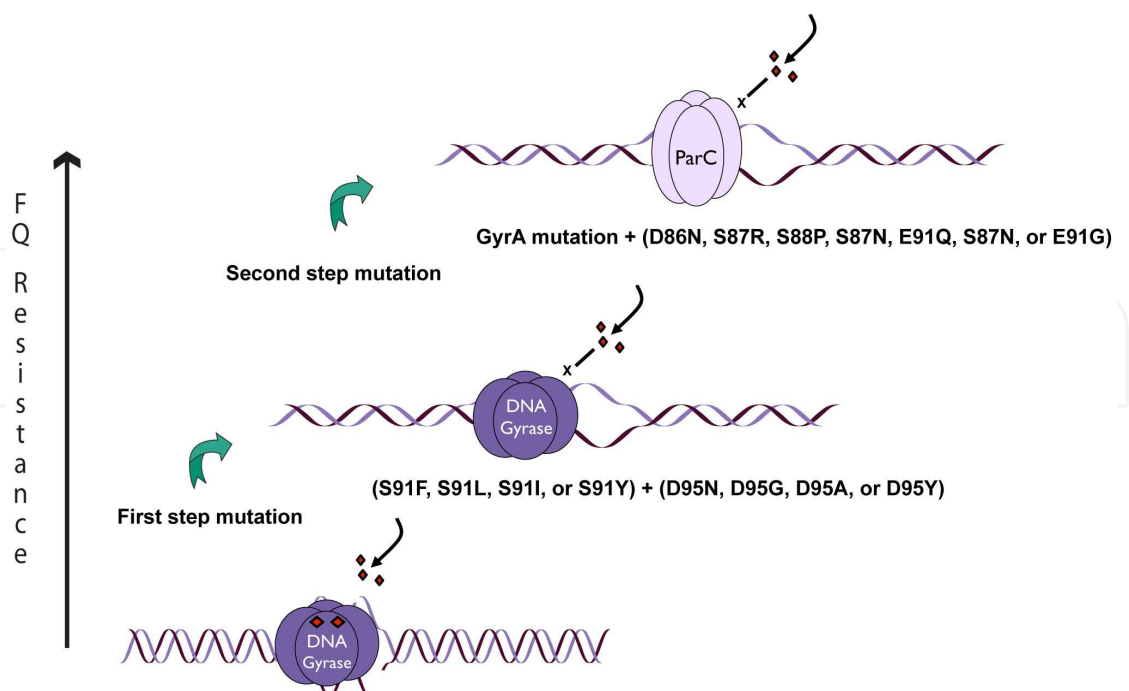


Fig. 2. Evolution of quinolone resistance in *N. gonorrhoeae*. Quinolone resistance in *N. gonorrhoeae* is a two-step process, beginning with point mutations in the QRDR of *gyrA*, which increase resistance to intermediate levels. Positions 91 and 95 are most often altered, with Ser91Phe and Asp95Asn the most common substitutions. Other substitutions have also been reported. High level resistance occurs upon mutation of the QRDR of *parC* mutation. *parC* mutations carried by Cip^R gonococci most often cause amino acid substations at position 91, 86, 87 or 88 (Kam et al., 2003; Ghanem et al., 2005; Morris et al., 2009; Ruiz 2001; Starnino et al., 2010; Tanaka et al., 2000; Trees et al., 2001; Vereshchagin et al., 2004; Vernel-Paulillac 2009).

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

Genotype	CI at day 3
<i>gyrA</i> _{91/95}	5-fold increase; 30-fold increase on day 5 compared to Cip ^S wild-type strain
<i>gyrA</i> _{91/95} , <i>parC</i> ₈₆	2-fold decrease compared to Cip ^S wild-type strain
<i>gyrA</i> _{91/95} , <i>mtrR</i> -79	40-fold increase compared to Cip ^S <i>mtr</i> -56 mutant parent strain
<i>gyrA</i> _{91/95} , <i>parC</i> ₈₆ , <i>mtrR</i> -79	50-fold decrease compared to Cip ^S <i>mtr</i> -56 mutant parent strain

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

As discussed, it is well established that *mtr* locus mutations increase gonococcal fitness in the mouse model, and we therefore wondered whether the fitness benefit conferred by *gyrA*_{91/95} mutations would enhance the fitness advantage afforded by increased efflux of host substrates through the MtrC-MtrD-MtrE active efflux pump. Our alternative hypothesis was that increasing numbers of resistance mutations would impair growth to such an extent as to abrogate the fitness benefits associated with either resistance mutation. To test this hypothesis, we constructed *gyrA*_{91/95} and *gyrA*_{91/95}, *parC*₈₆ mutants in an *mtr* mutant of strain FA19 that carries a commonly isolated *mtrR* promoter mutation (the single base pair deletion in the *mtrR* promoter termed hereafter as *mtrR*-79). The *gyrA*_{91/95}, *mtrR*-79 mutant (Cip^I) showed no fitness difference compared to the *mtrR*-79 mutant parent strain *in vitro*, but significantly out-competed the *mtrR*-79 mutant during experimental murine infection. In contrast, the highly Cip^R *gyrA*_{91/95}, *parC*₈₆, *mtrR*-79 mutant was severely attenuated both *in vitro* and *in vivo* relative to the *mtrR*-56 mutant, with only *mtrR*-56 mutant gonococci recovered from a majority of mice 5 days after inoculation (Table 2) (Kunz et al., In Press).

From these studies we conclude that the *gyrA*_{91/95} mutation confers a fitness benefit to *N. gonorrhoeae* that is independent of the MtrC-MtrD-MtrE efflux pump system, but that an additional *parC*₈₆ mutation results in a net fitness cost. These data are intriguing and may help to explain the frequent isolation of Cip^R gonococci that also carry *mtrR* promoter or *mtrR* structural gene mutations, which has been interpreted by others as evidence that active efflux through the MtrC-MtrD-MtrE pump is another mechanism of fluoroquinolone resistance in *N. gonorrhoeae* (Dewi et al., 2004; Vereshchagin et al., 2004). The fact that we found no difference in the Cip MICs of the *gyrA*_{91/95} versus *gyrA*_{91/95}, *MtrR*-79 mutants or of *gyrA*_{91/95}, *parC*₈₆ versus *gyrA*_{91/95}, *parC*₈₆, *mtrR*-79 mutants (Kunz et al., submitted), is strong genetic evidence that *mtr* mutations do not contribute to Cip^R in *N. gonorrhoeae*. Instead, the prevalence of Cip^R *mtr* strains may reflect increased microbial fitness conferred by these mutations.

It is important to remember that while mutations in both *gyrA* and *parC* led to reduced fitness in the mouse model, compensatory mutations may occur in nature that restore fitness while maintaining high level Cip^R. There is much evidence that fitness compensation can occur in bacteria without loss of antibiotic resistance (Balsalobre et al., 2011; Bjorkholm et al., 2001; Bjorkman et al., 1998; Giraud et al., 1999; Komp Lindgren et al., 2005; Marcussen et al., 2009; Nagaev et al., 2001). In support of this possibility for QRNG, we have observed that while Cip^R gonococci were outcompeted by Cip^S (wild-type) or Cip^I bacteria in a majority of mice tested, only Cip^R gonococci were recovered from some mice (10-17%) as infection progressed in each of several experiments (Figure 3). To further investigate this observation, we analyzed Cip^R bacteria isolated on day 5 in pure culture from a mouse inoculated with a mixture of Cip^I (*gyrA*_{91/95}, *mtrR*-79) and Cip^R (*gyrA*_{91/95}, *parC*₈₆, *mtrR*-79) mutants. Interestingly, these Cip^R bacteria grew better than the Cip^I and Cip^R strains used to inoculate the mouse and the Cip^S *mtr* parent of the Cip^I and Cip^R strains. Unlike either of these strains, the *in vivo*-selected Cip^R mutant had a wild-type *mtr* locus and a *gyrA* allele that was predicted to encode a leucine instead of phenylalanine residue at position 91 (Phe91Leu) (Kunz et al., In Press). We conclude that one or more compensatory mutations occurred during infection that allowed highly Cip^R gonococci to out-compete Cip^I bacteria *in vivo*.

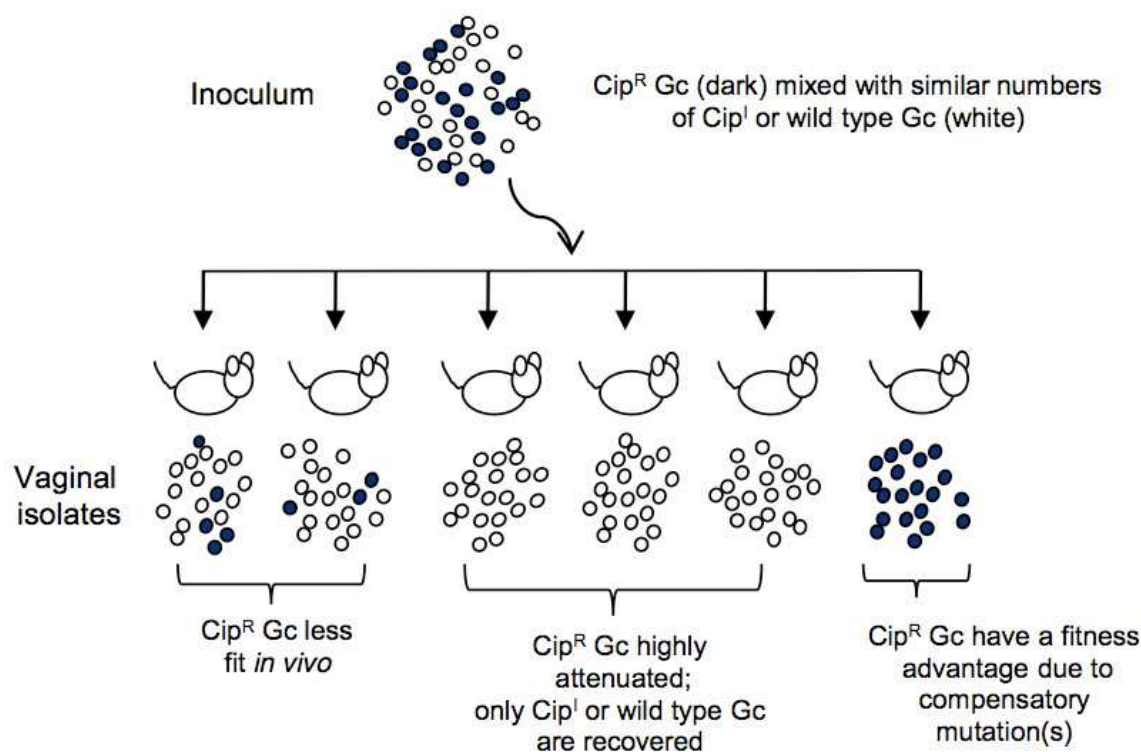


Fig. 3. The fitness disadvantage of Cip^R gonococci can be overcome by selection for compensatory mutations. Vaginal inoculation of estradiol-treated BALB/c mice with populations of Cip^S or Cip^I gonococci (white) mixed with similar numbers of Cip^R gonococci (black) results in the recovery of a higher proportion of Cip^S or Cip^I CFU, with some mice clearing the Cip^R bacteria. However, in 10-17% of mice tested, high numbers of only Cip^R CFU were recovered as infection progressed, most likely due to compensatory mutations (Kunz et al, In Press).

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

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

4. Conclusion

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

Less clear, however, was whether in the absence of selective pressure, a resistant strain would have a fitness benefit during an infection over a sensitive counterpart. In this respect, the report of Luo *et al.* (2005) dealing with the increased fitness of a ciprofloxacin resistant strain of *C. jejuni* over a sensitive parent strain *in vivo* was a “game-changer” for antibiotic resistance researchers. Briefly, it forced us to consider the rather scary possibility that a mechanism of antibiotic resistance can actually enhance the ability of a pathogen to survive in the community. This possibility has a number of important implications for our understanding of bacterial pathogenesis and bacterial infections that should be considered. First, are there

“antibiotic substitutes” *in vivo* that the resistance mechanism recognizes, allowing the resistant strain to out-compete the sensitive strain? Might these “host antibiotics” provide the selective pressure in the community? This is certainly likely for the fitness benefit imparted to those *N. gonorrhoeae* strains that over-express the Mtr efflux pump system. This pump, along with similar pumps produced by other Gram-negatives (Shafer et al., 2010), recognizes host antimicrobials (e.g., antimicrobial peptides) in addition to antibiotics such as beta-lactams and macrolides. In this context, efflux pump inhibitors (Lomovskaya & Bostian, 2006) may have clinical use as they would increase bacterial susceptibility to classical antibiotics as well as host antimicrobials. A second issue that requires further investigation is whether a resistance mechanism has secondary effects on the physiology of the resistant strain that results in an advantage during infection. This hypothesis may help to explain why *gyrA*_{91/95} mutations can enhance the fitness of Cip^I strains of *N. gonorrhoeae*. Hopefully, ongoing transcriptional profiling studies that compare isogenic Cip^I and Cip^S strains will provide insights that will help us to understand fitness differences. A third point merits consideration: stable mutations that decrease bacterial susceptibility to a given antibiotic, but not to an extent that it pushes them across the MIC breakpoint, may be more advantageous for the bacteria than previously thought. In this respect, as emphasized throughout this text, mutations in *mtrR* or *gyrA* provide gonococci with a fitness advantage *in vivo*, but do not push them across the MIC breakpoint for either beta-lactams or quinolones, respectively. Importantly, both are necessary for clinically significant levels of resistance imparted by other mutations. Accordingly, strains bearing *mtrR* and/or *gyrA* mutations may not only be more fit during infection, but also more likely to subsequently develop clinical resistance to beta-lactams and quinolones than fully sensitive strains. This issue is of greater urgency now because the gonococcal strain that caused a ceftriaxone-resistant infection in Japan is an *mtrR* mutant (Ohnishi et al., 2011) even though the mutation by itself has little impact on the level of beta-lactam resistance (Veal & Shafer, 2003). Finally, if a resistance mutation enhances fitness and is stably maintained in a bacterial pathogen for years, it may be yet another reason why antibiotic re-cycling after extended absence from the treatment regimen may not be a viable option to combat the emergence and spread of antibiotic resistant bacteria.

We have used *N. gonorrhoeae* as a model human pathogen for studies on how bacterial fitness can be impacted by mechanisms of antibiotic resistance. Having been intimately associated with humans for thousands of years, it is of no surprise that the gonococcus has evolved novel ways to evade or resist the multitude of toxic agents that it encounters during infection. The continued emergence of strains expressing decreased susceptibility or even clinical resistance to frontline antibiotics used today (e.g., ceftriaxone) in therapy emphasizes the remarkable adaptive ability of this pathogen. The examples provided herein with the gonococcus emphasize that mechanisms of antibiotic resistance can enhance bacterial virulence, as defined by increased *in vivo* fitness. Understanding the processes that lead to increased fitness of the gonococcus (or any other pathogen) due to antibiotic resistance may result in novel strategies that could be used to inhibit bacterial replication *in vivo* directly or indirectly by enhancing the efficacy of the defensive systems of the host that operate locally.

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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