MAJOR ARTICLE

Impact of Fluoroquinolone Resistance Mutations on Gonococcal Fitness and In Vivo Selection for Compensatory Mutations

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(See the editorial commentary by Dillon and Parti, on pages 1775–7.)

Background. Quinolone-resistant *Neisseria gonorrhoeae* (QRNG) arise from mutations in *gyrA* (intermediate resistance) or *gyrA* and *parC* (resistance). Here we tested the consequence of commonly isolated $gyrA_{91/95}$ and $parC_{86}$ mutations on gonococcal fitness.

Methods. Mutant $gyrA_{91/95}$ and $parC_{86}$ alleles were introduced into wild-type gonococci or an isogenic mutant that is resistant to macrolides due to an $mtrR_{-79}$ mutation. Wild-type and mutant bacteria were compared for growth in vitro and in competitive murine infection.

Results. In vitro growth was reduced with increasing numbers of mutations. Interestingly, the $gyrA_{91/95}$ mutation conferred an in vivo fitness benefit to wild-type and $mtrR_{-79}$ mutant gonococci. The $gyrA_{91/95}$, $parC_{86}$ mutant, in contrast, showed a slight fitness defect in vivo, and the $gyrA_{91/95}$, $parC_{86}$, $mtrR_{-79}$ mutant was markedly less fit relative to the parent strains. A ciprofloxacin-resistant (Cip^R) mutant was selected during infection with the $gyrA_{91/95}$, $parC_{86}$, $mtrR_{-79}$ mutant in which the $mtrR_{-79}$ mutation was repaired and the $gyrA_{91}$ mutation was altered. This in vivo–selected mutant grew as well as the wild-type strain in vitro.

Conclusions. $gyrA_{91/95}$ mutations may contribute to the spread of QRNG. Further acquisition of a $parC_{86}$ mutation abrogates this fitness advantage; however, compensatory mutations can occur that restore in vivo fitness and maintain Cip^R.

Neisseria gonorrhoeae is a Gram-negative diplococcus that plays a major role in urogenital tract and perinatal infections [1]. Gonorrhea is the second most frequently reported bacterial sexually transmitted infection (STI) in the United States, with an estimated 700 000 new cases each year. The rate of gonorrhea is highest among females aged <25 years old, especially those that engage in high-risk sexual behaviors [2]. The high prevalence of gonorrhea is particularly concerning because it is a major cause of pelvic inflammatory disease and can lead to serious outcomes such as ectopic pregnancy, infertility, and chronic pelvic pain. Gonorrhea is also a cofactor in human immunodeficiency virus transmission [3].

Antibiotic resistance emerges rapidly in *N. gonorrhoeae*, which challenges treatment options and threatens current control measures. Currently, only a single class of antibiotics, the third-generation cephalosporins, is recommended for routine treatment of gonorrhea in the Centers for Disease Control and Prevention (CDC) sexually transmitted disease guidelines [2]. The status of *N. gonorrhoeae* as a "superbug" continues to increase with the recent isolation of a ceftriaxone-resistant

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strain [4]. Fluoroquinolones were removed from the CDC list of recommended first-line antibiotics for treatment of gonorrhea in 2007 [5]. First used clinically in the mid-1980s [6], over 80% of gonococcal isolates in the western Pacific region were ciprofloxacin resistant (Cip^R) by 2002 [7, 8]. A steady increase in quinolone-resistant *N. gonorrhoeae* (QRNG) followed in the United States, with 891 of 6009 (14.8%) clinical isolates identified as QRNG in 2007 [9]. Resistance to this relatively inexpensive class of antibiotics, which target topoisomerase II (DNA gyrase) and topoisomerase IV [6], in *N. gonorrhoeae* is due to amino acid substitutions in the quinolone resistance–determining regions (QRDRs) of the A subunits of DNA gyrase (GyrA) and ParC, the A subunit of DNA topoisomerase IV [10–13].

Traditionally, antibiotic resistance is accompanied by a fitness loss, particularly when it occurs via mutation of genes that are important for basic cellular functions [14]. Some resistance mutations, however, provide a growth benefit in vitro or increase fitness when tested in pathogenesis models [15–19]. This phenomenon can be due to compensatory mutations that balance the detrimental effects of resistance mutations [20]. Increased microbial fitness during infection can also be a direct consequence of the resistance mutation, as shown for mutations that increase the efflux of antimicrobial substances [15, 18]. For example, in *N. gonorrhoeae*, multitransferable resistance (*mtr*) mutations, defined here as mutations in the *mtrR* repressor gene or its promoter region, increase resistance to macrolide antibiotics and high levels of penicillin G through overexpression of the MtrC-MtrD-MtrE active efflux pump. The *mtr* mutations also confer a fitness advantage during experimental infection of female mice [18], which is most likely due to increased resistance to host innate effectors that are also substrates of the MtrC-MtrD-MtrE active efflux pump [21–23]. Whether fluoroquinolone resistance mutations also promote increased in vivo fitness of *N. gonorrhoeae* is not known. This question warrants testing based on the wide prevalence of QRNG strains and reports that *gyrA* and *gyrA, parC* mutations in some pathogens confer a fitness advantage in animal models [16, 17].

To further explore the rapid spread of QRNG worldwide, here we introduced commonly isolated *gyrA* and *parC* mutations into a wild-type *N. gonorrhoeae* strain and tested the consequence on gonococcal fitness in vitro and in the mouse infection model. We also examined the effect of these mutations in an *mtr* mutant of the same strain background to determine if the fitness benefit conferred by overproduction of the MtrC-MtrD-MtrE active efflux pump is lost or further increased in QRNG.

METHODS

Bacterial Strains and Culture Conditions

Bacterial strains are described in Table 1. Streptomycin-resistant (Sm^R) derivatives of wild-type *N. gonorrhoeae* strain FA19 and mutant KH15 were used in this study [22]. Mutant KH15 overexpresses the *mtrCDE* operon due to a single base pair (bp) deletion in a 13-bp inverted repeat in the *mtrR* promoter that maps 79 bps upstream of the *mtrR* start codon ($mtrR_{-79}$) [18, 21, 24]. *Neisseria gonorrhoeae* strain C29 is a Cip^R isolate from an Israeli outbreak [25] that carries mutated *gyrA* (Ser91Phe and Asp95Asn) and *parC* (Asp86Asn) alleles. DNA from strain C29 (provided by Jonathan Zenilman, Johns Hopkins University) was used to polymerase chain reaction (PCR) amplify a 1.3-kb PCR fragment that carries the desired $gyrA_{91/95}$ mutations using primers gyrA1-for and gyrA1-rev. The PCR product was cloned into pCR-Blunt and transformed into *Escherichia coli* Top10 (Invitrogen). Transformants were selected on Luria agar with 50 μg/mL of kanamycin. The same mutated *gyrA* sequence was amplified from a positive clone and transformed into $FA19Sm^R$ and KH15 bacteria [26]. Transformants were selected on GC agar with 0.125 μg/mL Cip to obtain mutants AK1 ($gyrA_{91/95}$) and AK11 ($gyrA_{91/95}$, $mtrR_{-79}$). The $gyrA_{91/95}$, $parC_{86}$ double mutants were constructed similarly using primers parC1-for and parC1-rev to obtain a 1.6-kb PCR fragment containing the $parC_{86}$ mutation from strain C29 and mutants AK1 and AK2 as the recipients. Mutants AK2 ($gyrA_{91/95}$, $parC_{86}$) and AK12 ($gyrA_{91/95}$, $parC_{86}$, $mtrR_{-79}$) were selected on GC agar with 2.0 μg/mL of Cip. All plasmid clones and mutations were confirmed by nucleotide sequence analysis. Primers gyrA1-for, gyrA1-rev, and gyrA2-for and primers parC1-for, parC1-rev, and parC2-for anneal within the *gyrA* and *parC* structural genes, respectively (Table 2). The *mtr* locus of mutant AK13 was sequenced using primers 5′mtrR and 3′mtrR [27]. Stocks of nonpiliated wild-type and mutant bacteria, as assessed by colony morphology, were used for in vitro cocultures to minimize clumping and in competitive infection experiments to reduce DNA transfer between the strains being compared. *Neisseria gonorrhoeae* was cultured on GC agar with Kellogg's supplements as described [28].

Table 1. Bacterial Strains

Strain	Relevant Characteristics	Reference
$FA19Sm^R$	Spontaneous Sm^R mutant of wild-type FA19	[22]
KH15	$mtrR_{-79}$ mutant; parent strain $FA19Sm^R$	[27]
AK1	$gyrA_{91/95}$; parent strain $FA19Sm^R$	Present study
AK2	$gyrA_{91/95}$, $parC_{86}$; parent strain AK1	Present study
AK11	$gyrA_{91/95}$, $mtrR_{-79}$; parent strain KH15	Present study
AK12	$gyrA_{91/95}$, $parC_{86}$, $mtrR_{-79}$; parent strain AK11	Present study
AK13	in vivo-selected mutant; $gyrA_{Ser91Leu/95}$, $parC_{86}$, wild-type *mtr*	Present study

Table 2. Oligonucleotide Primers Used in This Study

Primer	Sequence (5′ to 3′)
gyrA1-for	GACTTCCTCATGCAGCAAATG
gyrA1-rev	CAACCATATTGATGCCGAAACTG
gyrA2-for	ACGAAACATTGAAACCATGAC
parC1-for	CATAGCGACGGTCTTTGTGTG
parC1-rev	GTTGATGAAGGTATCGGTATCGATG
parC2-for	ACGCTTCCCATACCGATTC

Minimal Inhibitory Concentration

The minimum inhibitory concentrations (MICs) of Cip and erythromycin (Em) against wild-type and mutant bacteria were determined by a standard agar dilution assay. Ciprofloxacin MICs of 0.125–0.5 μg/mL were reported as intermediate resistance (Cip^I); Cip MIC values ≥1 μg/mL were reported as resistance (Cip^R), as per CDC guidelines [29, 30].

Growth Kinetics and In Vitro Competition Experiments

Wild-type and mutant bacteria were harvested from GC agar plates after 18–20 hours incubation and inoculated into 30 mL of supplemented GC broth with 5 mM $NaHCO_3$ to a starting absorbance value at 600 nm (A600) = A_{600} = 0.07–0.075. Cultures were incubated in a shaking air incubator at 37°C as described [31]. The average time required to reach an A_{600} = 1.0 was determined from 3 independent experiments for each strain and compared by a 1-way analysis of variance. For in vitro competition experiments, similar numbers of the strains being compared were inoculated into GC broth, and aliquots were quantitatively cultured on GC agar with 100 μg/mL Sm [total number of colony forming units (CFUs)] and 100 μg/mL Sm plus 0.125 μg/mL Cip [total number of mutant CFUs] over time. The number of mutant CFUs was subtracted from the total number to estimate the number of parent bacteria. The ratio of mutant to parent CFUs at each time point was divided by the ratio of mutant to parent CFUs at time 0 to obtain the competitive index (Ic).

Competitive Experimental Murine Infection

Female BALB/c mice (approximately 6–8 weeks old) were treated with water-soluble 17-β-estradiol [32] and antibiotics (2.4 mg Sm and 0.4 mg vancomycin intraperitoneally twice daily and 0.04 g trimethoprim sulfate per 100 mL drinking water) to increase susceptibility to *N. gonorrhoeae* and reduce the overgrowth of commensal flora. None of the antibiotics used is a substrate of the MtrC-MtrD-MtrE efflux pump system or target GyrA or ParC. Bacteria were cultured on GC agar plates (18–20 hours), and isolated colonies were suspended in saline and passed through a 1.2-μm filter to remove aggregates. Suspensions with similar numbers of the bacteria to be compared were combined, and 20 μL of the mixed suspensions were inoculated vaginally into mice (total dose, approximately 10^6 CFUs) (n = 5–8 mice per test group). Vaginal mucus was quantitatively cultured on GC agar with Sm (total CFUs) and GC agar with Sm plus 0.125 μg/mL Cip (to isolate AK1 and AK11) or 2 μg/mL Cip (to isolate AK2 and AK12) on days 1, 3, 5, and 7 postinoculation, as described [31]. The number of parent CFUs was calculated by subtracting the number of mutant CFUs from the total number of CFUs. Data were expressed as an Ic [$(\text{mutant CFUs/parent CFUs})_{output}/(\text{mutant CFUs/parent CFUs})_{input}$]. The limit of detection (4 CFUs/100 μL vaginal swab suspension) was used for cultures from which no gonococci were isolated. All animal infection experiments were conducted at the Uniformed Services University of the Health Sciences (USUHS), which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the university's Institutional Animal Care and Use Committee.

RESULTS

Characterization of $gyrA_{91/95}$ and $gyrA_{91/95}$, $parC_{86}$ Mutants

Fluoroquinolone resistance in *N. gonorrhoeae* is a 2-step process in which mutations in *gyrA* lead to a Cip^I phenotype and an additional mutation in *parC* confers a Cip^R phenotype [10]. To test the consequence of intermediate-level Cip resistance on gonococcal fitness, we introduced 2 commonly isolated *gyrA* mutations that encode Ser91Phe and Asp95Asn substitutions into the Cip^S *N. gonorrhoeae* strain $FA19Sm^R$. We also introduced these mutations into strain KH15, which is an isogenic mutant of $FA19Sm^R$ that carries a commonly isolated *mtr* promoter mutation [27]. As expected, mutants AK1 ($gyrA_{91/95}$) and AK11 ($gyrA_{91/95,}$ $mtrR_{-79}$) were Cip^I (Table 3). Introduction of a $parC_{86}$ mutation that encodes an Asp86Asn substitution into mutants AK1 and AK11 resulted in mutants AK2 ($gyrA_{91/95,}$ $parC_{86}$) and AK12 ($gyrA_{91/95,}$ $parC_{86,}$ $mtrR_{-79}$), respectively, and increased the Cip MIC to a clinically relevant level (6 μg/mL). The $mtrR_{-79}$ mutation did not alter the Cip MIC, but as expected, Em MICs against parent strain KH15 ($mtrR_{-79}$) and mutants AK11 and AK12 were 2.5-fold higher than against $FA19Sm^R$, AK1, and AK2 (Table 3).

Mutations in the QRDR of *gyrA* and/or *parC* alter growth rates in other bacterial species [33–38]. When cultured separately in GC broth under standard conditions, growth of strains AK1 and AK11, which carry the $gyrA_{91/95}$ alleles, appeared slower than the respective parent strains (Figure 1), although there was no significant difference in the time it took the mutant and parent strains to reach mid to late logarithmic phase (A_{600} = 1.0) (Table 3). In contrast, mutants that carry both $gyrA_{91/95}$ and $parC_{86}$ mutations (AK2 and AK12) grew more slowly than the respective parent bacteria and required a

Table 3. Antibiotic Sensitivity and In Vitro Growth of Wild-Type and Mutant Strains

	MIC (μg/mL)		Time to Reach A_{600} = 1.0[a]	
Strain	Cip	Em	Minutes (±SE)[a]	*P* Value
FA19Sm^R	<0.0625	0.5	287 (±13)	…
AK1	0.5	0.5	325 (±6)	.08[b]
AK2	6	0.5	376 (±17)	<.01[c]
KH15	<0.0625	4	340 (±7)	.01[d]
AK11	1	4	360 (±8)	.08[e]
AK12	6	4	410 (±16)	<.01[f]
AK13	6	0.5	294 (±6)	<.05[g]

Abbreviations: Cip, ciprofloxacin; Em, erythromycin; MIC, minimum inhibitory concentration; SE, standard error.

[a] All broth cultures were started at an absorbance reading at 600 nm (A600) an A_{600} = 0.075–0.085 with the starting A_{600} for each strain within 0.005 units within each experiment. The A_{600} was measured at hourly time points and plotted vs time. The number of minutes required to reach an A_{600} = 1.0 was calculated from the linear part of the curve. Results were compared by a 1-way analysis of variance.

[b] AK1 compared with parent strain FA19Sm^R.

[c] AK2 compared with FA19Sm^R; *P* value < .05 for AK2 vs AK1.

[d] KH15 compared with parent strain FA19Sm^R.

[e] AK11 compared with parent strain KH15.

[f] AK12 mutant compared with FA19Sm^R, KH15, AK1, or AK11.

[g] AK13 mutant compared with KH15; *P* value < .01 for AK13 vs AK2, AK11, or AK12.

significantly longer time to reach an A_{600} = 1.0, with AK12 the most attenuated (Figure 1; Table 3). We conclude that Ser91Phe and Asp95Asn substitutions in GyrA do not detectably affect growth under these conditions. However, mutants that express both an altered GyrA and ParC protein grow significantly more slowly and are further attenuated by *mtrR*$_{-79}$.

Effect of *gyrA*$_{91/95}$ and *parC*$_{86}$ Mutations on In Vivo Fitness

We next examined whether the *gyrA*$_{91/95}$ or *gyrA*$_{91/95}$, *parC*$_{86}$ mutants exhibited altered fitness in vivo using a well-characterized female mouse model of lower genital tract infection [39]. Mice were inoculated with mixed suspensions of FA19Sm^R and AK1 or AK2 bacteria. The relative number of each strain among vaginal isolates was determined over time and normalized to the ratio of mutant and parent gonococci in the inocula. A significantly higher proportion of AK1 bacteria was isolated as infection progressed, with a >10-fold increase in the mean Ic observed on days 5 and 7 pos-inoculation (Figure 2*B*). In contrast, mutant AK1 showed a slight growth disadvantage when cocultured with FA19Sm^R in broth (Ic, approximately 3-fold decreased at 3 and 5 hours; 10-fold decreased at 7 hours [stationary phase]) (Figure 2*A*, closed symbols).

Unlike the Cip^I *gyrA*$_{91/95}$ mutant, the Cip^R mutant AK2 (*gyrA*$_{91/95}$, *parC*$_{86}$) exhibited an approximately 2-fold reduced recovery from infected mice relative to FA19Sm^R on days 1

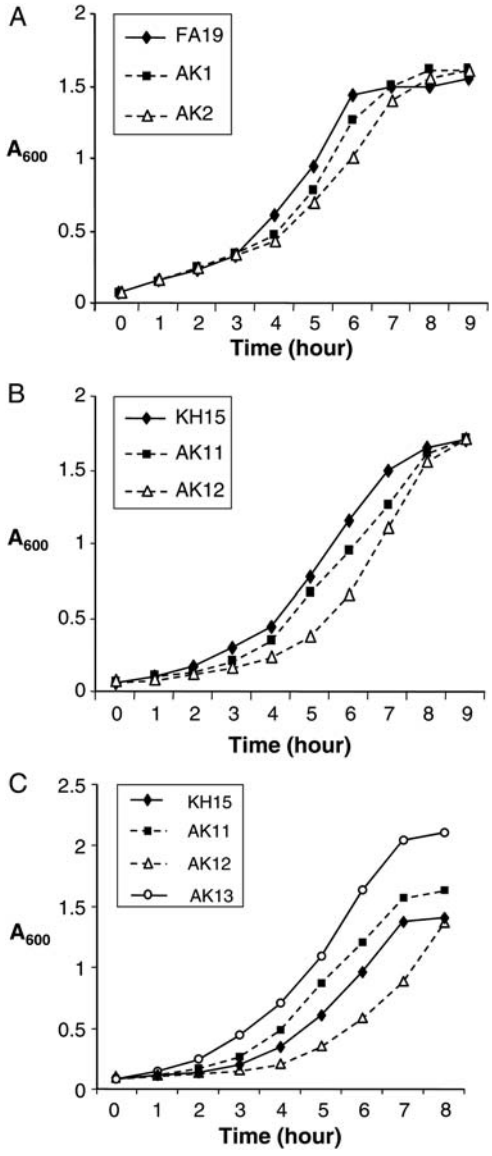

Figure 1. In vitro growth kinetics of wild-type and mutant strains. Representative growth curves for FA19Sm^R, AK1 (*gyrA*$_{91/95}$), and AK2 (*gyrA*$_{91/95}$, *parC*$_{86}$) (*A*); KH15 (*mtr*$_{-79}$), AK11 (*mtr*$_{-79}$, *gyrA*$_{Ser91Phe/95}$), and AK12 (*mtr*$_{-79}$, *gyrA*$_{Ser91Phe/95}$, *parC*$_{86}$) (*B*); and KH15, AK11, AK12, and AK13 (*gyrA*$_{Leu91Phe/95}$, *parC*$_{86}$) (*C*) when cultured separately in GC broth. Mutants AK2 and AK12, but not AK1 and AK11 grew more slowly than the respective parent strains. The in vivo–selected mutant AK13 grew significantly faster than mutants KH15, AK11, and AK12. Mutants were cultured separately in GC broth at a starting absorbance reading at 600 nm (A600) A_{600} = 0.070–0.075 and the A_{600} reading at each hour plotted. Experiments were performed 3 times per strain set.

and 3 postinoculation. A slight increase in the mean Ic occurred over time, with similar numbers of mice exhibiting increased or reduced fitness on days 5 and 7 (Figure 2*C*). Mutant AK2 also showed reduced fitness when cocultured with strain FA19Sm^R in vitro (Ic, approximately 5- and 10-fold decreased at 5 and 7 hours, respectively) (Figure 2*A*, open

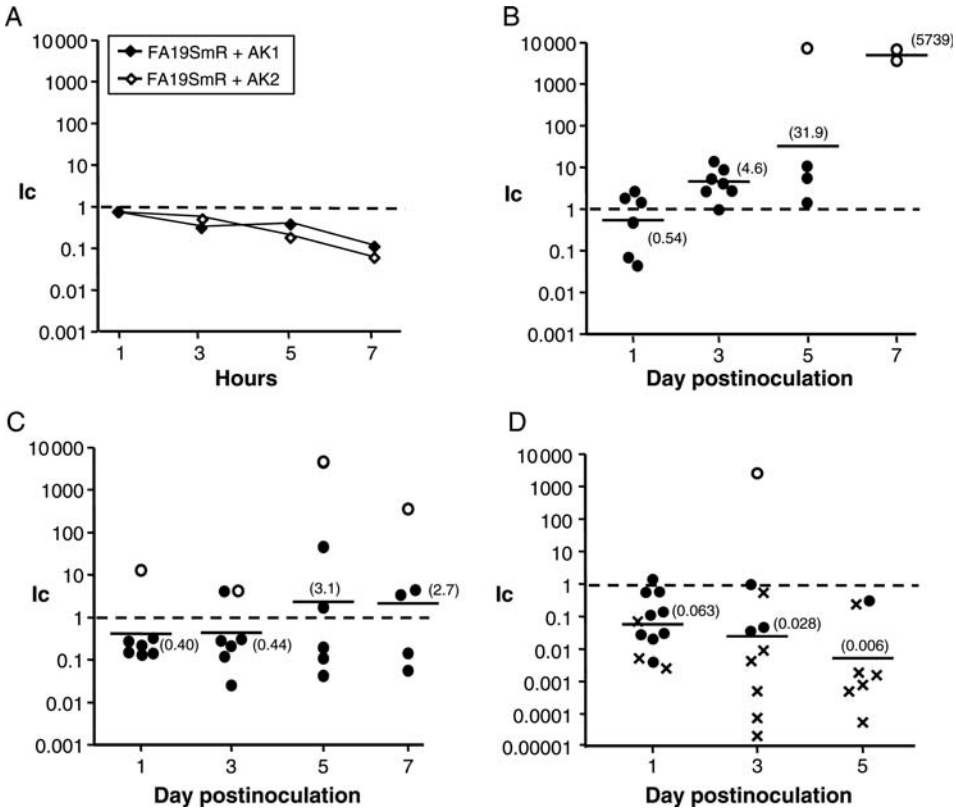

Figure 2. A $gyrA_{91/95}$ mutant is more fit than the parent wild-type strain in vivo. Strains FA19Sm^R, AK1 and AK2 were cocultured in vitro and coinoculated into mice. The competitive index (Ic) for in vitro and in vivo competition experiments was calculated as described in the Methods. Ic values = 1.0 indicate no fitness difference; <1 = reduced fitness; and >1 = increased fitness. (*A*) Ic values for FA19Sm^R vs AK1 or AK2 when cocultured in GC broth. *B–D* show results from competitive murine infection experiments with FA19Sm^R vs AK1 (*B*), FA19Sm^R vs AK2 (*C*), and AK1 vs AK2 (*D*). Symbols represent the Ic for individual mice at each time point. AK1 but not AK2 bacteria showed a marked in vivo fitness advantage relative to FA19Sm^R, and consistent with this result, AK2 was highly attenuated compared with AK1 (*D*). In *B* and *C*, open circles correspond to mice from which only AK1 or AK2 bacteria were recovered. In *D*, cross hatches correspond to mice from which only AK1 was recovered. The open circle in *D* corresponds to a mouse from which only ciprofloxacin-resistant colonies were recovered. Results are combined data from 2 independent experiments.

symbols). Competitive infection experiments with mutants AK1 and AK2 confirmed the in vivo fitness differences of these mutants. Only AK1 bacteria were recovered from a majority of mice on days 3 and 5 postinoculation (Figure 2*D*, cross hatches). We conclude that $gyrA_{91/95}$ mutant bacteria have a growth or survival advantage within the murine genital tract. This fitness benefit does not occur during broth culture in vitro. Introduction of the $parC_{86}$ allele abrogates the in vivo fitness benefit associated with $gyrA_{91/95}$ alone and significantly slows growth in vitro.

Impact of *gyrA* and *gyrA, parC* Mutations on *mtr* Mutant Gonococci

Quinolone-resistant *N. gonorrhoeae* frequently carry other resistance mutations [40–42]. We were particularly interested in the consequence of *gyrA* and *parC* mutations on the fitness of *mtr* mutant gonococci because we previously showed that *mtr* mutations increased gonococcal fitness in the mouse model [18]. We therefore performed competitive infections between strain KH15 ($mtrR_{-79}$) and either AK11 or AK12, which carry the $gyrA_{91/95}$ or the $gyrA_{91/95}$, $parC_{86}$ mutations, respectively, in the KH15 background. Strains AK11 and KH15 showed no fitness difference when cocultured in vitro (Figure 3*A*, open symbols). However, when inoculated into mice, significantly more AK11 ($gyrA_{91/95}$, $mtrR_{-79}$) bacteria were recovered relative to KH15 within 1 day postinoculation (mean Ic, approximately 40-fold increased on days 1 and 3), and only AK11 bacteria were recovered from several mice over the course of infection (Figure 3*B*, open circles).

In contrast, in vivo competition of strains KH15 and AK12 demonstrated a clear fitness disadvantage to having $mtrR_{-79}$, $gyrA_{91/95}$ and $parC_{86}$ relative to $mtrR_{-79}$ alone (Ic, approximately 50-fold decreased on days 3 and 5) (Figure 3*C*). When cocultured in vitro, AK12 bacteria were approximately 5-fold

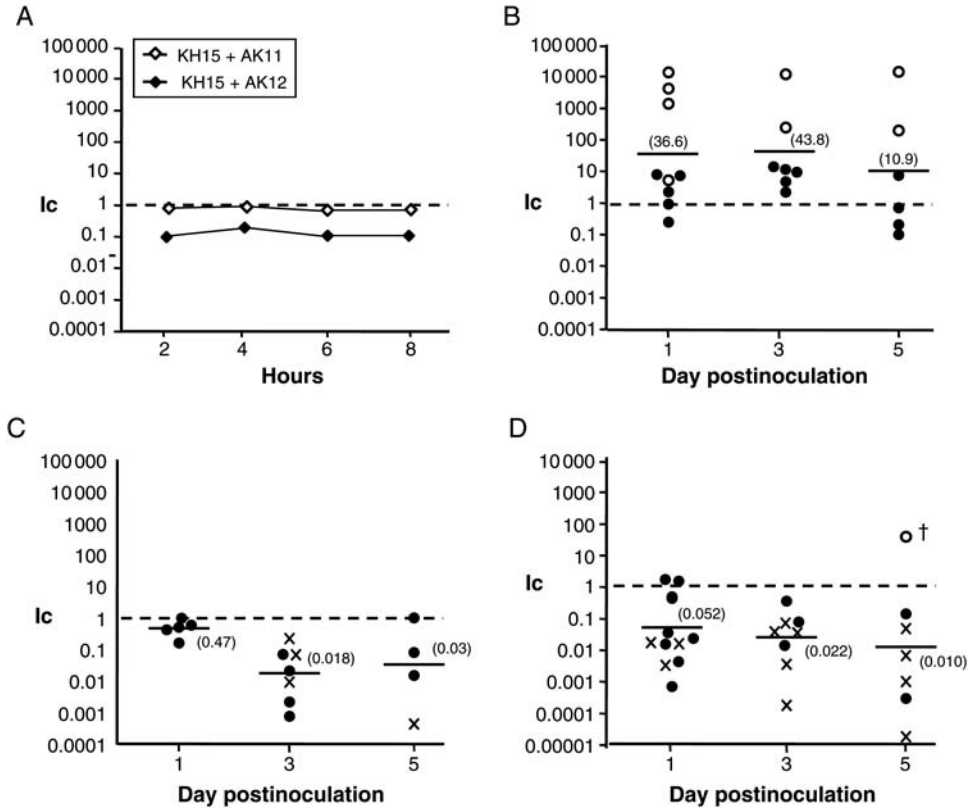

Figure 3. The mutant $gyrA_{91/95}$ allele increases the in vivo fitness of an *mtr* mutant. In vitro and in vivo competition experiments were performed between strains KH15, AK11, and AK12 and competitive index (Ic) values were calculated as described in the legend for Figure 2. *A*, The Ic values for KH15 vs AK11 or AK12 when cocultured in GC broth. *B–D*, results from competitive murine infections with KH15 vs AK11(*B*), KH15 vs AK12 (*C*), and AK11 vs AK12 (*D*). AK11 bacteria showed a marked in vivo fitness advantage relative to the *mtr* mutant parent strain KH15 early in infection. In contrast, AK12 was attenuated in vivo. This result is consistent with the increased fitness of AK11 over AK12 shown in *D*. In *B*, open circles correspond to mice from which only AK11 bacteria were recovered. In *C*, cross hatches refer to mice from which only KH15 bacteria were recovered. In *D*, cross hatches correspond to mice from which only AK11 colony-forming units were recovered. The open circle on day 5 in *D* corresponds to the mouse from which equal numbers of bacteria were recovered on GC media with streptomycin (Sm) and GC media with Sm and 2.0 ug/mL ciprofloxacin, as occurs with strain AK12. A ciprofloxacin-resistant isolate from this mouse was colony purified and later characterized as mutant AK13. All results are combined data from 2 independent experiments.

less fit than the parent strain (Figure 3*A*, closed symbols). As predicted, strain AK11 out-competed AK12 during mouse infections (Figure 3*D*). We conclude that the mutant $gyrA_{91/95}$ allele confers an in vivo fitness advantage to gonococci that already benefit from a mutation that derepresses the *mtrCDE* operon, but that additional acquisition of $parC_{86}$ confers a fitness cost to the gonococcus.

In Vivo Selection of a High-Level Cip^R Mutant With Increased Fitness

In the experiments described above, Cip^I mutants demonstrated an in vivo fitness advantage, regardless of the *mtr* genotype, but Cip^R mutants were compromised. We noted, however, that occasionally only Cip^R colonies were recovered from mice that were inoculated with Cip^R (AK2 or AK12) gonococci mixed with either wild-type or CipI strains (Figure 2*C* [day 5 and 7], 2*D* [day 3], and 3*D*, [day 5], open circles). To further investigate this finding, we analyzed Cip^R bacteria isolated on day 5 from a mouse inoculated with strains AK11 and AK12. Significantly more AK11 (3.3×10^4 CFUs) than AK12 (3.3×10^2 CFUs) were isolated from this mouse on day 1 (Ic, 0.018), followed by isolation of only 8 Cip^I CFUs on day 3, and high numbers (3.3×10^6 CFUs) of only Cip^R bacteria on day 5 (Figure 3*D*, open circle on day 5). One colony from day 5 was propagated further and frozen as mutant AK13. Because both AK11 and AK12 were constructed in the KH15 background, we expected AK13 bacteria to be Em^R. However, surprisingly, Em MICs against FA19Sm^R, AK13, and several other isolates from this mouse were similar (Table 3). Consistent with this finding, the nucleotide sequence of the *mtr* locus of mutant AK13 was identical to the wild-type sequence found in the parent strain FA19Sm^R in that the single bp deletion in the *mtrR* promoter was repaired. The *parC* gene of mutant AK13 carried the same $parC_{86}$ mutation found in

mutant AK12, but interestingly, the *gyrA* gene of AK13 was predicted to encode Ser91Leu instead of Ser91Phe due to a single bp change. The in vivo–selected mutant AK13 grew more rapidly than KH15, AK11, and AK12 bacteria in vitro (Figure 1*C*; Table 3). We conclude that ≥1 compensatory mutations occurred that allowed Cip^R gonococci to outcompete Cip^I bacteria in the murine genital tract.

DISCUSSION

Selection for antibiotic resistance mutations in the face of increasing antibiotic pressure is a commonly observed phenomenon. Resistance mutations often reduce the fitness of bacterial pathogens in the absence of antibiotics [37, 43–46]. Resistance mutations can also confer a fitness benefits. Examples of mutations that increase in vivo fitness include repressor gene mutations that result in overproduction of multidrug active efflux pumps that expel both antibiotics and antimicrobial substances of the host innate defense [15, 18, 27] and topoisomerase mutations in *Campylobacter jejuni* [16] and *E. coli* [17], which increased bacterial colonization in chicken and mouse infection models, respectively.

Here we report that *gyrA* mutations that confer a Cip^I phenotype to *N. gonorrhoeae* cause a fitness advantage in a genital tract infection model and that this advantage is abrogated by an additional mutation in *parC* that increases the Cip MIC to a clinically relevant level. This finding is intriguing because it suggests Cip^I strains may serve as a reservoir for Cip^R in *N. gonorrhoeae* because a single-step mutation in *parC* is all that is then required for resistance. It is important to note that the mutant strains used here may not accurately reflect all the adaptive mutations that are likely to accumulate in antibiotic-resistant clinical isolates in response to the resistance mutations or other pressures; such mutations may indirectly modify fitness costs to the organism, as proposed by Marcusson et al [17] in studies with highly Cip^R *E. coli*. Such a process could also occur with *N. gonorrhoeae*, especially in environments with constant low levels of antibiotic pressure, as seen with indiscriminate use of fluoroquinolones in clinical practice or where self-medication is common.

We do not know why the *gyrA* mutant studied here displayed a fitness benefit in vivo. The mechanism for increased fitness of *mtr* mutant gonococci is likely increased resistance to host innate defenses [18, 22, 23]; whether $gyrA_{91/95}$ mutations further protect against host defenses or alter a different aspect of gonococcal adaptation to the host is not known. Changes in gene expression due to alterations in supercoiling have been proposed to explain the fitness benefit of topoisomerase mutations in other bacterial pathogens [17, 19]. Because secondary compensatory mutations can reverse the detrimental effects of resistance mutations [14, 20], it is possible that compensatory mutations may have occurred in the g*yrA* mutants that increase survival or growth of the gonococcus in vivo. With regard to the observed in vivo fitness cost of high level Cip^R, this result may simply reflect the net balance of the in vivo advantage due to the $gyrA_{91/95}$ mutation and the more severe growth defect construed by mutation in $parC_{86}$. It is also possible 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. Identification of the mechanism by which *gyrA* mutations enhance the fitness of *N. gonorrhoeae* during experimental murine infection might help delineate how *parC* mutations and/or compensatory mutations balance fitness costs or benefits.

An important question is: can compensatory mutations that influence fitness occur during infection? We believe this to be the case based on the isolation of a highly Cip^R mutant from a mouse in pure culture in which growth in vitro was restored to wild-type levels. The likely parent strain, AK12, exhibited the most pronounced in vitro fitness cost and carried 2 mutated alleles, *gyrA*, *parC,* and an *mtr* mutation. Loss of the $mtrR_{-79}$ mutation in strain AK13 may have reversed or contributed to reversing this growth defect, although because the $mtrR_{-79}$ mutation is known to enhance in vivo fitness in this model [18], one might predict that selection against this mutation would be rare. Other compensatory mutations also may be present and whether the altered mutant GyrA subunit produced by mutant AK13 also impacts fitness is not known. Although the Ser91Phe *gyrA* mutation is most frequently identified among QRNG strains [8, 47], Cip^R isolates with the Ser91Leu GyrA substitution have been identified [48]. Other factors not yet defined must increase fluoroquinolone resistance beyond that conferred by *gyrA* and *parC* mutations. Quinolone-resistant *N. gonorrhoea* clinical isolates often have a Cip MIC > 16 ug/mL, as was the case for strain C29 used as the source of the *gyrA* and *parC* mutations examined in our study. The consequence of high level Cip^R on microbial fitness is not known and can be studied in the murine model used herein once the responsible mutations are identified.

In summary, the demonstration that gonococci with commonly isolated *gyrA* mutations exhibit increased fitness in a genital tract infection model suggests that Cip^I strains may serve as a reservoir for QRNG. Due to the overabundant use of fluoroquinolones for other disease processes, a low level of antibiotic pressure is likely to contribute to the persistence of these strains. Additionally, we have shown that although *gyrA, parC* mutants are attenuated in vivo, compensatory mutations can occur that restore fitness while maintaining Cip^R. Continued investigation of the frequency and nature of compensatory mutations is crucial in understanding the spread of QRNG. Additionally, defining the basis of the fitness advantage observed in *gyrA* mutants may lead to new insights into survival mechanisms utilized by *N. gonorrhoeae* during infection.

Notes

Disclaimer. The opinions or assertions contained in this article are the private views of the authors and are not to be construed as reflecting the views of the US Army, Air Force, or the Department of Defense.

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