# Mutation Supply and Relative Fitness Shape the Genotypes of Ciprofloxacin-Resistant *Escherichia coli*

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## Abstract

**Ciprofloxacin is an important antibacterial drug targeting Type II topoisomerases, highly active against Gram-negatives including *Escherichia coli*. The evolution of resistance to ciprofloxacin in *E. coli* always requires multiple genetic changes, usually including mutations affecting two different drug target genes, *gyrA* and *parC*. Resistant mutants selected *in vitro* or *in vivo* can have many different mutations in target genes and efflux regulator genes that contribute to resistance. Among resistant clinical isolates the genotype, *gyrA* S83L D87N, *parC* S80I is significantly overrepresented suggesting that it has a selective advantage. However, the evolutionary or functional significance of this high frequency resistance genotype is not fully understood. By combining experimental data and mathematical modeling, we addressed the reasons for the predominance of this specific genotype. The experimental data were used to model trajectories of mutational resistance evolution under different conditions of drug exposure and population bottlenecks. We identified the order in which specific mutations are selected in the clinical genotype, showed that the high frequency genotype could be selected over a range of drug selective pressures, and was strongly influenced by the relative fitness of alternative mutations and factors affecting mutation supply. Our data map for the first time the fitness landscape that constrains the evolutionary trajectories taken during the development of clinical resistance to ciprofloxacin and explain the predominance of the most frequently selected genotype. This study provides strong support for the use of *in vitro* competition assays as a tool to trace evolutionary trajectories, not only in the antibiotic resistance field.**

***Key words:*** **ciprofloxacin, multistep evolution, population bottleneck, modeling evolution, clinical isolates.**

## Introduction

Fluoroquinolones are an important group of antimicrobial drugs used to treat a variety of indications, including urinary tract infections (UTI) that are primarily caused by *Escherichia coli* (Naber et al. 2001; Kaper et al. 2004; Alteri and Mobley 2015). The most commonly used fluoroquinolone for Gram-negative infections, including UTIs, is ciprofloxacin, but resistance is increasing in frequency among clinical isolates (Fasugba et al. 2015; Bryce et al. 2016). Ciprofloxacin targets two essential enzymes, DNA gyrase (two subunits encoded by *gyrA* and *gyrB*), and topoisomerase IV (two subunits encoded by *parC* and *parE*), with DNA gyrase being the primary target in *E. coli* (Hooper 1999).

The *in vitro* selection of *E. coli* mutants with a reduced susceptibility to ciprofloxacin, or to the related drug norfloxacin, led to the identification of several different amino acid substitutions at a small number of positions in the quinolone resistance determining regions (QRDR) of DNA gyrase (Yoshida et al. 1990; Heisig and Tschorny 1994; Komp Lindgren et al. 2005) and topoisomerase IV (Heisig 1996; Kumagai et al. 1996). In addition, mutations that cause upregulation of drug efflux pumps were also shown to reduce susceptibility to ciprofloxacin (Li et al. 2015; Pietsch et al. 2017). The *in vitro* selection data, from single and multistep selections, suggests that the initial event in resistance evolution is a mutation in *gyrA* usually affecting residue S83 or D87. In clinical isolates of *E. coli* the genetics of ciprofloxacin resistance evolution is complicated because multiple genetic changes must occur before the clinical breakpoint, ciprofloxacin MIC of 1 mg/L (van der Bij et al. 2012), is reached. Sequence analysis of clinical isolates has identified several different amino acid substitutions, in particular at residues S83 and D87 in *gyrA*, and residues S80 and E84 in *parC* that are strongly associated with fluoroquinolone resistance in clinical *E. coli* isolates (Everett et al. 1996; Komp Lindgren et al. 2003; Hopkins et al. 2005; Johnson et al. 2010; Nazir et al. 2011; Baudry-Simner et al. 2012; Johnson et al. 2013; Betitra et al. 2014). Clinical isolates can also carry additional mutations that upregulate drug efflux (Komp Lindgren et al. 2003; Li et al. 2015), and may additionally carry a plasmid-borne resistance gene (Nazir et al. 2011; Jacoby et al. 2014). However, there is no individual resistance mutation or resistance gene that is sufficient to confer clinical resistance. The common denominator of almost all resistant clinical isolates is that they carry mutations at three or four key residues in *gyrA* and *parC*, with a strong bias in favor of a few specific







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allele combinations. This suggests that during the process of evolving clinical resistance there is a strong selection for a subset of genotypes with the most favorable allele combinations. However, it is not currently understood why these particular allele combinations are favored in resistant clinical isolates.

To address this question we combined experimental measurements of mutation rates, drug susceptibility, and relative fitness, with mathematical modeling of evolution under a variety of conditions with different population sizes, transmission bottlenecks, and degrees of drug selective pressure. The data were used to map evolutionary trajectories from a susceptible wild-type to the multiple-mutant genotypes most frequently found in resistant clinical isolates. We identified favored trajectories of evolution that point to the critical importance of relative fitness, and of mutation supply, in selecting the most frequent clinical genotypes.

## Results

### Analysis of Ciprofloxacin-Resistant Clinical Isolate Datasets

Published datasets for which ciprofloxacin MIC values, and the QRDR sequences of *gyrA* and *parC* for individual clinical isolates of *E. coli* have been reported, were examined. The analysis was confined to the key resistance-associated residues S83, D87 in *gyrA*, and S80, E84 in *parC*. The data (supplementary table S1, Supplementary Material online) were taken from five publications, reporting a total of 195 resistant clinical isolates from Europe, North and South America, Asia, and Africa (Everett et al. 1996; Komp Lindgren et al. 2003; Nazir et al. 2011; Baudry-Simner et al. 2012; Betitra et al. 2014). Most isolates, regardless of origin, have *gyrA* S83L in combination with *parC* S80I (79%, 154/195), and the great majority (73%, 142/195) have the double mutation *gyrA* S83L together with D87N. The single most frequent genotype (37%, 73/195), with a high frequency across all five collections, is *gyrA* S83L D87N, *parC* S80I (and if an additional *parC* mutation, E84K/V/G/A, is included, the frequency of this genotype class is 65%, 126/195). From analyzing the data for these four key residues, we concluded (i) that the primary event affecting the drug target in clinical resistance evolution is most likely the occurrence of a single mutation in *gyrA*; and (ii) that the evolution of clinical resistance usually results in a multiply mutant drug target genotype with *gyrA* S83L D87N together with a single or double mutation in *parC* (most often S80I, and/or E84K/V/G/A). This conclusion is also in agreement with a recent analysis of the genotype of the widespread fluoroquinolone-resistant ST131 clone of *E. coli* (Johnson et al. 2013). The fact that the successful fluoroquinolone-resistance genotype is so widespread, and is found in genetically distinct lineages, argues strongly that this genotype has intrinsic advantages and is not simply hitchhiking with putative virulence factors associated with a single successful clone.

### Whole Genome Sequence Analysis of Ciprofloxacin-Resistant Clinical Isolates

Mutations in additional target enzyme genes (*gyrB* and *parE*), as well as in genes that regulate ciprofloxacin efflux (*acrR*, *marR*, and *soxR*), have previously been linked to ciprofloxacin resistance (Komp Lindgren et al. 2003; Piddock 2006; Li et al. 2015). We did whole genome sequencing of 30 resistant strains from one of the collections with phylogenetically diverse clinical isolates (Komp Lindgren et al. 2003) to determine the frequency of additional mutations in these other genes of interest. The data (supplementary table S2, Supplementary Material online) showed that all 30 isolates carried a *gyrA* S83L mutation as well as a mutation in *parC* at position S80 and/or E84. Additionally, 28/30 carried a mutation of D87 in *gyrA*. Only one isolate carried a putative resistance mutation in *gyrB*, while seven carried putative resistance mutations in *parE*. In a previous study (Komp Lindgren et al. 2003), 15 of these 30 isolates were shown to have organic solvent tolerance (OST), a phenotypic measure of increased efflux pump activity. There is a very good correlation between the OST phenotype and the occurrence of putative resistance mutations in one or more of the efflux regulator genes, *acrR*, *marR*, and *soxR* (mutations in 14 of 15 isolates with an OST phenotype). Most of the mutations are in *acrR* (13 in *acrR*, 8 in *marR*, and 1 in *soxR*). The efflux regulator mutations identified in at least 6 of the 15 isolates are predicted to be knockout mutations of their respective regulators (frameshift mutations, nonsense mutations, or IS element insertions) supporting the conclusion that they cause an increase in ciprofloxacin efflux. Interestingly, the presence of efflux regulator mutations correlates strongly with isolates that have the highest MIC values. Twelve of the 16 isolates with efflux regulator mutations have MICs of $\geq$ 32 mg/L (supplementary table S2, Supplementary Material online). This correlation between efflux mutations and higher MIC values is compatible with the efflux mutations becoming part of the genotypes of these isolates at a late stage in their evolution. We concluded that the dominant resistance genotype (*gyrA* S83L D87N, *parC* S80I) is found in phylogenetically diverse isolates, strongly suggesting that this genotype arises independently because it has intrinsic advantages over other resistance genotypes.

Based on this analysis of the genotypes of ciprofloxacin-resistant clinical isolates, we determined that the most relevant drug target mutations were *gyrA* S83L D87N, and *parC* S80I, with the frequent addition of a fourth target mutation in *parC* or *parE* and/or a mutation in *acrR* or *marR*. We decided to experimentally measure parameters that we hypothesized could be relevant to the accumulation of the genotypic changes associated with the evolution of this genotype. We took advantage of a collection of isogenic strains, carrying relevant resistance mutations, and previously characterized in terms of MIC and relative fitness (supplementary table S3, Supplementary Material online).

### Selection of the Initial Mutation: *gyrA* S83L

Almost every ciprofloxacin resistant clinical isolate of *E. coli* carries a mutated *gyrA* allele (99%, 194/195, supplementary table S1, Supplementary Material online). This indicates that the initial step in the evolution of clinical ciprofloxacin resistance is the selection of a mutation in *gyrA*. To test this hypothesis, we performed a fluctuation test on

wild-type *E. coli* to measure the spontaneous rate of mutations for reduced susceptibility to ciprofloxacin at 4-fold MIC (Materials and Methods). The rate of mutation was $3.6 \times 10^{-9}$, in the range expected for amino acid substitution mutations. The QRDR of *gyrA* was sequenced from 50 independently selected mutants. Eight different amino acid substitution mutations in *gyrA* were identified in the QRDR in 43 of the 50 mutants (supplementary table S4, Supplementary Material online) with *gyrA* S83L, D87G, and D87Y each accounting for 25–28% of the mutants. D87N, the second most frequent *gyrA* allele in clinical isolates occurred infrequently (1/43 *gyrA* mutations). Next, we constructed a set of isogenic *E. coli* strains that each carry one of the *gyrA* mutations most frequently found in clinical isolates (*gyrA* S83L, D87G, D87Y, and D87N), the most common *parC* allele (*parC* S80I), as well as deletions of the drug efflux regulators AcrR and MarR (Δ*acrR* and Δ*marR*) and measured ciprofloxacin MICs of the constructed strains. The different *gyrA* mutations conferred a 10- to 20-fold higher increase in ciprofloxacin MIC than mutations in *parC*, *acrR*, or *marR* (supplementary table S3, Supplementary Material online), consistent with their predominance in the selection of resistant mutants. To assess the fitness of a strain carrying the most frequent clinical allele, *gyrA* S83L, relative to other alleles of *gyrA* frequently found in clinical isolates we did growth competition experiments as a function of ciprofloxacin concentration (fig. 1). The strain carrying *gyrA* S83L outcompeted strains with alternative *gyrA* alleles across a range of ciprofloxacin concentrations, and the relative advantage was greatest at the higher concentrations (fig. 1). This selective advantage could explain the very high frequency of *gyrA* S83L among resistant clinical isolates (98%, 191/195, supplementary table S2, Supplementary Material online) and supports that its selection is the first step towards a clinical resistant phenotype.

## Selection of the Second Mutation: *parC* S80I

We asked which mutation is most frequently selected after the initial selection of *gyrA* S83L. Analysis of the genotypes of resistant clinical isolates strongly suggests that the mutation most frequently selected subsequent to *gyrA* S83L is either *parC* S80I (78%, 149/191) or *gyrA* D87N (74%, 142/191) (supplementary table S1, Supplementary Material online), but second-step mutations in the drug efflux regulators AcrR and MarR would also significantly increase ciprofloxacin MIC (supplementary table S3, Supplementary Material online). We used a fluctuation test to measure the mutation rate at 4-fold MIC using the *gyrA* S83L strain as a starting point. The mutation rate measured was $3.8 \times 10^{-6}$. This relatively high mutation rate is expected if the mutational target size is large, for example, because it includes mutations that inactivate genes encoding repressors of drug efflux pumps. We analyzed 11 independently selected mutants by whole genome sequencing. Each mutant had acquired a mutation in a gene coding for a regulator of the AcrAB-TolC efflux pump. Over half of the mutations affected *marR*, and in most cases they were predicted to be gene-inactivating mutations (supplementary table S5, Supplementary Material online). This result is in agreement with the mutation rate data, but contradicts the expectation, based on the clinical data, that the second-step mutation would be in *gyrA* or *parC* (supplementary table S2, Supplementary Material online). To address this discrepancy, we assayed MIC and competitive fitness of four isogenic strains that carry the primary *gyrA* S83L mutation in combination with each class of potential second-step mutation (*gyrA* D87N, *parC* S80I, Δ*marR*, and Δ*acrR*). Two of the four strains, *gyrA* S83L *parC* S80I, and *gyrA* S83L Δ*marR* had identical ciprofloxacin MICs of 0.75 mg/L, significantly higher than the MICs of the other two strains (supplementary table S3, Supplementary Material online). Next we compared the relative fitness of the double mutants, competing the *gyrA* S83L *parC* S80I strain against each of the other strains. The results (fig. 2) showed that the *gyrA* S83L *parC* S80I double

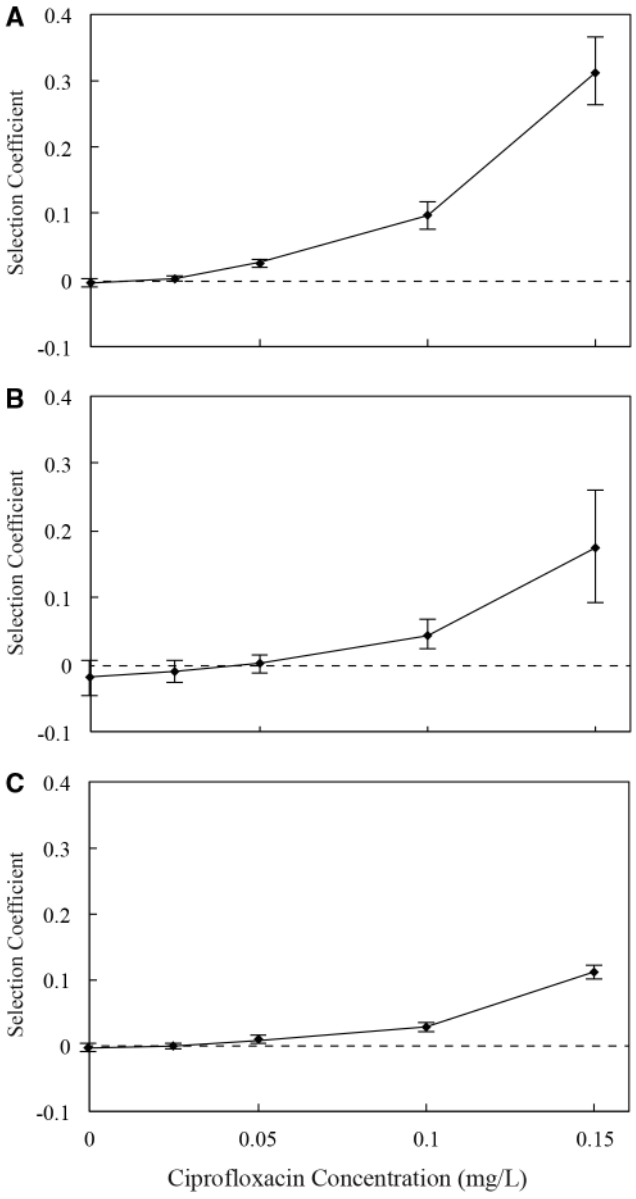


FIG. 1. Competitive fitness of the *gyrA* S83L mutant as function of ciprofloxacin concentration in competition against (**A**) *gyrA* D87G; (**B**) *gyrA* D87Y; and (**C**) *gyrA* D87N. Values are mean ± standard deviation.

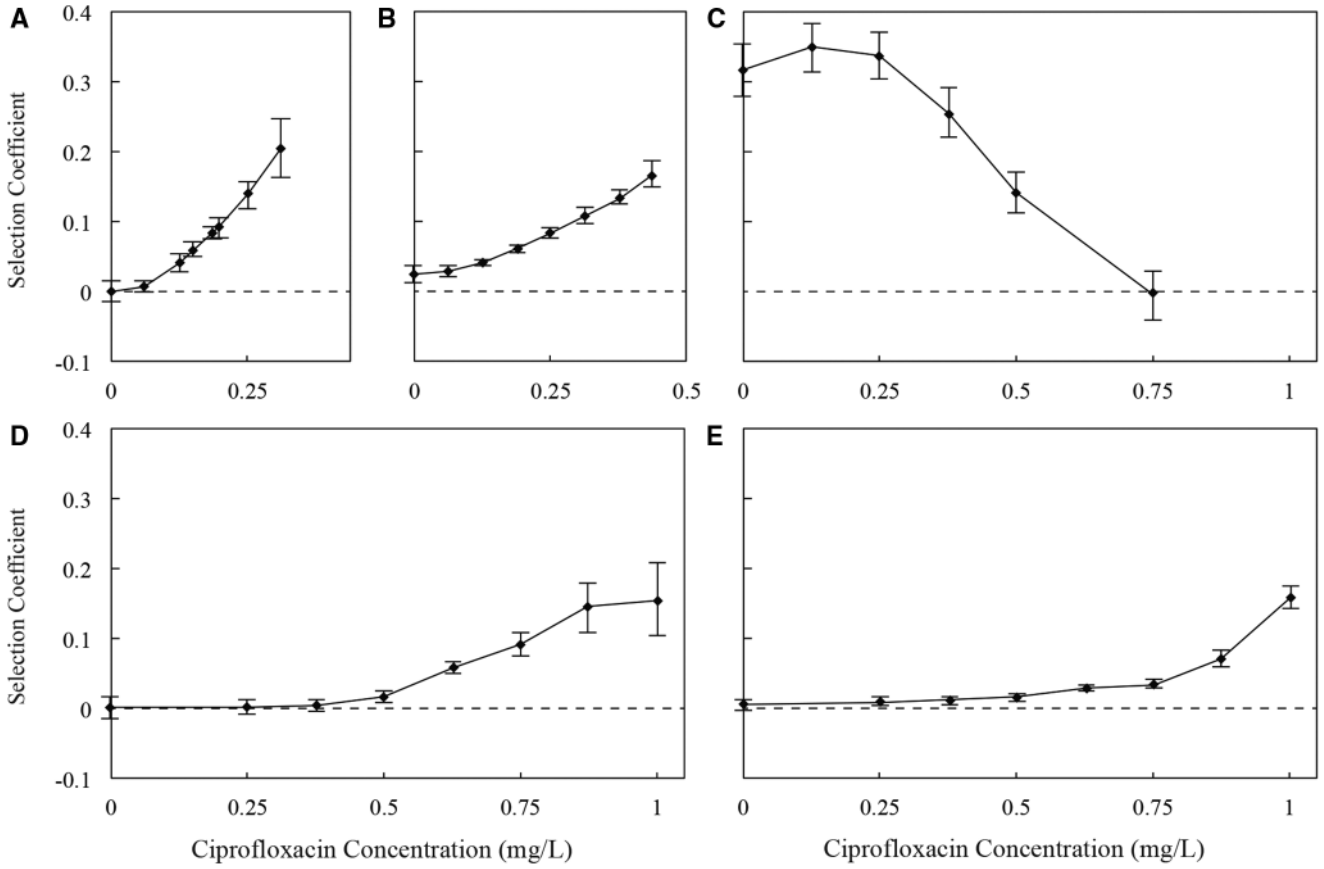


**Fig. 2.** Competitive fitness of the *gyrA* S83L *parC* S80I double mutant as function of ciprofloxacin concentration in competition against (***A***) *gyrA* S83L D87N; (***B***) *gyrA* S83L Δ*acrR*; (***C***) *gyrA* S83L Δ*marR*; (***D***) *gyrA* S83L *parC* S80R; and (***E***) *gyrA* S83L *parC* E84K. Values are mean ± standard deviation.

mutant has a significant competitive advantage over the *gyrA* S83L D87N (fig. 2*A*) and *gyrA* S83L Δ*acrR* strains (fig. 2*B*) that increased as a function of increasing drug concentration. This result is consistent with the *gyrA* S83L *parC* S80I double mutant having a higher MIC than either of the other two strains. Importantly, the *gyrA* S83L *parC* S80I double mutant also had a large fitness advantage over the *gyrA* S83L Δ*marR* strain (fig. 2*C*). In this case, the advantage was evident even in the absence of drug and was maintained in the presence of drug until a concentration at which the growth of both strains was suppressed. This result is compatible with these two strains having identical ciprofloxacin MIC values. We concluded that the *gyrA* S83L *parC* S80I double mutant has a competitive growth advantage over each of the alternative double mutants.

Based on the superior MIC and relative fitness values, we conclude that mutation in *parC* is the most likely second step in the evolution of ciprofloxacin resistance. We next conducted an experimental evolution experiment with the *gyrA* S83L mutant strain, to test whether rare second-step mutations in drug target genes (such as the *parC* S80I mutation) could be selected and enriched if mutation supply was less limiting, even though mutants such as the *gyrA* S83L Δ*marR* double mutant would arise spontaneously much more frequently. The expectation was that if the conditions allowed the *gyrA* S83L *parC* S80I double mutant to occur, then it would ascend to fixation under drug selection because of its superior growth fitness. We evolved ten independent lineages of a strain carrying the *gyrA* S83L mutation at a ciprofloxacin concentration of 0.5-fold MIC. The total population size of each lineage was approximately $4 \times 10^{11}$ cells with a bottleneck of $4 \times 10^{10}$ cells at every transfer. After twelve cycles (38 generations of growth) we plated dilutions of the cultures on LA and isolated two random colonies from each lineage. The ciprofloxacin MIC of most isolates was 0.5–0.75 mg/L (supplementary table S6, Supplementary Material online), consistent with each isolate having a second resistance mutation. We sequenced the *parC* gene of these isolates and found, in contrast to the results of previous selection with the smaller population bottleneck (supplementary table S5, Supplementary Material online), that almost every isolate (18/20) had acquired a mutation in *parC* (supplementary table S6, Supplementary Material online). The majority of these mutations (10/18) were mutations at amino acid *parC* S80 and the mutation *parC* S80I was found in three independent lineages. Whole genome sequencing was done on eight clones carrying *parC* S80I, S80R, or E84K (from independent selections) in each case confirming the presence of *gyrA* S83L and the selected *parC* mutation, and the absence of any efflux regulator mutations. This shows that under conditions where the population size is sufficiently large, that evolution of resistance preferentially selects second-step mutations in *parC*.

Since the evolution experiment resulted in the selection of several different *parC* alleles we asked whether the allele most frequently found in clinical isolates, *parC* S80I, had any competitive advantage over other *parC* alleles found in clinical isolates (*parC* S80R, E84K; supplementary tables S1 and S2, Supplementary Material online). The double mutant *gyrA* S83L, *parC* S80I was competed against isogenic double mutants carrying either *parC* S80R or *parC* E84K. The results show that the *parC* S80I allele has no detectable fitness advantage in the absence of ciprofloxacin but an increasing selective advantage over the alternative *parC* alleles as a function of increasing drug concentrations (fig. 2*D* and *E*).

From these experiments, we concluded that after the selection of *gyrA* S83L the trajectory of ciprofloxacin resistance development could take two paths that were equivalent in terms of MIC (mutations in *marR* or *parC*). Although *marR* mutations arise at a much higher rate than mutations in *parC* they confer a significant fitness cost. Resistant clinical isolates of *E. coli* show a clear predominance of *parC* mutations over *marR* mutations, suggesting that the superior fitness of the *parC* mutation is of paramount importance during the selection of ciprofloxacin resistance. We conclude that the selection of the *parC* S80I allele represents the major second step mutation in clinical ciprofloxacin resistance development.

## Evolution from a Double Mutant to a Triple Mutant: *gyrA* D87N

Sequence data from resistant clinical isolates strongly suggests that the predominant trajectory of evolution from a *gyrA* S83L *parC* S80I double mutant is to acquire a second mutation in *gyrA* at residue D87 but that alternative trajectories could involve the acquisition of mutations in *acrR* or *marR* (supplementary table S2, Supplementary Material online). The mutation rate of the double mutant to higher-level resistance at 4-fold MIC was measured at $1.2 \times 10^{-9}$ consistent with the third mutation being in a drug target gene (small mutational target) rather than in an efflux regulator gene. The alternative mutational trajectory, via acquisition of high-frequency efflux regulator mutations, may be effectively blocked off because the resulting increase in MIC is significantly lower (supplementary table S3, Supplementary Material online). We picked 16 independently selected mutants and sequenced the QRDR of *gyrA*. We found that 14 of the 16 mutants had acquired an additional mutation at position D87 in *gyrA* (7 examples of D87Y, 5 of D87G, and 2 of D87N). Thus, the selection of the double mutant *gyrA* S83L *parC* S80I for increased resistance to ciprofloxacin selects in the majority of cases for a mutant with three drug target alterations. The one surprise from this experiment is that the most frequent genotype found clinically, *gyrA* S83L D87N *parC* S80I, was not the most frequent genotype selected. The low spontaneous frequency of mutation to *gyrA* D87N relative to D87Y or D87G was also noted in the selection of the initial target mutation (supplementary table S4, Supplementary Material online). We asked whether a difference in relative fitness in the presence of ciprofloxacin could account for the greater frequency of the *gyrA* S83L D87N *parC* S80I genotype among clinical isolates (supplementary tables S1 and S2, Supplementary Material online). To test this, we measured the competitive growth fitness of the *gyrA* S83L D87N *parC* S80I triple mutant, at different concentrations of ciprofloxacin, against the *gyrA* S83L D87Y *parC* S80I (fig. 3*A*) and against the *gyrA* S83L D87G *parC* S80I (fig. 3*B*) triple mutant strains. We found no detectable fitness difference between the mutant variants in the absence of ciprofloxacin but *gyrA* S83L D87N *parC* S80I has an increasing selective advantage over *gyrA* S83L D87G *parC* S80I with increasing drug concentrations (fig. 3*B*). Interestingly, we could not detect any difference in fitness for the triple mutants *gyrA* S83L D87N *parC* S80I and *gyrA* S83L D87Y *parC* S80I (fig. 3*A*) at any of the tested drug concentrations. We conclude that selection of the clinically frequent double target mutant, *gyrA* S83L *parC* S80I, for further reduced susceptibility, selects for a secondary mutation in *gyrA*. Our data do not show a clear advantage of the *gyrA* D87N mutation over *gyrA* D87Y under the tested conditions but the clinical data clearly point towards *gyrA* D87N as the most common third-step mutation.

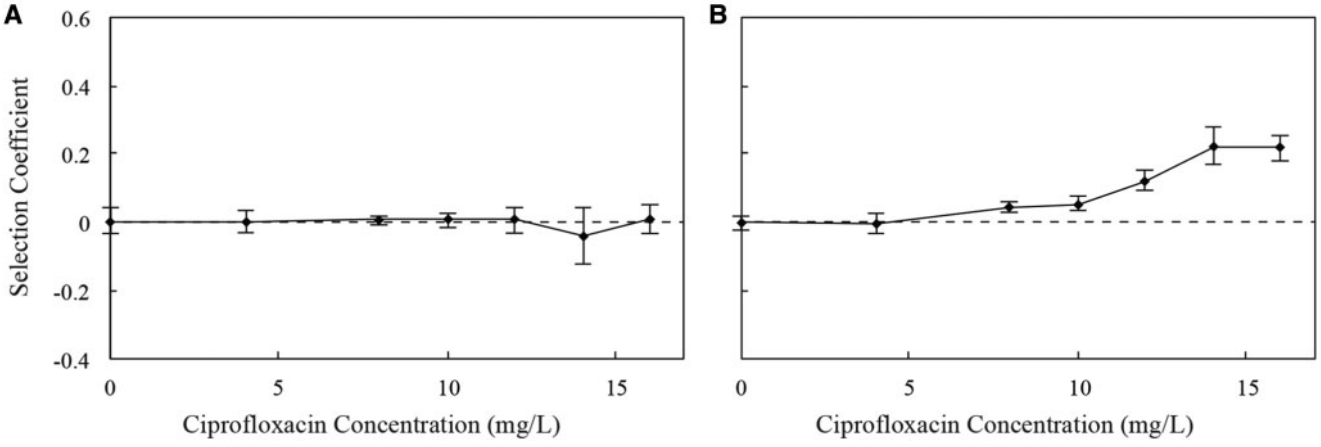


FIG. 3. Competitive fitness of the *gyrA* S83L D87N *parC* S80I triple mutant as function of ciprofloxacin concentration in competition against (***A***) *gyrA* S83L D87Y *parC* S80I and (***B***) *gyrA* S83L D87G *parC* S80I. Values are mean ± standard deviation.

### Subsequent Evolution: *parC*, *parE*, Δ*acrR*, or Δ*marR*

Our data on the selection of a triple mutant, in agreement with clinical sequence data, strongly suggests that the evolution of ciprofloxacin resistance starts with the acquisition of three target mutations, most commonly *gyrA* S83L D87N, *parC* S80I. When constructed in the lab, a strain that carries this genotype confers a ciprofloxacin MIC of 16 mg/L (supplementary table S3, Supplementary Material online), consistent with ciprofloxacin MIC values obtained from clinical isolates that carry the same resistance alleles (supplementary table S2, Supplementary Material online). Interestingly, all clinical isolates with a ciprofloxacin MIC greater than 16 mg/L carry at least one additional mutation, either a fourth target mutation (*parC* or *parE*) and/or one to three efflux regulator mutations (*acrR*, *marR*, or *soxR*) (supplementary table S2, Supplementary Material online). Unlike the first three steps of evolution to ciprofloxacin resistance, there seems to be no apparent bias towards any particular mutation or gene in the further course of evolution. The 17 strains with a ciprofloxacin MIC greater than 16 mg/L carry a total of 28 mutations additional to the first three target mutations. These 28 mutations consist of nine additional target mutations (2× *parC* and 7× *parE*) as well as 19 mutations in the three efflux regulator genes (6× *marR*, 12× *acrR*, 1× *soxR*) and none of the mutations is found more than twice (supplementary table S2, Supplementary Material online).

We conclude that the evolution of ciprofloxacin resistance up to and beyond the clinical breakpoint is severely constrained during the selection of the first three mutations but that a variety of different efflux and target mutations can arise at the fourth and subsequent steps, increasing the MIC to more than 30-fold above the clinical breakpoint.

### Modeling the Development of Ciprofloxacin Resistance

We decided to model the resistance development using only a small number of experimentally determined parameters (relative mutation rates, competitive growth fitness, and MIC) to get a better understanding of the conditions under which the clinical mutant genotype *gyrA* S83L D87N *parC* S80I could evolve (Materials and Methods). The model is based on a wild-type *E. coli* population that is passaged in increasing concentrations of ciprofloxacin starting at 0.004 mg/L ciprofloxacin (0.25-fold MIC of wild-type) and increasing in a step-wise fashion up to 4 mg/L (∼250-fold MIC of wild-type). To model various selective conditions we allowed a total population size of $4 \times 10^9$ with different bottleneck sizes ($4 \times 10^6$–$4 \times 10^8$ cells per passage) and different relative increases in ciprofloxacin concentration (1.1- to 2.0-fold increases per step) as described in Materials and methods. In total, 70 distinct conditions were modeled and for each model 1000 independent experiments were run. In each of these experiments, five different mutations were allowed to appear in any combination and order: (i) *gyrA* S83L, (ii) *gyrA* D87N, (iii) *parC* S80I, (iv) Δ*marR*, and (v) Δ*acrR*. Mutations occurred at random and according to experimentally determined mutation rates, and were selected according to experimentally determined relative fitness and ciprofloxacin MIC values (supplementary table S3, Supplementary Material online). As expected, general resistance development was highly dependent on both bottleneck size and step size in ciprofloxacin concentration increase, such that larger bottleneck sizes and smaller increases in drug concentration led to a higher frequency of resistance development (fig. 4*A*). Next, we decided to focus on the clinically relevant mutant genotype *gyrA* S83L D87N, *parC* S80I. This particular phenotype was generally very rare or not present at all but was more frequently found under conditions where bottleneck sizes were larger and increases in drug concentration were smaller. At the combination of largest bottleneck size and lowest drug concentration increase this specific triple mutant appeared in 22% of independent experiments (fig. 4*B*). We decided to map the alternative trajectories leading to resistance under the conditions with the largest bottleneck size and smallest step size, where the clinically relevant genotype arises most frequently. Fifty independent experiments were analyzed and 10 distinct trajectories were identified of which eight led to ciprofloxacin resistance of 4 mg/L and two reached dead ends at lower concentrations (fig. 5 and supplementary fig. S1, Supplementary Material online). Interestingly, the vast majority (85%) of experiments that led to a clinically relevant resistance level (>1 mg/L) acquired the *gyrA* S83L mutation as the first resistance mutation which is in agreement with the observation that almost all clinically resistant *E. coli* isolates carry this specific mutation. Furthermore, all experiments that led to the clinically relevant triple-mutation genotype followed the same mutational order: first *gyrA* S83L, second *parC* S80I, and third *gyrA* D87N. This particular order is highly consistent with our experimental data and the sequencing data of clinical resistant isolates.

Our modeled data showed that large bottleneck sizes favor the clinically relevant genotype. We decided to test if a larger population size and a subsequently larger bottleneck size would lead to an increased frequency of the clinically relevant triple mutant. Resistance development was modeled with the same parameters as before but the population size was increased 10-fold to $4 \times 10^{10}$ cells, which also led to a 10-fold increase in bottleneck sizes ($4 \times 10^7$–$4 \times 10^9$ cells per passage). We observed that the larger population size significantly increased the frequency of resistance development (fig. 4*C*). The clinically relevant triple mutant genotype *gyrA* S83L D87N, *parC* S80I appeared more frequently and was the only resistant genotype found at several of the tested combinations of bottleneck size and increases in drug concentration (fig. 4*D*).

Taken together the modeling results, based on experimentally determined parameters, indicate that clinical resistance to ciprofloxacin develops under conditions where mutation supply is not limiting, conditions that could be fulfilled by either a large number of cells, or a high mutation rate. Under these conditions, resistance can develop frequently even if drug concentrations increase rapidly. The modeling also suggests that there is one main trajectory of mutational evolution that leads to clinically relevant resistance, namely, (i) *gyrA* S83L, (ii) *parC* S80I, and finally (iii) *gyrA* D87N. This trajectory

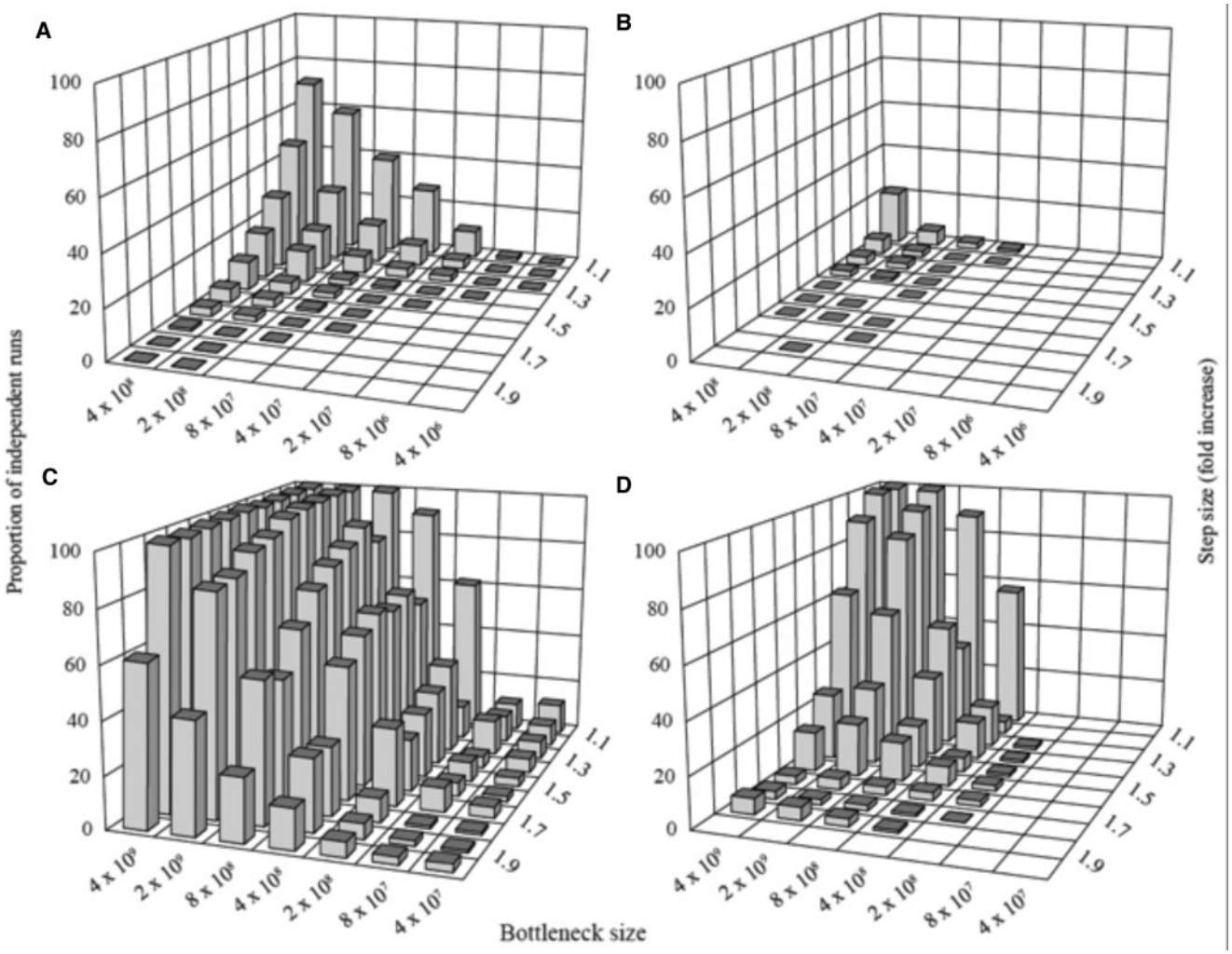


**Fig. 4.** Outcome of population modeling at ciprofloxacin concentration of 4 mg/L. The resistance development model (Materials and Methods) was run for 70 distinct combinations of bottleneck sizes and stepwise increases in ciprofloxacin concentrations with a total population size of $4 \times 10^9$ (***A***,***B***) or $4 \times 10^{10}$ (***C***,***D***). Results are averages of 1000 independent runs. (*A*, *C*) Proportion of runs that survive up to a concentration of 4 mg/L. (*B*, *D*) Proportion of runs that develop the clinically relevant genotype *gyrA* S83L D87N, *parC* S80I.

is in full agreement with the sequencing results of clinical *E. coli* isolates as well as our *in vitro* selection data.

## Discussion

We combined sequence analysis of resistant clinical isolates, and experimental measurements of resistance-related parameters (mutation rate, MIC, and relative fitness), with *in silico* modeling, to develop an understanding of the major trajectories of ciprofloxacin-resistance evolution in *E. coli*. The results of these three approaches are highly consistent with each other, and they explain the prevalence of a few dominant genotypes and the order in which the mutations are accumulated. In addition, the modeling suggests that clinical resistance evolves preferentially under conditions where mutation supply is large. However, even small populations can evolve the clinical resistance genotype if the drug selection pressure is very low.

Under the conditions predicted to drive resistance evolution the relative fitness of competing mutant genotypes is paramount. This probably explains why the early stages in resistance evolution, from wild type to triple mutant, are dominated by rare but relatively cost-free drug-target mutations (fig. 5). The high-frequency efflux pump regulator mutations are in contrast costly and only accumulate late in evolution, in agreement with genome sequence analysis of clinical isolates (supplementary table S2, Supplementary Material online). An implication of this data is that the interplay between mutation supply and relative fitness is potentially limiting for resistance development during the early stages of resistance development, where three successive specific point mutations must occur and be selected in two different drug target genes. Deviations from this set of mutations in clinical strains may represent cases of genetic drift allowing suboptimal mutations to become fixed in populations subject to narrow bottlenecks. This requirement for three successive specific point mutations might be part of the explanation for the previously observed correlation between clinical resistance to ciprofloxacin in *E. coli* and greater than wild-type mutation rates (Komp Lindgren et al. 2003). Selection for very specific point mutations in target genes can

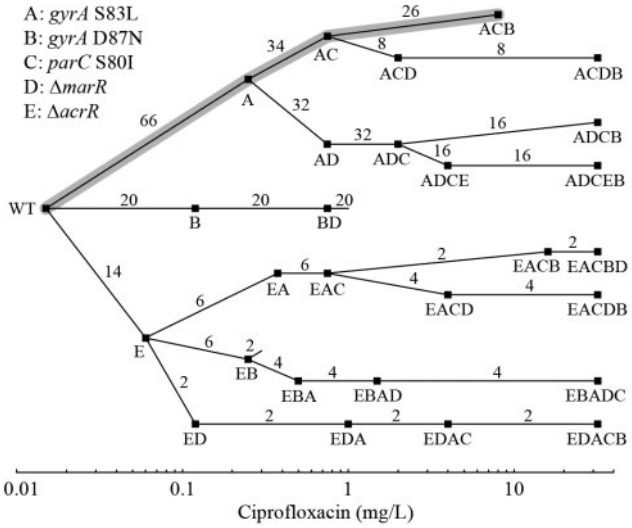


**Fig. 5.** Trajectories of resistance development. The resistance development model (Materials and Methods) was run fifty times with a bottleneck of $4 \times 10^8$ cells per passage and a 1.1-fold increase per cycle in ciprofloxacin to a final concentration of 4 mg/L. Filled squares show genotypes of strains that appear during the evolution. All strains are places at their respective MIC values and labeled in the order of mutation appearance (A: *gyrA* S83L, B: *gyrA* D87N, C: *parC* S80I, D: Δ*marR*, and E: Δ*acrR*). Lines that connect two mutants indicate a mutational event and lines with an open end indicate lineages that went extinct. Numbers on top of the lines show percentage of runs that followed the particular trajectory. The main trajectory (wild type to clinical relevant triple mutant ACB) is highlighted with in grey.

be satisfied either by having very large population sizes or by smaller populations with higher mutation rates.

One could argue that because the experiments were performed under optimal conditions in broth *in vitro* the results might not reflect the competitive situation during an infection in the urinary tract, where the environment might be more hostile, and where for example, a low pH could decrease the efficacy of fluoroquinolones. However, the mathematical model is based on relative fitness values compared with the wild-type. Although these values were measured under optimal conditions, the modeling also reflects all conditions that affect the absolute fitness of the various mutants to the same extent, as urine conditions are also expected to do. Slow growth or other conditions that decreased ciprofloxacin activity would accordingly shift the selection of mutants towards a higher absolute ciprofloxacin concentration but would not be predicted to necessarily affect the order in which mutants/mutations were selected.

A possible implication of our data and models is that clinical resistance evolution might be selected in the wider environment at very low drug concentrations (Gullberg et al. 2011; Hughes and Andersson 2012). Although our data and modeling do not specifically address environmental selection, they show that drug concentrations only slightly higher than MIC can preferentially select the evolution of the common clinical resistance genotype. This situation could also occur in patients, for example when drug therapy (for whatever reason) fails to achieve the concentration required to clear the infection, or when the affected bacteria reside in a body compartment where they are exposed to sub-lethal drug doses. One could ask whether different mutational types or classes would occur and be selected under conditions where the bacteria are exposed to gradients of drugs, for example mutations affecting outer membrane porins. We have found no evidence that other types of mutation with a low-level resistance/low cost phenotype, play an important role in the selection of ciprofloxacin resistance. In addition, whole genome sequencing of clinical and experimentally evolved mutants has not revealed mutations affecting the outer membrane. However, importantly, our data and modeling show that the clinically observed resistance genotypes can also be effectively selected at relatively high drug concentrations, as long as the mutation supply is not limiting (large population sizes and/or high mutations rates).

In conclusion our experimental data and modeling show how high-level mutational resistance to ciprofloxacin in *E. coli* can evolve under conditions of low drug selective pressure (as found environmentally), but also under conditions of relatively high drug selective pressure as long as mutation supply is not limiting. In addition, we have shown that the interplay between susceptibility and relative fitness associated with different genotypes is the most critical factor determining which mutations are selected during the evolution of clinically successful resistant mutants. Our data suggest that therapeutic regimens that reduce bacterial population sizes and maximize bacterial exposure to high drug concentrations would be the most effective in slowing down the rate of resistance evolution, at least for drugs where the resistance requires the successive accumulation of multiple mutations. Finally, the robustness and clarity of the data generated in this study support the use of *in vitro* competition assays more generally, as an important tool to trace evolutionary trajectories, and not only in the antibiotic resistance field.

## Materials and Methods

### Bacterial Strains and Growth Conditions

Bacterial strains are isogenic with *E. coli* MG1655 and are listed in supplementary table S3, Supplementary Material online. Selected mutants are referred to in the text. Bacteria were grown in Luria Broth (LB) and on Luria Agar (LA, LB supplemented with 1.5% agar), or Mueller Hinton agar (Becton Dickinson & Company, France) where indicated in the text, with incubation at 37°C. Chromosomal mutations were moved between strains as previously described (Näsvall et al. 2016) with appropriate selections as detailed in the text.

### Antibiotics and Susceptibility Testing

Ciprofloxacin was purchased from Sigma Aldrich (Stockholm, Sweden) and was dissolved in 0.1 M HCl at 1 mg/mL before use. Ciprofloxacin was added to liquid and solid media for selections at the concentrations stated in the text. Minimal inhibitory concentrations (MIC) were determined in diffusion assays using M.I.C.E.valuator strips (Oxoid, Basingstoke, UK) on Mueller Hinton II agar (Becton Dickinson & Company, France). Plates were incubated at 37°C overnight.

## Mutation Rates

Mutation rates to reduced susceptibility at different concentrations of ciprofloxacin were measured in fluctuation tests (Luria and Delbruck 1943) as previously described (Abdulkarim and Hughes 1996). Each mutation rate is based on a fluctuation test with 40 independent bacterial cultures.

## PCR and DNA Sequencing

The genome sequence of MG1655 was used to design primers for PCR amplification and DNA sequencing. DNA was amplified using Fermentas 2× PCR Mastermix (Thermo Scientific, Waltham, USA) according to the protocol of the manufacturer with 0.5 μM forward and reverse primers for the QRDR of *gyrA* and *parC* as appropriate (supplementary table S7, Supplementary Material online) and 1 μl of DNA sample using a S1000 Thermal Cycler (Bio-Rad Laboratories, Berkley, California, USA). PCR was initiated by denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Amplification products were visualized by agarose gel electrophoresis and GelRed staining to assess the sizes of the gene fragments. DNA sequencing of PCR products was performed at Macrogen Europe, Amsterdam, The Netherlands. Sequences were analysed using CLC Main Workbench 6.6.2 (CLCbio, Qiagen, Denmark).

## Whole Genome Sequencing

Genomic DNA was prepared using the MasterPure DNA Purification Kit (Epicentre, Illumina Inc., Madison, Wisconsin). Final DNA was resuspended in EB buffer. Genomic DNA concentrations were measured in a Qubit 2.0 Fluorometer (Invitrogen via ThermoFisher Scientific). DNA was diluted to 0.2 ng/mL in water (Sigma-Aldrich, Sweden) and the samples were prepared for whole genome sequencing according to Nextera® XT DNA Library Preparation Guide (Illumina Inc., Madison, Wisconsin). After the PCR cleanup-step, samples were validated for DNA fragment size distribution using the Agilent High Sensitivity D1000 ScreenTape System (Agilent Technologies, Santa Clara, California). Sequencing was performed using a MiSeq™ desktop sequencer, according to the manufacturer's instructions (Illumina Inc., Madison, Wisconsin). The sequencing data were aligned and analyzed in CLC Genomics Workbench version 8.0.3 (CLCbio, Qiagen, Denmark).

## Growth Competition Experiments

Strains for competition experiments were constructed by P1 transduction from donors containing *galK::mTagBFP2-kanR*, *galK::dTomato-kanR*, or *galK::SYFP2-kanR* cassettes. For each competition, independent cultures of each strain were grown for 16 h at 37 °C in LB. The fluorescently marked strains to be competed were mixed 1:1 and then used to inoculate 180 μL LB cultures (1:128) at indicated ciprofloxacin concentrations in a 96-well round bottom microwell plate (Thermo Scientific 262162). Plates were grown for 24 h with vigorous agitation (900 rpm) at 37 °C using a Microplate Thermoshaker (Grant-Bio PHMP-4). Following growth, the resultant cultures were passaged to an identical plate (1:128), and also diluted in PBS (1:142) for fluorescent population analysis with a MACSQuant VYB (Miltenyi Biotec). For population analysis 20,000 cells were counted, and the ratio of SYFP:BFP or SYFP:dTomato expressing cells was determined. This was repeated the following day, and for each culture the ratios were used to calculate the selective coefficient using the equation $s = [\ln(R(t)/R(0))]/[t]$ as described previously (Dykhuizen 1990). Each selective coefficient represents the results of six independent competitions with one fluorescent cassette pair and an identical set of six competitions with the fluorescent cassettes switched, thus 12 total competitions.

## Population Model

Five distinct resistance mutations (*gyrA* S83L, *gyrA* D87N, *parC* S80I, Δ*marR*, and Δ*acrR*) were chosen to model the development of ciprofloxacin resistance. Experimental measurements show that each of the three point mutations appears with a rate of $\sim 10^{-10}$ per generation and the two deletions with a rate of $\sim 10^{-7}$ per generation. Twenty-nine distinct strains (0–5 mutations per strain) were defined to contribute to the resistance development. Fitness and MIC were determined for all strains (supplementary table S3, Supplementary Material online) and used for the population model. The bacterial growth rate is a monotonically increasing function of the concentration of a limiting resource, R (mg/L) (Monod 1949)

$$\psi_i(R) = V_i(Ab)\left(\frac{R}{R+k}\right) \quad (1)$$

where $V_i(Ab)$ is the maximum relative fitness of the $i$th strain of bacteria as a function of the antibiotic concentration Ab [Equation (2)] and $k$ is the concentration of the resource at which the $V_i(Ab)$ is at half its maximum value (the "Monod constant").

The relative fitness of bacteria is an approximately linear decreasing function of the antibiotic concentration at sub-MIC level (Gullberg et al. 2011, 2014) so that

$$V_i(Ab) = \begin{cases} V_i\left(\dfrac{MIC_i - Ab}{MIC_i}\right) & if\ Ab < MIC_i \\ 0 & if\ Ab \geq MIC_i \end{cases} \quad (2)$$

where $V_i$ is the relative fitness in the absence of antibiotic and $MIC_i$ is the MIC of the $i$th strain of bacteria. With these definitions the change in densities of bacterial populations and the concentration of resources are given by the following two coupled differential equations:

$$\frac{dR}{dt} = -\sum_{i=1}^{29} n_i * \psi_i(R) * e_n \quad (3)$$

$$\frac{dn_i}{dt} = n_i * \psi_i(R) \quad (4)$$

where $n_i$ is the density of strain $i$ (cfu/mL) and e is the conversion efficiency parameter (μg/cell). The standard parameters $R_{t=0} = 100$ mg/L, $k = 1$ mg/L, $e_1 = 2.5 \times 10^{-8}$ μg/cell and $e_2 = 2.5 \times 10^{-9}$ μg/cell result in a growth cycle that leads to a final density of approximately $4 \times 10^9$ cfu/mL for $e_1$

and $4 \times 10^{10}$ cfu/mL for $e_2$. The initial culture is inoculated with $10^6$ cfu/mL of wild-type population and ciprofloxacin at a concentration of 0.004 mg/L. After every cycle the culture is diluted with the dilution factor d into fresh media with an antibiotic concentration that increase by the factor s (per cycle). A Monte Carlo procedure is used to determine the appearance of mutant populations. The probability $p_i(t)$ that the $i$th strain is generated by a mutational event at time point $t$ is

$$p_i(t) = \sum_{j=1}^{N} g_j * \mu_j \quad (5)$$

where $N$ is the number of bacterial strains that can give rise to strain $i$, $g_j$ is the number of generations of growth of the $j$th strain at time point $t$, and $\mu_j$ is the mutation rate (mutations per generation) for the mutation that changes strain $j$ to strain $i$. A random number $x$ ($0 < x < 1$) is generated. A single cell of strain $i$ is generated at time point $t$ if $x < p_i(t)$. The simulation was programmed in Berkeley Madonna (Version 9.0.100) and run with varying bottlenecks and increases in antibiotic concentrations to a final antibiotic concentration of 4 mg/L. All results are averages of 1000 independent simulations.

## Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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