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Weak Mutators Can Drive the Evolution of Fluoroquinolone Resistance in *Escherichia coli*

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Weak mutators are common among clinical isolates of *Escherichia coli*. We show that the relative mutation rate and the "evolvability of fluoroquinolone resistance" are related by a power law slope of 1.2 over 3 orders of magnitude. Thus, even weak mutators can drive the evolution of fluoroquinolone resistance under selection pressure.

Fluoroquinolones are a widely used group of antimicrobial agents. The development of resistance to clinically achievable levels of fluoroquinolones in most organisms, including *Escherichia coli*, is a multistep mutational process. Although several plasmid-borne resistance determinants have been described (10, 13, 14, 20), the great majority of the genetic alterations associated with fluoroquinolone resistance are mutations of chromosomal genes, including *gyrA*, *gyrB*, *parC*, *parE*, *marR*, and *acrR* (11, 12). *E. coli* isolates with MICs above the CLSI (formerly NCCLS)-defined breakpoint for ciprofloxacin (4 μg/ml) usually have four or more mutations distributed among these genes (17). This implies that resistant lineages have gone through several cycles of mutation and selection. The relative mutation rate might be a significant factor that determines the probability that resistance will evolve in a lineage by increasing the rate of supply of rare new mutations.

The focus of interest regarding the influence of mutators on evolution has been on strong mutators such as *mutS* mutants (3, 7, 21, 24, 25). These increase mutation rates ~500-fold and are found in up to 1% of natural *E. coli* isolates (2, 9, 17, 18, 21). They have strong selective benefits in experimental models (8, 19, 24, 27) but in vivo also lead to the accumulation of detrimental mutations that severely reduce fitness, measured as reduced competitiveness during transmission to and recolonization of eukaryotic hosts (8). In contrast, studies of hundreds of clinical *E. coli* isolates, also from a variety of sources, have found that about 25% are weakly hypermutable (up to 13-fold increase in the mutation rate) (2, 21). The relative preponderance of weak and moderate hypermutators among clinical isolates, including multiply resistant isolates (5), may reflect their better ability to evolve resistance, be transmitted to new hosts, and persist longer than strong mutators without incurring major fitness costs (4, 28).

We have previously noted an increased mutation rate correlated with fluoroquinolone resistance in clinical isolates of *E. coli* (17). The increases are modest, with the mutation rate in 80% of resistant isolates increasing by less than 20-fold the rate for the wild type. The influence of small increases in the mutation rate on the evolution of antibiotic resistance has not previously been tested experimentally. This raises the question of whether the link between fluoroquinolone resistance and a moderately increased mutation rate (17) is fortuitous or causal. We tested whether small and moderate increases in the mutation rate were sufficient to proportionately increase the ability of *E. coli* to evolve fluoroquinolone resistance.

Seven different mutator alleles were introduced into *E. coli* K12 MG1655 by P1 transduction (Table 1). These mutator alleles have been described in the literature, and their reported mutator phenotypes differ widely in magnitude (1, 15, 16, 22, 26, 29). Fifty independent lineages of each strain were evolved by serial passage in Luria-Bertani medium with successively higher concentrations of ciprofloxacin (Bayer AG, Wuppertal, Germany) in a BioscreenC machine (Oy Growth Curves Ab Ltd., Helsinki, Finland). The ciprofloxacin concentration steps were 0, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640, 1,280, and 2,560 μg/ml. Each cycle was 23 to 24 h of growth in a 200 μl, with the transfer of 5 μl at each step (initially 10^6 to 10^7 CFU). During the course of the evolution experiments, different lineages grew to different cell concentrations, reflecting their independent evolutionary trajectories; and thus, biological bottleneck sizes were lineage and step specific. The optical density at 600 nm (OD_{600}) of each well was measured at 10-min intervals throughout the culture period. Extinction of a lineage was defined as the failure to obtain growth ($OD_{600} < 0.03$) after overnight incubation.

TABLE 1. Bacterial strains and mutator genotypes

Strain	Genotype[a]	Source of mutator allele
MG1655	Wild type	Coli Genetic Stock Center
CH188	Δ*mutS*::FRT	This laboratory
CH286	*mutY*::Tn*10*dTet^r	Jeffrey H. Miller (23)
CH287	*dam*Δ*16*::Kan^r	Martin Marinus (26)
CH289	*miaA*::ΩCam^r	Malcolm Winkler (29)
CH290	*ung*::Spc^r	Murat K. Saparbaev (15)
CH291	*mutM*::Tn*10*dTet^r	Ashok S. Bhagwat (16)
BS1511	*mutA*~*ampC*::Kan^r	M. Zafri Humayun (1)

[a] "~" means the markers are linked by P1 transduction.

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TABLE 2. Relationship between mutation rate and K_E

Genotype	MIC (μg/ml)	Mutation rate	Relative mutation rate	K_E	Relative K_E
Wild type	0.016	1.8×10^{-8}	1	0.04	1
mutM	0.016	3.3×10^{-8}	2	0.64	16
mutA	0.023	6.4×10^{-8}	4	0.64	16
miaA	0.023	1.4×10^{-7}	8	0.04	1
ung	0.016	1.8×10^{-7}	10	1.25	31
mutY	0.023	1.8×10^{-7}	10	1.25	31
dam	0.016	7.2×10^{-7}	41	10	250
mutS	0.016	9.2×10^{-6}	525	160	4,000

The extinction coefficient (K_E) is defined as the lowest drug concentration step at which ≥50% of the lineages were extinct.

The MIC of ciprofloxacin was determined by Etest, according to the instructions of the manufacturer (AB BIODISK, Solna, Sweden). The mutator mutations do not themselves significantly affect the MICs (Table 2).

Mutation rates were measured by fluctuation tests with 40 independent cultures of each strain, assaying for rifampin resistance as described previously (17). This is a standard assay and measures the occurrence of at least 69 different base substitutions in *rpoB* (6). The results (Table 2) are close to those expected on the basis of the measurements for the parental strains. The range of mutation rates covered by these strains is ~500-fold, with an emphasis on the lower end of the range up to 10-fold the rate for the wild type.

All lineages of each strain, with the exception of a few *mutS* lineages, went to extinction within the course of the experiment. At 1,280 and 2,560 μg/ml ciprofloxacin, 20% and 13% of the *mutS* lineages were still living, respectively. The K_E values for each strain (Table 2) ranged over 3 orders of magnitude. When the K_E values were plotted against the relative mutation rate, the data fit a straight line (linear regression R^2 value, 0.77; P value, <0.001 at the 95% confidence level) to a power law with a slope of 1.2 (Fig. 1). Thus, "evolvability" (K_E) increases as the 1.2 power of the mutation rate. The results for one *miaA* strain deviated notably from this regression line, possibly because it failed to generate an increased rate of some mutation required for the evolution of fluoroquinolone resistance, although pleiotropic side effects due to the absence of this tRNA modification cannot be ruled out. Removal of the data for the *miaA* strain from the analysis gives a slope of 1.2 for the remaining seven strains (linear regression R^2 value, 0.95; P value, < 0.001). This particular power law relationship may reflect the average value of synergistic epistasis between different mutations and their effects on the resistance level. For example, a *parC* mutation does not show a resistance phenotype except in the presence of an appropriate *gyrA* mutation (30).

In conclusion, we found a good correlation between the mutation rate and K_E that extended over a wide range of mutation rates. This shows that even small increases in the mutation rate have a positive and measurable effect on the evolution of fluoroquinolone resistance by mutation and that the effect is in proportion to the magnitude of the mutation rate increase. There is no minimum threshold of mutability for increasing the rate of evolution to drug resistance.

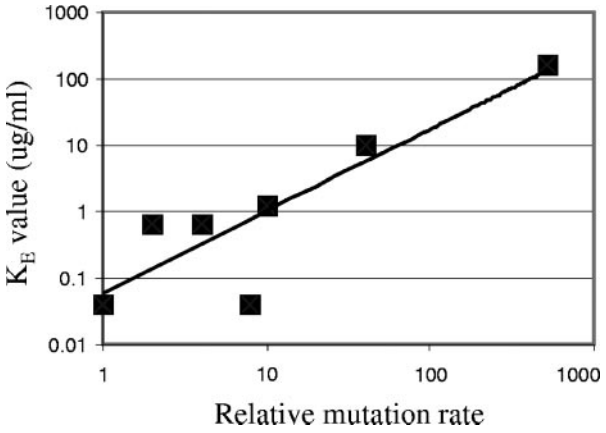

FIG. 1. K_E values for wild-type and mutator strains plotted as a function of relative mutation rate.

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