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Adaptive Mutations Produce Resistance to Ciprofloxacin

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Mutation to ciprofloxacin resistance continually occurred in nondividing *Escherichia coli* cells during a 7-day exposure to ciprofloxacin in agar, while no accumulation of rifampin resistance mutations was detected in those cells. We propose that the resistance mutations result from adaptive mutations, which preferentially produce phenotypes that promote growth in nondividing cells.

It is generally assumed that antibiotic-resistant strains emerge by selection for preexisting mutants in populations of bacteria exposed to antibiotics. Although this is an important mechanism for generating resistance, this study considers an alternate mechanism involving the occurrence of late-appearing mutations during prolonged antibiotic exposure, i.e., adaptive mutations.

Adaptive mutations differ from growth-dependent mutations in two key respects. First, adaptive mutations occur in nondividing or slowly dividing cells which are under selection for a particular phenotype, whereas growth-dependent mutations occur in dividing cells that are not under strong selection. Second, adaptive mutations seem to produce only those phenotypes which allow the cells to grow, whereas growth-dependent mutations occur randomly with respect to their effects on fitness. The mechanisms for producing adaptive mutations remain unknown, although numerous speculative models have been proposed. The different mutational spectra produced by adaptive mutations and growth-dependent mutations (2, 3) and the different responses to lesions in excision repair functions (4) suggest that the mechanisms for producing these two classes of mutation differ.

Adaptive mutations are typically observed by spreading a population of bacteria or yeast onto medium upon which growth cannot occur unless a known mutation reverts. The first revertants to appear are presumed to be the result of mutations that were present in the population prior to plating. Typically, additional revertant colonies continue to appear for periods ranging from a few days up to a month, and it is those late-appearing colonies that are said to result from adaptive mutations.

It is proposed that because adaptive mutations allow growth-inhibited bacteria to resume growth, bacteria that are inhibited by antibiotics may be able to resume growth as the consequence of adaptive mutations to antibiotic resistance.

Ciprofloxacin is a highly potent, broad-spectrum, bactericidal, fluoroquinolone antibiotic whose primary target in *Escherichia coli* is DNA gyrase. Ciprofloxacin and other quinolones interfere with cellular processes, including DNA replication, chromosomal segregation, transcription, and recombination (6). Fluoroquinolones have been used to treat a variety of *E. coli* infections, including those of the urinary tract.

The MIC of ciprofloxacin at 37°C was determined by the standard doubling dilution method to be 0.03 μg/ml for wild-type *E. coli* MG1655 ($F^-\lambda^-$) in Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter).

The growth-dependent mutation rate to ciprofloxacin resistance was estimated from a Luria-Delbrück fluctuation test (5) by spreading 40 independent 0.1-ml cultures of MG1655 onto LB agar containing ciprofloxacin at 0.06 μg/ml (twice the MIC) (LB-ciprofloxacin agar). That rate was 2.6×10^{-9} mutations per cell division.

To determine whether adaptive mutations to ciprofloxacin resistance could occur, MG1655 cells from an exponentially growing culture in LB broth were spread onto 40 LB-ciprofloxacin agar plates at 2×10^7 cells per plate and the plates were incubated at 37°C for 7 days. The colonies which appeared each day were marked and counted (Table 1). Each day a few newly arisen ciprofloxacin-resistant colonies were subcultured for storage until later study. Daily, after the colonies were counted, the resistant colonies were eliminated. This was done with little disturbance of the surrounding cells through the use of a diathermy probe (Hyfrecator Plus model 7-796; Birtcher Medical Supplies), an electrosurgery device that delivers an intense spark which kills the cells in the colony. The number of ciprofloxacin-sensitive cells per plate was estimated daily by resuspending two plates in 10 ml of 61 mM sodium phosphate buffer, pH 7.0, and spreading serial dilutions onto both LB agar and LB-ciprofloxacin agar plates. The number of viable cells per plate was estimated for each suspension and corrected for ciprofloxacin-resistant cells that escaped the diathermy probe, and the counts of the two suspensions were averaged. The number of viable cells dropped from 2×10^7 to about 1×10^5 per plate within 24 h and remained at that level, with neither detectable growth nor death, for the next 6 days (Table 1). The MIC of ciprofloxacin for such colonies was unchanged; i.e., the colonies were susceptible. Although it is bactericidal, ciprofloxacin leaves a residual population of ciprofloxacin-susceptible cells whose growth is inhibited by the drug. Concentrations above 0.5 μg/ml did not leave a detectable residual population of cells.

The susceptibilities of the selected colonies to ciprofloxacin ranged from 0.12 to 1 μg/ml for early-arising mutants and from 0.12 to 0.5 μg/ml for late-arising mutants. To determine whether the late-arising mutants were the result of preexisting growth-dependent mutations or the result of mutations that occurred on the LB-ciprofloxacin agar plates during selection, cells of the ciprofloxacin-resistant mutants that had been iso-

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TABLE 1. Details of the adaptive mutagenesis experiment

Day	Mean no. of ciprofloxacin-resistant colonies/plate	Cumulative no. of ciprofloxacin-resistant colonies/plate	No. of viable ciprofloxacin-susceptible cells/plate	No. of mutations/viable cell[a]
0			2.9×10^7	2.5×10^{-8}
1	0.44	0.44	2.6×10^4	1.7×10^{-5}
2	0.28	0.72	1.2×10^5	6.8×10^{-5}
3	1.67	2.39	6.2×10^4	1.6×10^{-6}
4	0.68	3.07	3.1×10^4	2.6×10^{-6}
5	0.16	3.23	1.3×10^5	2.5×10^{-6}
6	0.27	3.5	2.3×10^5	
7	0.25	3.85		

[a] On day 0 the value is estimated as the sum of the numbers of colonies appearing on days 1 and 2 divided by the number of viable cells on day 0, i.e., the number of cells plated. On subsequent days it is estimated as the number of new colonies per plate 2 days later divided by the average number of viable ciprofloxacin-susceptible cells per plate on days 1 to 6.

lated over the 7-day period were spread onto LB-ciprofloxacin agar plates and the time required for the emergence of visible colonies was determined. Both the early-appearing and the late-appearing ciprofloxacin-resistant mutants formed colonies on LB-ciprofloxacin agar plates within 1 to 2 days. Therefore, colonies that appeared on days 1 or 2 are assumed to have arisen from preexisting mutations and colonies that arose on or after day 3 are assumed to have arisen from adaptive mutations.

Adaptive mutation rates were calculated daily by dividing the mean number of new colonies that appeared 2 days later by the average number of viable ciprofloxacin-sensitive cells per plate that were present on days 1 through 6. A second experiment gave results that were virtually identical with those described in Table 1.

Mutation to rifampin resistance was studied to determine whether exposure to ciprofloxacin was generally mutagenic or whether mutations only occurred at loci that conferred ciprofloxacin resistance. Rifampin is an antibiotic, unrelated to ciprofloxacin, that inhibits RNA polymerase, thereby blocking RNA synthesis (9). Rifampin resistance arises via mutation at *rpoB*, and the growth-dependent mutation rate to rifampin resistance was estimated from a fluctuation test to be 2.8×10^{-9} mutations per cell division. As in the experiment described in Table 1, 2×10^7 cells were spread onto 40 LB-ciprofloxacin agar plates and onto a few 200-μg/ml LB-rifampin agar plates to determine the initial frequency of rifampin-resistant mutants. Each day, ciprofloxacin-resistant mutant colonies on the LB-ciprofloxacin agar plates were killed with the diathermy probe. On days 1, 3, 5, and 7 cells were collected from eight LB-ciprofloxacin agar plates and resuspended in 10 ml of LB broth. After about two generations of growth to permit expression of the rifampin resistance phenotype, the entire suspensions were concentrated and spread onto LB-rifampin agar plates. No rifampin-resistant mutants were detected among the cells collected from LB-ciprofloxacin agar plates on days 1, 3, 5, or 7. If ciprofloxacin were generally mutagenic and the mutation rates at all genes were increased to comparable levels, we would have expected to see an accumulation of rifampin-resistant mutants similar to the accumulation of ciprofloxacin-resistant mutants. That no such accumulation of rifampin-resistant mutants was observed is evidence that the mutations to ciprofloxacin resistance are adaptive in the sense of being specific to the selective challenge of exposure to ciprofloxacin. Although quinolones are not thought to be mutagenic when examined by conventional mutagenicity assays, these agents clearly induce the SOS response (8). Conventional mutagenicity assays (Ames test, V79, MNT, and DLT) have also indicated that ciprofloxacin is not mutagenic (7).

During the 7-day experiment adaptive mutants contributed more to the cumulative number of mutations than preexisting mutants, suggesting that adaptive mutations may be a significant source of antibiotic-resistant mutants during prolonged exposure. There was an average of 0.72 preexisting mutants per plate, while an additional 3.0 colonies per plate appeared to result from adaptive mutations (Table 1).

These in vitro results suggest that adaptive antibiotic resistance mutations may occur in vivo under conditions where ciprofloxacin concentrations are similar to those used in this study. Such concentrations are likely to occur following cessation of ciprofloxacin treatment, when the drug is eliminated slowly over a matter of weeks. Because *E. coli* is very susceptible to fluoroquinolones, the mutations obtained in this study are unlikely to immediately produce clinically significant resistance in this species. However, adaptive mutation may be the first step to developing clinically resistant organisms such as those recently described (1).

The mutations which occurred after exposure to ciprofloxacin exhibited both characteristics of adaptive mutations: occurrence in nondividing cells and specificity for beneficial phenotypes. The observation that adaptive antibiotic resistance mutations seem to occur suggests that exposure to other antibiotics may also select mutants via this mechanism. If adaptive antibiotic resistance mutations prove to be a general phenomenon, this may be another reason to move toward short-course antimicrobial chemotherapy. It may also be a reason to consider the duration of low concentrations of antibiotics following cessation of therapeutic treatment.

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