ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 4205–4208 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00647-07


# Contributions of the Combined Effects of Topoisomerase Mutations toward Fluoroquinolone Resistance in *Escherichia coli*[▽]

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Received 16 May 2007/Returned for modification 3 July 2007/Accepted 27 July 2007

**In defined, isogenic strains, at least three mutations, two of which must be in *gyrA*, were required to exceed the CLSI breakpoint for fluoroquinolone resistance. Strains with double mutations in both *gyrA* and *parC* had even higher MICs of fluoroquinolones than strains with totals of three mutations.**

Fluoroquinolones are widely prescribed antibiotics used to treat a broad range of bacterial infections (13). Fluoroquinolones target the type 2 topoisomerases, gyrase (*gyrA* and *gyrB*), and topoisomerase IV (*parC* and *parE*) (5, 6). Mutations in the target genes increase the MICs of fluoroquinolones (16). *Escherichia coli* clinical isolates with high MICs frequently contain double *gyrA* mutations in the codons for amino acid positions 83 and 87; some also contain double *parC* mutations in the codons for amino acid positions 80 and 84 (7, 11, 19). The genetic background of clinical isolates is undefined and highly variable; therefore, it is not possible to conclude that the analyzed mutations caused the observed increases in MIC. Indeed, isolates with the same *gyrA* and *parC* mutations can have MICs that differ >10-fold (11).

The strains and plasmid used in this study are listed in Table 1, and the oligonucleotides used in this study are listed in Table 2. The effects of double *gyrA* mutations on MIC in isogenic strains created by drug selection of an *E. coli* clinical isolate were measured previously (2, 17). Double *gyrA* mutations combined with a single *parC* mutation caused 12-fold-increased MICs for ciprofloxacin, the only drug tested (2, 17). However, in defined, isogenic *Salmonella* strains, the same combinations of mutations caused 30- and 85-fold (depending upon the *gyrA* mutation)-increased ciprofloxacin MICs (18). Because the genetic background of the *E. coli* clinical isolate is unknown, it is impossible to conclude whether the differences between the *E. coli* and *Salmonella* data are species specific. In addition, no one has measured directly the effect of double *parC* mutations on the MICs of fluoroquinolones in defined, isogenic strains.

Here, we determined the effects of *gyrA* and *parC* double mutations on fluoroquinolone susceptibility in defined, isogenic *E. coli* strains by Etest (AB Biodisk, Solna, Sweden) per the manufacturer's instructions. MICs exceeding Etest detection limits were determined by broth dilution (macrodilution), following CLSI (formerly NCCLS) guidelines (15). Where indicated, mutants were constructed using the λ Red temperature-sensitive

TABLE 1. Bacterial strains and plasmid[a]

| Strain | Genotype or relevant features | Construction type or reference |
|---|---|---|
| Strains | | |
| C600 | F$^-$ *thr-1 leu-6 thi-1 lacY1 supE44 tonA21* | 1 |
| SKM9 | 1597 except *gyrA*(*L83*,*Y87*) | λ Red mutagenesis |
| SKM11 | C600 except *gyrA*(*L83*,*Y87*) *zei-723*::Tn*10* | P1 (SKM9), C600, Tet[b] |
| SKM13 | 1597 except *gyrA*(*Y87*) | λ Red mutagenesis |
| SKM15 | 1608 except *parC*(*I80*) | λ Red mutagenesis |
| SKM16 | 1608 except *parC*(*I80*,*G84*) | λ Red mutagenesis |
| SKM17 | 1609 except *gyrA*(*Y87*) | λ Red mutagenesis |
| SKM18 | SKM11 except *parC*(*I80*,*G84*) | λ Red mutagenesis |
| 1596 | C600 except *gyrA*(*L83*) *zei-723*::Tn*10 parC*(*L80*) Kan$^r$ | 10 |
| 1597 | C600 except *zei-723*::Tn*10 parC*(*L80*) Kan$^r$ | 10 |
| 1608 | C600 except *gyrA*(*L83*) *zei-723*::Tn*10 parC*$^+$ Kan$^r$ | 10 |
| 1609 | C600 except *zei-723*::Tn*10 parC*$^+$ Kan$^r$ | 10 |
| 1983 | C600 except *zei-723*::Tn*10 parC*(*K84*) Kan$^r$ | 10 |
| Plasmid | | |
| pSIM5 | λ Red *exo bet gal*; *repA*(Ts) Cam$^r$ | 4 |

[a] Cam, chloramphenicol; Kan, kanamycin; P1, source of transducing lysate; r, resistant; Tet, tetracycline; Ts, temperature sensitive.
[b] P1 transduction; donor (SKM9), recipient (C600), and selection (Tet).

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▽ Published ahead of print on 6 August 2007.

TABLE 2. Oligonucleotides used in this study

| Primer or oligonucleotide | Sequence |
|---|---|
| *parC* QRDR[a] | |
| Forward | 5′GTACGTGATCATGGACCGTGCG |
| Reverse | 5′GCTCGCTCAATAGCAGCTCGG |
| *gyrA* QRDR | |
| Forward | 5′TACACCGGTCAACATTGAGG |
| Reverse | 5′TTAATGATTGCCGCCTCGG |
| λ Red | |
| *gyrA*(*Y87*) | 5′GTAAATACCATCCCCATGGTGACTCGGCGGTCTATTACACGATCGTCCGCATGGCGCAGCCATTCTCGCT |
| *gyrA*(*L83*,*Y87*) | 5′GTAAATACCATCCCCATGGTGACTCGGCGGTCTATTACACGATCGTCCGCATGGCGCAGCCATTCTCGCT |
| *parC*(*I80*) | 5′TACTGGGTAAATACCATCCGCACGGCGATATCGCCTGTTATGAAGCGATGGTCCTGATGGCGCAACCGTTCTC |
| *parC*(*I80*,*G84*) | 5′TACTGGGTAAATACCATCCGCACGGCGATATCGCCTGTTATGGAGCGATGGTCCTGATGGCGCAACCGTTCT |

[a] QRDR, quinolone resistance-determining region.

plasmid pSIM5 as described previously (3), except that cells were allowed to recover overnight following electroporation. Strains were cured of the plasmid at the nonpermissive temperature 37°C, and plasmid loss was confirmed by lack of growth on LB agar containing 30 μg/ml chloramphenicol.

The parental strain MICs were the same as those previously reported (Fig. 1A) (10, 14). The effects of *gyrA* mutations on the MICs are shown relative to the MIC of the parental strain (1609) to allow direct comparison of the different fluoroquinolones and control for drug-specific and cellular factors (Fig. 1B). *gyrA*(*L83*,*Y87*) increased the MICs of the fluoroquinolones ~5- to 15-fold, approximately the same result as that for single mutants. Thus, double *gyrA* mutations, by themselves, do not increase the MICs of the fluoroquinolones in *E. coli*.

We measured the effects of *parC* mutations on MIC in the *gyrA*(*L83*) background (Fig. 1C). *gyrA*(*L83*) *parC*(*I80*,*G84*) showed no additional MIC increases compared to *gyrA*(*L83*) with *parC* single mutations. The norfloxacin MIC increased the most (~5-fold); the moxifloxacin MIC was not significantly increased. Surprisingly, when we analyzed strains carrying only *parC* mutations, as controls, we found that *parC*(*K84*) had significantly ($P < 0.01$) decreased sparfloxacin, grepafloxacin, gemifloxacin, and moxifloxacin MICs (averages of 0.008, 0.014, 0.007, and 0.028 μg/ml, respectively).

Double *gyrA* mutations with *parC*(*L80*) caused high MIC increases for the fluoroquinolones. Shown (Fig. 1D) is the severalfold increase for *gyrA*(*L83*,*Y87*) *parC*(*L80*) (SKM9) relative to that for *gyrA*(*L83*) *parC*(*L80*) (1596). *gyrA*(*L83*,*Y87*) *parC*(*L80*) MICs increased 9- to 60-fold, depending upon the fluoroquinolone (Fig. 1D). The ciprofloxacin MIC increased the most (~60-fold), and the moxifloxacin MIC increased the least (~9-fold). This magnitude of increase is the same as that for defined, isogenic *Salmonella* strains (18); therefore, the previous difference was not species specific but was likely a consequence of the unknown *E. coli* background (2, 17).

To determine the effects of double *parC* mutations on MICs, we compared *gyrA*(*L83*,*Y87*) *parC*(*I80*,*G84*) (SKM18) to *gyrA*(*L83*,*Y87*) *parC*(*L80*) (SKM9) (Fig. 1E). The gatifloxacin MIC increased the least (twofold). The moxifloxacin MIC increased the most (~10-fold), which is interesting because the first *parC* mutation did not significantly increase the moxifloxacin MIC over that for the single *gyrA* mutation. Double *parC* mutations increased MICs, but only with double *gyrA* muta-

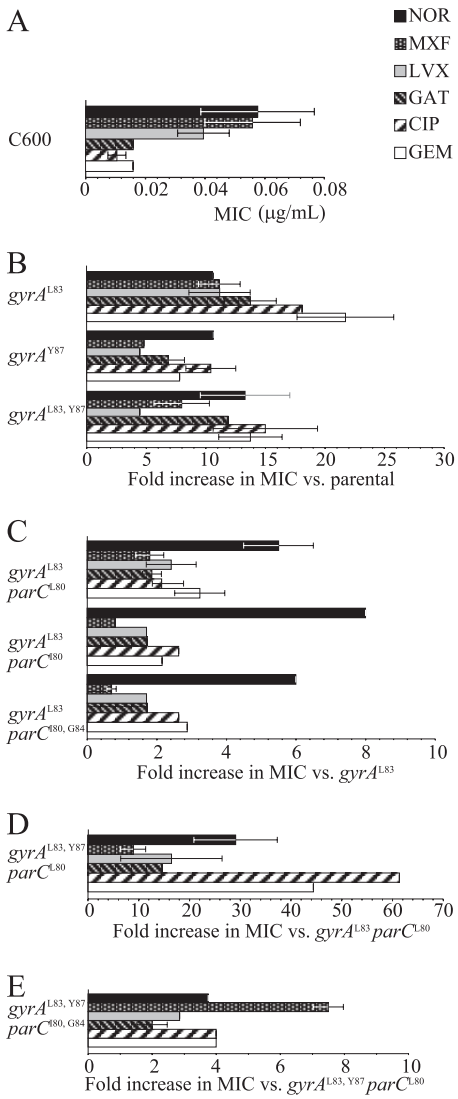


FIG. 1. Effects of topoisomerase mutations on fluoroquinolone resistance. (A) Fluoroquinolone MICs for the parental C600 strain (1609). The cumulative effects of gyrase and topoisomerase IV mutations were determined by calculating the severalfold increase in the MICs of fluoroquinolones relative to the MICs for the parent strain (1609) (B), the *gyrA*(*L83*) strain (1608) (C), the *gyrA*(*L83*) *parC*(*L80*) strain (1596) (D), or the *gyrA*(*L83*,*Y87*) *parC*(*L80*) strain (SKM9) (E). NOR, norfloxacin; MXF, moxifloxacin; LVX, levofloxacin; GAT, gatifloxacin; CIP, ciprofloxacin; GEM, gemifloxacin.

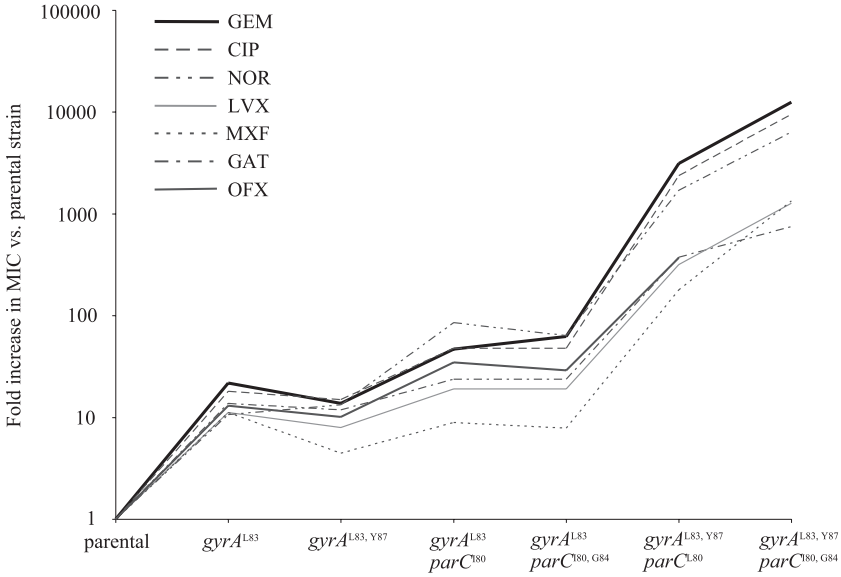


FIG. 2. Comparison of fluoroquinolone resistance in mutants. The *x* axis shows strains. The *y* axis denotes the severalfold increases in the MICs of all fluoroquinolones for each strain compared to the MICs for the parental C600 strain (1609). Ofloxacin (OFX) MICs were not measured for the *gyrA*(*L83*,*Y87*) *parC*(*L80*) and *gyrA*(*L83*,*Y87*) *parC*(*I80*,*G84*) strains. GEM, gemifloxacin; CIP, ciprofloxacin; NOR, norfloxacin; LVX, levofloxacin; MXF, moxifloxacin; GAT, gatifloxacin.

tions; otherwise, the second *parC* mutation was "silent." Additionally, the magnitudes of increase were significantly lower for *parC* mutations than for *gyrA* mutations. Thus, although *parC* mutations themselves did not greatly increase MICs, they were required for *gyrA* mutations to cause high MICs.

Plotting the severalfold increases in MIC versus the MICs for the parental strain revealed informative trends with regard to fluoroquinolone- and mutation-specific differences (Fig. 2). In the double *gyrA*, combined with single or double *parC*, mutants, the increases in MIC for gemifloxacin, ciprofloxacin, and norfloxacin grouped together at ~10,000-fold. Likewise, the increases for levofloxacin, moxifloxacin, and gatifloxacin grouped together at ~1,000-fold (Fig. 2). Levofloxacin, moxifloxacin, and gatifloxacin all have C-8 substitutions, which may explain their lower MIC increases (22).

In *E. coli*, gyrase is the primary target for all fluoroquinolones; topoisomerase IV is a secondary target (reviewed in reference 9). Purified gyrase is more susceptible to fluoroquinolones, which may explain why mutations in topoisomerase IV are not effective until gyrase is mutated (reviewed in reference 8). As the organism acquires mutations, the primary target of the fluoroquinolones switches between gyrase and topoisomerase IV. This idea is supported by previous studies examining stepwise mutants in which the first-, second-, third-, and fourth-step mutations occurred in *gyrA*, *parC*, *gyrA*, and *parC*, respectively (12). A single mutation in gyrase decreases its susceptibility sufficiently that topoisomerase IV becomes targeted. The silent phenotype of a second gyrase mutation in a *parC* wild-type background implies that the fluoroquinolones are targeting primarily topoisomerase IV; however, once *parC* has a single mutation, gyrase once again becomes targeted, explaining why double gyrase mutants have up to 60-fold-increased MICs over those for single *gyrA*, single *parC* mutants. An additional *parC* mutation in the double *gyrA* mutant strain increased MICs, indicating that topoisomerase IV becomes the target once again.

We thank Hana M. El Sahly, Richard J. Hamill, and Sheila I. Hull for critically reading the manuscript.

L.Z. was supported by National Institutes of Health grant R01-AI054830 and The Burroughs Wellcome Fund. S.K.M. was supported by a Houston Area Molecular Biophysics Program predoctoral fellowship, NIH T32-GM008280.

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