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Detection of Mutations in *parC* in Quinolone-Resistant Clinical Isolates of *Escherichia coli*

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The gene *parC* encodes the A subunit of topoisomerase IV of *Escherichia coli*. Mutations in the *parC* region analogous to those in the quinolone resistance-determining region of *gyrA* were investigated in 27 clinical isolates of *E. coli* for which ciprofloxacin MICs were 0.007 to 128 μg/ml. Of 15 isolates for which ciprofloxacin MICs were ≥1 μg/ml, 8 showed a change in the serine residue at position 80 (Ser-80), 4 showed a change in Glu-84, and 3 showed changes in both amino acids. No mutations were detected in 12 clinical isolates for which ciprofloxacin MICs were ≤0.25 μg/ml. These findings suggest that ParC from *E. coli* may be another target for quinolones and that mutations at residues Ser-80 and Glu-84 may contribute to decreased fluoroquinolone susceptibility.

Fluoroquinolones are broad-spectrum antibiotics which inhibit DNA gyrase activity (11, 31, 37). DNA gyrase is a type II DNA topoisomerase that catalyzes the negative supercoiling of DNA (10, 12, 35) and the separation of interlocked replicated daughter chromosomes (30). These processes are important for DNA replication and transcription and for the segregation of replicated chromosomes (21, 36). DNA gyrase is a tetrameric enzyme with two A subunits and two B subunits encoded by the genes *gyrA* and *gyrB*, respectively (1, 12, 28, 32).

In *Escherichia coli* quinolone resistance has been linked mainly to mutations located in a region of *gyrA* known as the quinolone resistance-determining region (4, 7, 13, 24, 34, 39, 41). Mutations in *gyrB* also have been associated with quinolone resistance in both clinical and laboratory *E. coli* isolates (34, 38, 40); however, the frequency of *gyrB* mutations in clinical isolates is much lower in comparison with the frequency of *gyrA* gene mutations (22, 25, 34). Changes in the permeation of quinolones by decreased uptake or increased efflux have also been linked to quinolone resistance acquisition (5, 6, 14–17).

Recently, a new topoisomerase (topoisomerase IV) has been purified (20, 27). The major role of this enzyme seems to be in decatenating daughter replicons following DNA replication (2, 21, 26), although it can also cause the relaxation of supercoiled DNA (20). Topoisomerase IV also has an A_2B_2 structure; the A and B subunits are encoded by the *parC* and *parE* genes, respectively (19, 27). This enzyme shows an amino acid sequence similarity to the corresponding subunits of DNA gyrase. The homology between subunits A from topoisomerase IV and DNA gyrase is higher in the NH_2-terminal region. This is the region of DNA gyrase where the quinolone resistance-determining mutations are located. This similarity implies that quinolones may be capable of inhibiting the activity of topoisomerase IV as well as that of DNA gyrase. Peng and Marians (27) have demonstrated that quinolones inhibit topoisomerase IV relaxation activity at concentrations 3- to 30-fold higher than those required to inhibit DNA gyrase. However, Hoshino et al. (18) have shown recently that measurement of the decatenation activity of topoisomerase IV is a better assay than measurement of topoisomerase IV relaxation for evaluating drug inhibition. Using this assay, they showed a positive correlation between inhibitory activity against topoisomerase IV decatenation and DNA gyrase supercoiling, suggesting that topoisomerase IV could also be a target for quinolones. Moreover, a quinolone resistance locus, *nfxD*, found in a multiply resistant mutant of *E. coli* has been mapped to the region of the *parC* and *parE* genes (29).

We investigated whether mutations in the region of *parC*, equivalent to the quinolone resistance-determining region in *gyrA*, could be involved in the acquisition of quinolone resistance in clinical isolates of *E. coli*. We determined the nucleotide sequence of that region of *parC* from 27 clinical isolates of *E. coli*. In these strains, susceptibility testing was performed by using an agar dilution method in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (23). Approximately 10^4 CFU of each isolate was inoculated onto freshly prepared medium containing serial dilutions of ciprofloxacin (Bayer, Leverkusen, Germany). For these strains, ciprofloxacin MICs ranged from 0.007 to 128 μg/ml. To identify *parC* mutations, PCR and direct DNA sequencing were used. Two oligonucleotide primers, 5′-AAACC TGTTCAGCGCCGCATT-3′ and 5′-GTGGTGCCGTTAAG CAAA-3′, were used to amplify the fragment of *parC* from nucleotides 115 to 509 (395 bp) by following the procedure described previously (33). Primers and free nucleotides from the PCR mixtures were removed by using the QiaQuick spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.). The purified PCR products were directly processed for DNA sequencing by using the *Taq* DyeDeoxiTerminator Cycle Sequencing Kit and were analyzed in an automatic DNA sequencer (373A; Applied Biosystems).

All mutations in *parC* between nucleotides encoding the lysine residue at position 49 (Lys-49) and Thr-170 which resulted in amino acid substitutions are listed in Table 1. For all strains analyzed, the other amino acid residues in this region were identical to the amino acid residues in the wild-type sequence reported by Kato et al. (19), with the modifications of Peng and Marians (27). All 15 isolates for which the ciprofloxacin MIC was ≥1 μg/ml showed mutations at amino acid codons Ser-80 or Glu-84, or both, whereas no amino acid changes were detected in the 12 clinical isolates for which the ciprofloxacin MIC was ≤0.25 μg/ml. Of these 15 quinolone-resistant clinical isolates, 8 had a change at Ser-80, 4 had a change at Glu-84, and 3 had changes at both amino acids. The

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TABLE 1. Mutations in the *parC* gene of quinolone-resistant clinical isolates of *E. coli*

Strain	Ciprofloxacin MIC (μg/ml)	Amino acid change		
		GyrA[a]	GyrB[a]	ParC
		Ser-83···Asp-87	Lys-447	Ser-80···Glu-84
C-20,[b] C-13[b]	0.007	—···—	—	—···—
C-1,[b] C-11[b]	0.06	—···—	—	—···—
C-8,[b] C-18[b]	0.125	—···—	—	—···—
C-5,[d] C-6,[b] C-7,[b] C-9[d]	0.25	—···—	—	—···—
C-4,[b] C-15[b]	0.25	Leu···—	—	—···—
C-10	1	Leu···—	—	Arg···—
1327[e]	2	Leu···—	—	Ile···Val
1289	4	Leu···—	—	Arg···—
1363[d]	4	Leu···—	Glu	—···Lys
1273[b]	8	Leu···Tyr	—	—···Lys
1331[b]	8	Leu···Asn	—	—···Lys
1574[e]	8	Leu···Asn	—	Ile···—
1283	16	Leu···Asn	—	Arg···—
1334[e]	16	Leu···Asn	—	Ile···—
1360[b]	32	Leu···Tyr	—	—···Lys
1416[e]	32	Leu···Asn	—	Ile···—
1323,[e] 1388[e]	64	Leu···Asn	—	Ile···—
1319[e]	64	Leu···Asn	—	Ile···Val
1383[e]	128	Leu···Tyr	—	Ile···Lys

[a] Results are from reference 34.
[b] Strains with codon AGT for amino acid Ser-80.
[c] No mutations seen.
[d] Strains with codon AGC for amino acid Ser-80.
[e] Strains with a G→T transversion from codon AGC (Ser-80).

16 strains with no mutations at amino acid codon Ser-80 showed two different codons for this amino acid, AGT (13 strains) and AGC (3 strains). For eight strains the mutations at amino acid codon Ser-80 were generated by a G→T transversion from codon AGC, leading to Ser-80-Ile substitution, whereas mutations at the amino acid codon Ser-80 of three strains were generated from codon AGT or AGC by a T→A or a C→A tranversion (strains 1283, C-10) or by a T→G or a C→G transversion (strain 1289), all leading to Ser-80-Arg substitutions. All strains retaining Glu-84 had the wild-type GAA codon. The *parC* genes from two strains exhibited a A→T transversion at nucleotide position 251, leading to a Glu-84-Val substitution, whereas five strains exhibited a G→A transition, leading to a Glu-84-Lys substitution. Mutations in the amino acids corresponding to Ser-80 and Glu-84 of *E. coli* have also been identified in the DNA topoisomerase IV gene, *grlA*, of quinolone-resistant *Staphylococcus aureus* (8, 9) and in the *parC* gene of quinolone-resistant *Neisseria gonorrhoeae* (3). In *S. aureus*, Ser-80 is replaced by Tyr and Glu-84 is replaced by Lys or Leu, whereas in *N. gonorrhoeae*, Ser-88 (equivalent to Ser-80 from *E. coli*) is replaced by Pro and Glu-91 (equivalent to Glu-84 in *E. coli*) is replaced by Lys.

We have found previously that a change in Ser-83 of the GyrA protein generated resistance to ciprofloxacin when ciprofloxacin was used at a level of between 0.25 and 4 μg/ml in clinical *E. coli* isolates, whereas a double mutation, in both Ser-83 and Asp-87, was associated with resistance to ciprofloxacin when ciprofloxacin was used at a level of ≥8 μg/ml (34). However, these GyrA mutations did not explain why the MIC of ciprofloxacin was 8 μg/ml for one strain and 128 μg/ml for another strain. One possibility was additional mutations in the *gyrB* gene; however, there were no changes in amino acids 426 and 447 of the GyrB protein (34), which is also implicated in resistance to quinolones. Although mechanisms related to drug permeation or drug efflux could account for this difference in MICs, it is also possible that amino acid substitutions in both ParC and GyrA led to an increased level of resistance.

The present work has shown that, with some exceptions, a specific substitution in GyrA is associated with a very low level of resistance to ciprofloxacin (0.25 μg/ml); two changes, one in GyrA and a second in ParC, were associated with a moderate level of resistance to ciprofloxacin (1 to 4 μg/ml), three amino acid substitutions (two in GyrA and one in ParC) were associated with a high level of resistance to ciprofloxacin (8 to 64 μg/ml), and four changes (two in GyrA and two in ParC protein) were associated with the highest level of resistance (128 μg/ml) (Table 1). Exceptions were strain 1327, which possesses three amino acid changes (two in ParC and one in GyrA) and for which the ciprofloxacin MIC is 2 μg/ml, and strain 1319, which showed four changes and for which the ciprofloxacin MIC is 64 μg/ml. These exceptions, in which resistance was lower than expected, could be explained by increased drug permeation or decreased drug efflux (accumulation studies with these strains are under way). However like strain 1383 (ciprofloxacin MIC, 128 μg/ml), strain 1319 has four amino acid changes; however, it shows Glu-84-Val, whereas strain 1383 showed Glu-84-Lys. If we consider that Glu-84 (−1 charge) could bind to a substituent at C-7 in ciprofloxacin (+1 charge), we would expect that a change of Glu-84 (−1 charge) to Lys (+1 charge) would present a larger decrease in the affinity of ciprofloxacin than a change of Glu-84 to Val, a nonpolar amino acid.

In the present study, no single *parC* mutation has been found without the concomitant presence of a mutation in the *gyrA* gene, suggesting that DNA topoisomerase IV could be a secondary target for quinolones. Those results are in agreement with those of Soussy et al. (29), who recently showed that a quinolone-resistant mutant of *E. coli* carrying a mutation in the region of *parC* and *parE* required an additional mutation in *gyrA* to express quinolone resistance. Belland et al. (3) also found that increasing numbers of mutations in *gyrA* and then in *parC* were associated with increasing levels of resistance in *N. gonorrhoeae*. However, in contrast, Ferrero et al. (9) found that low-level quinolone-resistant clinical isolates of *S. aureus*

lacked mutations in the quinolone resistance-determining region of the *gyrA* gene, whereas mutations at amino acid codon Ser-80 of *grlA* were found in both high- and low-level quinolone-resistant isolates, suggesting that topoisomerase IV is a primary target of fluoroquinolones in *S. aureus*.

In the present study, we examined only the region of *parC* equivalent to the quinolone resistance-determining region of the *gyrA* gene. Therefore, it is possible that alterations in drug permeation or drug efflux could contribute to the level of resistance in these strains. Moreover, the two amino acids of GyrB involved in quinolone resistance are conserved in ParE; thus, mutations in *parE* could also modulate the ultimate MICs for these strains. However, these results do suggest that ParC from *E. coli* is a secondary target for quinolones and mutations in amino acid codons Ser-80 and Glu-84 are associated with higher levels of quinolone resistance.

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