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Genetic Evidence for a Role of *parC* Mutations in Development of High-Level Fluoroquinolone Resistance in *Escherichia coli*

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Fifteen strains of *Escherichia coli* with MICs of ciprofloxacin (CIP) between 0.015 and 256 μg/ml were examined for the presence of mutations in the quinolone resistance-determining region of the *gyrA* gene and in an analogous region of the *parC* gene. No mutation was found in a susceptible isolate (MIC of CIP, 0.015 μg/ml). Four moderately resistant strains (MIC of CIP 0.06 to 4 μg/ml) carried one *gyrA* mutation affecting serine 83, but in only one strain was an additional *parC* mutation (Gly-78 to Asp) detected. All ten highly resistant strains examined (MIC of CIP, >4 μg/ml) carried two *gyrA* mutations affecting residues serine 83 and aspartate 87, and at least one *parC* mutation. These *parC* mutations included alterations of serine 80 to arginine or isoleucine and glutamate 84 to glycine or lysine. The $parC^+$ and two mutant alleles ($parC^{I\text{-}80}$ and $parC^{I\text{-}80,G\text{-}84}$) were inserted into the mobilizable vector pBP507. Transfer of a plasmid-coded $parC^+$ allele into $parC^+$ strains did not alter the susceptibilities towards ciprofloxacin or nalidixic acid, while a significant increase in susceptibility was detectable for *parC* mutants. This increase, however, did not restore wild-type susceptibility, whereas transfer of a plasmid-coded $gyrA^+$ allele alone or in combination with $parC^+$ did. These data are in agreement with the view that topoisomerase IV is a secondary, less sensitive target for quinolone action in *Escherichia coli* and that the development of high-level fluoroquinolone resistance in *E. coli* requires at least one *parC* mutation in addition to the *gyrA* mutation(s).

The development of clinical resistance to fluoroquinolones (i.e., MIC of ciprofloxacin [CIP] of ≥4 μg/ml) in *Escherichia coli* involves multiple mutations. These include (i) alterations associated with reduced accumulation of fluoroquinolones in the cell as well as (ii) alterations of the bacterial type II topoisomerase DNA gyrase which, being bound to its substrate double-stranded DNA, is believed to be the molecular target for quinolone action (for a recent review, see reference 19).

Mutations resulting in reduced drug accumulation in *E. coli* affect loci like *mar* involved in the regulation of the expression of outer membrane proteins, e.g., OmpF porin (6, 20). However, the impact of these mechanisms on the resistance to quinolones is still being investigated.

Alterations affecting DNA gyrase in the genes *gyrA* and *gyrB*, coding for the two subunits GyrA and GyrB, have been identified (summarized in reference 19). This bacterial enzyme has the tetrameric structure A_2B_2 and is unique in its ability to catalyze the negative supercoiling of covalently closed circular DNA double strands in an ATP-consuming reaction (for a recent review, see reference 36). While only two different *gyrB* mutations have been associated with low-level quinolone resistance in *E. coli* (47, 50), several single-step mutations in the *gyrA* gene which cause high-level resistance towards unfluorinated but not towards fluorinated quinolones have been identified. These mutations are clustered near the 5′ end of the *gyrA* gene in a region highly conserved among all known *gyrA* genes between residues Ala-67 and Gln-106 of the *E. coli* gene. This region is called the quinolone resistance-determining region (QRDR) (7, 11, 32, 49). Among these mutations, those altering residue Ser-83 of the *E. coli gyrA* gene were most frequently found and are associated with a significant increase in the resistance towards all quinolones (32, 37, 49). This resistance seems to be due to reduced affinities of the drugs to the complex of DNA and the mutant enzyme (45, 51).

The increase in quinolone resistance due to gyrase mutations can be reversed after introduction of a second copy of the respective *gyr* wild-type (WT) or quinolone-susceptible allele into the cells (dominance of the WT [susceptible] over the mutant [resistant] allele [12, 30]). Examining the dominance of the $gyrA^+$ allele in high-level fluoroquinolone-resistant clinical isolates of *E. coli* provided evidence for the involvement of *gyrA* mutations (18). The CIP concentration necessary to inhibit in vitro the supercoiling activity of such a mutant gyrase by 90% was shown to be increased to over 4,000-fold compared with that of the WT. Sequencing of the respective gene revealed a *gyrA* double mutation, S-83 to L (S83L) and D87G (16). In addition, reduced CIP accumulation associated with reduced amounts of OmpF porin was found not only for this clinical isolate but also for a *gyrA* double mutant selected in three steps in vitro from an *E. coli* WT isolate (17). Similar *gyrA* double mutations affecting Ser-83 and Asp-87 in fluoroquinolone-resistant isolates of *E. coli* (44) were also found by others.

On the basis of these findings, a plausible explanation for the development of high-level fluoroquinolone resistance seemed to be a combination of two *gyrA* mutations and a mutation reducing the accumulation of CIP. However, by a gene replacement strategy, exchange of the WT *gyrA* gene by an allele carrying the double mutation resulted in only a slight increase of quinolone resistance (14). This observation is supported by recent results of Ouabdesselam et al. (33) from an indirect approach with conditional *gyrA* mutants for the expression of a plasmid-coded copy of a mutant *gyrA* allele. These data together suggest that additional, non-*gyr* mutations are involved.

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TABLE 1. List of plasmids used in this study

Plasmid	Size (kb)	Relevant markers	Source or reference
RP1H	61	*bla*, *tet*, Tra$^+$	18
pBP507	9.8	*aacA*, Mob$^+$, Tra$^-$, *aphA*	13
pBP517	12.3	*aacA*, Mob$^+$, Tra$^-$, *aphA*, *gyrA*$^+$	13
pBP567	10.7	*aacA*, Mob$^+$, Tra$^-$, *parC*$^+$	This study
pBP567-4	10.7	*aacA*, Mob$^+$, Tra$^-$, *parC*$^{I\text{-}80}$	This study
pBP567-9	10.7	*aacA*, Mob$^+$, Tra$^-$, *parC*$^{I\text{-}80,G\text{-}84}$	This study
pBP56717	13.9	*aacA*, Mob$^+$, Tra$^-$, *parC*$^+$, *gyrA*$^+$	This study

One likely candidate for such non-*gyr* mutations is topoisomerase IV (Topo IV), another type II topoisomerase detected in *E. coli* (23), *Salmonella typhimurium* (28, 39), *Neisseria gonorrhoeae* (4), and *Staphylococcus aureus* (9). Like gyrase, Topo IV consists of two pairs of subunits: ParC (GrlA in *S. aureus*), which has significant homology with GyrA, especially in the N-terminal region including the QRDR, and ParE (GrlB in *S. aureus*), which contains long stretches of homology to the nucleotide binding region of GyrB (9, 25, 34, 40).

Both topoisomerases are able to relax supercoiled DNA and to decatenate intertwined double-stranded DNA rings (1, 23, 39, 42). The decatenating activity of Topo IV from *E. coli* can be inhibited in vitro by quinolone drugs. However, the quinolone concentrations necessary to inhibit 50% of the decatenation activity are at least eightfold higher for *E. coli* Topo IV than those for *E. coli* DNA gyrase (21). While no *par* mutation associated with quinolone resistance has been detected in *E. coli* so far, fluoroquinolone-resistant mutants of *S. aureus* (MIC of CIP, 2 to 32 μg/ml) were found to carry a *grlA* but no *gyrA* mutation (8, 9). These data indicate that Topo IV is a primary target in this species and that *grlA* mutations play a major role for the development of fluoroquinolone resistance in *S. aureus*. The accumulation of multiple mutations in both *gyrA* and *parC* is associated with high-level fluoroquinolone resistance in isolates of *N. gonorrhoeae* selected in vitro. In this species, however, DNA gyrase seems to be the primary target, since *parC* mutations were detected only in *gyrA* double mutants (4).

The goal of the present study was to investigate the possible role of Topo IV in the development of high-level resistance to fluoroquinolones in *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and antimicrobial agents. The *E. coli* K-12 derivatives used were JM83 (48), C600SN (18), and C600 (2). EJ812 [C600 *parC1215* (Ts)] (24) was generously provided by L. Zechiedrich. Isolates of *E. coli* included WT and its in vitro-selected quinolone-resistant mutants MI, MII, MIII, MIVb, and R17 (17), as well as CIP-resistant clinical isolates 205096 (18) and 3204917 obtained from C. Krasemann, 4469 from B. Wiedemann, U12987 from R. Reinert, 129801, 130162, 133700, and 136437 from M. Lontic, and HP24704-1 from M. López-Brea (27).

The plasmids used in this study are listed in Table 1.

Amikacin (AMI) (Grünenthal, Aachen, Germany), nalidixic acid (NAL) (Sterling-Winthorp, Guildford, UK), and ampicillin and CIP (Bayer, Wuppertal, Germany) were kindly supplied by the manufacturers.

Chemicals, biochemicals, and media. Unless otherwise stated, all chemicals as well as Standard broth No I (NI agar and NI broth) were purchased from Merck (Darmstadt, Germany). All biochemicals except for restriction nucleases and *Taq* DNA polymerase (Life Technologies, Eggenstein, Germany) and the ligation kit (Stratagene, Heidelberg, Germany) were from Boehringer (Mannheim, Germany). Mueller-Hinton broth was obtained from Difco (Detroit, Michigan).

Susceptibility testing. MICs were determined in a broth microdilution test, using unsupplemented Mueller-Hinton broth, according to the recommendations of the National Committee for Clinical Laboratory Standards (31). The susceptibilities of plasmid-bearing strains for the detection of dominance were determined as single-cell MICs (scMICs) (18). In brief, three different inoculum sizes (about 10^0, 10^1, and 10^2 CFU) of a strain were spotted onto agar plates containing twofold serial dilutions of the respective quinolone. By definition, the scMIC was determined after 18 h of incubation as that concentration leading to a >10-fold reduction in the viable cell count compared with that of a drug-free control.

Isolation and cloning of *parC* genes from different *E. coli* isolates. Standard recombinant DNA techniques followed the protocols of Sambrook et al. (38). Chromosomal DNA was isolated from *E. coli* isolates according to the protocol of Ausubel et al. (3). About 250 ng of chromosomal DNA was used as the template for amplification of a 2.4-kb DNA fragment carrying the *parC* gene. PCRs were carried out in a total volume of 100 μl containing 50 pmol each of primers PARC5-2, 5′-ACCGGAATTCGGCGCTATTCACGTTGATCTCCTG-3′ (nucleotides −106 to −84), and PARC3-2, 5′-GCCTAATGCATTACTCTTCGCTATCACCGCTGCTG-3′ (complementary to nucleotides 2235 to 2259), on the basis of sequences reported previously (25, 34). Thirty-five cycles of the following temperature profile were run: 95°C for 30 s, 55°C for 45 s, and 72°C for 4 min, with time increments of 5 s per cycle in the polymerization step. The amplified DNA fragments were purified by gel elution, digested with *Eco*RI and *Nsi*I, and ligated to plasmid vectors pBP507 and pBP517 (13), respectively, which had been treated with the same restriction nucleases. A 0.83-kb DNA fragment carrying part of the *aphA* gene was thereby deleted. Ligation mixtures containing the resulting plasmids pBP567 and pBP56717, respectively, were used to transform competent cells of *E. coli* JM83. From transformants growing in the presence of AMI (20 μg/ml) plasmid DNA was isolated and characterized by restriction endonuclease analysis with *Eco*RI-*Nsi*I or *Pvu*II. With plasmid DNA from positive clones, *E. coli* C600SN cells carrying mobilization helper plasmid RP1H, strain CR1H, were transformed to yield the respective donor for subsequent conjugation experiments. In order to prevent cloning of artifacts, the *parC* wild-type gene (*parC*$^+$) was cloned twice with chromosomal DNA from two different colonies. *E. coli* K-12 strain C600 and its *parC*(Ts) derivative EJ812 were transformed with either plasmid pBP567, pBP567-4, pBP567-9, or pBP56717 at a permissive temperature. Serial dilutions of liquid cultures of the different transformants were replica plated on NI agar containing AMI (20 μg/ml) and were incubated at 30, 37, and 42°C, respectively. Growth of transformants at nonpermissive temperatures indicated the expression of a functionally active ParC protein.

Test for dominance of *parC* WT (*parC*$^+$) over mutant allele (*parC*) in the absence and presence of *gyrA*$^+$. Recombinant plasmids of the pBP567 series as well as plasmid pBP56717 (Table 1) were transferred to the different *E. coli* isolates by mobilization following a mating protocol, carried out essentially as described previously (18). Transconjugants were selected on M9 minimal agar containing glucose and AMI (20 μg/ml). Three to five individual colonies of the purified culture were used to prepare the inoculum for susceptibility testing (scMIC). Each determination was done at least in duplicate.

DNA sequencing. To determine the DNA sequence of a 5′ region of the *parC* gene, a 418-bp fragment was amplified with primers PARC5-1, 5′-GCGAATAAGTTGAGGAATCAG-3′ (nucleotides −26 to −6), and PARC3-1, 5′-AGCTCGGAATATTTCGACAAC-3′ (complementary to nucleotides 372 to 392) (numbering is based upon the DNA sequences published by Kato et al. [25] and Peng and Marians [34]). Thirty-five cycles of the following temperature profile were run: 30 s at 95°C, 45 s at 55°C, and 90 s at 72°C. The resulting DNA fragments were purified with Qiaquick purification cartridges (Qiagen, Hilden, Germany). About 100 fmol, corresponding to about 30 ng, of fragment DNA were used for subsequent cycle sequencing with the Silver Sequencing kit (Promega, Heidelberg, Germany), used according to the manufacturer's recommendations, with nested primers parC-S, 5′-GTATGCGATGTCTGAACTGGGCCTG-3′ (nucleotides 138 to 162), and parC-U, 5′-ACCGGGATTCGGTGTAACGCATTGC-3′ (complementary to nucleotides 349 to 373) (25, 34).

For the sequencing of the 5′ region of the *gyrA* gene the same amplification protocol using amplification primers 5-1 and 3-1 and sequencing primers A and B (16, 17) was applied.

Sequencing reactions were run on a 6% polyacrylamide gel containing 8 M urea. DNA bands were visualized with the Silver Staining kit (Promega) used according to the manufacturer's instructions.

RESULTS

Identification of *gyrA* and *parC* mutations in clinical isolates of *E. coli*. Each set of amplification reactions yielded a single DNA fragment identical in size: about 0.42 kb in the case of *parC* and about 0.52 kb in the case of *gyrA*. The results of the DNA sequencing of *gyrA* and those of *parC* were consistent and provided information from both strands for a region between nucleotides 193 and 318 (corresponding to codons 65 to 106 of the *gyrA* gene) and from nucleotides 178 to 324 (corresponding to codons 60 to 108) of the *parC* gene, respectively. By comparison with the DNA sequences of the respective genes from *E. coli* K-12 (25, 34, 43), mutations affecting codons

TABLE 2. Mutations in genes *gyrA* and *parC*

E. coli strain(s)	*gyrA* mutation			*parC* mutation		
	Nucleotide position	Nucleotide exchange	Amino acid exchange	Nucleotide position	Nucleotide exchange	Amino acid exchange
WT						
MI, MII, 4469	248	TCG→TTG	S83L			
3204917	248	TCG→TGG	S83W	233	GGC→GAC	G78D
MIII, MIVb, R17	248	TCG→TTG	S83L	239	AGT→ATT	S80I
	260	GAC→GGC	D87G			
129801	248	TCG→TTG	S83L	250	GAA→AAA	E84K
	259	GAC→AAC	D87N			
133700	248	TCG→TTG	S83L	239	AGC→ATC	S80I
	259	GAC→AAC	D87N			
HP24704-1	248	TCG→TTG	S83L	240	AGC/T→AGA	S80R
	259	GAC→AAC	D87N			
130162	248	TCG→TTG	S83L	239	AGC→ATC	S80I
	259	GAC→TAC	D87Y			
205096	248	TCG→TTG	S83L	250	GAA→AAA	E84K
	260	GAC→GGC	D87G			
U12987, 136437	248	TCG→TTG	S83L	239	AGC→ATC	S80I
	259	GAC→AAC	D87N	251	GAA→GGA	E84G

83 and 87 of the *gyrA* gene and those affecting codons 78, 80, and 84 of *parC* were identified (Table 2). In addition, in *parC* a few silent mutations were found at wobble positions in codons 80 (AGT instead of AGC, detected in *E. coli* WT, its derivatives, and isolate 129801), 91 (CAG instead of CAA, detected in all isolates examined), and 107 (GGT instead of GGC, detected in *E. coli* WT and its derivatives). A silent mutation in the *gyrA* genes of all isolates investigated was detected at the wobble position of codon 89 (ATC instead of ATT). Except for isolate U12987, all other isolates carry additional wobble mutations in codons 85 (GTT instead of GTC), 91 (CGT instead of CGC), and 100 (TAC instead of TAT).

Test for dominance of *parC*$^+$ over its resistant allele in heterodiploids. Since *parC* mutations were found only in isolates carrying at least one *gyrA* mutation and showing elevated MICs of CIP (≥2 μg/ml), the involvement of *parC* mutations in the development of fluoroquinolone resistance seemed possible. If Topo IV acts as secondary target in these isolates, it should be inhibited by quinolones in vivo, analogous to observations made from in vitro experiments (21). This, in turn, should result in cell death, because conditional mutations affecting either *parC* or *parE* can be isolated (28, 39, 40). To investigate whether a dominance effect analogous to that observed for the gyrase genes is detectable in *parC* mutants, the WT *parC* gene (*parC*$^+$) was cloned with the broad-host-range vectors pBP507 and pBP517 to yield recombinant plasmids pBP567 (*parC*$^+$) and pBP56717 (*parC*$^+$ *gyrA*$^+$), respectively (Fig. 1). In addition, two mutant *parC* genes were isolated from laboratory mutant MIII to yield plasmid pBP567-4 carrying a single mutation (S80I) and from clinical isolate U12987 to yield plasmid pBP567-9 carrying a double mutation (S80I and E84G), respectively. The PCR fragments obtained were identical in size (about 2.4 kb). Physical characterization of recombinant DNA from transformants by restriction mapping and sequencing confirmed the presence of a DNA fragment of the expected size. The functional expression of the cloned *parC* genes was confirmed by growth of the conditional *parC*(Ts) mutant EJ812 carrying plasmid pBP567 or a derivative (data not shown) at nonpermissive temperatures. Each plasmid DNA from four individual clones was used to retransform *E. coli* K-12 strain C600SN carrying plasmid RP1H to yield donor strains CR567 (C600SN [RP1H/pBP567]), CR567-4, CR567-9,

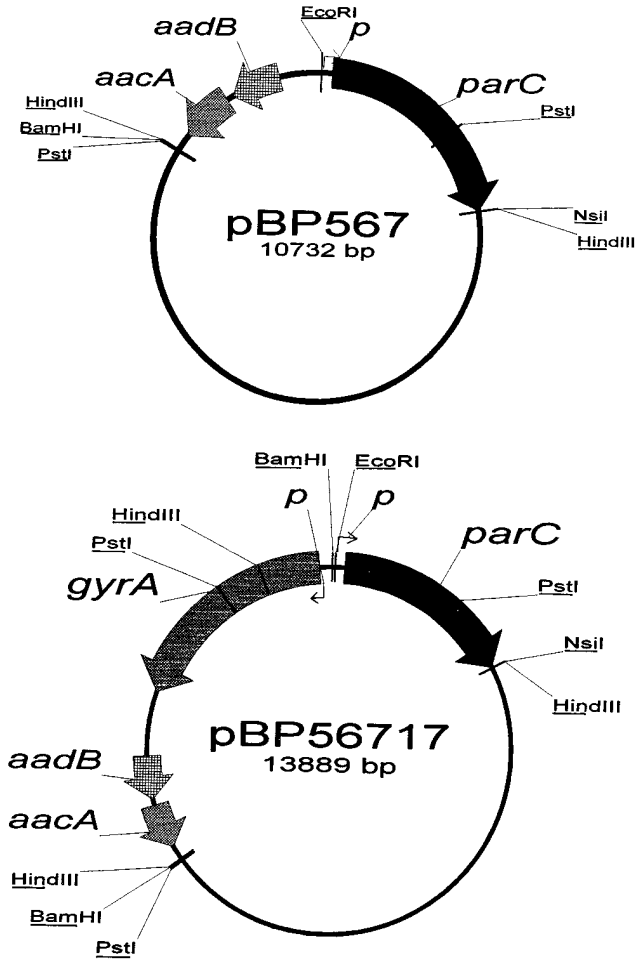

FIG. 1. Physical maps of plasmids pBP567 and pBP56717. The arrows indicate positions and orientations of the genes *parC*, coding for subunit A of Topo IV from *E. coli*, *aadB*, coding for an aminoglycoside-2″-adenosyltransferase mediating gentamicin resistance, *aacA*, coding for aminoglycoside-6′-acetyltransferase (AAC6′-Ia) mediating AMI resistance, and *gyrA*, coding for subunit A of DNA gyrase. The lengths of the arrows are to scale. *p* indicates the positions of the promoters of *parC* and *gyrA*. The recognition sites of some selected restriction nucleases are indicated.

TABLE 3. Dominance test results

Clone	Mutation(s) in:		MIC (μg/ml) of CIP (NAL)	scMIC (μg/ml) of CIP (NAL) for clone with:					
	gyrA	*parC*		pBP507 (vector)[a]	pBP517 (*gyrA*$^+$)	pBP567 (*parC*$^+$)	pBP56717 (*gyrA*$^+$ *parC*$^+$)	pBP567-4 (*parC*$^{I\text{-}80}$)	pBP567-9 (*parC*$^{I\text{-}80,G\text{-}84}$)
WT			0.015 (2)	0.03 (2)	0.03 (4)	0.03 (2)	0.015 (4)	0.03 (2)	0.03 (4)
MI	S83L		0.5 (512)	1 (256)	0.03 (4)	1 (256)	0.03 (4)	2 (256)	1 (256)
MII	S83L		2 (2,048)	4 (1,024)	0.125 (16)	4 (1,024)	0.06 (8)	8 (1,024)	4 (1,024)
MIII	S83L, D87G	S80I	64 (>2,048)	128 (>1,024)	0.25 (32)	8 (1,024)	0.25 (32)	128 (>1,024)	128 (>1,024)
MIVb	S83L, D87G	S80I	256 (>2,048)	256 (>1,024)	0.25 (32)	8 (1,024)	0.25 (64)	128 (>1,024)	256 (>1,024)
R17	S83L, D87G	S80I	256 (>2,048)	256 (>1,024)	0.06 (2)	1 (256)	0.03 (2)	256 (>1,024)	256 (>1,024)
4469	S83L		4 (2,048)	2 (1,024)	0.06 (4)	1 (512)	0.06 (4)	4 (>1,024)	2 (>1,024)
3204917	S83W	G78D	2 (256)	8 (128)	0.06 (8)	1 (128)	0.06 (8)	8 (>1,024)	8 (>1,024)
129801	S83L, D87N	E84K	16 (2,048)	32 (1,024)	0.03 (4)	0.5 (128)	0.03 (4)	32 (>1,024)	64 (1,024)
133700	S83L, D87N	S80I	64 (2,048)	64 (1,024)	0.06 (4)	4 (128)	0.06 (4)	64 (1,024)	128 (>1,024)
HP24704-1	S83L, D87N	S80R	256 (>2,048)	128 (>1,024)	0.06 (8)	8 (512)	0.06 (8)	128 (>1,024)	128 (>1,024)
130162	S83L, D87Y	S80I	32 (>2,048)	32 (>1,024)	0.125 (8)	2 (128)	0.125 (8)	32 (>1,024)	64 (>1,024)
205096	S83L, D87G	E84K	64 (1,024)	64 (1,024)	0.03 (4)	2 (256)	0.015 (4)	64 (1,024)	32 (1,024)
U12987	S83L, D87N	S80I, E84G	128 (>2,048)	128 (>1,024)	0.06 (8)	2 (256)	0.06 (4)	16 (>1,024)	64 (>1,024)
136437	S83L, D87N	S80I, E84G	64 (>2,048)	128 (>1,024)	0.06 (8)	0.5 (128)	0.06 (8)	16 (>1,024)	128 (>1,024)

[a] The scMICs were identical in the presence and absence of vector plasmid pBP507.

and CR56717. These were used together with strains CR507 and CR517 for conjugational mobilization of the different pBP-plasmids into the *E. coli* isolates. This transfer technique yielded recombinant cells from all isolates, while plasmid transfer by transformation failed in several cases. This failure could be due to restriction-modification interference. The scMIC determinations were done at least in duplicate with two transconjugants obtained from separate mobilization experiments. Thus, the data in Table 3 show representative results of multiple determinations.

Introduction of the *parC*$^+$ gene from *E. coli* WT (plasmid pBP567) into different quinolone-resistant isolates caused a reduction of the MIC of CIP by three to eight dilution steps for those strains carrying a *parC* mutation(s), while no changes were observed for *parC*$^+$ strains. The differences observed with NAL could not be determined for all isolates because of the high MICs already conferred by a single *gyrA* mutation. Increases in the CIP susceptibilities (5 to 12 dilution steps) after introduction of the *gyrA*$^+$ allele alone or in combination with the *parC*$^+$ allele were identical for both plasmids pBP517 and pBP56717 and were three to seven dilution steps greater than those obtained for pBP567. Introducing the *parC*$^{I\text{-}80}$ allele, which is derived from mutant strain MIII and, therefore, is assumed to differ from the *parC*$^+$ allele in pBP567 by only one mutation, did not result in changes of the quinolone susceptibilities of one-step *parC* mutants except for isolate 3204917: these cells carrying plasmid pBP567-4 or pBP567-9 showed an increase in the resistance towards NAL acid by more than four dilution steps, but showed no increase in resistance towards ciprofloxacin. Introducing the *parC*$^{I\text{-}80}$ allele into isolates U12987 and 136437, both carrying two *parC* mutations, resulted in an increase in the CIP susceptibilities by three dilution steps. In some strains, the introduction of a resistant *parC* allele on a plasmid caused an increase in the scMIC by one dilution step, e.g., *parC*$^{I\text{-}80}$ in *parC*$^+$ strains MI, MII, and 4469, and *parC*$^{I\text{-}80,G\text{-}84}$ in strains WT (*parC*$^+$), and 129801, 130162, and 133700 carrying one-step mutations in *parC*.

DISCUSSION

While it is generally accepted that *gyrA* mutations play a major role in the development of fluoroquinolone resistance in *E. coli*, the data presented here for clinical isolates as well as for mutants selected in vitro demonstrate that *parC* mutations are additionally associated with resistance.

All mutations detected in *parC* alter residues in the QRDR

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GYRASE A  *                          *  * *    *                              *     Ref.
E.coli   67A R V V G D V I G K Y H P H G D S A V Y D T I V R M A Q P F S L R Y M L V D G Q106   5,11,43,49
S.tym.   67A R V V G D V I G K Y H P H G D S A V Y D T I V R M A Q P F S L R Y M L V D G Q106   15,35
N.gon.   75A R I V G D V I G K Y H P H G D S A V Y D T I V R M A Q N F A M R Y V L I D G Q114   4
S.aur.   68A R I V G D A M G K Y H P H G D S S I Y E A M V R M A Q D F N Y R Y M L V D G H107   10,29,41
TOPO IV A                            *  * *    *
E.coli   64A R T V G D V L G K Y H P H G D S A C Y E A M V L M A Q P F S Y R Y P L V D G Q103   25,26,34
S.tym.   64A R T V G D V L G K Y H P H G D S A C Y E A M V L M A Q P F S Y R Y P L V D G Q103   28
N.gon.   71A R V V G E I L G K Y H P H G D S S A Y E A M V R M A Q D F T I R Y P L I D G I110   4
S.aur.   64A K T V G D V I G Q Y H P H G D S S V Y E A M V R L S Q D W K L R H V L I E M H103   8,9
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FIG. 2. Comparison of amino acid sequences of QRDRs of the A subunits of DNA gyrase and Topo IV from *E. coli*, *S. typhimurium* (*S.tym.*), *N. gonorrhoeae* (*N.gon.*), and *S. aureus* (*S.aur.*) The numbers at the beginning and end of each sequence indicate the first and last amino acids of the QRDR. Amino acids which differ from the respective residue of the *E. coli* DNA gyrase A subunit are shown in boldface letters. Residues which are altered in quinolone-resistant isolates of the respective species are marked by an asterisk.

(49), a region highly conserved among various type II topoisomerases (46) (Fig. 2, Table 2). While the *gyrA* mutations which affect residues serine 83 and aspartate 87 are known from previous studies on fluoroquinolone resistance in *E. coli* (7, 16, 32, 33, 44, 49), no naturally occurring *parC* mutation in *E. coli* has been reported. The *parC* mutations presented here which affect residues serine 80 and glutamate 84 (Table 2) are comparable not only to those of *gyrA* but also to those detected in *parC* of fluoroquinolone-resistant mutants of *N. gonorrhoeae* (4) and *grlA* of *S. aureus* (Fig. 2) (8, 9).

The G78D mutation in the *parC* gene of isolate 3204917 is newly detected and is analogous to a G81D mutation detected previously in the *gyrA* gene of an isolate of *E. coli* resistant to fluoroquinolones but not to NAL (5). Interestingly, for isolate 3204917 the MIC of CIP (2 μg/ml) is elevated significantly above that of a single-step *gyrA* mutation (e.g., 0.5 μg/ml for MI [Table 3]), while the MIC of NAL is low (comparable to that of a single-step *gyrA* mutation). Obviously, this *parC* mutation (G78D) confers a similar quinolone resistance phenotype as its *gyrA* analog.

The finding that all mutations in *gyrA* and *parC* affect analogous residues in highly conserved regions is in agreement with the assumption of Reece and Maxwell (36) that quinolones interact with some kind of pocket characteristic for eubacterial type II topoisomerases. Thus, the QRDR is likely to be involved in forming such a pocket.

Mutant MIII obtained in three consecutive selection steps from a quinolone-susceptible WT isolate in vitro, has been shown to carry a second *gyrA* mutation (D87G) in addition to an S83L mutation (MI) and an as yet unidentified mutation reducing the amount of OmpF (MII). Concomitantly, the MIC of CIP increased by an additional five dilution steps from 2 to 64 μg/ml (17). The present investigation demonstrated that MIII but not MII carries an additional mutation in *parC*, S80I (Table 2). The occurrence of two mutations in one selection procedure (MII to MIII) is unusual but has been observed by others during the selection of fluoroquinolone-resistant mutants of *S. aureus* in vitro (22). Maintaining the isolated mutant under selective pressure, i.e., in the case of MII cells growing in the presence of 1 μg of CIP per ml for the next selection step, may have favored the establishment of a clone carrying the second *gyrA* mutation or the *parC* mutation. This view is supported by the finding that the mutation frequency determined for the third selection step was the lowest of all steps (17).

The most striking evidence for a role of *parC* mutations in fluoroquinolone resistance comes from the results of the dominance tests (Table 3). As has been observed for *gyrA* and *gyrB* heterozygotes the plasmid-coded $parC^+$ allele is dominant over the chromosomally coded *parC* allele. However, introduction of the $gyrA^+$ allele alone (plasmid pBP517) or in combination with the $parC^+$ allele (plasmid pBP56717) yielded identical quinolone MICs (within one dilution step) significantly lower than those obtained with $parC^+$ alone. These findings provide a reasonable explanation for the apparent lack of *parC* mutations in *E. coli*: the introduction of the $gyrA^+$ gene restores in the cell the target with a higher degree of quinolone sensitivity, irrespective of the presence or absence of *parC* mutations. Moreover, a combination of one *gyrA* mutation with one mutation affecting CIP accumulation (MII) results in a level of resistance to CIP similar to that resulting from the combination of one *gyrA* and one *parC* mutation (3204917 [Table 3]). Thus, in *E. coli* the detection of *parC* mutations by a dominance test requires a *gyrA* but not a $gyrA^+$ background.

The alterations of the quinolone susceptibilities after transfer of the $parC^{I\text{-}80}$ allele (plasmid pBP567-4) were dependent on the respective chromosomal allele: while those of one-step *parC* mutants did not change (except for isolate 3204917), those of two-step *parC* mutants increased. This dominance of a plasmid-coded susceptible allele over a chromosomally coded (more) resistant allele is in agreement with data obtained for pBP567.

In contrast, transfer of $parC^{I\text{-}80}$ or $parC^{I\text{-}80,G\text{-}84}$ into a $parC^+$ background showed a slight increase in the MIC of CIP for some strains (Table 3). Since this inversion of the dominance effect is not found for all strains and the differences are within the range of one dilution step only, the significance of these results remains speculative. However, recent data from Khodursky et al. (26) are in agreement with the present findings. Khodursky et al. (26) assume that, in contrast to gyrase, which mainly operates during the elongation phase of DNA replication, Topo IV acts in the termination step (1). Thus, in the presence of inhibitory concentrations of quinolones, Topo IV, like gyrase, remains fixed to DNA. However, topo IV-mediated blockage does not become lethal before a prolonged lag phase allowing repair. Consequently, the magnitude of the dominance effect of the plasmid-coded *parC* allele is influenced by the copy number of the respective plasmid vector, as is demonstrated by Khodursky et al. (26). However, the observed differences in the quinolone susceptibilities were so small that a detection by the scMIC method is difficult.

The clinical importance of *parC* mutations can be estimated only from a comparison of *E. coli* WT and its consecutive mutants: the scMIC of CIP increases by five and two dilution steps for mutants MI and MII, respectively. This effect cannot be reversed by the introduction of the $parC^+$ allele (Table 3). Mutant MIII isolated in the following step carries two additional mutations (D87G in *gyrA* and S80I in *parC* [Table 2]) which are associated with an additional increase in the MIC of CIP by five dilution steps. Introduction of $parC^+$ partly reverses this increase by four steps. The remaining difference of one dilution step corresponds to the increase in the MIC of CIP observed after the introduction of the *gyrA* D87G mutation into the chromosome of MII ($parC^+$ $gyrA^{S83L}$ *mar*-like) (14). Thus, it is reasonable to assume that alteration of the natural primary target gyrase by one mutation makes Topo IV the primary target (MI) of quinolone action. The MIC of CIP for MI reflects the susceptibility of Topo IV. The situation is only slightly affected by a second *gyrA* mutation, but a *parC* mutation would reduce the accessibility of Topo IV (isolate 3204917). In this situation, the mutant gyrase becomes the primary target, whose susceptibility to CIP is reflected by the MIC for this isolate. High-level resistance towards CIP is found only if a second mutation accumulates in *gyrA*. This renders the mutant Topo IV the primary target (MIII). Still uncertain in this proposed "ping-pong" scenario is the role of additional mutations in *parC*, like those detected in isolates U12987 and 136437; these strains are as resistant as isolate HP24704-1, which carries only one *parC* mutation (Table 2). Perhaps nonspecific mutations which reduce the accumulation of quinolones, like those found in MII or in 205096, are sufficient to increase the resistance to a level similar to that obtained by a second *parC* mutation.

The data presented here implicate Topo IV as a secondary target in *E. coli* for quinolone antibacterial agents in vivo. From the clinical point of view, a prolonged exposure of the bacteria to drug concentrations transiently high enough to select for this resistance phenotype as well as environmental conditions in the patient (e.g., compromised immune defense) permitting the accumulation of large bacterial populations could set the stage for the development of such consecutive mutations. To preserve for the future the still-uncontested

antibacterial potency of fluoroquinolone antibacterial agents against *E. coli*, it is necessary to prevent the single-agent use of these drugs for long-term treatment of such patients. Moreover, routinely screening for low-level fluoroquinolone-resistant isolates should allow the early adjustment of the antibacterial chemotherapy.

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