ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1999, p. 62–66 Vol. 43, No. 1
0066-4804/99/$04.00+0

Type II Topoisomerase Mutations in Ciprofloxacin-Resistant Strains of *Pseudomonas aeruginosa*

HYAM MOUNEIMNÉ, JÉROME ROBERT, VINCENT JARLIER, AND EMMANUELLE CAMBAU*

Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Paris VI, Faculté de Médecine Pitié-Salpêtrière, 75634 Paris Cedex 13, France

Received 20 March 1998/Returned for modification 28 May 1998/Accepted 30 September 1998

We determined the sequences of the quinolone resistance-determining regions of *gyrA*, *gyrB*, and *parC* genes for 30 clinical strains of *Pseudomonas aeruginosa* resistant to ciprofloxacin that were previously complemented by wild-type *gyrA* and *gyrB* plasmid-borne alleles and studied for their coresistance to imipenem (E. Cambau, E. Perani, C. Dib, C. Petinon, J. Trias, and V. Jarlier, Antimicrob. Agents Chemother. 39:2248–2252, 1995). In the present study, we found mutations in type II topoisomerase genes for all strains. Twenty-eight strains had a missense mutation in *gyrA* (codon 83 or 87). Ten of them had an additional mutation in *parC* (codon 80 or 84), including a novel mutation of Ser-80 to Trp, but all were fully complemented by a plasmid-borne wild-type *gyrA* allele. The remaining two strains harbored the first *gyrB* mutation described in *P. aeruginosa*, leading to the substitution of phenylalanine for serine 464. The strains which had two mutations in type II topoisomerase genes (i.e., *gyrA* and *parC*) were significantly more resistant to fluoroquinolones than those with a single mutation in *gyrA* or *gyrB* (geometric mean MICs of ciprofloxacin, 39.4 versus 10.9 μg/ml, $P < 0.01$; geometric mean MICs of sparfloxacin, 64.0 versus 22.6, $P < 0.01$). No mutant with a *parC* mutation alone was observed, which favors DNA gyrase being the primary target for fluoroquinolones. These results demonstrate that *gyrA* mutations are the major mechanism of resistance to fluoroquinolones for clinical strains of *P. aeruginosa* and that additional mutations in *parC* lead to a higher level of quinolone resistance.

The intrinsic resistance of *Pseudomonas aeruginosa* to a wide variety of antibiotics represents a major therapeutic challenge. Ciprofloxacin has emerged as one of the most effective quinolones against *P. aeruginosa* (28). Imipenem has also been widely used for the treatment of *P. aeruginosa* infections in hospitals. The intensive use of these antibiotics has led to the emergence of resistant strains, some of which are coresistant to quinolones and carbapenems (20, 25, 28). The question of a common mechanism of resistance to quinolones and carbapenems has been raised by several authors (18, 21). The mechanisms of quinolone resistance described in *P. aeruginosa* are mutations in the DNA gyrase *gyrA* gene (15, 32, 34) and, recently, in the topoisomerase IV *parC* gene (19), decreased permeability of the cell wall, and multidrug efflux systems (17). The latter mechanisms can affect both quinolones and a wide variety of antibiotics other than quinolones, including carbapenems (5, 17). However, we have previously demonstrated that in 20 clinical strains of *P. aeruginosa*, coresistance to ciprofloxacin and imipenem was due to two distinct mechanisms (2). In these strains, imipenem resistance was associated with a lack of a specific outer membrane protein, OprD (4, 24), whereas ciprofloxacin resistance was essentially related to *gyrA* or *gyrB* mutations, as suggested by complementation tests with plasmids containing either wild-type *gyrA* or *gyrB* genes. However, the wide range of ciprofloxacin MICs (2 to 128 μg/ml) among the strains suggested the association of several mechanisms of resistance in those with the highest level of resistance. Indeed, an additive effect of several mutations affecting *gyrA* or both *gyrA* and *parC* has been described in other gram-negative bacteria (3, 9, 26). To characterize the mutations in type II topoisomerase genes, we analyzed the quinolone resistance-determining regions (QRDR) of the *gyrA*, *gyrB*, and *parC* genes of the 30 clinical ciprofloxacin-resistant *P. aeruginosa* strains previously studied by complementation tests (2).

* Corresponding author. Mailing address: Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Paris VI, Faculté de Médecine Pitié-Salpêtrière, 91 bd de l'hôpital, 75634 Paris Cedex 13, France. Phone: (33) 1 40 77 97 46. Fax: (33) 1 45 82 75 77. E-mail: bacterio@biomath.jussieu.fr.

MATERIALS AND METHODS

Strains. Thirty *P. aeruginosa* strains resistant to ciprofloxacin (MIC, 2 to 128 μg/ml), collected between 1990 and 1992 from clinical specimens in our hospital, were included in the study. Twenty of these strains were resistant to imipenem (MICs, ≥8 μg/ml). MIC determination and transformation with plasmids carrying the wild-type *gyrA* gene (pPAW207) or *gyrB* gene (pPBW801) had been previously carried out in these strains and were detailed elsewhere (2). Two wild-type strains were included as controls: strain 65, which was a clinical strain, and the reference strain PAO1, kindly given by E. Collatz.

PCR amplification and DNA sequencing. For DNA extraction, 2 ml of an 18-h culture in brain heart infusion broth was centrifuged (13,000 × *g*, 1 min). The pellet was suspended in 500 ml of H_2O, and cells were lysed by the freeze-thaw procedure, alternately 1 min at 100°C and 1 min at 0°C, 10 times (29). Two 21-mer primers, PSE1 (5′-GACGGCCTGAAGCCGGTGCAC-3′) and PYO1 (5′-GCCCACGGCGATACCGCTGGA-3′), constructed on the basis of the nucleotide sequence of *P. aeruginosa* PAO1 *gyrA* (15), were used to amplify a 417-bp fragment of *P. aeruginosa gyrA* from positions 115 to 531. Two primers, GYRB1 (5′-GCGCGTGAGATGACCCGCCGT-3′) and GYRB2 (5′-CTGGC GGTAGAAGAAGGTCAG-3′), designed to be homologous to the *gyrB* gene of *Escherichia coli* (30) from nucleotides 1162 to 1182 and from nucleotides 1531 to 1551, respectively, were used to amplify a 390-bp fragment of *P. aeruginosa gyrB*. Two primers, PARC1 (5′-CTGGATGCCGATTCCAAGCAC-3′) and PARC2 (5′-GAAGGACTTGGGATCGTCCGG-3′), constructed on the basis of the partial sequence of *P. aeruginosa parC* (19), were used to amplify a 186-bp fragment of *parC*. All amplifications were carried out in a 100-μl volume containing 0.4 μM each nucleotide primer, 0.25 mM each 2′-deoxynucleoside 5′-triphosphate (Pharmacia Biotech, Orsay, France), 10 μl of reaction buffer (Boehringer Mannheim, Meylan, France), 2 μl of a template DNA sample, and 2 U of *Taq* DNA polymerase (Boehringer Mannheim). The reactions were performed on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 40 cycles, each cycle consisting of 1 min at 94°C for denaturation, 1 min at 65°C (*gyrA* and *gyrB*) or 61°C (*parC*) for annealing, and 1 min at 72°C for polymerization. For each *P. aeruginosa* strain, the amplified DNA was pooled from two different PCR assays and purified by using the Geneclean kit (Bio 101, La Jolla, Calif.) in accordance with the manufacturer's recommendations.

Sequencing of the *gyrB* and *parC* fragments was done with the same primers as

TABLE 1. Clinical strains of *P. aeruginosa*: patterns of susceptibility to fluoroquinolones and imipenem, results of complementation tests with wild-type *gyrA* or *gyrB* alleles, and DNA mutations found in *gyrA*, *gyrB*, or *parC* QRDR

Strain	MIC (μg/ml) of[a]:					Amino acid (codon) encoded by indicated gene at position:				
	CPX			SPX	IPM	*gyrA*		*gyrB*	*parC*	
	NT[b]	*gyrA*$^{+}$[c]	*gyrB*$^{+}$[d]	NT	NT	83	87	464	80	84
PAO1	0.12	0.12	0.12	0.25	1	Thr (ACC)	Asp (GAC)	Ser (TCC)	Ser (TCG)	Glu (GAG)
65	0.25	0.25	0.25	1	1	—[e]	—	—	—	—
21	2	0.12	2	4	8	Ile (ATC)	—	—	—	—
119	2	0.5	4	8	4	Ile (ATC)	—	—	—	—
1311	2	0.5	4	8	8	Ile (ATC)	—	—	—	—
44	4	0.25	4	8	64	Ile (ATC)	—	—	—	—
47	4	0.5	4	8	32	Ile (ATC)	—	—	—	—
128	4	4	0.06	16	2	—	—	Phe (TTC)	—	—
1273	4	0.25	4	16	1	—	Gly (GGC)	—	—	—
92	4	0.5	8	32	1	Ile (ATC)	—	—	—	—
74	8	0.12	4	32	2	Ile (ATC)	—	—	—	—
2120	8	4	0.12	32	1	—	—	Phe (TTC)	—	—
P136	8	0.12	16	16	16	Ile (ATC)	—	—	Leu (TTG)	—
2	16	0.25	16	32	16	Ile (ATC)	—	—	—	—
57	16	0.25	16	32	16	Ile (ATC)	—	—	—	—
78	16	0.5	16	32	16	Ile (ATC)	—	—	—	—
134	16	0.12	16	32	16	Ile (ATC)	—	—	Leu (TTG)	—
D	32	0.25	16	32	32	Ile (ATC)	—	—	Leu (TTG)	—
6243	32	0.5	32	32	32	Ile (ATC)	—	—	—	—
C6241	32	0.25	16	64	16	Ile (ATC)	—	—	Leu (TTG)	—
J15	32	0.5	16	32	16	Ile (ATC)	—	—	—	—
J388	32	0.5	32	32	32	Ile (ATC)	—	—	—	—
C5904	32	0.5	16	128	2	Ile (ATC)	—	—	Trp (TGG)	—
76	32	0.25	16	32	16	Ile (ATC)	—	—	—	—
5299	64	0.12	16	64	32	Ile (ATC)	—	—	—	—
8	64	0.5	32	64	16	Ile (ATC)	—	—	Leu (TTG)	—
77	64	0.5	64	128	16	Ile (ATC)	—	—	Leu (TTG)	—
137	64	0.25	64	128	8	Ile (ATC)	—	—	—	Lys (AAG)
1746	64	0.5	32	64	2	Ile (ATC)	—	—	Trp (TGG)	—
2863	64	0.25	32	128	4	Ile (ATC)	—	—	—	—
J431	64	0.5	32	32	32	Ile (ATC)	—	—	—	—
J1375	128	0.12	128	128	0.5	Ile (ATC)	—	—	Leu (TTG)	—

[a] CPX, ciprofloxacin; SPX, sparfloxacin; IPM, imipenem.
[b] NT, not transformed.
[c] Strain transformed with plasmid pPAW207 carrying the wild-type *gyrA* gene of *E. coli*.
[d] Strain transformed with plasmid pPBW801 carrying the wild-type *gyrB* gene of *E. coli*.
[e] —, amino acid and codon identical to those of strain PAO1.

those used for PCR. For the *gyrA* fragments, we had to design internal primers, PYO2 (5′-ATGAGCGAGCTGGGCAACGAC-3′) and PSE2 (5′-CAGGTCCG CCAGCAGTTCGTG-3′) from positions 154 to 414, to facilitate its sequencing. For *gyrA* and *gyrB*, sequence reactions were performed on both DNA strands with the T7 sequencing kit (Pharmacia Biotech) by the sequencing technique of Tabor and Richardson (23) modified as previously described (16). For *parC*, the purified DNA was sequenced by the Genome Express Society (Paris, France). For each DNA fragment, mutations were confirmed on a new PCR product.

Antibiotic resistance patterns. The MICs of ciprofloxacin, sparfloxacin, and imipenem were determined in a previous study (2). The $\log_{10}$ MICs were compared by use of the Mann-Whitney U test. Differences between groups were considered to be statistically significant for P values of <0.05.

Nucleotide accession number. The partial sequence of *gyrB* from *P. aeruginosa* PAO1 was submitted to GenBank, EMBL and DDJB under accession no. Y16286.

RESULTS

The mutations found in *gyrA*, *gyrB*, and *parC* QRDR of the 30 clinical ciprofloxacin-resistant *P. aeruginosa* strains are described in Table 1.

***gyrA* mutations.** The nucleotide sequence of the *gyrA* fragment obtained from the wild-type ciprofloxacin-sensitive *P. aeruginosa* strain 65, used as a control, was identical to the sequence of strain PAO1 (15), except for two silent mutations in codons 91 (CGT instead of CGC) and 132 (CAT instead of CAC). Twenty-eight of the 30 ciprofloxacin-resistant *P. aeruginosa* strains harbored a *gyrA* mutation. Twenty-seven of them had an ACC-to-ATC mutation in codon 83, leading to the amino acid substitution of an isoleucine for a threonine. The remaining strain had a GAC-to-GGC mutation in codon 87, leading to the substitution of a glycine for an aspartic acid. A polymorphism was found in codon 132, with the nucleotides encoding histidine 132 being CAT for 17 strains, as already mentioned for strain 65, and CAC for the remaining 13 strains, as described for PAO1 (15).

***gyrB* mutations.** Since the *gyrB* sequence of *P. aeruginosa* was not available, we used the *gyrB* sequence of *E. coli* (30) to design primers for PCR. Primers were chosen in order to amplify a fragment including the putative QRDR (amino acids 426 to 447 in *E. coli*) (30). PCR amplification of total DNA from *P. aeruginosa* with the primers GYRB1 and GYRB2 resulted in several DNA fragments. Only one fragment had the predicted size (390 bp), and it was subsequently purified from the agarose gel and sequenced, leading to the analysis of a 303-bp DNA fragment (Fig. 1). The nucleotide sequence had 86% identity with the corresponding *gyrB* fragment of *Pseudomonas putida* and 83% with that of *E. coli* and *Salmonella enterica* serovar Typhimurium, which have the same sequence. The deduced amino acid sequence had 98% identity

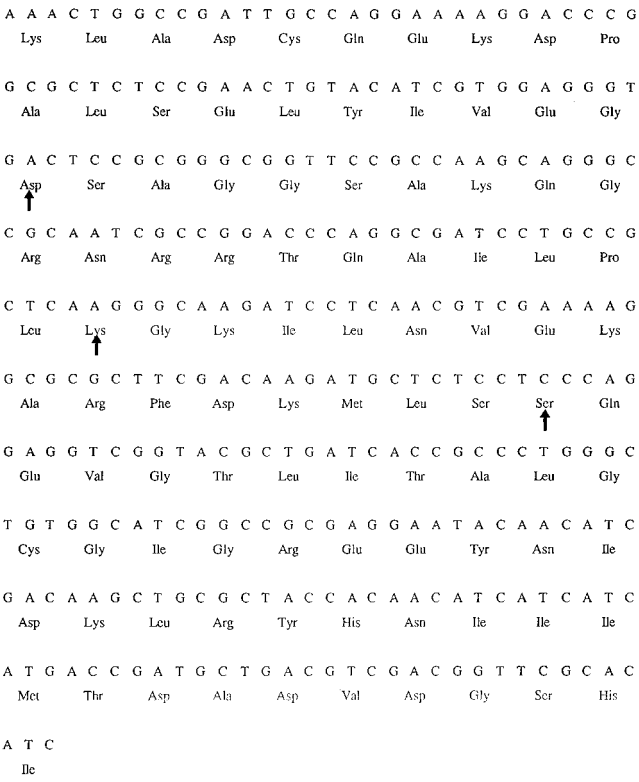

FIG. 1. Nucleotide sequence of the 303-bp fragment of the *gyrB* gene of *P. aeruginosa* PAO1 (corresponding to nucleotides 1216 to 1518 in *E. coli gyrB*), including the putative QRDR. The arrows indicate codons 426, 447, and 464.

with the corresponding GyrB sequence of *P. putida* (31) and 92% with that of *E. coli* (Fig. 2).

The nucleotide sequence of the *gyrB* fragment from the wild-type *P. aeruginosa* strain 65 was identical to that of PAO1. Strains 128 and 2120, which have been complemented by a wild-type *gyrB* gene, did not have mutations in codon 426 or 447, where mutations were described in *E. coli* (30). However, they had a TCC-to-TTC mutation in codon 464, leading to the amino acid substitution of a phenylalanine for a serine. In all of the other *P. aeruginosa* strains, we did not find any mutation in the *gyrB* fragment leading to amino acid changes. Still, compared to the sequence of the *gyrB* fragment obtained from PAO1, silent mutations were found in codons 406 (AAA to AAG, 7 strains), 407 (CTG to CTT, 1 strain), 436 (CGC to CGT, 1 strain), 454 (GAA to GAG, 6 strains), 456 (GCG to GCA, 4 strains), 472 (ACC to ACT, 12 strains), 479 (GGC to GGT, 6 strains), and 482 (GAA to GAG, 3 strains).

Overall, we found 28 strains with a *gyrA* mutation and 2 strains with a *gyrB* mutation. These results were consistent with the results of the complementation tests performed previously (2) except for those for two strains, 128 and J1375, for which there had been a mix of plasmids carrying *gyrA* or *gyrB* at the time of complementation. New complementation tests performed with the original clinical strains showed that complementation of strain 128 occurred only with the wild-type *gyrB* plasmid and that of strain J1375 occurred only with the wild-type *gyrA* plasmid.

***parC* mutations.** The nucleotide sequences of the *parC* fragments from the ciprofloxacin-susceptible strains PAO1 and 65 were identical to that described by Nakano et al. (19). Ten *P. aeruginosa* strains harbored a mutation in *parC*. For nine of them, the mutation was located in the codon corresponding to Ser-80 in *E. coli parC* and led to the amino acid substitution of leucine for serine (seven strains) or of tryptophan for serine (two strains). The remaining strain had a mutation in the codon corresponding to Glu-84 in *E. coli*, leading to the amino acid substitution of lysine for glutamic acid. All of the strains with *parC* mutations also had a mutation in the *gyrA* codon corresponding to Thr-83. In contrast to *gyrA* and *gyrB*, no polymorphism was found in the sequences of the *parC* fragments.

Correlation between antibiotic resistance patterns and topoisomerase mutations. For all strains, resistance to ciprofloxacin was well correlated with resistance to sparfloxacin (Table 1). Ciprofloxacin and sparfloxacin MICs were significantly higher for strains with a double *gyrA*-parC mutation than for isolates with a single mutation in *gyrA* or *gyrB* (geometric mean MICs of ciprofloxacin, 39.4 versus 10.9 μg/ml, $P = 0.008$; geometric mean MICs of sparfloxacin, 64.0 versus 22.6 μg/ml, $P = 0.003$). Ciprofloxacin and sparfloxacin MICs were not statistically different for imipenem-resistant (Imi^r) and imipenem-susceptible (Imi^s) strains. However, ciprofloxacin MICs were slightly higher for Imi^r strains than for Imi^s ones (geometric mean MICs, 19.0 versus 13.0 μg/ml, $P = 0.53$), particularly for the strains with a single mutation in *gyrA* or *gyrB* (14.4 versus 6.5 μg/ml, $P = 0.21$), although these differences did not reach statistical significance.

DISCUSSION

Mutations in topoisomerases genes are the main mechanism of fluoroquinolone resistance in many bacteria (28). Previous studies have shown that this mechanism is involved in quinolone resistance of *P. aeruginosa* in clinical practice (2, 15, 19, 34). Herein, we screened for mutations in *gyrA*, *gyrB*, and *parC*

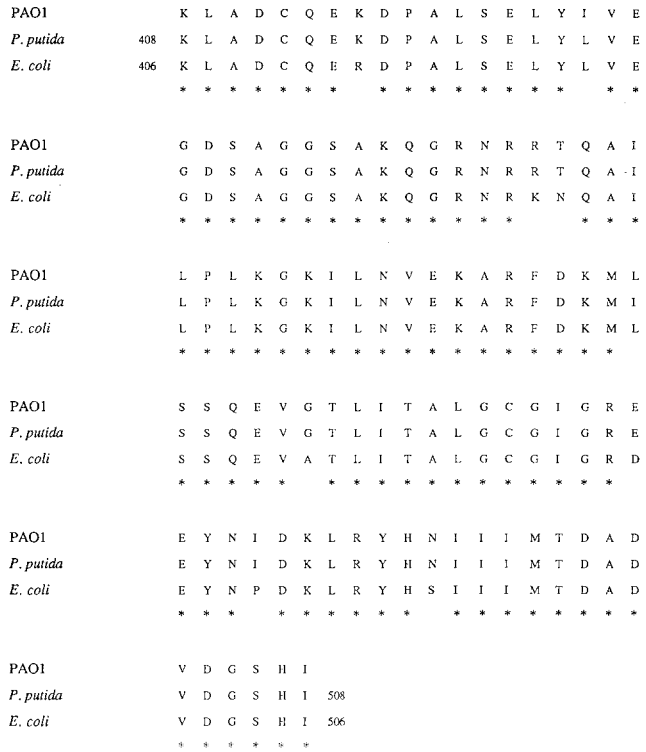

FIG. 2. Comparison of the amino acid sequences encoded by the 303-bp *gyrB* fragments of *P. aeruginosa* PAO1, *P. putida*, and *E. coli*. The conserved residues are indicated by asterisks.

in 30 clinical ciprofloxacin-resistant *P. aeruginosa* strains which were shown to be fully complemented by a plasmid carrying the wild-type *gyrA* or *gyrB* allele in a previous work (2).

DNA sequencing of the *gyrA* QRDR showed a high frequency of point mutations in codon 83 (27 of 30 strains), whereas only one strain had a point mutation in codon 87. These results are consistent with those reported in previous studies on clinical fluoroquinolone-resistant strains of *P. aeruginosa* in which 13 of 22 strains (19) and 10 of 18 strains (32) harbored a single *gyrA* mutation in codon 83 and 1 of 22 strains (19) and 1 of 18 (32) harbored only a single mutation in codon 87. We did not find any strain with a double mutation in codons 83 and 87 such as that described by Nakano et al. (19). Strikingly, in our study as well as in others dealing with *P. aeruginosa* (15, 19, 32) or *Campylobacter jejuni* (27), the point mutation at position 83 always led to the substitution of an isoleucine for a threonine, despite several other possible amino acid substitutions encoded by a point mutation in an ACC codon (Pro, Ala, Ser, and Asn). This could not be attributed to a clonal relation between the strains of *P. aeruginosa* studied, since these strains had different antibiotypes and serotypes (4) and were isolated over a long period of time (3 years). In *E. coli*, mutagenesis studies have shown that the mutation Ser-83 to Ala was responsible for a low level of ciprofloxacin resistance compared to the Ser-to-Leu or Ser-to-Trp mutations described in strains selected in vivo (8, 33). Thus, we assume that the putative mutation Thr-83 to Ala in *gyrA* would lead to a poor selective advantage in *P. aeruginosa* in vivo, in contrast to the mutation Thr-83 to Ile. This point could be further investigated in a directed mutagenesis study.

We sequenced a 303-bp *gyrB* fragment from *P. aeruginosa* which included the QRDR. This fragment showed a high homology with the corresponding *gyrB* fragment of *Pseudomonas putida* (i.e., same genus) but also with that of other members of the family *Enterobacteriaceae* such as *E. coli* or *S. enterica* serovar Typhimurium. Since some regions of the ParE subunit of topoisomerase IV are highly homologous to the GyrB subunit of DNA gyrase in *E. coli* (13), we compared our sequence to the ParE amino acid sequence of *E. coli*. Amino acid identity was only 52% with ParE, in contrast to 92% identity with GyrB, suggesting that our DNA fragment corresponded to *gyrB* and not to *parE*.

We described the first mutation in the *gyrB* gene of *P. aeruginosa*, Ser-464 to Phe, located outside the *gyrB* QRDR described in *E. coli* (30) and in *Staphylococcus aureus* (11). Similar mutations have been described in quinolone-resistant strains of *S. enterica* serovar Typhimurium: TCC(Ser)-464 to TAC-(Tyr) (6, 7) and TCC(Ser)-464 to TTC(Phe) (GenBank accession no. Y08383) (14a). Considering the absence of mutations in codons 426 and 447 and the full complementation of these two strains by a wild-type *gyrB* allele, we assume that the mutation found in codon 464 is responsible for quinolone resistance in *P. aeruginosa*. The hydrophobicity and the bulkiness of the R group of phenylalanine in comparison to serine could modify the conformation of the B-subunit domain involved in quinolone interaction. Since there are now four strains belonging to two different genera (*S. enterica* serovar Typhimurium and *P. aeruginosa*) resistant to quinolones and harboring a mutation in codon 464 of the *gyrB* gene, we propose to extend the QRDR in the B subunit of DNA gyrase to the domain of codons 426 to 464.

Analysis of the partial sequences of *gyrA* and *gyrB* showed a few silent base substitutions, resulting in a polymorphism of the sequences of the gyrase genes among *P. aeruginosa* strains. Kureishi et al. mentioned the existence of silent nucleotide changes in *P. aeruginosa gyrA* but did not provide a detailed description (15). Wobble base changes were reported in *C. jejuni* (27) and *P. putida* (31). The polymorphism that we found in *P. aeruginosa gyrB* is similar, for some codons, to that described in *P. putida* (codons 406, AAG or AAA; codons 436, CGC or CGT; codons 456, GCA or GCG; codons 472, ACT or ACC). Yamamoto et al. proposed analysis of the *gyrB* polymorphism as a tool for phylogenetic studies in *P. putida*, since the base substitution frequency in *gyrB* in this species turned out to be higher than that in 16S ribosomal DNA (31).

TABLE 2. Distribution of *P. aeruginosa* strains by susceptibility to ciprofloxacin and imipenem and number of mutations in topoisomerase genes

MIC of ciprofloxacin (μg/ml)	No. of strains with:			
	One mutation[a]		Two mutations[b]	
	Imi^{s} [c]	Imi^{r} [d]	Imi^{s}	Imi^{r}
2	1	2	0	0
4	3	2	0	0
8	2	0	0	1
16	0	3	0	1
32	0	4	1	2
64	1	2	1	3
128	0	0	1	0

[a] Strains with a single mutation in *gyrA* or *gyrB*.
[b] Strains with one mutation in *gyrA* and one mutation in *parC*.
[c] Imi^{s}, susceptible to imipenem at MIC range of 0.5 to 4 μg/ml.
[d] Imi^{r}, resistant to imipenem at MIC range of 8 to 64 μg/ml.

DNA sequencing of the *parC* QRDR showed the higher frequency of point mutations at codon 80 (nine strains), which is homologous to codon 83 in *gyrA*, than in codon 84 (one strain), which is homologous to codon 87 in *gyrA*. Nakano et al. recently reported mutations in codon 80 (Ser to Leu) of *P. aeruginosa parC* for eight clinical strains and in codon 84 (Glu to Lys) for only two strains (19). In the present study, we found a novel substitution of tryptophan for serine encoded by codon 80 for two strains. The mutations Ser to Leu and Ser to Trp which are frequent at position 83 in *E. coli* GyrA are known to be involved in quinolone resistance (33). Since the A subunit of gyrase and the ParC subunit of topoisomerase IV are highly homologous (13), our results emphasize the key role of the serine at position 83 (or at position corresponding to) in both DNA gyrase and topoisomerase IV for determining quinolone resistance.

Only 10 *P. aeruginosa* strains had a mutation in *parC*, whereas all of the strains had a mutation in *gyrA* or *gyrB*. Overall, the strains with a double *gyrA-parC* mutation were three to four times more resistant to ciprofloxacin and sparfloxacin than the strains with a single mutation in *gyrA* or in *gyrB*. We thus hypothesize that in *P. aeruginosa*, mutations in *parC* occur at a second step in strains already harboring mutations in *gyrA* or *gyrB* and lead to a fourfold increase in their fluoroquinolone resistance level. All of the strains with a double *gyrA-parC* mutation were fully complemented by the wild-type *gyrA* gene, as already reported for *E. coli* (9). These findings support the hypothesis that in *P. aeruginosa*, as in *E. coli*, DNA gyrase is the primary target for quinolones (9) and that topoisomerase IV is a secondary target.

There was a large overlap in the distribution of fluoroquinolone MICs within both strains with a double *gyrA-parC* mutation and those with a single mutation in *gyrA* or *gyrB* (Table 2). An additional mechanism(s) of resistance could be implicated in some strains, particularly in those with a single mutation and for which the ciprofloxacin MIC was above 8 μg/ml. Mutations in *parE* can be one of these mechanisms, since *E. coli* strains with double mutations in *gyrA* and *parE* are fully

complemented by a plasmid carrying the wild-type *gyrA* allele (1, 22). Unfortunately, we have not yet succeeded in amplifying the *parE* gene of *P. aeruginosa*.

Twenty of the 30 *P. aeruginosa* strains studied were coresistant to imipenem and fluoroquinolones (ciprofloxacin and sparfloxacin). We have previously demonstrated that in those strains, imipenem resistance was related to the lack of OprD (2), and we demonstrated in the present work that quinolone resistance was related to mutations in DNA topoisomerase genes. For strains having a single mutation in *gyrA* or *gyrB*, not associated with a mutation in *parC*, the level of resistance to ciprofloxacin, but not that to sparfloxacin, was slightly but not significantly higher in Imi^r than in Imi^s strains. Multidrug efflux systems were described in clinical fluoroquinolone-resistant isolates (5, 12, 14), and some of them, such as MexEF-OprN corresponding to the phenotype NfxC, lead to coresistance to quinolones and imipenem (5, 17). The hypothesis that some of the Imi^r strains used in this study overexpress this efflux system is unlikely, since *gyrA-nfxC* double mutants are known to be partially complemented with a plasmid carrying a wild-type *gyrA* gene (5) in contrast to our strains which were all fully complemented as previously shown. For the strains with a double *gyrA-parC* mutation, the fluoroquinolone MICs were not higher in Imi^r than in Imi^s strains. Thus, despite the limited number of strains studied, we assume that the imipenem resistance mechanism has no effect at all on ciprofloxacin resistance when two mutations in topoisomerase genes are present. Taken together, the results of the present work support the hypothesis that the main mechanisms of quinolone resistance in clinical strains of *P. aeruginosa* are mutations in DNA gyrase or topoisomerase IV genes and that the mechanisms conferring quinolone resistance and those conferring imipenem resistance are independent of each other.

ACKNOWLEDGMENT

This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale (CRI no. 950601).

REFERENCES

1. **Breines, D. M., S. Ouabdessalam, E. Y. Ng, J. Tankovic, S. Shah, C. J. Soussy, and D. C. Hooper.** 1997. Quinolone resistance locus *nfxD* of *Escherichia coli* is a mutant allele of the *parE* gene encoding a subunit of topoisomerase IV. Antimicrob. Agents Chemother. **41:**175–179.
2. **Cambau, E., E. Perani, C. Dib, C. Petinon, J. Trias, and V. Jarlier.** 1995. Role of mutations in DNA gyrase genes in ciprofloxacin resistance of *Pseudomonas aeruginosa* susceptible or resistant to imipenem. Antimicrob. Agents Chemother. **39:**2248–2252.
3. **Deguchi, T., M. Yasuda, T. Kawamura, M. Nakano, S. Ozeki, E. Kanematsu, Y. Nishino, and Y. Kawada.** 1997. Improved antimicrobial activity of DU-6859a, a new fluoroquinolone, against quinolone-resistant *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates with alterations in GyrA and ParC proteins. Antimicrob. Agents Chemother. **41:**2544–2546.
4. **Dib, C., J. Trias, and V. Jarlier.** 1995. Lack of additive effect between mechanisms of resistance to carbapenems and other beta-lactam agents in *Pseudomonas aeruginosa*. Eur. J. Clin. Microbiol. Infect. Dis. **14:**979–986.
5. **Fukuda, H., M. Hosaka, S. Iyobe, N. Gotoh, T. Nishino, and K. Hirai.** 1995. *nfxC*-type quinolone resistance in a clinical isolate of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**790–792.
6. **Gensberg, K., Y. F. Jin, and L. J. V. Piddock.** 1995. A novel *gyrB* mutation in a fluoroquinolone-resistant clinical isolate of *Salmonella* typhimurium. FEMS Microbiol. Lett. **132:**57–60.
7. **Gensberg, K., Y. F. Jin, and L. J. V. Piddock.** 1996. Corrigendum to "A novel *gyrB* mutation in a fluoroquinolone-resistant clinical isolate of *Salmonella* Typhimurium." FEMS Microbiol. Lett. **137:**293.
8. **Hallett, P., and A. Maxwell.** 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. Antimicrob. Agents Chemother. **35:**335–340.
9. **Heisig, P.** 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **40:**879–885.
10. **Huang, H., and R. E. W. Hancock.** 1993. Genetic definition of the substrate selectivity of outer membrane porin protein OprD of *Pseudomonas aeruginosa*. J. Bacteriol. **175:**7793–7800.
11. **Ito, H., H. Yoshida, M. Bogaki-Shonai, T. Niga, H. Hattori, and S. Nakamura.** 1994. Quinolone resistance mutations in the DNA gyrase *gyrA* and *gyrB* genes of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38:**2014–2023.
12. **Jakics, E. B., S. Iyobe, K. Hirai, H. Fukuda, and H. Hashimoto.** 1992. Occurrence of the *nfxB* type mutation in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2562–2565.
13. **Kato, J. I., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *E. coli*. Cell **63:**393–404.
14. **Köhler, T., M. Michéa-Hamzehpour, P. Plésiat, A. L. Kahr, and J. C. Pechère.** 1997. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2540–2543.

14a. **Kratz, B.** Unpublished results.

15. **Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan.** 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **38:**1944–1952.
16. **Mabilat, C., S. Goussard, W. Sougakoff, R. C. Spencer, and P. Courvalin.** 1990. Direct sequencing of the amplified structural gene and promoter for the extended-broad-spectrum β-lactamase TEM-9 (RHH-1) of *Klebsiella pneumoniae*. Plasmid **23:**27–34.
17. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
18. **Michéa-Hamzehpour, M., Y. X. Furet, and J. C. Pechère.** 1991. Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**2091–2097.
19. **Nakano, M., T. Deguchi, T. Kawamura, M. Yasuda, M. Kimura, Y. Okano, and Y. Kawada.** 1997. Mutations in the *gyrA* and *parC* genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2289–2291.
20. **Ogle, J. W., L. B. Reller, and M. L. Vasil.** 1988. Development of resistance in *Pseudomonas aeruginosa* to imipenem, norfloxacin, and ciprofloxacin during therapy: proof provided by typing with a DNA probe. J. Infect. Dis. **157:**743–748.
21. **Rådberg, G., L. E. Nilsson, and S. Svensson.** 1990. Development of quinolone-imipenem cross resistance in *Pseudomonas aeruginosa* during exposure to ciprofloxacin. Antimicrob. Agents Chemother. **34:**2142–2147.
22. **Soussy, C. J., J. S. Wolfson, E. Y. Ng, and D. C. Hooper.** 1993. Limitations of plasmid complementation test for determination of quinolone resistance due to changes in the gyrase A protein and identification of conditional quinolone resistance locus. Antimicrob. Agents Chemother. **37:**2588–2592.
23. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84:**4767–4771.
24. **Trias, J., and H. Nikaïdo.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**52–57.
25. **Troillet, N., M. H. Samore, and Y. Carmeli.** 1997. Imipenem-resistant *Pseudomonas aeruginosa*: risk factors and antibiotic susceptibility patterns. Clin. Infect. Dis. **25:**1094–1098.
26. **Vila, J., J. Ruiz, P. Goñi, and T. Jimenez de Anta.** 1997. Quinolone-resistance mutations in the topoisomerase IV *parC* gene of *Acinetobacter baumanii*. J. Antimicrob. Chemother. **39:**757–762.
27. **Wang, Y., W. N. Huang, and D. E. Taylor.** 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob. Agents Chemother. **37:**457–463.
28. **Wolfson, J. S., and D. C. Hooper.** 1989. Fluoroquinolone antimicrobial agents. Clin. Microbiol. Rev. **2:**378–424.
29. **Woods, S. A., and S. T. Cole.** 1989. A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol. Lett. **65:**305–310.
30. **Yamagishi, J. I., H. Yoshida, M. Yamayoshi, and S. Nakamura.** 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Mol. Gen. Genet. **204:**367–373.
31. **Yamamoto, S., and S. Harayama.** PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Environ. Microbiol. **61:**1104–1109.
32. **Yonezawa, M., M. Takahata, N. Matsubara, Y. Watanabe, and H. Narita.** 1995. DNA gyrase *gyrA* mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1970–1972.
33. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34:**1271–1272.
34. **Yoshida, H., M. Nakamura, M. Bogaki, and S. Nakamura.** 1990. Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**1273–1275.