ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1999, p. 530–536 Vol. 43, No. 3
0066-4804/99/$04.00+0
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

Cloning, Expression, and Enzymatic Characterization of *Pseudomonas aeruginosa* Topoisomerase IV

TAKAAKI AKASAKA,* YOSHIKUNI ONODERA, MAYUMI TANAKA, AND KENICHI SATO

New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan

Received 5 October 1998/Returned for modification 12 November 1998/Accepted 30 December 1998

The topoisomerase IV subunit A gene, *parC* homolog, has been cloned and sequenced from *Pseudomonas aeruginosa* PAO1, with cDNA encoding the N-terminal region of *Escherichia coli parC* used as a probe. The homolog and its upstream gene were presumed to be *parC* and *parE* through sequence homology with the *parC* and *parE* genes of other organisms. The deduced amino acid sequence of ParC and ParE showed 33 and 32% identity with that of the *P. aeruginosa* DNA gyrase subunits, GyrA and GyrB, respectively, and 69 and 75% identity with that of *E. coli* ParC and ParE, respectively. The putative ParC and ParE proteins were overexpressed and separately purified by use of a fusion system with a maltose-binding protein, and their enzymatic properties were examined. The reconstituted enzyme had ATP-dependent decatenation activity, which is the main catalytic activity of bacterial topoisomerase IV, and relaxing activities but had no supercoiling activity. So, the cloned genes were identified as *P. aeruginosa* topoisomerase IV genes. The inhibitory effects of quinolones on the activities of topoisomerase IV and DNA gyrase were compared. The 50% inhibitory concentrations of quinolones for the decatenation activity of topoisomerase IV were from five to eight times higher than those for the supercoiling activities of *P. aeruginosa* DNA gyrase. These results confirmed that topoisomerase IV is less sensitive to fluoroquinolones than is DNA gyrase and may be a secondary target of new quinolones in wild-type *P. aeruginosa*.

Bacterial DNA topoisomerases are enzymes responsible for controlling the topological states of DNA in DNA replication and transcription (23). They act upon DNA to alter the level of supercoiling, as well as to catenate and decatenate chromosomes (7, 28). Four DNA topoisomerases have been isolated from *Escherichia coli*: topoisomerase I (44), DNA gyrase (11), topoisomerase III (6), and topoisomerase IV (18). DNA gyrase and topoisomerase IV are classified as type II topoisomerases based on similarities in amino acid sequences and enzymatic mechanisms. The mechanism of these enzymes involves DNA cleavage and DNA strand passage through the break, followed by rejoining of the cleaved DNA (36). DNA gyrase is unique among known DNA topoisomerases because of its ability to introduce negative supercoils into DNA molecules (11). DNA gyrase, a heterotetramer, is composed of two subunits, GyrA and GyrB, which are encoded by the *gyrA* and *gyrB* genes, respectively (1, 41, 45). GyrA is responsible for the DNA strand binding, cleavage, and rejoining, and GyrB is responsible for ATPase activity. The N-terminal region of GyrA is where the covalent attachment of a tyrosine residue to the 5′ end of cleaved DNA is formed (14).

Topoisomerase IV, the other type II DNA topoisomerase, is encoded by the *parC* and *parE* genes in *E. coli* (18). Topoisomerase IV was reported previously to relax superhelical DNA and to decatenate kinetoplast DNA (19, 34, 35). Unlike gyrase, it shows no supercoiling activity. In vitro studies using purified ParC and ParE proteins showed that the decatenation activity of topoisomerase IV was five times more effective than its relaxing activity (15).

The ParC and ParE proteins are homologous to GyrA (36%) and GyrB (40%), respectively, in *E. coli*, and the amino acids around the DNA-binding site (tyrosine at position 122 in GyrA) are particularly well conserved (18, 19, 34). In spite of the high sequence homology between the respective DNA gyrase and topoisomerase IV subunit genes, they cannot complement each other (19, 34). The genes encoding homologs of *E. coli* DNA gyrase and topoisomerase IV subunits have since been identified in many phylogenetic branches of bacteria (16).

Type II topoisomerases have become critical targets of drugs for the treatment of various diseases. Bacterial type II topoisomerases have proven to be important targets for two classes of antimicrobial agents; the A subunit is considered to be a target of quinolones, whereas the B subunits are considered to be that of coumarins (26). Quinolone antibacterial agents have been used in therapy for various bacterial infections (8, 26). In vitro and in vivo studies showed that the activity of DNA gyrase and topoisomerase IV is inhibited by quinolones (8). DNA gyrase is a primary target of quinolones in the gram-negative species, such as *E. coli*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (3, 12, 13, 21). For the *E. coli* enzymes, the inhibition of the decatenating activity of topoisomerase IV requires a 15- to 50-times-higher concentration of quinolones than does the inhibition of the supercoiling activity of DNA gyrase (15). In contrast, the topoisomerase activity of topoisomerase IV is more sensitive than that of DNA gyrase to some quinolones such as levofloxacin and ciprofloxacin in *Staphylococcus aureus* (43).

Pseudomonas aeruginosa is an opportunistic human pathogen and is intrinsically resistant to a wide variety of antibiotics, because of the low outer membrane permeability and drug efflux systems (32). Especially in patients with cystic fibrosis, emergence of antibiotic-resistant *P. aeruginosa* strains is observed (29). The major mechanisms of bacterial resistance to quinolones are the modifications of the target sites of DNA gyrase and topoisomerase IV. Alterations in DNA gyrase or

* Corresponding author. Mailing address: New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 16-13, Kitakasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan. Phone: 81-3-3680-0151. Fax: 81-3-5696-8344. E-mail: akasa94k@daiichipharm.co.jp.

TABLE 1. Nucleotide sequences of primers used in PCR

Primer	Nucleotide sequence	Position	Comment
Pr-PAPARE01	5′-ATGATCGCCTGGAATCCTTTCCCG-3′	*parE* 1310–1285	Amplification of a 1.5-kb fragment containing the 5′ region of *parE*
Pr-PAPARC05	5′-TCATCACCCCGCGCGCCGACCTGCAG-3′	*parC* 683–708	
Pr-PAPARC06	5′-TCTCCGTGAGGGATCCATGAGCGAAT-3′	*parC* −16–10	Amplification of a 2.5-kb fragment containing *parC*
Pr-PAPARC07	5′-ACCAGCGCCAAAGCTTCAGAGGCAGA-3′	*parC* 2450–2425	
Pr-PAPARE03	5′-TTGACTGTCTAGAGACCCCATGGCTA-3′	*parE* −19–7	Amplification of a 1.9-kb fragment containing *parE*
Pr-PAPARE04	5′-GAACAAGCTTAGGATGGCCAGCAGGC-3′	*parE* 1922–1897	
Pr-PAGYRA31	5′-GAAAAAGGATCTAGACTTCTC-3′	*gyrA* −21–1	Amplification of a 2.8-kb fragment containing *gyrA*
Pr-PAGYRA32	5′-CCGAAGCTTACTCTTCGTT-3′	*gyrA* 2778–2759	
Pr-PAGYB103	5′-ACGACCATCGGGAATTCAGCATGAGCGAGA-3′	*gyrB* −20–5	Amplification of a 2.2-kb fragment containing *gyrB*
Pr-PAGYB104	5′-CCGTATCCAAGCTTCCTGGCGCAA-3′	*gyrB* 2502–2478	

topoisomerase IV caused by mutations in the so-called quinolone resistance-determining region (QRDR) (47) of *gyrA* or *parC* appear to provide the resistance in many species of bacteria (8). The *gyrA* gene of *P. aeruginosa* was identified by Kureishi et al. (22), and many mutations of the QRDR of *gyrA* have been found in the quinolone-resistant *P. aeruginosa* (5, 22, 46). Although many studies have focused on DNA gyrase (17, 20, 25, 48, 49), the studies on topoisomerase IV are less advanced for quinolone-resistant *P. aeruginosa*. Recently, Nakano et al. (31) determined QRDR sequences of the *gyrA* and *parC* genes of 22 clinical isolates of *P. aeruginosa* and reported that the accumulation of alterations in GyrA and the simultaneous presence of alterations in ParC may be associated with the development of higher-level fluoroquinolone resistance. However, it remains to be determined whether topoisomerase activity of DNA gyrase is more sensitive to inhibition by quinolones than that of topoisomerase IV in *P. aeruginosa*.

Insofar as *P. aeruginosa* is an important bacterium for ecology and infectious disease, we attempted to clarify the role of topoisomerase IV in the mechanism of action of quinolone on *P. aeruginosa*. We report here the sequence of topoisomerase IV *parC* and *parE* genes of *P. aeruginosa* PAO1. We focused on and compared the inhibitory activities of quinolones against gyrase and topoisomerase IV purified by the same method from *P. aeruginosa* PAO1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. *P. aeruginosa* PAO1 was used to construct genomic libraries and as a reference strain having wild-type DNA gyrase and topoisomerase IV. *E. coli* MC1061 and plasmids pUC19 and pUC118 were used to construct libraries and to subclone DNA inserts. Plasmid pCRII (Invitrogen, San Diego, Calif.) was employed to clone PCR products in *E. coli* JM109. Supercoiled pBR322 plasmid DNA (Boehringer Mannheim GmbH, Mannheim, Germany) and kinetoplast DNA (Nippongene, Toyama, Japan) were used for enzyme assays. Bacteria were grown routinely in Luria-Bertani broth or on Luria-Bertani agar plates (27). SOC medium (Gibco BRL, Grand Island, N.Y.) was used for transformation. The antibiotic used for plasmid selection in *E. coli* was ampicillin (50 μg/ml). All other chemicals were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Plasmid preparation, agarose gel electrophoresis, DNA ligation, transformation, and other cloning procedures were done by standard methods (37).

Southern blot analysis. Chromosomal DNA or cloned DNA fragments were digested with restriction enzymes, separated by 0.8% agarose gel electrophoresis, and blotted onto Hybond-N$^+$ membranes (Amersham Pharmacia Biotech) according to standard procedures (37). Filters were hybridized to ^{32}P-radiolabeled DNA probes obtained by random priming with the Quick Prime kit (Amersham Pharmacia Biotech) with [α-^{32}P]dCTP. After hybridization, filters were washed twice in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) for 2 h at 65°C.

PCR amplification and DNA sequence determination and analysis. The oligonucleotide primers used for PCR amplification were synthesized in our laboratories and are listed in Table 1. The amplification procedure comprised denaturation at 92°C for 2 min; this was followed by 35 cycles including denaturation for 1 min at 92°C, annealing for 1 min at 55°C, and polymerization for 1 to 5 min at 68 or 72°C. The reactions were performed in a final volume of 50 μl with 2.5 U of *LA Taq* DNA polymerase (Takara, Kyoto, Japan). DNA fragments were subcloned into plasmid pUC19 and sequenced by the dideoxy chain termination method (38) with a T7 sequencing kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions and by a Pharmacia automatic sequencer. DNA and protein sequences were analyzed by use of the GENETYX program (Software Development Co., Ltd., Tokyo, Japan).

Construction of fusion plasmids. Four sets of 26-mer oligonucleotide primers were designed to allow amplification of *parC*, *parE*, *gyrA*, and *gyrB* genes (Table 1). These genes were digested by *Bam*HI-*Hin*dIII (*parC*), *Xba*I-*Hin*dIII (*parE* and *gyrA*), or *Eco*RI-*Hin*dIII (*gyrB*); ligated with the pMAL-c2 plasmid (New England Biolabs, Beverly, Mass.), yielding plasmids pMPPC203 (*parC*), pMPPE72 (*parE*), pMPGA417 (*gyrA*), and pMPGB512 (*gyrB*), respectively; and used to produce the fusion protein.

Purification of the ParC, ParE, GyrA, and GyrB proteins. Proteins encoded by the *parC*, *parE*, *gyrA*, and *gyrB* genes were purified by a protein fusion and purification system for maltose-binding protein (MBP) fusion proteins (New England Biolabs). Purification of the fusion proteins was carried out according to the manufacturer's protocol.

Inhibitory activities of quinolones against topoisomerase IV and DNA gyrase. Supercoiled pBR322 plasmid DNA was purchased from Boehringer Mannheim GmbH and was relaxed by topoisomerase I (Fermentas Ltd., Vilnius, Lithuania) before testing for the supercoiling activity of DNA gyrase. Inhibitory activities of quinolones against type II topoisomerases were assayed electrophoretically as described previously (2).

Determination of MICs. *P. aeruginosa* PAO1 was cultivated overnight at 37°C in Mueller-Hinton broth, and the MICs of quinolones were determined by the standard agar dilution method with Mueller-Hinton agar (Difco). The inoculum size was approximately 10^4 CFU/spot. The MIC was defined as the lowest drug concentration that prevented visible bacterial growth of the inoculum after incubation for 18 h at 37°C.

Nucleotide sequence accession numbers. The nucleotide sequence data of *parC* and *parE* will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following respective accession numbers: AB003428 and AB003429.

RESULTS

Cloning of the *parC* and *parE* homologs from *P. aeruginosa* PAO1. The genomic DNA from *P. aeruginosa* PAO1 was partially digested with *Sau*3AI. The digested DNA was size fractionated (2.0 to 6.5 kb) and ligated into pUC19, which was digested with *Bam*HI. Transformants derived from *E. coli*

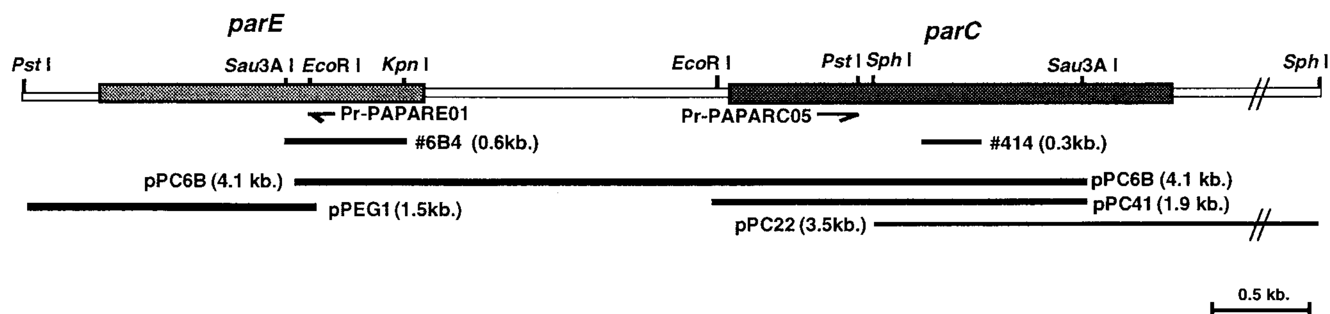

FIG. 1. Restriction map of the *parC* and *parE* genes in *P. aeruginosa* PAO1 and alignment of plasmid clones. The *parC* and *parE* genes are indicated by shaded regions. Plasmid pPC6B (3.8-kb insert in pUC19) and plasmid pPC41 (2.0-kb insert in pUC19) were obtained by colony hybridization with the 0.6-kb probe encoding the N-terminus of *E. coli parC*. Plasmid pPC22, containing a 3.5-kb *Sph*I insert, was isolated from a size-selected library with a 0.3-kb fragment (no. 414) as probe. Plasmid pPEG1 was obtained by PCR of a 4.7-kb *Pst*I fragment.

MC1061 transformed with the resultant plasmids were screened with the probe of 0.6-kb *E. coli* KL-16 *parC* (N-terminal region) (positions 1 to 599 in *parC*). Plasmids were isolated from colonies that showed a hybridization signal, and plasmid pPC6B (4.1-kb insert in pUC19) and plasmid pPC41 (1.9-kb insert in pUC19) were isolated (Fig. 1). DNA sequence analysis indicated that plasmid pPC6B contained parts of the *parC* N-terminal and *parE* C-terminal regions and that plasmid pPC41 contained part of the *parC* N-terminal region (Fig. 1).

To obtain full-length *parC* and *parE* genes, we performed Southern blot analysis. *Sph*I digestion of genomic DNA from *P. aeruginosa* PAO1 produced a single band of 3.5 kb that hybridized with part of the *P. aeruginosa parC* N-terminal (no. 414; positions 1105 to 1315 in *parC*; 0.3 kb) probe. DNA fragments after *Sph*I digestion were ligated into pUC19 digested with *Sph*I, and the resulting plasmids were transformed into *E. coli* MC1061. After screening with probe 414, plasmid pPC22 (3.5-kb insert in pUC19) was isolated from a colony that showed a hybridization signal. The subsequent sequence analysis revealed that plasmid pPC22 contained part of the *parC* C-terminal region (Fig. 1).

P. aeruginosa PAO1 genomic DNA after *Pst*I digestion contained a single band of 4.7 kb that hybridized with probe 6B4 (positions 1168 to 1794 of *parE*). The genomic DNA digested with *Pst*I was size fractionated and circularized by self-ligation with T4 DNA ligase. Self-ligated circular DNA was used as a PCR template to obtain a fragment corresponding to part of the *parE* N-terminal region. PCR was done with forward primer Pr-PAPARC05 and reverse primer Pr-PAPARE01 (Table 1). The PCR product was ligated into pCRII, and plasmid pPEG1 was isolated (Fig. 1).

Nucleotide sequence of the *P. aeruginosa parC* and *parE* homologs. The DNA fragments shown in Fig. 1 were subcloned for sequence analysis. The subcloned plasmids were sequenced by the dideoxy chain termination method with either vector-specific primers or primers chosen from the internal sequence. An open reading frame (ORF1) of 2,265 nucleotides coded for a polypeptide of 754 amino acids (Fig. 2) with a calculated molecular mass of 83.3 kDa. Putative −10 (TCGAAT) and −35 (TCGGCA) regions and ribosome binding signals were found upstream of the initiation ATG codon. The deduced protein had a general amino acid identity of 33% with *P. aeruginosa* GyrA (22) and exhibited homology with known topoisomerase IV subunit A proteins of *E. coli* (18, 34), *Salmonella typhimurium* (24), *H. influenzae* (10), *S. aureus* (i.e., GrlA) (9), *Streptococcus pneumoniae* (33), *Bacillus subtilis* (i.e., GrlA) (accession no. Z73234), and *N. gonorrhoeae* (3) at 69, 68, 64, 31, 33, 31, and 44%, respectively (Table 2). These results suggested that ORF1 might be identified as *parC*. *P. aeruginosa* ParC-like protein was compared with ParC of *E. coli* and GrlA of *S. aureus*. A region with high homology was found in the N-terminal DNA breakage-reunion region of *P. aeruginosa* ParC-like protein and its counterparts. The catalytic tyrosine residue present in the active site of the type II topoisomerases was identified putatively as Tyr-127 in *P. aeruginosa* ParC by alignment of a conserved AAMRYTE sequence with catalytic Tyr-120 of *E. coli* ParC. Serine (equivalent to Ser-80 in *E. coli* ParC and *S. aureus* GrlA) in the QRDR sequence was at amino acid position 87 (reported as Ser-80 by Nakano et al. [31]). From the results, it was concluded that ORF1 might be the *parC* gene of *P. aeruginosa* PAO1.

Analysis of the sequenced regions upstream of the putative *parC* gene revealed a region homologous with the *parE* sequence of *E. coli*: an open reading frame (ORF2) of 1,890 nucleotides coding for a polypeptide of 629 amino acids with a predicted molecular mass of 69.2 kDa. Putative −10 (CTGA AT) and −35 (CCGACA) promoter regions were found upstream of the initiation ATG codon (Fig. 3). The deduced amino acid sequence exhibited 75% identity with the ParE subunit of *E. coli* (18, 34). Comparison of ORF2 with the GyrB subunits of *P. aeruginosa* (accession no. AB00581) and *E. coli* (1, 45) revealed 32% identity with each of them. On this basis, the 629-residue *P. aeruginosa* protein was identified putatively as ParE. The *P. aeruginosa* ParE homolog is identical to known ParE proteins of *S. typhimurium* (40), *H. influenzae* (10), *S. aureus* (i.e., GrlB) (9), *S. pneumoniae* (33), and *B. subtilis* (i.e., GrlB) (accession no. Z73234) at 70, 64, 33, 37, and 36%, respectively (Table 2). Compared with its counterparts, *P.*

TABLE 2. Comparison of protein homology among *P. aeruginosa* topoisomerase IV and its counterparts

Bacterium	% Homology of *P. aeruginosa*	
	Subunit A (ParC)	Subunit B (ParE)
E. coli	69	75
S. typhimurium	68	73
H. influenzae	64	70
N. gonorrhoeae	44	ND[a]
S. aureus	31	33
S. pneumoniae	33	36
B. subtilis	31	37
P. aeruginosa (DNA gyrase)	33	32

[a] ND, not determined.

```
-151  GATATCCGCCTGTCCGGGGTTCGCGCGCTGATTGCGCCGGGCATGGACGGCGATGAGGTGCTACTCGGCATGAGCGCCCTGAAACAACTCGAATTCACCCAGCGCGACGGCACCCTGGTG  -32

 -31  CTGCGCCAAAACACTTCTCCGTGAGGTACGCATGAGCGAATCCCTCGATCTGAGCCTGGAAGGGGTCGAACGCCGGTCGTTGGCCGAGTTCACCGAGCAGGCCTATCTGAACTATTCCAT  89
                                         M  S  E  S  L  D  L  S  L  E  G  V  E  R  R  S  L  A  E  F  T  E  Q  A  Y  L  N  Y  S  M

  90  GTACGTGATCATGGACCGCGCCCTGCCGCATATCGGCGACGGCCTGAAACCGGTGCAGCGACGCATCGTCTACGCCATGAGCGAACTGGGGCTGGATGCCGATTCCAAGCACAAGAAGTC  209
       Y  V  I  M  D  R  A  L  P  H  I  G  D  G  L  K  P  V  Q  R  R  I  V  Y  A  M  S  E  L  G  L  D  A  D  S  K  H  K  K  S

 210  GGCGCGCACCGTCGGCGACGTGCTCGGCAAGTTCCACCCGCACGGCGACTCGGCCTGCTACGAGGCCATGGTGCTGATGGCGCAGCCGTTCTCCTATCGCTATCCGCTGGTGGACGGCCA  329
       A  R  T  V  G  D  V  L  G  K  F  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  Q

 330  GGGCAACTGGGGGGCTCCGGACGATCCCAAGTCCTTCGCCGCCATGCGTTATACCGAGGCGCGCCTGTCGCGCTATTCCGAGGTGCTGCTCAGCGAACTGGGCCAGGGTACCGTGGACTG  449
       G  N  W  G  A  P  D  D  P  K  S  F  A  A  M  R  Y  T  E  A  R  L  S  R  Y  S  E  V  L  L  S  E  L  G  Q  G  T  V  D  W

 450  GGTACCGAACTTCGACGGCACCCTCGACGAGCCGGCCGTGCTGCCGGCCCGCCTGCCCAACCTGCTGCTCAACGGCACCACCGGCATCGCGGTGGGCATGGCCACCGACGTGCCGCCGCA  569
       V  P  N  F  D  G  T  L  D  E  P  A  V  L  P  A  R  L  P  N  L  L  L  N  G  T  T  G  I  A  V  G  M  A  T  D  V  P  P  H

 570  CAACCTGCGGGAAGTCGCGTCGGCCTGCGTGCGCCTGCTCGACCAGCCGGGCGCGACGGTCGCCGAATTGTGCGAACACGTGCCGGGCCCGGACTTCCCCACCGAAGCCGAGATCATCAC  689
       N  L  R  E  V  A  S  A  C  V  R  L  L  D  Q  P  G  A  T  V  A  E  L  C  E  H  V  P  G  P  D  F  P  T  E  A  E  I  I  T

 690  CCCGCGCGCCGACCTGCAGAAGGTCTACGAGACCGGCCGCGGTTCGGTGCGCATGCGCGCGGTGTACCGCTTCGAGGACGGCGATATCGTCATCCACGCCCTGCCGCACCAGGTGTCCGG  809
       P  R  A  D  L  Q  K  V  Y  E  T  G  R  G  S  V  R  M  R  A  V  Y  R  F  E  D  G  D  I  V  I  H  A  L  P  H  Q  V  S  G

 810  TTCCAAGGTGCTGGAACAGATCGCCGGGCAGATGCAGGCCAAGAAGCTGCCGATGGTGGCCGACCTGCGCGACGAGTCGGACCACGAGAACCCGACCCGCATCGTCATCATCCCGCGTTC  929
       S  K  V  L  E  Q  I  A  G  Q  M  Q  A  K  K  L  P  M  V  A  D  L  R  D  E  S  D  H  E  N  P  T  R  I  V  I  I  P  R  S

 930  GAACCGGGTCGATGTCGAAGAGCTGATGACCCATCTGTTCGCCACCACCGACCTGGAGACCAGCTACCGGGTCAACCTGAACATCATCGGCCTCGACGGCAAGCCGGCAGTCAAGGACCT  1049
       N  R  V  D  V  E  E  L  M  T  H  L  F  A  T  T  D  L  E  T  S  Y  R  V  N  L  N  I  I  G  L  D  G  K  P  A  V  K  D  L

1050  GCGCCAGTTGCTCTCGGAGTGGCTGCAGTTCCGCATCGGCACCGTGCGTCGACGCCTGCAGTTCCGCCTGGACAAGGTCGAGCGCCGCCTGCATCTGCTGGATGGCTTGCTGATCGCCTT  1169
       R  Q  L  L  S  E  W  L  Q  F  R  I  G  T  V  R  R  R  L  Q  F  R  L  D  K  V  E  R  R  L  H  L  L  D  G  L  L  I  A  F

1170  CCTCAACCTCGACGAGGTGATCCACATCATCCGCACCGAGGACCAGCCCAAGGCGGTGCTGATGGAGCGCTTCGAACTCAGCGAGGTGCAGGCCGACTACATCCTCGACACCCGCCTGCG  1289
       L  N  L  D  E  V  I  H  I  I  R  T  E  D  Q  P  K  A  V  L  M  E  R  F  E  L  S  E  V  Q  A  D  Y  I  L  D  T  R  L  R

1290  CCAGTTGGCACGCCTGGAAGAGATGAAGATCCGCGGCGAGCAGGAAGAGTTGCTGAAGGAGCAGAAGCGCCTGCAGACCCTGCTCGGCAGCGAGGCCAAGCTGAAGAAGCTGGTGCGCGA  1409
       Q  L  A  R  L  E  E  M  K  I  R  G  E  Q  E  E  L  L  K  E  Q  K  R  L  Q  T  L  L  G  S  E  A  K  L  K  K  L  V  R  E

1410  GGAGCTGATCAAGGACGCCGAGACCTACGGCGACGACCGCCGTTCGCCGATCGTCGCCCGCGCCGAGGCCCGCGCGCTGTCGGAAACCGAGCTGATGCCCACCGAACCGGTGACCGTGGT  1529
       E  L  I  K  D  A  E  T  Y  G  D  D  R  R  S  P  I  V  A  R  A  E  A  R  A  L  S  E  T  E  L  M  P  T  E  P  V  T  V  V

1530  GCTCTCGGAAAAAGGCTGGGTGCGTTGCGCCAAGGGCCACGACATCGACGCCGCCGGCCTCTCCTACAAGGCCGGCGACGGCTTCAAGGCCGCCGCGCCGGGACGCTCGAACCAGTATGC  1649
       L  S  E  K  G  W  V  R  C  A  K  G  H  D  I  D  A  A  G  L  S  Y  K  A  G  D  G  F  K  A  A  A  P  G  R  S  N  Q  Y  A

1650  GGTGTTCATCGACTCCACCGGGCGCAGCTACTCGCTGCCGGCCCACAGCCTGCCGTCCGCGCGAGGCCAGGGCGAGCCACTCAGCGGCCGGCTGACGCCGCCGCCGGGGGCCAGCTTCGA  1769
       V  F  I  D  S  T  G  R  S  Y  S  L  P  A  H  S  L  P  S  A  R  G  Q  G  E  P  L  S  G  R  L  T  P  P  P  G  A  S  F  E

1770  ATGCGTGCTGCTGCCGGACGACGATGCGCTGTTCGTGATCGCTTCCGACGCCGGCTATGGTTTCGTGGTCAAGGGCGAGGACCTGCAGGCCAAGAACAAGGCCGGCAAGGCCCTGCTCAG  1889
       C  V  L  L  P  D  D  D  A  L  F  V  I  A  S  D  A  G  Y  G  F  V  V  K  G  E  D  L  Q  A  K  N  K  A  G  K  A  L  L  S

1890  CCTGCCCAACGGCTCCGCCGTGGTGGCGCCGCGCCCGGTGCGCGATGTGGAGCAGGATTGGCTGGCGGCCGTGACGACCGAGGGCCGTCTGCTATTGTTCAAGGTCTCCGACCTGCCGCA  2009
       L  P  N  G  S  A  V  V  A  P  R  P  V  R  D  V  E  Q  D  W  L  A  A  V  T  T  E  G  R  L  L  L  F  K  V  S  D  L  P  Q

2010  GCTCGGCAAGGGCAAGGGCAACAAGATCATCGGCATCCCCGGCGAACGCGTGGCCAGCCGCGAGGAATACCTCACCGACCTGGCTGTTCTGCCAGCCGGGGCGACGTTGGTCCTGCAGGC  2129
       L  G  K  G  K  G  N  K  I  I  G  I  P  G  E  R  V  A  S  R  E  E  Y  L  T  D  L  A  V  L  P  A  G  A  T  L  V  L  Q  A

2130  CGGAAAGCGTACCCTGTCGCTCAAGGGCGACGACCTGGAACACTACAAGGGGGAGCGAGGCCGGCGAGGCAACAAGCTGCCGCGCGGTTTCCAGCGCGTCGACAGCCTGCTGGTGGATAT  2249
       G  K  R  T  L  S  L  K  G  D  D  L  E  H  Y  K  G  E  R  G  R  R  G  N  K  L  P  R  G  F  Q  R  V  D  S  L  L  V  D  I

2250  TCCGCCACAGGATTGA  2265
       P  P  Q  D  *
```

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *P. aeruginosa parC* region. A methionine initiation codon and putative −10 and −35 regions are shown by underlining. An asterisk indicates the translation stop codon.

aeruginosa ParE showed highly conserved EGDSA and N-terminal sequences, including the G-loop ATP-binding moiety.

Purification of *P. aeruginosa* topoisomerase IV subunits. The putative ParC and ParE proteins were overexpressed and were purified separately with a fusion system with MBP and then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). The putative *parC* and *parE* genes from *P. aeruginosa* PAO1 were cloned into pMAL-c2, a *tac* promoter-based expression vector, yielding plasmids pMPPC203 and pMPE72, respectively. *E. coli* MC1061 harboring pMPPC203 and pMPPE72 overproduced MBP-ParC and MBP-ParE proteins, respectively, after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 4, lanes 2). MBP-ParC and MBP-ParE were purified by affinity chromatography (Fig. 4, lanes 3), and the ParC and ParE proteins were then obtained by digestion with protease factor Xa (Fig. 4, lanes 4). The molecular size of the purified proteins was in agreement with the molecular weight calculated from the deduced protein sequences of ParC and ParE.

Enzymatic activities of *P. aeruginosa* topoisomerase IV. Putative ParC and ParE were examined for decatenating activity (Fig. 5). Enzymatic activity was dependent on ATP, Mg^{2+} (data not shown), and the presence of both subunits (Fig. 5, lane 2), because the omission of ATP (Fig. 5, lane 4) or either single subunit (Fig. 5, lanes 1 and 3) did not lead to DNA decatenation activity. In addition, relaxing activity of superhelical DNA was detected with the combination of ParC and ParE (results not shown). No supercoiling activity was detected with ATP when relaxed DNA was incubated with ParC, ParE, or both (data not shown), indicating that the combination of ParC and ParE catalyzes reactions similarly to *E. coli* and other topoisomerase IV proteins (19, 34).

When *P. aeruginosa* ParC or ParE was combined with *E. coli*

```
-198  CTGCAGACCGCCGACGAAACCCTCGATTATCGCGTCGCGCAGGCCTTCTACCGAGCCTGCGCGCTACGTATCCAGGCCGGCGGCGACCACGGTTTCCAGGGCTTCGCCGGCACCTGCCGG  -79

 -78  CGCTGCTGGCGTTCGCCGGCTACCTGTCGGCGCTGTGGCCGACACCGACTTTTCGACTTTTGACTGAATAGAGACCCCATGGCTACTTACAACGCAGACGCCATCGAAGTCCTTTCCGGC  42
                                                                                     M  A  T  Y  N  A  D  A  I  E  V  L  S  G

  43  CTCGACCCGGTGCGCAAGCGCCCGGGGATGTACACCGACACCACCCGCCCCAACCATCTGGCCCAGGAAGTCATCGACAACAGCGTCGACGAAGCCCTGGCCGGCCATGCGAAGAGCGTG  162
      L  D  P  V  R  K  R  P  G  M  Y  T  D  T  T  R  P  N  H  L  A  Q  E  V  I  D  N  S  V  D  E  A  L  A  G  H  A  K  S  V

 163  CAGGTGATCCTGCACCAGGACAACTCGCTGGAAGTCATCGACGATGGCCGCGGCATGCCGGTGGACATCCACCCGGAAGAGGGCGTGCCGGGCGTCGAGCTGACCCTTATCAAGCTGCAT  282
      Q  V  I  L  H  Q  D  N  S  L  E  V  I  D  D  G  R  G  M  P  V  D  I  H  P  E  E  G  V  P  G  V  E  L  T  L  I  K  L  H

 283  GCCGGCGGCAAGTTCTCGAACAAGAACTACCAGTTCTCCGGCGGCTTGCACGGGGTCGGCATCTCGGTGGTGAACGCGCTCTCGACCCGGGTCGAGGTACGCGTCAAGCGCGACGCCAAC  402
      A  G  G  K  F  S  N  K  N  Y  Q  F  S  G  G  L  H  G  V  G  I  S  V  V  N  A  L  S  T  R  V  E  V  R  V  K  R  D  A  N

 403  GAGTACCGGATGACCTTCGCCGACGGCTTCAAGGACAGCGATCTGGAAGTCATCGGCACGGTCGGCAAGCGCAATACCGGTACCAGCGTGCATTTCTGGCCGGATCCGAAGTATTTCGAT  522
      E  Y  R  M  T  F  A  D  G  F  K  D  S  D  L  E  V  I  G  T  V  G  K  R  N  T  G  T  S  V  H  F  W  P  D  P  K  Y  F  D

 523  TCGGCGAAGTTCTCGGTCAGCCGCCTCAAGCATGTGCTCAAGGCCAAGGCGGTGCTGTGCCCGGGCCTGAGCGTGGTGTTCGAGGACAAGAACACCGGCGAGCGCGTCGAGTGGCACTTC  642
      S  A  K  F  S  V  S  R  L  K  H  V  L  K  A  K  A  V  L  C  P  G  L  S  V  V  F  E  D  K  N  T  G  E  R  V  E  W  H  F

 643  GAGGACGGCCTGCGCTCCTACCTGACCGACGCGGTCGCCGAGCTGCCGCGCCTGCCCGATGAACCCTTCTGCGGCAACCTCGAAGGTTCCAAGGAAGCGGTGAGCTGGGCCCTGCTGTGG  762
      E  D  G  L  R  S  Y  L  T  D  A  V  A  E  L  P  R  L  P  D  E  P  F  C  G  N  L  E  G  S  K  E  A  V  S  W  A  L  L  W

 763  CTGCCCGAGGGCGGTGAGTCGGTGCAGGAAAGCTACGTCAACCTGATTCCCACGGCCCAGGGCGGCACCCATGTGAACGGCCTGCGCCAGGGCCTGCTCGACGCCATGCGCGAGTTCTGC  882
      L  P  E  G  G  E  S  V  Q  E  S  Y  V  N  L  I  P  T  A  Q  G  G  T  H  V  N  G  L  R  Q  G  L  L  D  A  M  R  E  F  C

 883  GAGTTCCGCAACCTGTTGCCGCGCGGCGTCAAGCTGGCGCCCGAGGACGTCTGGGAGCGGATCGCCTTCGTCCTCTCGATGAAGATGCAGGAGCCGCAGTTCTCCGGGCAGACCAAGGAG  1002
      E  F  R  N  L  L  P  R  G  V  K  L  A  P  E  D  V  W  E  R  I  A  F  V  L  S  M  K  M  Q  E  P  Q  F  S  G  Q  T  K  E

1003  CGCCTGTCGTCCCGCGAGGCGGCGGCGTTCGTCTCGGGCGTGGTGAAGGACGCCTTCAGCCTGTGGCTCAACGAGCACGCCGAAATCGGCCTGCAACTGGCGGAACTGGCGATCAGCAAC  1122
      R  L  S  S  R  E  A  A  A  F  V  S  G  V  V  K  D  A  F  S  L  W  L  N  E  H  A  E  I  G  L  Q  L  A  E  L  A  I  S  N

1123  GCCGGGCGTCGCCTCAAGGCGGGCAAGAAGGTCGAGCGCAAGAAGATCACCCAGGGGCCGGCGCTGCCCGGCAAACTGGCCGACTGCGCCGGACAGGAACCGATGCGCGCGGAACTGTTC  1242
      A  G  R  R  L  K  A  G  K  K  V  E  R  K  K  I  T  Q  G  P  A  L  P  G  K  L  A  D  C  A  G  Q  E  P  M  R  A  E  L  F

1243  CTGGTCGAGGGCGACTCCGCCGGCGGCTCGGCGAAGCAGGCGCGGGACAAGGAATTCCAGGCGATCATGCCGCTGCGCGGAAAGATCCTGAACACCTGGGAAGTGGACGGCGGCGAGGTG  1362
      L  V  E  G  D  S  A  G  G  S  A  K  Q  A  R  D  K  E  F  Q  A  I  M  P  L  R  G  K  I  L  N  T  W  E  V  D  G  G  E  V

1363  CTCGCCAGCCAGGAGGTCCACGACATCGCGGTGGCCATCGGCGTCGATCCGGGTGCCAGTGACCTGGCCCAGCTGCGCTACGGCAAGATCTGTATCCTCGCGGATGCCGACTCCGACGGG  1482
      L  A  S  Q  E  V  H  D  I  A  V  A  I  G  V  D  P  G  A  S  D  L  A  Q  L  R  Y  G  K  I  C  I  L  A  D  A  D  S  D  G

1483  CTGCACATCGCCACGCTGCTCTGCGCGCTGTTCGTCCGCCATTTCCGCCCGCTGGTGGAAGCCGGCCACGTCTACGTGGCGATGCCGCCGCTGTACCGCATCGACCTCGGCAAGGACATC  1602
      L  H  I  A  T  L  L  C  A  L  F  V  R  H  F  R  P  L  V  E  A  G  H  V  Y  V  A  M  P  P  L  Y  R  I  D  L  G  K  D  I

1603  TACTACGCCCTCGACGAAGCCGAGCGCGACGGCATCCTCGAGCGCCTGGCCGCAGAGAAGAAGCGCGGCAAGCCGCAGGTCACCCGCTTCAAGGGCCTTGGCGAAATGAATCCGTTGCAA  1722
      Y  Y  A  L  D  E  A  E  R  D  G  I  L  E  R  L  A  A  E  K  K  R  G  K  P  Q  V  T  R  F  K  G  L  G  E  M  N  P  L  Q

1723  CTGCGCGAGACCACCATGGATCCGAATACCCGGCGGCTGGTCCAGCTCACCCTGGAGGACGCCACCGGTACCCTGGAGATCATGGACATGCTGCTGGCCAAGAAGCGCGCCGGTGACCGC  1842
      L  R  E  T  T  M  D  P  N  T  R  R  L  V  Q  L  T  L  E  D  A  T  G  T  L  E  I  M  D  M  L  L  A  K  K  R  A  G  D  R

1843  AAGTCCTGGCTGGAAAGCAAGGGCAACCTGGCCGAGGTGCTGGTCTGA  1890
      K  S  W  L  E  S  K  G  N  L  A  E  V  L  V  *
```

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *P. aeruginosa parE* region. The *P. aeruginosa parE* nucleotide sequence is shown along with the predicted amino acid sequence. Symbols are the same as those defined for Fig. 2.

ParE or ParC, respectively, the decatenating activity was detected (Fig. 5, lanes 5 and 6). These results indicate that a complementation occurs between topoisomerase IV subunits of *P. aeruginosa* and *E. coli* in vitro.

Comparison of inhibitory activities of quinolones against DNA gyrase and topoisomerase IV. The inhibitory effects of quinolones on the topoisomerase activities of topoisomerase IV and DNA gyrase were determined by quantitative electrophoresis with kinetoplast DNA and relaxed DNA as a substrate. The 50% inhibitory concentrations (IC_{50}s) of quinolones were calculated from the quantification of bands corresponding to fully decatenated substrate or supercoiled DNA. MICs and the IC_{50}s of quinolones on the topoisomerase activities are shown in Table 3. The IC_{50}s against the decatenation of topoisomerase IV were higher than those against the supercoiling activity of DNA gyrase. Among the quinolones tested, sitafloxacin showed the highest level of inhibitory activity against DNA gyrase and topoisomerase IV. There was a high correlation between the inhibitory effects of the quinolones on bacterial growth and the inhibitory activity against the topoisomerase activity of DNA gyrase (correlation coefficient, 0.942) and of topoisomerase IV (correlation coefficient, 0.972). The correlation between the IC_{50}s of quinolones against DNA gyrase and topoisomerase IV is presented in Fig. 6. The inhibitory activities of quinolones against type II topoisomerases were correlated well, with the correlation coefficient being 0.977.

DISCUSSION

A *P. aeruginosa parC* homolog was cloned and sequenced from *P. aeruginosa* PAO1, by use of cDNA encoding the N-

TABLE 3. Inhibitory activities of quinolones against topoisomerase IV and DNA gyrase

Drug	MIC (μg/ml)[a]	IC_{50} (μg/ml)	
		DNA gyrase, supercoiling	Topoisomerase IV, decatenation
Sitafloxacin	0.10	0.42	2.34
Levofloxacin	0.39	0.88	6.08
Ofloxacin	0.78	1.47	9.72
Ciprofloxacin	0.10	0.55	3.90
Sparfloxacin	0.39	0.75	5.93

[a] Tested against *P. aeruginosa* PAO1.

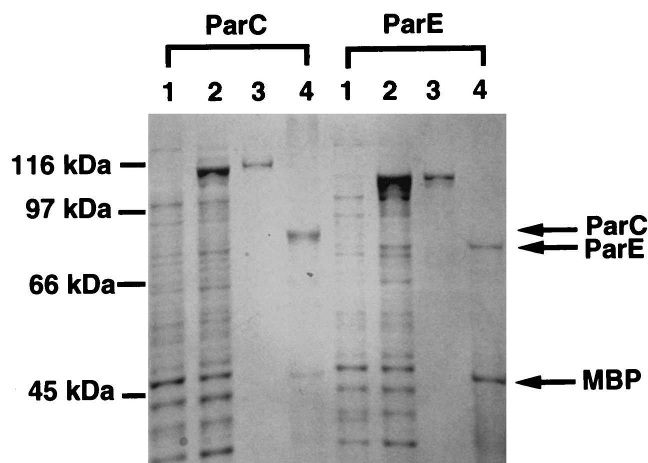

FIG. 4. SDS-polyacrylamide gel electrophoresis analysis of ParC and ParE. Proteins at various purification steps were electrophoresed in an SDS–12.5% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes 1, soluble extracts from uninduced cells; lanes 2, soluble extracts from IPTG-induced cells, lanes 3, affinity-purified MBP-ParC or MBP-ParE protein; lanes 4, factor Xa digest of MBP-ParC or MBP-ParE.

terminal region of *E. coli parC* as a probe. The homolog and its upstream gene were identified putatively as *parC* and *parE*, respectively, through sequence homology with other *parC* and *parE* genes. The *parC* homolog of 2,265 bp coded for a protein of 754 amino acids. The deduced amino acid sequence of the ParC homolog showed 69% identity with that of *E. coli* ParC and 33% identity with that of *P. aeruginosa* GyrA. The *parE* homolog of 1,890 bp encoded 629 amino acids, and this gene product was 75% identical to *E. coli* ParE and 32% identical to *P. aeruginosa* GyrB.

The putative ParC and ParE proteins were overexpressed and separately purified with a fusion system involving an MBP, and their enzymatic properties were examined. The combined putative ParC and ParE proteins catalyzed decatenation and relaxing reactions but had no supercoiling reaction. Not only from the sequence homologies but also from the characteristics of the enzyme, isolated *P. aeruginosa* genes were confirmed as *parC* and *parE* genes of *P. aeruginosa* topoisomerase IV.

When *P. aeruginosa* ParC or ParE was combined with *E. coli* ParE or ParC, respectively, decatenation activity was detected in vitro. This result is not surprising given the high degree of homology between the *P. aeruginosa* and *E. coli* ParC proteins and the fact that *P. aeruginosa* GyrA protein can functionally complement the *E. coli* GyrA protein in vivo (22). However,

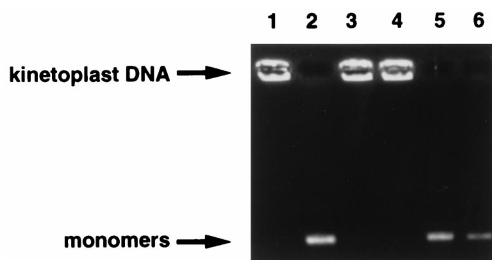

FIG. 5. Topoisomerase IV activities of ParC and ParE proteins. Lane 1, *P. aeruginosa* ParC (1 U); lane 2, *P. aeruginosa* ParC (1 U) and *P. aeruginosa* ParE (1 U); lane 3, *P. aeruginosa* ParE (1 U); lane 4, *P. aeruginosa* ParC (1 U) and *P. aeruginosa* ParE (1 U) but with ATP omitted; lane 5, *P. aeruginosa* ParC (1 U) and *E. coli* ParE (1 U); lane 6, *E. coli* ParC (1 U) and *P. aeruginosa* ParE (1 U).

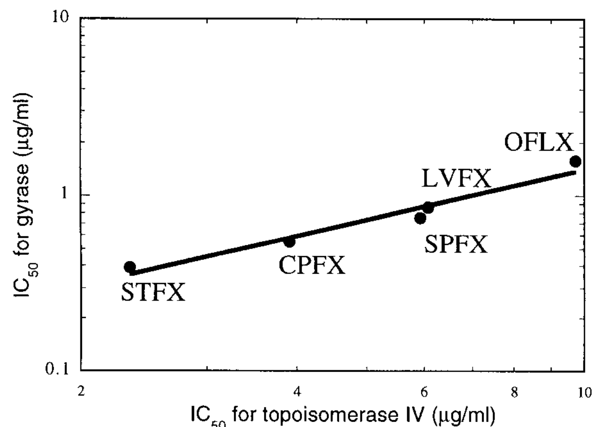

FIG. 6. Correlation between inhibition of topoisomerase IV and that of DNA gyrase. Abbreviations: STFX, sitafloxacin; CPFX, ciprofloxacin; SPFX, sparfloxacin; LVFX, levofloxacin; OFLX, ofloxacin.

when *S. aureus* GrlA or GrlB was combined with *E. coli* ParE or ParC, respectively, no decatenation activity was detected (4), and the temperature-sensitive phenotype of *S. typhimurium parC* and *parE* mutants was complemented by the *S. aureus grlA* and *grlB* genes only when the two genes were coexpressed (9).

The decatenation activity of *P. aeruginosa* topoisomerase IV was inhibited by quinolones. There was a high correlation between the inhibitory activity against the topoisomerase activity of DNA gyrase and that of topoisomerase IV.

Sitafloxacin (DU-6859a), a newly developed quinolone antibacterial agent, showed more potent activity against a wide spectrum of bacteria (30, 39, 42) than did levofloxacin and ciprofloxacin. Our previous study (20) showed the inhibition by sitafloxacin of purified DNA gyrases from only clinical isolates of *P. aeruginosa*. In this study, sitafloxacin had a lower MIC against *P. aeruginosa* PAO1 than most of the other quinolones and the lowest IC_{50}s for DNA gyrase and topoisomerase IV of *P. aeruginosa* among the quinolones tested, and a good correlation existed between the inhibitory effects on bacterial growth (MICs) and those on the type II topoisomerases of *P. aeruginosa* PAO1. From these results, sitafloxacin appears to have higher activity against *P. aeruginosa* than do other available quinolones, probably because of its higher inhibitory effects against type II topoisomerases.

In this study, we purified topoisomerase IV and DNA gyrase of *P. aeruginosa* PAO1 in the same manner and compared the inhibitory activities of quinolones against the purified enzymes. The supercoiling activity of DNA gyrase was more sensitive to quinolones than was the decatenation activity of topoisomerase IV. Our results, obtained by enzymatic methods, support the view that DNA gyrase is the primary target of new quinolones and have shown that topoisomerase IV may act as a secondary target in the quinolone-susceptible *P. aeruginosa* strain.

ACKNOWLEDGMENTS

We thank Yuki Nagano for preparation of the primers. We are thankful to Kenji Hayata for sharing with us the sequence of the *gyrB* gene of *P. aeruginosa*.

REFERENCES

1. **Adachi, T., M. Mizuuchi, E. Robinson, E. Appella, M. O'Dea, M. Gellert, and K. Mizuuchi.** 1987. DNA sequence of the *E. coli gyrB* gene: application of a new sequencing strategy. Nucleic Acids Res. **15:**771–783.
2. **Akasaka, T., S. Kurosaka, Y. Uchida, M. Tanaka, K. Sato, and I. Hayakawa.**

1998. Antibacterial activities and inhibitory effects of sitafloxacin (DU-6859a) and its optical isomers against type II topoisomerases. Antimicrob. Agents Chemother. **42:**1284–1287.

3. **Belland, R. J., S. G. Morrison, C. Ison, and W. M. Huang.** 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Mol. Microbiol. **14:**371–380.
4. **Blanche, F., B. Cameron, F. X. Bernard, L. Maton, B. Manse, L. Ferrero, N. Ratet, C. Lecoq, A. Goniot, D. Bisch, and J. Crouzet.** 1996. Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases. Antimicrob. Agents Chemother. **40:**2714–2720.
5. **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.
6. **Dean, F., M. A. Krasnow, R. Otter, M. M. Matzuk, S. J. Spengler, and N. R. Cozzarelli.** 1983. *Escherichia coli* type-1 topoisomerases: identification, mechanism, and role in recombination. Cold Spring Harbor Symp. Quant. Biol. **2:**769–777.
7. **Drlica, K.** 1992. Control of bacterial DNA supercoiling. Mol. Microbiol. **6:**425–433.
8. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
9. **Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13:**641–653.
10. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
11. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.
12. **Georgiou, M., R. Munoz, F. Roman, R. Canton, R. Gomez-Lus, J. Campos, and A. G. De La Campa.** 1996. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. Antimicrob. Agents Chemother. **40:**1741–1744.
13. **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.
14. **Horowitz, D., and J. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **262:**5339–5348.
15. **Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J. Kato, and H. Ikeda.** 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob. Agents Chemother. **38:**2623–2627.
16. **Huang, W. M.** 1996. Bacterial diversity based on type II DNA topoisomerase genes. Annu. Rev. Genet. **30:**79–107.
17. **Inoue, Y., K. Sato, T. Fujii, K. Hirai, M. Inoue, S. Iyobe, and S. Mitsuhashi.** 1987. Some properties of subunits of DNA gyrase from *Pseudomonas aeruginosa* PAO1 and its nalidixic acid-resistant mutant. J. Bacteriol. **169:**2322–2325.
18. **Kato, J., 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.
19. **Kato, J., H. Suzuki, and H. Ikeda.** 1992. Purification and characterization of DNA topoisomerase IV in *Escherichia coli*. J. Biol. Chem. **267:**25676–25684.
20. **Kitamura, A., K. Hoshino, Y. Kimura, I. Hayakawa, and K. Sato.** 1995. Contribution of the C-8 substituent of DU-6859a, a new potent fluoroquinolone, to its activity against DNA gyrase mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1467–1471.
21. **Kumagai, Y., J. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda.** 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. Antimicrob. Agents Chemother. **40:**710–714.
22. **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.
23. **Luttinger, A.** 1995. The twisted 'life' of DNA in the cell: bacterial topoisomerases. Mol. Microbiol. **15:**601–606.
24. **Luttinger, A., A. Springer, and M. Schmid.** 1991. A cluster of genes that affects nucleoid segregation in *Salmonella typhimurium*. New Biol. **3:**687–697.
25. **Masecar, B. L., R. A. Celesk, and N. J. Robillard.** 1990. Analysis of acquired ciprofloxacin resistance in a clinical strain of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**281–286.
26. **Maxwell, A.** 1997. DNA gyrase as a drug target. Trends Microbiol. **5:**102–109.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Miller, W. G., and R. W. Simons.** 1993. Chromosomal supercoiling in *Escherichia coli*. Mol. Microbiol. **10:**675–684.
29. **Mouton, J. W., J. G. den Hollander, and A. M. Horrevorts.** 1993. Emergence of antibiotic resistance amongst *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. J. Antimicrob. Chemother. **31:**919–926.
30. **Nakane, T., S. Iyobe, K. Sato, and S. Mitsuhashi.** 1995. In vitro antibacterial activity of DU-6859a, a new fluoroquinolone. Antimicrob. Agents Chemother. **39:**2822–2826.
31. **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.
32. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
33. **Pan, X. S., and L. M. Fisher.** 1996. Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: role in fluoroquinolone resistance. J. Bacteriol. **178:**4060–4069.
34. **Peng, H., and K. J. Marians.** 1993. *Escherichia coli* topoisomerase IV: purification, characterization, subunit structure, and subunit interactions. J. Biol. Chem. **268:**24481–24490.
35. **Peng, H., and K. J. Marians.** 1993. Decatenation activity of topoisomerase IV during *oriC* and pBR322 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **90:**8571–8575.
36. **Roca, J.** 1995. The mechanisms of DNA topoisomerases. Trends Biochem. Sci. **20:**156–160.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
39. **Sato, K., K. Hoshino, M. Tanaka, I. Hayakawa, and Y. Osada.** 1992. Antimicrobial activity of DU-6859, a new potent fluoroquinolone, against clinical isolates. Antimicrob. Agents Chemother. **36:**1491–1498.
40. **Springer, A. L., and M. B. Schmid.** 1993. Molecular characterization of the *Salmonella typhimurium parE* gene. Nucleic Acids Res. **21:**1805–1809.
41. **Swanberg, S., and J. Wang.** 1987. Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. J. Mol. Biol. **197:**729–736.
42. **Tanaka, M., K. Hoshino, M. Hohmura, H. Ishida, A. Kitamura, K. Sato, I. Hayakawa, and T. Nishino.** 1996. Effect of growth conditions on antimicrobial activity of DU-6859a and its bactericidal activity determined by the killing curve method. J. Antimicrob. Chemother. **37:**1091–1102.
43. **Tanaka, M., Y. Onodera, Y. Uchida, K. Sato, and I. Hayakawa.** 1997. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV purified from *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:**2362–2366.
44. **Wang, J. C.** 1971. Interaction between DNA and an *Escherichia coli* protein omega. J. Mol. Biol. **55:**523–533.
45. **Yamagishi, J., 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.
46. **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.
47. **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.
48. **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.
49. **Yoshida, T., T. Muratani, S. Iyobe, and S. Mitsuhashi.** 1994. Mechanisms of high-level resistance to quinolones in urinary tract isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **38:**1466–1469.