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Cloning and Nucleotide Sequence of *Pseudomonas aeruginosa* DNA Gyrase *gyrA* Gene from Strain PAO1 and Quinolone-Resistant Clinical Isolates

AMAR KUREISHI,[1,2] JONATHAN M. DIVER,[1]* BRENDA BECKTHOLD,[1] TINEKE SCHOLLAARDT,[1] AND LARRY E. BRYAN[1,2]

Department of Microbiology and Infectious Diseases, University of Calgary,[1] and Foothills Hospital,[2] Calgary, Alberta, Canada T2N 4N1

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The *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene was cloned and sequenced from strain PAO1. An open reading frame of 2,769 bp was found; it coded for a protein of 923 amino acids with an estimated molecular mass of 103 kDa. The derived amino acid sequence shared 67% identity with *Escherichia coli* GyrA and 54% identity with *Bacillus subtilis* GyrA, although conserved regions were present throughout the sequences, particularly toward the N terminus. Complementation of an *E. coli* mutant with a temperature-sensitive *gyrA* gene with the PAO1 *gyrA* gene showed that the gene is expressed in *E. coli* and is able to functionally complement the *E. coli* DNA gyrase B subunit. Expression of PAO1 *gyrA* in *E. coli* or *P. aeruginosa* with mutationally altered *gyrA* genes caused a reversion to wild-type quinolone susceptibility, indicating that the intrinsic susceptibility of the PAO1 GyrA to quinolones is comparable to that of the *E. coli* enzyme. PCR was used to amplify 360 bp of *P. aeruginosa gyrA* encompassing the so-called quinolone resistance-determining region from ciprofloxacin-resistant clinical isolates from patients with cystic fibrosis. Mutations were found in three of nine isolates tested; these mutations caused the following alterations in the sequence of GyrA: Asp at position 87 (Asp-87) to Asn, Asp-87 to Tyr, and Thr-83 to Ile. The resistance mechanisms in the other six isolates are unknown. The results of the study suggested that mechanisms other than a mutational alteration in *gyrA* are the most common mechanism of ciprofloxacin resistance in *P. aeruginosa* from the lungs of patients with cystic fibrosis.

The enzyme DNA gyrase is a type II DNA topoisomerase that is able to introduce negative superhelical coils into covalently closed bacterial DNA in an ATP-dependent process. Gyrase also plays a role in DNA replication, recombination, decatenation, and transcriptional regulation of some supercoiling-sensitive promoters (for a review, see reference 24). The gyrase holoenzyme is a tetramer of two A and two B subunits, the products of the *gyrA* and *gyrB* genes, respectively. The A subunits are responsible for DNA breakage and reunion, while the B subunits are the site of ATP hydrolysis. The enzyme has proven to be a good target for antimicrobial agents; the actions of the A subunits are inhibited by quinolones such as nalidixic acid and ciprofloxacin, while those of the B subunits are inhibited by coumarins such as coumermycin A_1 and novobiocin. The peptide antibiotic microcin B17 and the glycocinnamoylspermidine agent cinodine have also been shown to be inhibitors of DNA gyrase function (22, 33). The contiguous *gyrB-gyrA* locus has been cloned and sequenced in *Bacillus subtilis* (19), *Staphylococcus aureus* (10), *Mycoplasma pneumoniae* (1), and *Haloferax* spp. (9). In *Escherichia coli* (32, 37), *Klebsiella pneumoniae* (3), *Pseudomonas putida* (23), *Neisseria gonorrhoeae* (31), and *Campylobacter jejuni* (34), the cloned *gyrA* and *gyrB* genes are not contiguous. The deduced amino acid sequences of the A and B subunits are very well conserved within procaryotes. The amino acids around the active-site tyrosine (amino acid 122 in *E. coli*) are particularly well conserved.

Pseudomonas aeruginosa is an important human pathogen and is intrinsically resistant to a number of antibiotics, likely owing in part to the low level of permeability of the outer membrane. The newer fluorinated quinolone derivatives show good in vitro activity against *P. aeruginosa* and have proven to be useful therapeutic agents against the organism (36). One problem, however, is the propensity of the organism to develop resistance to these agents. In the case of lung infections in patients with cystic fibrosis, pathogen resistance appears to be a persistence phenomenon: resistant organisms emerge during drug therapy and disappear when therapy is withdrawn (5). In *E. coli*, *S. aureus*, and *C. jejuni*, specific mutations in *gyrA* (or less frequently in *gyrB*) are associated with quinolone resistance (2, 6, 34, 37, 38); however, mutational alterations in *gyrA* of *P. aeruginosa* have not been described at the nucleotide level. The aim of the study described here was to clone, sequence, and characterize the wild-type *gyrA* gene from PAO1. We also sequenced regions of *gyrA* genes from quinolone-resistant *P. aeruginosa* obtained from patients with cystic fibrosis undergoing ciprofloxacin therapy and compared the sequences with those of quinolone-susceptible isolates obtained from the same patient pre- and posttherapy.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The nine sets of pretherapy ciprofloxacin-susceptible, during-therapy ciprofloxacin-resistant, and posttherapy ciprofloxacin-susceptible isolates of *P. aeruginosa* have been described previously (5). Each set of strains (named P, Q, R, S, U, V, W, X, and Y) consists of three strains named, for example, P-pre, P-during, and P-post. The assumption that each set of strains is isogenic is based on genomic DNA probing data (5). *E. coli* and *P.*

* Corresponding author. Mailing address: Genta Inc., 3550 General Atomics Ct., San Diego, CA 92122. Phone: (619) 455 2772. Fax: (619) 455 2795.

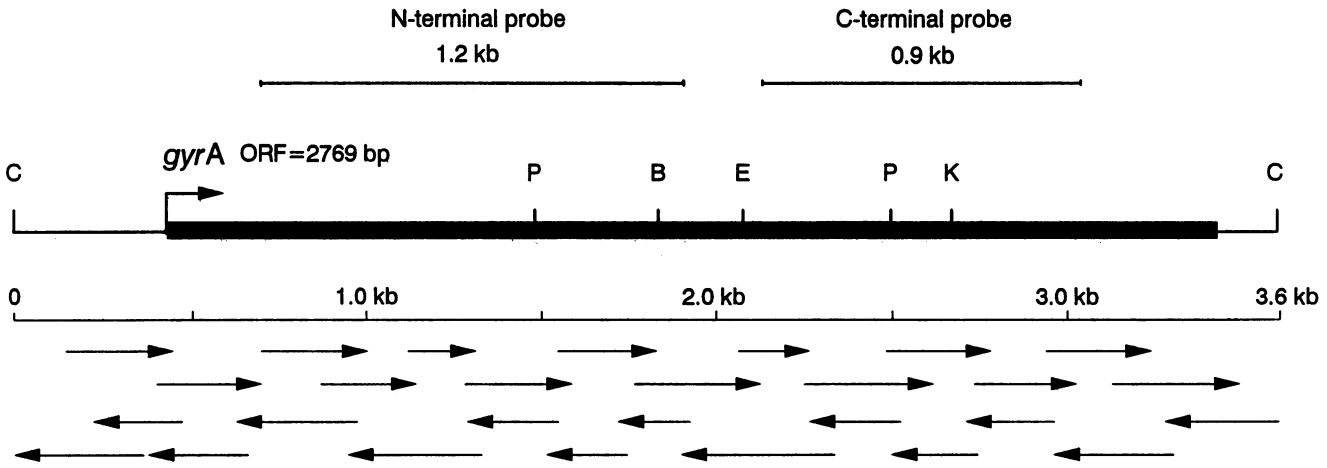

FIG. 1. Restriction map and sequencing strategy for pGL2B5. The PAO1 *gyrA* open reading frame (ORF) is indicated by the thick line. K, P, C, B, and E represent restriction sites of *Kpn*I, *Pst*I, *Cla*I, *Bam*HI, and *Eco*RI, respectively.

aeruginosa strains were grown in LB medium or tryptic soy broth (Difco Laboratories). The antibiotics used for plasmid selection were ampicillin (100 μg/ml) for *E. coli* and carbenicillin (500 μg/ml) and tetracycline (400 μg/ml) for *P. aeruginosa*. Other antibiotics used were nalidixic acid (Sigma Chemical Co.) and ciprofloxacin (Miles Laboratories, Rexdale, Ontario, Canada). The chemicals used for molecular biological techniques were BRL Ultra Pure (BRL Life Technologies Inc.). All other chemicals were from Sigma.

DNA manipulations and other molecular techniques. Plasmid isolation, agarose gel electrophoresis, DNA ligation, and other cloning procedures were done by standard methods (28). DNA restriction endonucleases, T4 DNA ligase, DNA size markers, and calf intestinal alkaline phosphatase were from BRL Life Technologies Inc. The transformation of *E. coli* and *P. aeruginosa* was done by electroporation as described previously (4).

P. aeruginosa PAO1 total genomic DNA (7) was digested to completion with restriction endonucleases according to the manufacturer's instructions other than the addition of 3 mM spermidine. DNA fragments were size fractionated on a 10 to 40% sucrose gradient by ultracentrifugation.

N- and C-terminal probes for *gyrA* were prepared as follows. As an N-terminal probe, a 1.2-kb DNA fragment of the N-terminal region (nucleotides 115 to 1363) of the *P. aeruginosa* Y4492 *gyrA* gene was amplified by PCR as described previously (15). For a C-terminal probe, a 0.95-kb *Bgl*II fragment of pSLS447 (32) containing the C terminus of the *E. coli gyrA* gene was used. These fragments were labeled with [α-^{32}P]dCTP by using a Pharmacia oligolabeling kit (Pharmacia LKB Biotechnology) according to the manufacturer's instructions and were used to probe Southern blots by standard methods. Transformants of *E. coli* DH5α were screened by in situ hybridization by using Colony/Plaque Screen filters (NEN/Dupont Research Products). All hybridizations were performed under high-stringency conditions at 65°C.

DNA base sequence determination and sequence analysis. Nucleotide sequences of PAO1 *gyrA* and PCR-amplified *gyrA* fragments from clinical isolates were determined by primer extension reactions by using the Sequenase kit (version 2; U.S. Biochemical Corp.). Band compressions caused by secondary structure formation in GC-rich areas were resolved either by using 40% formamide plus 7 M urea polyacrylamide gels or by substituting a 7-deaza-GTP analog (U.S. Biochemicals) in the extension reaction. To avoid any possible sequence errors introduced during the PCRs, the 360-bp amplicons from the quinolone resistance-determining regions were sequenced in duplicate by using the Circumvent kit (New England Biolabs) and the *fmol* DNA sequencing system (Promega). Separate PCRs were used for each sequencing reaction. Sequencing primers were provided by the Regional DNA Synthesis Laboratory, University of Calgary.

DNA sequence data were analyzed by using the IBI Pustell sequence analysis program. GenBank searches used the PC/Gene nucleic acid and protein sequence analysis software.

Nucleotide and protein sequence accession number. The nucleotide and protein sequences of the PAO1 DNA gyrase have been deposited in GenBank with the accession number L29417.

PCR. A region of the *P. aeruginosa gyrA* gene analogous to the region known to be the site of mutations causing quinolone resistance in *E. coli* and *S. aureus* was amplified from chromosomal DNA preparations of the *P. aeruginosa* clinical isolates by a two-step nested PCR method. As a first amplification, a 1.2-kb region of the *gyrA* gene was amplified by using the primers 5′-GAC/TGGCCTG/CAAGCCG/CGTC/GCAC/TCG and 5′-CTC/GAGGATC/GGCCTGG/CGCC/TTG (set 1 primers) and the conditions described previously (15), except that the amplified product was purified by using the Magic Prep purification kit (Promega). A second amplification used primers (set 2 primers) internal to the 1.2-kb fragment derived from the known sequence of *P. aeruginosa gyrA*. The primer sequences were 5′-TTATGCCATGAGCGAGCTGGGCAAC GACT beginning at nucleotide 147 and 5′-AACCGTTGAC CAGCAGGTTGGGAATCTT beginning at nucleotide 484 (see Fig. 2). PCR was performed by using the Gene Amp kit and DNA thermal cycler model 9600 (Perkin-Elmer Cetus). *Taq* polymerase (Amplitaq) was added to purified template DNA, and the sample was heated to 94°C for 3 min. The reaction was then cycled for 1 min at temperatures of 94, 57, and 72°C for a total of 20 cycles. The reaction mixture was incubated for an additional 7 min at 72°C before storage at 4°C. This second PCR amplification yielded a 360-bp fragment which was used in the DNA sequencing reactions described below.

***E. coli* minicell expression of cloned PAO1 *gyrA*.** Minicell-

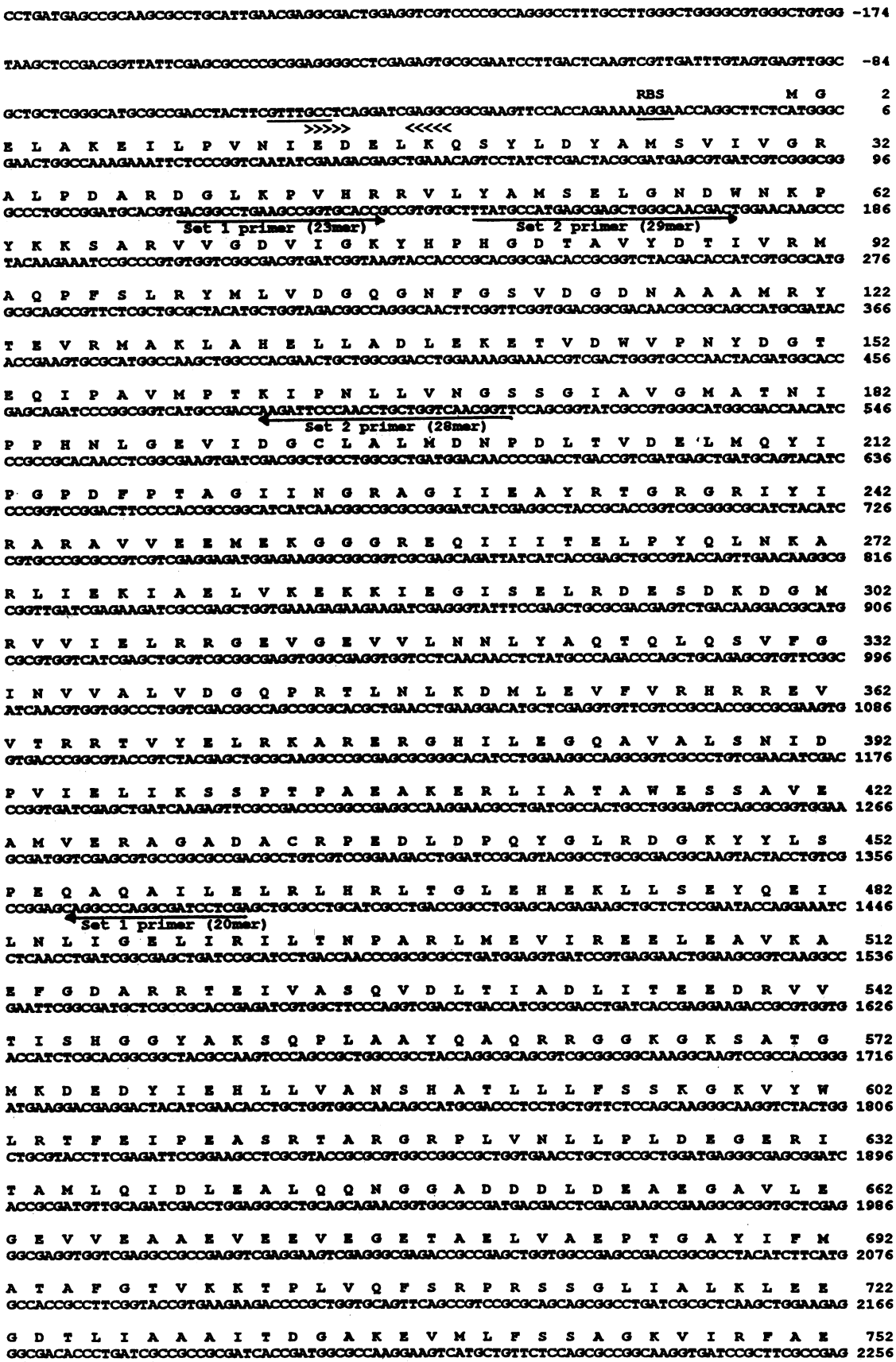

FIG. 2. Nucleotide and predicted amino acid sequences of the PAO1 *gyrA* gene. The underlined sequences are a putative ribosome-binding site (RBS) and a possible topoisomerase promoter region motif (see text). Potential hairpin loops upstream and downstream of the open reading frame are indicated by the symbols > and < under inverted repeats. Also shown are the locations of the nested PCR primers.

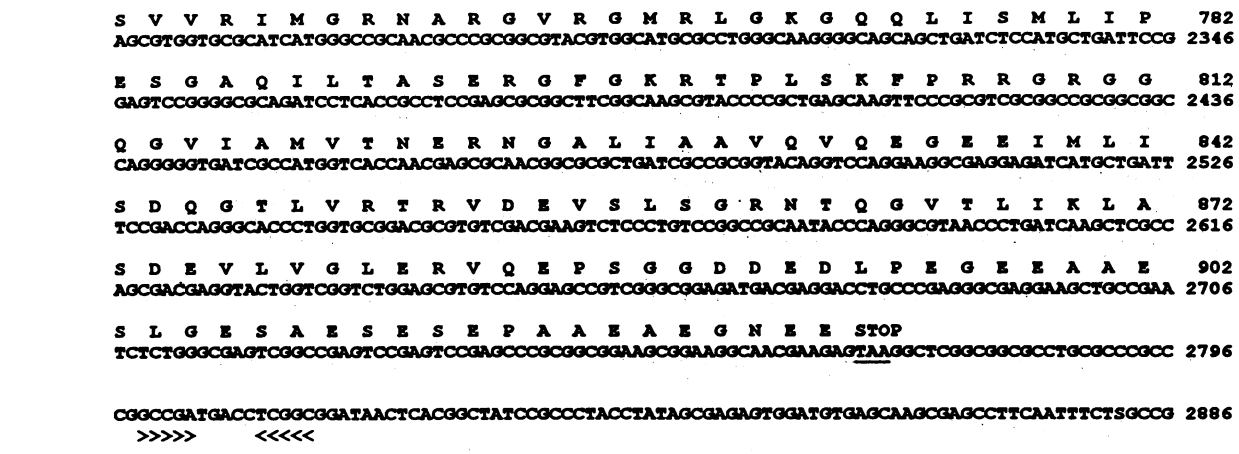

FIG. 2—*Continued.*

producing *E. coli* SA-2742 was transformed with plasmids containing the *P. aeruginosa* PAO1 and *E. coli gyrA* genes. Minicells were obtained by sucrose gradient centrifugation. Nascent polypeptides were labeled with [^{35}S]methionine for 1 h. Minicells were washed with M9 medium and boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. Proteins were separated on an SDS–10% polyacrylamide gel, and the locations of the radiolabeled bands were determined by autoradiography.

MIC determination. The MIC of ciprofloxacin was determined by a standard agar dilution method described previously (5). Five independently isolated transformants were tested in the presence and absence of the selecting antibiotic for the plasmid.

RESULTS

Cloning the *gyrA* gene from *P. aeruginosa* PAO1. Digestion of genomic DNA from *P. aeruginosa* PAO1 with *Cla*I produced a single hybridizing band of 3.6 kb when the DNA was probed with the *P. aeruginosa* N-terminal probe and the *E. coli* C-terminal probe. The digested DNA was size fractionated and ligated into pUC19 digested with *Acc*I. Transformants of *E. coli* DH5α were screened with the 1.2-kb *P. aeruginosa gyrA* N-terminal probe under high-stringency conditions. Hybridization of probe to the chromosomal *E. coli gyrA* gene was minimal under these conditions. Plasmids were isolated from colonies that gave a strong hybridization signal, and a restriction map was prepared. Plasmid pGL2B5 (3.6 kb *Cla*I-*Cla*I insert in pUC19) was isolated, and subsequent DNA base sequence analysis and functional studies (see below) revealed that pGL2B5 contained the PAO1 *gyrA* gene. Insert DNA from pGL2B5 hybridized with the 1.2-kb *P. aeruginosa gyrA* N-terminal-coding probe and the 0.9-kb *E. coli gyrA* C-terminal-coding probe (data not shown). An additional clone, pGL1A16, was isolated after *Kpn*I digestion of genomic DNA and was subsequently found to contain a truncated PAO1 *gyrA* gene missing the C-terminal 225 amino acid residues. This plasmid was used in complementation experiments with *E. coli* KNK453 (temperature-sensitive *gyrA* mutant; *gyrA*[Ts]). (see below).

Nucleotide sequence of the PAO1 *gyrA* gene. A strategy for the nucleotide sequencing of PAO1 *gyrA* from plasmid pGL2B5 is shown in Fig. 1. Initial sequence information was obtained by sequencing the 1.2-kb PCR-generated N-terminal region of the *gyrA* gene from a *P. aeruginosa* clinical isolate, isolate Y4492 (15). This information was used to construct sequencing primers for dideoxy-chain termination reactions by using double-stranded, CsCl-purified pGL2B5 DNA as the template. The nucleotide sequence of the PAO1 *gyrA* gene and the predicted amino acid sequence are shown in Fig. 2. An open reading frame of 2769 nucleotides coding for a polypeptide of 923 amino acids was found. The calculated mass of the GyrA polypeptide was 101,135 Da. The DNA had a G+C content of 65.5%, which is typical of *P. aeruginosa* (35). Located between 12 and 22 nucleotides upstream of the AUG translation initiation codon is a homopurine region containing a possible ribosome-binding site. Between nucleotides −56 and −50 is a seven-base sequence (GTTTGCC) which bears a strong resemblance to the conserved sequence (GTTTACC) found in the promoter regions of *E. coli* and *K. pneumoniae gyrA* (3, 17) and *E. coli gyrB* (17). Interestingly, a hairpin loop may form in this region, as has been predicted to occur in the corresponding region of the *K. pneumoniae gyrA* promoter. This may be an indication of supercoiling-dependent regulation of *gyrA* transcription, as has been shown for other promoters (11). Located 26 bases downstream of the TAA stop codon is another potential hairpin loop which may act as a transcription termination signal.

The derived amino acid sequence of the PAO1 *gyrA* protein is shown in Fig. 3 and is compared with those of *E. coli* and *B. subtilis*. PAO1 GyrA shares an overall amino acid identity of 67% (83% similarity) with the *E. coli* GyrA protein and 54% identity (70% similarity) with the *B. subtilis* GyrA protein. Regions of high amino acid homology are present throughout the sequences, particularly toward the N terminus. Amino acid identity with *E. coli* GyrA is 73% at the N terminus (amino acids 1 to 633) and 52% at the C terminus (amino acids 690 to 923). The sequence around the catalytic tyrosine residue (amino acid 122) present at the active site of the *E. coli* enzyme is identical in PAO1 GyrA. Amino acid residues close to the active site (between amino acids 67 and 106) which, when mutated from the wild type in *E. coli* GyrA, confer quinolone resistance were conserved in PAO1 GyrA with the exception of position 83, where the serine in *E. coli* is replaced by a threonine in PAO1.

Analysis of the sequenced regions upstream and downstream of PAO1 *gyrA* did not reveal any homologies with *gyrB* sequences. It thus appears unlikely that the *gyrA* and *gyrB* genes of *P. aeruginosa* are contiguous as is the case in *B. subtilis*, *S. aureus*, *M. pneumoniae*, and *Haloferax* spp. The

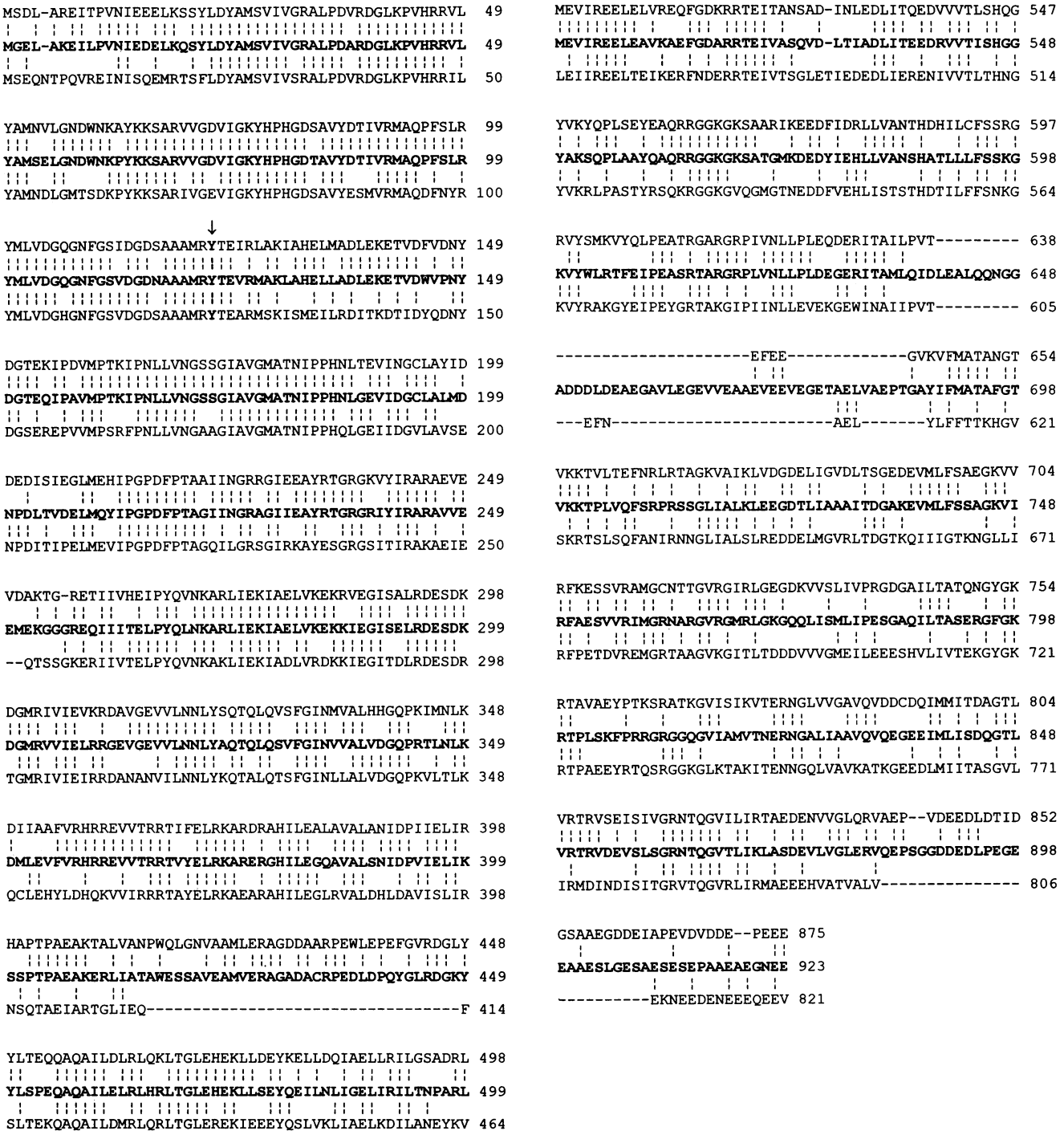

FIG. 3. Alignment of the derived amino acid sequences of the *gyrA* genes of *P. aeruginosa* PAO1 (middle line), *E. coli* (top line), and *B. subtilis* (lower line). Identical amino acids are joined by a dash. The active-site tyrosine at position 122 is indicated by an arrow.

arrangement of DNA gyrase genes on the *P. aeruginosa* chromosome thus appears to be noncontiguous, as is the case in *E. coli*, *K. pneumoniae*, and *P. putida*.

Expression of PAO1 *gyrA*. The PAO1 GyrA protein was expressed in *E. coli* KNK453, a strain with a *gyrA*(Ts) gene (14). Figure 4 shows that pGL2B5 was shown to functionally complement the *E. coli gyrA*(Ts) mutation by allowing full growth at 43°C. This result strongly supports the identification of the insert DNA in pGL2B5 as *gyrA* and shows that a *P. aeruginosa* GyrA–*E. coli* GyrB holoenzyme complex is functional. A truncated PAO1 *gyrA* gene (pGL1A16) missing the C-terminal 225 amino acid residues did not complement the *E. coli* gyrA(Ts) mutant indicating that the C terminus of GyrA is essential for full gyrase function or that these amino acids are important in holoenzyme subunit association.

The PAO1 *gyrA* protein was also expressed in minicells of *E.*

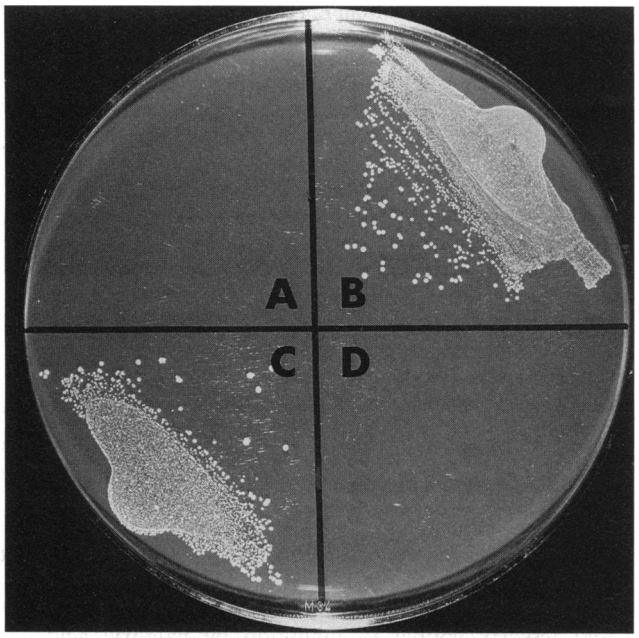

FIG. 4. Agar plate growth at 43°C of *E. coli* KNK453 (*gyrA*[Ts]) transformed with the following; untransformed (A), pSLS447 (*E. coli gyrA*) (B), pGL2B5 (PAO1 *gyrA*) (C), and pGL1A16 (C-terminus-truncated PAO1 *gyrA*) (D).

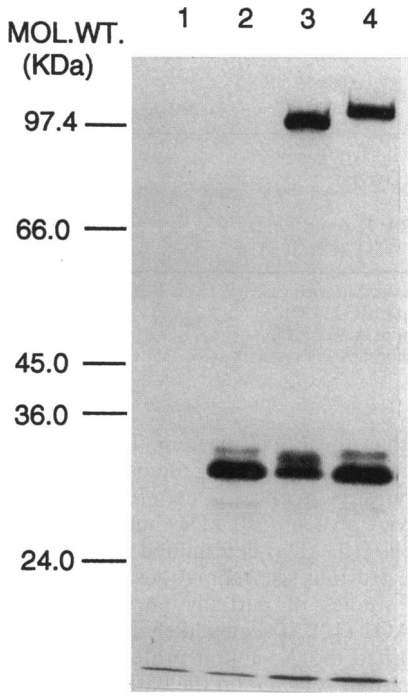

FIG. 5. *E. coli* SA-2742 minicell expression following transformation with plasmids containing *gyrA*. Autoradiogram of [^{35}S]methionine-labeled protein separated by SDS–10% PAGE. Lanes: 1, no plasmid; 2, pUC19 vector; 3, pSLS447 (*E. coli gyrA*); 4, pGL2B5 (PAO1 *gyrA*). The bands at approximately 30 kDa are plasmid-encoded β-lactamase.

coli SA-2742 and was found to have a molecular mass of approximately 103 kDa, in good agreement with the estimated molecular mass from the amino acid sequence predictions (Fig. 5).

Complementation of quinolone resistance in *E. coli* and *P. aeruginosa*. In *E. coli* and *P. aeruginosa*, the nalidixic acid resistance phenotype (GyrA^r) is recessive in merodiploids (8, 26). Therefore, strains with a mutation in the chromosomal *gyrA* gene causing quinolone resistance are transformed to nalidixic acid susceptibility by the introduction of a plasmid-encoded wild-type *gyrA* gene. It is thus possible to distinguish phenotypically nalidixic acid-resistant strains which have mutationally altered chromosomal *gyrA* genes from those which have an alternative mechanism of resistance such as altered permeability or mutations in *gyrB*. We therefore cloned the PAO1 *gyrA* gene into the broad-host-range vector pUCP19 (29). This construct (pUCP19-3.6) and pNJR3-2 containing the wild-type *E. coli gyrA* gene on the cosmid vector pLA2917 (26) were introduced into quinolone-resistant laboratory mutants of *E. coli* and *P. aeruginosa*. Table 1 shows the decreases in the MICs of ciprofloxacin for *E. coli* DH5α (GyrA^r) and *P. aeruginosa* PAO4702 (GyrA^r) after transformation with plasmids containing wild-type *gyrA* genes. The MIC of ciprofloxacin decreased eightfold for *E. coli* DH5α and 16-fold for *P. aeruginosa* PAO4702 after introduction of either the wild-type *E. coli* or PAO1 *gyrA* gene. Decreases in the MICs were the same after the introduction of PAO1 *gyrA* or *E. coli gyrA*. This indicates that *P. aeruginosa* GyrA is as susceptible as *E. coli* GyrA to inhibition by quinolones. For *E. coli* DH5α, the MIC of ciprofloxacin was decreased to less than that for the parent strain. This may be due to the *recA* genotype of this strain because *recA* mutants have been shown to be more susceptible to quinolones (16). The introduction of PAO1 *gyrA* into *P. aeruginosa* PK1013E (*nfxB*) (20), a laboratory-derived quinolone-resistant strain thought to be quinolone resistant because of its decreased level of drug accumulation, had no effect on the MIC for the strain (data not shown).

Sequencing of the quinolone resistance-determining region of ciprofloxacin-resistant clinical isolates of *P. aeruginosa*. Table 2 shows the nucleotide changes in PCR-amplified fragments of the *gyrA* gene from three sets of pre- and during-ciprofloxacin therapy isolates. Each 360-bp fragment was sequenced four times to check for errors introduced during PCR. No errors of amplification or sequencing were detected. In three of nine resistant isolates, a nucleotide change leading to an amino acid substitution was detected. In isolate sets P and Y, the aspartic acid at position 87 was substituted with either an asparagine or a tyrosine, respectively. In isolate set S, the threonine at position 83 was substituted by an isoleucine. Amino acid substitutions at both of these positions have been found in quinolone-resistant *E. coli*, although the substitutions to tyrosine at position 87 and isoleucine at position 83 have not been described before. In all isolates of *P. aeruginosa*, the amino acid at position 83 was threonine rather than serine, as in *E. coli*. This conservative substitution of one polar amino acid for another is also seen in the GyrA sequence of quinolone-susceptible strains of *K. pneumoniae* and *C. jejuni*, and thus appears unlikely to affect the susceptibility of PAO1 GyrA to quinolones (3, 34). All other detected nucleotide changes were silent.

DISCUSSION

DNA gyrase subunits have been cloned and sequenced or have been purified from a number of diverse bacteria. In general there is good homology at the amino acid level between the *gyrA* amino acid sequences from these species. The *P. aeruginosa gyrA* protein is the largest, having 48 more

TABLE 1. Alterations in MIC of ciprofloxacin on introduction of cloned DNA gyrase genes from *E. coli* and *P. aeruginosa* into quinolone-susceptible and -resistant strains of *E. coli* and *P. aeruginosa*

Host strain	Ciprofloxacin MIC (μg/ml)[a]		
	Vector	PAO1 *gyrA*	*E. coli gyrA*
E. coli C600 (wild type)	0.016 (pUC19)	0.016 (pGL2B5)	0.016 (pSLS447)[b]
E. coli DH5α (GyrA^r)	0.032 (pUC19)	0.004 (pGL2B5)	0.004 (pSLS447)
P. aeruginosa PAO1 (wild type)	0.128 (pUCP19 or pLA2917)	0.128 (pUCP19-3.6)	0.128 (pNJR3-2)[c]
P. aeruginosa PAO4702 (GyrA^r)[d]	2 (pUCP19 or pLA2917)	0.128 (pUCP19-3.6)	0.128 (pNJR3-2)

[a] The plasmids used in each case are given in parentheses.
[b] *E. coli gyrA* in pBR322 (32).
[c] *E. coli gyrA* in pLA2917 (26).
[d] *cfxA3 ser-3* (ciprofloxacin resistant) (26).

amino acids than the *E. coli* protein. This is mainly due to a stretch of 43 amino acids showing almost no aligned homology with other gyrase sequences. The predicted size of the *P. aeruginosa* GyrA protein (101 kDa) and the measured size in *E. coli* minicells (103 kDa) determined in the present study do not match the previous size estimations of 79 kDa obtained on the basis of studies of partially purified enzyme from *P. aeruginosa* PAO1 (12). It seems likely that those investigators wrongly identified the A subunit protein in partially purified gyrase preparations selected on the basis of DNA supercoiling activity or that a proteolytic cleavage product of the complete polypeptide was present in large quantities. Assuming the size of the *P. aeruginosa* gyrase B subunit to be similar to those of *E. coli* and *P. putida* (90 kDa), the size of the A_2B_2 gyrase holoenzyme would be 386 kDa, in approximate agreement with the 360-kDa size estimation of the holoenzyme by density gradient centrifugation (18).

Expression of PAO1 *gyrA* in a *gyrA*(Ts) *E. coli* mutant showed that gyrase A subunits from *P. aeruginosa* could combine with B subunits from *E. coli* to produce an active holoenzyme able to complement the *gyrA*(Ts) mutation in *E. coli* KNK453. This result is not surprising given the high degree of homology between the *P. aeruginosa* and the *E. coli gyrA* proteins and the fact that the less homologous *B. subtilis* GyrB protein can functionally complement the *E. coli* GyrA protein (21). The C terminus of *E. coli* GyrA has been postulated to be involved in gyrase-DNA complex stability (25). The lack of complementation of the *gyrA*(Ts) mutant with a C-terminus-truncated PAO1 *gyrA* clone supports the importance of the region for full gyrase function.

TABLE 2. Nucleotide changes and predicted amino acid alterations in PCR-amplified 360-bp fragments of the *gyrA* genes of *P. aeruginosa* isolated from ciprofloxacin-resistant, during-therapy isolates[a]

Strain[b]	Ciprofloxacin MIC (μg/ml)	Nucleotide change (position)	Amino acid change (position)
Set P			
Pretherapy (Cip^s)	0.5		
During-therapy (Cip^r)	4	G→A (259)	Asp→Asn (87)
Set S			
Pretherapy (Cip^s)	0.25		
During-therapy (Cip^r)	8	C→T (248)	Thr→Ile (83)
Set Y			
Pretherapy (Cip^s)	1		
During-therapy (Cip^r)	32	G→T (259)	Asp→Tyr (87)

[a] All sequences were compared with those from the corresponding ciprofloxacin-susceptible, pretherapy isolate.
[b] Cip^s, ciprofloxacin susceptible; Cip^r, ciprofloxacin resistant.

Comparison of the intrinsic susceptibilities of DNA gyrases from different species to quinolone inhibition has so far relied on comparisons of in vitro reactions with purified enzymes. It has been reported that the DNA gyrase from *P. aeruginosa* is 3-fold less susceptible to inhibition by nalidixic acid and between 20- and 60-fold less susceptible to newer fluoroquinolones in in vitro DNA supercoiling reactions than the enzyme from *E. coli* (13, 18). Other data, however, suggest that the two enzymes have the same susceptibilities to quinolone inhibition (27). The data obtained in the present study show that introduction of the cloned PAO1 *gyrA* into a GyrA^r mutant of *E. coli* decreased the quinolone MIC to slightly below that for the wild type. This result shows that the PAO1 *gyrA* protein has susceptibility comparable to that of the *E. coli gyrA* protein. This result is not surprising given the almost identical amino acid sequences of the two genes at the N terminus, the region shown to be important for mutationally altered quinolone susceptibility in *E. coli* and *S. aureus*. Conversely, the introduction of wild-type *gyrA* from *E. coli* or *P. aeruginosa* into wild-type *P. aeruginosa* did not reduce the MIC to that for wild-type *E. coli*, indicating that an intrinsic factor such as low membrane permeability is responsible for susceptibility differences between the two species. It could be argued that the introduction of a large number of plasmid-encoded *gyrA* genes may increase the concentration of drug targets and thus affect susceptibility; however, this seems unlikely given the lack of an effect on the MIC for wild-type *E. coli* or *P. aeruginosa* and the limited number of preferred gyrase-binding sites on the chromosome (30).

The major mechanisms of bacterial resistance to quinolones are target site modification (alteration in DNA gyrase) and decreased intracellular accumulation. The results of the present study show that *P. aeruginosa* may modify DNA gyrase in a manner similar to the way that *E. coli* and *S. aureus* modify their enzymes. Alterations in DNA gyrase caused by mutations in the so-called quinolone resistance-determining region (38) of *gyrA* appear to provide the highest level of resistance in *E. coli* and *S. aureus*. In the present study, alterations in DNA gyrase were found in only three of nine resistant isolates tested. In each of these cases, a coding change occurred at a nucleotide previously shown to be a mutation site in *gyrA* of *E. coli*, *S. aureus*, and *C. jejuni*. It remains possible that resistance-causing mutations in *gyrA* occur outside of the 360-bp region sequenced; however, the similarity in the clustering of mutations in *E. coli*, *S. aureus*, and *C. jejuni* strongly suggests that this region is especially important in intermolecular interac-

tions in the quinolone-gyrase-DNA complex. It should be noted, however, that a functional demonstration of the altered susceptibility caused by these mutations has so far been demonstrated only in *E. coli* by the construction and expression of chimeric constructs of wild-type and mutationally altered *gyrA* genes (2).

In all nine sets of clinical isolates examined, the pre- and posttherapy ciprofloxacin-susceptible isolates had identical *gyrA* sequences, indicating either an in vivo reversion of the mutation found in the corresponding during-therapy strain or an overgrowth with a subpopulation of cells with the pre-therapy sequence which may have resisted the action of ciprofloxacin by another mechanism. Evidence for this latter mechanism was provided in set P, in which the pre- and posttherapy strains had a silent mutation at nucleotide 252 which was not present in the during-therapy strain. Interestingly, attempts to isolate revertants of the during-therapy, ciprofloxacin-resistant isolates of sets P, S, and Y on ciprofloxacin-free agar in the laboratory were unsuccessful, despite approximately 40 repeated subcultures, whereas other resistant isolates quickly reverted (5).

The cloning of PAO1 *gyrA* reported here should allow the construction and expression of chimeric genes from parts of *gyrA* genes isolated from quinolone-resistant strains of *P. aeruginosa* and wild-type PAO1 genes to confirm and quantitate the contribution of the *gyrA* alterations to resistance as has been done in *E. coli* (2). The data obtained from the present study indicate that mutational alterations in DNA gyrase may be a relatively minor component of clinical resistance to quinolones in *P. aeruginosa*. In another study (39), 12 of 17 clinical isolates of *P. aeruginosa* were considered to have mutational alterations in *gyrA*, as shown by phenotypic characterization by using complementation with an *E. coli gyrA* gene. Other resistance mechanisms such as decreased intracellular accumulation may be operating in resistant isolates in which changes in *gyrA* have not been detected. Our previous phenotypic characterization of these strains (5) found differences in the outer membrane protein and lipopolysaccharide profiles of susceptible and resistant isolates; however, no significant differences in the uptake of ^{14}C-labeled ciprofloxacin could be shown other than in set Y, in which drug binding was lower in the resistant isolate. Final elucidation of the quinolone resistance mechanisms in these strains will require further study.

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