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Ciprofloxacin-Resistant *Haemophilus influenzae* Strains Possess Mutations in Analogous Positions of GyrA and ParC

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The nucleotide sequences of the quinolone resistance-determining regions of the *gyrA* and *parC* genes from five ciprofloxacin-resistant strains of *Haemophilus influenzae* (MICs, 2 to 32 μg/ml) isolated from patients with cystic fibrosis and three ciprofloxacin-susceptible strains of *H. influenzae* (MICs, ≤0.1 μg/ml) were determined. Four of the five resistant strains possessed at least one amino acid substitution in each of the GyrA and ParC fragments studied. The mutations identified in GyrA were a serine at residue 84 (Ser-84) to Leu or Tyr and Asp-88 to Asn or Tyr. ParC mutations were in positions exactly analogous to those identified in GyrA, namely, Ser-84 to Ile and Glu-88 to Lys. The Glu-88 to Lys ParC substitution was identified only in high-level ciprofloxacin-resistant strains. These mutations have been shown to be the origin of the observed resistance after transformation into ciprofloxacin-susceptible *H. influenzae* isolates. These results suggest that *H. influenzae* isolates require at least one amino acid substitution in both GyrA and ParC in order to attain significant levels of resistance to quinolones.

Fluoroquinolones are a relatively new class of potent, broad-spectrum antimicrobial agents (20). The principal targets of the quinolones are DNA gyrase and topoisomerase IV, members of the topoisomerase family of enzymes, which are responsible for the maintenance of superhelical tension of DNA in the cell (19). Both of these enzymes function by passing a DNA strand through another strand by using a transient double-strand break (13). DNA gyrase is an essential bacterial enzyme; it catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (18). The enzyme consists of two A and two B subunits, which are encoded by the genes *gyrA* and *gyrB*, respectively. Topoisomerase IV, recently described in *Escherichia coli* strains (12), is encoded by the closely linked genes *parC* and *parE*, and it is believed that this enzyme has an essential role in partitioning replicated chromosomes (13). The deduced amino acid sequences of ParC and ParE have been found to be homologous to GyrA and GyrB (the two subunits of DNA gyrase), respectively (12).

Bacterial resistance to quinolones can arise through mutations in DNA gyrase; single point mutations in both subunits have been shown to play a role in quinolone resistance (for a review, see reference 15). However, in *E. coli* strains, high-level resistance mutations map primarily to the quinolone resistance-determining region (QRDR) located between residues 67 and 106 on the GyrA sequence (22) and where sequence similarities between GyrA and ParC are highest. Recent studies have identified similar mutations in the analogous region of ParC. Point mutations producing amino acid changes in the serine at residue 88 (Ser-88) and Glu-91 (equivalent to Ser-81 and Glu-84 of *E. coli* ParC, respectively) in *Neisseria gonorrhoeae* (3) and Ser-80 in *Staphylococcus aureus* (6) have been identified. In the gram-negative bacterium *N. gonorrhoeae*, GyrA was identified as the primary target of ciprofloxacin because amino acid changes in *parC* were observed only with the simultaneous presence of one or more resistance mutations in *gyrA*. Interestingly, the opposite was observed in the gram-positive bacterium *S. aureus.*

A recent development has been the identification of *Haemophilus influenzae* strains resistant to a number of fluoroquinolones (2, 9); however, studies investigating the mechanisms behind this observed resistance have not been attempted. Our laboratory identified five *H. influenzae* strains exhibiting various levels of ciprofloxacin resistance (Cpx^r). These strains were isolated from three cystic fibrosis patients, all of whom had previously been treated with ciprofloxacin and other fluoroquinolones (3a). We report here on the characterization of the QRDRs of the *gyrA* and *parC* genes in these and other strains in an effort to identify possible mechanisms of resistance.

Sequencing of *gyrA* and *parC* QRDRs of *H. influenzae*. The *gyrA* and *parC* genes were identified by an approach that has proved to be successful for the isolation of *gyrA* homologs from other bacterial species (3, 6). Degenerated oligonucleotide primers were designed on the basis of sequence similarities identified among *gyrA* genes corresponding to regions 39 to 45 and 173 to 180 of the amino acid sequence of *E. coli* GyrA. Genomic DNA was obtained from *H. influenzae* MAP (4) as described elsewhere (1) and was amplified by PCR with primers B-98 [5′-gcgctctaGA(C/T)GGT(C/T)TNAAACCNGTN CA-3′], coding for DGLKPVH, and B-96 (5′-gcgcaagcTTTG TAGCCATACCNACNGCAATNCC-3′), the complementary strand of the one coding for GIAVGMAT. The 5′ ends of the primers contained sequences including either an *Xba*I (B-98) or a *Hin*dIII (B-96) restriction site (lowercase letters). Amplifications were performed with a GeneAmp kit (Perkin-Elmer Cetus) with 1 μg of chromosomal DNA and 1 μM (each) primer and were carried out as described elsewhere (5), except that an initial cycle of 5 min at 95°C, 20 min at 55°C, and 6 min at 72°C was performed. PCR products of 444 bp were run on agarose gels (16), and fragments of the appropriate sizes were purified by using the GeneClean kit (Bio 101), treated with *Xba*I and *Hin*dIII, and cloned into pUC18 (21) cut with the same endonucleases. Transformation into *E. coli* DH5 was as

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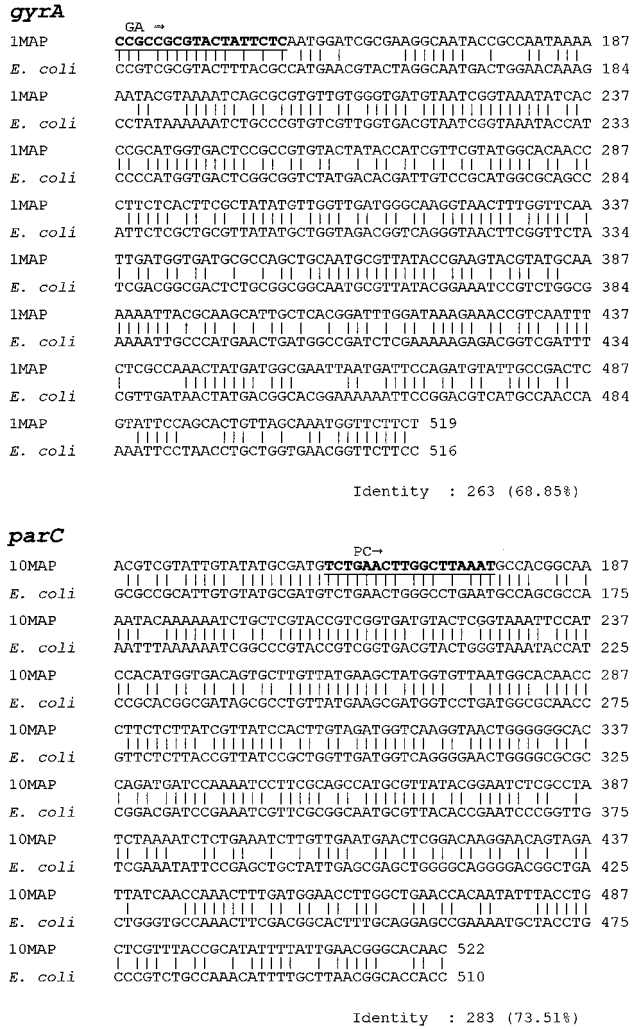

FIG. 1. Nucleotide sequences of the inserts of plasmids p1MAP and p10MAP compared with the equivalent regions of *E. coli*. The oligonucleotide primers GA and PC used for the amplification of the specific regions of *gyrA* and *parC* genes are underlined and are in boldface type. The Nalign program from PC/Gene 6.60 was used.

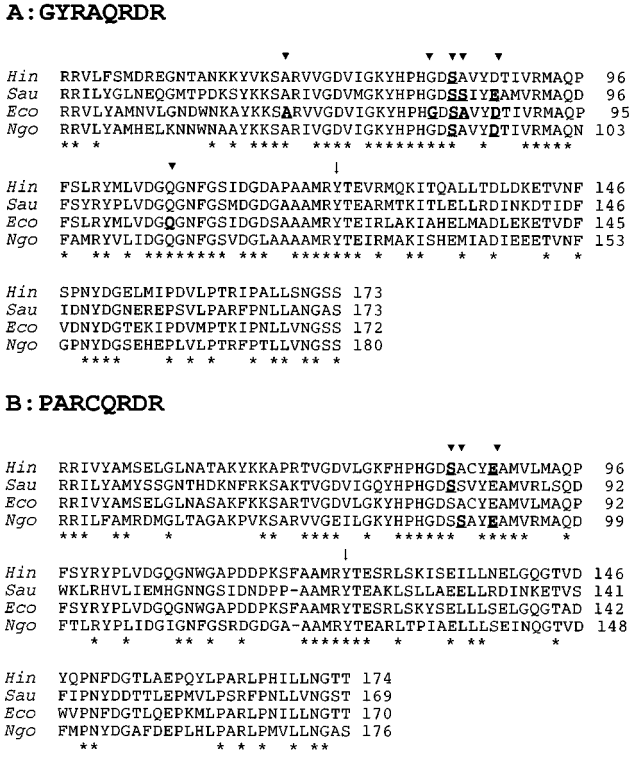

FIG. 2. Comparison between the QRDRs of GyrA (A) and ParC (B) from *H. influenzae* (*Hin*), *S. aureus* (*Sau*) (11), *E. coli* (*Eco*) (17), and *N. gonorrhoeae* (*Ngo*) (3). The clustal program from PC/Gene 6.60 was used to compare predicted sequences. Residues involved in quinolone resistance (▼) are underlined and are in boldface type. Arrows indicate the active Tyr involved in DNA binding. Asterisks indicate amino acid identity, and dashes indicate gaps introduced to maximize similarities.

described elsewhere (16). *E. coli* transformants were selected in Luria-Bertani (LB) medium containing ampicillin at 100 μg/ml. The inserts from two recombinant plasmids (p1MAP and p10MAP) showing different restriction patterns with the *Ase*I restriction endonuclease were sequenced in both DNA strands with the Sequenase kit from U.S. Biochemicals and synthetic oligonucleotides. Analysis revealed two distinct sequences (Fig. 1) which shared 60% identity at the nucleotide level. These sequences were tentatively identified as the *gyrA* (insert of p1MAP) and *parC* (insert of p10MAP) QRDRs of *H. influenzae* because of their high levels of identity (higher than 68%) to the corresponding regions in *E. coli gyrA* and *parC* genes (Fig. 1). The comparison of these sequences with that of the recently published *H. influenzae* Rd strain (7) showed complete identity, bar a nucleotide change in *gyrA* which corresponds to a resistance mutation, as will be described below.

Identification of *gyrA* and *parC* mutations responsible for fluoroquinolone resistance. Once the sequence from *H. influenzae* MAP was identified, oligonucleotides specific for the 5′ ends of the *gyrA* and *parC* genes were designed (Fig. 1 and 2). The oligonucleotides were as follows: for *gyrA* (oligonucleotide GA), 5′-gcgctctag**aagtgcggtca**CCGCCGCGTACTATTCTC-3′, coding for RRVLFS, and for *parC* (oligonucleotide PC), 5′-gcgctctag**aagtgcggtca**TCTGAACTTGGCTTAAAT-3′, coding for SELGLN (lowercase letters indicate bases not present in the original nucleotide sequence). These oligonucleotides were chosen to allow for maximum pairing with the gene sequences after the incorporation of the 11-bp sequence (boldface type) into both primers to aid in the transformation of *H. influenzae* (8). PCR products from five Cpx^r and two highly susceptible strains (Table 1) were obtained through amplifications of chromosomal DNA by using oligonucleotide GA and B-96 or PC and B-96. The PCR products were purified, and the region spanning residues 53 to 173 of GyrA and ParC were directly sequenced with the *fmol* system (Promega) by using the same two oligonucleotides as primers. Cpx^r strains were isolated from three patients with cystic fibrosis. The MICs for the strains were determined by the microdilution method (Sensititre Ltd., East Grinstead, West Sussex, England) with Haemophilus Test Medium (HTM) and were verified by a standard agar dilution method (14). Both susceptible strains had identical amino acid sequences. Strain MAP, although clinically susceptible, is 10-fold less susceptible to ciprofloxacin than the highly susceptible strains (MIC, 0.1 μg/ml) and shows a single amino acid substitution in the GyrA QRDR compared with the sequences of the susceptible strains, namely, an Asp-88 to Tyr substitution (Table 1). This was the only strain in the study with a single amino acid alteration. No changes were observed in strain 19594. However, all other resistant strains possessed

TABLE 1. Amino acid changes of QRDRs of GyrA and ParC homologs of *H. influenzae* isolates[a]

Strain	Ciprofloxacin MIC (μg/ml)	Amino acid change (codon change)	
		GyrA	ParC
H93/12	0.01	^{80}PHGD**S**AVY**D**TIVR^{92}	^{80}PHGDSACYEAMVL^{92}
H93/25	0.01	None	None
MAP	0.10	^{88}D→Y (GAT→**T**AT)	None
24194	2.00	^{88}D→N (GAT→**A**AT)	^{84}S→I (AGT→A**T**T)
19594	2.00	None	None
0694	4.00	^{84}S→L (TCC→T**T**A)	^{84}S→I (AGT→A**T**C)
2495B	16.00	^{88}D→N (GAT→**A**AT)	^{88}E→K (GAA→**A**AA)
2495A	32.00	^{84}S→Y (TCC→T**A**C) ^{88}D→N (GAT→**A**AT)	^{88}E→K (GAA→**A**AA)
$R1^{2495B-A}$	0.80	^{88}D→N (GAT→**A**AT)	
$R1^{24194-C}$	2.00	^{88}D→Y (GAT→**T**AT)	^{84}S→I (AGT→A**T**T)
$R2^{0694-A/-C}$	4.00	^{84}S→L (TCC→T**T**A)	^{84}S→I (AGT→A**T**C)
$R2^{2495A-A/-C}$	32.00	^{84}S→Y (TCC→T**A**C) ^{88}D→N (GAT→**A**AT)	^{88}E→K (GAA→**A**AA)

[a] $R1^{24194-C}$ is the nomenclature for transformants of MAP with the *parC* PCR product of strain 24194, and $R1^{2495B-A}$ with the *gyrA* PCR product of strain 2495A. $R2^{0694-A/-C}$ and $R2^{2495A-A/-C}$ are transformants obtained by two successive transformations with the *gyrA* and *parC* PCR products from strains 0694 and 2495A, respectively. Boldface type indicates the base change.

amino acid alterations in both *gyrA* and *parC* genes. Mutations in *gyrA* were Ser-84 to Leu or Tyr and Asp-88 to Asn. Substitutions in these amino acid positions of GyrA are the most common mutations involved in fluoroquinolone resistance, with mutations in these positions being found in a number of bacteria (3). The mutations identified in *parC* were Ser-84 to Ile and Glu-88 to Lys. The location of the *parC* mutations are at positions exactly analogous to those found in the *gyrA* gene.

Genetic exchange of ciprofloxacin resistance by transformation. Further evidence that the amino acid changes identified in the GyrA and ParC fragments are responsible for ciprofloxacin resistance was obtained by transformation. Both whole-cell DNAs and PCR products encoding the QRDRs of *parC* and *gyrA*, obtained as described above, from Cpx^r and Cpx^s strains were used to transform competent *H. influenzae* MAP cells. Competence was developed by the methods described by Herriott et al. (10). Selection for transformants was on HTM plates containing 0.3 μg of ciprofloxacin per ml. The plates were incubated for 24 to 48 h at 37°C with 5% CO_2. The spontaneous mutation frequency to ciprofloxacin resistance was determined for strains MAP and H93/12 by plating 10^9 cells on HTM agar containing either 0.3 μg (MAP) or 0.03 μg (H93/12) of ciprofloxacin per ml. The mutation frequency was undetectable ($<10^{-9}$) for the two strains after 36 h of incubation. Transformation experiments gave frequencies of transformation of between 7×10^{-6} and 1×10^{-4} for chromosomal DNA and between 1×10^{-7} and 7×10^{-5} for PCR products. All PCR fragments encoding the QRDR of *gyrA* from Cpx^r strains, bar that from strain 19594, transformed strain MAP and in all cases increased the MIC of ciprofloxacin for MAP to about 0.8 μg/ml. The sequence of the *gyrA* region of one of these transformants, $R1^{2495B-A}$, is given in Table 1. Subsequently, PCR fragments encoding the QRDR of *parC* were used to transform the earlier MAP transformants containing the *gyrA* mutations. Again, all PCR products from Cpx^r strains, bar that from strain 19594, transformed MAP to further resistance. For all new transformants the MIC was equal to that for the donor strain. Two of these transformants, $R2^{0694-A/-C}$ and $R2^{2495A-A/-C}$ (Table 1), have all of the nucleotide changes that were detected in the *gyrA* and *parC* genes of the donor strains.

The *parC* PCR fragments were also used to transform susceptible strain MAP. In this case, only *parC* fragments from strains 24194 and 0694 were able to increase the level of ciprofloxacin resistance. These fragments shared a Ser-84 to Ile substitution. The sequences of the *gyrA* and *parC* regions of one of these transformants, $R1^{24194-C}$ (Table 1), confirms that when the Asp-88 to Tyr GyrA change of strain MAP is combined with the Ser-84 to Ile ParC change, resistance is seen. We can, however, find no explanation as to why DNAs from strains possessing the Glu-88 to Lys ParC change (strains 2495A and 2495B) did not transform MAP.

The results from the transformation experiments suggest that *gyrA* is the primary target of ciprofloxacin in *H. influenzae* strains, because an amino acid alteration in the GyrA protein is necessary before ParC mutations can influence resistance levels. The only strain to possess a single mutation was strain MAP (an Asp-88 to Tyr substitution in GyrA). Evidence that both GyrA and ParC must be altered in order to gain significant levels of resistance is provided by the experiment transforming MAP with *gyrA* fragments. When *gyrA* mutations were introduced into strain MAP, MICs did not exceed 1 μg/ml in any case unless a *parC* mutation was also introduced. Table 1 illustrates how the type and number of mutations are important to the overall level of ciprofloxacin resistance. High-level resistance seems to be due to mutations causing the Asp-88 to Lys ParC change, which is present only in high-level Cpx^r strains. Strain 19594 possessed no mutations in either QRDR, and furthermore, PCR products could not transform MAP to ciprofloxacin resistance. It is likely that other mechanisms of resistance are involved in providing the observed phenotype of this strain. Examples include a GyrB amino acid substitution, decreased drug permeation, or quinolone efflux.

Although detailed three-dimensional structures of the GyrA and ParC proteins are not available, these observations provide evidence that Ser-84 and Asp-88 of both GyrA and ParC are essential amino acids for interactions with DNA and fluoroquinolones. The levels of homology between these four amino acids and their respective mutations also suggest that the GyrA and ParC interactions with these molecules are very similar in nature.

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