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Involvement of Topoisomerase IV and DNA Gyrase as Ciprofloxacin Targets in *Streptococcus pneumoniae*

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Ciprofloxacin-resistant mutants of *Streptococcus pneumoniae* 7785 were generated by stepwise selection at increasing drug concentrations. Sequence analysis of PCR products from the strains was used to examine the quinolone resistance-determining regions of the GyrA and GyrB proteins of DNA gyrase and the analogous regions of the ParC and ParE subunits of DNA topoisomerase IV. First-step mutants exhibiting low-level resistance had no detectable changes in their topoisomerase quinolone resistance-determining regions, suggesting altered permeation or another novel resistance mechanism. Nine of 10 second-step mutants exhibited an alteration in ParC at Ser-79 to Tyr or Phe or at Ala-84 to Thr. Third- and fourth-step mutants displaying high-level ciprofloxacin resistance were found to have, in addition to the ParC alteration, a change in GyrA at residues equivalent to *Escherichia coli* GyrA resistance hot spots Ser-83 and Asp-87 or in GyrB at Asp-435 to Asn, equivalent to *E. coli* Asp-426, part of a highly conserved EGDSA motif in GyrB. No ParE changes were observed. Complementary analysis of two *S. pneumoniae* clinical isolates displaying low-level resistance to ciprofloxacin revealed a ParC change at Ser-79 to Phe or Arg-95 to Cys but no changes in GyrA, GyrB, or ParE. A highly resistant isolate, in addition to a ParC mutation, had a GyrA alteration at the residue equivalent to *E. coli* Asp-87. Thus, in both laboratory strains and clinical isolates, ParC mutations preceded those in GyrA, suggesting that topoisomerase IV is a primary topoisomerase target and gyrase is a secondary target for ciprofloxacin in *S. pneumoniae*.

Effective treatment of respiratory tract infections is a major focus of antibiotic therapy (8). The gram-positive bacterium *Streptococcus pneumoniae* is one of the most important respiratory pathogens, being the leading cause of community-acquired pneumonia. It is also responsible for acute otitis media and meningitis (8, 27). At the dawning of the antibiotic era, most *S. pneumoniae* strains encountered in the clinic were exquisitely susceptible to penicillins. However, therapy with β-lactam antibiotics has been compromised by the emergence of highly resistant strains (20). The paucity of effective antipneumococcal drugs has led to the widespread prescribing of fluoroquinolones, particularly ciprofloxacin (CIP). Resistance to CIP whose molecular basis is unknown has rapidly emerged. It is important to address this issue, given that a new generation of fluoroquinolones, e.g., sparfloxacin, is being introduced for pneumococcal infections (5, 27).

In *Escherichia coli*, several fluoroquinolones are known to target DNA gyrase, an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes, which is responsible for ATP-dependent DNA supercoiling by a double-strand DNA break mechanism (10, 12, 24). The drugs cause inhibition of DNA synthesis and trigger cell killing by interfering with double-strand breakage-reunion mediated by catalytic Tyr-122 of the GyrA subunits (9–11, 15). Previous studies of *E. coli* have identified quinolone resistance mutations in a discrete region of GyrA and GyrB, termed the quinolone resistance-determining region (or QRDR). Mutations in *E. coli* GyrA map between residues 67 and 106, predominantly at Ser-83 and less so at Asp-87 (4, 10, 25, 35). For GyrB, mutations at Asp-426 and Lys-447 confer low-level resistance (34, 37). Work from several groups has shown that in development of high-level resistance, gyrase mutations precede those in the related enzyme DNA topoisomerase IV, a C_2E_2 complex that is encoded by genes *parC* and *parE* and is essential for chromosome partitioning (1, 18). The data suggest that in *E. coli*, gyrase and topoisomerase IV are respective primary and secondary targets for CIP (14, 16, 19, 22). Similar observations have been reported for *Neisseria gonorrhoeae* (2). Interestingly, the inverse is true for *Staphylococcus aureus* (6, 7) and possibly for *Enterococcus faecalis* (21). Thus, *gyrA* mutations have been identified in CIP-resistant *S. aureus* (17, 23, 29, 30) but are preceded by mutations in the *parC* (*grlA*) gene that confer low-level resistance (6, 7, 33). It appears that topoisomerase IV is the primary target of CIP in *S. aureus.*

Recently, we cloned and sequenced the *parE-parC* and *gyrB* genes from *S. pneumoniae* and showed that mutation of the conserved Ser-79 residue in ParC occurred in four laboratory mutants, 2C1 to 2C4, selected for low-level resistance to CIP (26). Although the results implicated ParC in resistance, lack of data on DNA gyrase did not allow us to investigate the role of topoisomerase IV as a target vis-a-vis DNA gyrase. Here we report a detailed analysis of the QRDRs of the *parC*, *parE*, *gyrA*, and *gyrB* genes in *S. pneumoniae* strains selected stepwise for resistance to CIP. In parallel studies, we also examined CIP-resistant *S. pneumoniae* clinical strains including sequential isolates from a patient with chronic obstructive airway disease.

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MATERIALS AND METHODS

Materials. *S. pneumoniae* clinical isolates were obtained at the North Middlesex Hospital, London, United Kingdom. Isolates D5, B10, and D11 were from the same patient treated for chronic obstructive airway disease with 500 mg of

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CIP at 12-h intervals for 8 days. Susceptible *S. pneumoniae* clinical isolate 7785 has been described previously (26). *E. coli* XL1 was from our strain collection and was used in conjunction with plasmid pCRII (Invitrogen) for cloning and sequence analysis of PCR products. Brain heart infusion medium and horse blood were from Gibco-BRL and Difco, Ltd., respectively. Chromosomal DNAs from pneumococcal isolates were purified as described previously (26). Restriction enzymes, T4 DNA ligase, and *Taq* polymerase were from Northumbria Biologicals, Cramlington, United Kingdom. CIP and sparfloxacin were kindly provided by Bayer, Wuppertal, Germany, and Dainippon Pharmaceutical Co., Suita, Japan. Ampicillin was from Sigma Co., Poole, United Kingdom.

Stepwise selection of CIP-resistant *S. pneumoniae* strains. Mutants were selected by plating approximately 2×10^{10} CFU of strain 7785 on brain heart infusion plates containing 10% horse blood with or without CIP. Incubation was aerobic at 37°C for 24 to 48 h. The frequency of spontaneous mutants resistant to CIP was determined from the number of colonies that grew on plates with the drug compared to that obtained without the drug. Individual resistant colonies were grown in brain heart infusion medium and used for subsequent rounds of selection. All drug-resistant isolates were confirmed as *S. pneumoniae* by their appearance and by their optochin sensitivity.

Drug susceptibilities. MICs were determined by the twofold dilution method using brain heart infusion medium supplemented with 5% horse blood. Plates were examined after overnight aerobic growth at 37°C.

PCR and restriction fragment length polymorphism (RFLP) analysis. Genomic DNA, isolated from confluent bacteria grown on brain heart infusion plates containing 10% horse blood, was used as a template for PCRs (26). PCRs were carried out in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1 μM each oligonucleotide primer, 2 U of *Taq* DNA polymerase, and 1 ng of genomic DNA. For *parC*, a 366-bp region encoding residues 35 to 157 was amplified by using primers M0363 (5′-TGGGTTGAAGCCGGTTCA; nucleotide positions 104 to 121) and M4271 (5′-TGCTGGCAAGACCGTTGG; positions 453 to 470). PCR conditions were 94°C for 1 min, 53°C for 1 min, and 72°C for 3 min (30 cycles). A 5′ *gyrA* gene segment was initially obtained from strain 7785 by PCR with primers based on conserved sequence motifs DGLKPV and GIVGMAT of *E. coli* GyrA. From the sequence of this PCR product, two nested primers were used to amplify the *gyrA* QRDR: VGA3, 5′-CCGTCGCATTCTTTACG, and VGA4, 5′-AGTTGCTCCATTAACCA. The resulting 382-bp PCR product encoded a region equivalent to residues 46 to 172 of *E. coli* GyrA. In the case of *parE*, PCR products encoding residues 398 to 483 were obtained by using primers S6398, 5′-AAGGCGCGTGATGAGAGC (nucleotides 1179 to 1196), and S6399, 5′-TCTGCTCCAACACCCGCA (complementary to nucleotides 1451 to 1468). To amplify a *gyrB* region encoding residues 371 to 512, primers H4025, 5′-TTCTCCGATTTCCTCATG (positions 1096 to 1113), and H4026, 5′-AGAAGGGTACGAATGTGG (complementary to 1536 to 1553), were used. PCR conditions for *parE* and *gyrB* were 92°C for 1 min, 48°C for 1 min, and 72°C for 2 min (30 cycles).

For RFLP analysis, *parC* and *gyrA* PCR products were digested with *Hin*fI and examined by electrophoresis in 2% low-gelling-temperature agarose (30). The 366-bp *parC* product from the wild-type gene carried *Hin*fI sites at nucleotide positions 232 and 288 and on digestion generated 183-, 127-, and 56-bp fragments. Loss of site 232 through mutation of codon 78 or 79 in resistant isolates generated two 183-bp fragments that ran as a single electrophoretic band. The 382-bp *gyrA* PCR product derived from the wild-type gene underwent cleavage at the single *Hin*fI site, producing 110- and 272-bp fragments: no breakage was seen for genes mutated at the codon equivalent to Ser-83 of *E. coli gyrA* (Fig. 1 and 2).

DNA sequence analysis. To identify mutations at the nucleotide sequence level, PCR products were cloned into pCRII and sequenced from SP6 and T7 primers by using Sequenase version 2 and [α-^{35}S]dATP as described previously (23, 26, 28). Two clones for each PCR product were sequenced on both strands, and complementary strand sequences were in accord.

RESULTS

QRDRs of *S. pneumoniae parC*, *gyrA*, *gyrB*, and *parE* genes. We have recently cloned and sequenced the *S. pneumoniae parC*, *parE*, and *gyrB* genes (26), allowing design of PCR primers for amplification of their QRDRs from resistant mutants. A 382-bp segment of the *S. pneumoniae gyrA* gene encompassing the *gyrA* QRDR was obtained by a standard PCR approach (see Materials and Methods) (Fig. 1). The deduced *S. pneumoniae* GyrA protein sequence is highly similar to that of *E. coli* GyrA (Fig. 2A): residues whose mutation is known to give resistance in *E. coli* are identical or conserved in *S. pneumoniae* GyrA, notably the residues equivalent to Ser-83 and Asp-87 in *E. coli*. Figure 2A also compares the sequence of the *S. pneumoniae* GyrA QRDR with that of the analogous region of *S. pneumoniae* ParC contained in a 366-bp PCR product.

```
CCGTCGCATTCTTTACGGAATGAATGAATTGGGTGTGACCCCAGACAAACCCCATAAAAA
 R  R  I  L  Y  G  M  N  E  L  G  V  T  P  D  K  P  H  K  K    65

ATCTGCTCGTATTACAGGGGATGTCATGGGTAAATATCACCCACACGGGGATTCCTCTAT
 S  A  R  I  T  G  D  V  M  G  K  Y  H  P  H  G  D  S  S  I    85

TTATGAAGCCATGGTTCGTATGGCTCAATGGTGGAGCTACCGTTACATGCTTGTAGATGG
 Y  E  A  M  V  R  M  A  Q  W  W  S  Y  R  Y  M  L  V  D  G   105

TCATGGGAATTTTGGTTCCATGGATGGAGATAGTGCTGCCGCTCAACGTTATACCGAGGC
 H  G  N  F  G  S  M  D  G  D  S  A  A  A  Q  R  Y  T  E  A   125

ACGTATGAGCAAGATTGCTCTGGAAATGCTTCGTGATATCAACAAAAATACAGTTGATTT
 R  M  S  K  I  A  L  E  M  L  R  D  I  N  K  N  T  V  D  F   145

CGTTGATAACTATGATGCCAATGAACGGGAACCCTTGGTCTTGCCAGCGCGTTTTCCAAA
 V  D  N  Y  D  A  N  E  R  E  P  L  V  L  P  A  R  F  P  N   165

CCTTTTGGTTAATGGAGCAACT
 L  L  V  N  G  A  T                                          172
```

FIG. 1. DNA sequence of a 382-bp PCR product encompassing the QRDR in the *gyrA* gene from *S. pneumoniae* 7785. Underlining highlights a *Hin*fI site spanning a sequence equivalent to the Asp-82 and Ser-83 codons in *E. coli gyrA*. Letters under the nucleotide sequence show the deduced protein sequence. Amino acid residues are numbered at the right by analogy with the *E. coli* GyrA protein.

Despite differences, the two sequences share considerable identity: ParC residues 79 and 83 are identical to their *E. coli* counterparts. Ser-79 in ParC is part of an HPHGDSSIY motif conserved in *S. pneumoniae* GyrA. In both cases, the nucleotide sequences of the adjacent Asp and Ser codons form a *Hin*fI site that is eliminated by resistance mutations affecting the conserved serine (Fig. 1). This observation allowed the use of a rapid *Hin*fI RFLP analysis to screen *S. pneumoniae parC* and *gyrA* PCR products for Ser-79 (Ser-83) resistance mutations (see Materials and Methods).

PCR was also used to amplify 457-bp *gyrB* and 289-bp *parE* regions from *S. pneumoniae* carrying a coding sequence equivalent to the QRDR region identified in *E. coli* GyrB (Fig. 2B). *S. pneumoniae* proteins GyrB and ParE share considerable homology with each other and with *E. coli* GyrB, notably conserved EGDSA and PL(R/K)GK motifs identified with quinolone resistance mutations at Asp-426 and Lys-447 in *E. coli* GyrB (Fig. 2B). Thus, comparison of ParE and GyrB sequences, as for ParC and GyrA (Fig. 2A), reveals considerable identity between their respective QRDRs.

***S. pneumoniae* mutants selected stepwise for CIP resistance acquire mutations in *parC* before *gyrA* and *gyrB*.** To examine the role of the two topoisomerases in the development of resistance to CIP, a series of *S. pneumoniae* mutants were generated by stepwise selection at various drug concentrations (Fig. 3). Approximately 10^{10} CFU of strain 7785 (CIP MIC of 1 μg/ml) were spread onto plates containing CIP at 2 μg/ml, yielding 17 drug-resistant colonies (no mutants were obtained on plates containing CIP at 4 or 6 μg/ml). Three of these first-step mutants, 1C1, 1C2 and 1C3, were characterized and independently exposed to CIP at either 4 or 6 μg/ml. Resistant colonies 2C1 to 2C4 from 1C1, 2C5 to 2C7 from 1C2, and 2C8 to 2C10 from 1C3 were isolated and characterized. Second-step mutant 2C1 was plated on CIP at 9 μg/ml, generating third-step mutants 3C1 to 3C3, and 2C2 was plated on CIP at 10, 12.5, and 15 μg/ml, generating third-step mutants 3C4 to 3C9. Finally, fourth-step mutants 4C1 to 4C3 were isolated after exposure of strain 3C1 to CIP at 15 μg/ml (Fig. 3). The procedure generated three independent sets of second-step mutants and four independent sets of third-step mutants. Mutation frequencies were similar for all steps of selection and were in the range of 9×10^{-7} to 9×10^{-8} (Table 1). Mutant strains were characterized in terms of susceptibility to CIP, and the status of the QRDRs of their *parC*, *gyrA*, *gyrB*, and *parE*

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(A)

                                67              83  87                106
                                 |               |   |                 |
Ec. GyrA  rrvlyamnvlgndwnkaykksarvvgdvigkyhphgdsavydtivrmaqpfslrymlvdgqgnk  109
Sp. GyrA  RRILYGMNELGVTPDKPHKKSARITGDVMGDYHPHGDSSIYEAMVRMAQWWSYRYMLVDGHGNF
Sp. ParC  RRILYSMNKDSNTFDKSYRKSAKSVGNIMGNFHPHGDSSIYDAMVRMSQNWKNREILVEMHGNN  105
          ***** **    * **   ***   *  **  ********* ***** * *  *  **  ***

                     122
                      o
Ec. GyrA  gsidgdsaaamryteirlakiahelmadleketvdfvdnydgtekipdvmptkipnllvngss   172
Sp. GyrA  GSMDGDSAAAQRYTEARMSKIALEMLRDINKNTVDFVDNYDANEREPLVLPARFPNLLVNGAT
Sp. ParC  GSMDGDPPAAMRYTEARLSEIAGYLLQDIEKKTVPFAWNFDDTEKEPTVLPAAFPNLLVNGST   168
          ******  ** ****** * **   * ** * ** *  * *  * ** **** ******** *

(B)

                                                426                 447
                                                 |                   |
Ec. GyrB  remtrrkgaldlaglpgkladcqerdpalselylvegdsaggsakggrnrknqailplkgkil  451
Sp. GyrB  REVTRKKSGLEISNLPGKLADCSSNNPAETELFIVEGDSAGGSAKSGRNREFQAILPIRGKIL  460
Sp. ParE  ESRNGKKNKKDKGLLSGKLTPAQSKNPAKNELYLVEGDSAGGSAKQGRDRKFQAILPLRGKVI  460
               **       * ***    * ***  **  *********** ** * ****** ***

Ec. GyrB  nvekarfdkmlssqevatlital                                          174
Sp. GyrB  NVEKASMDKILANEEIRSLFTAM                                          483
Sp. ParE  NTAKAKMADILKNEEINTMIYTI                                          483
          *  ** *  ** ****
```

FIG. 2. Comparison of the *S. pneumoniae* (Sp.) GyrA-ParC (A) and GyrB-ParE (B) regions homologous to *E. coli* (Ec.) QRDRs. Asterisks indicate identity between *S. pneumoniae* sequences (uppercase): conserved EGDSA and PL(R/K)GK motifs are underlined. *E. coli* sequences are in lowercase: the GyrA residues 67, 83, 87, and 106 and GyrB residues 426 and 447 mutated in quinolone-resistant *E. coli* strains are indicated, as is catalytic GyrA residue Tyr-122 (open circle).

genes was ascertained by DNA sequence analysis of PCR products (Table 2).

For each of the first-step mutants 1C1 to 1C3, there was a threefold increase in the CIP MIC (Table 2). However, none of the mutants carried a change in the QRDR of either *parC* or *gyrA*. Moreover, no change was seen in the QRDR of gene *gyrB* or *parE* of strain 1C1. This observation was reproducible: no *parC* alterations were detected by PCR-*Hin*fI RFLP in 10 first-step mutants from two other independent experiments (data not shown). It appears that neither topoisomerase IV nor gyrase undergoes a mutation in the first stage of selection. For the 10 second-step mutants, 2C1 to 2C10, the CIP MICs were 8 to 16 μg/ml (Table 2). Nine of the 10 had acquired single mutations in the ParC QRDR: eight had a Ser-79 (TCT)-to-Tyr (TAT) or -Phe (TTT) change, whereas 2C10 had an Ala-84 (GCC)-to-Thr (ACC) substitution. Given that 2C1 to 2C10 were obtained by three independent drug challenges, it seems that *parC* mutations are frequently selected in the second step.

None of the strains 2C1 to 2C10 examined had a mutation in *gyrA*, *gyrB*, or *parE* (Table 2). However, *gyrA* mutations did appear in third-step mutants which were selected at 10, 12.5, or 15 μg/ml: 3C4 and 3C6 to 3C9 all carried *gyrA* mutations of either Ser (TCC) to Tyr (TAC) or Glu (GAA) to Lys (AAA) at residues corresponding, respectively, to Ser-83 and Asp-87 in *E. coli* GyrA (Table 2). Furthermore, strain 3C8 carried additional changes of Lys-93 (AAA) to Glu (GAA) in ParC and of Asp-435 (GAC) to Asn (AAC) in GyrB. Interestingly, strains 3C1 to 3C3 and 3C5, for which the CIP MICs were

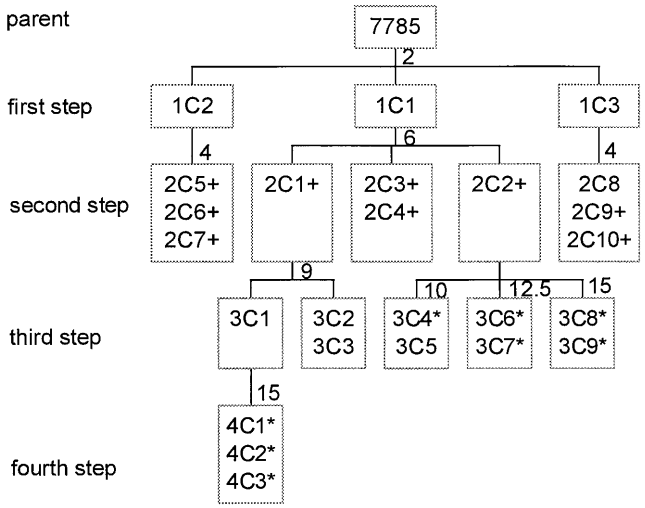

FIG. 3. Flow chart showing the relationships among *S. pneumoniae* 7785 and its quinolone-resistant mutants 1C1 to 4C3, which were selected by stepwise exposure to CIP. First-, second-, third-, and fourth-step mutants are designated by the prefixs 1, 2, 3, and 4. The numbers outside the boxes show the concentrations of CIP (in micrograms per milliliter) used in each selection step. Plus signs and asterisks denote the first appearance of mutations in *parC* and *gyrA*, respectively (see Table 1).

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TABLE 1. Frequencies of CIP-resistant mutants obtained from *S. pneumoniae* 7785 and its derivatives

Strain	CIP MIC (μg/ml)	CIP concn (μg/ml) for selection	Mutant frequency
7785	1	2	5.8×10^{-8}
1C1	3	6	3.8×10^{-8}
2C2	8	15	9.0×10^{-7}
3C1	10	15	8.5×10^{-8}

much lower, did not carry *gyrA* mutations. It is noteworthy that the drug concentration used to select 3C1 to 3C3 (i.e., CIP at 9 μg/ml) was only marginally higher than the CIP MIC for parent strain 2C1. It is significant that further selection using 3C1 and CIP at 15 μg/ml generated mutants 4C1 to 4C3 displaying high-level resistance and each had acquired a Ser-83-to-Tyr alteration in GyrA (Table 2). Thus, mutations in GyrA occurred after those in ParC and are associated with high-level resistance to CIP.

***parC* and *gyrA* mutations in sequential CIP-resistant *S. pneumoniae* isolates.** Rapid development of clinical resistance has been reported during CIP therapy of pneumococcal infections. Therefore, we investigated a number of resistant clinical *S. pneumoniae* isolates (Table 2). Of particular interest were three strains, D5, B10, and D11, which were sequential isolates obtained from the same patient before, during, and after CIP treatment for chronic obstructive airway disease. D5, the pretherapy strain, was susceptible to CIP, with an MIC typical for *S. pneumoniae* of 1 μg/ml. B10 and D11 exhibited low-level and high-level CIP resistance, respectively. PCR and DNA sequence analysis were used to amplify and characterize the QRDRs of genes *parC* and *gyrA* in these and other isolates (Table 2).

The *parC* nucleotide sequences of susceptible strains D5 and 7785 were identical, except for two differences: a silent change at codon 128 (GGG in D5 and GGC in 7785) and a Lys-137-to-Asn change (AAT for D5 and AAG for 7785). *parC* PCR products for strains D5, B10, and D11 were identical, except for an additional nucleotide change detected in B10 and D11: a codon 79 TCT-to-TTT change leading to a Ser-to-Phe substitution at the protein level. The sequences of the B10 and D5 *gyrA* PCR products were identical. However, D11 *gyrA* encoded a Glu→Lys substitution at the position equivalent to Asp-87 in *E. coli* GyrA. These ParC and GyrA changes involve mutations known to confer resistance in other species. Thus, in the patient, low-level resistance was associated with mutation of *S. pneumoniae parC*: development of high-level resistance involved subsequent mutation of *gyrA*. No changes were detected in *gyrB* or *parE* (Table 2).

Several other *S. pneumoniae* clinical isolates exhibiting low-level CIP resistance were examined. Isolate E4, for which the CIP MIC was 4 μg/ml, i.e., similar to that for B10, also carried a ParC mutation, the novel codon 95 CGT-to-TGT mutation replacing a conserved Arg residue with Cys (Table 2). As with B10, no change was detected in *gyrA*. Finally, *Hin*fI RFLP analysis was used to examine the *parC* PCR products from six *S. pneumoniae* isolates (A4, A6, F7, F8, B12, and H4), for which the CIP MIC is 2 μg/ml, i.e., slightly elevated over the 0.5- to 1-μg/ml MIC seen for susceptible isolates. Similar to low-level laboratory mutants 1C1 to 1C3, no changes in *parC* were detected (results not shown). Thus, overall, results for the clinical isolates parallel those obtained with laboratory mutants.

DISCUSSION

We have characterized the QRDRs of the *parC*, *gyrA*, *parE*, and *gyrB* genes in *S. pneumoniae* mutants selected stepwise for resistance to CIP. No mutations were detected in the topoisomerase genes of first-step mutants displaying low-level resistance. *parC* mutations occurred in the second step of selection and preceded those in *gyrA* (and *gyrB*) found in third- or fourth-step mutants exhibiting high-level resistance. Statistical bias is unlikely to account for this specific pattern of mutations: it was seen reproducibly in several independent drug challenges and also in sequential *S. pneumoniae* clinical isolates (Table 2 and Fig. 3). The selection of *parC* mutations before those in *gyrA* suggests that topoisomerase IV is a primary target for CIP in *S. pneumoniae.*

We do not know the identity of the first-step mutation(s), but three alternative explanations can be considered. First, it is conceivable that the initial mutation arose elsewhere in *parC* or *parE* or could affect another topoisomerase. More likely, it

TABLE 2. Mutations identified in the QRDRs of topoisomerase IV and gyrase proteins in CIP-resistant *S. pneumoniae* strains and clinical isolates[a]

Strain	CIP MIC (μg/ml)	Mutation(s) in QRDR of:			
		ParC	GyrA	GyrB	ParE
7785	1				
1C1	3[b]	None	None	None	None
1C2	3[b]	None	None		
1C3	3[b]	None	None		
2C1	8	Ser-79→Tyr	None	None	None
2C2	8	Ser-79→Tyr	None	None	None
2C3	8	Ser-79→Tyr	None		
2C4	8	Ser-79→Tyr	None		
2C5	8	Ser-79→Tyr	None	None	None
2C6	8	Ser-79→Tyr	None	None	None
2C7	8	Ser-79→Phe	None	None	None
2C8	8	None	None	None	None
2C9	16	Ser-79→Tyr	None	None	None
2C10	8	Ala-84→Thr	None	None	None
3C1	8–10	Ser-79→Tyr	None	None	None
3C2	8–10	Ser-79→Tyr	None		
3C3	8–10	Ser-79→Tyr	None		
3C4	64	Ser-79→Tyr	Ser-83*→Tyr	None	
3C5	16	Ser-79→Tyr	None	None	None
3C6	64	Ser-79→Tyr	Glu-87*→Lys	None	
3C7	64	Ser-79→Tyr	Ser-83*→Tyr	None	
3C8	64	Ser-79→Tyr, Lys-93→Glu	Ser-83*→Tyr	Asp435→Asn	
3C9	64	Ser-79→Tyr	Ser-83*→Tyr		
4C1	100[b]	Ser-79→Tyr	Ser-83*→Tyr	None	None
4C2	100[b]	Ser-79→Tyr	Ser-83*→Tyr		
4C3	100[b]	Ser-79→Tyr	Ser-83*→Tyr		
D5	1	None	None	None	None
B10	4	Ser-79→Phe	None	None	None
D11	64	Ser-79→Phe	Glu-87*→Lys	None	None
E4	4	Arg-95→Cys	None		

[a] Laboratory strains 1C1 to 4C3 were derived from parent wild-type strain 7785 by stepwise selection. D5, B10, and D11 were sequential clinical isolates from the same patient. E4 was isolated from a different patient. MICs were determined in brain heart infusion medium containing 5% horse blood. Asterisks identify *S. pneumoniae* GyrA residues by analogy with the equivalent residue in *E. coli* GyrA.

[b] From reference 26.

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could involve altered drug permeation, perhaps through a homolog of *norA*, an efflux transporter for hydrophilic quinolones described in *S. aureus* (31, 36). NorA is not particularly effective against hydrophobic quinolones consistent with the susceptibility of first-step mutants to sparfloxacin (MIC, 0.4 μg/ml). A third possibility is that *S. pneumoniae* has a homolog of the multiple antibiotic resistance (*mar*) operon found in *E. coli*, whose induction (through mutation) confers pleiotropic effects, including low-level quinolone resistance, and is known to protect against the rapid bactericidal effects of fluoroquinolones (13). The presence of a *mar* mutation in *E. coli* has been shown to increase the selection frequency of subsequent highly quinolone-resistant mutants by a factor of 10^3 (13). Conceivably, the presence of *mar* mutations in first-step *S. pneumoniae* mutants could allow the multistep development of high-level resistance involving *parC* and *gyrA* changes. However, the similar mutation frequencies we observed at each stage of stepwise selection in *S. pneumoniae* appears to argue against *mar* involvement (Table 1). These various possibilities are under investigation.

In terms of the QRDR status of topoisomerase IV and gyrase examined in both laboratory and clinical isolates, Table 2 represents the most complete analysis yet reported for a gram-positive organism. Previous studies have focused predominantly on *E. coli*, in which resistance most often involves mutation of the two highly conserved GyrA residues Ser-83 and Asp-87 with less frequent changes at other positions in the QRDR (Fig. 2). Mutations of the equivalent Ser-80 and Glu-84 residues have been seen in the *E. coli* ParC QRDR. Mutation of *E. coli* GyrB has been reported at conserved residues Asp-426 and Arg-447: relatively few changes in ParE have been documented. The equivalent ParC, GyrA, and GyrB residues also undergo substitution in the *S. pneumoniae* mutants (Table 2 and Fig. 2). Identification of these mutations with resistance is in accord with the usual inference that the stepwise selection procedure is likely to generate single-step mutations. However, scrutiny of the data for some mutants in Table 2 indicates that this inference need not always hold. For example, strain 3C4 contains topoisomerase mutations identical to those of strains 4C1 to 4C3 but the respective CIP MICs are different, indicating the presence of additional undetected mutations in strains 4C1 to 4C3. Similarly, strain 3C8, isolated from parent strain 2C2 by the single-step plating procedure (Fig. 2), had acquired three new mutations (Table 2): at Ser-83 of GyrA and Asp-435 of GyrB, conserved positions whose mutation gives rise to resistance in other species, and at Lys-93 in ParC. Despite these peculiarities and the need of genetic proof, where there are close parallels with known resistance mutations, the data strongly suggest that the identified ParC, GyrA, and GyrB mutations are responsible for resistance in *S. pneumoniae*. Moreover, the results indicate that there is close structural similarity in the CIP-binding pockets among gyrases, among topoisomerases IV, and between gyrases and topoisomerases IV.

The exact structural role of the conserved residues in ParC, GyrA, and GyrB that are mutated in resistant strains is unknown. In the case of *E. coli* DNA gyrase, we have suggested that there are two quinolone-binding pockets and that some resistance mutations, e.g., Ser-83 to Trp, may act by reducing drug binding (4, 10). Recent studies have confirmed this idea for mutant gyrase complexes reconstituted with Trp-83 or Leu-83 in GyrA (32, 38). In principle, reduced drug binding could result from loss of critical drug-enzyme contacts, from steric interference, or by long-range conformational changes that alter the drug-binding site. Although there is no structural information on the QRDRs of topoisomerase IV and gyrase, the equivalent regions are present in a recent crystal structure of yeast topoisomerase II (3). Topoisomerase II residues equivalent to resistance hot spots in the ParC and GyrA QRDRs lie in or adjacent to an α helix (A′α4) that is part of a CAP-like structure thought to interact directly with DNA. The GyrB EGDSA and PLRGK motifs found to be mutated in resistant strains are directly conserved in topoisomerase II. They are present in loops that interact with each other in topoisomerase II. However, these sequences appear to be spatially removed from the DNA-binding cleft. Clearly, the structure of a ternary drug-topoisomerase-DNA complex is required to understand the relationship between the QRDRs and quinolone-binding pockets.

Although the overwhelming majority of *S. pneumoniae* laboratory strains and clinical isolates exhibiting moderate or high-level resistance had GyrA and/or ParC mutations, alteration of topoisomerase QRDRs was not obligatory for the resistance phenotype. We note that one *S. pneumoniae* clone, 2C8, was unique among 10 second-step mutants in not carrying a mutation in any of the ParC, GyrA, GyrB, or ParE QRDRs (Table 2). Thus, although ParC mutations appear to be the predominant route to high-level CIP resistance in *S. pneumoniae*, clone 2C8 (like first-step mutants 1C1 to 1C3) suggests that other novel mechanisms remain to be uncovered. Interestingly, stepwise development of CIP resistance in *S. aureus* has been reported to involve mutations affecting *parC*, then permeation, then *gyrA* (6). However, it is not clear whether permeation changes must necessarily precede alterations in *gyrA* of *S. aureus.*

Finally, the presence of at least two quinolone targets in bacteria has interesting implications. The primacy of gyrase as the quinolone target in *E. coli* has been attributed to two factors: greater intrinsic sensitivity of gyrase over topoisomerase IV and gyrase inhibition being more lethal than topoisomerase IV inhibition (19). The greater lethality of gyrase inhibition is ascribed to gyrase acting ahead of the replication fork, whereas topoisomerase IV is presumed to act behind the fork, giving scope for repair of drug-promoted DNA lesions. We do not know whether greater enzyme sensitivity or greater lethality is the factor operating in *S. pneumoniae* to favor topoisomerase IV as the primary CIP target. Were the respective enzyme affinities for the drug to be a key determinant, it follows that preference for one enzyme or the other would depend on the molecular structure of the drug. Therefore, it may be simplistic and misleading to generalize the target preference of a particular congener to all fluoroquinolone derivatives. For chemotherapy, a drug displaying similar affinities for topoisomerase IV and gyrase may be especially desirable. Development of resistance would require simultaneous alteration of two targets, a genetically rare event. This approach could minimize the emergence of clinical resistance. The studies reported here establish *S. pneumoniae* as a well-characterized gram-positive system with which to explore these issues.

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