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ParC Subunit of DNA Topoisomerase IV of *Streptococcus pneumoniae* Is a Primary Target of Fluoroquinolones and Cooperates with DNA Gyrase A Subunit in Forming Resistance Phenotype

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The genes encoding the ParC and ParE subunits of topoisomerase IV of *Streptococcus pneumoniae*, together with the region encoding amino acids 46 to 172 (residue numbers are as in *Escherichia coli*) of the pneumococcal GyrA subunit, were partially characterized. The *gyrA* gene maps to a physical location distant from the *gyrB* and *parC* loci on the chromosome, whereas *parC* is closely linked to *parE*. Ciprofloxacin-resistant (Cp^r) clinical isolates of *S. pneumoniae* had mutations affecting amino acid residues of the quinolone resistance-determining region of ParC (low-level Cp^r) or in both quinolone resistance-determining regions of ParC and GyrA (high-level Cp^r). Mutations were found in residue positions equivalent to the serine at position 83 and the aspartic acid at position 87 of the *E. coli* GyrA subunit. Transformation experiments suggest that ParC is the primary target of ciprofloxacin. Mutation in *parC* appears to be a prerequisite before mutations in *gyrA* can influence resistance levels.

Fluoroquinolones are a relatively new class of potent, broad-spectrum antimicrobial agents. The principal targets of the fluoroquinolones are DNA gyrase and topoisomerase IV, members of the topoisomerase family of enzymes that control bacterial DNA topology. Both enzymes function by passing a DNA double helix through another, using a transient double-strand break (8). DNA gyrase is an essential bacterial enzyme that consists of two A and two B subunits, which are encoded by the *gyrA* and *gyrB* genes, respectively. It catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (19). Topoisomerase IV, recently described in *Escherichia coli* (7, 11), is encoded by two closely linked genes, *parC* and *parE*, and it is believed that this enzyme plays an essential role in partitioning replicated chromosomes (8). The deduced amino acid sequences of ParC and ParE are homologous to those of GyrA and GyrB, respectively (7). Bacterial resistance to quinolones can arise through mutations in DNA gyrase; single point mutations in any subunit have been shown to play a role in quinolone resistance (for a review, see reference 12). However, in *E. coli*, high-level resistance mutations map primarily to the quinolone resistance-determining region (QRDR); the QRDR spans residues 67 to 106 of the GyrA sequence (23) and is where sequence similarities between GyrA and ParC are the highest. Recent studies have identified similar mutations in the analogous region of ParC of *Neisseria gonorrhoeae* (1) and *Staphylococcus aureus* (4). In *N. gonorrhoeae*, GyrA was identified as the primary target of ciprofloxacin because amino acid changes in ParC were only observed with the simultaneous presence of one or more resistance mutations in *gyrA*. Interestingly, the opposite was observed in the gram-positive bacterium *S. aureus.*

Streptococcus pneumoniae is one of the main human bacterial pathogens. Penicillin-resistant strains, which are often multiply resistant, are becoming widespread (18). Pneumococci show a relatively high level of resistance to most quinolones (21), and these drugs are generally less active against gram-positive bacteria (including *S. pneumoniae*), whereas coumarins, which inhibit the GyrB subunit, are less active against gram-negative bacteria. However, it is not clear whether these differences in drug susceptibility are due to drug accessibility or to the structures of their DNA topoisomerase type II enzymes, or both. The characterization of the genes responsible for the synthesis of these enzymes will help to resolve this issue and will help investigators develop new antibiotics active against *S. pneumoniae*. We have recently characterized mutations in the pneumococcal *gyrB* gene responsible for novobiocin resistance (10). In this report we describe the characterization of the *parE*, *parC*, and *gyrA* genes of *S. pneumoniae* in relation to the formation of the fluoroquinolone resistance phenotype. Sequence analysis and transformation experiments indicated that ParC is the primary target of ciprofloxacin in pneumococcus and that it cooperates with GyrA mutants to increase the resistance level.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strain used for plasmid transformation was DH5α (6). Strain Y1090r$^-$ was used for recombinant phage λ propagation. The ciprofloxacin-susceptible (Cp^s) strains of *S. pneumoniae* used were wild-type strain R6, M11 (like R6, but *hex-4 end-1 exo-2 ery*), and M22 (13) and three clinical isolates (937, 5145, and 3073). The ciprofloxacin-resistant (Cp^r) clinical isolates were obtained from sputum samples. The plasmid used for cloning was pUC18 (22).

Growth and transformation of bacteria. *E. coli* was grown in Luria-Bertani (LB) medium (14), and pneumococci were grown in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco). Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood were used for ciprofloxacin susceptibility tests. *S. pneumoniae* was also grown in liquid C medium containing 0.08% yeast extract, and transformation was performed as described by Tomasz (17). Transformants of *S. pneumoniae* were selected in Mueller-Hinton agar plates containing ciprofloxacin (Bayer) at different concentrations and were incubated at 37°C in a 5% CO_2 atmosphere. Transformants of *E. coli* were selected in LB medium containing ampicillin at 100 μg/ml.

DNA isolation and manipulation. *S. pneumoniae* chromosomal DNA was obtained as described previously (3). Plasmids were prepared from *E. coli* by the

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alkaline lysis method or by equilibrium centrifugation in CsCl-ethidium bromide gradients (14). Restriction endonucleases, DNA ligase, T4, and Klenow fragment DNA polymerases were obtained commercially and were used as specified by the suppliers. Gel electrophoresis of plasmids, restriction fragments, and PCR products was carried out in agarose gels as described previously (14). DNA was recovered from gel slices with the GeneClean II Kit (Bio 101). Unidirectional DNA deletions were generated by *Exo*III and S1 nucleases by using the Double-Stranded Nested Deletion Kit (Pharmacia). A commercial library of *S. pneumoniae* DNA in λgt11 (Clontech) was used for screening with the gyrA46-172 probe that was radiolabelled with 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) by using the Multiprime DNA Labelling System (Amersham). That library has been constructed by shearing mechanically the genomic DNA of type 2 capsule strain D39 (the parental strain of R6), the addition of *Eco*RI linkers, and ligation to λgt11 digested with the same endonuclease. Isolation of recombinant phages and extraction of their DNAs were performed as described previously (14).

PCR amplification and DNA sequence determination and analysis. The oligonucleotide primers used for PCR amplification and for sequencing the genes were synthesized in a Pharmacia LKB Gene Assembler Plus DNA synthesizer. PCR amplifications were performed with a GeneAmp kit (Perkin-Elmer Cetus) with 1 μg of target DNA and 1 μM (each) synthetic oligonucleotide primer and were carried out as described elsewhere (3). For the amplification of the gyrA46-172 fragment, the following synthetic oligonucleotide primers were used: gyrA46 [5′-gcgctctaGA(C/T)GGT(C/T)TNAAACCNGTNCA-3′], coding for DGL KPVH, and gyrA172 (5′-gcgcaagcTTTGTAGCCATACCNACNGCAATNCC-3), the complementary strand of the primer coding for GIAVGMAT. For the amplification of the C terminus of *gyrB*, the following primers were used: gyrB376 (5′-cgcgtctagaTTGCCAAACGTATCGTAGA-3′), coding for IAKRIV, and gyrB512 (5′-cgcgaagctTGGGCTCCATCGACATCGGC-3′), the complementary strand of the primer coding for AGDVA. For the amplification of parC50-152, the following primers were used: parC50 (5′-cgcgaagcttGTTGGTTCTTTC TCCGTATCG-3′), coding for PEKETD, and parC152 (5′-gcgctctagAAGGAT AGCAATACTTTT-3′), the complementary strand of the primer coding for KDSNTF. The 5′ ends of the primers contained sequences including either an *Xba*I (gyrA46, gyrB376, and parC152) or *Hin*dIII (gyrA172, gyrB512, and parC50) restriction sites (lowercase letters). For the amplification of the C terminus of *parC* from λA3 DNA, the following oligonucleotides were used: lambda2 (5′-GGTGGCGACGACTCCTGGAGCCCGTCAGTA-3′), from the λgt11 DNA sequence, and parC448 (5′-GCTGGCGGCTATTATCGG-3′), coding for LAAIIG. DNA sequencing was carried out by using the protocols and materials from the Sequenase system (U.S. Biochemicals) or the *fmol* DNA Sequencing System (Promega). For all sequences presented in this report, the sequences of both strands of the DNA were determined. DNA and protein sequence comparisons were done by using Intelligenetics PC Gene software, version 6.0.

Pulsed-field gel electrophoresis of chromosomal DNA. DNA embedded in agarose plugs was prepared from *S. pneumoniae* R6 as described previously (5). Gels were cast and run as described previously (3). Labelling of specific DNA was carried out with the PolarPlex Chemiluminescent Blotting Kit (Millipore). Southern blotting and hybridization were done according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The DNA sequences corresponding to the *parE-parC* genes and to the partial *gyrA* sequence have been assigned GenBank accession numbers X95717 and X95718, respectively.

RESULTS

Cloning and sequencing the *gyrA* QRDR of *S. pneumoniae*. The *gyrA* gene was identified by an approach successfully used for the isolation of *gyrA* homologs from other bacterial species (1, 4). Degenerate oligonucleotide primers were designed on the basis of the sequence conservation found among known *gyrA* genes corresponding to regions 39 to 45 and 173 to 180 of the amino acid sequence of *E. coli gyrA*. Amplification with DNA from the *S. pneumoniae* wild-type strain R6 resulted in the production of a PCR product of 444 bp, designated gyrA46-172. This product was isolated from agarose gels, digested with *Xba*I and *Hin*dIII (targets included in the primers), and cloned by using pUC18 previously digested with the same endonucleases. The nucleotide sequences of six inserts from different recombinant plasmids were identical and should encode 127 amino acid residues (excluding those encoded by the primers), corresponding to positions 46 to 172 of the *E. coli* GyrA subunit, which includes the QRDR (see above). This amino acid sequence showed homology with those of other previously reported GyrA proteins (Fig. 1). Subsequently, DNAs from clinical isolates of *S. pneumoniae*, either Cp^s or

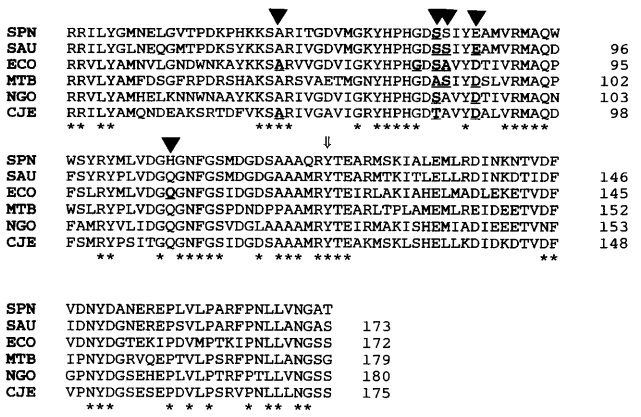

FIG. 1. Comparison of the amino acid sequences of a region of DNA gyrase A containing the QRDR from *S. pneumoniae* (SPN), *S. aureus* (SAU) (9), *E. coli* (ECO) (15), *Mycobacterium tuberculosis* (MTB) (16), *N. gonorrhoeae* (NGO) (1), and *Campylobacter jejuni* (CJE) (20). The clustal program from PC Gene, version 6.60, was used to compare the predicted sequences. The residues involved in quinolone resistance (▼) are set in boldface type and underlined; double-tailed arrow, active residue which links to DNA; asterisks, amino acid identity.

Cp^r, were amplified as described above, and the nucleotide sequence of the corresponding *gyrA* region was determined directly from the PCR products. All except two clinical strains had a sequence identical to that of the wild-type strain R6 or had silent nucleotide changes; two strains had high-level ciprofloxacin resistance (MICs, ≥64 μg/ml), and point mutations were observed in the sequences of the strains (Table 1). Mutations in these two strains would result in S to Y (strain 517) or S to F (strain 1244) changes (Table 1) in a residue position equivalent to the S at position 83 (S-83) of *E. coli* GyrA (Fig. 1). Transformation experiments were performed by using competent cells of Cp^s pneumococcal strains M22 and M11 as recipients and both chromosomal DNAs and the gyrA46-172 PCR products from strains 517 and 1244 as donor DNAs. Only chromosomal DNAs were able to transform the strains to Cp^r (see below). Unexpectedly, no Cp^r transformants were observed when the selection was carried out at concentrations of ciprofloxacin ranging from 1 to 16 μg/ml. To discard the possibility of a very low efficiency of transformation because of the small size of the PCR fragments, these were cloned into pUC18, and again, no transformation to Cp^r was observed.

Since it has been described that mutations in the C terminus of GyrB would contribute to quinolone resistance (12), the region encoding residues 376 to 512 of *S. pneumoniae* GyrB (10) was amplified with specific oligonucleotides, and subsequently, the PCR products were sequenced with the same oligonucleotides. As indicated in Table 1, only silent nucleotide changes were found. These results suggest that other regions of *gyrA* or *gyrB* (different from the ones sequenced here) would be involved in ciprofloxacin resistance in pneumococci. Alternatively, other genes could also be involved.

Cloning and sequencing *parE* and *parC* genes of *S. pneumoniae*. We approached the cloning of the complete *gyrA* gene by using the amplified gyrA46-172 DNA as a probe to screen a λgt11 library of *S. pneumoniae* DNA, and several recombinant phages were identified (Fig. 2). The inserts of these phages were subcloned into plasmids and were sequenced with synthetic oligonucleotides. We found two large open reading frames (ORFs). One putative promoter for transcription was detectable, with its corresponding ribosome-binding site being upstream of the second ORF (Fig. 3). Amino acid sequence comparisons showed that the first ORF was homologous to

TABLE 1. *S. pneumoniae* clinical strains and transformants investigated in the study[a]

Strain	Ciprofloxacin MIC (μg/ml)	*gyrA* mutation		*parC* mutation		*gyrB* mutation	
		Nucleotide change	Amino acid change	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change
937	1	C-231-T	No	ND[b]	ND	ND	ND
		C-333-T	No	ND	ND	ND	ND
5145	1	C-231-T	No	ND	ND	ND	ND
3073	2	C-231-T	No	G-411-T	K-137-N	No	No
		C-333-T	No				
13925	8	No	No	G-247-T	D-83-Y	A-1152-G	No
				C-384-T	No	G-1158-A	No
				G-411-T	K-137-N		
3429	8	C-231-T	No	C-236-A	S-79-Y	T-1416-C	No
3305	8	C-231-T	No	C-236-A	S-79-Y	No	No
				G-411-T	K-137-N	No	No
517	64	C-231-T	No	C-236-T	S-79-F	C-1356-T	No
		C-248-A	S-83-Y			C-1383-T	No
						G-1416-A	No
1244	128	C-231-T	No	C-236-T	S-79-F	A-1152-G	No
		C-248-T	S-83-F	C-384-T	No	G-1158-A	No
				G-411-T	K-137-N		
$R1^{13925\text{-}C}$	4	No	No	G-247-T	D-83-Y	ND	ND
$R1^{3429\text{-}C}$	4	No	No	C-236-A	S-79-Y	ND	ND
$R2^{13925\text{-}C/517\text{-}A}$	16	C-231-T	No	G-247-T	D-83-4	ND	ND
		C-248-A	S-83-Y				
$R2^{3429\text{-}C/517\text{-}A}$	32	C-231-T	No	C-236-A	S-79-Y	ND	ND
		C-248-A	S-83-Y				
$R2^{1244\text{-genomic}}$	32	C-231-T	No	C-236-T	S-79-F	ND	ND
		C-248-T	S-83-F	C-384-T	No	ND	ND
				G-411-T	K-137-N	ND	ND

[a] The nucleotide positions indicated for *gyrA* refer to the corresponding positions in the *E. coli gyrA* sequence. Nucleotide positions for *parC* and *gyrB* are numbered by taking the first nucleotide as nucleotide 1. $R1^{13925\text{-}C}$ and $R1^{3429\text{-}C}$ is the nomenclature for transformants of M22 to the first level of ciprofloxacin resistance obtained with the parC50-152 PCR products of strains 13925 and 3429, respectively. $R2^{13925\text{-}C/517\text{-}A}$, $R2^{517\text{-}C/1244\text{-}A}$, and $R2^{3429\text{-}C/517\text{-}A}$ are transformants to the second level of ciprofloxacin resistance obtained from $R1^{13925\text{-}C}$ and $R1^{3429\text{-}C}$ by using the gyrA46-172 PCR products of strains 517, 1244, and 517, respectively. $R2^{1244\text{-genomic}}$ is a transformant obtained from M22 with chromosomal DNA from the 1244 strain.

[b] ND, not determined.

ParE (Fig. 4) and that the second ORF was homologous to ParC (Fig. 5). These allowed us to name these genes *parE* and *parC*, respectively. The region homologous between the gyrA46-172 probe and *parC* is presented in Fig. 3. The 65.5% identity at the nucleotide level could account for the nonspecific hybridization between the *gyrA* probe and the 780-bp *Eco*RI fragment included in *parC* (Fig. 2). On the other hand, ParE of *S. pneumoniae* showed a higher degree of identity with ParE of *S. aureus* (68.4%) than with GyrB of *S. pneumoniae* (47.5%) (Fig. 4).

Location of *gyrA* and *parC* genes in the *S. pneumoniae* genome. Chromosomal DNA prepared from strain R6 was digested with different restriction endonucleases and was subjected to pulsed-field gel electrophoresis, and the resulting fragments were blotted and hybridized with probes specific for *parC*, *gyrA*, and *gyrB*: parC449-620 (Fig. 3), gyrA46-172 (Fig. 1) (amino acid numbers are as in *E. coli*), and gyrB10-330 (Fig. 4), respectively. Both *gyrB* and *parC* probes detected a *Sma*I fragment of 380 kb, an *Apa*I fragment of 330 kb, and a *Sac*II fragment of 160 kb (Fig. 6), corresponding to fragment numbers 1/2, 1, and 5, respectively, of the *S. pneumoniae* genome (5). However, when *gyrA* was used as a probe, a 340-kb *Sma*I fragment, a 330-kb *Apa*I fragment, and a 310-kb *Sac*II fragment were detected, corresponding to fragment numbers 2, 1, and 1, respectively (Fig. 6). These results indicate that *gyrA* is located (at least 90 kb apart) in a region different from the region where *gyrB* and *parC* are located, according to the published *S. pneumoniae* R6 chromosomal map.

Characterization of ciprofloxacin-resistant isolates of *S. pneumoniae*. To learn if the Cp^r pneumococcal isolates had

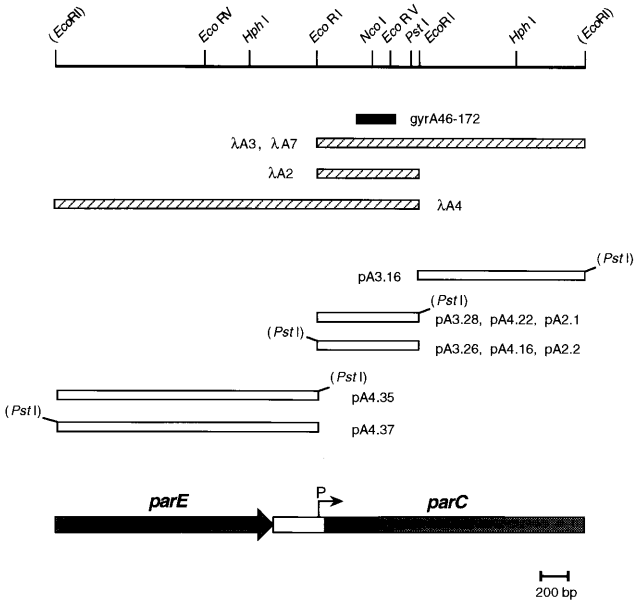

FIG. 2. Restriction map of *S. pneumoniae* genomic DNA region containing the *parE* and *parC* genes. The inserts of λA3, λA2, and λA7 (hatched bars) and the gyrA46-172 fragment used as a probe (black bars) are indicated. Inserts subcloned in pUC18 are indicated by white bars, together with the location of the pUC18 *Pst*I target. The *Eco*RI targets in parentheses are derived from the cloning strategy and do not represent real targets in the pneumococcal DNA.

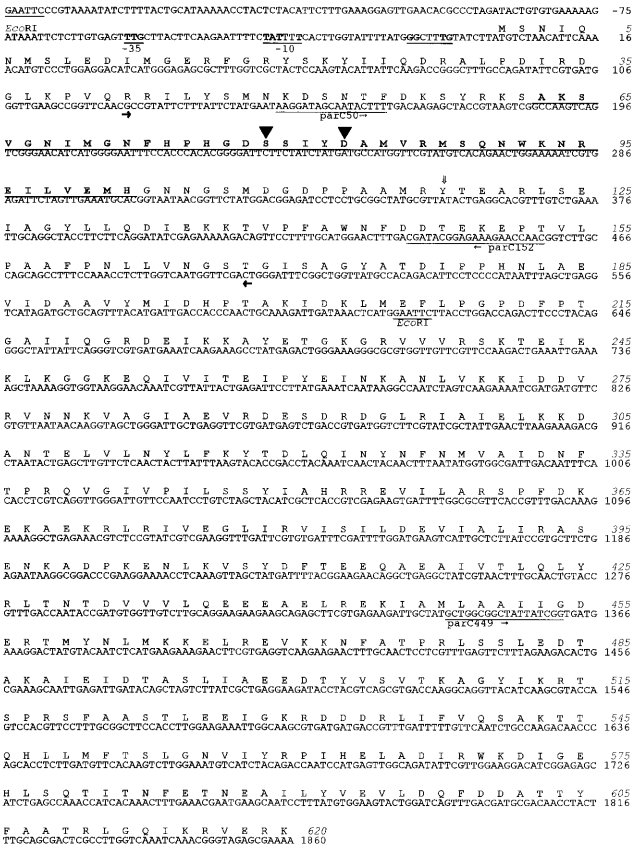

FIG. 3. Nucleotide sequence of a 1,860-bp fragment which contains most of the *parC* gene. The strand corresponding to the mRNA sequence is shown. Nucleotides and amino acids (*italics*) are numbered by taking the first *parC* nucleotide as nucleotide 1 and the first ParC residue as number 1. Putative consensus regulatory elements are underlined and set in boldface type. Oligonucleotides and *Eco*RI sites are indicated and underlined. The amino acid residues that constitute the QRDR are underlined and set in boldface type. The nucleotide sequence between the two black arrows corresponded to the region homologous to the gyrA46-172 probe. The residues involved in ciprofloxacin resistance (▼) and the active Y-118 site which links DNA (double-tailed arrow) are indicated.

FIG. 4. Comparison of ParE sequences of *S. pneumoniae* (SPNPARE) and *S. aureus* (SAUPARE) (4) with DNA gyrase B from *S. pneumoniae* (SPNGYRB) (10). Identical amino acids are indicated by asterisks. Dashes indicate gaps introduced to maximize similarities.

mutations in the *parC* gene, as has been described in other bacteria, PCR fragments spanning amino acids 50 to 152 of ParC (designated parC50-152) were obtained after amplification with oligonucleotides parC50 and parC152 (Fig. 3), and the region encoding residues 56 to 146 was sequenced with these same oligonucleotides. Analysis of the nucleotide sequences (Table 1) indicated that some Cp^s and Cp^r clinical isolates had a nucleotide change from a G to a T at position 411 (G-411-T) that would produce a K-137-N amino acid change. The importance of this mutation will be discussed later. All Cp^r strains (MICs, ≥8 μg/ml) had mutations altering amino acids S-79 or D-83, whereas susceptible strains (MICs, ≤2 μg/ml) did not. Most Cp^r strains have alterations at S-79, a position analogous to S-83 of GyrA. The nature of the changes affecting these two S residues were equivalent: S-to-Y or S-to-F amino acid changes.

Genetic exchange of ciprofloxacin resistance by genetic transformation. Direct evidence indicating that the *parC* and *gyrA* mutations are responsible for ciprofloxacin resistance was obtained by genetic transformation. Both chromosomal DNAs and the gyrA46-172 and parC50-152 PCR products were used as donor DNAs. Only chromosomal DNAs and the *parC* PCR fragments from Cp^r strains were able to transform the competent strain *S. pneumoniae* M22 (Cp^s; MIC, 0.50 μg/ml). The frequencies of transformation to Cp^r achieved with both DNAs (1×10^5 to 5×10^5 transformants per ml) were similar when the selection was done at 0.55 μg/ml. However, when selection was performed at 8 μg/ml, only chromosomal DNAs were able to transform, although at frequencies of 2×10^3 to 3×10^3 transformants per ml, which are about 100 times lower. This latter frequency is consistent with transformation by two unlinked markers. One of these transformants, $R^{1244\text{-genomic}}$ (Table 1) was shown to have all the nucleotide changes that were detected in *parC* (three changes) and *gyrA* (two changes) of donor strain 1244 (Table 1). Transformants obtained with parC50-152 from Cp^r isolates 13925 and 3429 increased the ciprofloxacin MIC; for recipient strain M22 the ciprofloxacin MIC increased to 4 μg/ml. The sequences of the *parC* and *gyrA* regions of two of these transformants, $R1^{13925\text{-C}}$ and $R1^{3429\text{-C}}$ (Table 1), showed some of the *parC* nucleotide changes present in the donor strains. Transformant $R1^{13925\text{-C}}$ had the G-247-T transversion that produced the D-83-Y change, but not the other two nucleotide changes present in donor strain 13925: neither the C-384-T change that would not produce any amino acid change nor the G-411-T change that would produce the K-137-N change (Table 1). This result identified the D-83-Y change as being responsible for Cp^r in strain 13925. Moreover, transformation with DNA from strain 3073 containing a ParC K-137-N change did not render any Cp^r transfor-

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SPNPARC     MSNI-QNMSLEDIMGERFGRYSKYIIQDRALPDIRDGLKPVQRRILYSMN    49
SAUPARC     **EIIQDLS*EDVLGDRFGR**K*I*QE****DVR**********L*A*Y    50
ECOPARC     **DMAERLA*HEFTENAYLN**M*V*MD****FIG**********V*A*S    50

SPNPARC     KDSNTFDKSYRKSAKSVGNIMGNFHPHGDSSIYDAMVRMSQNWKNREILV    99
SAUPARC     SSGNTHDKNFR***KT**DVI*QY******SV*E***RLS*DWKL*HV*I   100
ECOPARC     ELGLNASAKFK***RT**DVL*KY******AC*E***LMA*PFSY*YP*V   100

SPNPARC     EMHGNNGSMDGDPP-AAMRYTEARLSEIAGYLLQDIEKKTVPFAWNF-DD   147
SAUPARC     EMH**N*SI*NDPP-*******AK**LLAEE**RDINKE*VSFIP*Y-*D   148
ECOPARC     DGQ**W*AP*DPKSF*******SR**KYSEL**SELGQG*ADWVP*FF*G   150

SPNPARC     TEKEPTVLPAAFPNLLVNGSTGISAGYATDIPPHNLAEVIDAAVYMIDHP   197
SAUPARC     *TL**MV**SRF**L*V**S***SA*Y**********A**IQ*TLKY**N*   198
ECOPARC     *LQ**KM**ARL**I*L**T***AV*M*********R**AQ*AIAL**Q*   200

SPNPARC     TAKIDKLMEFLPGPDFPTGA-IIQGRDEIKKAYETGKGRVVVRSKTEIEK   246
SAUPARC     DITVNQ*MKYIK***F**GG-**QGIDG*K*A**S*K*RIIV*SKVEEET   247
ECOPARC     KTTLDQ*LDIVQ***Y**EAE**TSRAE*R*I**N*R*SVRM*A-----V   245

SPNPARC     LKGGKEQIVITEIPYEINKANLVKKIDDVRVNNKVAGIAEVRDESDRDG-   295
SAUPARC     LRNGRKQLI*TEI*YEVNKGSLVKR*DELRADK*VDGIVEV***T*RTG-   296
ECOPARC     WKKEDGAVV*SAL*HQVSGARVLEQ*AAQMRNK*LPMVDDL***S*HENP   295

SPNPARC     LRIAIELKKD-ANTELVLNYLFKYTDLQINYNFNMVAID-NFTPRQVGIV   343
SAUPARC     L*IA*ELKKD-VNSESIK*Y*YKNS**QIS*NF*MVA*S-DGR*KLMGIR   344
ECOPARC     T*LV*VPRSNRVDMDQVM*H*FATT**EKS*RI*LNM*GLDGR*AVKNLL   345

SPNPARC     PILSSYIAHRREVILARSPFDKEAKEKRLRIVEGLIRVISILDEVIALIR   393
SAUPARC     Q*IDSYLNHQIEVVAN*TKFELDNAE**MH*V***IKALSIL*K**EL**   394
ECOPARC     E*LSEWLVFRRDTVRR*LNYRLEKVL**LH*L***LVAFLNI*E**EI**   395

SPNPARC     ASENKADPKENLKVSYDFTEEQAEAIVTLQLYRLTNTDVVVLQEEEAELR   443
SAUPARC     SSKNKRDA*EN*IEVYEF**E*****VM*Q*YR*TNTDIVALEG*HK**E   444
ECOPARC     NED---EP*PA*MSRFGL**T*****LE*K*RH*AKLEEMKIRG*QS**E   442

SPNPARC     EKIAMLAAIIGDERTMYNLMKKELREVKKNFATPRLSSLEDTAKAIEIDT   493
SAUPARC     ALIKQ*RH*LDNHDALL*VI*E**NEIKKKFKSE*L*LIEAEIEEIKIDK   494
ECOPARC     KERDQ*QG*LASERKMN*LL*K**QADAQAYGDD*R*PLQEREEAKAMSE   492

SPNPARC     ASLIAEEDTYVSVTKAGYIKRTSPRSFAASTLEEIGKRDDDRLIFVQSAK   543
SAUPARC     EVMVPS*EVILSMTRH*YIKRTSIRSFN*SGVEDIGLKDG*SLLKHQEVN   544
ECOPARC     HDMLPS*PVTIVLSQM*WVRSAKGHDID*PGLN---YKAG*SFKAAVKGK   539

SPNPARC     TTQHLLMFTSLGNVIYRPIHELADIRWKDIGEHLSQ--TITNFETNEAIL   591
SAUPARC     TQDTVLVFTNK*RYLFIPVHK*RDI*WKEL*QHVSQ---IVPIEEDEVVI   591
ECOPARC     SNQPVVFVDST*RSYAIDPIT*PSA*--GQ*EPLTGKLTLPPGATVDHML   587

SPNPARC     YVEVLDQF--DDATTY-----------------FAATRL-----------   620
SAUPARC     NVYNEKDF--NTDAF*VFA-TQNGMIKKSTVPLFKTT*FNKPLIATKVKE   638
ECOPARC     MESDDQKLLMASDAG*GFVCTFNDLVARN--------*AGKALIT--LPE   627

SPNPARC     ----------------------------------------------------   611
SAUPARC     NDDLISVMRFEKD-QLITVITNKGMSLTYNTSELSDTGLRAAGVKSINLK   687
ECOPARC     NAHVMPPVVIEDASDMLLAITQAGRMLMFPVSDLPQLS-KGKGNKIINIP   676

SPNPARC     ----------------------GQIKRVERK------------------   620
SAUPARC     VEDFVVMTEGVSENDTILMATQRGSLKRISFKILQVAKRAQRGITLLKEL   737
ECOPARC     SAEAARGEDGLAQ---LYVLPPQSTLT------IHVGKR---------KI   708

SPNPARC     ----------------------------------------------------   620
SAUPARC     KKNPHRIVAAHVVTGEHSQYTLYSKSNEEHGLINDIHKSEQYTNGSFIVD   787
ECOPARC     KLRPEEL---QKVTGERGRRGTLMRGLQRIDRV-EIDSPRRASSG----D   750

SPNPARC     ---   620
SAUPARC     TDD   790
ECOPARC     SEE   753
```

FIG. 5. Comparison of ParC sequences of *S. pneumoniae* (SPNPARC), *S. aureus* (SAUPARC) (4), and *E. coli* (ECOPARC) (7). Identical amino acids are indicated by asterisks. Dashes indicate gaps introduced to maximize similarities.

mant (data not shown), indicating that this mutation is not involved in ciprofloxacin resistance. On the other hand, S-79 is a residue involved in ciprofloxacin susceptibility, as deduced from the *parC* sequence of transformant $R1^{3429\text{-}C}$ (Table 1).

Once the cells have acquired low-level resistance to ciprofloxacin by gaining mutations in *parC*, it was possible to transform strains to a higher level of resistance by using the gyrA46-172 DNAs from the high-level Cp^r strains. Table 1 presents the sequence data that confirmed that the second-level transformants $R2^{13925\text{-}C/517\text{-}A}$ and $R2^{3429\text{-}C/517\text{-}A}$ contained mutations in both *parC* and *gyrA* that are present in the donor DNAs. For the new transformants, MICs were 16 μg/ml (fourfold increase) if the S-83-Y or S-83-F changes of GyrA were combined with the D-83-Y change of ParC or 32 μg/ml (eightfold increase) if the GyrA changes were combined with mutations in equivalent positions of ParC (S-79-Y or S-79-F). Taken together, these results indicate that both *parC* and *gyrA* mutations are involved in ciprofloxacin resistance in pneumococci and that transformation to low-level resistance is achieved by mutations in *parC*.

DISCUSSION

In the present study we cloned and partially sequenced the genes coding for the ParE and ParC homologs of *S. pneumoniae*. These two genes are located contiguously in the chromosome, as is the case in the gram-positive bacterium *S. aureus* (4). The pneumococcal *parC* homolog mapped to a position different from *gyrA*, and *gyrA* was also mapped to a region of the pneumococcal chromosome of strain R6 (5) distant from *gyrB* (Fig. 6).

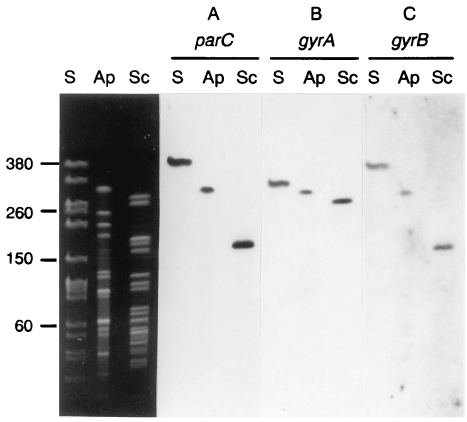

FIG. 6. Locations of the *gyrA*, *gyrB*, and *parC* genes in the *S. pneumoniae* genome. Chromosomal DNA from strain R6 was cleaved with *Sma*I (S), *Apa*I (Ap), or *Sac*II (Sc), and the fragments were separated by pulsed-field gel electrophoresis. The gels were blotted and the blots were probed with biotinylated DNA as follows: lanes A, parC449-620 PCR product; lanes B, same blot as in lanes A but stripped and reprobed with the gyrA46-172 PCR product; lanes C, same blot as in lanes B but stripped and reprobed with the gyrB10-330 restriction fragment. The sizes (in kilobases) of some of the fragments generated by *Sma*I are indicated on the left.

Amino acid sequence comparisons between the QRDRs (corresponding to positions 67 to 106 of the *E. coli* GyrA subunit) of the pneumococcal ParC and GyrA subunits with those of the gram-negative bacteria (*E. coli* and *N. gonorrhoeae*) and the gram-positive organism *S. aureus* were performed. In the gram-negative organisms the primary target for fluoroquinolones is GyrA, while in *S. aureus* the primary target for these drugs is ParC. The highest degrees of identity among ParC and GyrA sequences were found between *S. pneumoniae* and *S. aureus* (65 and 87.5%, respectively); the levels of identity between the ParC and GyrA sequences from the other organisms were less than 53 and 70%, respectively. This high degree of amino acid sequence similarity is consistent with the intrinsic resistance of *S. pneumoniae* and *S. aureus* (ca., 1 μg/ml) to ciprofloxacin and with the fact that ParC is more susceptible to ciprofloxacin than GyrA in these species. These comparisons support the idea that the amino acid sequences of the QRDRs of ParC and GyrA are mainly responsible for the different susceptibilities of topoisomerase IV and DNA gyrase to fluoroquinolones. It is tempting to speculate that, given the evolutionary relationship between gram-positive and gram-negative bacteria, the primary target for fluoroquinolones in the former would be ParC (as happens in *S. aureus* and *S. pneumoniae*), whereas GyrA would be the primary target in gram-negative bacteria.

In the *E. coli* GyrA subunit, S-83 is the position most commonly associated with high-level quinolone resistance (2, 23). In other bacteria, mutations affecting the residue equivalent to S-83 of *E. coli* have also been found (Fig. 1). In low-level Cp^r pneumococcal clinical isolates (MICs, 8 μg/ml), we found no substitution in the QRDR of GyrA; however, a change of S-79 of ParC (equivalent to S-83 of GyrA) does occur. Mutations in *S. pneumoniae gyrA* were only observed in high-level Cp^r isolates. In these cases, the amino acid substitutions observed

were S-83 to Y or F. These same substitutions have been associated with *gyrA* mutation in Cp^r strains of *N. gonorrhoeae* (S to F) (1). The absence of a substitution in the QRDR of GyrA in low-level fluoroquinolone-resistant isolates suggests that ParC is the primary target for fluoroquinolones in *S. pneumoniae* and that a mutation in *parC* is a prerequisite before mutations in *gyrA* occur. All five Cp^r isolates have a mutation in the QRDR of ParC. Low-level Cp^r isolates showed single mutations in *parC*, whereas the high-level Cp^r isolates have double mutations affecting both GyrA and ParC (Table 1). Mutations in *parC* produce amino acid changes at positions equivalent to that found to be altered in GyrA subunits of fluoroquinolone-resistant isolates of other bacteria (Fig. 1), including the ParC subunits of *N. gonorrhoeae* (1) and *S. aureus* (4). The data in Table 1 suggest that *gyrB* is not involved in ciprofloxacin resistance in pneumococci, since only silent mutations were found in the strains studied.

To address the importance of the changes detected in *gyrA* and *parC* in the development of ciprofloxacin resistance, we transferred the resistance determinants from a resistant to a susceptible strain of *S. pneumoniae*. The results from these transformation experiments (Table 1) support the fact that ParC is the primary target for fluoroquinolones, since an amino acid substitution in the ParC protein is necessary before *gyrA* mutations can influence resistance levels. Three strains have single mutations in *parC* responsible for Cp^r (Table 1). These mutations conferred a MIC of 8 μg/ml for the clinical isolates, an eightfold increase compared with those for susceptible clinical isolates (for which the MIC is usually 1 μg/ml). The same mutations conferred the same eightfold increase in resistance to laboratory strains M22 or M11. A similar effect was also observed with clinical isolates carrying mutations affecting the two equivalent positions S-79 of ParC and S-83 of GyrA. The combination of the two mutations (S-83-Y in GyrA and S-79-F in ParC) in strain 517 (Table 1) resulted in a MIC of 64 μg/ml (a 64-fold increase); likewise, for strain M22, for which the MIC is 0.5 μg/ml, these two mutations raised the MIC to 32 μg/ml. However, for strain 1244, the differences in the increase in the MICs for the clinical isolate (128 μg/ml) (Table 1) and the transformants possessing the same mutations ($R2^{517\text{-}C/1244\text{-}A}$ and $R^{1244\text{-genome}}$; MICs, 32 μg/ml) were only two times. The difference between the MIC for the clinical isolate and those for the transformants could be attributed to alterations in drug permeation or other mutations in regions of the genes encoding the subunits of the type II topoisomerases of *S. pneumoniae* not sequenced in the present study.

Table 1 illustrates how both the type and number of mutations are important to the overall level of ciprofloxacin resistance. High-level resistance is due to mutations causing an S-to-F or -Y changes in both of the two equivalent S residues of ParC and GyrA. Although detailed three-dimensional structures of the GyrA and ParC proteins are not available, these observations provide evidence that S-83 of GyrA and S-79 of ParC are essential amino acids for interactions with the DNA and the fluoroquinolones.

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