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Cloning and Characterization of the *parC* and *parE* Genes of *Streptococcus pneumoniae* Encoding DNA Topoisomerase IV: Role in Fluoroquinolone Resistance

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DNA topoisomerase IV mediates chromosome segregation and is a potential target for antibacterial agents including new antipneumococcal fluoroquinolones. We have used hybridization to a *Staphylococcus aureus gyrB* probe in concert with chromosome walking to isolate the *Streptococcus pneumoniae parE-parC* locus, lying downstream of a putative new insertion sequence and encoding 647-residue ParE and 823-residue ParC subunits of DNA topoisomerase IV. These proteins exhibited greatest homology respectively to the GrlB (ParE) and GrlA (ParC) subunits of *S. aureus* DNA topoisomerase IV. When combined, whole-cell extracts of *Escherichia coli* strains expressing *S. pneumoniae* ParC or ParE proteins reconstituted a salt-insensitive ATP-dependent decatenase activity characteristic of DNA topoisomerase IV. A second *gyrB* homolog isolated from *S. pneumoniae* encoded a 648-residue protein which we identified as GyrB through its close homology both to counterparts in *S. aureus* and *Bacillus subtilis* and to the product of the *S. pneumoniae nov-1* gene that confers novobiocin resistance. *gyrB* was not closely linked to *gyrA*. To examine the role of DNA topoisomerase IV in fluoroquinolone action and resistance in *S. pneumoniae*, we isolated mutant strains stepwise selected for resistance to increasing concentrations of ciprofloxacin. We analysed four low-level resistant mutants and showed that Ser-79 of ParC, equivalent to resistance hotspots Ser-80 of GrlA and Ser-84 of GyrA in *S. aureus*, was in each case substituted with Tyr. These results suggest that DNA topoisomerase IV is an important target for fluoroquinolones in *S. pneumoniae* and establish this organism as a useful gram-positive system for resistance studies.

Streptococcus pneumoniae is the major cause of community-acquired pneumonia and is also implicated in acute otitis media and meningitis (9). In recent years, pneumococci resistant to penicillins and other beta-lactam antibiotics have emerged that pose a serious medical challenge (20). This situation has suggested the use of fluoroquinolones such as ciprofloxacin in the treatment of pneumococcal infection, including those due to beta-lactam resistant strains. However, ciprofloxacin has marginal clinical activity against *S. pneumoniae* and, inevitably, quinolone-resistant strains are now encountered (6, 9, 30). The molecular basis of quinolone resistance in *S. pneumoniae* is currently unknown.

Studies in *Escherichia coli* have shown that quinolones such as ciprofloxacin act by targeting DNA gyrase, an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes that catalyzes ATP-dependent DNA supercoiling by a double-strand DNA break mechanism (4, 11, 12, 23). Quinolones interrupt DNA breakage-resealing mediated by the two GyrA subunits (10). By contrast, coumarin drugs, including novobiocin and coumermycin, act as competitive inhibitors of ATP binding to the GyrB proteins (13, 35). *gyrA* (and *gyrB*) mutations have been shown to confer quinolone resistance (5, 11, 28, 38). Recent studies have confirmed our original suggestion (5, 10) that the Ser-83→Trp (serine-to-tryptophan mutation at position 83) GyrA resistance mutation acts by diminishing binding of quinolones to the gyrase complex (37, 39). Mutations in the related type II enzyme, DNA topoisomerase IV, a C_2E_2 complex encoded by *parC* and *parE* genes and essential for chromosome segregation, are a secondary event (1, 3, 17–19). Interestingly, in *Staphylococcus aureus*, mutations in the *parC* (*grlA*) gene confer low-level resistance and precede those in *gyrA* (7, 8, 34). From the limited work thus far, it is unresolved whether the primacy of topoisomerase IV as a quinolone target is a unique conserved feature of gram-positive bacteria.

Given the importance of *S. pneumoniae* as a respiratory pathogen and the recent introduction of antipneumococcal fluoroquinolones (6, 30), we have sought to develop the basic molecular genetics of *S. pneumoniae* type II topoisomerases and to understand the mechanism of fluoroquinolone action and resistance in this organism. As a first step, we report here the cloning and characterization of *parE-parC* and *gyrB* loci from ciprofloxacin-susceptible *S. pneumoniae* 7785 and an examination of the role of DNA topoisomerase IV in the stepwise acquisition of fluoroquinolone resistance in vitro. This work complements a recent genetic study identifying the *nov-1* gene responsible for novobiocin resistance in *S. pneumoniae* as an allele of *gyrB* (27).

MATERIALS AND METHODS

Bacterial strains, plasmids, and enzymes. *S. pneumoniae* 7785 is a ciprofloxacin-susceptible clinical strain isolated at St. George's Hospital Medical School. *E. coli* XL1 Blue and plasmid Bluescript SK were used to construct libraries and to subclone DNA inserts. Plasmid pET29a was used to construct plasmids for overexpression of ParC and ParE proteins of *S. pneumoniae* in *E. coli* host BL21(DE3)plysE (obtained from Novagen). Plasmid pCRII (Invitrogen) was used to clone PCR products in *E. coli* XL-Blue. DNA gyrase A and B proteins were purified to homogeneity from overexpressing *E. coli* strains (24) and were kindly provided by Martin Goble of this research group.

Drug susceptibility of *S. pneumoniae* strains. *S. pneumoniae* 7785 was grown on brain heart infusion agar plates containing 10% horse blood. Susceptibility testing of mutant strains was done by streaking the bacteria (about 10^5 CFU) onto plates containing dilutions of ciprofloxacin or sparfloxacin and incubating aerobically overnight at 37°C.

Stepwise selection of ciprofloxacin-resistant *S. pneumoniae* mutants. Strain 7785 (about 10^{10} CFU) was spread on plates containing ciprofloxacin at concentrations of 2, 3, and 4 μg/ml and incubated aerobically at 37°C overnight. The

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first-step resistance mutants appeared only on plates containing 2 μg of drug per ml and were used to carry out a second round of selection. Second-step mutants appeared at a concentration of 6μg of ciprofloxacin per ml. Third- and fourth-step mutants were obtained by challenge at concentrations of 9 and 15 μg/ml, respectively.

DNA preparation. Plasmid DNA was obtained by the standard mini-prep procedure (32). Genomic DNA from *S. pneumoniae* 7785 and its ciprofloxacin-resistant mutants was prepared as follows. Pneumococci were plated at high density on five brain heart infusion agar plates containing 10% horse blood and incubated aerobically overnight at 37°C. The confluent bacteria were collected and washed in a solution containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA. Bacteria were spun down, resuspended in 10 ml of the same solution and sodium dodecyl sulfate (SDS) and proteinase K (Sigma) were added to final concentrations of 1% and 50 μg/ml, respectively. Incubation of the mixture at 37°C for 2 h was followed by phenol and phenol-chloroform extraction and precipitation with ethanol. The chromosomal DNA pellet was dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 4°C.

PCR and inverse PCR (IPCR) techniques. 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 to 5 U of *Taq* or Vent DNA polymerase, and 1 to 10 ng of DNA template (32). The *S. aureus gyrB* probe used in Southern hybridization was obtained by using forward primer RH5, 5′ TAACAGAAAGCCATGGTGACTGCA, and reverse primer RH6, 5′ TCCTTCAAAGGTACCGTTCACAGC (where internal *Nco*I and *Kpn*I sites are underlined) (22). Conditions for PCR were 30 cycles of 92°C for 1 min, annealing at 50°C for 1 min, and 74°C for 3 min.

Plasmid pXP1 was used as a PCR template to obtain a fragment corresponding to the putative insertion sequence element. A forward primer, 5′ AATACGAC TCACTATAG, was made to vector T7 sequence lying upstream of the *Hin*dIII site into which the *S. pneumoniae* DNA had been inserted. The reverse primer M0357, 5′ ATCATTCGTCATAAGGAC, was complementary to nucleotide sequence 559 to 576 of pXP1. PCR conditions were 30 cycles of 92°C for 1 min, 47°C for 1 min, and 74°C for 3 min. The PCR product was digested with *Hin*dIII (to remove vector sequence), purified by agarose gel electrophoresis, and used as a radiolabeled probe for Southern analysis.

Amplification of a 5′ region of the *parC* gene from *S. pneumoniae* 7785 and its ciprofloxacin-resistant mutants was carried out by using genomic DNA as template. The forward primer M0363 was 5′ TGGGTTGAAGCCGGTTC (*parC* positions 105 to 121) and the reverse primer M4721 was 5′ TGCTGGCAAGAC CGTTGG (454 to 471). Conditions for PCR were 30 cycles of 94°C for 1 min, 51°C for 1 min, and 74°C for 3 min. The 366-bp PCR products (codons 35 to 157) were cloned into pCRII for DNA sequence analysis.

The IPCR conditions were similar to those of PCR except that pretreatment of DNA templates was required. *S. pneumoniae* 7785 chromosomal DNA was digested with appropriate restriction enzymes and the DNA fragments were circularized by self-ligation at low DNA concentration by using T4 DNA ligase. IPCR at the 3′ end of *parC* (to yield IPCR1) was done by using forward primer V003, 5′ ATCAAACGGGTAGAGCG (positions 1838 to 1855 in *parC*), and reverse primer V009, 5′ ACTCATCACGAACCTCAGC (*parC* positions 851 to 870). IPCR conditions were 30 cycles of 92°C for 1 min, 50°C for 1 min, and 74°C for 3 min. IPCR at the 3′ end of the *gyrB* gene (to yield IPCR2) was carried out by using primers V002, 5′ ATTGCCCAACCACCAATCT (1609 to 1627 of *gyrB*), and V006, 5′ ACATCCTTGTTCTCGTTG (713 to 731 of *gyrB*).

Southern blot analysis. Chromosomal DNA or cloned DNA fragments were digested with restriction enzymes, separated by electrophoresis in 0.8% agarose, and blotted onto Hybond-N filters (Amersham) following standard procedures (15). Filters were hybridized to ^{32}P-radiolabeled DNA probes obtained by random priming with [α-^{32}P]dCTP and the Multiprime labeling kit (Amersham). For low stringency probing, filters were hybridized at 50 to 55°C in a solution containing 900 mM NaCl and 90 mM sodium citrate and then washed at 55°C in a solution containing 15 mM NaCl and 1.5 mM sodium citrate, with two changes each time for 15 min. High stringency hybridization was carried out analogously but at 60 to 65°C and filters were washed at 65°C for 1 h with several changes of washing buffer.

Construction and screening of size-selected plasmid libraries. Genomic DNA (10 μg) was digested at 37°C overnight with appropriate restriction enzymes in a 100-μl reaction volume. After electrophoresis in 0.8% low gelling agarose, DNA fragments in the desired size range were isolated (in the absence of ethidium bromide staining) and purified by phenol extraction and precipitation with ethanol. Plasmid pBluescript SK was digested with appropriate restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). Ligations were set up with T4 DNA ligase by using a molar insert-to-plasmid ratio of 1:2, and after overnight incubation at 16°C, the ligation mix was used to transform *E. coli* XL1 Blue (14). Colorless colonies were transferred individually to a 132-mm-diameter circular Hybond-N filter which was placed on top of a Luria-Bertani–agar plate containing 50 μg of ampicillin per ml and incubated overnight at 37°C. Two replica filters were made by replica plating from the master, and the latter was then stored on a fresh Luria-Bertani–agar plate containing 20% glycerol and 50 μg of ampicillin per ml at −20°C. Bacteria on replica filters were grown for 6 h at 37°C, and after SDS-alkali treatment and Tris neutralization, filters were baked at 80°C for 1 h. Before prehybridization, the filters were prewashed at 50°C for 30 min in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS, 1 mM EDTA, pH 8.0. The conditions used for colony hybridization were the same as those for Southern blotting described above. Positive colonies indicated by hybridization of duplicate filters were taken by aligning the pre-oriented replica to the master filter.

DNA sequence analysis. DNA fragments were subcloned into plasmid pBluescript SK and sequenced by the chain termination method (33) employing the Sequenase version 2.0 kit (United States Biochemical Corp.) according to the manufacturer's instructions. A combination of internal primers and universal primers (T3 and T7) that anneal to vector DNA flanking the multicloning site was used to obtain complete sequence information for both DNA strands. DNA and protein sequences were analyzed by using the PC-GENE software package.

Protein expression. Two sets of 24-mer oligonucleotide primers were designed to allow amplification of *parE* and *parC* genes in a form suitable for insertion into pET overexpression vectors. In each case, the sequences of the forward primer were chosen to introduce an *Nde*I site overlapping the initiation codon. The putative translation start codon of *parE*, GTG (Val), was substituted to ATG (Met) for convenience of cloning. For reverse primers, a *Bam*HI or *Xho*I site was introduced just downstream of the *parE* and *parC* stop codon. For *parE*, the forward primer was N7043, 5′ AGGAGGTTCCATATGTCAAAAAAG (*Nde*I), and the reverse primer was N7044, 5′ TATTTGGATCCATTAAAACACTGTC (*Bam*HI). Primers for the *parC* gene were N6894, 5′ TGGGCTTTGTATCA TATGTCTAAC (*Nde*I), and N6895, 5′ AGAACTTATTGAGCTCTTCACTTA (*Xho*I). PCR was carried out on genomic DNA from strain 7785 by using Vent DNA polymerase as follows: 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. PCR products were digested with restriction enzymes, ligated into expression plasmid pET29a, and transformed into *E. coli* expression host BL21(DE3)plysE. Strains were grown, induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and soluble whole-cell extracts were prepared (29). The conditions for induction and purification of ParC and ParE proteins remain to be optimized and will be reported elsewhere (28a).

Topoisomerase assays. Decatenation of kinetoplast DNA (kDNA) from *Crithidia fasciculata* by *E. coli* cell extracts was carried out as described (8). DNA gyrase activity was determined in a supercoiling assay (12, 24).

Nucleotide sequence accession numbers. The nucleotide sequences shown in Fig. 2 and Fig. 4 will appear in the EMBL Data Library under accession nos. Z67740 and Z67739, respectively.

RESULTS

Cloning two gyrB homologs from *S. pneumoniae*. Southern blot analysis of genomic DNA from *S. pneumoniae* 7785 revealed that two *Hin*dIII fragments of 4.3 and 2.2 kb hybridized to an *S. aureus gyrB* probe (data not shown). These fragments were isolated by colony hybridization of two size-selected *S. pneumoniae* 7785 *Hin*dIII fragment libraries in plasmid pBluescript SK yielding plasmids pXP1 and pXP4 (Fig. 1). DNA sequence analysis (see below) indicated plasmid pXP1 contained the entire *parE* gene and part of *parC*; the pXP4 *Hin*dIII insert contained all but the extreme 3′ end of the *S. pneumoniae gyrB* gene. To obtain full-length *parC* and *gyrB* genes and information on their chromosomal context, a combination of local chromosome walking and IPCR was used to isolate overlapping clones pXP2 and pXP3 (for *parC*) and pXP5 and pXP6 (for *gyrB*) (for details, see Fig. 1 legend and Materials and Methods).

Nucleotide sequence analysis of *S. pneumoniae* gyrB. DNA fragments in pBluescript or IPCR products in plasmid pCRII were sequenced directly by the chain termination method (33) by using either vector-specific T3, T7, and SP6 primers or primers made to internal sequence. The full nucleotide sequence of a 3.6-kb region of the *S. pneumoniae* genome specified by the overlapping inserts of pXP4, pXP5, and pXP6 is presented in Fig. 2. Two open reading frames (ORFs) were present. The 5′ end of the insert in plasmid pXP4 had an incomplete ORF coding for a 144-residue protein with no homologs in the PC-GENE database. The second ORF encoded a 648-residue protein with a predicted molecular weight of 72.3 kDa. Putative −10 (TATCGT) and −35 (TTGATA) regions and ribosome binding signals were found upstream of the initiation ATG codon. The deduced protein sequence exhibited strong homology with all known GyrB proteins i.e., 62 and

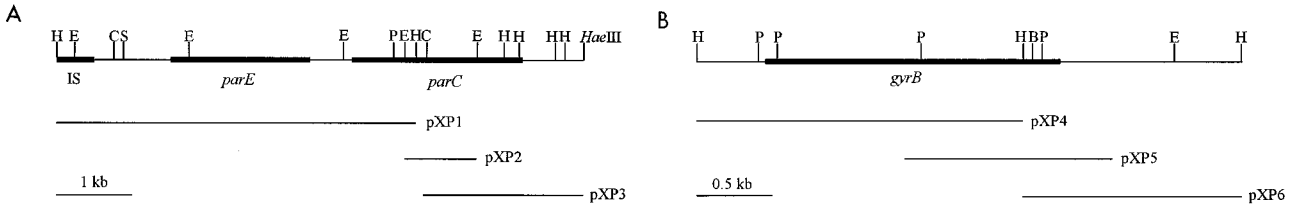

FIG. 1. Restriction maps of the *parE-parC* (A) and *gyrB* (B) loci in *S. pneumoniae* 7785 and alignment of plasmid clones. The *parE*, *parC*, and *gyrB* genes are indicated by heavy lines. IS denotes a region upstream of *parE* displaying strong homology to the insertion sequence IS*861*. B, C, E, H, P, and S indicate restriction sites for *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I and *Sac*I, respectively. The topoisomerase IV and gyrase genes were identified and restriction maps were obtained by Southern blotting of digested genomic DNA with an *S. aureus gyrB* probe. Plasmids pXP1 and pXP4, carrying the indicated *Hin*dIII genomic inserts, were isolated from size-selected plasmid libraries by colony hybridization to an *S. aureus gyrB* probe. Plasmid clone pXP2, containing a 1.2-kb *Eco*RI insert, was obtained by Southern blot analysis and colony hybridization of size-selected libraries with a subfragment of pXP1 insert as probe. The 3′ end of the *S. pneumoniae parC* gene could not be cloned directly and was obtained by IPCR of a 1.4-kb *Hae*III fragment, yielding plasmid pXP3. Plasmid clone pXP6, containing a 1.5-kb *Hin*dIII fragment, was obtained from a size-selected library by using a 600-bp *Hae*II IPCR fragment (from pXP5) as probe.

48% identity with GyrB subunits of *S. aureus* and *E. coli*, respectively (22) (Fig. 3). Homology to the ParE subunits of *S. aureus* (i.e., GrlB) and *E. coli* was lower at 49 and 35% identity, respectively (8). On this basis, we assigned the 648-residue *S. pneumoniae* protein as GyrB. Moreover, the sequence of our *gyrB* gene is closely similar to that recently reported for the *S. pneumoniae nov-1* gene that confers resistance to novobiocin and which has been identified as a *gyrB* allele (27). The 1944-bp

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      A  L  K  V  S  T  P  F  A  I  E  T  F  A  P  N  L  E  N  F  L  E  K  Y  K  E  N  E  A  R  E  L  E  H  P  I  L  F  E  G
1     AAGCTTTAAAGGTTTCTACTCCTTTTGCGATTGAGACATTCGCTCCCAATTTAGAGAATTTTTTAGAAAAGTACAAGGAAAATGAAGCCAGAGAGCTTGAACACCCGATTTTATTTGAAG
      V  S  D  L  L  E  D  I  L  N  Q  G  G  R  H  F  L  V  S  H  R  N  D  Q  V  L  E  I  L  E  K  T  S  I  A  A  Y  F  T  E
121   GAGTTTCTGACCTATTGGAAGACATTTTAAATCAAGGTGGCCGTCATTTTTTGGTCTCTCATCGAAATGATCAGGTTTTGGAAATTTTAGAAAAAACCTCTATAGCAGCTTATTTTACAG
      V  V  T  S  S  S  G  F  K  R  K  P  N  P  E  S  M  L  Y  L  R  E  K  Y  Q  I  S  S  G  L  Y  I  G  D  R  P  I  D  I  E
241   AAGTGGTGACTTCTAGCTCAGGCTTTAAGAGAAAGCCAAATCCCGAATCCATGCTTTATTTAAGAGAAAAGTATCAGATTAGCTCTGGTCTTGTCATTGGTGATCGGCCGATTGATATCG
      A  G  Q  A  A  G  L  D  T  H  L  F  T  S  I  V  N  L  R  Q  V  L  D  I  ***                  M  T  E  E  I  K  N  L  Q  A
361   AAGCAGGTCAAGCTGCAGGACTTGATACCCACTTGTTTACCAGTATCGTGAATTTAAGACAAGTATTAGACATATAAGAAAAAGGAATAAGATGACAGAAGAAATCAAAAATCTGCAGGC
      Q  D  Y  D  A  S  Q  I  Q  V  L  E  G  L  E  A  V  R  M  R  P  G  M  Y  I  G  S  T  S  K  E  G  L  H  H  L  V  W  E  I
481   ACAGGATTATGATGCCAGTCAAATTCAAGTTTTAGAGGGCTTAGAGGCTGTTCGTATGCGTCCAGGGATGTACATTGGATCAACCTCAAAAGAAGGTCTTCACCATCTAGTCTGGGAAAT
      V  D  N  S  I  D  E  A  L  A  G  F  A  S  H  I  Q  V  F  I  E  P  D  D  S  I  T  V  V  D  D  G  R  G  I  P  V  D  I  Q
601   TGTTGATAACTCAATTGACGAGGCCTTGGCAGGATTTGCCAGCCATATTCAAGTTTTTATTGAGCCAGATGATTCGATTACTGTTGTGGATGATGGGCGTGGTATCCCAGTCGATATTCA
      E  K  T  G  R  P  A  V  E  T  V  F  T  V  L  H  A  G  G  K  F  G  G  G  G  Y  K  V  S  G  G  L  H  G  V  G  S  S  V  V
721   GGAAAAAACAGGCCGTCCTGCTGTTGAGACCGTCTTTACAGTCCTTCATGCTGGAGGAAAGTTCGGCGGTGGTGGATACAAGGTTTCAGGTGGTCTTCACGGGGTGGGGTCGTCAGTAGT
      N  A  L  S  T  Q  L  D  V  H  V  H  K  N  G  K  I  H  Y  Q  E  Y  R  R  G  H  V  V  A  D  L  E  V  V  G  D  T  D  R  T
841   TAATGCCCTTTCCACTCAATTAGACGTTCATGTCCATAAAAACGGTAAGATTCATTACCAAGAATACCGTCGTGGTCATGTTGTTGCAGACCTTGAGGTGGTTGGGGATACAGATAGAAC
      G  T  T  V  H  F  T  P  D  P  E  I  F  T  E  T  T  I  F  D  F  D  K  L  N  K  R  I  Q  E  L  A  F  L  N  R  G  L  Q  I
961   TGGAACAACGGTTCACTTCACACCGGACCCAGAAATTTTCACTGAAACAACAATCTTTGATTTTGATAAATTAAATAAACGGATTCAAGAGTTGGCCTTTCTAAATCGCGGTCTTCAAAT
      S  I  T  D  K  R  Q  G  L  E  Q  T  K  H  Y  H  Y  E  G  G  I  A  S  Y  V  E  Y  I  N  E  N  K  D  V  I  F  D  T  P  I
1081  TTCAATTACAGATAAGCGCCAAGGTTTGGAACAAACCAAGCATTATCATTATGAAGGTGGGATTGCTAGTTACGTTGAATATATCAACGAGAACAAGGATGTAATCTTTGATACACCAAT
      Y  T  D  G  E  M  D  D  I  T  V  E  V  A  M  Q  Y  T  T  G  Y  H  E  N  V  M  S  F  A  N  N  I  H  T  H  E  G  G  T  H
1201  CTATACAGACGGTGAGATGGATGATATCACAGTTGAGGTAGCCATGCAGTACACAACAGGTTACCATGAAAATGTCATGAGTTTCGCCAATAATATTCATACCCATGAAGGTGGAACGCA
      E  Q  G  F  R  T  A  L  T  R  V  I  N  D  Y  A  R  K  N  K  L  L  K  D  N  E  D  N  L  T  G  E  D  V  R  E  G  L  T  A
1321  TGAACAAGGTTTCCGTACGGCACTGACTCGTGTTATCAATGATTATGCTCGTAAGAATAAGTTACTGAAAGACAATGAAGATAATTTAACAGGGGAAGATGTTCGCGAAGGATTAACTGC
      V  I  S  V  K  H  P  N  P  Q  F  E  G  Q  T  K  T  K  L  G  N  S  E  V  V  K  I  T  N  R  L  F  S  E  A  F  S  D  F  L
1441  AGTTATCTCAGTTAAACACCCAAATCCACAGTTTGAAGGACAAACCAAGACCAAATTGGGAAATAGCGAAGTGGTCAAGATTACCAATCGCCTCTTCAGTGAAGCCTTCTCCGATTTCCT
      M  E  N  P  Q  I  A  K  R  I  V  E  K  G  I  L  A  A  K  A  R  V  A  A  K  R  V  R  E  V  T  R  K  K  S  G  L  E  I  S
1561  CATGGAAAATCCACAGATTGCCAAACGTATCGTGGAAAAAGGGATTTAGCTGCCAAGGCTCGTGTGGCTGCCAAGCGTGCGCGTGAAGTCACACGTAAAAAATCTGGTTTGGAAATTTC
      N  L  P  G  K  L  A  D  C  S  S  N  N  P  A  E  T  E  L  F  I  V  E  G  D  S  A  G  G  S  A  K  S  G  R  N  R  E  F  Q
1681  CAACCTTCCAGGGAAACTAGCAGACTGTTCTTCTAATAACCCTGCTGAAACAGAACTCTTCATCGTCGAAGGAGACTCAGCTGGTGGATCAGCCAAATCTGGTCGTAACCGTGAGTTTCA
      A  I  L  P  I  T  G  K  I  L  N  V  E  K  A  S  M  D  K  I  L  A  N  E  E  I  R  S  L  F  T  A  M  G  T  G  F  G  A  E
1801  GGCTATCCTTCCAATTCGCGGTAAGATTTTGAACGTTGAAAAAGCAAGTATGGATAAGATTCTAGCTAACGAAGAAATTCGTAGTCTTTTCACAGCCATGGGAACAGGATTTGGCGCAGA
      F  D  V  S  K  A  R  Y  Q  K  L  V  L  M  T  D  A  D  V  D  G  A  H  I  R  T  L  L  L  T  L  I  Y  R  Y  M  K  P  I  L
1921  ATTTGATGTTTCGAAAGCCCGTTACCAAAAACTCGTTTTGATGACCGATGCCGATGTCGATGGAGCCCACATTCGTACCCTTCTTTTAACCTTGATTTATCGTTATATGAAACCAATCCT
      E  A  G  Y  V  Y  I  A  Q  P  P  I  Y  G  V  K  V  G  S  E  I  K  E  Y  I  Q  P  G  A  D  Q  E  I  K  L  Q  E  A  L  A
2041  AGAAGCTGGTTATGTTTATATTGCCCAACCACCAATCTATGGTGTCAAGGTTGGAAGCGAGATTAAAGAATATATCCAGCCGGGTGCAGATCAAGAAATCAAACTCCAAGAAGCTTTAGC
      R  Y  S  E  G  R  T  K  P  T  I  Q  R  Y  K  G  L  G  E  M  D  D  H  Q  L  W  E  T  T  M  D  P  E  H  R  L  M  A  R  V
2161  CCGTTATAGTGAAGGTCGTACCAAACCGACTATTCAGCGTTATAAGGGGCTAGGTGAAATGGACGATCATCAGCTGTGGGAAACAACCATGGATCCCGAACATCGCTTGATGGCTAGAGT
      S  V  D  D  A  A  E  A  D  K  I  F  D  M  L  M  G  D  R  V  E  P  R  R  E  F  I  E  E  N  A  V  Y  S  T  L  D  V  ***
2281  TTCTGTAGATGATGCTGCAGAAGCAGATAAAATCTTTGATATGTTGATGGGGGATCGAGTAGAGCCTCGTCGTGAGTTTATCGAAGAAAATGCTGTCTATAGTACACTTGATGTCTAAAA
2401  AATAGGGAGAAGTAGTTCCCTTGGTCTAAAAAATATGATATAATCGTTACGATTGTTTCAAGTAAAAAAGGAGTTTGATATGTCTAATGGACAACTAATTTATTTAATGGTTGCAATTGC
2521  AGTCATTTTAGTTCTGGCTTATGTAGTGGCAATCTTTCTACGTAAGCGAAACGAGGGGAGTTAGAGGCGCTAGAAGAAAGAAAAGAAGAACTATACAATCTTCCAGTAAATGATGAAGTA
2641  GAAGCTGTAAAAAAATATGCACTTGATTGGACAAAGTCAAGTGGCTTTCCGTGAATGGAATCAAAAATGGGTCGATTTACTCTCAACTCTTTTGCCGATATTGAAAATAATCTCTTTGAAG
2761  CAGAAGGCTATAACCATTCATTTCGTTTTCTCAAGGCCAGTCATCAAATTGACCAAATTGAGAGTCAAATTACTTTGATTGACGAAGATATTGCGGCAATTCGCAATGCTTTGGCAGACT
2881  TAGAGAAGCAAGAATCTAAAAATAGTGGTCGTGTTCTTCATGCTTTGGATTTATTTGAGGAACTTCAGCATAGAGTTGCTGAAAATTCAGAACAGTATGGTCAAGCCTTGGATGAAATTG
3001  AAAAACAATTAGAAAATATCCAATCTGAATTTTCACAATTTGTAACCTTGAATTCATCGGGTGACCCTGTGGAAGCCGCAGTGATTTTGGATAATACAGAAAATCACATTTTGGCCTTAA
3121  GTCATATTGTGGATCGTGTTCCAGCCTTGGTTACGACGCTTTCTACAGAATTGCCAGATCAATTACAGGATTTGGAAGCCGGTTATCGTAAACTAATTGATGCTAATTATCATTTTGTTG
3241  AAACGGATATTGAAAGCGCGTTTCCACTTGCTTTATGAAGCATTCAAGAAAAACCAAGAGAATATTCGTCAGTTGGAATTGGATAATGCCGAATATGAGAATGGACAGGCACAAGAGGAA
3361  ATCAATGCCTTGTATGATATTTTTACTCGAGAAATTGCTGCTCAGAAAGTAGTGGAAAATCTACTTGCAACTCTTCCAACTTATCTTCAACATATGAAAGAGAATAATACTTTATTGGGA
3481  GAAGATATTGCACGTTTGAACAAGACCTATTTACTTCCTGAGACAGCTGCAAGCCATGTTCGTCGTATTCAGACAGAATTAGAGAGTTTTGAGGCAGCTATTGTTGAGGTAACTTCAAAT
3601  CAAGAAGAACCAACCCAAGCTT
```

FIG. 2. Nucleotide sequence of a 3.6-kb region of the *S. pneumoniae* genome containing the *gyrB* gene. The inferred amino acid sequences for two ORFs are shown above the DNA sequence. A methionine initiation codon is underlined, as are putative −10 and −35 regions. ***, stop codons.

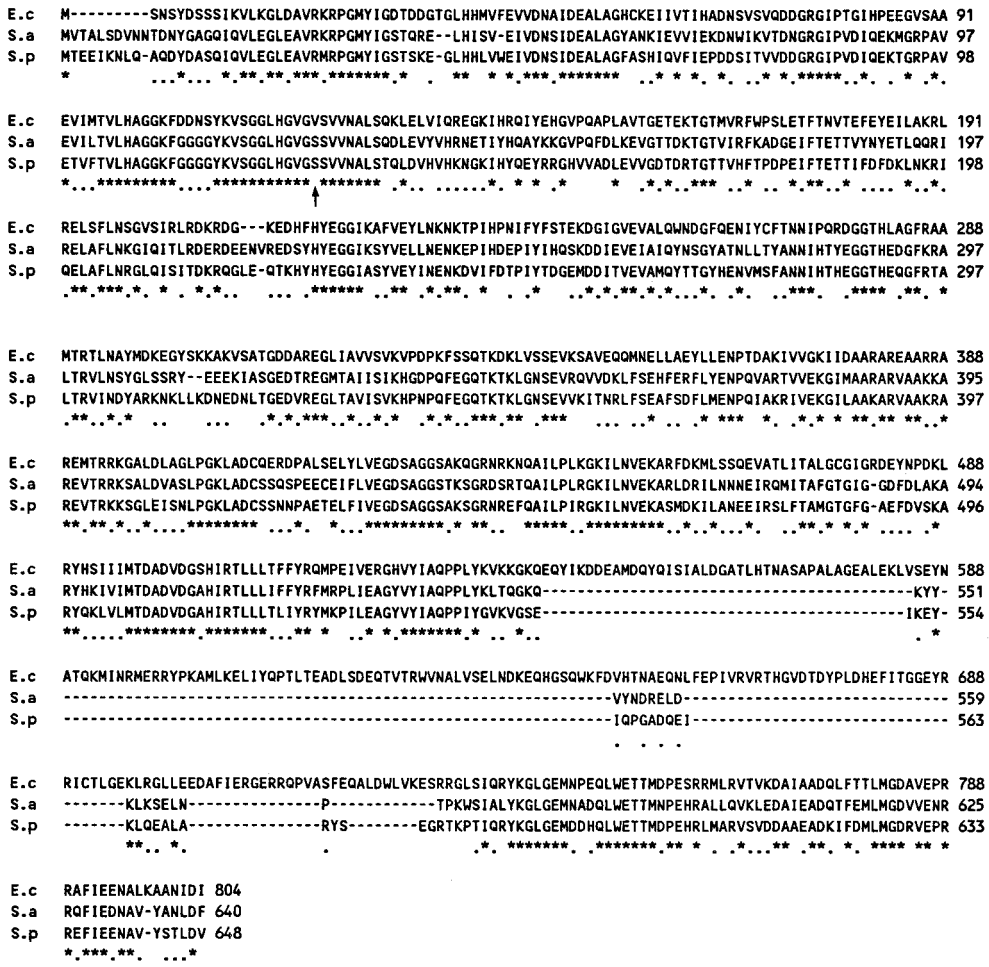

FIG. 3. Alignment of *S. pneumoniae* (S.p.) gyrase B protein with counterparts in *E. coli* (E.c.) and *S. aureus* (S.a.). An asterisk indicates a residue identical in all three proteins, a dot denotes similar residues. Numbers at right indicate amino acid residues. Arrow denotes Ser-127 mutated to Leu in novobiocin-resistant strain 533 (27).

nov-1 gene and *gyrB* sequences (Fig. 3) differed at only 38 nucleotide positions, resulting in only six differences at the protein level (results not shown). Conserved features in the *S. pneumoniae* GyrB protein are the N-terminal ATP-binding region (residues 1 to 140), containing a glycine-rich segment (G-loop), and also EGDSA and YKGL moieties. Typical of gram-positive GyrB proteins, compared with the *E. coli* protein, *S. pneumoniae* GyrB lacks an approximately 160-residue segment in the C-terminal region. GyrB in strain 7785 displayed the wild-type Ser-127 found in novobiocin-susceptible *S. pneumoniae* (27) (Fig. 3). Isolation of clones yielding sequence downstream of that shown in Fig. 2 did not reveal a *gyrA* homolog (results not shown).

***S. pneumoniae parC* and *parE* genes lie downstream of a novel insertion sequence.** Figure 4 presents the sequence of a 6.8-kb segment of the *S. pneumoniae* chromosome contained in plasmids pXP1, pXP2, and pXP3 (Fig. 1A). One partial and two complete ORFs are identified, specifying proteins of 144, 647, and 823 amino acid residues, respectively. The incomplete ORF at the very 5′ end of the pXP1 insert suggests the presence of an insertion sequence which we termed IS-SP (Fig. 5A). First, the deduced protein sequence showed strong homology to the C-terminal portion of the transposase of several insertion sequences, including IS*3* and IS*150* of *E. coli*. The element was not homologous to IS*1202*, previously described in *S. pneumoniae* (26), a copy of which was detected 4 kb downstream of *gyrB* (results not shown). However, the greatest homology was observed to the transposase of a group B Streptococcal insertion sequence, IS*861* (31). Of the 144 residues in the C-terminal partial ORF, 116 were identical to the residues in IS*861*. Second, a 576-bp IS-SP probe from the 5′ end of the pXP1 insert hybridized to many different restriction fragments of *S. pneumoniae* 7785 genomic DNA, suggesting that, typical of an insertion element, the sequence is present in multiple copies on the chromosome (Fig. 5B). Thus, IS-SP appears to be representative of a previously unreported insertion sequence in *S. pneumoniae*.

The two complete ORFs, separated by 419 bp, encoded 647- and 823-residue proteins with predicted molecular masses of 71.7 and 93.1 kDa, assuming that translation of ORF1 initiates at a GTG (Val) codon immediately downstream of a Shine-Dalgarno sequence (AGGAGG) (Fig. 4). The protein sequences deduced for these ORFs exhibited 68 and 51% identity to GrlB (ParE) and GrlA (ParC) subunits of *S. aureus* DNA topoisomerase IV (6) but only 43 and 30% identity to the GyrB and GyrA proteins of *S. aureus* DNA gyrase, respectively (22) (Fig. 6; Table 1). From these sequence homologies, we provisionally identified ORF1 and ORF2 with the *parE* and *parC* genes encoding subunits of *S. pneumoniae* topoisomerase IV.

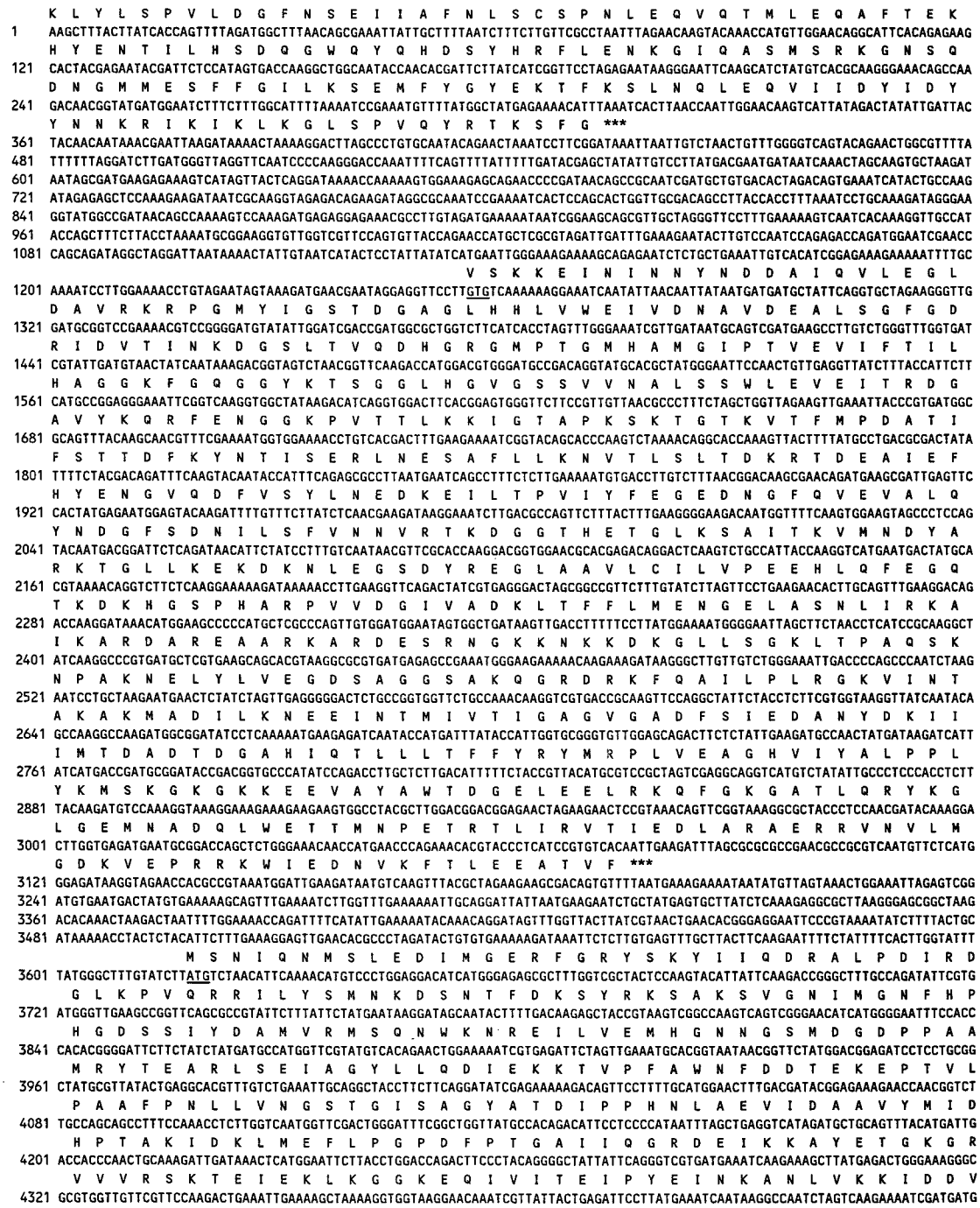

FIG. 4. DNA sequence of the *S. pneumoniae parE-parC* locus and deduced amino acid sequences of the ParE and ParC subunits of DNA topoisomerase IV. Symbols are the same as those defined in the legend for Fig. 2.

Similar to the GyrB proteins, *S. pneumoniae* ParE has highly conserved EGDSA and N-terminal sequences, including the G loop ATP binding moiety (Fig. 6). Compared with its counterparts in *E. coli* and *S. aureus*, the ParC protein shows greatest homology in its N-terminal DNA breakage-reunion region. Ser-79 (equivalent to Ser-80 in *E. coli* and *S. aureus* ParC, hotspots for mutation to quinolone resistance [8]) is part of a conserved HPHGDS sequence. Finally, the catalytic tyrosine of ParC engaged in DNA breakage-reunion is identified as Tyr-118 by alignment of a conserved AAMRYTE sequence with catalytic Tyr-122 of *E. coli* (Fig. 6) (16).

ParC and ParE proteins expressed in *E. coli* reconstitute a salt-insensitive DNA decatenase activity. To confirm our assignment of *gyrB* homologs, we overexpressed the putative ParC and ParE proteins and examined their enzymatic properties. *parC* and *parE* genes were cloned into plasmid pET29a downstream of an inducible T7 promoter yielding plasmids pXP7 and pXP8 (see Materials and Methods). *E. coli* strain

```
      R  V  N  N  K  V  A  G  I  A  E  V  R  D  E  S  D  R  D  G  L  R  I  A  I  E  L  K  K  D  A  N  T  E  L  V  L  N  Y  L
4441  TTCGTGTTAATAACAAGGTAGCTGGGATTGCTGAGGTTCGTGATGAGTCTGACCGTGATGGTCTTCGTATCGCTATCGAACTTAAGAAAGACGCTAATACTGAGCTTGTTCTCAACTACT
      F  K  Y  T  D  L  Q  I  N  Y  N  F  N  M  V  A  I  D  N  F  T  P  R  Q  V  G  I  V  P  I  L  S  S  Y  I  A  H  R  R  E
4561  TATTTAAGTACACCGACCTACAAATCAACTACAACTTTAATATGGTGGCGATTGACAATTTCACACCTCGTCAGGTTGGGATTGTTCCAATCCTGTCTAGCTACATCGCTCACCGTCGAG
      V  I  L  A  R  S  R  F  D  K  E  K  A  E  K  R  L  H  I  V  E  G  L  I  R  V  I  S  I  L  D  E  V  I  A  L  I  R  A  S
4681  AAGTGATTTTGGCGCGTTCACGCTTTGACAAAGAAAAGGCTGAGAAACGTCTCCATATCGTCGAAGGTTTGATTCGTGTGATTTCGATTTTGGATGAAGTCATTGCTCTTATCCGTGCTT
      E  N  K  A  E  A  K  E  N  L  K  V  S  Y  D  F  T  E  E  Q  A  E  A  I  V  T  L  Q  L  Y  R  L  T  N  T  D  V  V  V  L
4801  CTGAGAATAAGGCGGACGCCAAGGAAAACCTCAAAGTTAGCTATGATTTTACGGAAGAACAGGCTGAGGCTATCGTAACTTTGCAACTGTACCGTTTGACCAATACCGATGTGGTTGTCT
      Q  E  E  E  A  E  L  R  E  K  I  A  M  L  A  A  I  I  G  D  E  R  T  M  Y  N  L  M  K  K  E  L  R  E  V  K  K  K  F  A
4921  TGCAGGAAGAAGAAGCAGAGCTTCGTGAGAAGATTGCTATGCTGGCGGCTATTATCGGTGATGAAAGGACTATGTACAATCTCATGAAGAAAGAACTTCGTGAGGTCAAGAAGAAATTTG
      T  P  R  L  S  S  L  E  D  T  A  K  A  I  E  I  D  T  A  S  L  I  A  E  E  D  T  Y  V  S  V  T  K  A  G  Y  I  K  R  T
5041  CAACTCCTCGTTTGAGTTCTTTAGAAGACACTGCGAAAGCAATTGAGATTGATACAGCTAGTCTTATCGCTGAGGAAGATACCTACGTCAGCGTGACCAAGGCAGGTTACATCAAGCGTA
      S  P  R  S  F  A  A  S  T  L  E  E  I  G  K  R  D  D  D  R  L  I  F  V  Q  S  A  K  T  T  Q  H  L  L  M  F  T  S  L  G
5161  CCAGTCCACGTTCCTTTGCGGCTTCCACCTTGGAAGAAATTGGCAAGCGTGATGATGACCGTTTGATTTTTGTTCAATCTGCCAAGACAACCCAGCACCTCTTGATGTTCACAAGTCTTG
      N  V  I  Y  R  P  I  H  E  L  A  D  I  R  W  K  D  I  G  E  H  L  S  Q  T  I  T  N  F  E  T  N  E  E  I  L  Y  V  E  V
5281  GAAATGTCATCTACAGACCAATCCATGAGTTGGCAGATATTCGTTGGAAGGACATCGGAGAGCATCTGAGCCAAACCATCACAAACTTTGAAACGAATGAAGAAATCCTTTATGTGGAAG
      L  D  Q  F  D  D  A  T  T  Y  F  A  V  T  R  L  G  Q  I  K  R  V  E  R  K  E  F  T  P  W  R  T  Y  R  S  K  S  V  K  Y
5401  TACTGGATCAGTTTGACGATGCGACAACCTACTTTGCAGTGACTCGCCTTGGTCAAATCAAACGGGTAGAGCGAAAAGAATTCACTCCATGGCGGACCTATAGATCTAAGTCTGTCAAGT
      L  S  S  K  T  I  Q  T  Q  I  V  A  V  A  P  I  K  L  D  D  V  V  L  V  S  Q  N  G  Y  A  L  R  F  N  I  E  E  V  P  V
5521  ATCTAAGCTCAAAGACGATACAGACTCAGATTGTAGCAGTGGCTCCGATTAAACTAGATGATGTTGTCTTGGTTAGTCAAAATGGTTATGCCCTGCGTTTCAATATCGAAGAGGTTCCGG
      V  G  A  K  A  A  G  V  K  A  M  N  L  K  E  D  D  V  L  Q  S  G  F  I  C  N  T  S  S  F  Y  L  L  T  Q  R  G  S  L  K
5641  TTGTCGGTGCTAAGGCAGCAGGTGTCAAGGCTATGAATTTGAAAGAAGATGATGTCCTCCAATCTGGCTTTATCTGTAATACTTCGTCCTTCTACCTCTTGACCCAGCGTGGAAGCTTGA
      R  V  S  I  E  E  I  L  A  T  S  R  A  K  R  G  L  Q  V  L  R  E  L  K  N  K  P  H  R  V  F  L  A  G  A  V  A  E  Q  G
5761  AACGTGTTTCTATTGAGGAAATTCTAGCAACCAGCCGTGCCAAACGAGGATTACAAGTCTTGCGTGAGTTGAAAAACAAACCGCATCGTGTCTTCTTGGCAGGAGCAGTTGCAGAGCAAG
      F  V  G  D  F  F  S  T  E  V  D  V  N  D  Q  T  L  L  V  Q  S  N  K  G  T  I  Y  E  S  R  L  Q  D  L  N  L  S  E  R  T
5881  GATTTGTTGGCGATTTCTTCAGTACGGAAGTGGATGTGAACGACCAAACTCTGCTTGTCCAATCCAATAAAGGAACAATCTATGAAAGCCGATTGCAAGACTTGAACTTGTCAGAACGCA
      S  N  G  S  F  I  S  D  T  I  S  D  E  E  V  F  D  A  Y  L  Q  E  V  V  T  E  D  K  ***
6001  CTAGCAATGGAAGCTTCATTTCTGACACGATTTCAGATGAAGAAGTTTTTGACGCTTATCTTCAGGAAGTAGTTACTGAAGATAAATAAGTGAAATGAAGAATAAGTTCTAAGAGCTGGT
6121  TTTTATTGGAGAAATTTTCTGAAAATTACAAAATATACTTGCTATTTTAGAAAATAGTGTAGAATATAAGAAATAGGTTTTTAGAATAAGGAGTGGAATATGACAGTAACGATTGATTGG
6241  GAAAACCTCGGTTTTTCCTATATGAAATTACCTTATCGCTATCTTGCTCATTTCAAAAATGGACAATGGGATCAAGGAGAGCTTACAGAGGATGCAACTTTGCATATTTCAGAGTCTTCT
6361  CCAAGTCTTCACTATGGACAACAAGCATTTGAAGGTTTGAAAGCTTATCGTACTAAGGATGGCAGTGTTCAACTGTTCCGTCCTGATGAAAATGCTAAACGTCTGCAACGTACATGTGAC
6481  CGTCTCTTGATGCCACAAGTTCCGACAGACATGTTTGTAGAAGCTTGTAAAGCAGTTGTCCGTGCGAATGAAGAATACGTACCACCATACGGAACAGGTGGAACCTTATATCTTCGCCCT
6601  CTTTTGATTGGTGTCGGAGATATTATCGGGGTAAAACCGGCAGAAGAGTACATTTTCACCATCTTTGCTATGCCAGTTGGAAATTACTTTAAAGGTGGTTTGGTCCCAACCAACTTCTTG
6721  ATTCAGGATGAGTACGACCGTGCAGCACCAAATGGTACAGGTGCGGCTAAGGTTGGTGGAAACTATGCTGCAAGTCTCTTACCAGGAAAAAT
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FIG. 4—*Continued.*

BL21(DE3)plysE containing pXP7 or pXP8 on induction with IPTG overexpressed 93-kDa or 72-kDa proteins in soluble form, as seen by SDS-polyacrylamide gel electrophoresis (PAGE) analysis of whole-cell extracts (Fig. 7A). These sizes are the same as those predicted from the deduced ParC and ParE protein sequences (Fig. 4). Extracts expressing ParC and ParE, when combined, generated a decatenase activity capable of unlinking kDNA in the presence of 200 mM potassium glutamate (Fig. 7B, lanes 8 and 9). Enzymatic activity was dependent on ATP and the presence of both subunits, as the omission of ATP (Fig. 7B, lane 5) or either subunit (lanes 6 and 7) did not lead to DNA unlinking activity. Under these high salt conditions, purified *E. coli* DNA gyrase did not promote ATP-dependent unlinking of kDNA, in agreement with previous work (compare lanes 1 and 2). The same gyrase preparation was fully active in DNA supercoiling assays conducted under low salt conditions (24) and run in parallel with kDNA decatenation experiments (not shown). The demonstration of a salt-insensitive decatenation activity typical of topoisomerase IV (29) confirms the identification of the *S. pneumoniae* 93- and 72-kDa proteins as ParC and ParE subunits.

Ser-79→Tyr ParC mutations in stepwise-selected ciprofloxacin-resistant mutants of *S. pneumoniae*. Characterization of the *parE-parC* locus (Fig. 4) allowed us to examine the role of topoisomerase IV in quinolone-resistant *S. pneumoniae*. First, we developed mutants of susceptible strain 7785 by stepwise exposure to ciprofloxacin (Table 2). In the first round of selection, isolate 7785 (approx 10^{10} CFU) was plated on brain heart infusion medium plates containing increasing concentrations of ciprofloxacin at multiples of the MIC. Seventeen colonies (first-step mutants) grew on the plate containing 2 μg of ciprofloxacin per ml; no growth was seen at higher drug concentrations. Three first-step mutants (1C1, 1C2, and 1C3) were selected for *parC* sequence analysis. Mutant 1C1 was exposed to increasing drug levels on plates. At a concentration of 6 μg/ml, six colonies (second-step mutants) were able to grow. Four of these second-step mutants, 2C1 to 4, were characterized. Third- and fourth-step mutants, which grew in the presence of 9 and 15 μg of ciprofloxacin per ml, respectively, were generated similarly. MIC determination showed that parental strain 7785 and mutant strains 1C1, 2C1, 3C1, and 4C1 exhibited a wide range of resistance to ciprofloxacin, e.g., strain 4C1 was 100 times more resistant than was strain 7785 (Table 2). Mutant strains were also cross-resistant to sparfloxacin (30), a new and more potent antipneumococcal fluoroquinolone (Table 2). Interestingly, although the third-step mutant 3C1 was only marginally (some twofold) more resistant to sparfloxacin than was strain 7785, the fourth-step strain was nearly 70-fold more resistant. Thus, the mutants responded differently to the two drugs.

A 366-bp *parC* fragment spanning codons 35 to 157 was amplified by PCR from strain 7785 and its ciprofloxacin-resistant mutants. This region encompasses sequence equivalent in ParC to the quinolone resistance-determining region of GyrA (residues 67 to 106 in the *E. coli* protein) (38). PCR products were ligated into plasmid pCRII and the inserts were sequenced on both strands by using T7 and SP6 primers. The nucleotide sequences of PCR products from 1C1, 1C2, and 1C3 were identical to that of 7785. However, PCR products from all four second-step mutants carried a single nucleotide change compared with wild-type, i.e., a TCT-to-TAT alteration at codon 79 resulting in a Ser-to-Tyr substitution in ParC (Table 2). Sequence analysis of PCR products from third- and fourth-step mutants (in each case three mutants were examined) did not reveal further mutations in the *parC* gene.

DISCUSSION

We have cloned and characterized two *gyrB* homologs from *S. pneumoniae* 7785. One gene was identified as *gyrB* through

A

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IS150   MK-VLNELRQF-YPLDELLRAAEIPRSTFYY---HLKALSKPDKY-ADVKKRISEIYHENRGRYGYRRVTLSLHREGKQINHKAVQRLMGTLSLKAAIKV 94
IS861   MV-------TGGFRLDLLLEITKIARATYYY---QLKKLNKPDK-DKAIKSDIQSIYDEHRGNYGYRRIYLELRNRGFVINHKRVQGLMKSMGLTARIRR 89
IS-S.P  ---------------------------------------------------------------------------------------------- 0

IS150   K-RYRSYRGEVGQTAPNVLQRDFKATRPNEKWVTDVTEFAVNGRKLYLSPVIDLFNNEVISYSLSERPVMNMVENMLDQAFKKLNPHEHPVLHSDQGWQY 193
IS861   KRKYASYKGEVGKKADNLIQRQFEGSKPYEKCYTDVTEFALPEGKLYLSPVLDGYNSEIIDFTLSRSPALKQVQTMLERAFPA-ASYSETILHSDQGWQY 188
IS-S.P  ---------------------------------------------KLYLSPVLDGFNSEIIAFNLSCSPNLEQVQTMLEQAFTE-KHYENTILHSDQGWQY 55
                                                      *******.* .*.*.*...** .*  . * .**. **       . .*********

IS150   RMRRYQNILKEHGIKQSMSAKGNCLDNAVVECFFGTLKSECFYLDE--FSNISELKDAVTEYIEY-YNSRRISLKLKGLTPIEYRNQTYMPRV 283
IS861   QHKSYHQFLEDKGIRPSMSRKGNSPDNGMMESFFGILKSEMFYGLEKSYKSLDDLEQAITDYIFY-YNNKRIKAKLKGLSPVQYRTKSF---T 277
IS-S.P  QHDSYHRFLENKGIQASMSRKGNSQDNGMMESFFGILKSEMFYGYEKTFKSLNQLEQVIIDYIDY-YNNKRIKIKLKGLSPVQYRTKSF---G 144
          *.  *. .**. *** ***. **. .*.*** **** **  *  . . . * ... .** * **. **  *****.* .**...
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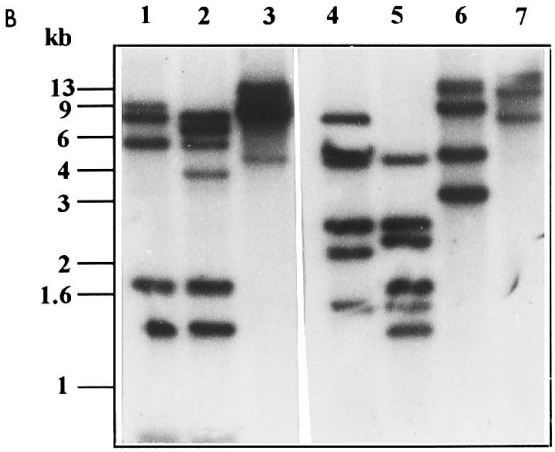

FIG. 5. *parE* gene lies downstream of a putative insertion sequence. (A) Partial ORF (IS-SP) in Fig. 4 is homologous to the transposases of insertion sequences IS*150* and IS*861* (31). Symbols are the same as those defined in the legend for Fig. 3. (B) Insertion sequence homolog IS-SP is present in multiple copies in *S. pneumoniae*. Genomic DNA from *S. pneumoniae* 7785 was digested (lanes 1 to 7) with *Eco*RI, *Eco*RI-*Eco*RV, *Eco*RV, *Hin*dIII, *Hin*dIII-*Hin*cII, *Hin*cII, and *Cla*I, respectively. DNA samples were electrophoresed in 0.8% agarose, transferred to a nylon filter, and hybridized to an IS-SP probe amplified by PCR from pXP1 (see Materials and Methods). Size markers are on the left.

sequence homology with other *gyrB* genes and its near identity to *nov-1*, a *gyrB* allele that confers novobiocin resistance in *S. pneumoniae* (27). Unlike *S. aureus* and *B. subtilis*, in which the *gyrB* and *gyrA* genes are adjacent (2, 22, 25), the *gyrB* gene was not closely linked (within 10 kb) to *gyrA*. Unlinked *gyrA* and *gyrB* genes in *S. pneumoniae* may have arisen by chromosomal rearrangement in this recombination proficient organism. The second homolog and its adjacent downstream gene were identified as *parE* and *parC*, specifying ParE and ParC subunits of *S. pneumoniae* DNA topoisomerase IV. Assignment was based on close sequence homology to the *S. aureus* topoisomerase IV subunits and the demonstration that when expressed in *E. coli*, the ParC and ParE proteins together reconstituted a salt-insensitive ATP-dependent DNA decatenase activity characteristic of a DNA topoisomerase IV. By examining *parC* genes in ciprofloxacin-resistant strain 7785 mutants, we found that mutation of Ser-79 to Tyr in the 823-residue ParC protein is implicated in low-level resistance. These results are the first molecular examination of fluoroquinolone resistance in *S. pneumoniae*.

We isolated a series of *S. pneumoniae* 7785 mutants resistant to ciprofloxacin by stepwise selection on plates containing increasing drug concentrations (Table 2). These strains also exhibited cross-resistance to sparfloxacin, a specific antipneumococcal fluoroquinolone. By PCR amplification of codons 35 to 157 of the *S. pneumoniae parC* genes, it was found that mutation of Ser-79 in ParC was absent in the parental strain or three first-step mutants (selected at a concentration of 2 μg/ml, i.e., two times the ciprofloxacin MIC) but appeared in each of four second-step mutants (selected at a concentration of 6 μg/ml) and was maintained in third- and fourth-step mutants (selected at concentrations of 9 and 15 μg/ml, respectively) (Table 2). DNA sequence analysis revealed in each case the ParC change was a Ser-79 to Tyr substitution. Thus, although not the first

TABLE 1. Comparison of sequence homologies among *S. pneumoniae* ParC and ParE proteins and subunits of *S. aureus* and *E. coli* topoisomerases[a]

Protein	% Amino acid identity							
	SA GyrB	SA GrlB	EC GyrB	EC ParE	SA GyrA	SA GrlA	EC GyrA	EC ParC
SP GyrB	62	49	48	35				
SP ParE	43	68	44	37				
SP ParC					30	51	33	30

[a] SA, *S. aureus*; EC, *E. coli*; SP, *S. pneumoniae.*

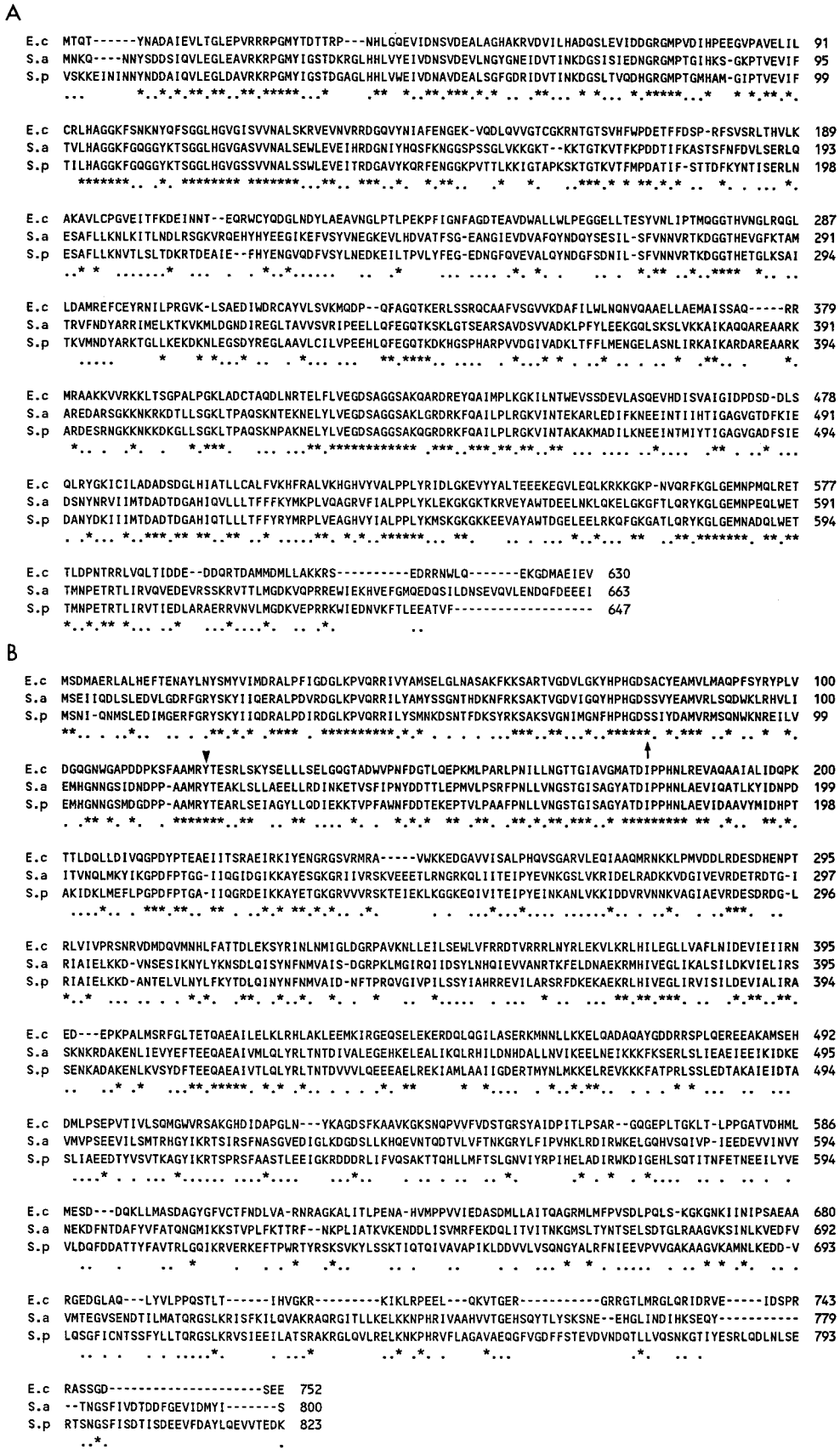

FIG. 6. Alignment of *S. pneumoniae* (S.p.) ParE (A) and ParC protein (B) sequences with their counterparts in *E. coli* (E.c.) and *S. aureus* (S.a.). Symbols are the same as those defined in the legend for Fig. 3 except that arrow denotes residue 79 in *S. pneumoniae* ParC and arrowhead indicates position of the catalytic tyrosine residue involved in DNA breakage-reunion (16). The sequences for ParE and ParC of *E. coli* and of ParE (GrlB) and ParC (GrlA) of *S. aureus* were taken from references 7 and 29, respectively.

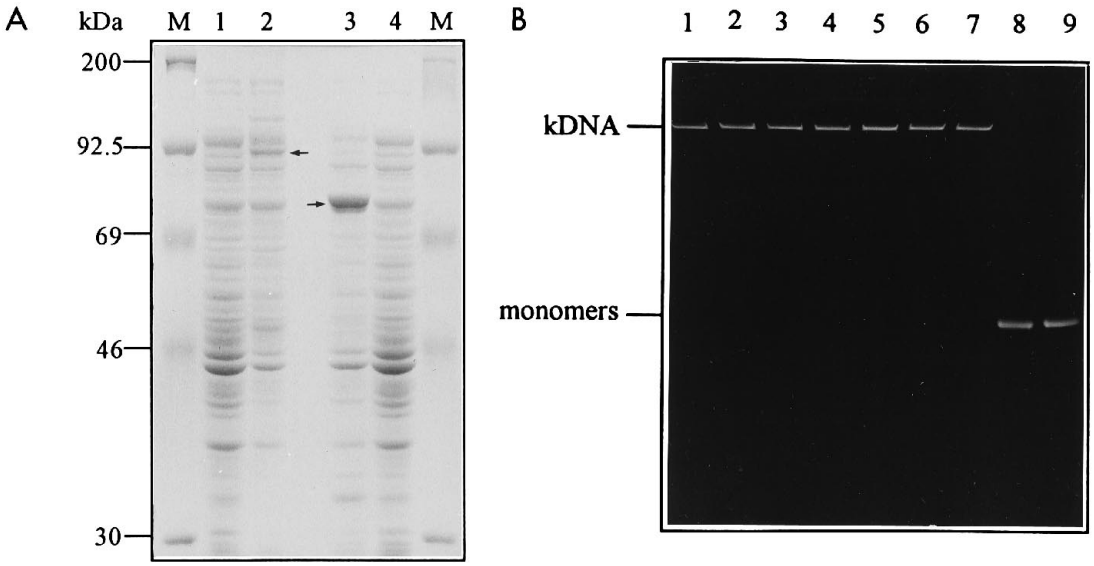

FIG. 7. ParC and ParE proteins reconstitute a salt-insensitive ATP-dependent DNA decatenase activity. (A) SDS-PAGE analysis of cell extracts from *E. coli* BL21 strains expressing *S. pneumoniae* ParE and ParC proteins. Lanes: 1 and 2, soluble extracts from uninduced and IPTG-induced *E. coli* containing pXP7; 3 and 4, extracts from IPTG-induced and uninduced *E. coli* containing pXP8. Proteins were electrophoresed in a 7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Arrows indicate the presence of overexpressed proteins. Sizes of protein markers (M) are indicated in kDa on the left. (B) Topoisomerase IV activity of ParC and ParE proteins. kDNA was incubated with topoisomerase proteins in the presence of 200 mM potassium glutamate. Lanes: 1, no addition; 2, *E. coli* DNA gyrase (10 U, 20 ng) 1 mM ATP; 3, parC extract (2 μg); 4, ParE extract (2 μg); 5, ParC and ParE extracts; 6 to 8, as in lanes 3 to 5 but 1 mM ATP included; lane 9, ParC and ParE extracts each diluted 10 times and with 1 mM ATP included. *E. coli* gyrase activity was determined in a supercoiling assay by using relaxed plasmid pBR322 as substrate (24).

event, ParC mutations occur early in the development of ciprofloxacin resistance in *S. pneumoniae*, suggesting that topoisomerase IV is highly sensitive to the drug in vivo.

These results may be compared with those observed in other bacteria. In the gram-negative species *E. coli* and *Neisseria gonorrhoeae*, quinolone resistance arises initially through mutations in *gyrA* (3, 5, 10, 28, 38) and additional mutation of *parC* leads to highly resistant isolates (1, 19). Thus, gyrase appears to be the primary target in these bacteria, with topoisomerase IV acting as a secondary target. By contrast, for the gram-positive bacterium *S. aureus*, mutations of codon 80 (Ser to Phe or Tyr) in the *grlA* (*parC*) gene of topoisomerase IV were found in clinical isolates exhibiting low-level resistance to ciprofloxacin (MIC, 2 to 16 μg/ml) and preceded those in *gyrA* of highly resistant isolates (MIC, >16 μg/ml) (7). Moreover, in studies on laboratory strains of *S. aureus*, *parC* mutations at Ser-80 or Glu-84 appeared in first step mutants selected at 2 μg of ciprofloxacin per ml; this was followed by *gyrA* mutations in subsequent steps (7). The observation of ParC mutations in first-step resistance mutants indicates that topoisomerase IV is the primary target for ciprofloxacin in *S. aureus*. Interestingly, studies of fluoroquinolone resistance in *Enterococcus faecalis*, another gram-positive species, showed that mutations in the *gyrA* gene were found only in highly resistant isolates (ciprofloxacin MIC, 32 to 64 μg/ml) (21). No *gyrA* mutations were present in laboratory isolates with low-level resistance (MIC, 8 to 16 μg/ml). Mutation of another locus, plausibly *parC*, precedes that of *gyrA* in *E. faecalis.*

S. pneumoniae is a gram-positive pathogen with a susceptibility to ciprofloxacin that is broadly similar to that of *E. faecalis* and *S. aureus* (30). However, unlike *S. aureus*, ParC mutations occurred in second-step rather than first-step quinolone-resistant mutants of *S. pneumoniae*. Moreover, no further mutations were found in the *parC* genes of more highly resistant mutants. Two models may be considered that explain this pattern of resistance development. In the first model, gyrase and topoisomerase IV are envisaged to be comparably sensitive to quinolone inhibition in vivo, with gyrase marginally more sensitive by a factor of two- to severalfold. First-step mutants could then arise through mutation of gyrase genes, but the level of resistance would be modest (even for Ser-84 GyrA mutants) because of the presence of topoisomerase IV, a drug-sensitive secondary target. Second-step mutations in ParC would then be necessary to achieve higher levels of resistance. In an alternate model, it is conceivable that first-step mutants exhibiting a twofold increase in resistance arise from changes in permeability or drug efflux or possibly elsewhere within ParC. Selection for ParC changes in second-step mutants is then followed by alterations in other genes in third- and fourth-step mutants, for which the *gyrA* gene would be an obvious

TABLE 2. Properties of mutant strains of *S. pneumoniae* 7785 selected for resistance by stepwise exposure in vitro to ciprofloxacin

Mutant[a]	Parent	MIC (μg/ml)[b]		parC mutation	
		CIP	SPAR	Nucleotide	Codon
	7785	1	0.3		
1st step					
1C1	7785	3	0.4	None	None
2nd step					
2C1	1C1	8	0.6	236(C→A)	Ser-79→Tyr
3rd step					
3C1	2C1	10	0.7	236(C→A)	Ser-79→Tyr
4th step					
4C1	3C1	100	20	236(C→A)	Ser-79→Tyr

[a] Ciprofloxacin at a concentration of 2 μg/ml was used in the first round of selection; this was followed by challenges at concentrations of 6, 9, and 15 μg/ml in subsequent steps. A minimum of three mutants were characterized in each round of selection (four second-step mutants were characterized).

[b] The MIC was the lowest drug concentration showing no growth on brain heart infusion plates containing 10% horse blood after aerobic incubation overnight at 37°C. CIP, ciprofloxacin; SPAR, sparfloxacin.

candidate. Clearly, information on *gyrA* will be important in distinguishing the models. Efforts thus far to isolate the *gyrA* gene, including repeated attempts at PCR amplification by using degenerate primers, have been unsuccessful (results not shown). However, whichever model applies, our data indicate that topoisomerase IV is a critical target for quinolones in *S. pneumoniae*. The Ser-79→Tyr ParC mutation found in second-step resistant mutants is associated with growth at a 6 μg/ml concentration of ciprofloxacin (up from 2 μg/ml), a level sufficient to confer clinical resistance to the drug (30). Consistent with this idea, we have found that *S. pneumoniae* clinical isolates exhibiting low-level ciprofloxacin resistance (MIC, 4 μg/ml) do indeed carry Ser-79 ParC mutations (unpublished data).

The work presented here suggests that topoisomerase IV plays a key role as a fluoroquinolone target in *S. pneumoniae*. This situation is similar to that in *S. aureus* but differs from gram-negative species in which gyrase is the prime target. One may speculate that these species differences arise simply from the relative fluoroquinolone sensitivities of gyrase and topoisomerase IV. Thus, unlike *E. coli* gyrase, which is very sensitive to drug action, *S. aureus* gyrase is much more resistant (36). An inherently resistant gyrase in *S. pneumoniae* and other gram-positive bacteria could bring topoisomerase IV into play as a target. Extensive characterization of the *S. pneumoniae parC*, *parE*, and *gyrB* genes reported here should facilitate further work in this important gram-positive respiratory pathogen.

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REFERENCES

1. **Adams, D. E., E. M. Shekhtman, E. L. Zechiedrich, M. B. Schmid, and N. R. Cozzarelli.** 1992. The role of topoisomerase IV in partitioning bacterial replicons and the structure of catenated intermediates in DNA replication. Cell **71:**277–288.
2. **Alonso, J. C., and L. M. Fisher.** 1995. Nucleotide sequence of the *recF* gene cluster from *Staphylococcus aureus* and complementation analysis in *Bacillus subtilis recF* mutants. Mol. Gen. Genet. **246:**680–686.
3. **Belland, R. J., S. G. Morrison, C. Ison, and W. M. Huang.** 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Mol. Microbiol. **14:**371–380.
4. **Brown, P. O., and N. R. Cozzarelli.** 1980. A sign inversion mechanism for enzymatic supercoiling of DNA. Science **206:**1081–1083.
5. **Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fisher.** 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrob. Agents Chemother. **33:**886–894.
6. **Eliopoulos, G. M.** 1995. *In vitro* activity of fluoroquinolones against gram positive bacteria. Drugs **49**(Suppl. 2)**:**48–57.
7. **Ferrero, L., B. Cameron, and J. Crouzet.** 1995. Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39:**1554–1558.
8. **Ferrero, L., B. Cameron, B. Manse, D. Lagneux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target for fluoroquinolones. Mol. Microbiol. **13:**641–653.
9. **Finch, R. G.** 1995. The role of new quinolones in the treatment of respiratory tract infections. Drugs **49**(Suppl. 2)**:**144–151.
10. **Fisher, L. M., M. Oram, and S. Sreedharan.** 1992. DNA gyrase: mechanism and resistance to 4-quinolone antibacterial agents, p. 145–155. *In* T. Andoh, H. Ikeda, and M. Oguro (ed.), Molecular biology of DNA topoisomerases and its application to chemotherapy. CRC Press, Boca Raton, Fla.
11. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. I. Tomizawa.** 1977. Nalidixic acid resistance: a second character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
12. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.
13. **Gellert, M., M. H. O'Dea, T. Itoh, and J. I. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling by DNA gyrase. Proc. Natl. Acad. Sci. USA **73:**4474–4478.
14. **Hanahan, D.** 1985. Techniques for transformation of *Escherichia coli*. *In* D. Glover (ed.), DNA cloning: a practical approach. IRL Press, Oxford.
15. **Hopewell, R., M. Oram, R. Briesewitz, and L. M. Fisher.** 1990. DNA cloning and organization of the *Staphylococcus aureus gyrA* and *gyrB* genes: close homology among gyrase proteins and implications for 4-quinolone action and resistance. J. Bacteriol. **172:**3481–3484.
16. **Horowitz, D. S., and J. C. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **269:**5339–5344.
17. **Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J. I. Kato, and H. Ikeda.** 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob. Agents Chemother. **38:**2623–2627.
18. **Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Higara, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *E. coli*. Cell **63:**393–404.
19. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli.** 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**11801–11805.
20. **Klugman, K. P.** 1990. Pneumococcal resistance to antibiotics. Clin. Microbiol. Rev. **3:**171–196.
21. **Korten, V., W. M. Huang, and B. E. Murray.** 1994. Analysis by PCR and direct DNA sequencing of *gyrA* mutations associated with fluoroquinolone resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **38:**2091–2094.
22. **Margerrison, E. E. C., R. Hopewell, and L. M. Fisher.** 1992. Nucleotide sequence of the *Staphylococcus aureus gyrB-gyrA* locus encoding the DNA gyrase A and B proteins. J. Bacteriol. **174:**1596–1603.
23. **Mizuuchi, K., L. M. Fisher, M. H. O'Dea, and M. Gellert.** 1980. DNA gyrase action involves the introduction of transient double-strand DNA breaks into DNA. Proc. Natl. Acad. Sci. USA **77:**1847–1851.
24. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea, and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259:**9199–9201.
25. **Moriya, S., N. Ogasawara, and H. Yoshikawa.** 1985. Structure and function of the region of the replication origin of the *Bacillus subtilis* chromosome. Nucleotide sequence of some 10,000 base pairs in the origin region. Nucleic Acids Res. **13:**2251–2265.
26. **Morona, J. K., A. Guidolin, R. Morona, D. Hansman, and J. C. Paton.** 1994. Isolation, characterization, and nucleotide sequence of IS*1202*, an insertion sequence of *Streptococcus pneumoniae*. J. Bacteriol. **176:**4437–4443.
27. **Muñoz, R., M. Bustamante, and A. G. de la Campa.** 1995. Ser-127-to-Leu substitution in the DNA gyrase B subunit of *Streptococcus pneumoniae* is implicated in novobiocin resistance. J. Bacteriol. **177:**4166–4170.
28. **Oram, M., and L. M. Fisher.** 1991. 4-quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrob. Agents Chemother. **35:**387–389.

28a. **Pan, X. S., and L. M. Fisher.** Unpublished data.

29. **Peng, H., and K. J. Marians.** 1993. DNA topoisomerase IV: purification, characterization, subunit structure and subunit interactions. J. Biol. Chem. **268:**24481–24490.
30. **Piddock, L. J. V.** 1994. New quinolones and gram-positive bacteria. Antimicrob. Agents Chemother. **38:**163–169.
31. **Rubens, C. E., L. M. Heggen, and J. M. Kupers.** 1989. IS*861*, a group B streptococcal insertion sequence related to IS*150* and IS*3* of *Escherichia coli*. J. Bacteriol. **171:**5531–5535.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Sreedharan, S., M. Oram, B. Jensen, L. R. Peterson, and L. M. Fisher.** 1990. DNA gyrase *gyrA* mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: close similarity with quinolone resistance mutations in *Escherichia coli*. J. Bacteriol. **172:**7260–7262.
35. **Sugino, A., N. P. Higgins, P. O. Brown, C. L. Peebles, and N. R. Cozzarelli.** 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. USA **75:**4838–4842.
36. **Tanaka, M., K. Sato, Y. Kimura, I. Hayakawa, Y. Osada, and T. Nishino.** 1991. Inhibition by quinolones of DNA gyrase from *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35:**1489–1491.
37. **Willmott, C. J. R., and A. Maxwell.** 1993. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrob. Agents Chemother. **37:**126–127.
38. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34:**1271–1272.
39. **Yoshida, H., M. Nakamura, M. Bogaki, H. Ito, T. Kojima, H. Hattori, and S. Nakamura.** 1993. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **37:**839–845.