

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 A₂B₂ 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 C₂E₂ 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 con-

fer 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 10⁵ CFU) onto plates containing dilutions of ciprofloxacin or sparfloxacillin and incubating aerobically overnight at 37°C.

Stepwise selection of ciprofloxacin-resistant *S. pneumoniae* mutants. Strain 7785 (about 10¹⁰ 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 6 µg 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 MgCl₂, 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 *NcoI* and *KpnI* 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 *HindIII* 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 *HindIII* (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' ATTGCCAACCAACCAATCT (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 ³²P-radiolabeled DNA probes obtained by random priming with [α -³²P]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 *NdeI* 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 *BamHI* or *XhoI* site was introduced just downstream of the *parE* and *parC* stop codon. For *parE*, the forward primer was N7043, 5' AGGAGGTTCCATATGTCAAAAAAG (*NdeI*), and the reverse primer was N7044, 5' TATTTGGATCCATTAAAAACACTGTC (*BamHI*). Primers for the *parC* gene were N6894, 5' TGGGCTTGTATCA TATGTCTAAC (*NdeI*), and N6895, 5' AGAACTTATTGAGCTCTTCACTTA (*XhoI*). 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 *Criethidia 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 *HindIII* 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 *HindIII* 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 *HindIII* 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

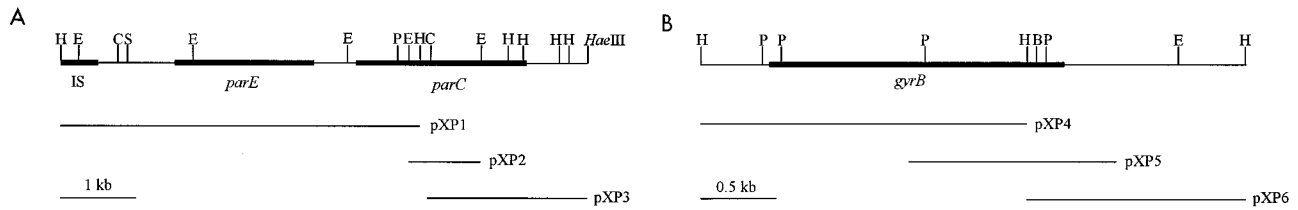


FIG. 1. Restriction maps of the *parE-parC* (A) and *gyrB* (B) loci in *S. pneumoniae* 7785 and alignment of plasmid clones. The *parE*, *parC*, and *gyrB* genes are indicated by heavy lines. IS denotes a region upstream of *parE* displaying strong homology to the insertion sequence IS861. B, C, E, H, P, and S indicate restriction sites for *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *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 *Hind*III 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 *Hind*III 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  AAGCTTTAAAGTTTCTACTCCTTTTGGCATTGAGACATTCGCTCCAATTTAGAGATTTTTAGAAAAGTACAAGGAAAATGAAGCCAGAGAGCTTGAACCCCGATTTATTTGAAG
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 GAGTTTCTGACCTATTGGAAGACATTTTAAATCAAGGTGGCCGTCATTTTTGGTCTCTCATCGAAATGATCAGGTTTTGGAATTTAGAAAAAACCTCTATAGCAGCTTATTTTACAG
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 AAGTGGTACTTCTAGCTCAGGCTTTAAGAGAAAGCCAAATCCGAATCCATGCTTTATTAAGAGAAAGTATCAGATTAGCTGTGGTCTTGGTATTGGTATTGGTATTGATATCG
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 AAGCAGGTCAAGCTGCAGGACTTGATACCCACTTGTTTACCAGTATCGTGAATTAAGACAAGTATAGACATATAAGAAAAAGGAATAAGATGACAGAGAAATCAAAATCTGCAGGCT
Q D V 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 L L V W E I
481 ACAGGATTATGATCCAGTCAAATCAAGTTTTAGAGGGCTTAGAGGCTGTTCGATTCGCTCCAGGATGTACATTGGATCAACCTCAAAAAGAGGCTTCCACCTAGTCTGGGAAAT
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 TGTTGATACTCAATTGACGAGGCTTGGCAGGATTTGCCAGCATATCAAGTTTTATTGAGCCAGATGATTCGATTACTGTTGGATGATGGCGTGGATCCAGCTCGATATTC
E K T G R P A V E T V F T V L H A G G K F G G G Y K V S G G L H G V G S S V V
721 GGAAAAACAGCCGCTCTGCTGTTGAGACCGTCTTACAGCTCTCATGCTGGAGGAAAGTTCGGCGGTGGTGATACAAGGTTTCAGTGGTCTTCCAGGGGTGGGTGCTCAGTAGT
N A L S T Q L D V H V H K N Q K I H Y Q E Y R R R G H V V A D L E V V D T D R T
841 TAATGCCCTTCCACTCAATAGACGTTTCATGCCATAAAAAAGGTAAGATTCATACCAAGAATACCGTCGTGGTTCATGTTGTCGACGCTTGGAGTGGTGGGGATACAGATAGAAC
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 TGGAACAACGGTTCACCTCACACGGACCCAGAAATTTCACTGAACAACAATCTTTGATTTGTATAAATAAACAAGGATTCAGAGGTTGGCCTTTCTAAATCCGCGCTTCAAAT
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 TTCAATTACAGATAGCGCCAAAGGTTTGAACAACAACAAGCATTATCATTGAAGGTGGGATGCTAGTTCAGTTGAATATATCAACGAGAAAGGATTAATCTTTGATACCAACCAAT
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 CTATACAGCGGTGAGATGATATCACAGTTGAGTAGCCATGACACACAGGTTACCATGAAAATGTATGAGTTTCGCCAATAATATTCATACCCATGAAGTGAACGCA
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 TGAAACAAGGTTCCGTCAGCGACTGACTGCTGTTCAATGATTTGCTGTAAGATAAAGTACTGAAAGACAATGAAGATAAATTAACAGGGGAGGATGTCGCGAAGGATTAACGTCG
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 AGTTATCTCAGTAAACACCAATCCACAGTTTGAAGGACAAACAAGGCAATTTGGGAAATAGCGAAGTGGTCAAGATTACCAATCGCTCTTCCAGTGAAGCTTCCGATTTCT
M E N P Q I A K R I V E K I L A K A R V A A K R V R E V T R K G S G L E I S
1561 CATGGAATAACACAGATGCCAAACGATCGTGGAAAAAGGATTTAGCTGCCAAGGCTCGTGTGGCTGCCAAGCGTGGCGTGAAGTACACGTAATAAATCTGGTTTGGAAATTC
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 CAACCTTCCAGGGAAGTACGACGACTGTTCTTAAACCTGCTGAAACAGAATCTTCATCGTGAAGGAGACTCAGCTGGTGGATCAGCAGCAATCTGGTCTGAACCGTGAAGTTCA
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 GGCTATCCTTCCAATTCGCGTAAGATTTTGAACGTTGAAAAGCAAGTATGATGAAGTTCAGTAACGAAAGAAATTCGTAGCTTTTCCACAGCAGTGGGAACAGGATTTGGCGAG
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 ATTTGATGTTTCCGAAAGCCGTTACCAAAAACCTGTTTATGACCGATGCCGATGTCGATGGAGCCACATTCGTACCTCTTTTAACTTGATTTATCGTTATATGAAACCAATCCT
E A G Y V 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 AGAAGCTGATGTTATGTTATATGCCCCAACCAACTATGTTGTTCAAGGTTGGAAGCGAGATTAAGAATATATCCAGCCGGTGCAGATCAAGAAATCAAATCCCAAGAGCTTAGC
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 CCGTTATAGTGAAGGCTACCAAAACGACTATTCAGCGTTATAAGGGGCTAGGTGAAATGGACGATCATCAGCTGTGGGAAACAACCATGGATCCCGAACATCGCTTGDGTTAGGCTAGAGT
S V D D A A E A D K I F D M L M G D V E P R R E F I E E N A V V ***
2281 TTCCTGATGATGCTGCAGAGCAGATAAATCTTGATATGTTGATGGGGATCGAGTAGAGCTCGTCTGAGTATATCGAAGAAAATGCTGTCTATAGTACACTTGATGTCAA
2401 AATGAGGGAAGTATCCCTGGTCAAAAATATGATATAATCGTTACGATGTTTCAAGTAAAAAGGAGTTGATATGCTAATGGACAACATAATTTAATGGTTGCAATGCA
2521 AGTCATTTTATGCTGCTTATGATGAGCAATCTTCTAGCTAAGCGAAACGAGGAGATTTAGAGGCTGAGAAGAAAAGAAAGAACTATACAATCTCCAGCTTAATGATGAAGT
2641 GAAGCTGAAAAAATATGACCTTGAATGACAAAAGTCAAGTGGCTTCCGTTGAATGAAATCAAAAATGGTTCGATTTACTCTCAACTCTTTTGGCGATTTGAAAATAATCTTTGAAG
2761 CAGAAGGCTATAACCATTCATTTCTGTTTCTCAAGGCCAGTCAATTAAGCAAAATGAGAGTCAAAATTACTTTGATGACGAAAGATTTGGCGCAATTCGAATGCTTTGGCAGACT
2881 TAGAAGAGCAAGAAATCAAAAATAGTGGTCTGTTCTTATGAGCAATCAAGAAAACCAAGAGAAATTTCTGTCAGTTGAAATGGATAATGCGGAATTCAGAACAGATTTGGTCAAGCTTGAATG
3001 AAAAAAATAGAAAATATCCAATCTGAATTTTCACAATTTGTAACCTTGAATTCATCGGTCAGCCCTGTGGAAGCCGAGTATTGGATAATACAGAAAATCACATTTGGCCCTTAA
3121 GTCATATTGGTGGTCTGTTCCAGCCTTGGTTACGACGCTTCTACAGAATGGCAGATCAATTACAGGATTTGGAAGCCGGTATCGTAACTAATGATGCTAATTCATTTGTTG
3241 AAACGGATTTGAAAGCCGCTTTCCACTGCTTTATGAGCAATCAAGAAAACCAAGAGAAATTTCTGTCAGTTGAAATGGATAATGCGGAATTCAGAACAGATTTGGTCAAGCTTGAATG
3361 ATCAATGCTTGTATGATATTTTACTCGAAGAAATGCTGCTCAGAAGTGTGGAAGAACTACTTGAACCTCTTCAACTATCTTCAACATATGAAAGAGATAATACTTTATTTGGGA
3481 GAAGATATTGACGCTTTGAACAAGACCTATTACTTCTGAGACAGCTGCAAGCCATGTTCTGCTGATTCAGACAGAATAGAGAGTTTGGGCGAGCTATTGTTGAGGTAACCTCAAAT
3601 CAAGAAGAACCAACCAAGCTT
    
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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.

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K L Y L S P V L D G F N S E I I A F N L S C S P N L E Q V Q T M L E Q A F T E K
 1 AAGCTTTACTTACCAGTTTAGATGGCTTAAACAGCGAAATTTGCTTTAATCTTCTGTTCGCCTAATTTAGAACAGTACAAACCATGTTGGAACAGGCATTCACAGAGAAG
 H Y E N T I L H S D Q G W Q Y Q H D S Y H R F L E N K G I Q A S M S R K G N S Q
 121 CACTACGAGAATACGATTCCTCATAGTACCAAGGCTGGCAATACCAACACGATCTTCTATCGKTCCTAGAGAAATAGGGAATTAAGCATCTATGTCACCGAAGGAAACAGCCAA
 D N G M M E S F F G I L K S E M F Y G Y E K T F K S L N Q L E Q V I D Y I D
 241 GACAACGGTATGGAATCTTCTTTGGCATTAAAATCCGAAATGTTTATGGCTATGAGAAAACATTTAAATCACTTAACCAATTTGAAACAAGTATATAGACTATTATTGATTAC
 Y N N K R I K I K L K G L S P V Q Y R T K S F G ***
 361 TACAACAATAAACGAATTAAGATAAACTAAAGGACTTAGCCCTGTGCAATACAGAACTAACTCTCGGATAAATTAATGTCTAACTGTTGGGGTCAGTACAGAACTGGCGTTTTA
 481 TTTTTAGGACTTGTAGGGTTAGGTTCAATCCCAAGGGACCAAAATTTTCAGTTTTAATTTTGATACGAGCTAATTTGCTTATGACGAATGATAATCAAATAGCAAGTGTAAAGAT
 601 AATAGCGATGAAGAGAAGTCATAGTTACTCAGGATAAAAACAAAAGTGGAAAGAGCAGAACCCCGATAACAGCCGCAATCGATGCTGTGACACTAGACAGTGAATCATACTGCCAAG
 721 ATAGAGAGCTCAAAGAAGATAATCGCAAGGTAGAGACAGAAGATAGGCGCAATCCGAAAATCACTCCAGCACTGGTTGGCAGAGCTTACCACCTTTAAATCTCGAAAGATAGGGAA
 841 GGTATGGCCGATAACAGCCAAAAGTCAAAGATGAGAGGAGAAACCGCTGTAGATGAAAAAATCGGAAGCAGCGTGTAGGGTTCCTTTGAAAAGTCAATCACAAGGTTGCCAT
 961 ACCAGCTTTCTTACCTAAAATGCGGAAGGTGTTGGTCTCCAGTGTACCAGAACCATGCTCGCGTAGATTGATTTGAAAAGTACTTGTCCAATCCAGAGCCAGATGGAATCGAAC
 1081 CAGCAGATAGGCTAGGATTAATAAACTATTGTAATCACTCTATTATATATCATGAATTTGGGAAAGAAAAGCAGAGAATCTCTGCTGAAATTTGCACATCGGAGAAAAGAAAATTTGC
 V S K K E I N I N N Y N D D A I Q V L E G L
 1201 AAAATCCTTGGAAAACCTGTAGAATAGTAAAGATGAACGAATAGGAGGTTCTTGTTCGAAAAGGAAATCAATATAAACAATTTAATGATGATGCTATTCAGGTGCTAGAGAGGTTG
 D A V R K R P G M Y I G S T D G A G L H L V W E I V D N A V F D E A L S G F G D
 1321 GATGCGGTCCGAAAACGTCGGGGATGTATTTGGATCGACCGATGGCGTGGTCTTACACCTAGTTTGGGAAATCGTTGATAATGCAGTCGATGAAGCCTTGTCTGGTGGTGGTGT
 R I D D V T I N K D G S L T V Q D H G R G M P T G M H A M G I P T V E V I F T I L
 1441 CGTATTGATGAACATCAATAAAGACGGTAGTCTAACGGTTCAGACCATGGACGGATGCGCAGAGTATGCGCCTATGGGAAATTCACCTTTTACCTTATCTTACCTTCT
 H A G G K F G Q G G Y K T S G G L H G V G S S V V N A L S S W L E V E I T R D G
 1561 CATGCCGAGGAAATTCGGTCAAGGTGGCTATAAGACATCAGTGGACTTCACGAGTGGGTCTTCGGTGTGTAACGCCCTTTCTAGCTGGTATAGAAATGAAATCCCGTATGGC
 A V Y K R F E N G G K P V T T L K K I G T A P K S K T G T K V T F M P D A T I
 1681 GCAGTTTACAAGCAACGTTTCAAATGTTGAAAACCTGTCAGACTTGAAGAAAATCGGTACAGCACCCAAAGTCTAAAACAGGCACCAAAAGTACTTTTATGCCTGACCGACTATA
 F S T T D F K Y N T I S E R L N E S A F L L K N V T L S L T D K R T D E A I E F
 1801 TTTTCTACGACAGATTTCAAGTACAATACCATTTCAGAGCGCTTAATGAATCACTAGCTTTCTTCAAATAATGTCAGCTTGTCTTAAAGGACAGCAACAGATGAAAGCAGTTAGTTC
 H Y E N G V Q D F V S Y L N E D K E I L T P V I Y F E G E D N G F Q V E V A L Q
 1921 CACTATGAAATGGATACAAGATTTGTTCTTATCTCAACGAAGATAAGGAAATCTTGACCGCAGTCTTACTTTGAAAGGGAAGACAAATGGTTTTCAAGTGGAAATGACCTCCAG
 Y N D G S F N D I L S F V N V R T K D G G G T H E T G L K S A I T K M N D Y A
 2041 TACAATGACGGATCTCAGATAACATCTATCTTGTCAATAACGTTCCGACCAAGGACGGTGAAGCAGCAGAGCAGGACTCAAGTCTGCCATTCCAAGTCTGAATGACTATGCA
 R K T G L L K E K D K N L E G S D Y R E G L A A V L C I L V P E E H L Q F E G Q
 2161 CGTAAAACAGGCTTCTCAAGGAAAAGATAAAAACCTTGAAGGTTCAAGACTATCGTGAAGGACTAGCGGCGTCTTTGTATCTAGTTCTGAAAGAACACTGCAAGTTGAAGGACAG
 T K D K H G S P H A R P V V D G I V A D K L T F F L M E N G E L A S N L I R K A
 2281 ACCAAGGATAAACATGGAAGCCCAATGCTCGCCAGTTGTGGATGGAATAGTGGCTGATAAGTTGACCTTTTCTTATGAAAATGGGAAATAGCTTCACTACCTATCCGCAAGGCT
 I K A R D E A A R K A R K A R D E S R N K K N K K D K G L L S G K L T P A Q S K
 2401 ATCAAGCCCGTGTGCTGTGAAGCAGCAGTAAAGCCGCTGATGAGAGCCGAAATGGGAAGAAAACAAGAAAGATAAGGGCTTGTGTCTGGGAAATGACCCACGCCCAATCTAAG
 N P A K N E L Y L V E G D S A G G S A K Q G R D R K F Q A I L P L R G K V I N T
 2521 AATCTGCTAAGAAATGAACCTATCTAGTTGAGGGGACTCTGCGGTGGTCTGCGAAACAAGTCTGACCGCAAGTCCAGGCTATCTACCTCTTCGTTGGTAAAGTATCAATACA
 A K A K M A D I L K N E E I N T M I V T I G A G V G A D F S I E D A N Y D K I I
 2641 GCCAAGCCAGATGGCGGATCTCAAATGAAGAGATCAATACCTGATTTATACCTTTGGTGGCGGTTGGAGCAGACTCTCTATTGAAGATGCCAATGATAAGATCATT
 I M T D A D T D G A H I Q D L L T F F Y R Y M R P L V E A G I A L P P L
 2761 ATCATGACCGATGCGGATACCGCAGTGGCCATATCCAGACCTTGTCTTACATTTTCTACCGTTACATGCGTCCGCTAGTCGAGGCAGGTCATGCTATATGCCCTCCACCTCTT
 Y K M S K G K G K K E E V A Y A W T D G E L E E L R K Q F G K G A T L Q R Y K G
 2881 TACAAGATGTCCAAAGGTAAGGAAAAGAAAGAAAGTGGCCTGACCTGTCGACGGACGGAAGTGAAGAAAGTCCGTAACACAGTTCGTAAGGCGCTACCCTCAAAGGATAACAAGGA
 L G E M N A D Q L W E T T M N P E T R T L I R V T I E D L A R A E R R V N V L M
 3001 CTGGTGAGATGAATGCGGACCGCTGCGGAAACAACCATGAACCCAGAACACGTCACCTCTCGTGTACAATTTGAAGATTTAGCGCGCCGCAACCCCGCTCAATGTTCTCATG
 G D K V E P R R K W I E D N V K F T L E E A T V F ***
 3121 GGAGATAAGGTAGAACCACCGCTAAATGGATTGAAGATAATGTCAAGTTACGCTAGAAGAGCGACAGTGTTTAATGAAAGAAAATTAATGTTAGTAACTGGAAATAGAGTCGG
 3241 ATGTGAATGACTATGTGAAAAGCAGTTGAAAATCTTGGTTTGAAAAATTTGCAAGTATTAATGAAGATCTGCTATGAGTCTTATCTCAAAGAGCGCTTAAGGAGCGGCTAAG
 3361 ACACAACTAAGACTAATTTGAAAACAGATTTTCATTTGAAAATACAAACAGGATAGTTGGTACTTATCGTAACAGAACCGGAGGAAATCCCGTAAAATCTTTTACTCG
 3481 AATAAACTACTCTACATCTTCTGAAAGAGTGAACACGCCCTAGATACTGTGTGAAAAGATAAATCTCTGTGAGTTGCTTACTCTCAAGAAATTTCTATTTTCACTGGTATTT
 M S N I Q N M S L E D I M G E R F G R Y S K Y I I Q D R A L P D I R D
 3601 TATGGCTTTGTATCTTATGTTTAAACATGTCCTCGGAGGACATCATGGAGAGCGCTTTGGTCCGCTACTCCAAGTACATTAATCAAGACCGGGCTTTGCCAGATATTCGTG
 G L K P V Q R R I L Y S M N K D S N T F D K S Y R K S A K S V G N I M G N F H P
 3721 ATGGTTGAAGCGGTTGAGCGGCTATCTTATCTTGAATGAAGATGCAACTTTTGAACAAGGCTACCGTAAGTCCGCAAGTCAAGTGGGAAACATCATGGGAAATTTCCACC
 H G D S S I Y D A M V R M S Q N W K N R E I L V E M H G N G S M D G P P A A
 3841 CACACGGGGATCTTCTATCTATGATGCCATGTTGCTGATGTCACAGAACTGGAAAATCGTGAGATCTAGTTGAAATGACCGGTAATAACGGTCTGATGGACGGAGATCTCCTCGCG
 M R Y L T E A G R L S E I A G Y L L Q D I E K K T V P F A W N F D D G T E K E P T V L
 3961 CTATGCGTTATCTAGGACCGTTGTCTGAAATTCGAGGCTACTTCTCAGGATATCGAGAAAAGACAGTTCCTTTGCTGATGGAATTTGACGATACGGAGAAAACCAAGGCTCT
 P A A F P N L L V N G S T G I S A G Y A T D I P P H N L A E V I D A A V Y M I D
 4081 TGCCAGAGCCTTTCAAACCTCTTGGTCAATGGTTCGACTGGATTTGGCTGGTATGCCACAGACATCTCCCAATAATTTAGCTGAGTATAGATGCTGAGTGTACATGATG
 H P T A K I D K L M E F L P G P D F P T G A I I Q G R D E I K K A Y T G K G R
 4201 ACCACCAACTGCAAGATGATAAACTCATGGAATCTTACCTGGACAGACTTCCCTACAGGGCTATTATTAGGGTCTGATGAAATCAAGAAGCTTATGAGACTGGGAAAGGGC
 V V V R S K T E I E K L G K G E Q I V I T E I P Y E I N K A L N V K I D D V
 4321 CGGTGGTGTCTGTTCAAGACTGAAATGAAAAGCTAAAAGTGGTAAGGAACAATCGTTATTACTGAGATTCCTTATGAAATCAATAAGGCCAATCTAGTCAAGAAAATCGATGATG

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

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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 TTCGTGTTAATAACAAGGTAGCTGGGATTGCTGAGGTTCTGATGAGTCTGACCGTGATGGTCTTCGTATCGCTATCGAACTTAAGAAGACCGTAACTACTGAGCTTGTCTCAACTACT
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 TATTTAAGTACACCGACCTACAAAACAACCTACAACCTTAAATATGGTGGCGATGCAAAATTCACACCTCGTCAAGTGGGATTGTCCAATCTGCTAGCTACATCGCTCACCGCTCGAG
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 AAGTGATTTTGGCGCTTACGCTTTGACAAAGAAAGGCTGAGAAACGCTCCATATCGTGGAAAGTTGATTCGTGTGATTTCGATTTGGATGAAGTCATTGCTTATCGGTGCTT
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 CTGAGAATAAGCGGACGCCAAGGAAACCTCAAAGTTAGCTATGATTTTACGGAAAGACAGGCTGAGGCTATCGTAACTTTGCAACTGTACCGTTTACCAATACCGATGTGGTTGTCT
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 TGCAGGAAGAAGCAGAGCTTCGTGAGAAGATTGCTATGCTGGCGCTATTATCGGTGATGAAAGGACTATGACAATCTCATGAAGAAAGAACTCGTGAGGTCAGAAGAAATTTG
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 CAACTCCTCGTTTGGATTCCTTAGAAGACACTGCGAAAGCAATTGAGATTGATACAGTCTTATCGCTGAGGAAGATACCTACGTCAGCGTGACCAAGGCGAGTTACATCAAGCGTA
S P R S F A A S T L E E I G K R D D R L I F V Q S A K T T Q H L L M F T S L G
5161 CCAGTCCAGCTTCTTTCGGCTCCACCTTGGAAAGAAATGGCAAGCGTATGATGACCGTTTGTATTTGTTCAACTGCCAAGACAACCCAGCACCTTGTGATTCACAAGTCTTG
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 GAAATGTCATCTACAGACCAATCCATGAGTGGCAGATATTCGTTGGAAGGACATCGGAGGACATCGAGCCAAACCATCACAACTTTGAAACGAAATGAAGAAATCCTTATGTGGGAG
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 TACTGGATCAGTTTGCAGTACGACCACTTTCAGTGACTCGCTTGGTCAAATCAAACGGGTAGAGCGAAAGAAATTCACCTCCATGGCGGACATAGATTAAGTCTGCAAGT
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 ATCTAAGCTCAAAGACGATACAGACTCAGATTGTAGCAGTGGCTCCGATTAACACTAGATGATGTGCTGGTGTAGTCAAATGGTTATGCCCTGCGTTTCAATATCGAAGGTTCCGG
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 T Q R G S L K
5641 TTGTCGGTGTAAAGCAGCGAGTGTCAAGGCTATGAATTTGAAAGAAAGATGATGCTCCCAACTGGCTTATCTGTAATACTTCGCTCTTACCTTTCACCTTTCACCCAGCGTGAAGCTTGA
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 AACGTGTTCTATTAGGAAATCTAGCAACCCGCTGACCAACCGAGGATTAACAAGTTCGCTGAGTTGAAAACAAACCCGATCGTGTCTTTCGGCAGGACAGTTCAGAGGCAAG
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 GATTTGTTGGCGATTCTTCAGTACGGAAGTGGATGACGACCAACTGCTGTTCAATCAAAGAAACCAATCTAAGAAAGCTTGAACCTTGAACCTTGTGAGAAGCGCA
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 CTAGCAATGGAAGCTTCAATTTCTGACAGATTCAGATGAAGAAGTTTTGACGCTTATCTTCAGGAAGTAGTTACTGAAGATAAATAAGTGAATGAAGAATAAGTCTAAGAGCTGGT
6121 TTTTATGGAGAAATTTCTGAAAATACAAAATATACTTCTGATTTTGAAGAAATAGTGTAGAATATAAGAATAGGTTTTAGAAATAAGGATGGAATATGACAGTACCGATTGATGGT
6241 GAAAACCTCGGTTTTCTATATGAAATACCTTATCGCTATCTGCTATTTCAAATAAGGATGGGATCAAGGAGAGCTTACAGAGGATGCAACTTTGCTATTTTCAGAGCTTCT
6361 CCAAGTCTCAGTATGGAACAACAGCTTTCAGGTTTGAAGCTTATCGTACTAAGGATGGCAGTGTCAACTGTTCCGTCTGATGAAAATGCTAAACGCTGCAACGCTACATGTGAC
6481 CGTCTCTGATGCCACAAGTTCGACAGACATGTTGTAGAAGCTTGAAGCAGTGTCCGTCGGAATGAAGAAATCGTACCACCATCGGAACAGGTTGAACCTTATATCTTCCGCTT
6601 CTTTGTATGGTGTGCGAGATATTATCGGGTAAAACCGGACAGAGTACATTTTACCATCTTGTATGCCAGTGGAAATTAAGGTTGGTTCACCAACTCTTCTG
6721 ATTCAGGATGATACGACCGTGCAGCACCAAATGGTACAGGTGCGGCTAAGTTGGTGGAAACTATGCTGCAAGTCTTACCCAGGAAAAAT
    
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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 10¹⁰ 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 sparflloxacin (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 sparflloxacin 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

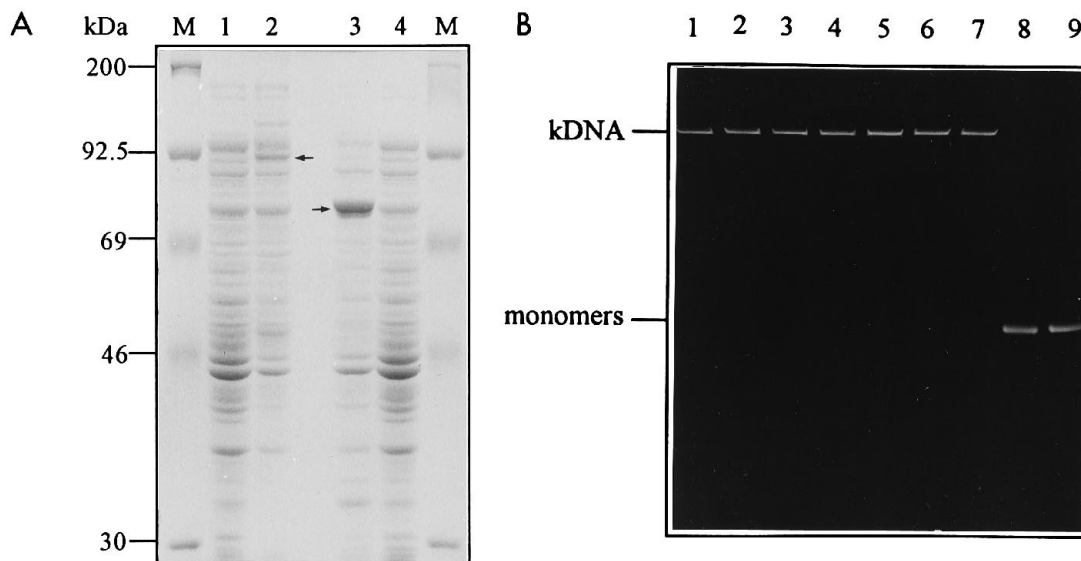


FIG. 7. ParC and ParE proteins reconstitute a salt-insensitive ATP-dependent DNA decatenase activity. (A) SDS-PAGE analysis of cell extracts from *E. coli* BL21 strains expressing *S. pneumoniae* ParE and ParC proteins. Lanes: 1 and 2, soluble extracts from uninduced and IPTG-induced *E. coli* containing pXP8; 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 *glaA* (*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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