ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2000, p. 840–847 Vol. 44, No. 4
0066-4804/00/$04.00+0

Horizontal Transfer of *parC* and *gyrA* in Fluoroquinolone-Resistant Clinical Isolates of *Streptococcus pneumoniae*

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Received 30 July 1999/Returned for modification 4 November 1999/Accepted 27 December 1999

We have analyzed genetically three clinical isolates (3180, 3870, and 1244) of *Streptococcus pneumoniae* with high-level ciprofloxacin resistance. Isolates 3180 and 3870 were atypical because of their insolubility in deoxycholate. However, they hybridized specifically with pneumococcal autolysin and pneumolysin gene probes and have typical pneumococcal *atpC* and *atpA* gene sequences. Analysis of the complete sequences of the *parC* and *gyrA* genes revealed total variations of 8 and 8.7% (isolate 3180) and 7.4 and 3.6% (isolate 3870), respectively, compared to the wild-type strain R6 sequence. The variations observed between the sequences of R6 and isolate 1244 were less than 0.9%. The structure of the *gyrA* and *parC* genes from isolates 3180 and 3870 was organized in sequence blocks that show different levels of divergence, suggesting a pattern of recombination. These results are evidence for recombination at the fluoroquinolone target genes in clinical isolates of *S. pneumoniae*. The genetically related viridans group streptococci could act as a reservoir for fluoroquinolone resistance genes.

Streptococcus pneumoniae is the most common bacterial cause of community-acquired pneumonia, meningitis, otitis media, and sinusitis. The emergence of resistance to antimicrobial agents commonly used for the treatment of pneumococcal diseases (5, 17, 25, 38) has made very difficult the selection of optimal antimicrobial therapies for the treatment of pneumococcal infections. A parallel increasing resistance to penicillin and macrolide antibiotics has been also observed for the viridans group streptococci (1, 2, 7, 8). These microorganisms are, like *S. pneumoniae*, commensals of the oropharyngeal tract. Nevertheless, they are causative organisms of infective endocarditis (12, 44, 47) and are also a major cause of bacteremia in neutropenic cancer patients (3, 7, 8, 16).

There is considerable interest in the use of alternative antimicrobial agents, such as the new fluoroquinolones, with good activity against streptococci for the treatment of respiratory tract infections (6). The prevalence of ciprofloxacin resistance in *S. pneumoniae* has been found to be low in Spain (<3%) (32, 33); similar data have been reported in Canada (9). The prior administration of fluoroquinolones could be an important risk factor for quinolone-resistant strain selection, as has been observed for respiratory tract infections caused by ciprofloxacin-resistant (Cp^r) *S. pneumoniae* (41). Likewise, fluoroquinolone resistance has been reported for blood isolates of viridans group streptococci from neutropenic cancer patients who received quinolone prophylaxis (22, 50). The prevalence of resistance to ciprofloxacin (MIC, ≥4 μg/ml) in viridans group streptococci consecutively isolated from different clinical sources from 1993 to 1998 at Hospital Princeps d'Espanya was as follows: 17.8% (135 of 756 clinical isolates) for *Streptococcus mitis*, 12.0% (10 of 83 isolates) for *Streptococcus salivarius*, 2.9% (11 of 378 clinical isolates) for *Streptococcus sanguis*, and 2.3% (13 of 575 isolates) for *Streptococcus anginosus* (unpublished data). These data are in accordance with those obtained in Canada, which showed a prevalence of resistance to ciprofloxacin of 11.4% (27 of 236 isolates) for the viridans group streptococci, *S. mitis* and *S. salivarius* being the most resistant (10).

The principal targets of the fluoroquinolones are DNA gyrase (gyrase) and DNA topoisomerase IV (topo IV), members of the topoisomerase family of enzymes that control bacterial DNA topology (15). Both enzymes function by passing one DNA double helix through another, using a transient double-strand break (35). Gyrase, an A_2B_2 complex encoded by the *gyrA* and *gyrB* genes, catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (53); topo IV, a C_2E_2 complex encoded by the *parC* and *parE* genes, is essential in chromosome partitioning (35). The deduced amino acid sequences of ParC and ParE are homologous to those of GyrA and GyrB, respectively (30). Genetic studies from a number of laboratories (23, 28, 36, 39, 49) have shown that topo IV is the primary target for ciprofloxacin in *S. pneumoniae* and that gyrase is the secondary target. Resistance mutations have been identified in a discrete region of ParC, ParE, GyrA, and GyrB termed the quinolone resistance-determining region (QRDR). We recently reported the same mechanism for viridans group streptococci (22): low-level Cp^r strains had mutations altering one of the two subunits of topo IV.

The viridans group streptococci could be a reservoir of fluoroquinolone resistance genes if we assume that resistance in viridans group streptococci and *S. pneumoniae* arose from horizontal transfer, as has been observed with penicillin resistance (46). A number of observations suggest that this transfer between viridans group streptococci and *S. pneumoniae* could be a possible mechanism for the spread of fluoroquinolone resistance. The viridans group streptococci and *S. pneumoniae* share the same mechanism of resistance (22). The nucleotide sequences of their gyrase and topo IV genes show high identity (20, 22), and it is possible to transform *S. pneumoniae* cells to

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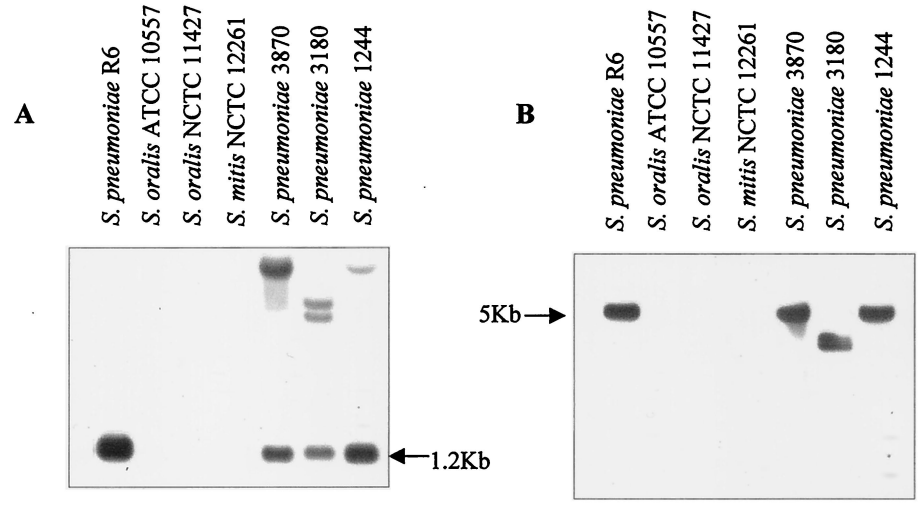

FIG. 1. Identification of *S. pneumoniae* isolates by hybridization with specific DNA probes. Chromosomal DNAs from the *S. oralis*, *S. mitis*, and *S. pneumoniae* strains indicated were cleaved with *Hin*dIII (A) or *Cla*I (B), and the fragments were separated in 1% agarose gels. The gels were blotted, and the blots were probed with biotinylated DNA as follows: A, insert of plasmid pCE3 containing the N terminus of the *lytA* gene; and B, insert of plasmid pJCP191 containing the *pnl* gene.

ciprofloxacin resistance with DNA from Cp^r viridans group streptococci (22, 27). Additionally, nucleotide sequence comparisons of the DNA topoisomerase genes of the viridans group streptococci (20, 22) show a high level of intraspecies variation. These observations suggest that the viridans group streptococci could be considered a group of species that interchange genetic material between them and possibly with *S. pneumoniae*. In this report, we describe the characterization of Cp^r *S. pneumoniae* isolates with a mosaic structure in their *parC* and *gyrA* genes, suggesting such interspecies recombination.

MATERIALS AND METHODS

Southern blot identification of *S. pneumoniae* strains. The ciprofloxacin-sensitive (Cp^s) strain of *S. pneumoniae* used was the wild-type strain R6. The Cp^r clinical isolates were obtained from sputum samples at the Hospital Princeps d'Espanya (Barcelona, Spain) in 1992 (strain 1244), 1994 (strain 3180), and 1996 (strain 3870). Plasmid pCE3 (18), containing a 0.65-kb fragment coding for the N terminus of the major pneumococcal autolysin (amidase), was used as a source of the *lytA* DNA probe. Plasmid pJCP191 (48), containing a 1.6-kb fragment coding for the complete pneumococcal pneumolysin gene, was used as a source of the *pnl* DNA probe and was kindly provided by S. Taira. The inserts of pCE3 and pJCP191 were isolated after digestion with *Hin*dIII-*Hin*cII and *Pvu*II, respectively. The resulting DNA inserts were labeled with the Phototope-Star Detection Kit (New England Biolabs). Southern blotting and hybridization were done by following the manufacturer's instructions.

Amplification and analysis of genes. Genes were amplified from genomic DNA by the PCR as described previously (22). The *atpCA* region and the *parC* and *gyrA* genes were amplified with the following primers, based on published sequences (4, 19, 36, 40): atpCUP (5′-dAAAGGAGAATTTGTTATGAA-3′), corresponding to nucleotides −15 to +5 of *atpC*, and atpB56 (5′-dGACGGGC TTCTTCAGCTCTGTC-3′), complementary to nucleotides 147 to 169 of *atpB*; parCUP (5′-dGAACACGCCCTAGATACTGTG-3′), corresponding to nucleotides −103 to −83 of *parC*, and parCDOWN (5′-dCGTTACTGTCATATTCC ACTCC-3′), complementary to nucleotides 120 to 142 downstream of *parC*; and gyrAUP1 (4) and gyrADOWN (4). DNA fragments were purified with Micro-Spin S400 HR columns (Pharmacia) and were sequenced on both strands by use of an Applied Biosystems Prism 377 sequencer with the primers used for PCR amplification and with internal primers. For nucleotide sequence comparisons, in addition to the Cp^s *S. pneumoniae* strain R6 (GenBank accession no. AF170996 and AF053121 for *parC* and *gyrA*, respectively), two other sequences were used: the sequences of the Cp^s strain 7785 (accession no. Z67739 and AJ005815 for *parC* and *gyrA*, respectively) and of another, unknown isolate, which we call AB (accession no. for *gyrA*, AB010387).

Nucleotide sequence accession numbers. The new DNA sequences reported in this paper have been assigned the following GenBank accession no.: AF170996 to AF170999 (*parC* genes), AF170993 to AF170995 (*gyrA* genes), and AF171000 to AF171002 (*atpCA* regions).

RESULTS AND DISCUSSION

Identification of strains. Three clinical isolates, 3180, 3870, and 1244, were analyzed in this work. These isolates were previously described as *Streptococcus oralis* 3180 (22), *S. oralis* 3870 (22), and *S. pneumoniae* 1244 (36). Initial characterization of the three isolates by colony morphology on blood agar and optochin susceptibility identified them as pneumococcal strains. However, strains 3180 and 3870 were insoluble in deoxycholate, while strain 1244 was soluble. Phenotypic characterization of isolates 3180 and 3870 by the API 32 Strep system classified them as *S. oralis* (22). Given the unreliability of this method for the identification of *S. pneumoniae* (20, 31), the isolates were studied by hybridization with two pneumococcal probes. One of the probes coded for the N terminus of the major pneumococcal autolysin (*lytA*), and the other coded for the complete pneumococcal pneumolysin (*pnl*). The *S. pneumoniae* strain R6 showed, as expected, hybridization with the *lytA* probe in a 1.2-kb *Hin*dIII chromosomal fragment (21), while *S. oralis* and *S. mitis* type strains did not (Fig. 1A). Strains 3180, 3870, and 1244 showed high-molecular-weight hybridization bands with the *lytA* probe, in addition to the 1.2-kb *Hin*dIII fragment (Fig. 1A). These bands could have resulted from hybridization with homologous *lytA* genes of pneumococcal prophages, which have been described to be very frequent in pneumococcal clinical isolates (43). Hybridization with the pneumolysin probe detected a single 5-kb band in *S. pneumoniae* R6 *Cla*I-digested DNA (Fig. 1B), as expected from the physical map of the *pnl* chromosomal region (48, 52), while no hybridization was observed with the *S. oralis* and *S. mitis* type strains. The three Cp^r *S. pneumoniae* clinical isolates (3180, 3870, and 1244) all hybridized with the *pnl* probe. Because both LytA and pneumolysin proteins have been demonstrated to be species specific (18, 24, 29, 42, 45, 51), these results identified the three isolates as *S. pneumoniae*.

Sequencing of the *atpC* and *atpA* genes allowed further characterization of the strains. The *atpC* gene is responsible for the characteristic optochin susceptibility phenotype of pneumococci (19, 37). The sequences of a region spanning 960 nucleotides, including *atpC* and *atpA*, showed high homogeneity (data not shown). The three Cp^r *S. pneumoniae* strains

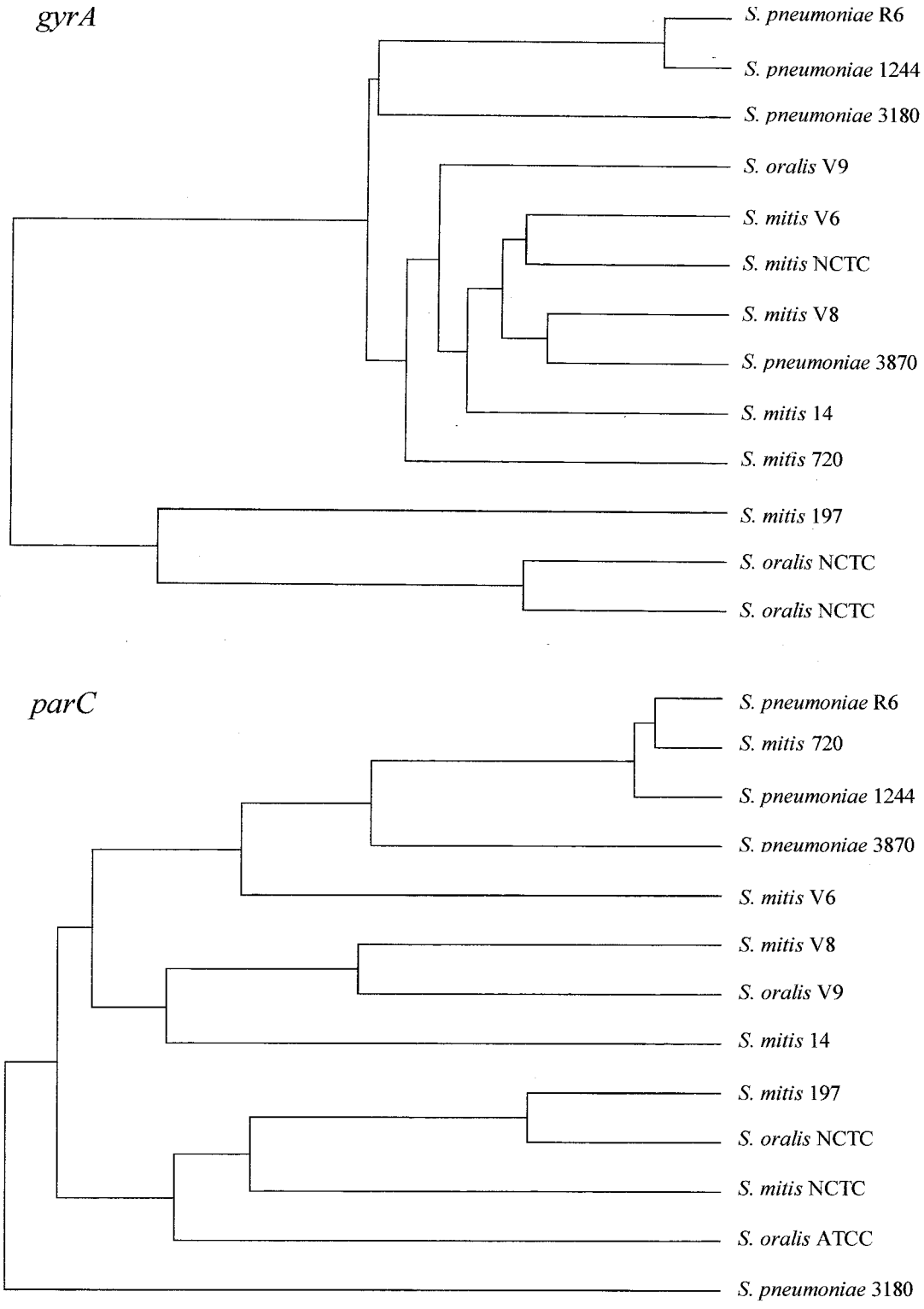

FIG. 2. Trees of nucleotide sequences of *parC* and *gyrA* QRDRs. The 185-nucleotide *parC* sequence included positions 213 to 397, and the 280-nucleotide *gyrA* sequence included positions 175 to 454. Nucleotides are numbered by taking the first *gyrA* and *parC* nucleotides as nucleotide 1. The trees were compiled by using the CLUSTAL multiple-alignment program from PCGENE with default parameters. The nucleotide sequences used have been previously reported (20, 22, 36).

showed less than 0.6% variation, compared with the wild-type strain R6, while the *S. oralis* NCTC 11427 type strain showed 20% variation. These values are in agreement with the results of comparisons of the amylomaltase gene sequences: ≤0.5% *S. pneumoniae* intraspecies variation (14) and 4 to 6% divergence between *S. pneumoniae* and *S. oralis* (13).

The results of comparisons of the *atpCA* region are consistent with the results of Southern blot hybridization and iden-

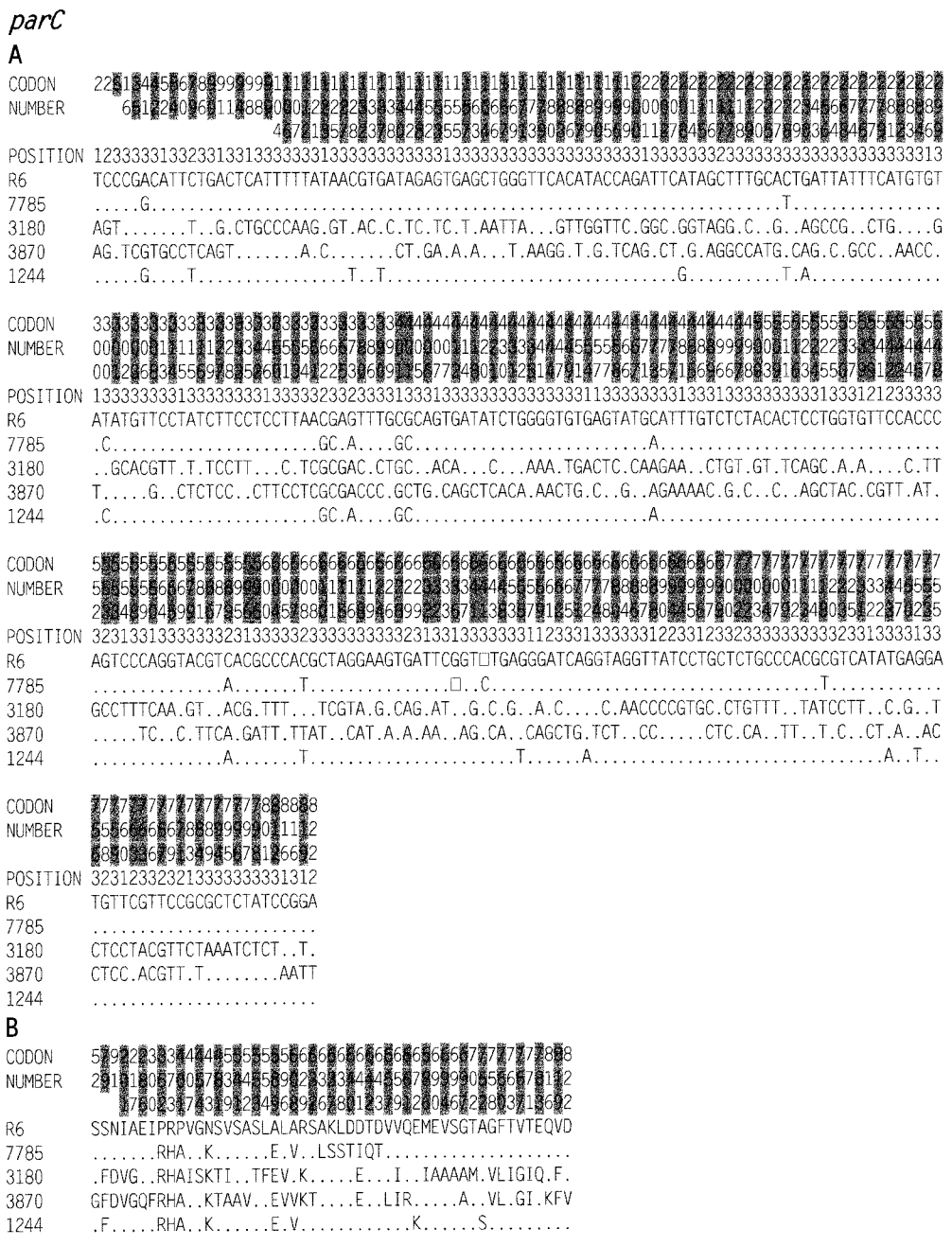

FIG. 3. Nucleotide (A) and amino acid (B) sequence variations in the *parC* genes of *S. pneumoniae* strains. The nucleotides and amino acids present at each polymorphic site are shown in full for strain R6, but only sites that differ are shown for the other strains. Nucleotides and amino acids that are the same as in R6 are shown by dots. The codon numbers are indicated in vertical format above the sequences. The different codons are alternatively shaded in grey for clarity. Positions 1, 2, and 3 in the fourth row refer to the first, second, and third nucleotides in the codon. The sequence in panel A is numbered from the initiation codon of the *parC* gene. Open squares denote nucleotide deletions. The strains used were R6 (GenBank accession no. AF170996), 7785 (accession no. Z67739), 3180 (accession no. AF170997), 3870 (accession no. AF170998), and 1244 (accession no. AF170999).

tified the clinical isolates as *S. pneumoniae*. Since *lytA* encodes the major pneumococcal autolysin (amidase), responsible for the sodium deoxycholate solubility of pneumococci (34 and references therein), the observed deoxycholate insensitivity of isolates 3870 and 1244 could be due to some alteration in the *lytA* gene, as has been described for another atypical pneumococcal isolate (11).

Analysis of the sequences of the *parC* and *gyrA* genes. We have previously determined the nucleotide sequences of portions of the *parC* and *gyrA* genes that included the QRDRs from strains 3180, 3870, and 1244 (22, 36). Comparison of the sequences of isolate 1244 to those of Cp^s isolates revealed two sense mutations, one in *parC* and the other in *gyrA*. The mutation found in *parC* produced the amino acid change Ser-79 to Phe (TCT to TTT), while the mutation in *gyrA* produced an alteration at the equivalent Ser-81 (TCC) residue: a change to Phe (TTC). By means of genetic transformation, it was shown that these amino acid changes were responsible for the Cp^r

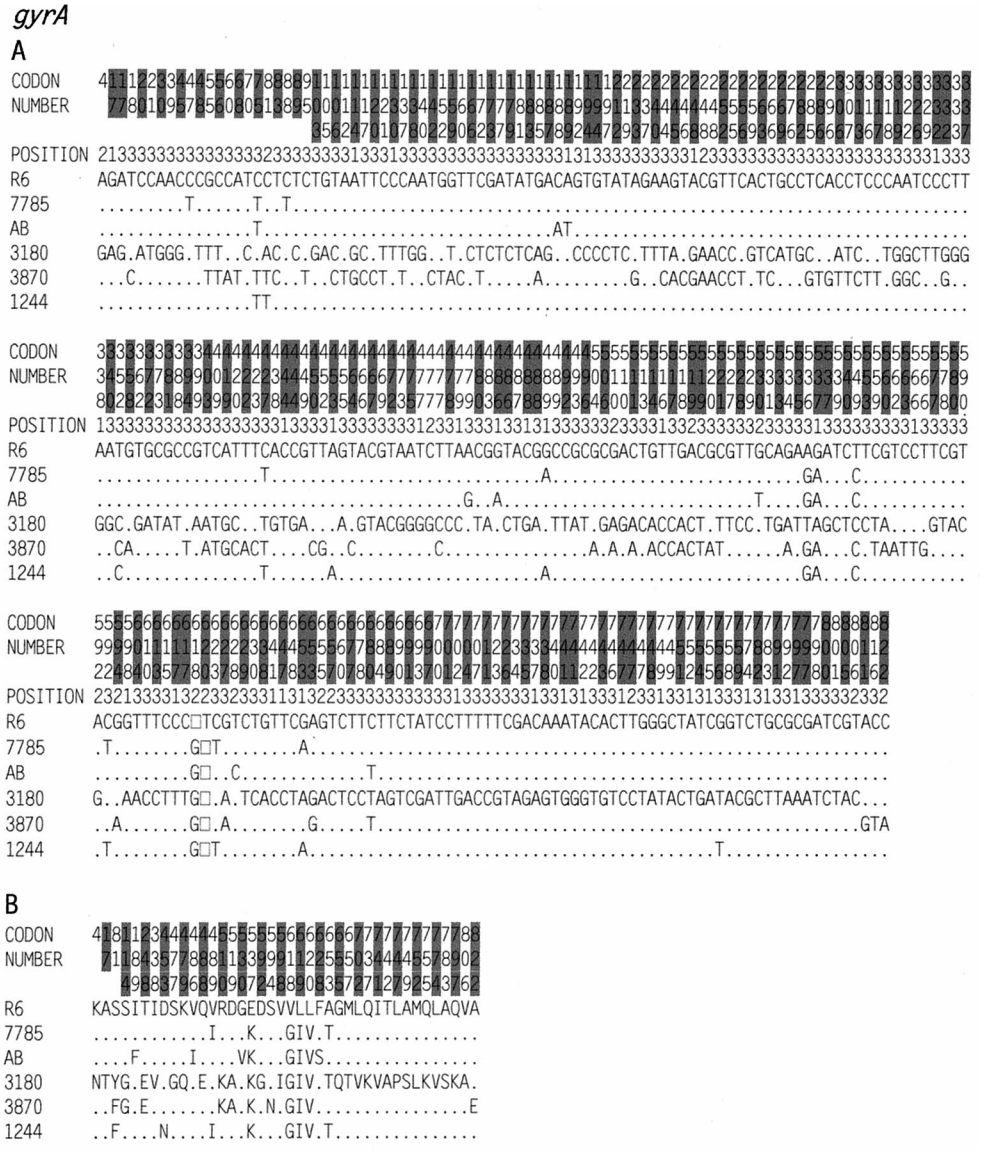

FIG. 4. Nucleotide (A) and amino acid (B) sequence variations in the *gyrA* genes of *S. pneumoniae* strains. See the legend to Fig. 3 for details. Codons are numbered according to the R6 sequence. The strains used were R6 (GenBank accession no. AF053121), 7785 (accession no. AJ005815), AB (accession no. AB010387), 3180 (accession no. AF170993), 3870 (accession no. AF170994), and 1244 (accession no. AF170993).

phenotype of isolate 1244 (36). We paid special attention to isolates 3180 and 3870, because they showed an unexpectedly high nucleotide sequence variation in their QRDRs compared to *S. pneumoniae* strain R6. A comparison of the *parC* and *gyrA* QRDR sequences of 13 independent clinical isolates of *S. pneumoniae* (including 1244) showed variations of ≤1% (unpublished results). However, these variations (excluding mutations involved in Cp^r) were 8.6 and 4.3% for the *parC* QRDRs of isolates 3180 and 3870 and 5% for their *gyrA* QRDRs. Despite this high nucleotide sequence variation, only two amino acid changes were found in the *parC* QRDRs of isolates 3180 and 3870: Ser-79 to Phe (TCT to TTT) and Asn-91 to Asp (AAC to GAC) (22). Biological evidence showing that the changes of Ser-79 to Phe were involved in ciprofloxacin resistance has been obtained by genetic transformation. A Cp^r recombinant strain was obtained by transformation of competent *S. pneumoniae* R6 cells with DNA encoding the *parC* QRDR of isolate 3180. This recombinant strain was shown to carry the Ser-79–to–Phe change but not the Asn-91–to–Asp change (22). On the other hand, the nucleotide changes observed in the *gyrA* QRDRs of isolates 3180 and 3870 also produced two amino acid changes. One of these changes was Ser-81 to Tyr (TCC to TAC) in isolate 3180 and Ser-81 to Phe

TABLE 1. Nucleotide sequence differences for *gyrA* and *parC* genes[a]

Strain	% (no.) of nucleotide differences in strain:					
	R6	7785	AB	3180	3870	1244
R6		0.5 (13)	0.5 (13)	8.7 (216)	3.6 (90)	0.6 (15)
7785	0.6 (14)		0.6 (14)	8.7 (216)	3.6 (89)	0.2 (6)
AB	—	—		8.8 (218)	3.6 (89)	0.6 (15)
3180	8.0 (198)	8.0 (196)	—		8.1 (200)	8.6 (213)
3870	7.4 (184)	7.2 (178)	—	7.6 (188)		3.5 (87)
1244	0.8 (20)	0.5 (12)	—	8.0 (197)	7.2 (175)	

[a] Differences were measured over sequences of 2,469 bp from *gyrA* (above the diagonal) and 2,472 bp from *parC* (below the diagonal).

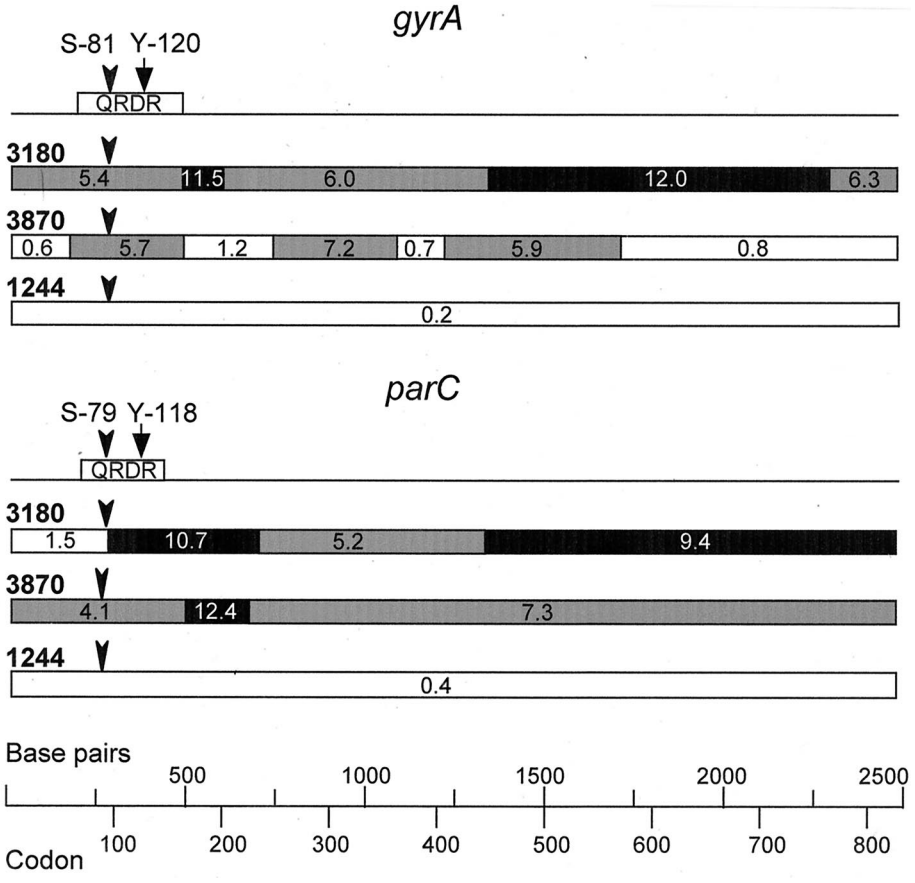

FIG. 5. Mosaic structure of the *gyrA* and *parC* genes of Cp^r *S. pneumoniae* isolates 3180 and 3870. The locations of the QRDRs are represented above the *gyrA* and *parC* sequences. The positions of the active Tyr residues (Y-120 in GyrA and Y-118 in ParC) that bind DNA and of the Ser residues that are changed in the Cp^r strains (S-81 in GyrA and S-79 in ParC) and are involved in resistance are marked. Blocks showing the percent sequence divergence from the corresponding regions in Cp^s pneumococci are indicated. White boxes, regions of sequence that differ by ≤1.5%; grey boxes, regions that differ by more than 1.5% but less than 9%; black boxes, regions that differ by >9%.

(TTC) in isolate 3870. The other change, present in both isolates, was Gly-144 to Ser (AGT to GGT). The analysis of Cp^r transformants obtained with DNA from the *gyrA* QRDRs of isolates 3180 and 3870 showed that the amino acid changes at Ser-81 were indeed involved in resistance (22).

A comparison of the nucleotide sequences of the *parC* and *gyrA* QRDRs of isolates 3180, 3870, and 1244, *S. pneumoniae* R6, and several *S. oralis* and *S. mitis* strains is shown in Fig. 2. While isolate 1244 was grouped with *S. pneumoniae* R6 within the *parC* or *gyrA* tree, the location of isolates 3180 and 3870 varied depending on the gene considered. These results suggested a recombinational origin for the genes encoding the fluoroquinolone target proteins of isolates 3180 and 3870. Such a situation would be due to genetic transformation with DNA from other bacterial species, probably the genetically closed related viridans group streptococci. The recombination machinery requires approximately 80% sequence identity between two homologous DNA molecules (19, 26). At least 87% identity was found between the *parC* and *gyrA* QRDRs of isolates 3180 and 3870 and those of the viridans group streptococci (Fig. 2). Similarly, horizontal transfer of altered penicillin-binding protein genes between *S. pneumoniae* and viridans group streptococci (46) has been observed.

To test this hypothesis, the nucleotide sequences of the complete *parC* and *gyrA* genes of the two isolates (3180 and 3870) that showed high divergence in their *parC* and *gyrA* QRDRs and one isolate (1244) that did not shown this divergence were determined. The results of sequence comparisons of Cp^s and Cp^r strains (Fig. 3 and 4 and Table 1) clearly showed three groups of strains. One group, with a nucleotide sequence variation of <1%, was formed by the sensitive strains and isolate 1244. Isolates 3180 and 3870 each formed separate groups, since the nucleotide sequence variations between the sequences of the two isolates were 7.6% for their *parC* sequences and 8% for their *gyrA* sequences. The average variations in the *parC* and *gyrA* sequences between isolate 3180 and the first group of strains (sensitive strains and isolate 1244) were ≥8%, while these values for isolate 3870 were about 7% for *parC* and about 3% for *gyrA* (Table 1). The variations found in isolates 3180 and 3870 could be organized in blocks (Fig. 5) with different degrees of relatedness. The limits of the blocks were determined by inspection, with the only limitation being at least a 4% difference in divergence between two contiguous blocks.

From these results, we can assume that the first group of strains (sensitive strains and isolate 1244) are nonrecombinant isolates, while isolates 3180 and 3870 show a recombinational pattern probably resulting from gene transfer events. The *gyrA* sequence of isolate 3870 clearly shows four blocks of low divergence (≤1.2%) and three blocks of high divergence (≥5.7%). This result suggests that the low-divergence blocks represent regions in which interspecies recombination events

had occurred. Another block with a 1.5% divergence was observed in *parC* of isolate 3180, although in this case the second recombination point should be located outside the gene. Likewise, recombination outside the genes would be the origin of the *gyrA* gene of isolate 3180 and of the *parC* gene of isolate 3870.

Because both gyrase and topo IV are tetrameric proteins, an interchange of *parC* would need an accompanying interchange of *parE*. Since both genes are contiguous in the pneumococcal chromosome, we cannot exclude the possibility of a recombinational event involving both genes. On the other hand, an interchange of both *gyrA* and *gyrB* genes would involve two independent recombinations, since the genes are separated by at least 90 kb in the chromosome (36).

Two different processes could lead to the acquisition of fluoroquinolone resistance: spontaneous mutation and transformation. A comparison of the frequencies of these two processes reveals that transformation could be several orders of magnitude more frequent than mutation. The frequency of mutation to Cp^r in *S. pneumoniae* has been shown to be in the range of 10^{-8} to 10^{-9} (39). However, the frequencies of transformation to Cp^r with chromosomal DNAs from Cp^r *S. pneumoniae* strains were in the range of 10^{-2} (36, 49) for monogenic transformation (low-level Cp^r) (36, 49). The acquisition of low-level Cp^r via transformation could then be 10^6 to 10^7 times more frequent than that via spontaneous mutation. Likewise, it has been shown that the frequency of transformation of *S. pneumoniae* competent cells to low-level Cp^r with DNA from Cp^r *S. mitis* is in the range of 10^{-3} (22, 27). Interspecies transformation could thus be 10^5 to 10^6 more frequent than spontaneous mutation. These differences are even higher when the acquisition of high-level resistance is considered. The frequencies of transformation with two unlinked markers that gave rise to high-level Cp^r were 10^{-4} when both donor and recipient cells were *S. pneumoniae* (36, 49) and 10^{-6} when competent *S. pneumoniae* cells were transformed with *S. mitis* DNA (27). However, two spontaneous mutations are necessary to obtain a high level of resistance (i.e., the frequency could be 10^{-14} to 10^{-16}). Nevertheless, these estimates are not necessarily true since, as pointed out above, an interchange of *parC* would need an accompanying interchange of *parE* and an interchange of *gyrA* would need an accompanying interchange of *gyrB*. Other factors to be considered for transformation in the natural environment are the availability of DNA and the competence state of the recipient cells.

ACKNOWLEDGMENTS

We thank P. A. Lazo for allowing us to use the PCGENE program and A. Rodriguez-Bernabé for excellent technical assistance.

M.J.F. has a fellowship from Comunidad Autónoma de Madrid. This work was supported by grant 97/2026 from Fondo de Investigación Sanitaria.

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