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The viridans streptococci are the donors in the horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*

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ABSTRACT

A total of 46 ciprofloxacin-resistant (Cip^R) *Streptococcus pneumoniae* strains were isolated from 1991 to 2001 at Hospital of Bellvitge. Five of these strains showed unexpected high nucleotide variations in the quinolone-resistance-determining region (QRDR) of their *parC*, *parE* and *gyrA* genes. The nucleotide sequence of the full-length *parC*, *parE*, and *gyrA* genes of one of these isolates revealed a mosaic structure compatible with interspecific recombination origin. Southern blot analysis and nucleotide sequence determinations showed the presence of an *ant*-like gene in the intergenic *parE-parC* region of the *S. pneumoniae* Cip^R isolates with high variations in their *parE* and *parC* QRDRs. The *ant*-like gene was absent in typical *S. pneumoniae* strains, whereas it was present in the intergenic *parE-parC* region of the viridans group streptococci (*Streptococcus mitis* and *Streptococcus oralis*). These results suggest that the viridans group streptococci are acting as donors in the horizontal transfer of fluoroquinolone-resistant genes to *S. pneumoniae*.

Streptococcus pneumoniae (the pneumococcus) remains the leading bacterial cause of community-acquired pneumonia, meningitis and otitis media. The emergence and spreading of resistance to penicillin and macrolide antibiotics (17, 25, 41), has made the selection of optimal antimicrobial therapy difficult. A parallel increasing resistance to those antibiotics has also been observed for the viridans group streptococci (VS) (1, 2, 7, 8), which are commensals of the oropharyngeal tract of healthy individuals, but also a major cause of endocarditis (46) and bacteremia in neutropenic patients (3, 7, 8, 15). Fluoroquinolones with increased activity against *S. pneumoniae*, such as levofloxacin, moxifloxacin and gatifloxacin are now being recommended for the treatment of patients with community-acquired pneumonia (5). Although the prevalence of ciprofloxacin resistance in *S. pneumoniae* is still low in Spain (3- 7%)(32, 43) and Canada (2%) (9), higher values have been found for the VS. Among 1,046 VS characterized as *S. mitis* isolated during 1993-2001 at Hospital of Bellvitge the prevalence of ciprofloxacin resistance was of 16.6 % (unpublished data), a figure very similar to the 11.4% reported in Canada (11). An increase in resistance to fluoroquinolones in both *S. pneumoniae* and VS would be expected as a consequence of a widespread use of these compounds. Prior fluoroquinolone administration is an important risk factor for resistant strain selection, as observed for respiratory tract infections caused by ciprofloxacin resistant (Cip^R) (44) and levofloxacin-resistant (10, 54) *S. pneumoniae*. Likewise, emergence of Cip^R VS blood isolates from neutropenic cancer patients that received fluoroquinolone prophylaxis has been reported (23, 55).

Bacterial resistance to fluoroquinolones occurs mainly by alteration of drug targets. The intracellular fluoroquinolone targets are DNA topoisomerase IV (topo IV) and DNA gyrase (gyrase), enzymes that function by passing a DNA double helix through another, using a transient

double-strand break (14). DNA gyrase, an A_2B_2 complex, encoded by *gyrA* and *gyrB,* catalyses ATP-dependent negative supercoiling of DNA to relieve the topological stress generated during DNA replication and transcription. Topoisomerase IV, a C_2E_2 complex encoded by *parC* and *parE,* is essential in chromosome partitioning. The amino acid sequences of ParC and ParE are homologous to those of GyrA and GyrB, respectively (29).

Genetic and biochemical studies have shown that topo IV is the primary target for ciprofloxacin and that gyrase is a secondary target in *S. pneumoniae* (20, 28, 42, 52). Resistance mutations have been identified in a discrete region of ParC, ParE and GyrA termed the quinolone resistance-determining region (QRDR). The VS share the same mechanism of ciprofloxacin resistance (23), and it has been possible to transform *S. pneumoniae* cells to ciprofloxacin resistance with DNA from Cip^R VS in the laboratory (23, 27). The VS could be as a reservoir of fluoroquinolone resistance by acting as DNA donors in the horizontal transfer to pneumococci, similarly to that observed for penicillin resistance (50). The high level of intra-species sequence variation in the DNA topoisomerase genes of VS (6, 23) and the mosaic structures of *parC* and *gyrA* in *S. pneumoniae* clinical isolates (6, 22, 57), led us to suggest that genetic interchange of the fluoroquinolone target genes occurs both into VS and between VS and pneumococci. In this work we present evidence supporting the hypothesis of VS being the donors in the recombination events yielding DNA topoisomerase mosaic genes in *S. pneumoniae* Cip^R clinical isolates.

MATERIALS AND METHODS

Bacterial strains, serotyping and susceptibility tests. The strains used in this work were isolated from sputum of adult patients. Only one isolate per patient was evaluated. Identification was according to standard methodology using the following tests: colonial morphology, Gram´s

stain, catalase reaction, optochin susceptibility and bile solubility. The strains were serotyped by detection of quellung reaction using antisera provided by the Staten Seruminstitut (Copenhagen) at the Spanish Pneumococcus Reference Laboratory (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda Madrid). Identification of VS was by standard methods (16, 48). MICs were determined by the microdilution method, using cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (40). The inoculum was prepared by suspension of several colonies from an overnight blood agar culture in Mueller-Hinton broth and adjusting the turbidity to the 0.5 McFarland standard (ca. 10^8 CFU/ml). The suspension was further diluted to provide a final bacterial concentration of 10^4 CFU/ml in each well of the microdilution trays. Plates were covered with plastic tapes and incubated in ambient atmosphere at 37ºC for 20-24 h. MIC was defined as the lowest concentration of drug that inhibited visible growth. *S. pneumoniae* ATCC 49619 and *S. pneumoniae* R6 strains were used for the quality control. Ciprofloxacin was kindly provided by Bayer.

Southern blot analysis. For identification of *S. pneumoniae* strains, 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 (51), containing a 1.6-kb fragment coding for the complete pneumococcal pneumolysin gene was used as a source of *pnl* DNA probe and was kindly provided by S. Taira. The *parC* and *parE* probes were obtained by PCR amplification of the R6 laboratory strain with oligonucleotides parCUP; parCDOWN; parEUP; and parEDOWN (Table 1). The *ant* probe was obtained by amplification of strain 3870 DNA with oligonucleotides antUP and antDOWN (Table 1). Al probes were labeled with the Phototope-Star Detection Kit (New England Biolabs). Southern blot and hybridization followed

the manufacturer's instructions.

PCR amplification and DNA sequence determination. *S. pneumoniae* chromosomal DNA was obtained as described previously (19). DNA topoisomerase QRDRs were amplified from genomic DNA by the polymerase chain reaction (PCR) as described (23, 38). The *parE*, *parC* and *gyrA* genes were amplified with the following primers, based on published sequences (4, 19, 21, 38): parEUP and parEDOWN; parCUP and parCDOWN; gyrAUP1, and gyrADOWN (Table 1). PCR amplifications were performed using 0.5 units of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 1 μg of chromosomal DNA, 0.4 μM (each) of the synthetic oligonucleotide primers, 0.2 mM of each dNTP, and 2 mM $MgCl_2$ in a final volume of 50 μl. To amplify the *atpC* and *atpA* genes, oligonucleotides atpWO and atpB56, were used (Table 1). Amplification was achieved with an initial cycle of 1 min denaturation at 94ºC, and 30 cycles of 30 seconds at 94ºC, 90 seconds at 55ºC and 80 seconds polymerase extension step at 72ºC, with a final 8 min 72ºC extension step and slow cooling at 4ºC. Gel electrophoresis of PCR products was carried out in agarose gels as described (49). DNA fragments were purified using MicroSpin™ S400 HR columns (Amersham Pharmacia Biotech) and sequenced on both strands with an Applied Biosystems Prism 377 DNA sequencer, accordingly to protocols provided by the manufacturer with both the primers used for the PCR amplifications and internal primers.

DNA sequence determination and phylogenetic analysis. DNA sequencing was carried out with an Applied Biosystems Prism 377 DNA sequencer. Sequencing reactions were performed accordingly to protocols provided by Applied Biosystems with the primers used for the PCR amplifications and with internal primers. Phylogenetic analysis was performed by using the MEGA 2.1 programs (31), available at http://www.megasoftware.net. Dendograms were constructed by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method

with the Kimura-2 parameter. The percentage bootstrap confidence levels of internal branches, as defined by MEGA (31), were calculated from 1000 random resamplings.

Nucleotide sequence accession numbers. The new DNA sequences reported in this paper have been assigned the following GenBank Accession numbers: AY166963, AY166965, AY167641 to AY167643, AY168409 to AY168412, AY184477 (*ant* regions), AY167637 and AY167640 (*atpCA* regions), AY157690 (*parC* of *S. pneumoniae* 4391), AY157689 (*gyrA* of *S. pneumoniae* 4391), AY157687, AY157688 and AY167691 (*parE* sequences). The *atpCA* region of *S. pneumoniae* 4589 is identical to that of *S. pneumoniae* 3180 (accession number: AF171000).

RESULTS

Characterization of *S. pneumoniae* isolates. An epidemiological study performed at Hospital of Bellvitge during an 11-year period (1991-2001) revealed 2.3 % (89 out of 3819) Cip^R (MICs ≥ 4 μg/ml) *S. pneumoniae* isolates (unpublished results). The *parC*, *parE*, *gyrA*, and *gyrB* QRDRs of a total of 46 Cip^R strains were characterized. All strains showed low variations (≤1%) in the nucleotide sequence of *gyrB* QRDR. However, although 41 of the 46 strains showed variations ≤1% in their *parE*, *parC* and *gyrA* QRDRs, 5 strains (numbers 3180, 3870, 4391, 4589, and 5237) exhibited unexpected high nucleotide variations (> 4%) in at least one of these sequences. Since high nucleotide sequence variations in the QRDRs have been associated with a mosaic structure in the *parC* and *gyrA* genes of strains 3180 and 3870 (21), we hypothesized that a gene showing QRDR nucleotide sequence variation higher than 4% will have a mosaic structure indicative of DNA interspecific horizontal transfer. Comparison of the *parC*, *parE* and *gyrA* QRDRs of these five mosaic strains with that of *S. pneumoniae* R6, the Cip^R *S. pneumoniae* 4638 typical isolate (not showing variations in their QRDRs), *S. mitis* and *S. oralis* type strains and

three Cip^R *S. mitis* isolates was performed (Fig. 1, Table 2). Mosaic strains showed nucleotide sequence variations higher than 4% for *parE* (5 out of 5 strains), *parC* (5 out of 5 strains) and *gyrA* (4 out of 5 strains, exception being strain 5237) when compared with *S. pneumoniae* R6 and *S. pneumoniae* 4638. All Cip^R *S. pneumoniae* and VS strains showed typical mutations yielding changes in ParC S79 (to F, N, or Y) and GyrA S81 (to F or Y) (Fig. 1, Table 2). Two additional amino acid changes (ParC K137N and ParE I460V), not involved in resistance, which are present both in Cip^S and Cip^R *S. pneumoniae* strains and that are consequently considered as polymorphisms, were found in *S. pneumoniae* 4638. All five *S. pneumoniae* strains showing high *parC* QRDR nucleotide sequence variations showed the same amino acid change (N91D) that is present in ParC of both Cip^S and Cip^R VS strains. When the GyrA QRDR was considered, the S114G amino acid change was observed in the four *S. pneumoniae* strains showing high nucleotide sequence variations in their *gyrA* QRDR and in both Cip^S and Cip^R VS strains. The presence of ParC N91D and GyrA S114G in VS strains and in Cip^R *S. pneumoniae* mosaic strains suggests that mosaic strains have been originated by recombination with VS. Additional amino acid changes were observed in *S. pneumoniae* 5237 (ParC I126V and E135D changes), *S. mitis* 181731-3 (GyrA M90G) and *S. mitis* 181732-2 (GyrA N150S change; ParE P424A, I460L, A463E, K466N and A468S changes). These changes are an indication of the high level of intra-species variation in the VS.

Identification of the strains as *S. pneumoniae* by using molecular tools. We have previously characterized *S. pneumoniae* 3180 and *S. pneumoniae* 3870 (21) by hybridization with *pnl* and *lytA* pneumococcal specific probes (18, 24, 45, 47, 56). In the present study identical tests were performed with the rest of the mosaic strains. Hybridization with the *pnl* probe detected single fragments of about 5-kb in *S. pneumoniae* strains *Cla*I-digested DNA, while no hybridization was

observed with the VS DNAs. All *S. pneumoniae* strains showed, as expected, hybridization with the *lytA* probe in a 1.2-kb *Hin*d III chromosomal fragment, while the VS strains did not. Sequencing of a region spanning 960 nucleotides including *atpC* and *atpA* genes that are responsible for the unique optochin susceptibility of the pneumococcus (19, 33, 39) allowed further characterization of the strains. All *S. pneumoniae* strains showed high homogeneity (less than 0.7% nucleotide sequence variation), while variations among *S. pneumoniae* R6 and VS type strains was higher than 20%. These values are in agreement with the comparisons of the amylomaltase gene sequences: ≤ 0.5% *S. pneumoniae* intraspecies variation (13), and 4-6% divergence between *S. pneumoniae* and *S. oralis* (12). A phylogenetic tree was constructed with the concatenated *atpC* and *atpA* genes of *S. pneumoniae* and VS strains and of *Bacillus halodurans* as outgroup (Fig. 2).The *S. pneumoniae* strains formed a monophyletic group within the tree.

Analysis of the sequences of the *parC*, *parE* and *gyrA* genes. To asses the recombinational origin of the mosaic strains, the *parE* sequence of *S. pneumoniae* 4391 and VS type strains was determined. Oligonucleotides based on the *S. pneumoniae* R6 sequence were used to obtain and sequence PCR products. Nucleotide sequence variations between 8 and 12% were observed among the strains (Fig. 3). Similar variations were observed between the *parE* sequence of strain 4391 and that of *S. pneumoniae* R6 or *S. pneumoniae* TIGR4. However, the *parE* sequences of R6 and TIGR4 were almost identical (1% variation). The variations found could be organized in blocks 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. Two blocks were detected in *S. pneumoniae* 4391 while no blocks were detected in VS type strains (Fig. 3). A WU-blast search with *S. pneumoniae* 4391

ParE on the Swiss-Prot sequence database was performed to select sequences to be used in the construction of the tree shown on Fig. 2. Only the nucleotide sequences of the more similar proteins, which corresponded to ParE subunits of *S. pneumoniae* TIGR4 and R6 strains, along with the sequences determined in this work and that of *B. halodurans* as an outgroup, were used. The *parE* sequences of *S. pneumoniae* 4391, *S. mitis* 12261, *S. pneumoniae* R6 and *S. pneumoniae* TIGR4 formed a statistically significant group within the tree.

The full-length sequences of *parC* and *gyrA* of strain 4391 were also determined. These sequences showed variations of 8.9% for *gyrA* and 5.8% for *parC* when compared to the *S. pneumoniae* R6 sequence that could be organized in blocks of divergence (Fig. 3).

Characterization of the *parE-parC* intergenic regions. Several PCR amplifications were performed to determine the sequences of *parE* and *parC* genes of *S. pneumoniae* 4391. When oligonucleotides parEdownR (coding for the last 4 ParE residues) and parC26R (complementaryto the strand coding for ParC residues 26 to 33) were used, a product of about 6 kb was obtained. Since the intergenic *parE-parC* region of *S. pneumoniae* R6 is 420-bp long (38), these results suggested a different genetic organization of the *parE-parC* chromosomal region in strain 4391. To know if this was the case for the rest of the Cip^R *S. pneumoniae* mosaic strains, the length of their *parE-parC* intergenic regions was determined and compared with that of *S. pneumoniae* R6, the typical Cip^R *S. pneumoniae* 4638 strain and both Cip^S and Cip^R VS strains. PCR amplifications with four pairs of oligonucleotides located upstream *parE*, and in the *parC* N-terminus (parEUP and parC26R), upstream of *parE* and downstream *parC* (parEUP and parCDOWN), downstream *parE* and in the *parC* N-terminus (parEDOWNR and parC26R), and downstream of *parE* and downstream *parC* (parERDOWNR and ParCDOWN) were performed. The size of these PCR products were compatible with intergenic regions for R6 and 4638 of 0.4

kb, for VS strains in the range of 1 to 2.5 kb, and for the *S. pneumoniae* Cip^{R} mosaic strains in the 2 to 6.2 kb-range. Southern blot experiments using *parC* and *parE* specific probes and digestions of PCR products were performed (not shown) to construct physical maps for *EcoR*V and *Nco*I of the *parE-parC* regions (Fig. 4). These experiments showed that, except for *S. pneumoniae* R6 and *S. pneumoniae* 4638, the estimated sizes on the intergenic *parE-parC* regions were in the 1- to-6.2 kb range. Among *S. pneumoniae* mosaic strains, 3870 and 4589 share the same physical maps of the *parE-parC* chromosomal region (Fig. 4). Among VS strains, the same *parE-parC* physical map was observed for *S. mitis*^{T} 12261 and *S. mitis* 181731-3.

Characterization of the *ant* gene. The nucleotide sequences of the *parE-parC* intergenic region of *S. oralis*^{T} 10557, *S. pneumoniae* 3870 and *S. pneumoniae* 4589 revealed the presence of an open reading frame, *ant*, recently described in the intergenic *parE-parC* region of the *S. mitis* isolate CIP 103335T (27). To check if this gene was also present in other *parE-parC* intergenic regions, a probe for *ant* of *S. pneumoniae* 3870 was constructed by PCR with oligonucleotides antUP and antDOWN. This probe was used to hybridize the Southern blots of DNA cut with *EcoR*V and *Nco*I described above. These experiments (Fig. 5) confirmed the presence of *ant* in the intergenic *parE-parC* region of all VS strains checked and in all *S. pneumoniae* strains with a suspected recombinational origin. However, not hybridization was observed with several *S. pneumoniae* strains, including R6 and ATCC strains 49619, 700669 and 700671, the two latter being representative of Spain^{23F}-1 and Spain^{9V}-3 clones, respectively (35). In addition, two low-level Cip^{R} (strains 3724, 4837) and two high-level Cip^{R} (strains 4638 and 4235) pneumococcal clinical isolates with typical ParC, ParE, and GyrA QRDRs (data not shown) did not show hybridization with the *ant* probe (Fig. 5). These results show that the presence of *ant* is a characteristic of VS and that is present in the *parE-parC* intergenic region of *S. pneumoniae*

mosaic strains. The *ant* open reading frame is homologous to *ant* genes encoding aminoglycoside adenylyltransferase enzymes from bacteria (36) but was not associated with any particular phenotype in the *S. pneumoniae* mosaic strains or in VS strains (data not shown). Comparisons of the *ant* nucleotide sequence of *S. pneumoniae* mosaic strains and VS strains showed similarities between 99.7% and 65% (Fig. 2) and identity among *S. pneumoniae* mosaic strains 4589 and 3870. The *ant* sequences determined in this work were analyzed along with *S. mitis* CIP and *B. halodurans* and used in the construction of a phylogenetic tree shown on Fig.2.The *S. pneumoniae* mosaic strains and VS strains formed a separate group within the tree, being *S. oralis* 10557 the only exception.

DISCUSSION

The QRDRs of the DNA topoisomerase genes of 46 Cip^R *S. pneumoniae* strains isolated during an 11-year period at Hospital of Bellvitge were characterized. Four isolates showed high nucleotide variations (>4%) in three genes (*parC*, *parE* and *gyrA*) and one isolate in two (*parE* and *parC*) (Fig. 1). We hypothesized that this unexpected QRDR variations were reflect of the variations present in the whole gene. An isolate with a nucleotide variation higher than 4% in the QRDR on a specific gene with respect to *S. pneumoniae* R6, will likely have a mosaic structure originated by recombination. This hypothesis was confirmed for all those genes that we have fully sequenced, such as *parC* of strains 3180, 3870 (21) and 4391 (Fig. 3), *parE* of strain 4391 (Fig. 3), and *gyrA* of strains 3180, 3870 (21) and 4391 (Fig. 3). Other authors have reported results compatible with that hypothesis (6, 57). Among the five mosaic strains, four would have a mosaic structure in their *parC*, *parE*, and *gyrA* genes and one strain in their *parC* and *parE* genes. The genetic organization of the *parE-parC* chromosomal region of the five *S. pneumoniae* mosaic strains isolated was different from that of typical *S. pneumoniae* strains, such as R6 and 4638. The size of the intergenic

parE- parC region in the mosaic strains and VS was longer than in typical *S. pneumoniae* strains (Fig. 4). While the sizes of the intergenic regions in VS strains varied between 1 and 2.5 kb, those from the *S. pneumoniae* mosaic strains studied in this work varied between 1.9 and 6.2 kb (Fig. 4). These figures are compatible with an interchange of genetic material between VS and pneumococci. Supporting this hypothesis, the *ant* gene is present in the intergenic *parE- parC* region, both in *S. pneumoniae* mosaic strains and in VS, but is absent in typical *S. pneumoniae* strains (Fig 5). In addition, the *ant* gene was not found in the *S. pneumoniae* R6 (26) or the *S. pneumoniae* TIGR4 (53) sequence databases. Although the nucleotide sequence of *ant* genes showed high heterogeneity, comparison of this gene from VS and *S. pneumoniae* mosaic strains with those present in the data bases showed that *ant* genes from *S. pneumoniae* mosaic strains and VS formed a separate group within the phylogenetic tree (Fig. 2). Similar results were obtained when the *parE* genes were compared (Fig. 2). Altogether these results show that *ant* is typical of VS and its presence in the *S. pneumoniae* mosaic strains indicates that a VS strain, probably a *S. oralis* or *S. mitis*, was the donor in the recombination event that originated the mosaic *parE* and *parC* genes. *S. oralis* and *S. mitis* are the most closely related species to *S. pneumoniae* on the basis of 16S rRNA sequence, which exhibits more than 99% identity with *S. pneumoniae*, although the DNA-DNA similarity values for their total chromosomal DNAs is less than 60% (30). Analysis of the genetic structure of the five *S. pneumoniae* mosaic strains (Fig. 4) suggests that the initial interchange that originated these mosaic strains included the whole *parE-ant-parC* chromosomal region. However, further reorganizations by recombination with VS or *S. pneumoniae* probably occurred, as deduced from the analysis of the *gyrA*, *parC* and *parE* genes of strain 4319 (Fig. 3) and of the *gyrA* and *parC* genes of strains 3180 and 3870 (21).

We have not found identity between the *ant* sequences of VS and *S. pneumoniae* mosaic

strains. However, identity was observed between *ant* of *S. pneumoniae* strains 3870 and 4589 (Fig. 2). Although these strains had identical *ant* sequence, they were isolated from unrelated patients in different years, had different pulse-field-gel electrophoresis patterns (data not shown) and different nucleotide sequences in their *parC*, *parE*, *gyrA*, and *atpCA* genes. However, both strains share the same physical structure in their *parE-ant-parC* region and *ant* sequences, suggesting that they have interchange the *parE-ant-parC* region with a closely-related VS strain.

We have observed a low prevalence (11%, 5 out of 46) of *S. pneumoniae* strains with mosaic structure in the *parE-parC* region among the Cip^R isolates. These strains were of serotype 23F (2 out of 5 strains) or not-typeable (3 out of 5 strains) and all of them were also resistant to penicillin and other drugs. Since the 23F serotype is one of the most common among penicillin-resistant isolates in Spain and worldwide (17, 34, 37), it would be possible for these mosaic strains to be spread in a near future.

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FIGURE LEGENDS

Figure 1. Nucleotide sequence variations in the ParC, ParE, and GyrA QRDRs. The nucleotides present at each polymorphic site are shown for strain R6, but only sites that differ are shown for the other strains. Changes yielding amino acid substitutions, including those involved in fluoroquinolone resistance, are showed in boldface. Codon numbers are indicated in vertical format above the sequences. Positions 1, 2, and 3 in the fourth row refer to the first, second, and third nucleotides in the codon. Strains whose sequences do not differ from that of R6 more than 1% are shadowed in gray.

Figure 3. Mosaic structure of the *gyrA*, *parC* and *parE* genes of the *S. pneumoniae* and VS strains indicated. The locations of the QRDRs are represented at the top of the *gyrA* and *parC* sequences. The position of the active Tyr residues (Y-120 in GyrA and Y- 118 in ParC) that bind DNA, and the Ser residues that are changed in strain 4391 (S-81 in GyrA and S-79 in ParC), and are involved in resistance, are marked. Blocks showing the percentage sequence divergence from the corresponding regions of *S. pneumoniae* R6 strain are indicated. White box, region of sequence that differ by ≤ 1.5%; gray boxes, regions that differ by more than 1.5% but less than 9%; black boxes, regions that differ by > 9%. Strains used were *S. pneumoniae* (SPN) R6 (GenBank accession number AE008451); SPN TIGR4 (accession number AE007391); SPN 4391; *S. mitis* (SMI) NCTC 12261; and *S. oralis* (SOR) NCTC 11427.

Figure 2. UPGMA trees of concatenated *atpC* and *atpA*, full-length *parE* genes , and *ant* genes. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 by using the UPGMA method. Only bootstrap confidence intervals exceeding 90% are shown. The accession numbers of each nucleotide sequence is shown in parentheses.

Figure 4 . Restriction map of the *parE-parC* region of *S. pneumoniae* and VS strains and its genetic organization as deduced from Southern blot experiments and nucleotide sequence analyses. E, *Eco*RV; N, *Nco*I. The *parE* and *parC* genes with a mosaic structure are showed with stripped arrows.

Figure 5 . Southern blot hybridization of *S. pneumoniae* and VS strains with an *ant* probe. Chromosomal DNAs from the strains indicated was cleaved with *EcoR*V + *Nco*I and the fragments separated in 0.8% agarose gels. The gels were blotted and the blot was probed with a biotinylated probe derived from *S. pneumoniae* 3870 containing positions 26 to 290 of the *ant* gene.

TABLE 1**.** Oligonucleotides used in this work

Name	Sequence (5'-3')[a]	Nucleotide positons[b]
antDOWN	TCATGAGTCTTCTCCTCTCGC	Complementary to 853 to 873 of *ant*
antUP	GCTGTCGCCATGTCTGGTTCACG	76 to 98 of *ant*
atpB56	GACGGGCTTCTTCAGCTCTGTC	Complementary to 169 to 147 of *atpB*
atpWO	gcgcatgcTTAAAGGAGAATTTGTTATGAA	–15 to 5 of *atpC*
gyrADOWN	gcgctctagAGTAATATCAGAAATCCTGCTAGG	Complementary to 2524 to 2501of *gyrA*
gyrAUP1	gcgctctagaTGGTTTAGAGGCTGAAATAGAC	–77 to –56 of *gyrA*
parCDOWN	CGTTACTGTCATATTCCACTCC	Complementary to 124 to 145 of *parC*
parCUP	GAACACGCCCTAGATACTGTG	–103 to –83 of *parC*
parC26R	GAATATCTGGCAAAGCCCGGTCTTG	Complementary to 76 to 100 of *parC*
parEDOWN	gcgcaagcTTAAAACACTGTCGCTTCTTCTAGCG	Complementary to 1919 to 1944 of *parE*
parEDOWNR	CGCTAGAAGAAGCGACAGTG	1919 to 1938 of *parE*
parEUP	cgcgcataTGTCAAAAAAGGAAATCAATATTAAC	2 to 27 of *parE*

[a] The 5' end of some of the primers contained a sequence including an *Nde*I (parEUP), *Hin*dIII (parEDOWN), *Sph*I (atpWO) or *Xba*I (gyrAUP1 and gyrADOWN) restriction sites, which are shown underlined.

[b] Nucleotide and amino acid numbering refers to the genes and proteins obtained from the *S. pneumoniae* R6 sequence, with the first nucleotide or amino acid at position 1.

TABLE 2**.** Phenotypes of fluoroquinolone-resistant *S. pneumoniae* and *S. mitis strains* and amino acid changes in their DNA topoisomerase genes

Strain	Date	Type	Optochin	Bile solubility	CIP MIC (μg/ml)	Amino acid change in the QRDR of:[c]		
						ParC	GyrA	ParE
SPN ATCC 49619		19F	S	+	1	None	None	None
SPN R6			S	+	0.5	None	None	None
SPN 3180	1994	NT	S	–	128	**S79F**, N91D	**S81Y,** S114G	None
SPN 3870	1996	NT	S	–	64	**S79F,** N91D	**S81Y,** S114G	None
SPN 4391	1997	23F	S	+	128	**S79Y,** N91D	**S81F,** S114G	None
SPN 4589	1998	NT	S	+	64	**S79F**, N91D	**S81F,** S114G	None
SPN 4638	1998	23F	S	+	32	**S79Y,** K137N	**S81F**	I460V
SPN 5237	1999	23F	S	+	8	**S79N,** N91D, I126V,E135D	**S81Y**	None
SOR ATCC 10557			R	–	2	N91D	S114G	None
SOR NCTC 11427			R	–	2	N91D	S114G	None
SMI NCTC 12261			R	–	1	N91D	S114G	None
SMI 75414-2	2000		R	–	>32	**D83Y**, N91D	**S81F**, S114G	None
SMI 181731-3	1999		R	–	64	**S79F,** N91D	**S81F,** M90G, S114G	None
SMI 181732-2	1999		R	–	>32	**S79F,** N91D	**S81Y,** S114G, N150S	P424A, I460L, A463E, K466N, A468S

[a] NT, not typeable

[b] CIP, ciprofloxacin

[c] Residues involved in ciprofloxacin resistance are showed in boldface, and when double-underlined, that are located in a gene with a mosaic structure. No changes in GyrB were found.