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Viridans Group Streptococci Are Donors in Horizontal Transfer of Topoisomerase IV Genes to *Streptococcus pneumoniae*

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A total of 46 ciprofloxacin-resistant (Cip^r) *Streptococcus pneumoniae* strains were isolated from 1991 to 2001 at the Hospital of Bellvitge. Five of these strains showed unexpectedly high rates of nucleotide variations in the quinolone resistance-determining regions (QRDRs) 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 an interspecific recombination origin. Southern blot analysis and nucleotide sequence determinations showed the presence of an *ant*-like gene in the intergenic *parE-parC* regions of the *S. pneumoniae* Cip^r isolates with high rates of variations in their *parE* and *parC* QRDRs. The *ant*-like gene was absent from typical *S. pneumoniae* strains, whereas it was present in the intergenic *parE-parC* regions 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 resistance genes to *S. pneumoniae*.

Streptococcus pneumoniae (the pneumococcus) remains the leading bacterial cause of community-acquired pneumonia, meningitis, and otitis media. The emergence and spread of resistance to penicillin and macrolide antibiotics (17, 25, 41) have made the selection of the optimal antimicrobial therapy difficult. Parallel increases in the rates of resistance to those antibiotics have also been observed among the viridans group streptococci (VS) (1, 2, 7, 8), which are commensal organisms of the oropharyngeal tracts of healthy individuals but which are also a major cause of endocarditis (46) and bacteremia in neutropenic patients (3, 7, 8, 15). Fluoroquinolones with increased levels of 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 among *S. pneumoniae* strains is still low in Spain (3 to 7%) (32, 43) and Canada (2%) (9), higher prevalences have been found among the VS. Among 1,046 isolates of VS characterized as *S. mitis* and isolated from 1993 to 2001 at the Hospital of Bellvitge, the prevalence of ciprofloxacin resistance was 16.6% (unpublished data), a rate very similar to the rate of 11.4% reported in Canada (11). An increase in the rates of resistance to fluoroquinolones in both *S. pneumoniae* and VS would be expected as a consequence of the widespread use of these compounds. Prior fluoroquinolone administration is an important risk factor for the selection of resistant strains, as observed for respiratory tract infections caused by ciprofloxacin-resistant (Cip^r) (44) and levofloxacin-resistant (10, 54) *S. pneumoniae* isolates. Likewise, the emergence of Cip^r isolates of VS in the blood of 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 and DNA gyrase (gyrase), enzymes that function by passing a DNA double helix through another by use of a transient double-stranded break (14). DNA gyrase, an A_2B_2 complex encoded by *gyrA* and *gyrB*, catalyzes 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 topoisomerase 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 act as a reservoir of fluoroquinolone resistance by acting as donors in the horizontal transfer of DNA to pneumococci, similar to the mechanism observed for penicillin resistance (50). The high level of intraspecies variation in the sequences of 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 among VS and between VS and pneumococci. In this work we present evidence supporting the hy-

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TABLE 1. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′-3′)[a]	Nucleotide positions[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 2501 of *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′ ends 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 site, which is underlined. Bases not present in *S. pneumoniae* R6 are lowercased.

[b] The nucleotide and amino acid numbering refers to the numbering for the genes and proteins of the *S. pneumoniae* R6 sequence, with the first nucleotide or amino acid being at position 1.

pothesis that VS are the donors in the recombination events yielding DNA topoisomerase genes with mosaic structures 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 the sputum of adult patients. Only one isolate per patient was evaluated. Identification was by a standard methodology by the following tests: colonial morphology, Gram staining, catalase reaction, optochin susceptibility, and bile solubility. The strains were serotyped at the Spanish Pneumococcus Reference Laboratory (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain) by detection of the Quellung reaction with antisera provided by the Staten Seruminstitut (Copenhagen, Denmark). Identification of VS was done by standard methods (16, 48). MICs were determined by the microdilution method with cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood, as recommended by the National Committee for Clinical Laboratory Standards (40). The inoculum was prepared by suspension of several colonies from an overnight blood agar culture in Mueller-Hinton broth and adjustment of the turbidity to a 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. The plates were covered with plastic tape and incubated in ambient atmosphere at 37°C for 20 to 24 h. The MIC was defined as the lowest concentration of drug that inhibited visible growth. Strains *S. pneumoniae* ATCC 49619 and *S. pneumoniae* R6 were used for quality control. Ciprofloxacin was kindly provided by Bayer.

Southern blot analysis. For identification of *S. pneumoniae* strains, plasmid pCE3 (18), which contains 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), which contains 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. Probes specific for *parC* and *parE* were obtained by PCR amplification of the R6 laboratory strain with oligonucleotides parCUP, parCDOWN, parEUP, and parEDOWN (Table 1). The *ant*-specific probe was obtained by amplification of strain 3870 DNA with oligonucleotides antUP and antDOWN (Table 1). All probes were labeled by use of the Phototope-Star detection kit (New England Biolabs). Southern blotting and hybridization were performed according to the instructions of the manufacturer.

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 PCR described previously (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, and gyrAUP1 and gyrADOWN, respectively (Table 1). PCR amplifications were performed with 0.5 U of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 1 μg of chromosomal DNA, the synthetic oligonucleotide primers at a concentration of 0.4 μM each, deoxynucleoside triphosphates at a concentration of 0.2 mM each, and 2 mM $MgCl_2$ in a final volume of 50 μl. Oligonucleotides atpWO and atpB56 (Table 1) were used to amplify the *atpC* and *atpA* genes. Amplification was achieved with an initial cycle of 1 min of denaturation at 94°C and 30 cycles of 30 s at 94°C, 90 s at 55°C, and a polymerase extension step for 80 s at 72°C, with a final extension step for 8 min at 72°C and slow cooling at 4°C. Electrophoresis of the PCR products was carried out in agarose gels as described previously (49). The DNA fragments were purified with MicroSpin S400 HR columns (Amersham Pharmacia Biotech), and both strands were sequenced with an Applied Biosystems Prism 377 DNA sequencer by the protocols provided by the manufacturer with both the primers used for the PCR amplifications and internal primers.

Phylogenetic analysis. Phylogenetic analysis was performed by using the MEGA program (version 2.1) (31), available at http://www.megasoftware.net. Dendrograms were constructed by the unweighted pair group method with arithmetic mean (UPGMA) method with the Kimura-2 parameter. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program (31), was calculated from 1,000 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, and AY184477 (*ant* regions); AY167637 and AY167640 (*atpCA* regions); AY157690 (*parC* of *S. pneumoniae* 4391); AY157689 (*gyrA* of *S. pneumoniae* 4391); and AY157687, AY157688, and AY167691 (*parE* sequences). The *atpCA* region of *S. pneumoniae* 4589 is identical to that of *S. pneumoniae* 3180 (GenBank accession number AF171000).

RESULTS

Characterization of *S. pneumoniae* isolates. An epidemiological study performed at the Hospital of Bellvitge during an 11-year period (1991 to 2001) revealed that 2.3% (89 of 3,819) of the *S. pneumoniae* isolates were Cip^r (MICs ≥ 4 μg/ml) (unpublished results). The *parC*, *parE*, *gyrA*, and *gyrB* QRDRs of a total of 46 Cip^r strains were characterized. All strains showed low levels of variation (≤1%) in the nucleotide sequences of their *gyrB* QRDRs. However, although 41 of the 46 strains showed variations of ≤1% in the sequences of their *parE*, *parC*, and *gyrA* QRDRs, the sequences of at least one of the QRDRs from 5 strains (strains 3180, 3870, 4391, 4589, and 5237) exhibited unexpectedly high levels of nucleotide sequence variation (>4%). Since high levels of nucleotide sequence variation 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 a level of QRDR nucleotide sequence variation greater than 4% will have a mosaic structure, indicative of interspecies horizontal DNA transfer. Comparison of the *parC*, *parE*, and *gyrA*

ParC QRDR

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                                        111111111111111 111111111111
CODON           666677777  8888899 999 9000000111112222 222222333333
                567813789  0346901 348 9245679024590123 456789235678
POSITION        333333331233133331333133333333333333333133313333333333

SPN R6          ACGCGCGTTCTTGCTAGACATCATCTCTTCATGTTGATTGTAAACCGTGAGA
SPN 3180        .........T.......G..CTGC.C.CA..A....G....G.T..AC...C
SPN 3870        .........T....C.AGT............A......C.............
SPN 4391        TA.T.T...A....C.AGT..TGC....A..A.C.................
SPN 4589        T.........T....CTAGT............G......C...........T
SPN 4638        .........A.....................................T.
SPN 5237        ......TCAA.......G......T......G.G..G....GG..T..T..C
SOR 10557       T.T...TCAGCC.TC..GT......C........C.T....G.TTTA.A...
SOR 11427       ......T..........G..C.G........G..C.T....G.TTTA.AG..
SMI 12261       T.T...TCAG.......G...TG........A..C.TC...G.TTT.....C
SMI 75414-2     T.T...T.AG..T..G.G.........A..TGTAC....A.G..T.A....C
SMI 181731-3    T...TTT..T....C..G.......C...T.G....T....G.TT....G.C
SMI 181732-2    ..A.TTA..T....CT.G.GC....CT....A..GAT...AG......T...
```

ParE QRDR

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CODON           444 4 4444444444 44444 4444444444444444444444 44 4444444 4444  44444
                011 1 1111122222 22233 3333333344444444455555 55 5556666 6666  77777
                801 2 3467801234 56801 2345678901245678901234 45 6780123 4568  13456
POSITION        331313333333333313333313333333333333333333333313313333313323333312333333

SPN R6          TCTGTGTGGCAATGTCTTGATCATGGCTCTTTCATTCCGCGTTCATCTTTGATACCGCGGCGCTAGC
SPN 3180        .A..C..TA..GC..........C.....A....C.............................A.....
SPN 3870        .A..C..TA..G.AC....G.TGC.A......T...TT.T....T.TA.......TA........A.
SPN 4391        C...C......G....A........AT..A....C.T.....................TA.....T.GA.
SPN 4589        ....C..TA..G.AC....G.TGC.A......T...TT.T....T.TA.......TA........A.
SPN 4638        ...........................................................G.............T
SPN 5237        ....C......G.....A.......AT..A..T.C.T..............................A.
SOR 10557       C...CT...T.G.AC..A..C..CAAT......G...TA.A.CTG...C........T.......A.
SOR 11427       C...CT.T.T.G.AC..A..C..CAAT......G.C.TA.A.CTG....C.......T.......A.
SMI 12261       C.C...A.AATGC...A.....GC..T............A.CTG.......C.........T.G..
SMI 75414-2     .A.........G....A.A....C..T..A....C......ACTG...........A........A.
SMI 181731-3    ......GTAG.G.................C...G........CTG..A..A......T...A.C.A.
SMI 181732-2    .ACT.A.T..GGC..G......GCAA.AT.AG.G.A.T...CC.TA..AG.C.TAA.TCAGC.CGA.
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GyrA QRDR

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                                          1 11111111111111111111111 11 11111111111111 11
CODON           66666677777788888889999990 00000011111111122222223 33 33333344444445 55
                02457801458913468901256 70 12356912345678901236890 13 456789023467 90 12
POSITION        333333333333233333233333133333333331333333333333313313333333333333332333

SPN R6          CATTTATCCCCGCTTCCTTTACCTCTATTGTCTATATCTATTCGACTTCGACTTTCCATATCTCTCATAG
SPN 3180        ......C.....AC...C.........GAC....G..C...T.....T.......TG.G..........
SPN 3870        A....T...T..TC.......T.........C..T.G..C..CT.............T...C........T
SPN 4391        ......C.....TC.A.C..........GAC....G..CG.CT......A...A..TG.G.....T...T
SPN 4589        A....T...T..TC.......T.........C..T.G..CG.CT............T....C........T
SPN 4638        ........T...T..................................................................
SPN 5237        ..........A.....................................................................C..
SOR 10557       T.AC...T...A..C.T...G.....T.GACTCGCG..GGGCT...........C.T.C...A.CT.C.T
SOR 11427       T.AC...T..TT..C.T.........T.GACT..CG..GGGCT......T......T.C...A.CT.C.T
SMI 12261       T........T...C.......T.........C..T.G..CG.CT.......G....T....C........T
SMI 75414-2     T...........TC...........C..GACT..CG.TCG..T.C...........TG...T.TC....T
SMI 181731-3    T........T..TC....G.......C.GACT..CGATCG..T....G...........G.....T..GT
SMI 181732-2    AGACC..T....A...TG.G.TTCTA.C.ACTCT.GCTA.G.AATTC..T.TGG..T.....A...G..T
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FIG. 1. Nucleotide sequence variations in the ParC, ParE, and GyrA QRDRs. The nucleotides present at each polymorphic site are shown for strain R6, but for the other strains, only the nucleotides that differ from those in R6 are shown. Changes yielding amino acid substitutions, including those involved in fluoroquinolone resistance, are shown in boldface. Codon numbers are indicated vertically above the sequences. Positions 1, 2, and 3 in the fourth row refer to the first, second, and third nucleotides in the codon, respectively. Strains whose sequences do not differ from that of R6 more than 1% are shaded in gray. SPN, *S. pneumoniae*; SOR, *S. oralis*; SMI, *S. mitis*.

QRDRs of these five strains with mosaic gene structures with that of *S. pneumoniae* R6 and the Cip^r *S. pneumoniae* 4638 typical isolate (which did not show variations in their QRDRs), the *S. mitis* and *S. oralis* type strains, and three Cip^r *S. mitis* isolates was performed (Fig. 1; Table 2). Strains with mosaic gene structures showed levels of nucleotide sequence variation greater than 4% for *parE* (five of five strains), *parC* (five of five strains), and *gyrA* (four of five strains, with the exception being strain 5237) compared with the sequences of *S. pneumoniae* R6 and *S. pneumoniae* 4638. All Cip^r strains of *S. pneumoniae* and VS 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

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

Strain	Yr isolated	Type	Susceptibility by the optochin assay[a]	Bile solubility	CIP[b] MIC (μg/ml)	Amino acid change in the QRDR of[c]:		
						ParC	GyrA	ParE
S. pneumoniae ATCC 49619		19F	S	+	1	None	None	None
S. pneumoniae R6			S	+	0.5	None	None	None
S. pneumoniae 3180	1994	NT[d]	S	−	128	**S79F**, N91D	**S81Y**, S114G	None
S. pneumoniae 3870	1996	NT	S	−	64	**S79F**, N91D	**S81Y**, S114G	None
S. pneumoniae 4391	1997	23F	S	+	128	**S79Y**, N91D	**S81F**, S114G	None
S. pneumoniae 4589	1998	NT	S	+	64	**S79F**, N91D	**S81F**, S114G	None
S. pneumoniae 4638	1998	23F	S	+	32	**S79Y**, K137N	**S81F**	1460V
S. pneumoniae 5237	1999	23F	S	+	8	**S79N**, N91D, I126V, E135D	**S81Y**	None
S. oralis ATCC 10557			R	−	2	N91D	S114G	None
S. oralis NCTC 11427			R	−	2	N91D	S114G	None
S. mitis NCTC 12261			R	−	1	N91D	S114G	None
S. mitis 75414-2	2000		R	−	>32	**D83Y**, N91D	**S81F**, S114G	None
S. mitis 181731-3	1999		R	−	64	**S79F**, N91D	**S81F**, M90G, S114G	None
S. mitis 181732-2	1999		R	−	>32	**S79F**, N91D	**S81Y**, S114G, N150S	P424A, I460L, A463E, K466N, A468S

[a] S, susceptible; R, resistant.
[b] CIP, ciprofloxacin
[c] Residues involved in ciprofloxacin resistance are indicated in boldface, and double underlining indicates that the residue is located in a gene with a mosaic structure. No changes in GyrB were found.
[d] NT, not typeable.

additional amino acid changes (ParC K137N and ParE I460V) that are not involved in resistance and that are present in both Cip[s] and Cip[r] *S. pneumoniae* strains, and which are consequently considered polymorphisms, were found in *S. pneumoniae* 4638. All five *S. pneumoniae* strains with high levels of nucleotide sequence variations in their *parC* QRDRs showed the same amino acid change (N91D) present in the ParC proteins of both Cip[s] and Cip[r] strains of VS. When the GyrA QRDR was considered, the S114G amino acid change was observed in the four *S. pneumoniae* strains with high levels of nucleotide sequence variations in their *gyrA* QRDRs and in both Cip[s] and Cip[r] strains of VS. The presence of ParC N91D and GyrA S114G in strains of VS and in Cip[r] *S. pneumoniae* strains with a mosaic gene structure suggests that strains with a mosaic gene structure have originated by recombination with VS. Additional amino acid changes were observed in *S. pneumoniae* 5237 (ParC I126V and E135D), *S. mitis* 181731-3 (GyrA M90G), and *S. mitis* 181732-2 (GyrA N150S and ParE P424A, I460L, A463E, K466N, and A468S). These changes are an indication of the high level of intraspecies variation in the VS.

Identification of strains as *S. pneumoniae* by use of molecular tools. We have previously characterized *S. pneumoniae* 3180 and *S. pneumoniae* 3870 (21) by hybridization of their DNA with pneumococcal *pnl*- and *lytA*-specific probes (18, 24, 45, 47, 56). In the present study identical tests were performed with the rest of the strains with mosaic gene structures. Tests with the *pnl*-specific probe detected hybridization with single fragments of about 5 kb of *Cla*I-digested DNA of the *S. pneumoniae* strains, while no hybridization was observed with the DNAs of the VS. As expected, a 1.2-kb *Hin*dIII-digested chromosomal fragment of all *S. pneumoniae* strains hybridized with the *lytA*-specific probe, while the DNA of the strains of VS did not. Sequencing of a region spanning 960 nucleotides, including the *atpC* and *atpA* gene sequences, which is responsible for the unique optochin susceptibility of the pneumococcus (19, 33, 39) allowed further characterization of the strains. The sequences of all *S. pneumoniae* strains showed a high degree of homogeneity (less than 0.7% nucleotide sequence variation), while the sequences of *S. pneumoniae* R6 and type strains of VS varied by greater than 20%. These values are in agreement with those obtained by comparison of amylomaltase gene sequences: ≤0.5% *S. pneumoniae* intraspecies variation (13) and 4 to 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 strains of VS, with the sequence of *Bacillus halodurans* used as the outgroup (Fig. 2). The *S. pneumoniae* strains formed a monophyletic group within the tree.

Analysis of *parC*, *parE*, and *gyrA* gene sequences. To assess the recombinational origin of the strains with mosaic gene structures, the *parE* sequences of *S. pneumoniae* 4391 and the type strains of VS were determined. Oligonucleotides based on the *S. pneumoniae* R6 sequence were used to obtain and sequence the PCR products. Nucleotide sequence variations of 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 into 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 the type strains of VS (Fig. 3). A WU-BLAST search of the Swiss-Prot sequence database with the *S. pneumoniae* 4391 ParE sequence was performed to select sequences to be used in the construction of the tree shown in Fig. 2. Only the nucleotide sequences of the more similar proteins, which corresponded to the ParE

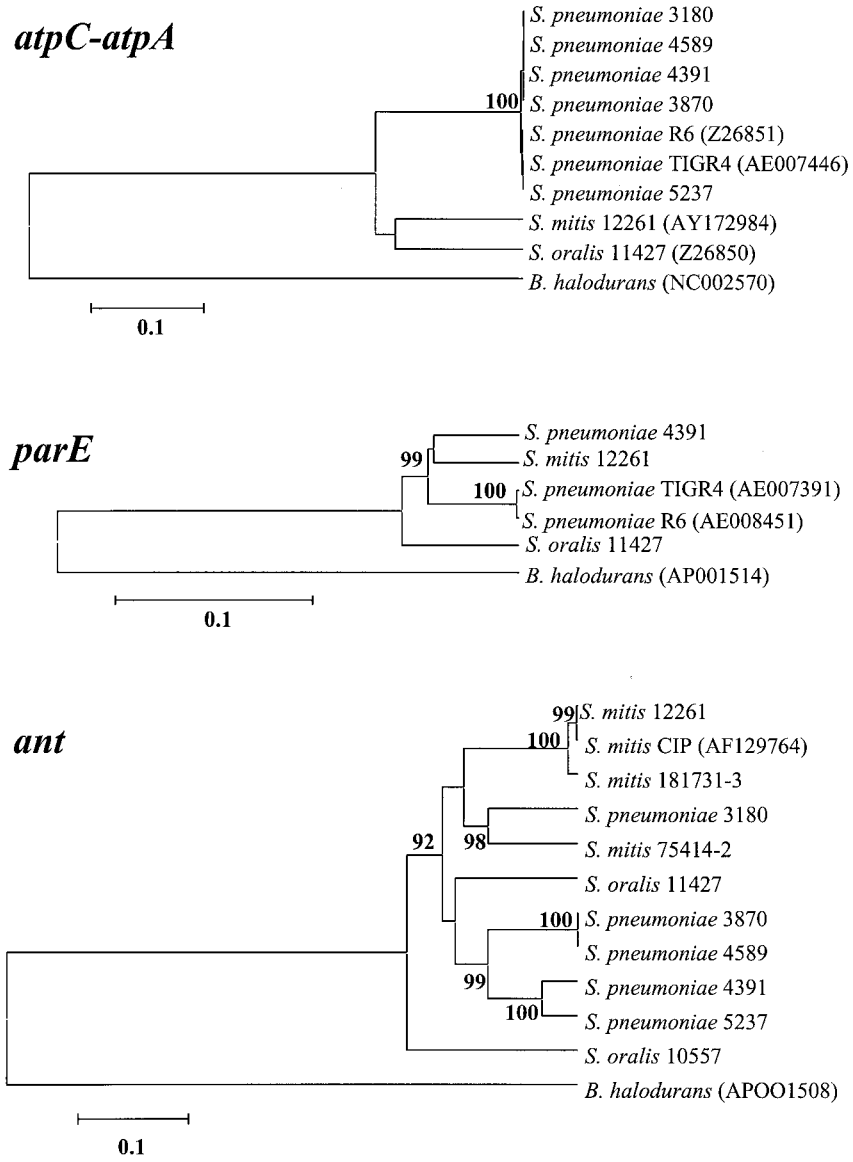

FIG. 2. UPGMA trees of concatenated *atpC* and *atpA* genes, full-length *parE* genes, and *ant* genes. Phylogenetic and molecular evolutionary analyses were conducted with the MEGA program (version 2.1) by the UPGMA method. Only bootstrap confidence intervals exceeding 90% are shown. The GenBank accession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

subunits of strains *S. pneumoniae* TIGR4 and R6, 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 *parC* and *gyrA* sequences of strain 4391 were also determined. The *gyrA* sequence showed 8.9% variation and the *parC* sequence showed 5.8% variation compared to the *S. pneumoniae* R6 sequences that could be organized into blocks of divergence (Fig. 3).

Characterization of *parE-parC* intergenic regions. Several PCR amplifications were performed to determine the sequences of the *parE* and *parC* genes of *S. pneumoniae* 4391. When oligonucleotides parEDOWNR (coding for the last 4 ParE residues) and parC26R (complementary to 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 determine whether this is the case for the rest of the Cip^r *S. pneumoniae* strains with mosaic gene structures, the lengths of their *parE-parC* intergenic regions were determined and compared with those of *S. pneumoniae* R6, the typical Cip^r strain *S. pneumoniae* 4638, and both Cip^s and Cip^r strains of VS. PCR amplifications with four pairs of oligonucleotides whose sequences are specific for sequences located upstream of *parE* and in the *parC* N terminus (parEUP and parC26R), upstream of *parE* and downstream of *parC* (parEUP and parCDOWN), downstream of *parE* and in the *parC* N terminus (parEDOWNR and parC26R), and downstream of *parE* and downstream of *parC* (parEDOWNR

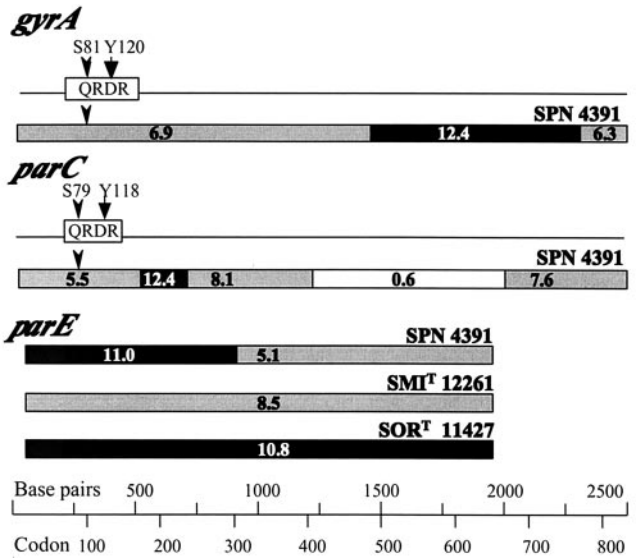

FIG. 3. Mosaic structures of the *gyrA*, *parC*, and *parE* genes of the indicated strains of *S. pneumoniae* and VS. The locations of the QRDRs are indicated at the tops of the *gyrA* and *parC* sequences. The positions of the active Tyr residues (Y120 in GyrA and Y118 in ParC) that bind to DNA and the Ser residues that are changed in strain 4391 (S81 in GyrA and S79 in ParC) and that are involved in resistance are marked. Blocks showing the percent sequence divergence from the corresponding regions of *S. pneumoniae* R6 are indicated. White box, region of the sequence that differed by ≤1.5%; gray boxes, regions that differed by more than 1.5% but less than 9%; black boxes, regions that differed by >9%. The strains used were *S. pneumoniae* (SPN) R6 (GenBank accession number AE008451), *S. pneumoniae* TIGR4 (GenBank accession number AE007391), *S. pneumoniae* 4391, *S. mitis* (SMI) NCTC 12261, and *S. oralis* (SOR) NCTC 11427.

and ParCDOWN) were performed. The sizes of these PCR products were compatible with the sizes of the intergenic regions for R6 and 4638 (0.4 kb), for strains of VS (range, 1 to 2.5 kb), and the *S. pneumoniae* Cip^r strains with mosaic gene structures (range, 1.9 to 6.2 kb). Southern blotting experiments with *parC*- and *parE*-specific probes and digestion of the PCR products were performed (data not shown) to construct physical maps for *Eco*RV and *Nco*I in the *parE-parC* regions (Fig. 4). These experiments showed that, except for *S. pneumoniae* R6 and *S. pneumoniae* 4638, the estimated sizes of the intergenic *parE-parC* regions were in the range of 1 to 6.2 kb. Among the *S. pneumoniae* strains with mosaic gene structures, strains 3870 and 4589 shared the same physical maps for the *parE-parC* chromosomal region (Fig. 4). Among the strains of VS, the same *parE-parC* physical map was observed for *S. mitis* 12261^T and *S. mitis* 181731-3.

Characterization of *ant* gene. The nucleotide sequences of the *parE-parC* intergenic regions of *S. oralis* 10557^T, *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 *S. mitis* isolate CIP 103335^T (27). To determine whether this gene was also present in other *parE-parC* intergenic regions, a probe specific 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 *Eco*RV and *Nco*I described above. These experiments (Fig. 5) confirmed the presence of *ant* in the intergenic *parE-parC* region of all strains of VS checked and in all *S. pneumoniae* strains with a suspected recombinational origin. However, no hybridization was observed with several *S. pneumoniae* strains, including R6 and strains ATCC 49619, ATCC 700669, and ATCC 700671; the last two strains are representative of clones Spain^{23F}-1 and Spain^{9V}-3, respectively (35). In addition, two pneumococcal clinical isolates with low-level Cip^r (strains 3724 and 4837) and two pneumococcal clinical isolates with high-level Cip^r (strains 4638 and 4235) and typical ParC, ParE, and GyrA QRDRs (data not shown) did not hybridize with the *ant*-specific probe (Fig. 5). These results show that the presence of *ant* is a characteristic of VS and that it is present in the *parE-parC* intergenic region of *S. pneumoniae* strains with a mosaic gene structure. The sequence of the *ant* open reading frame is homologous to those of *ant* genes encoding aminoglycoside adenylyltransferase enzymes from bacteria (36) but was not associated with any particular phenotype in the *S. pneumoniae* strains with mosaic gene structures or in the strains of VS (data not shown). Comparison of the *ant* nucleotide sequences of *S. pneumoniae* strains with mosaic gene structures and those of the strains of VS showed similarities between 99.7 and 65% (Fig. 2) and identity among *S. pneumoniae* strains 4589 and 3870 with mosaic gene structures. The *ant* sequences determined in this work were analyzed along with those of *S. mitis* CIP 103335^T and *B. halodurans* and used in the construction of the phylogenetic tree shown in Fig. 2. The *S. pneumoniae* strains with mosaic gene structures and the strains of VS formed a separate group within the tree, with *S. oralis* 10557 being 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 the Hospital of Bellvitge were characterized. Four isolates showed high levels of nucleotide variation (>4%) in three genes (*parC*, *parE*, and *gyrA*), and one isolate showed high levels of nucleotide variation in two genes (*parE* and *parC*) (Fig. 1). We hypothesized that these unexpected variations in the QRDRs reflect the variations present in the whole gene. An isolate with a level of nucleotide variation of more than 4% in the QRDR of a specific gene compared with the sequence of *S. pneumoniae* R6 will likely have a mosaic gene structure that originated by recombination. This hypothesis was confirmed for all those genes that we have fully sequenced, such as *parC* of strains 3180 and 3870 (21) and strain 4391 (Fig. 3), *parE* of strain 4391 (Fig. 3), and *gyrA* of strains 3180 and 3870 (21) and strain 4391 (Fig. 3). Other investigators have reported results compatible with that hypothesis (6, 57). Among the five strains with mosaic gene structures, four would have a mosaic structure in their *parC*, *parE*, and *gyrA* genes and one strain would have a mosaic structure in its *parC* and *parE* genes. The genetic organization of the *parE-parC* chromosomal region of the five *S. pneumoniae* strains with mosaic gene structures isolated was different from that of typical *S. pneumoniae* strains, such as strains R6 and 4638. The size of the intergenic *parE-parC* region in the strains with mosaic gene structures and the strains of VS was longer than that in typical *S. pneumoniae* strains (Fig. 4). While the sizes of the intergenic regions in VS strains

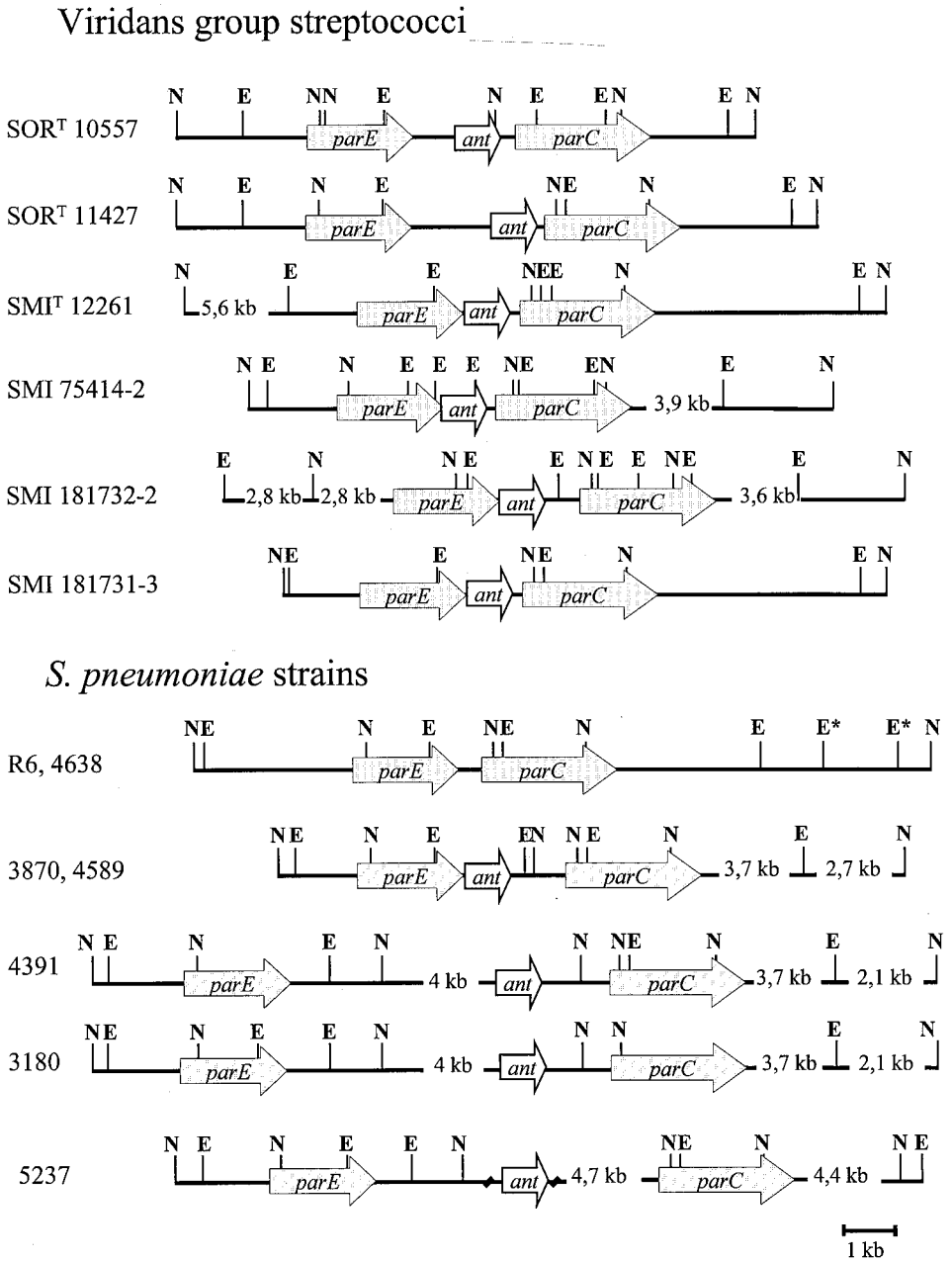

FIG. 4. Restriction map of the *parE-parC* region of *S. pneumoniae* strains and strains of VS and its genetic organization as deduced from Southern blotting experiments and nucleotide sequence analyses. E, *Eco*RV; N, *Nco*I. The *parE* and *parC* genes with mosaic structures are indicated by with striped arrows. SOR, *S. oralis*; SMI, *S. mitis*.

varied between 1 and 2.5 kb, those from the *S. pneumoniae* strains with mosaic gene structures studied in this work varied between 1.9 and 6.2 kb (Fig. 4). These values 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* strains with mosaic gene structures and in strains of VS but is absent from 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) sequences in databases. Although the nucleotide sequences of the *ant* genes showed high levels of heterogeneity, comparison of the sequence of this gene from strains of VS and *S. pneumoniae* strains with mosaic gene structures and the *ant* gene sequences present in the databases showed that *ant* genes from *S. pneumoniae* strains with mosaic gene structures and strains of 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* strains with mosaic gene structures indicates that a strain of VS, probably an *S. oralis* or *S. mitis* strain, was the donor in the recombination event that

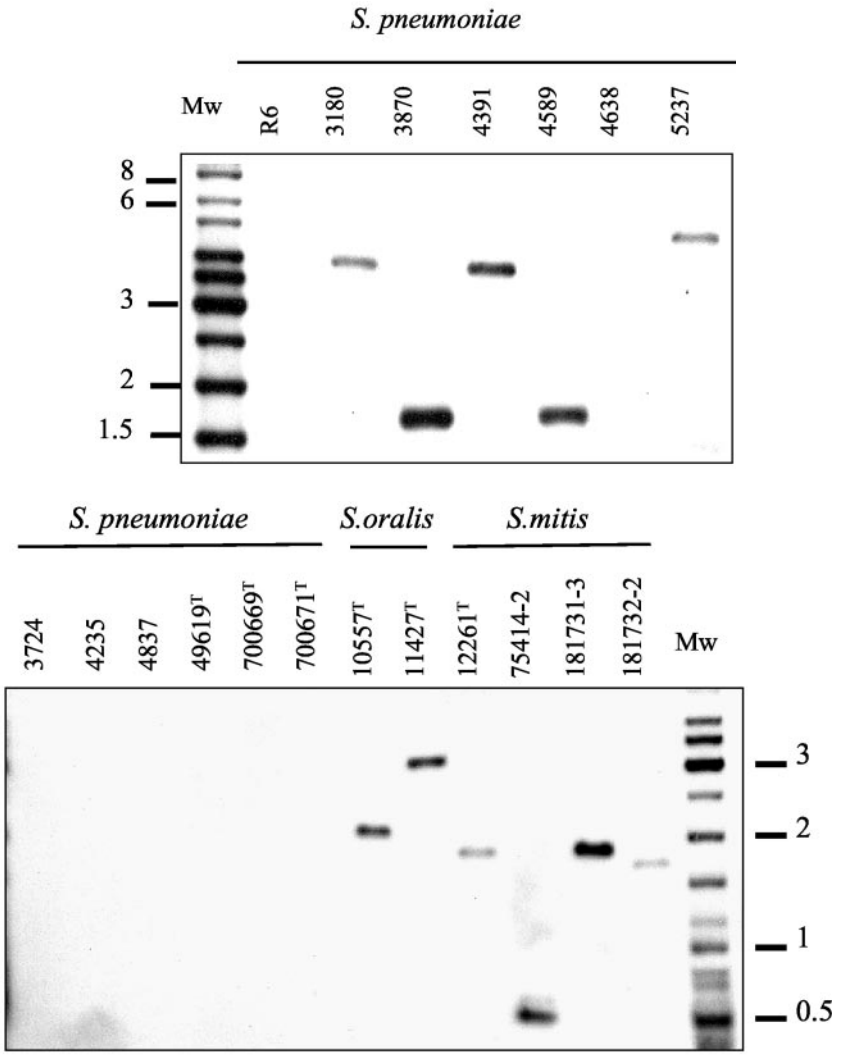

FIG. 5. Southern blot hybridization of *S. pneumoniae* strains and strains of VS with an *ant*-specific probe. Chromosomal DNAs from the indicated strains were cleaved with *Eco*RV-*Nco*I, and the fragments were separated in 0.8% agarose gels. The gels were blotted, and the blot was probed with a biotinylated probe derived from *S. pneumoniae* 3870 containing positions 26 to 290 of the *ant* gene. The numbers on the bottom right are molecular sizes (in kilobases).

originated the mosaic *parE* and *parC* genes. *S. oralis* and *S. mitis* are the species the most closely related to *S. pneumoniae* on the basis of their 16S rRNA sequences, which exhibit more than 99% identity with the 16S rRNA sequence of *S. pneumoniae*, although the DNA-DNA similarity for the total chromosomal DNAs of *S. oralis* and *S. mitis* with the chromosomal DNA of *S. pneumoniae* is less than 50% (30). Analysis of the genetic structures of the five *S. pneumoniae* strains with mosaic gene structures (Fig. 4) suggests that the initial interchange that originated these strains with mosaic gene structures 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* strains with mosaic gene structures. However, identity was observed between the *ant* genes of *S. pneumoniae* strains 3870 and 4589 (Fig. 2). Although these strains had identical *ant* sequences, they were isolated from unrelated patients in different years, had different pulsed-field gel electrophoresis patterns (data not shown), and had different nucleotide sequences in their *parC*, *parE*, *gyrA*, and *atpCA* genes. However, both strains shared the same physical structure in their *parE-ant-parC* regions and *ant* sequences, suggesting that they have interchanged the *parE-ant-parC* region with that of a closely related VS strain.

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

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