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New Mutations and Horizontal Transfer of *rpoB* Among Rifampicin-Resistant *Streptococcus pneumoniae* from four Spanish Hospitals

María José Ferrándiz,[1] Carmen Ardanuy,[2] Josefina Liñares,[2] José María García-Arenzana,[3] Emilia Cercenado, [4] Ana Fleites,[5] Adela G. de la Campa, [1]* and the Spanish Pneumococcal Infection Study Network[6]

Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid[1]; Hospital Universitari de Bellvitge, IDIBELL, L'Hospitalet de Llobregat, Barcelona[2]; Hospital Donostia, San Sebastián, Guipuzcoa[3]; Hospital Gregorio Marañón, Madrid[4]; and Hospital Central de Asturias, Oviedo, Asturias, Spain[5].

*Corresponding author. Mailing address: Unidad de Genética Bacteriana, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain. Phone: (34) 91-5097057; Fax: (34) 91-5097919; E-mail: agcampa@isciii.es

[5]Spanish Pneumococcal Infection Study Network G03/103—general coordination: Román Pallarés; participants and centers: Ernesto García, (Centro de Investigaciones Biológicas, Madrid); Julio Casal, Asuncion Fenoll, Adela G. de la Campa, (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid); Emilio Bouza, (Hospital Gregorio Marañon, Madrid); Fernando Baquero, (Hospital Ramón y Cajal, Madrid); Francisco Soriano,

José Prieto (Fundación Jiménez Díaz y Hospital Clínico, Madrid); Román Pallarés, Josefina Liñares, (Hospital Universitari de Bellvitge, Barcelona); Javier Garau, Javier Martínez Lacasa, Hospital Mutua de Terrassa, Barcelona); Cristina Latorre, (Hospital Sant Joan de Deu, Barcelona); Emilio Pérez-Trallero, (Hospital Donostia, San Sebastian); Juan García de Lomas, (Hospital Clínico, Valencia); and Ana Fleites, (Hospital Central de Asturias).

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ABSTRACT

A total of 103 (0.7%) of 14,236 *Streptococcus pneumoniae* isolates collected in four Spanish hospitals from 1989 to 2003 were resistant to rifampicin (MICs 4-512 μg/ml). Only sixty one (59.2%) of these isolates were available for molecular characterization. Resistance was mostly related to HIV-infected adult patients and to children with conjunctivitis. Thirty six different pulse-field gel-electrophoresis patterns were identified among resistant isolates, five of which were related to international clones (Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, Spain^{14}-5 and clone C of serotype 19F) and accounted for 49.2% of resistant isolates. Single sense mutations at cluster N or I of the *rpoB* gene were found in 39 isolates, while double mutations, either at cluster I, at cluster I and II or at cluster N and III were found in 14 isolates. The involvement of the mutations in rifampicin resistance was confirmed by genetic transformation. Single mutations at cluster N and I conferred MICs of 2 μg/ml and 4-32 μg/ml, respectively. Eight isolates showed high nucleotide sequence variations (2.3 to 10.8%) in *rpoB*, suggesting a recombinational origin for these isolates, being viridans streptococci potential gene donors. Although the majority of rifampicin-resistant isolates were isolated from individual patients without temporal or geographical relationship, clonal dissemination of rifampicin-resistant isolates was observed among 12 HIV-infected patients in the two hospitals with higher rates of resistance.

Global increase in resistance of *Streptococcus pneumoniae* to penicillin and multiple antibacterial agents has emerged worldwide in the 1980s and 1990s severely complicating the treatment of pneumococcal infections (12, 17, 20, 23, 25). The use of rifampicin (RIF) combined with either ß-lactams or vancomycine is recommended for the treatment of meningitis caused by multiresistant pneumococcal isolates (5, 26, 33). The rates of RIF resistance among pneumococci are low and range from 0.1% in the United States (12), to 0.4% in Spain (23) and to 1.4% in Italy (25). RIF resistance is usually preceded by RIF therapy, either for tuberculosis (18) or for prophylaxis or treatment of multidrug resistant pneumococci (36, 38). RIF is also used, in combined therapy, to treat staphylococcal infections, and it is extensively used in the prophylaxis of *Neisseria meningitidis* exposure.

The bactericidal properties of RIF are due to its high-affinity to bind the bacterial DNA-dependent RNA polymerase and inhibit its function (6), which is essential for bacterial growth (21). Structural and biochemical studies of the essential catalytic core of the RNA polymerase (subunit composition $\alpha_2\beta\beta'\omega$) of *Thermus aquaticus* have revealed that RIF interacts with a pocket of the RNA polymerase β subunit within the DNA-RNA channel and blocks the path of the elongating RNA when the transcript becomes two or three nucleotides long (6, 40). RIF-resistance has been described in Gram positive and Gram negative bacteria. The mutations responsible for this phenotype are localized in highly conserved regions, termed clusters N, I, II and III, of the *rpoB* gene encoding the β subunit, (6) (Fig. 1). Residues involved in RIF-resistance in several bacteria (2, 3, 9, 19, 31) form part of the RIF binding pocket and twelve of these residues interact directly with the RIF molecule (6). Few studies describing RIF-resistant pneumococcal clinical isolates have been reported (7, 15, 29, 32, 38), and all the mutations

identified were localized in clusters N, I and II. In this study we report the epidemiological and molecular characteristics of sixty-one RIF-resistant *S. pneumoniae* clinical isolates collected during a 15 year period (1989 to 2003) in four Spanish hospitals.

MATERIALS AND METHODS

Bacterial isolates and susceptibility tests. Identification was according to standard methodology and serotypes were determined by a quellung reaction. MICs of penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol, cotrimoxazole and RIF were determined by the microdilution method (Sensititre commercial plates) according to the National Committee for Clinical Laboratory Standards (NCCLS) methods (30). Rifampicin MICs of transformants were determined by microdilution (30) and by a macrodilution method (1), using a casein hydrolysate-based medium with 0.2% sucrose (AGCH) (22). *S. pneumoniae* ATCC 49619 and *S. pneumoniae* R6 strains were used for the quality control.

Genetic transformation. *S. pneumoniae* strain R6 was grown in AGCH and used as recipient in transformation experiments performed as described (27). Two DNA fragments were used as donors: a 1,662 bp fragment (RpoB residues M1-L554, taking the first residue of RpoB as residue number 1) and a 1,038 bp fragment (RpoB residues A428-M773). These fragments were obtained by PCR amplification from the RIF-resistant isolates and from R6, which was used as a control. Colonies were counted after 24 h growth at 37 ºC in a 5% CO_2 atmosphere in AGCH medium with 1% agar containing 1 μg/ml of RIF.

Pulsed-field-gel-electrophoresis (PFGE) and multilocus sequence typing (MLST). Genomic DNA embedded in agarose plugs was digested with *Sma*I, and fragments were separated by PFGE (28). PFGE patterns were compared to 26 representative international clones

of Pneumococcal Molecular Epidemiology Network (28). Isolates with patterns varying by three or less bands were considered to represent the same PFGE type (37). MLST was carried out (14) in one representative RIF-resistant isolate of each dominant PFGE pattern.

PCR amplification and DNA sequence determination. The RpoB L1-M773 region, was amplified with oligonucleotides rpob1 (5'-TTGACAAGGCTTGGAACTTAT-3') and rpob773R (5'-GTCATGTAGGCAACGAATTGGG-3'). To amplify the 1,662 bp and the 1,038 bp fragments used in transformation experiments, oligonucleotides rpob1 and rpob554R (5'-CAAGTGTCCGTAAGATGACAAG-3'), and rpob428 (5'-CGGTTGGTGAATTGCTTGCCAACCA-3') and rpob773R were used, respectively. Amplifications were performed using 0.5 U of *Thermus thermophilus* thermostable DNA polymerase (Biotools, Madrid, Spain), 0.1 μg of chromosomal DNA, 1 μM (each) of the synthetic oligonucleotide primers, 0.2 mM of each deoxynucleoside triphosphate (dNTP) in the buffer recommended by the manufacturers. Amplification was achieved with an initial cycle of 1 min denaturation at 94ºC; 25 cycles of 30 s at 94ºC, 45 s at 55ºC and 90 s or 180 s polymerase extension step at 72ºC; and a final 3-min 72ºC extension step. PCR fragments were purified using MicroSpin S400 HR columns (Amersham Pharmacia Biotech, Pistcatway, NJ) and sequenced, using the oligonucleotides used in PCR experiments and internal primers, with an Applied Biosystems Prism 377 DNA sequencer, accordingly to protocols provided by the manufacturer.

Nucleotide sequence accession numbers. Sequences submitted to GeneBank were assigned with the following accession numbers: AY695455 to AY695495, AY695497 to AY695516 and AY785246 (RIF resistant isolates); AY695496 (*Streptococcus oralis* ATCC 10557); AY785247 (*S. oralis* NCTC 11427).

RESULTS AND DISCUSSION

Epidemiological characterization of *S. pneumoniae* isolates. The overall prevalence of RIF-resistance among 14,236 pneumococci isolated from clinical specimens in four Spanish hospitals from 1989 to 2003, was 0,7% (103 isolates with MICs ≥4µg/ml). However, geographical variations in resistance rates were found: 0.3% in Hospital Central de Asturias (HCA) in the North West of Spain, 0.4% in Hospital Universitario de Bellvitge (HUB) in the North East of Spain (Barcelona), 1.1% in Hospital Gregorio Marañón (HGM) in the Central part of Spain (Madrid), and 1.1% in Hospital de Donostia (HD) in the North of Spain (Guipuzcoa). The frequency of RIF-resistant isolates in each hospital from 1989 to 2003 is shown in Fig. 2. Two hospitals, HCA and HUB, presented low rates during the study period (0-1.1%) whereas HD and HGM had higher rates, 6% in the 1993-94 period and 3.2% in the 1995-96 period. These increases of resistance were associated to a dissemination of four RIF-resistant clones among HIV-infected patients in HD and HGM. From 1999 to 2003 the rates of RIF resistance were lower than 1.1% in the four hospitals (Fig.2).

Only sixty-one (59.2%) out of 103 RIF-resistant isolates (one per patient) were recovered for further studies from the stock cultures, accounting for more than 50% of RIF-resistant isolates collected annually in each hospital. Among the 61 RIF-resistant isolates studied, 47 were isolated from adults (21-76 years) and 14 from children (<15 years). The sources of the isolates from adults were: sputum (n = 28), conjunctiva swab (n = 1), blood (n = 11), pleural fluid (n = 2), broncho-alveolar fluid (n = 2), catheter protected brush specimen (n = 1), pus (n = 1) and ascitic fluid (n = 1). The majority of pediatric isolates (12 of 14) were from children with conjunctivitis younger than 1 year-old, and the remaining two isolates were from the blood of an 8 year-old HIV-infected girl and from conjunctivitis in a 14 year-old child. More than half (32 of 61, 52.4%)

of RIF-resistant isolates were from HIV-infected adult patients. Moreover, the majority of invasive isolates (15 of 19, 79.0%) and isolates from sputum (18 of 28, 64.3%) were also from HIV-infected patients.

The *in vitro* activity of seven antimicrobial agents against the 61 RIF-resistant isolates was determined and the results are summarized in Table 1. Forty seven (77.1%) isolates were resistant to penicillin (29 intermediate resistant and 18 resistant) and thirty (49.2 %) were erythromycin resistant. Multi-drug resistance (resistance to 3 or more chemically unrelated drugs) was detected in forty-one isolates (67.2%) and 14 of them were resistant to 6 drugs (penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol and co-trimoxazole). Eight of the RIF-resistant isolates (13.1%) were susceptible to the other six antibiotics studied.

Next the serotype of the 61 RIF-resistant isolates was determined. Eighteen different serotypes were identified with the following distribution: 6B (12 isolates), 23F (11 isolates), 14 (8 isolates), 19F (6 isolates), 11 (4 isolates), 6A (3 isolates), 4 (2 isolates), 9N (2 isolates), 9V (2 isolates), and one isolate of each of the remaining serotypes (3, 7F, 15F, 18C, 20, 21, 23A, 31, and 34). Two isolates were non-typable.

The 61 RIF-resistant isolates were also characterized by their PFGE pattern. A total of 36 different PFGE patterns were identified, 5 PFGE patterns accounted for 30 (49.2%) isolates: Spain^{23F}-1 (11 isolates), Spain^{6B}-2 (9 isolates), Spain^{9V}-3 (2 isolates), Spain^{14}-5 (2 isolates) and clone C of serotype 19F (6 isolates). These four international multiresistant epidemic clones have been common in Spain since the 1980s (8, 16, 34). The association between PFGE patterns and global international clones (28) was confirmed by multilocus sequence typing (MLST) of one representative isolate of each PFGE listed in Table 2: Rif-36 (Spain^{23F}-1) had sequence typing (ST) ST81; Rif-19 (Spain^{6B}-2) had ST90; Rif-55 (Spain^{9V}-3) had ST156; Rif-15 (Spain^{14}-5) had

ST17 that is a single locus variant of the reference isolate; and Rif-3 (clone C of serotype 19F) had a ST89. Clone C of serotype 19F (ST89) has been identified in Spain among isolates from meningitis (16) and among ciprofloxacin-resistant isolates (10), and it has also been found sporadically in Italy (11) and Denmark (http://www.mlst.net).

In general, those isolates that shared the same PFGE pattern have the same *rpoB* polymorphisms with respect to the reference sequence of strain R6 (Table 2). However, there were four exceptions: two isolates with *rpoB* mosaic genes (Rif-13 of the Spain^{6B}-2 clone and Rif-31 of clone C of serotype 19F); one isolate (Rif-40 of Spain^{6B}-2 clone) which had those polymorphisms corresponding to the Spain^{23F}-1 clone; and one isolate (Rif-75 of the Spain^{23F}-1 clone) with an additional polymorphism at codon S498.

Twelve RIF-resistant isolates were isolated from twelve HIV-infected patients, and grouped in four clusters (Table 2). One cluster included 5 isolates of the Spain^{23F}-1 clone (Rif-34, -35, -36, -38 and -39) isolated from invasive samples in HGM (Madrid) in 1996. The characteristics of these isolates and the presence of two amino acid changes in RpoB (M488I, H499Y) suggest a cross-transmission of RIF-resistant pneumococci among HIV-infected patients. The remaining three clusters were identified at HD (Guipuzcoa) from 1993 to 1995 and included two isolates of the Spain^{23F}-1 clone (Rif-14 and -22); 3 isolates of Spain^{6B}-2 clone (Rif-18, -19 and -30), and 2 isolates of the Spain^{14}-5 clone (Rif-15 and -16). Temporal or geographical relationship could not be found among the remaining isolates, suggesting that RIF-resistance among pneumococci is mainly a sporadic event that occurs in individual patients.

Mapping of mutations involved in RIF-resistance. Two RpoB regions: L42-V175 (including cluster N) and region Q464-T700 (including clusters I, II and III) were sequenced (Fig. 1).

Nucleotide sequence comparisons of the Q464-T700 region among RIF-resistant isolates and R6 revealed 53 isolates with low nucleotide sequence variations (≤0.7%), and 8 with high nucleotide sequence variations (2.0 to 10.9%). Among the 53 isolates with low variation, 39 had single sense mutations and 14 double sense mutations (Table 3). Single mutations would produce amino acid changes at cluster N (Q150) or cluster I (S481, S482, Q486, D489, S495, H499 or L506)(Fig. 1). The only amino acid change found at cluster N was Q150R, which is involved in low-level resistance as it has been reported before (29). Twelve residues of clusters I and II (shadowed in Fig. 1) that are conserved in pneumococci and in other bacteria, are directly involved in the interaction with RIF in the *T. aquaticus* enzyme (6). Most RIF-resistant isolates had changes at 5 of these residues (Q486, D489, H499, R501, and L506, Table 3). Position H499 was the most frequently affected (26 of 39 single mutants), as previously found in pneumococci, either clinical isolates (15, 32) or laboratory mutants (27), as well as in other bacteria (2, 3, 9, 19, 31). The prevalence of substitutions at H499 could be due to the low biological cost imposed by the presence of changes at this residue, as has been reported for the H499N change in *S. aureus* (39) and for the H499Y change in *S. aureus* (39) and *E. coli* (35). Isolates with the highest MICs (128 μg/ml) carried Q486L, D489V or H499Y changes (Table 3). Two of the single changes, L506S, and H499S, have not been reported before. Double mutations would produce changes at one residue of cluster N and one residue of cluster III (isolate Rif-52, see below), two residues of cluster I, or one residue of cluster I plus a residue of cluster II (Table 3). Although S481, S482, S485, M488, S495, and P537 may not be in direct contact with the RIF molecule (Fig. 1), these residues are located in the vicinity of the RIF-binding pocket. Alteration of these residues may modify the conformation of the pocket and consequently, the binding of the antibiotic.

To establish the contribution of the mutations identified above to RIF resistance, genetic

transformation experiments were performed. PCR products from the RIF-resistant isolates were able to transform the susceptible R6 strain (MIC 0.015 μg/ml) to resistance at high frequency (about 1×10^{-2}) whereas PCR products from R6 transformed at frequencies 10^3-fold lower (about 2×10^{-5}). Transformants containing single mutations had MICs equivalent to that of the corresponding isolate, with a one- or two-fold dilution margin (Table 3). The Q150R change at cluster N conferred a MIC of 2 μg/ml ($T^{Q150R/Rif-42}$), equivalent to the MIC of 4-8 μg/ml for the clinical isolates carrying the same mutation (Rif-42, -52, -61 and -67). Although Rif-52 carried the Q150R change plus the V638G change at cluster III, no RIF-resistant transformants with PCR products carrying V638G were obtained. Moreover, since the MIC of $T^{Q150R/Rif-42}$ is equivalent to that of Rif-52, it could be assumed that V638G is not involved in RIF resistance. On the other hand, single mutations at cluster I conferred MICs 4-32 μg/ml (Table 3). To determine the contribution of the double mutations to resistance, the MICs for transformants containing one or two mutations were compared (Table 3). These comparisons confirmed the contribution of S485P, M488I and L506V to resistance: MIC of $T^{S481P,\ S485P/Rif-59}$ was 8-fold higher than that of $T^{S481P/Rif-59}$; MIC of $T^{M488I,\ H499Y/Rif-34}$ was 8-fold higher than that of $T^{H499Y/Rif-12}$. However, the N547S and D489G changes would not be involved in resistance given that MIC for $T^{H499Y,\ N547S/Rif-11}$ was 2-fold higher than that for $T^{H499Y/Rif-12}$, and MIC of $T^{D489G,\ L506V/Rif-18}$ was equal than that for $T^{L506V/Rif-18}$. On the other hand, the contribution of D489N, R501H, P537S to RIF-resistance could not be discerned, since no transformants with those single changes were obtained (Table 3).

Furthermore, the deduced amino acid sequences of the 8 RIF-resistant recombinant isolates contained several additional changes, besides the H499N and D489V changes involved in resistance (Fig. 3B). All recombinant isolates, except Rif-65, share the Y589F change present in *Streptococcus mitis* NCTC 12261 and *S. oralis* NCTC 11427 and *S. oralis* ATCC 10557 RIF-

susceptible isolates (Fig. 3B), indicating that this residue is not involved in resistance. Likewise, the I624V, Q671K, N623E, and N669D changes would not be implicated in resistance since they are also present in RIF-susceptible *S. mitis* and/or *S. oralis* strains. However, although it is not possible to assign the contribution of each residue to resistance, the comparison of the RIF MICs conferred by the D489V and H499N changes to the non-recombinant pneumococcal isolates (Table 3), suggest that these mutations are indeed responsible for the RIF-resistance phenotypes of the recombinant isolates.

Phylogeny of isolates with high nucleotide sequence variation. As pointed out above, a majority (86.9%) of RIF-resistant isolates showed low-level nucleotide sequence variation (≤ 0.7%) at their Q464-T700 *rpoB* sequences with respect to strain R6. Similar intraspecific variations have been determined for *atpC-atpA* (<0.7%) and for the quinolone-resistance determining regions of *parC*, *parE gyrA* and *gyrB* (≤1%) (4). However, 8 RIF-resistant isolates showed high nucleotide sequence variation (2.0-10.9% in the Q464-T700 fragment and 2.3-10.8 in the L42-T700 fragment). The existence of *S. pneumoniae* RIF-resistant isolates with high nucleotide sequence variations have been previously described (7, 15, 32). This variation was in accordance with the divergence found between *S. pneumoniae* and *S. oralis* for the amylomaltase gene (4-6%) (13) and between *S. pneumoniae* and viridans group streptococci for the *parE* gene (≥ 8.5%) (4). High nucleotide sequence variations (Fig. 3) suggested that these isolates would have a mosaic structure in *rpoB* as a consequence of recombination with viridans group streptococci. To establish the mosaic structure, a 1,977-bp region (residues L42-T700) was sequenced and compared with that from R6. Five isolates (Rif-13, -15, 16, -25, and -56) showed a continuous block of divergence, suggesting that the recombination points are located outside the region analyzed (Fig. 4). Isolates Rif-24 and Rif-65 showed two blocks whereas isolate Rif-31 showed three blocks. The blocks with

divergence lower than 1% could represent the recombination sites.

To establish the origin of the gene donor to the Rif-65 isolate (the isolate showing the highest divergence with respect R6 strain, 10.8%), several WU-Blast2 analysis of the EMBL database using were performed. The sequences more similar to *rpoB* Rif-65 were chosen to perform a phylogenetic analysis. Since the block structure of the *rpoB* genes revealed a common variable region of 357 bp (A474-A592) for the 8 recombinant isolates (Fig. 4A), this fragment was used for the detection of different clusters within the isolates. All recombinant isolates, except Rif-65, formed a monophyletic group including *S. pneumoniae* and viridans streptococcal (*S. oralis* and *S. mitis*) strains (Fig. 4B). These results suggest that the donors in the horizontal transfer of *rpoB* genes to *S. pneumoniae* are the viridans streptococci belonging to *S. mitis* or *S. oralis* species, as described for the penicillin- and fluoroquinolone -resistance determinants (10, 13). Three of these recombinant isolates belonged to the Spain^{14}-5 and Spain^{6B}-2 clones (Table 2), suggesting a possible dissemination of RIF-resistance through these clones.

In conclusion, the incidence of RIF-resistance among *S. pneumoniae* isolates is rare in Spain and was mainly related to HIV-infected patients and children with conjunctivitis, suggesting a prior treatment with this drug. Although cross-transmission of RIF-resistant isolates was demonstrated among HIV-infected patients, the majority of RIF-resistant isolates were isolated from individual patients without temporal or geographical relationship. This resistance was acquired either by point mutation in *rpoB* gene or by recombination with *viridans* group streptococci. Continuous surveillance of resistance to rifampicin among invasive pneumococci is important, because in serious pneumococcal infections a combination of rifampicin and third generation cephalosporin or vancomycin is recommended.

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REFERENCES

1. **Amsterdam, D.** 1986. Susceptibility testing of antimicrobials in liquid media. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Lippincott Williams & Wilkins, Baltimore.
2. **Aubry-Damon, H., M. Galimand, G. Gerbaud, and P. Courvalin.** 2002. *rpoB* mutation conferring rifampin resistance in *Streptococcus pyogenes*. Antimicrob. Agents Chemother. **46:**1571-1573.
3. **Aubry-Damon, H., C. J. Soussy, and P. Courvalin.** 1998. Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **42:**2590-2594.
4. **Balsalobre, L., M. J. Ferrándiz, J. Liñares, F. Tubau, and A. G. de la Campa.** 2003. Viridans Group Streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **47:**2072-2081.
5. **Bradley, J. S., and W. M. Scheld.** 1997. The challenge of penicillin-resistant *Streptococcus pneumoniae* meningitis: current antibiotic therapy in the 1990s. Clin. Infect. Dis. **24:**213-221.
6. **Campbell, E. A., N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb, and S. A. Darst.** 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell **104:**901-912.

7. **Chen, J. Y., C. P. Fung, F. Y. Chang, L. Y. Huang, J. C. Chang, and L. K. Siu.** 2004. Mutations of *rpoB* gene in rifampicin-resistant *Streptococcus pneumoniae* in Taiwan. J. Antimicrob. Chemother. **53:**375-378.

8. **Coffey, T., S. Berron, M. Daniels, M. García-Leoni, E. Cercenado, E. Bouza, A. Fenoll, and B. G. Spratt.** 1996. Multiply antibiotic-resistant *Streptococcus pneumoniae* recovered from Spain hospitals (1988-1994): novel major clones of serotypes 14, 19F, and 15F. Microbiol. **142:**2747-2757.

9. **Cruchaga, S., M. Pérez-Vázquez, F. Román, and J. Campos.** 2003. Molecular basis of rifampicin resistance in *Haemophilus influenzae*. J. Antimicrob. Chemother. **56:**1011-1014.

10. **de la Campa, A. G., L. Balsalobre, C. Ardanuy, A. Fenoll, E. Pérez-Trallero, J. Liñares, and the Spanish Pneumococcal Infection Study Network G03/103.** 2004. Fluoroquinolone resistance in penicillin-resistant *Streptococcus pneumoniae* clones, Spain. Emerg. Infect. Dis. **10:**1751-1759.

11. **Dicuonzo G, G. Gerardi, R. E. Gertz, F. D'Ambrosio, A. Goglio, G. Lorino, S. Recchia, A. Pantosti, and B. Beall.** 2002. Genotypes of invasive pneumococcal isolates recently recovered from Italian patients. J. Clin. Microbiol. **40:**3660-3665.

12. **Doern, G. V., K. P. Heilmann, H. K. Huynh, P. R. Rhomberg, S. L. Coffman, and A. B. Brueggemann.** 2001. Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999-2000, including a comparison of resistance rates since 1994-1995. Antimicrob. Agents Chemother. **45:**1721-1729.

13. **Dowson, C. G., A. Hutchinson, N. Woodford, A. P. Johnson, R. C. George, and B. G. Spratt.** 1990. Penicillin-resistant viridans streptococci have obtained altered penicillin-

binding protein genes from penicillin-resistant strains of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **87:**5858-5862.

14. **Enright, M., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiol. **144:**3049-3060.
15. **Enright, M., P. Zawadski, P. Pickerill, and C. G. Dowson.** 1998. Molecular evolution of rifampicin resistance in *Streptococcus pneumoniae*. Microb. Drug Resist. **4:**65-70.
16. **Enright, M. C., A. Fenoll, D. Griffiths, and B. G. Spratt.** 1999. The three major Spanish clones of penicillin-resistant *Streptococcus pneumoniae* are the most common clones recovered in recent cases of meningitis in Spain. J. Clin. Microbiol. **37:**3210-3216.
17. **Fenoll, A., I. Jado, D. Vicioso, A. Pérez, and J. Casal.** 1998. Evolution of *Streptococcus pneumoniae* serotypes and antibiotic resistance in Spain: update (1990-1996). J. Clin. Microbiol. **36:**3447-3454.
18. **García-Arenzana J. M., M. Montes, and E. Pérez-Trallero.** 1994. Are rifampin-resistant *Streptococcus pneumoniae* strains a consequence of the increase in cases of tuberculosis? Clin. Infect. Dis. **19:**360-361.
19. **Herrera, L., S. Jiménez, A. Valverde, M. A. García-Aranda, and J. A. Sáez-Nieto.** 2003. Molecular analysis of rifampicin-resistant *Mycobacterium tuberculosis* isolated in Spain (1996-2001). Description of new mutations in the *rpoB* gene and review of the literature. Int. J. Antimicrob. Agents **21:**403-408.
20. **Jacobs, M. R., D. Felmingham, P. C. Applelbaum, R. N. Grüneberg, and The Alexander Project Group.** 2003. The Alexander project 1998-2000: susceptibility of pathogens

isolated from community-acquired respiratory tract infection to commonly used antimicrobial agents. J. Antimicrob. Chemother. **52:**229-246.

21. **Jin, D. J., and Y. N. Zhou.** 1996. Mutational analysis of structure-function relationship of RNA polymerase in *Escherichia coli.* Methods Enzymol. **273:**300-319.

22. **Lacks, S. A.** 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53:**207-35.

23. **Liñares, J., R. Pallarés, T. Alonso, J. L. Pérez, J. Ayats, F. Gudiol, P. F. Viladrich, and R. Martín,** 1992. Trends in antimicrobial resistance of clinical isolates of *Streptococcus pneumoniae* in Bellvitge Hospital, Barcelona, Spain (1979-1990). Clin. Infect. Dis. **15:**99-105.

24. **Liñares, J., F. Tubau, and M. A. Dominguez.** 1999. Antibiotic resistance in *Streptococcus pneumoniae* in Spain: an overview in the 1990s. *In* A. Tomasz (ed.), *Streptococcus pneumoniae.* Molecular biology and mechanisms of disease-update for the 1990s. Mary Ann Liebert Inc., New York.

25. **Marchese, A., S. Mannelli, E. Tonoli, F. Gorlero, M. Toni, and G. C. Schito.** 2001. Prevalence of antimicrobial resistance in *Streptococcus pneumoniae* circulating in Italy: results of the Italian Epidemiological Observatory Survey. Microb. Drug Resist. **7:**277-287.

26. **Martínez-Lacasa J, C. Cabellos, A. Martos, A. Fernández, F. Tubau, P. F. Viladrich, J. Liñares, and F. Gudiol.** 2002. Experimental study of the efficacy of vancomycine, rifampicin and dexamethasone in the therapy of pneumococcal meningitis. J. Antimicrob. Chemother. **49:**507-513.

27. **Martín-Galiano, A. J., and A. G. de la Campa.** 2003. High-efficiency generation of antibiotic-resistant strains of *Streptococcus pneumoniae* by PCR and transformation. Antimicrob. Agents Chemother. **47:**1257-1261.

28. **McGee L, L. McDougal, J. Zhou, B.G. Spratt, F.C. Tenover, R. George, R. Hakenbeck, W. Hryniewicz, J. C. Lefevre, A. Tomasz, and K. P. Klugman.** 2001. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. J. Clin. Microbiol. **39:**2565-2571.

29. **Meier, P. S., S. Utz, S. Aebi, and K. Mühlemann.** 2003. Low-level resistance to rifampin in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **47:**863-868.

30. **National Committee for Clinical Laboratory Standars.** 2004. Performance standards for antimicrobial susceptibility testing. Fourteenth informational supplement. NCCLS document M100-S14. The Committee, Wayne (PA).

31. **Nielsen, K., P. Hindersson, N. Hoiby, and J. M. Bangsborg.** 2000. Sequencing of the *rpoB* gene in *Legionella pneumophila* and characterization of mutations associated with rifampin resistance in the legionellaceae. Antimicrob. Agents Chemother. **44:**2679-2683.

32. **Padayachee, T., and K. P. Klugman.** 1999. Molecular basis of rifampin resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **43:**2361-2365.

33. **Paris, M. M., S. M. Hickey, M. I. Uscher, S. Shelton, K. D. Olsen, and G. H. McCracken,** 1994. Effect of dexamethasone on therapy of experimental penicillin- and cephalosporin-resistant pneumococcal meningitis. Antimicrob. Agents Chemother. **38:**1320-1324.

34. **Pérez-Trallero, E., J. M. Marimón, A. González, and L. Iglesias.** 2003. Spain 14-5 international multiresistant clone resistant to fluoroquinolones and other families of antibiotics. J. Antimicrob. Chemother. **51:**715-719.

35. **Reynolds, M. G.** 2000. Compensatory evolution in rifampin-resistant *Escherichia coli*. Genetics **156:**1471-1481.

36. **Subramanian, D., J. A. T. Sandoe, V. Keer, and M. H. Wilcox.** 2003. Rapid spread of penicillin-resistant *Streptococcus pneumoniae* among high-risk hospital inpatiens and the role of molecular typing in outbreak confirmation. J. Hosp. Infect. **54:**99-103.

37. **Tenover, F. C., R. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns productiond by pulse-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233-2239.

38. **van Tilburg, P. M. B., D. Bogaert, M. Sluijter, A. R. Jansz, R. de Groot, and P. W. M. Hermans.** 2001. Emergence of rifampin-resistant *Streptococcus pneumoniae* as a result of antimicrobial therapy for penicillin-resistant strains. Clin. Infect. Dis. **33:**e93-e96.

39. **Wichelhaus, T. A., B. Böddinghaus, S. Besier, V. Schäfer, V. Brade, and A. Ludwig.** 2002. Biological cost of rifampin from the perspective of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46:**3381-3385.

40. **Zhang, G., E. A. Campbell, L. Minakhin, C. Ritcher, K. Severinov, and S. A. Darst.** 1999. Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Ä resolution. Cell **98:**811-824.

FIGURE LEGENDS

FIG. 1. Regions of *S. pneumoniae* RpoB and mutations conferring RIF-resistance. RpoB is represented as a bar showing clusters N, I, II and III (black boxes) and sequenced regions (stripped area). PCR fragments used for transformations (black bars) and primers (black arrows) are indicated above. Amino acids that constitute clusters N, I and II of *S. pneumoniae* (SPN), *Staphylococcus aureus* (SAU), *Mycobacterium tuberculosis* (MTB), *Escherichia coli* (ECO), and *T. aquaticus* (TAQ) are indicated. Amino acid changes found in resistant isolates are shown above the *S. pneumoniae* sequence, identical residues are indicated with a star below the *T. aquaticus* sequence, residues that changed in RIF-resistant isolates are underlined, the 12 residues involved in RIF binding are shadowed.

FIG. 2. Frequencies of RIF-resistant isolates in four hospitals during the 1989-2003 period. HD, hospital Donostia (n = 4211); HUB, Hospital Universitario de Bellvitge (n = 5381): HCA, Hospital Central de Asturias (n = 1056); HGM, Hospital Gregorio Marañón (n = 3588).

FIG. 3. Nucleotide (A) and amino acid (B) sequence variations at RpoB T464-T700 region of RIF-resistant recombinant isolates. The nucleotides and amino acids present at each polymorphic site are shown in full for the R6 strain, but for the other isolates, only sites that differ from those of R6 are shown. Codon numbers are indicated vertically above the sequences. Positions 1, 2, and 3 refer to the first, second, and third nucleotides in the codon, respectively. Sense mutations and a amino acid changes involved in RIF-resistance are showed in boldface and underlined. Nucleotides of clusters I, II and III are shadowed. SPN R6, *S. pneumoniae* R6; SMI 12261, *S. mitis* NCTC 12261; SOR 10557, *S. oralis* ATCC 10557; SOR 11427, *S. oralis* NCTC 11427.

FIG. 4. The pneumococcal recombinant isolates interchanged parts of their *rpoB* genes with viridans streptococci. (A) Mosaic structure of a 1,977-bp region (L42-T700) of *rpoB*. The

divergence of each block with respect the R6 sequence is indicated. (B) Phylogenetic tree of a 357-bp region including RpoB residues A474-A592 in which all isolates showed nucleotide sequence variations in the 4%-9% range. Phylogenetic and molecular evolutionary analysis were conducted with the MEGA program (version 2.1) by the Neighbor-Joining method. Only bootstrap confidence intervals exceeding 90% are shown.

TABLE 1. *In vitro* activity of 7 antimicrobial drugs against 61 rifampicin-resistant *Streptococcus pneumoniae* isolates[a].

Drug	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC Range (µg/mL)	Susceptible breakpoints	%S	%I	%R	%I+R
Penicillin	0.5	2	0.03-8	≤0.06	22.9	47.5	29.5	77.1
Erythromycin	0.06	≥256	0.06-≥256	≤0.25	50.8	0	49.2	49.2
Clindamycin	0.06	≥256	0.06-≥256	≤0.25	52.5	0	47.5	47.5
Tetracycline	16	64	0.12-64	≤2	34.4	0	65.6	65.6
Chloramphenicol	4	16	2-32	≤4	56.4	0	43.6	43.6
Cotrimoxazole	≥4/76	≥4/76	0.5/9.5-≥4/76	≤0.5/9.5	32.2	1.6	66.1	67.7
Rifampicin	32	512	4- 512	≤1	0	0	100	100

[a] S, susceptible; I, intermediate; and R, resistant, according to National Committee for Clinical Laboratory Standards (NCCLS) 2004 interpretative criteria.

TABLE 2. Summary of phenotypic characteristics and changes in RpoB among the most prevalent RIF-resistant pneumococcal clones

PFGE[a]	Isolate	Sero-type	Site[b]	Year	Origin[c]	Resistance pattern [d]	Nucleotide polymorphisms at *rpoB*[e]	Amino acid changes at RpoB[f]
Spain^{23F}-1	Rif-7	23F	HD	1991	Sputum	PTCSxTR	A516, V520, Q535, G551	**D489N, R501H**
	Rif-14	23F	HD	1993	Sputum	PTCSxTR	A516, V520, Q535, G551	**H499N**
	Rif-22	23F	HD	1994	Blood	PTCSxTR	A516, V520, Q535, G551	**H499N**
	Rif-34	23F	HGM	1996	Blood	PTCEClSxTR	A516, V520, Q535, G551	**M488I, H499Y**
	Rif-35	23F	HGM	1996	Blood	PTCEClSxTR	A516, V520, Q535, G551	**M488I, H499Y**
	Rif-36	23F	HGM	1996	Blood	PTCEClSxTR	A516, V520, Q535, G551	**M488I, H499Y**
	Rif-38	23F	HGM	1996	Blood	PTCEClSxTR	A516, V520, Q535, G551	**M488I, H499Y**
	Rif-39	23F	HGM	1996	Pus	PTCEClSxTR	A516, V520, Q535, G551	**M488I, H499Y**
	Rif-63	23F	HD	2001	Eye	PTCEClSxTR	A516, V520, Q535, G551	**L506S**
	Rif-75	23F	HUB	1995	Sputum	PTEClSxTR	A516, V520, Q535, G551, S498	**H499N**
	Rif-76	23F	HUB	1997	Sputum	PTCSxTR	A516, V520, Q535, G551	**H499Y**
Spain^{6B}-2	Rif-40	6B	HD	1996	Sputum	PTCR	A516, V520, Q535, G551	**H499Y**
	Rif-42	6B	HGM	1997	BAL	PTEClSxTR	I468, Q535	**Q150R**
	Rif-18	6B	HD	1994	Sputum	PTEClR	I468, Q535	**D489G, L506V**
	Rif-19	6B	HD	1994	Blood	PTEClR	I468, Q535	**D489G, L506V**
	Rif-30	6B	HD	1995	Blood	PTEClR	I468, Q535	**D489G, L506V**
	Rif-17	6B	HUB	1994	Sputum	PTCEClSxTR	I468, Q535	**D489N, P537S**

	Rif-23	6B	HUB	1995	Sputum	PTCEClSxTR	I468, Q535	**H499Y**
	Rif-53	6B	HD	1999	Sputum	PTCEClSxTR	I468, Q535	**H499Y**
	Rif-13	6B	HCA	1993	BPS	PTCSxTR		Y589F, I608V, I624V, N669D, Q671K, **H499N**,
Spain[9V]-3	Rif-55	14[g]	HCA	2000	Pleural	PSxTR	I468, Q535,	**D489V**
	Rif-1	9V	HD	1990	Sputum	PSxTR	I468, Q535,	**H499Y**
Spain[14]-5	Rif-15	14	HD	1993	Sputum	PTCEClSxTR		Y589F, **H499N**,
	Rif-16	14	HD	1993	Sputum	PTCEClSxTR		Y589F, **H499N**,
Clone C[h]	Rif-2	19F	HUB	1991	Sputum	PTSxTR	A474, V520, Q535,	**S481P, H499Y**
	Rif-3	19F		1991	Sputum	PTSxTR	A474, V520, Q535,	**D489V**
	Rif-12	19F		1999	Eye	PTCEClSxTR	A474, V520, Q535,	**H499Y**
	Rif-28	19F		1995	Sputum	PCSxTR	A474, V520, Q535,	**H499N**
	Rif-37	19F		1996	Sputum	PTEClSxTR	A474, V520, Q535,	**H499N**
	Rif-31	19F		1995	Blood	PTCSxTR		Y589F, **D489V**

[a] PFGE, pulse-field gel electrophoresis *Sma*I patterns.

[b] HD, Hospital de Donostia; HUB, Hospital Universitario de Bellvitge; HCA, Hospital Central de Asturias; HGM, Hospital Gregorio Marañón.

[c] BAL, bronchial alveolar wash specimen; BPS, bronchial protected catheter brush specimen.

[d] P, resistant to penicillin (MIC 0.12-4 μg/ml); T, resistant to tetracycline (MIC ≥4 μg/ml); C, resistant to chloramphenicol (MIC ≥8μg/ml); E, resistant to erythromycin (MIC ≥0.5 μg/ml); Cl,

resistant to clindamycin (MIC ≥0.5 μg/ml); SxT, resistant to trimethropin-sulfamethoxazole (MICs ≥ 4/ 76 μg/ml), R, resistant to RIF (MIC ≥ 4 μg/ml).

[e] RpoB regions sequenced were L42-V175 and Q464-T700. Nucleotide polymorphisms are indicated by the residue number, changes observed were: I468 (ATT instead ATC); A474 (GCG instead GCA); A516 (GCT instead GCC); V520 (GTA instead GTG); Q535 (GAG instead GAA); G551 (GGT instead GGA), and S498 (TCA instead TCT).

[f] Residue changes involved in RIF resistance are showed in boldface, and double-underlining indicates that the residue is located in a gene with a mosaic structure.

[g] Capsular switching.

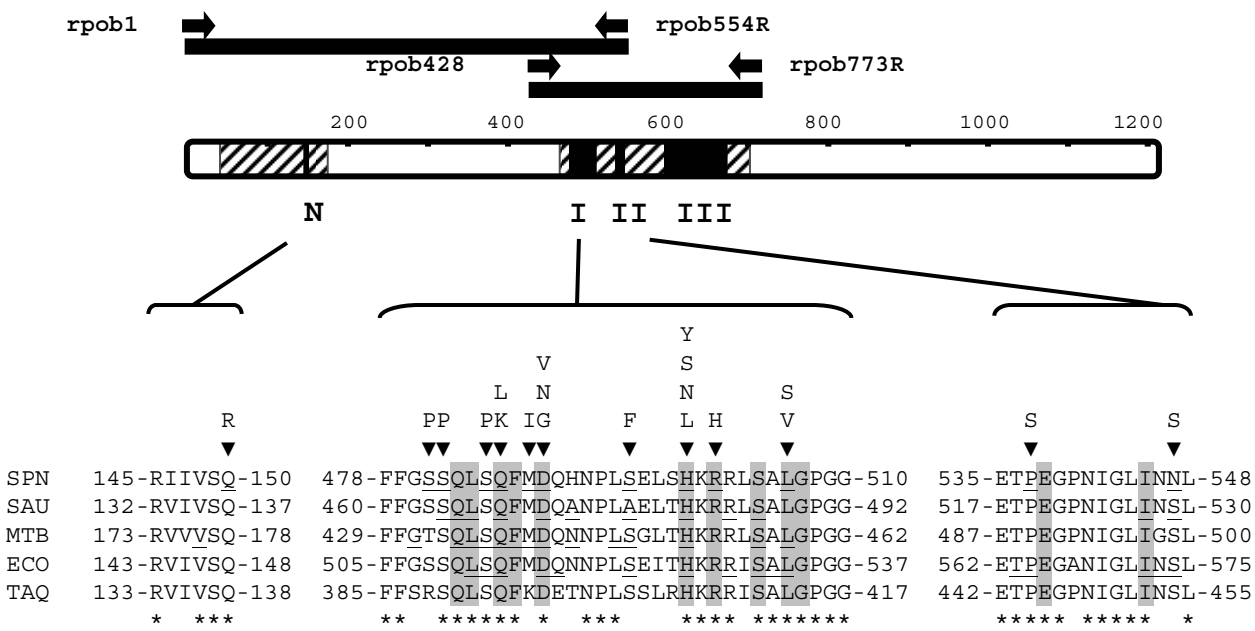
[h] C, PFGE type related to serotype 19F with MLST 89.

TABLE 3. Relationship between RIF MICs and amino acid changes in RpoB in 53 non-recombinant RIF-resistant isolates[a]

RpoB change at clusters:			No	Isolate or transformant strain	Rifampicine
N	**I**	**II** **III**	isolates		MIC (μg/ml)
Q150R			3	Rif-42, -61, -67	4-8
				$T^{Q150R/Rif-42}$	2
	S481P		1	Rif-26	8
				$T^{S481P/Rif-59}$	4
	S482P		1	Rif-10	32
				$T^{S482P/Rif-10}$	16
	Q486K		1	Rif-33	64
				$T^{Q486K/Rif-33}$	32
	Q486L		1	Rif-8	128
				$T^{Q486L/Rif-8}$	32
	D489V		2	Rif-3, -55	128
				$T^{D489V/Rif-31}$	32
	S495F		2	Rif-51, -68	16-32
				$T^{S495F/Rif-51}$	32
	H499L		1	Rif-29	64
				$T^{H499L/Rif-29}$	16
	H499N		11	Rif-9, -14, -22, -28, -32, -37, -45, -49, -58, -60, -75	8-16
				$T^{H499N/Rif-9}$	8

H499S		2	Rif-69, -71	64
			$T^{H499S/Rif-69}$	16
H499Y		12	Rif-1, -12, -23, -40, -50, -53, -62, -64, -66, -70, -74, -76	128
			$T^{H499Y/Rif12}$	32
L506S		2	Rif-57, -63	8-16
			$T^{L506S/Rif-57}$	8
Q150R	V638G	1	Rif-52	4
S481P, S485P		1	Rif-59	128
			$T^{S481P, S485P/Rif-59}$	32
S481P, H499Y		1	Rif-2	512
			$T^{S481P, H499Y/Rif-2}$	128
M488I, H499Y		5	Rif-34, -35, -36, -38, -39	512
			$T^{M488I, H499Y/Rif-34}$	256
D489G, L506V		3	Rif-18, -19, -30	16-32
			$T^{L506V/Rif-18}$	16
			$T^{D489G, L506V/Rif-18}$	16
D489N,R501H		1	Rif-7	32
			$T^{D489N, R501H/Rif-7}$	16
D489N	P537S	1	Rif-17	16
			$T^{D489N, P537S /Rif-17}$	16
H499Y	N547S	1	Rif-11	256
			$T^{H499Y, N547S /Rif-11}$	64

[a] Transformant strains (T) are R6-derivatives that carry the indicated mutations from the indicated donor isolates. For instance, $T^{Q150R/Rif\text{-}42}$ carries the Q150R change of isolate Rif-42.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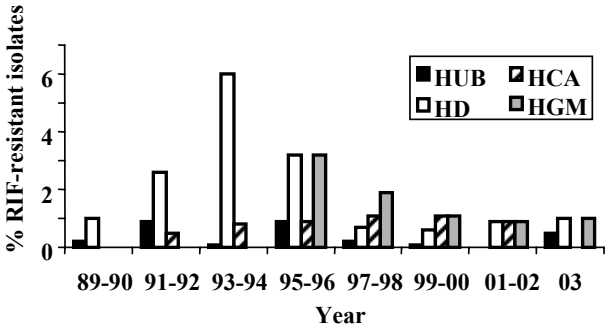
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03
Year

A

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Rif-15,-16  C.......T.....AA...A.T.A.CCG.T.......................C.CT..GG
Rif-25      C.......T.....AA...A.T...CAG..T...G...T.......G.....CTC..T...A
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SOR 11427   ......TC.GCTT.AGT.G..AG.AG.G..G..T.C..T.GAAA..GT...TC..C.G...A
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```

B

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Rif-15,-16  ...N...F..........   8-16     14
Rif-25      ...N...F..V.V.K...      8     NT
Rif-13      ...N...F..V.VDK...     16      6B
Rif-56      ...N...F..VEVDK.V.     64     14
Rif-24      ...N...FH.VEVDK.V.     64     23A
SMI12261    .......F..V.V.K...  0.015
SOR10557    .......F..VEVDK...  0.015
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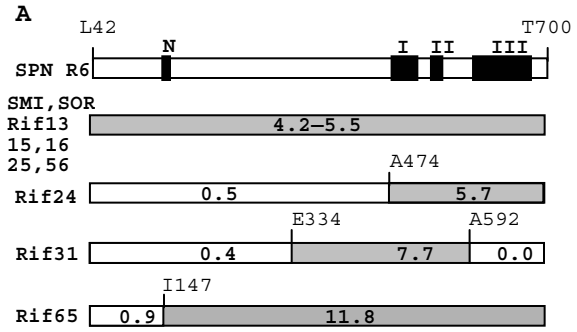
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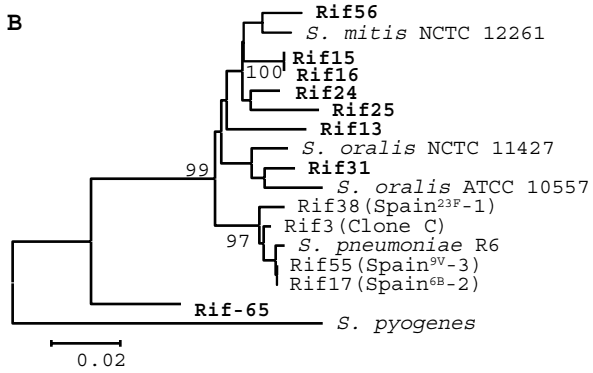
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