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New species genetic approach to identify strains of streptococci mitis group that are donors of rifampin resistance to *Streptococcus pneumoniae*

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Eight rifampin resistant streptococci of the mitis group were identified at the species level by using a concatenated 16S rDNA-*sodA*-*rpoB*-*hlpA* sequence. Characterization of their *rpoB* alleles showed single amino acid changes involved in rifampin resistance. Comparison of RpoB sequences from pneumococcal recombinant isolates, viridans isolates and type strains revealed a species-specific amino acid signature, which allowed ascertaining that recombinant RpoBs were originated in genetic interchanges with *Streptococcus mitis* and *Streptococcus oralis*.

Viridans streptococci (VS) form part of the microbiota of the oropharynx, and the gastrointestinal and female genital tracts (13, 37). However, they cause endocarditis in native valves, and pneumonia in neutropenic cancer patients (7, 8, 43). By their 16S ribosomal DNA (rDNA) sequences, VS can be classified into five groups: mutans, salivarius, anginosus, sanguinis and mitis (18). Species of the mitis group (SMG) includes *Streptococcus mitis, Streptococcus sanguinis, Streptococcus parasanginis, Streptococcus gordonii, Streptococcus oralis, Streptococcus cristatus, Streptococcus infantis, Streptococcus peroris*, *Streptococcus pneumoniae,* and *Streptococcus pseudopneumoniae*. Clinical features, together with their optochin susceptibility and bile solubility distinguish *S. pneumoniae* from other SMG species (27, 30, 39) although optochin susceptible VS have been found (6, 31).

SMG isolated from blood cultures of cancer patients are commonly resistant to antibiotics (2, 16, 21, 22, 25, 42) and constitute a reservoir of resistance by acting as donors in the horizontal transfer of DNA to pneumococci, as observed for penicillin and fluoroquinolones (5, 17, 35, 38, 40). Rifampin is used in the treatment of tuberculosis and in meningitis caused by multiresistant pneumococcal strains, combined with either ß-lactams or vancomycin (9, 33, 36). Rifampin binds to the DNA-dependent RNA polymerase (RpoB) inhibiting its function (10), which is essential for bacterial growth (15, 26). Resistance changes have been identified in four conserved regions (N, I, II and III) of RpoB in several bacteria (3, 4, 14, 24, 34). This resistance in *S. pneumoniae* is due to spontaneous mutations and it has been suggested to be also acquired by recombination with SMG species (19). In this study we have characterized rifampin-resistant SMG isolates, complementing the unique study of *S. mitis* (1), to ascertain the origin of the recombinant *rpoB* genes found in *S. pneumoniae* isolates.

Identification of viridans streptococci isolates to the species level. Among 1,272 VS isolates collected from adult patients at Hospital de Bellvitge (Barcelona) during ten years (1998-2007), 10 (0.79%) were rifampin-resistant as determined by broth microdilution and agar dilution (11, 12). Eight of them with high resistance level (MIC≥ 32µg/ml) were available for this study (Table 1). Although one VS isolate per patient was recovered, isolate 113 collected from patient 3 also yielded a rifampin-resistant *S. anginosus* isolate (113A) that was used for sequence comparisons. The global incidence of rifampin resistance observed in this study was similar to that found in Spain for *S. pneumoniae* (0.70%) (19), although a higher rate (3%) has been found in SMG isolated from hematologic cancer patients (1).

The 8 VS isolates were identified by phenotypic (39) and molecular methods. We used concatenated 16S rDNA-*sodA*-*rpoB*-*hlpA* sequences made with partial 16S rDNA, *rpoB* and *sodA* (1,198, 344, and 324-bp, respectively) and full *hlpA* (276-bp). To amplify 16S rDNA and *hlpA* we used the following primers: 16SDNAF1 (5'-GAGTTGCGAACGGGTGAGT-3') and 16SDNAR1 (5'-AGCGATTCCGACTTCAT-3'); huATG (5'-ATGGCAAACAAACAAGATT-3') and huTAA (5'-TTATTTAACAGCGTCTTTAAGAGC-3'). Partial *sodA* and *rpoB* were amplified and sequenced as described (28, 19). These genes were selected for their polymorphism among streptococci, and because they have been used as part of the *ddl*-*gdh*-*rpoB*-*sodA* sequence to differentiate SMG isolates (29). We assumed that *hlpA,* (coding the histone-like DNA binding protein HU) would improve our concatenated sequence discrimination capacity since HU, as an architectural cofactor, may require different DNA binding geometries (41), and probably sequence specificity. Clustering (bootstrap values ≥ 92%) in a phylogenetic

tree of the 2,142-bp 16S rDNA-*sodA-rpoB-hlpA* sequences of the eight isolates and type strains allowed species identification (Fig. 1, Table 1). Among the six clusters observed (plus the out-group), all except *S. pneumoniae*/ *S. pseudopneumoniae* and *S. sanguinis*/ *S. gordonii* formed species-specific groups.

The within-group sequence diversity (mean ± standard deviation) for *S. pneumoniae*/ *S. pseudopneumoniae* (0.4% ± 0.1%), *S. mitis* (1.2% ± 0.3%), and *S. oralis* (2.0% ± 0.5%) clusters, reflected low sequence diversity. Our *S. pneumoniae*/ *S. pseudopneumoniae* value was nearly half to that obtained using the *ddl-gdh-rpoB-sodA* concatenates (29) and for *S. mitis*, it was 4-to-5 fold lower than the value obtained by MLST (20, 23). Additionally, *S. pneumoniae*/ *S. pseudopneumoniae*, *S. mitis*, and *S. oralis* clusters were clearly separated, as their genetic distances to the node formed with the branch of *S. pneumoniae* / *S. pseudopneumoniae* were: 1.1% ± 0.7%, 1.9% ± 0.0%, and 3.2% ± 0.4% (mean ± SD), which are statistically significant ($p < 0.0001$).

Determination of mutations involved in rifampin resistance. RpoB regions L42-V175 and Q464-T702 were sequenced as described (19) and compared. Changes were found in the Q464- T702 region (Table 1 and Fig. 2). Among them, only H499N had been described in rifampin resistant in SMG (1), while the rest, with the exception of S504F (isolate 395), had been involved in resistance in *S. pneumoniae* (19), To test its role in resistance, transformation of *S. pneumoniae* R6 with the Q464-T700 fragment carrying S504F was performed as described previously (32). The transformant had the same rifampin MIC of isolate 395 showing that this change was indeed involved in resistance. Additional changes in cluster III, which were present in both susceptible and resistant strains (Fig. 2), are supposed not to be involved in resistance.

RpoB sequence comparisons revealed that most changes not involved in rifampin

resistance were conserved among the species (no more than two amino acid differences in regions I, II and III) (Fig. 2). These changes could be considered as a species-specific amino acid signature that give information about the phylogenetic origin of the isolates, as observed for ComC (29). On the basis of similarity scores with type strains (ClustalW), six groups could be deduced (Fig. 2) coinciding with the six clusters of the phylogenetic tree based on 16S rDNA-*sodA*-*rpoB*-*hlpA* sequences (Fig. 1). Two exceptions were observed: *S. pseudopneumoniae* type strain that shared the same similarity with *S. pneumoniae* and *S. mitis*; and isolate 889 (*S. parasanguinis* by the concatenated sequence) that shared the same similarity with *S. gordonii* and *S. sanguinis*. Furthermore, this amino acid signature allowed us to ascertain the origin of recombinant RpoBs. Six rifampin-resistant *S. pneumoniae* recombinant isolates, which we had previously characterized (19), were compared with other VS. Four of them grouped with *S. mitis* and *S. oralis* (RIF13, -25, -24, and 56) (Fig. 2). The source for isolates RIF31 and RIF65 could not be deduced because of the partial recombinational nature of the first (19) and poor scores with any of the type strains for the last, due to either the donor is not included in this comparison or several recombination events have occurred. In conclusion, *S. pneumoniae* and SMG share the same mechanisms of rifampin resistance and recombination events in *S. pneumoniae* take place mostly with *S. mitis* and *S. oralis* species.

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FIGURE LEGENDS

FIG. 1. Phylogenetic tree of concatenated sequences of 16S rDNA, *sodA*, *rpoB,* and *hlpA*. Analysis was conducted with the MEGA program (version 4.0.2) with the Neighbor Joining algorithm. Bootstrap confidence intervals exceeding 90% are shown in italics. The scale bar calculated by the MEGA program indicates the genetic divergence. Eight *S. pyogenes* strains were used as outgroup. Shadowed in grey are clusters that identified *S. pneumoniae* (Spn) plus *S. pseudopneumoniae* (Sps); *S. mitis* (Smi); *S. oralis* (Sor); *S. parasanguinis* (Spa); *S. sanguinis* (Ssa) and *S. gordonii* (Sgo); *S. anginosus* (San); and *S. pyogenes* (Spy) strains. SMG isolates characterized in this work appear in boldface and followed by an asterisk. The arrow indicates the node that separates *S. pneumoniae* plus *S. pseudopneumoniae* from the rest of the clusters.

FIG. 2. Amino acid sequence variations in RpoB (V475- A702) of rifampin-resistant recombinant isolates of *S. pneumoniae* (Spn-M) and SMG rifampin-resistant isolates characterized in this work (boldface and marked with an asterisk). RpoB is represented as a bar with clusters N, I, II, and III as black boxes and zigzagged areas showing sequenced areas. The amino acids present at each polymorphic site are shown in full for *S. pneumoniae* strains (R6, P1031, Hungary, Taiwan1, TIGR4, and JJA). For the other strains only sites that differ from those are shown. Residue numbers are indicated vertically above the sequences and black boxes below numbers localize clusters I and III. Amino acid changes involved in rifampin resistance are shown in boldface and underlined. Species nomenclature as in Fig. 1. ^{T} indicates type strain. Recombinant sequences are shadowed in

1 grey. Squares group sequences with highest similarity according to scores obtained by
2 ClustalW alignments.

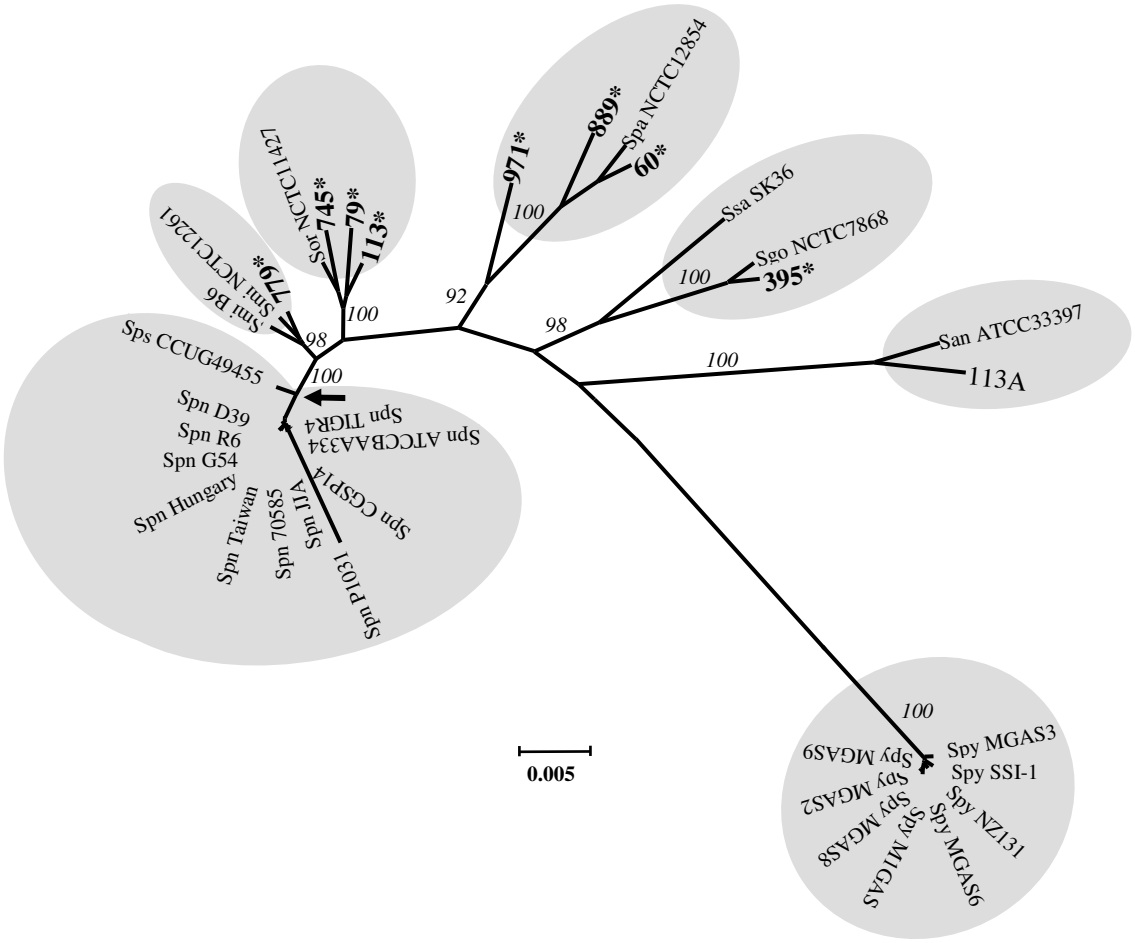
TABLE 1. Summary of isolation data, resistance characteristics, and identification.

Isolate	Origin[a]	Resistance Pattern[b]	Phenotypic characterization	Molecular characterization[c]
60	DB	PEN, ERY, RIF	*S. sanguinis*	*S. parasanguinis* (98.6)
79	AF	ERY, CLI, SXT, RIF	*S. sanguinis*	*S. oralis* (97.6)
113	WS	PEN, RIF	*S. oralis*	*S. oralis* (98.1)
395	AF	RIF	*S. sanguinis*	*S. gordonii* (98.9)
745	AF	ERY, CLI, TET, SXT, RIF	*S. sanguinis*	*S. oralis* (98.4)
779	BL	RIF	*S. mitis*	*S. mitis* (98.7)
889	B	PEN, SXT, RIF	*S. parasanguinis*	*S. parasanguinis* (97.4)
971	E	ERY, TET, RIF	*S. sanguinis*	*S. parasanguinis* (95)

[a] DB, duodenal biopsy; AF, ascitic fluid; WS, wound swab; BL, bronchoalveolar lavage; B, blood; E, eye.

[b] PEN, intermediate or high resistant to penicillin (MIC ≥ 0.25 μg/ml); TET, resistant to tetracycline (MIC ≥8 μg/ml); ERY, resistant to erythromycin (MIC ≥1 μg/ml); CLI, resistant to clindamycin (MIC ≥1 μg/ml); SXT, resistant to trimethropin-sulfamethoxazole (MICs, ≥4/76 μg/ml); RIF, resistant to Rifampin (MIC ≥4 μg/ml).

[c] The species identification was based on clustering with type strains in a phylogenetic tree obtained with a concatenated of partial sequences of 16S rDNA, *sodA*, *rpoB* and *hlpA*. Numbers in parentheses indicate the identity percentage with the corresponding type strain.


Sor NCTC11427
745*
79*
113*
971*
889*
Spa NCTC12854
60*
Ssa SK36
Sgo NCTC7868
395*
Sml B6
Smi NCTC12261
779*
Sps CCUG49455
Spn D39
Spn R6
Spn G54
Spn Hungary
Spn Taiwan
Spn 70585
Spn JJA
Spn P1031
Spn CGSp14
Spn ATCCBAA334
Spn TIGR4
San ATCC33397
113A
Spy MGAS3
Spy SSI-1
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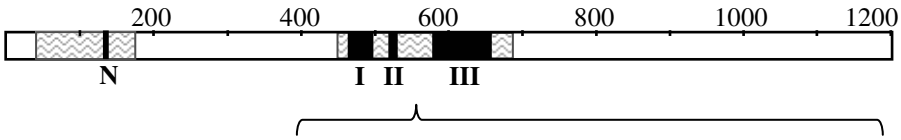
Spy MGAS6
Spy MIGAS
Spy MGAS8
Spy MGAS2
Spy MGAS9
92
98
100
0.005



RESIDUE		4**444445**5555555556666666666666666666677 7**888990**66778999900000112222267789990 5**689594**08139047901378460234791431560 **I** **III**	Rifampin MIC (μg/ml)
Spn	strains	V**Q**M**DSHS**VDTVYTARNEDTKIQVYANIYNQYAVYDTA	0.015
Spn-M	RIF31	...**V**.....F.....	512
Sps^T	CCUG4955	.....V.....V.....	0.015
Smi^T	NCTC12261	.....F.....V.....V..K.....	0.015
Smi	**779***	.....**Y**.....F.....V.....V..K.....	128
Spn-M	RIF25	.....**N**.....F.....V.....V..K.....	8
Spn-M	RIF13	.....**N**.....F.....V.....V.DK.....	16
Sor^T	NCTC11427	.....F.....V....EV.DK.....	0.015
Sor	**745***	.....**N**.....F.....V....EV.DK.....	32
Sor	**113***	.**L**.....F.....V....EV.DK.....	32
Sor	**79***	....**F**.....AF.....T....EV.DK...H...	32
Spn-M	RIF24	.....**N**.....F.....V....EV.DK.V.....	64
Spn-M	RIF56	.....**N**.....F.....V....EV.DK.V.....	64
Spa^T	NCTC12854	I.....I.K..I.K..EGP.RNFSDQ.DK.....	0.03
Spa	**60***	I....**Y**.I.K..I.K..EGP.RNFSDQ.DK.....	64
Spa	**971***	I....**N**.I.K..ISK..KGP.RNFSDQ.DK...H...	32
Ssa^T	SK36	I.....I.K.FI.K..KGP..NFSDQ.DK...H...	0.06
Spa	**889***	I..**V**...I.K.FI.K..EGP..NFSDQ.DK...H...	128
Sgo^T	NCTC7868	I.....I.A.FI.K..EGP..NFSDQ.DK...H...	0.125
Sgo	**395***	I.....**F**I.A.FI.K..EGP..NFSDQ.DK...H...	128
San^T	ATCC33397	I.....I..LFI.KT..RA..NFSDQFDK..IH.VS	0.125
San	113A	I.**I**..**Y**.IN.LFI.K...RA..NFSDQFDH...H.VS	256
Spn-M	RIF65	.....**N**.IAK....K.A...V...SS..DKF.I....	16