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Molecular Characterization of Disease-Associated Streptococci of the Mitis Group That Are Optochin Susceptible▽

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Eight optochin-susceptible (Opt^s) alpha-hemolytic (viridans) streptococcus isolates were characterized at the molecular level. These isolates showed phenotypic characteristics typical of both viridans streptococci and *Streptococcus pneumoniae*. Comparison of the sequence of housekeeping genes from these isolates with those of *S. pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus pseudopneumoniae* suggested that the Opt^s isolates corresponded to streptococci of the mitis group. Besides, the Opt^s streptococci were negative by a Gen-Probe AccuProbe pneumococcus test and hybridized with specific pneumococcal probes (*lytA* and *ply*) but also with *ant*, a gene not present in most *S. pneumoniae* strains. Moreover, the isolates were insoluble in 1% sodium deoxycholate but completely dissolved in 0.1% deoxycholate. Sequence analysis of the *lytA* gene revealed that the Opt^s streptococci carried *lytA* alleles characteristic of those present in nonpneumococcal streptococci of the mitis group. The determination of the partial nucleotide sequence embracing the *atp* operon encoding the F_oF_1 H^+-ATPase indicated that the optochin susceptibility of the isolates was due to the acquisition of *atpC*, *atpA*, and part of *atpB* from *S. pneumoniae* by horizontal gene transfer.

Streptococcus pneumoniae (the pneumococcus) is an important cause of morbidity and mortality worldwide, remaining a major etiological agent of community-acquired pneumonia, meningitis, and acute otitis media (6). Three phenotypic characteristics are used for identification of the pneumococcus among alpha-hemolytic (viridans) streptococci: optochin (Opt) susceptibility, bile (sodium deoxycholate [Doc]) solubility, and reaction with type-specific antisera (21). Although their colony morphologies can be very similar, the alpha-hemolytic streptococci of the mitis group (SMG), such as *Streptococcus mitis* or *Streptococcus oralis*, are classically Opt resistant (Opt^r), insoluble in 1% Doc, and nontypeable (21).

In most countries, the Opt susceptibility test remains the only clinical test carried out for the identification of the pneumococcus, although Doc solubility is also currently inspected in many U.S. laboratories. It is well known that the typical Opt susceptibility of pneumococci is due to the characteristics of the F_o complex of its F_oF_1 H^+-ATPase (12), an enzyme essential for the viability of this organism (13). The primary roles of this enzyme are to create a proton gradient with the energy provided by ATP hydrolysis and to maintain the intracellular pH via proton extrusion (24). Hydrolysis of ATP on the cytoplasmic F_1 sector (α, β, δ, ξ, and γ subunits) drives proton transport through the F_o cytoplasmic membrane sector (*a*, *b*, and *c* subunits) by long-range conformational changes (1, 34, 36). The activity of the F_oF_1 ATPase of *S. pneumoniae* is pH inducible and is regulated at the level of initiation of transcription (24).

Resistance to Opt or other amino-alcohol antimalarial drugs (e.g., quinine or mefloquine) in pneumococcus has been reported as a consequence of point mutations that change amino acid residues located in either one of the two transmembrane α-helices of the *c* subunit or one of the two last α-helices of the *a* subunit (8, 12, 25, 28, 33). This fact suggests that the abovementioned α-helices of these subunits interact and that the mutated residues are important for the structure of the F_o complex and, consequently, for proton translocation. Although several *S. pneumoniae* Opt^r isolates have been reported previously (19, 27, 32, 33, 39), to the best of our knowledge there are only three reports describing Opt^s SMG (5, 23, 26). Only a single Opt^s SMG strain has been characterized as having a recombinant structure in the genes encoding the F_oF_1 H^+-ATPase, that is, it had acquired *atpC*, *atpA*, and part of *atpB* from *S. pneumoniae* (23).

A new member of the mitis group (*Streptococcus pseudopneumoniae*) has been described recently (3). Isolates of this species exhibit an Opt^r phenotype when they are incubated under an atmosphere of increased CO_2, but Opt susceptibility increases in the presence of O_2 (3). For this reason, this previous report recommended the use of a CO_2-enriched atmosphere for this test to prevent misidentification of SMG Opt variants as truly pneumococci. The same authors described *S. pseudopneumoniae* as Doc insoluble, although solubility in 0.1% Doc has been documented recently (20). It has also been found that some SMG isolates, including *S. pseudopneumoniae* strains, carry atypical alleles of the *lytA* gene (encoding the major pneumococcal autolysin LytA) and that most of them are insoluble in 1% Doc but dissolve completely when 0.1%

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TABLE 1. Relevant characteristics of the strains used in this study

Strain	Origin(s) and patient condition(s)[a]	Organism(s) identified by API (% identification)	Opt susceptibility					Doc phenotype	AP[c,d]	Presence of[c]:		
			Zone size (mm) in:		MIC (μg/ml) in:		Phenotype (CO_2/O_2)[b]					
			CO_2	O_2	CO_2	O_2				*lytA*	*ply*	*ant*
SMG 578	Nose, sinusitis	*S. oralis* (98.5)	15	22	1.5	0.75	S/S	+/−	−	+	+	+
SMG 1237	BAA, pneumonia, HIV$^+$	*S. oralis* (84.4)	18	21	1.5	0.37	S/S	+/−	−	+	+	+
SMG 1504	LRT	*S. oralis* (99.0)	20	23	1.5	0.75	S/S	+/−	−	+	+	+
SMG 1956	Sputum, fever	*S. oralis* (94.9)	17	24	1.5	0.75	S/S	+/−	−	+	+	+
SMG 2410	Pus, abscess	*S. oralis* (89.9)	15	20	1.5	0.75	S/S	+/−	−	+	+	+
SMG 2859	Ascites	*S. oralis* (82.4)	18	20	1.5	0.75	S/S	+/−	−	+	+	+
SMG 3137	BAA, pneumonia	*S. oralis* (53.4), *S. pneumoniae* (32.6)	16	20	1.5	0.75	S/S	+/−	−	+	+	+
SMG 3198	Sputum, bronchitis	*S. oralis* (93.5)	12	18	3	0.75	IR/S	+/−	−	+	+	+
S. mitis NCTC 12261^T			6	6	96	48	R/R	−	−	−	−	+
S. oralis NCTC 11427^T			6	6	96	48	R/R	−	−	−	−	+
S. pneumoniae R6			12	19	3	1.5	IR/S	+	+	+	+	−
S. pneumoniae ATCC 6303			16	22	1.5	0.75	S/S	+	+	+	+	−
S. pseudopneumoniae CCUG 49455^T			10	20	6	1.5	IR/S	+/−	+	+	+	−
S. pseudopneumoniae CCUG 48465			12	21	6	0.75	IR/S	+/−	+	+	+	−

[a] BAA, bronchoalveolar aspirate; HIV$^+$, human immunodeficiency virus positive; LRT, lower respiratory tract.

[b] Opt susceptibility phenotypes were categorized as follows: resistant (R), MIC of ≥6 μg/ml or zone size diameter of <10 mm; intermediate resistant (IR), MIC of 3 to 6 μg/ml or zone size diameter of 10 to 14 mm; susceptible (S), MIC of ≤1.5 μg/ml or zone size diameter of >14 mm.

[c] +, positive; −, negative.

[d] AP, AccuProbe.

Doc is used (20). This behavior is in agreement with the finding that 1% Doc (but not 0.1% Doc) inhibited the activity of the LytA-like amidases from SMG, whereas the LytA enzyme synthesized by typical pneumococci was not affected by the detergent (30).

In this work we have gained more insight into the knowledge of the Opt^s phenotype in SMG by characterizing eight Opt^s SMG strains isolated during 2002 at the Spanish Pneumococcal Reference Laboratory and have determined a partial sequence of the *atp* operon from two *S. pseudopneumoniae* strains.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and determination of optochin sensitivity. The bacterial strains used in this work are listed in Table 1. Unless otherwise stated, streptococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract at 37°C without shaking. For determination of Opt sensitivity, each isolate was cultured onto Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood (BA plates). Several colonies were suspended in liquid culture, and 10^7 CFU was applied to BA plates in which 5-μg Opt disks of 6 mm (Becton Dickinson Microbiology Systems) were placed. After overnight incubation at 37°C in either 5% CO_2 or O_2 atmosphere, inhibition zones around the disk were measured. MICs were determined by the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (29), except that plates were incubated in either 5% CO_2 or ambient atmosphere. The MIC was defined as the lowest concentration of drug that inhibited visible growth. *S. pneumoniae* ATCC 6303, *S. pneumoniae* R6, *S. mitis* NCTC 12261^T, *S. oralis* NCTC 11427^T, and two *S. pseudopneumoniae* strains (CCUG 49455^T and CCUG 48465) were used as controls. Opt was purchased from Sigma Chemical, St. Louis, Mo.

Deoxycholate solubility tests. Aliquots (0.5 ml) of exponentially growing cultures received 50 μl of 1 M potassium phosphate buffer (pH 8.0) and 50 μl of a 10% or 1% Doc solution in water. The mixtures were incubated for up to 15 min at 37°C. Lysis was followed by decrease of the turbidity. The strains that lysed with 1% Doc were designated Doc$^+$. This was the case for true pneumococcal isolates. However, those SMG strains that lysed with 0.1% Doc but not with 1% Doc were designated Doc$^{+/-}$.

Gen-Probe (AccuProbe). For detection of the rRNA genes, an AccuProbe *S. pneumoniae* culture identification test (Gen-Probe, San Diego, Calif.) was used according to the manufacturer's instructions with four colonies from an overnight culture on 5% BA plates.

PCR amplification and DNA sequence determination and analysis. PCR amplifications were performed using 0.5 to 1 U of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 0.1 μg of chromosomal DNA, 1 μM (each) of the synthetic oligonucleotide primers, and 0.2 mM of each deoxynucleoside triphosphate in a final volume of 50 μl in the buffer recommended by the manufacturers. Amplification was achieved with an initial cycle of 1 to 5 min of denaturation at 94°C, 25 to 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 to 4 min of polymerase extension at 72°C, with a final 8-min extension at 72°C and slow cooling at 4°C. PCR products were purified using MicroSpin S400 HR columns (Amersham-Pharmacia-Biotech) and sequenced on both strands with an Applied Biosystems Prism 377 DNA sequencer. The following *atp* operon regions were amplified. Fragments of about 3 kb from SMG isolates 1504, 1237, 1956, 2859, and 3137 were amplified with oligonucleotides pepti101 (23) and atpδ110 (24), located in spr1284 and in the gene of the delta subunit of the F_oF_1 H^+-ATPase, respectively. Fragments of 1,935 bp from isolates 578, 2410, and 3198 were amplified with oligonucleotides pepti368 (5′-CCAGGTTTCCGTCATTTTGAAAC-3′) and atpBEND (5′-GGCTTCTCCTAGCTGATCGATATAC-3′), located in spr1284 and in *atpB*, respectively. To amplify the *atpCAB* region from *S. pseudopneumoniae* strains, PCR fragments were obtained by using oligonucleotides atpWO (23) and atpBEND. The upstream *atpC* regions from those strains were amplified with oligonucleotides spr1370-196 (5′-ATTCCTTGCCTTGCTCTCAGCAG-3′) and spr1368END2 (5′-GACACAATTTCTAGGTTATAACTGG-3′), located in spr1370 and downstream spr1368, respectively, yielding fragments of about 3 kb. PCR fragments were sequenced with the same oligonucleotides and with several internal oligonucleotides. In addition, direct sequencing of chromosomal DNAs by use of oligonucleotide atpCorf2 (24) was performed. PCR amplification and sequencing of the *lytA* alleles from SMG strains were carried out using conditions and oligonucleotide primers described elsewhere (20). Multilocus sequence typing

(MLST) was carried out exactly as described elsewhere (11) with the tools provided by the MLST website (http://www.mlst.net) for the molecular characterization of pneumococci. Multiple sequence alignments were created with PILEUP (9) or CLUSTAL W (38). Pairwise evolutionary distances (PEDs) (estimated number of substitutions per 100 bases) were determined using the Distances program (9) with the Kimura two-parameter distance correction (18). Sequence comparisons were carried out by running the BLAST program (2) with data from the EMBL/UniProtKB database and preliminary genomic data for *S. pneumoniae* 670 and the type strain of *S. mitis* (The Institute for Genomic Research website at http://www.tigr.org) and for other pneumococcal strains (The Sanger Institute; http://www.sanger.ac.uk/Projects/S_pneumoniae).

Southern blot identification of strains. Restriction fragments carrying *lytA* and *ply* (pneumolysin) DNA probes and PCR products carrying the *ant* probe (a homolog of genes encoding aminoglycoside adenylyltransferases) were obtained as described previously (14, 23). Probes were labeled with a Phototope-Star detection kit (New England Biolabs). Southern blotting and hybridization were carried out by following the manufacturer's instructions.

Nucleotide sequence accession numbers. The sequences of the *atpCAB* genes reported here have been deposited in the EMBL/GenBank/DDBJ databases. The *lytA* alleles have been assigned accession numbers AM113498, AM113499, AM113501, AM113502, and AM1113505, the housekeeping fragments are accession numbers DQ659935 to DQ659964 and DQ665241 to DQ665250, and the *atp* genes and their surrounding regions are accession numbers DQ659925 to DQ659934.

RESULTS

Phenotypic characteristics of Opt^s SMG. The Spanish Pneumococcal Reference Laboratory receives pneumococcal isolates for typing purposes and antibiotic resistance surveillance. Isolates are routinely confirmed to be *S. pneumoniae* isolates by means of the Opt susceptibility and Doc solubility tests. Among the isolates received during 2002, 2.4% (78 out of 3,240) showed any discrepancy in those tests and did not show hybridization with AccuProbe. Among the 78 AccuProbe-negative isolates, 11 exhibited a clear Opt inhibition zone ($\geq$12 mm) in the presence of 5% CO_2. Eight of these 11 isolates were chosen for further studies (Table 1). Phenotypic characterization of these Opt^s SMG isolates by use of a Rapid ID 32 STREP kit (bioMerieux, La Balme les Grottes, France) system classified them as *S. oralis* isolates. This was not completely unexpected since it is documented that phenotypic characterization is of limited value for identification of many species of nonhemolytic streptococci and particularly of SMG (16).

As previously reported (3), *S. pseudopneumoniae* showed Opt susceptibility (zones of inhibition of $\geq$14 mm) with Opt disks when the test was carried out in ambient air and Opt intermediate resistance in a CO_2-enriched atmosphere. Unexpectedly, a similar reaction was also observed for *S. pneumoniae* R6 (Table 1), making it difficult to evaluate the significance of the results. As it remains unclear whether incubation in CO_2, as recommended in the *Manual of Clinical Microbiology* (35), or ambient atmosphere, as recommended by the manufacturers of Opt test disks, is optimal for the identification of pneumococci, we decided to establish the Opt susceptibilities of various strains by following the standard procedures used to determine the MIC. The Opt MICs were always higher in the presence of 5% CO_2 than in ambient air. The lower Opt susceptibility in an atmosphere of increased CO_2 may be related to the activation of the promoter of the *atp* operon in response to acid, which would produce a twofold increase in the amount of the F_oF_1 H^+-ATPase (24), increasing the Opt MIC by titration.

The eight isolates showed a Doc$^{+/-}$ phenotype, that is, they did not lyse in the presence of 1% Doc but were solubilized when 0.1% Doc was used (not shown). Furthermore, they autolyzed after a prolonged incubation at 37°C, confirming previous results obtained with strains 578 and 1504, which synthesize a defective but functional LytA autolysin (20). These results taken together strongly suggested the presence of an atypical *lytA* allele not only in strains 578 and 1504 but also in the other SMG strains (see below).

Genetic relatedness of the Opt^s SMG isolates. The eight Opt^s Doc$^{+/-}$ AccuProbe-negative SMG isolates were inspected to determine the presence of pneumococcal-specific genes, such as *ply* or *lytA* (not shown). Southern blot hybridization experiments showed that all eight strains harbored both genes (Fig. 1 and unpublished observations). Quite surprisingly, however, they also hybridized with the *ant* probe, a gene that is not normally present in *S. pneumoniae* but that is found in *S. mitis* and *S. oralis* (4).

Taking into account these results, the genetic relationship between the Opt^s SMG strains and other strains was investigated by using MLST as previously suggested (15). Partial sequences of *gdh*, *gki*, *recP*, and *spi* were determined from the Opt^s SMG and *S. pseudopneumoniae* strains. The concatenated allelic profiles were compared to those present at the pneumococcal MLST website (15) and to that of *S. mitis* NCTC 12261^T (The Institute for Genomic Research; http://www.tigr.org). The eight Opt^s SMG isolates grouped with the nontypeable, nonpneumococcal isolates, as did *S. pseudopneumoniae* and *S. mitis* type strains. Six out of eight Opt^s SMG isolates formed a clade with *S. mitis* NCTC 12261^T (Fig. 2).

Genetic analysis of *lytA* alleles carried by Opt^s SMG. The *lytA* alleles from the isolates studied here were PCR amplified and sequenced, except those from isolates 578 and 1504 and the two *S. pseudopneumoniae* strains that had been already reported (20). Three new, different *lytA* alleles were found (data not shown). Strains 1237 and 2859 carried the same *lytA* allele, whereas alleles from strains 3137 and 2410 differed from each other and from those included in the EMBL database. The *lytA* alleles from strains 1956 and 3198 were identical, respectively, to those present in Opt^s SMG isolates 1504 and 3072 previously reported (20). All sequences matched the signatures characteristic of *lytA* alleles from nonpneumococcal SMG isolates, including the 6-bp deletion located near the 3′ end of the gene (20), and explained why the Opt^s SMG isolates studied here showed a Doc$^{+/-}$ phenotype (Table 1).

Susceptibilities of isolates to optochin and organization and sequence of the *atp* chromosomal region. Nucleotide sequences of 1,499-bp fragments containing *atpC*, *atpA*, part of *atpB*, and 114 bp located upstream of *atpC* from the 10 SMG strains, including the two strains of *S. pseudopneumoniae*, were determined. The sequences from strains 578 and 1504 were identical to those from strains 1237 and 3137, respectively. Besides, all of the isolates showed identical sequences from nucleotide positions 77 to 1217, which included the *atpC* and *atpA* genes as well as the first 138 bp of *atpB* (Fig. 3A). Moreover, sequence comparisons with those included in the EMBL database (21 March 2006, last date accessed) also showed that the *atpC* gene of the SMG strains was identical to that of strain R6 (EMBL accession numbers Z26851, AE008506, and AF368465) and to 15 other entries from pneumococcal strains. Moreover, we also found that the *atpA* alleles of R6 and the

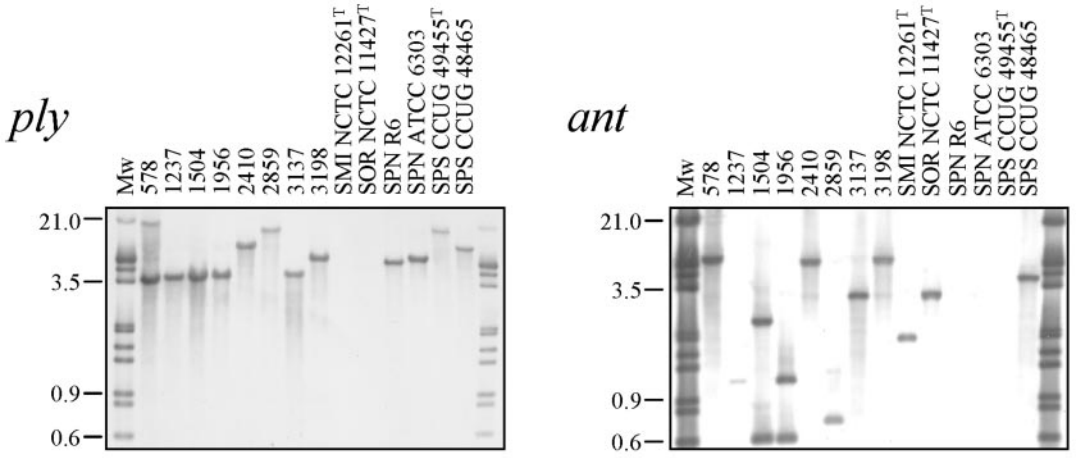

FIG. 1. Southern blot hybridization of streptococcal isolates by hybridization with *ply*- and *ant*-specific probes. Chromosomal DNAs were cleaved with ClaI (A) and EcoRV plus NcoI (B), and the fragments were separated in 1% agarose gels. The gel was blotted, and the blot was probed with biotinylated DNA as follows: an insert of plasmid pJCP191 containing the *ply* gene and a PCR fragment from *S. pneumoniae* 3870 containing most of the *ant* gene. Far-left and far-right lanes, biotinylated DNA ladder. Numbers at left are molecular size markers in kilodaltons. SMI, *S. mitis*; SOR, *S. oralis*; SPN, *S. pneumoniae*; SPS, *S. pseudopneumoniae*.

Opt^s SMG strains differed at only three positions and always at the third position of the corresponding codon: T-to-C transitions at codons 24 and 220 (positions 421 and 1009 in Fig. 3A) and a C-to-T transition at codon 84 (position 601 in Fig. 3A). None of these differences produced changes in the predicted primary sequence of the ATPase *a* subunit.

Upstream of *atpC* (nucleotide positions 1 to 114) and downstream of nucleotide 138 of *atpB* (nucleotide positions 1218 to 1499), the sequences of SMG strains clearly diverged from that of the pneumococcal strain R6 (Fig. 3A). Moreover, sequence divergence in those regions was found among the Opt^s SMG strains, being more evident upstream of *atpC* (PEDs up to 15.6%) (Fig. 3B) than at the 3′ end of *atpB* (PEDs lower than 6%) (Fig. 3F). Besides, evolutionary divergence ranged between 3.6% (for the *S. pseudopneumoniae* strains) and 17.8% (for strains 1504, 1956, 2410, and 3137) compared with the *S. pneumoniae* R6 strain (Fig. 3B). It should be noted that, in this region, strain 1504 and relatives were much more evolutionarily related to the type strain of *S. mitis* (PED of 6.43%) than to *S. pneumoniae*, although other Opt^s SMG strains clearly diverged (PEDs higher than 11%) from both *S. pneumoniae* R6 and the type strain of *S. mitis* (Fig. 3B). The type strain of *S. oralis* was only distantly related to the Opt^s SMG strains (PEDs higher than 19%). Taking together all these results, it is assumed that the Opt^s SMG strains acquired their *atpC* and *atpA* genes from *S. pneumoniae* via horizontal transfer.

Early reports had suggested that an insertion sequence (IS) was located immediately upstream of the *S. pneumoniae atp* operon (12). Nucleotide sequencing has since confirmed the presence in this region of the pneumococcal chromosome of a copy of IS*1239* (spr1367, or SP1515) in every strain tested, namely, R6 (17), TIGR4 (37), G54 (10), 670 (http://www.tigr.org), and Spain^{23F}-1, OXC14, and INV104B (http://www.sanger.ac.uk/Projects/S_pneumoniae) (data not shown). On the other hand, Martín-Galiano et al. recently reported that, in the type strains of *S. mitis* and *S. oralis*, *atpC* is preceded by a gene highly similar to *S. pneumoniae* spr1284, or SP1429, putatively encoding a protease (23). In *S. pneumoniae*, however, *atpC* and spr1284 are located approximately 65 kb apart (23, 37). Moreover, both genes are apparently arranged in an inverted position in type strains of *S. mitis* and *S. oralis* with respect to *S. pneumoniae* (where spr1284 is located downstream of *atpC*). PCR amplification and nucleotide sequencing with appropriate oligonucleotide primers showed that, with the significant exception of the *S. pseudopneumoniae* strains, the gene spr1284 is located immediately upstream of *atpC* in the Opt^s SMG strains (Fig. 4). In *S. pseudopneumoniae* strains, the gene spr1368 is located immediately upstream of *atpC*, which is equivalent to the gene organization in *S. pneumoniae* isolates but lacking the spr1367 copy of IS*1239* (Fig. 4).

DISCUSSION

The chromosomal organization of the *atp* operon region in *S. pneumoniae*, *S. mitis*, *S. pseudopneumoniae*, and Opt^s SMG (Fig. 4) and the nucleotide sequences of the *atpC-atpA-atpB* regions strongly suggest a recombinational origin for the eight Opt^s isolates analyzed in this work. This recombination resulted in the acquisition of a region of about 1.3 kb that included the complete *atpC* and *atpA* genes and 200 to 300 bp of *atpB* from *S. pneumoniae*. The same region was involved in the interchanges yielding the Opt^r *S. pneumoniae* strain M222 (12) and the Opt^s *S. mitis* isolate 1162/99 (23), suggesting that the reconstitution of a functional F_oF_1 H^+-ATPase requires a *b* subunit with an N terminus compatible with the *a* subunit and a C terminus compatible with the δ subunit. Besides, the gene organization upstream of *atpC* appears to be quite variable depending on the particular species and/or isolate studied. The comparison of the genomic regions of *S. pneumoniae* and *S. mitis* embracing the *atp* operon revealed the existence of significant differences between both species, with an obvious loss of synteny (Fig. 4B). Most of these differences were related to genomic rearrangements, suggesting frequent events of inversion and/or translocation of either individual genes or clusters

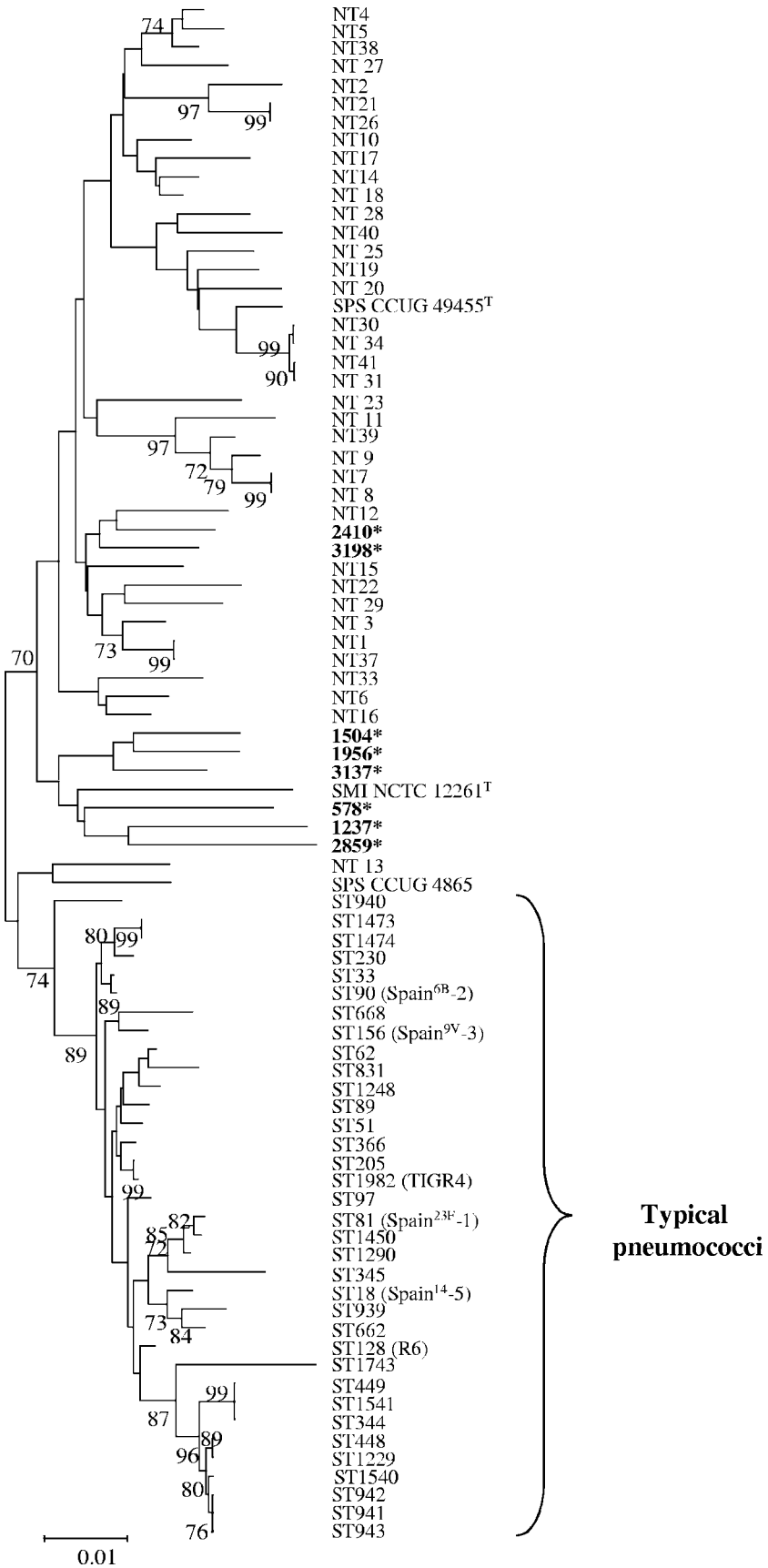

FIG. 2. Dendrogram of genetic relationships between the SMG isolates examined in this study (shown in boldface type and labeled with an asterisk) and other streptococcal isolates. The dendrogram was constructed from housekeeping gene sequence data by using the neighbor-joining method. Only bootstrap confidence values of ≥70% are shown. NT and ST denote nontypeable SMG and sequence type, respectively (11). The scale represents the number of nucleotide substitutions per site. SPS, *S. pseudopneumoniae*; SMI, *S. mitis*.

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                         146012222222333333333333333334444444444
      1122334444446667120001234999011123333334666890133345688
      123481412490123450146111938024258104751456787049839205681657
Spn   TAGCAGATTCAGCGGTTTTACTTCTATAACTTCTTAAAAGGAAACACCGACTTTAGTCTA
1504  AGTATT:CCTGTT:::ACCTACCTCG:::::::::::::::::::::AGACCCTACAC:
1956  AGTATT:CCTGTT:::ACCTACCTCG::::::::::G::::::::::AGACCCTACAC:
2410  AGTATT:CCTGTT:::ACCTACCTCG:::::::::::::::C::::AGACCCTACAC:
578   A:AG::G:C:GTGCAAA:::CCTCGATT::::CG:GGA:C::AGTTAGACCCTACAC:
3198  A::G::G:C:GTGCAAA:::CCTCGTTT::::CG:G:A:C:TAG:TAGACCCTACAC:
2859  A:AG::G:C:GTGCAAA:::CCTCGATT::::CG:G::C:::AGT:AGACCCTACAC:
49455 A:AG::::::::::::::::CCTCGATTT:::CG:GGAAC::AGTTAGACCCTACAC:
48465 A:AG::::::::::::::::CCTCGATT:AC::C::::::::::::AGACCCTACT:C
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B	1504	3198	48465	Spn	Smi	Sor
578	15.60	0.88	8.35	12.39	18.97	26.09
1504		15.60	15.65	17.81	6.43	19.99
3198			9.35	11.35	18.97	26.09
48465				3.60	20.09	24.75
Spn					22.38	27.21
Smi						13.42

C	Smi	Sor
Spn	17.36	26.95
Smi		12.46

D	Spn	Smi	Sor
48465	0.42	32.66	22.24
Spn		32.19	22.64
Smi			25.19

E	Spn	Smi	Sor
48465	0.73	19.89	19.18
Spn		18.92	18.19
Smi			22.06

F	1504	1956	2410	2859	3198	48465	49455	Spn	Smi	Sor
578	4.77	5.16	5.15	1.80	1.43	5.93	0.71	8.32	7.51	21.32
1504		0.36	0.36	3.64	4.01	4.01	5.54	4.77	7.53	20.75
1956			0.71	4.01	4.39	4.39	5.93	5.16	7.94	21.26
2410				4.01	4.39	4.38	5.92	5.15	7.92	20.69
2859					2.52	4.77	2.16	7.11	7.92	21.32
3198						5.91	2.16	8.29	8.70	22.23
48465							6.73	6.33	10.34	19.74
49455								9.15	7.92	21.32
Spn									10.36	21.71
Smi										20.30

FIG. 3. Polymorphism in the *atpC*, *atpA*, and *atpB* genes of Opt^s SMG. (A) The nucleotides present at each polymorphic site are shown for *S. pneumoniae* R6, but for the other strains, only nucleotides that differ from those in R6 are shown. Nucleotide positions at the 1,499-bp fragments are indicated vertically above the sequences. Nucleotide positions located upstream of *atpC*, in *atpA*, or in *atpB* are indicated on a white, gray, or black background, respectively. Colons indicate nucleotides identical to those of strain R6. SMG strains are identified by numbering corresponding to that in Table 1. (B to F) Pairwise comparison of the nucleotide sequences located upstream of *atpC* (nucleotide positions 1 to 114) (B), in *atpC* (nucleotide positions 115 to 315) (C), in *atpA* (nucleotide positions 350 to 1066) (D), in the conserved part of *atpB* (nucleotide positions 1080 to 1217) (E), and in the most divergent region of *atpB* (nucleotide positions 1218 to 1499) (F). Matrices of PEDs between aligned sequences are shown. Abbreviations: Spn, *S. pneumoniae* R6; Smi, type strain of *S. mitis*; Sor, type strain of *S. oralis*.

of genes. It has been proposed previously that the observed genomic plasticity of the pneumococcal genome (7) may be related, at least in part, to the abundant presence of ISs and repeat elements, such as those named BOX (22) and RUP (repeat unit of pneumococcus) (31). As shown in Fig. 4B, transposase genes, RUPs, and/or BOX elements were very frequently placed at the boundaries of the translocated/inverted DNA regions. We propose that one such genomic re-

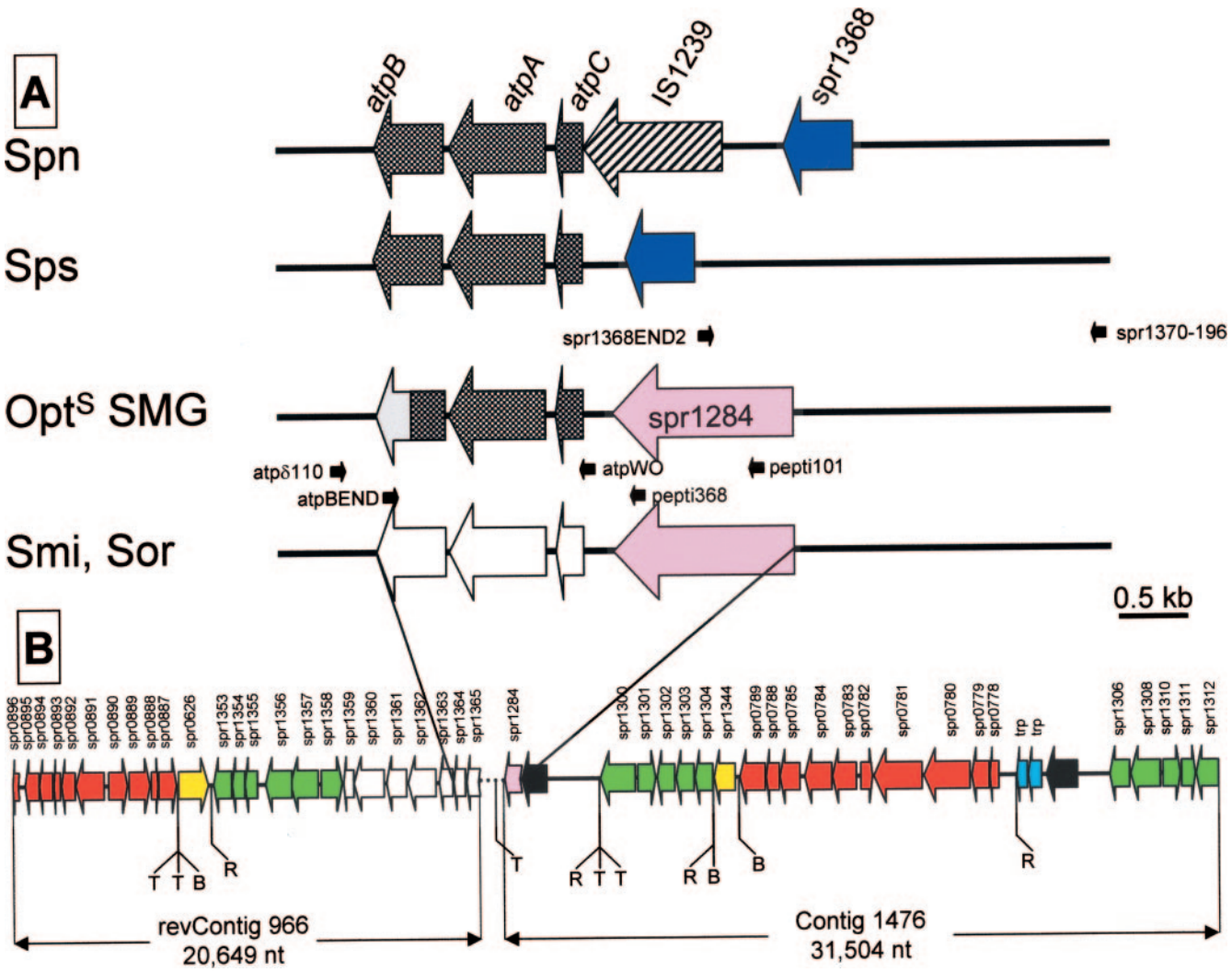

FIG. 4. Genetic structure of the *atp* region and its surrounding regions in SMG. Big arrows indicate the genes and their direction of transcription, using as a reference the *S. pneumoniae* genome (17, 37). The oligonucleotides used in PCR experiments are indicated by small black arrows. (A) Crosshatched and open arrows correspond to the *atp* genes of *S. pneumoniae* (Spn)/*S. pseudopneumoniae* (Sps) or *S. mitis* (Smi)/*S. oralis* (Sor), respectively. The *atpB* gene from Opt^s SMG is represented as a doubly shaded arrow, where the gray color indicates sequence divergence from either *S. pneumoniae* or *S. mitis* genes. (B) Partial genomic map of the DNA regions flanking the *S. mitis* type strain operon. The DNA region linking contig 966, whose complementary and inverted sequence is represented (revContig 966), and contig 1476 is represented as a dotted line; it has been sequenced previously (23), and it corresponds to the *S. mitis*/*S. oralis* scheme shown in panel A. Genes are named according to their *S. pneumoniae* R6 homologues. Yellow and pink arrows indicate genes that are translocated compared with their location and orientation in *S. pneumoniae*. Red arrows indicate gene or gene clusters that are inverted. *S. mitis* genes showing synteny with those of *S. pneumoniae* are indicated by green or open (for the *atp* genes) arrows. Light-blue and filled arrows represent transposase genes and genes lacking any significant similarity with those included in the databases, respectively. T, B, and R indicate the locations of transposase genes/IS-like elements and BOX or RUP repeats, respectively, as annotated in the *S. pneumoniae* R6 genome sequence (17). nt, nucleotides.

arrangement might be responsible for the differences observed in the genes located upstream of *atpC* in the closely related Opt^r/Opt^s SMG. It is not known if there is any advantage for selecting for Opt^s SMG, but the involvement of the F_oF_1 H^+-ATPase in the maintenance of the intracellular pH and of the membrane potential (24) suggests a relation between Opt susceptibility and the adaptation of *S. pneumoniae* to the different habitats in which it causes infection.

The eight Opt^s SMG isolates examined in this study represent a kind of streptococcus that could not be classified as *S. pneumoniae* on the basis of being AccuProbe negative or from their location in the phylogenetic tree constructed with housekeeping genes (Fig. 2). However, those isolates carry at least two pneumococcal virulence determinants (*lytA* and *ply*), and it was found that all of them perform an atypical bile solubility behavior, according to the genetic characteristics of their *lytA* alleles. At least seven of them were associated with disease, and all were penicillin resistant. These data and the description of three SMG isolates from the United Kingdom associated with respiratory diseases and also carrying *lytA* and *ply* (40) reflect the genetic interchange that takes place among SMG and between SMG and *S. pneumoniae* in nature.

Currently, most clinical laboratories depend on the Opt susceptibility test for *S. pneumoniae* identification. Accord-

ingly, the eight Opt^s SMG isolates described in this work were identified as *S. pneumoniae* isolates by the clinical laboratories that had sent them to the reference laboratory. Since misidentification of Opt^s SMG isolates as *S. pneumoniae* isolates may have significant implications for the management of patients, the performance of at least two tests for the identification of *S. pneumoniae*, i.e., Opt susceptibility and bile solubility, should be required. Nevertheless, the results presented here, together with other previous reports of Opt variant and/or Doc variant phenotypes in SMG strains, reflect the importance of describing accurately the conditions used for both tests, that is, CO_2 atmosphere composition and percentage of Doc, respectively.

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