Molecular characterization of disease-associated streptococci of the mitis group that are optochin susceptible.
Balsalobre L, Hernández-Madrid A, Llull D, Martín-Galiano AJ, García E, Fenoll A, de la Campa AG.
J Clin Microbiol. 2006 Nov;44(11):4163-71. Epub 2006 Sep 13.

Molecular Characterization of Disease-Associated Streptococci of the Mitis Group that are Optochin-Susceptible

Luz Balsalobre[1], Antonia Hernández-Madrid[1], Daniel Llull[2], Antonio J. Martín-Galiano[1]†, Ernesto García[2], Asunción Fenoll[3], and Adela G. de la Campa[1]*

[1]Unidad de Genética Bacteriana and [3]Servicio de Bacteriología , Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain. [2]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain

*Corresponding author. Phone: (+34) 91 509 7904. Fax: (+34) 91 509 7919. E-mail: agcampa@isciii.es

†Present address: Lehrstuhl für Genomorientierte Bioinformatik. Wissenschaftszentrum Weihenstephan, Am Forum 1, 85354 Freising, Germany

Running title: Optochin-sensitive streptococci of the mitis group

Eight optochin susceptible (Opt^S) α-hemolytic (viridans) streptococci 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 the 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 the nonpneumococcal streptococci of the mitis group. The determination of the partial nucleotide sequence embracing the *atp* operon encoding the F_0F_1 H^+-ATPase, indicated that the Opt 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 (3). Three phenotypic characteristics are used for identification of the pneumococcus among α-hemolytic (viridans) streptococci: optochin (Opt) susceptibility, bile (sodium deoxycholate; Doc) solubility, and reaction with type-specific antisera (21). Although their colony morphology can be very similar, the α-hemolytic streptococci of the mitis group (SMG) 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 still 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_0 complex of its F_0F_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_0 cytoplasmic membrane sector (*a*, *b*, and *c* subunits) by long-range conformational changes (1, 34, 36). The activity of the F_0F_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 (i.e., quinine, 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 mentioned α-

helices of these subunits interact and that the mutated residues are important for the structure of the F_0 complex and, consequently, for proton translocation. Although several *S. pneumoniae* Opt^R isolates have been reported (19, 27, 32, 33, 39), to the best of our knowledge there are only three reports describing Opt^S SMG (6, 23, 26), but only a single Opt^S SMG has been characterized as having a recombinant structure in the genes encoding the F_0F_1 H^+-ATPase, this is, it had acquired the *atpC*, *atpA* and part of *atpB* from *S. pneumoniae* (23).

A new member of the mitis group (*Streptococcus pseudopneumoniae*) has been recently described (4). Isolates of these 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 (4). In this sense, this 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 recently documented (20). It has also been found that some SMG, including *S. pseudopneumoniae* strains, carry atypical alleles of the *lytA* gene (encoding the major pneumococcal autolysin LytA), and most of them are insoluble in 1% Doc but completely dissolved when 0.1% Doc was 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 insights into the knowledge of the Opt^S phenotype in SMG by characterizing eight Opt^S SMG 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 37ºC without shaking. For determination of the 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 were applied to BA plates in which 5-μg OPT disks of 6 mm (Becton Dickinson Microbiology Systems) were placed. After overnight incubation at 37ºC either in 5% CO_2 or O_2 atmospheres, inhibition zones around the disk were measured. MICs were determined by the agar dilution method as recommended by the Clinical Laboratory Standards Institute (29) except that plates were incubated either in 5% CO_2 or ambient atmosphere. MIC was defined as the lowest concentration of drug that inhibited visible growth. *S. pneumoniae* ATCC strains 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.

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 37ºC. Lysis was followed by decrease of the turbidity. The strains that lysed with 1% Doc were designated as Doc$^+$. This was the case for true pneumococcal isolates. However, those SGM that lysed with 0.1% Doc but not with 1% Doc were named Doc$^{+/-}$.

Gene-Probe (AccuProbe). For detection of the ribosomal RNA genes, the 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 0.5–1 units of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 0.1 μg of chromosomal DNA, 1 μM (each) of the synthetic oligonucleotide primers, 0.2 mM of each dNTP 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 denaturation at 94ºC, 25–30 cycles of 1 min of denaturation at 94ºC, 1 min of annealing at 55ºC, and 1 to 4 min of polymerase extension at 72ºC, with a final 8 min extension at 72ºC and slow cooling at 4ºC. PCR products were purified using MicroSpin^{TM} 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 with oligonucleotides pepti101 (23) and atpδ110 (24), located in *spr1284* and in the gene of the delta subunit of the F_0F_1 H^+-ATPase, respectively. Fragments of 1935 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 spr1368END-2 (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 using oligonucleotide atpCorf2 (24) was performed.

PCR amplification and sequencing of the *lytA* alleles from SGM strains were carried out using the 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 Web site for the molecular characterization of pneumococci (http://www.mlst.net). Multiple sequence alignments were created with PILEUP (9) or CLUSTALW (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) and the EMBL/UniProtKB databases as well as preliminary genomic data for *S. pneumoniae* 670 and *S. mitis*^{T} (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 the Phototope-Star Detection Kit (New England Biolabs). Southern blot and hybridization followed 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, 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*, by means of the Opt-susceptibility and Doc solubility tests. Among the isolates received during 2002, 2.4% (78 out of 3240) showed any discrepancy in those tests and did not show hybridization with the AccuProbe. Among the 78 AccuProbe-negative isolates, 11 exhibited a clear Opt inhibition zone (≥ 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 SGM using the Rapid ID 32 STREP kit (Bio Merieux, La Balme les Grottes, France) system classified them as *S. oralis*. 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 (4), *S. pseudopneumoniae* showed Opt susceptibility (zones of inhibition ≥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 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 susceptibility of various strains following the standard procedures used to determine the MIC. The Opt MIC values 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 that would produce a 2-fold increase in the amount of the F_0F_1 H^+-ATPase (24), increasing the Opt MIC by titration.

The eight isolates showed a $Doc^{+/-}$ phenotype, this is, they did not lyse in the presence of 1% Doc, but were solubilized when 0.1% Doc was used (not shown). Furthermore, they autolysed after a prolonged incubation at 37°C confirming previous results obtained with strains 578 and 1504 that 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 (see below).

Genetic relatedness of the Opt^S SMG. 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 not normally present in *S. pneumoniae* but that is found in *S. mitis* and *S. oralis* (5).

Taking into account these results, the genetic relationship between the Opt^S SMG 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 Web site (15) and to that of *S. mitis* NCTC 12261^T (The Institute for Genomic Research; http://www.tigr.org). The eight Opt^S SMG grouped with the nontypeable, nonpneumococcal isolates, as did *S. pseudopneumoniae*^{T} and *S. mitis*^{T} strains. Six out of eight Opt^S SMG 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, excepting 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 $lytA_{1956}$ and $lytA_{3198}$ alleles were identical, respectively, to those present in the Opt^S SMG 1504 and 3072 previously reported (20). All sequences matched the signatures characteristic of *lytA* alleles from nonpneumococcal SMG including the 6-bp deletion located near the 3' end of the gene (20) and explained why the Opt^S SMG 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 1499-bp fragments containing the *atpC*, *atpA*, part of *atpB*, and 114 bp located upstream of *atpC* from the 10 SMG, 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 position 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 SMG was identical to that of strain R6 (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 Opt^S SMG only differed at 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 1–114) and downstream of nucleotide 138 of *atpB* (nucleotide positions 1218-1499) the sequences of SMG clearly diverged from that of the

pneumococcal strain R6 (Fig. 3A). Moreover, sequence divergence was found among the Opt^S SMG in those regions 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) when compared with the *S. pneumoniae* R6 strain (Fig. 3B). It should be noted that, in this region, strain 1504 and their relatives were much more evolutionarily related to *S. mitis*^{T} (PED, 6.43%) than to *S. pneumoniae*, although other Opt^S SMG clearly diverged (PEDs higher than 11%) from both *S. pneumoniae* R6 and *S. mitis*^{T} (Fig. 3B). *S. oralis*^{T} was only distantly related to the Opt^S SMG (PEDs higher than 19%). Taking together all these results, it is assumed that the Opt^S SMG acquired their *atpC* and *atpA* genes from *S. pneumoniae via* horizontal transfer.

Early reports had suggested that an insertion sequence (IS) element was located immediately upstream of the *S. pneumoniae atp* operon (12). Nucleotide sequencing has confirmed now the presence in this region of the pneumococcal chromosome of a copy of IS*1239* (*spr1367/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 *S. mitis*^{T} and *S. oralis*^{T}, *atpC* is preceded by a gene highly similar to the *S. pneumoniae spr1284/SP1429* putatively encoding a protease (23). In *S. pneumoniae*, however, *atpC* and spr1284 are located 65 kb apart approximately (23, 37). Moreover, both genes are apparently arranged in an inverted position in *S. mitis*^{T} *and S. oralis*^{T} 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 (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 of 200–300 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_0F_1 H^+-ATPase requires a *b* subunit with a 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 of 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 with genomic rearrangements suggesting frequent events of inversion and/or translocation of either individual genes or clusters of genes. It has been proposed that the observed genomic plasticity of the pneumococcal genome (7) may be related, at least in part, with 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, transposases, RUPs and/or BOX elements were very frequently placed at the boundaries of the translocated/inverted DNA regions. We propose that one of such genomic rearrangements 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_0F_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 examined in this study represent a kind of streptococci that could not be classified as *S. pneumoniae* on the basis of being AccuProbe-negative and 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 with 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 United Kingdom associated with respiratory diseases carrying also *lytA* and *ply* (40), reflects the genetic interchange that is taken 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. Accordingly, the eight Opt^S SMG described in this work were identified as *S. pneumoniae* by the clinical laboratories that had sent them to the reference laboratory. Since misidentification of Opt^S SMG as *S. pneumoniae* may have significant implications for the management of patients, to perform at least two tests for the identification of *S. pneumoniae*, i.e., Opt susceptibility and bile solubility should be a must. 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.

ACKNOWLEDGMENTS

We thank M. J. Ferrándiz for the critical reading of the manuscript. The technical assistance of Alicia Rodriguez-Bernabé and E. Cano is greatly acknowledged.

L. B. and A. H-M received fellowships from Instituto de Salud Carlos III. This study was supported by grants BIO2005-02189 and BMC2003-00074 from the Dirección General de Investigación Científica y Técnica. Sequencing of *S. mitis* NCTC 12261^T was accomplished with support from the National Institute of Dental and Craniofacial Research.

REFERENCES

1. **Abrahams, J. P., A. G. W. Leslie, and J. E. Walker.** 1994. Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature **370:**621-628.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
3. **Anonymus.** 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee of Immunization Practices. MMWR Recomm. Rep **46:**1-24.
4. **Arbique, J. C., C. Poyart, P. Trieu-Cuot, G. Quesne, G. Carvalho Mda, A. G. Steigerwalt, R. E. Morey, D. Jackson, R. J. Davidson, and R. R. Facklam.** 2004. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J. Clin. Microbiol. **42:**4686-4696.
5. **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.

6. **Borek, A. P., D. C. Dressel, J. Hussong, and L. R. Peterson.** 1997. Evolving clinical problems with *Streptococcus pneumoniae*: increasing resistance to antimicrobial agents, and failure of traditional optochin identification in Chicago, Illinois, between 1993 and 1996. Diagn. Microbiol. Infect. Dis. **29:**209-214.

7. **Claverys, J. P., M. Prudhomme, I. Mortier_Barriere, and B. Martin.** 2000. Adaptation to the environment: *Streptococcus pneumoniae,* a paradigm for recombination-mediated genetic plasticity? Mol. Microbiol. **35:**251-259.

8. **Cogne, N., J. Claverys, F. Denis, and C. Martin.** 2000. A novel mutation in the α-helix 1 of the C subunit of the F_1/F_0 ATPase responsible for optochin resistance of a *Streptococcus pneumoniae* clinical isolate. Diagn. Microbiol. Infect. Dis. **38:**119-121.

9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387-395.

10. **Dopazo, J., A. Mendoza, J. Herrero, F. Caldara, Y. Humbert, L. Friedli, M. Guerrier, E. Grand-Schenk, C. Gandin, M. de Francesco, A. Polissi, G. Buell, G. Feger, E. García, M. Peitsch, and J. F. García-Bustos.** 2001. Annotated draft genomic sequence from a *Streptococcus pneumoniae* type 19F clinical isolate. Microb. Drug Resist. **7:**99-125.

11. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology **144:**3049-3060.

12. **Fenoll, A., R. Muñoz, E. García, and A. G. de la Campa.** 1994. Molecular basis of the optochin-sensitive phenotype of pneumococcus: characterization of the genes encoding

the F_0 complex of the *Streptococcus pneumoniae* and *Streptococcus oralis* H^+-ATPases. Mol. Microbiol. **12:**587-598.

13. **Ferrándiz, M. J., and A. G. de la Campa.** 2002. The membrane-associated F_0F_1 ATPase is essential for the viability of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **212:**133-138.

14. **Ferrándiz, M. J., A. Fenoll, J. Liñares, and A. G. de la Campa.** 2000. Horizontal transfer of *parC* and *gyrA* in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **44:**840-847.

15. **Hanage, W. P., T. Kaijalainen, E. Herva, A. Saukkoriipi, R. Syrjanen, and B. G. Spratt.** 2005. Using multilocus sequence data to define the pneumococcus. J. Bacteriol. **187:**6223-6230.

16. **Hoshino, T., T. Fujiwara, and M. Kilian.** 2005. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J. Clin. Microbiol. **43:**6073-6085.

17. **Hoskins, J., W. E. Alborn, J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O_Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young_Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, P. L. Skatrud, and J. I. Glass.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709-5717.

18. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111-1120.

19. **Kontiainen, S., and A. Sivonen.** 1987. Optochin resistance in *Streptococcus pneumoniae* strains isolated from blood and middle ear fluid. Eur. J. Clin. Microbiol **6:**422-424.

20. **Llull, D., R. López, and E. García.** 2006. Characteristic signatures of the *lytA* gene provide a rapid and reliable diagnosis of *Streptococcus pneumoniae* infections. J. Clin. Microbiol. **44**:1250-1256

21. **Lund, E., and J. Herichsen.** 1978. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*. Methods Microbiol. **12:**241-262.

22. **Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J. and Claverys, J.P.** 1992. A higly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. **20:**3479-3483.

23. **Martín-Galiano, A. J., L. Balsalobre, A. Fenoll, and A. G. de la Campa.** 2003. Genetic characterization of optochin-susceptible viridans group streptococci. Antimicrob. Agents Chemother. **47:**3187-3194.

24. **Martín-Galiano, A. J., M. J. Ferrándiz, and A. G. de la Campa.** 2001. The promoter of the operon encoding the F_0F_1 ATPase of *Streptococcus pneumoniae* is inducible by pH. Mol. Microbiol. **41:**1327-1338

25. **Martín-Galiano, A. J., B. Gorgojo, C. M. Kunin, and A. G. de la Campa.** 2002. Mefloquine and new related compounds target the F_0 complex of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. **46:**1680-1687.

26. **Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. Am. J. Clin. Pathol. **109:**55-61.

27. **Muñoz, R., A. Fenoll, D. Vicioso, and J. Casal.** 1990. Optochin-resistant variants of *Streptococcus pneumoniae*. Diagn. Microbiol. Infect. Dis. **13:**63-66.

28. **Muñoz, R., E. García, and A. G. de la Campa.** 1996. Quinine specifically inhibits the proteolipid subunit of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae*. J. Bacteriol. **178:**2455-2458.

29. **National Committee for Clinical Laboratory** 2004. Performance standards for antimicrobial susceptibility testing. Fourteenth informational supplement. NCCLS document M100-S14. Wayne, Pa..

30. **Obregón, V., P. García, E. García, A. Fenoll, R. López, and J. L. García.** 2002. Molecular peculiarities of the *lytA* gene isolated from clinical pneumococcal strains that are bile insoluble. J. Clin. Microbiol. **40:**2545-2554

31. **Oggioni, M. R., and J. P. Claverys.** 1999. Repeated extragenic sequences in prokaryotic genomes: a proposal for the origin and dynamics of the RUP element in *Streptococcus pneumoniae*. Microbiology **145:**2647-2653.

32. **Phillips, G., R. Barker, and O. Brogan.** 1988. Optochin-resistant *Streptococcus pneumoniae*. Lancet **2:**281.

33. **Pikis, A., J. M. Campos, W. J. Rodriguez, and J. M. Keith.** 2001. Optochin resistance in *Streptococcus pneumoniae*: mechanism, significance, and clinical implications. J. Infect. Dis. **184:**582-590.

34. **Rastogi, V. K., and M. E. Girvin.** 1999. Structural changes linked to proton translocation by subunit *c* of the ATP synthase. Nature **402:**263-268.

35. **Ruoff, K. L., R. A. Whiley, and D. Beighton.** 2003. Streptococcus, p. 405-421. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Phaller, and R. H. Yolken (ed.), Manual of Clinical Microbiology, 8th ed. American Society for Microbiology Press, Washington D. C.

36. **Stock, D., A. G. W. Leslie, and J. E. Walker.** 1999. Molecular architecture of the rotary motor in ATP synthase. Science **286:**1700-1705.

37. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498-506.

38. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673-4680.

39. **Tsai, H.-Y., P.-R. Hsueh, L.-J. Teng, P.-I. Lee, L.-M. Huang, C.-Y. Lee, and K.-T. Luh.** 2000. Bacteremic pneumonia caused by a single clone of *Streptococcus pneumoniae* with different optochin susceptibilities. J. Clin. Microbiol. **38:**458-459.

40. **Whatmore, A. M., A. Efstratiou, A. P. Pickerill, K. Broughton, G. Woodard, D. Sturgeon, R. George, and C. G. Dowson.** 2000. Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*:

characterization of "atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. Infect. Immun. **68:**1374-1382.

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. Mw, biotinylated DNA ladder. The gel was blotted and the blot was probed with biotinylated DNA as follows: insert of plasmid pJCP191 containing the *ply* gene, and a PCR fragment from *S. pneumoniae* 3870 containing most of the *ant* gene.

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

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 (Spn), but for the other strains, only nucleotides that differ from those in R6 are shown. Nucleotide positions at the 1499-bp fragments are indicated vertically above the sequences. Nucleotides 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 the strain R6. SMG are identified by the corresponding numbering shown in Table 1. Pairwise comparison of the nucleotide sequences located upstream of *atpC* (nucleotide positions 1–114) (B), in *atpC* (nucleotide positions 115–315) (C), *atpA* (nucleotide positions 350–1066) (D), the conserved part of *atpB* (nucleotide positions 1080–1217) (E), and the most divergent region of *atpB* (nucleotide positions 1218–1499) (F). Matrices of PEDs between aligned sequences are shown. Abbreviations: Spn, *S. pneumoniae* R6; Smi, *S. mitis*^{T}; Sor, *S. oralis*^{T}.

FIG. 4. Genetic structure of the *atp* region and its surrounding regions in streptococci of the mitis group. Big arrows indicate the genes and their direction of transcription taking as 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*/*S. oralis* (Smi, Sor), respectively. The *atpB* gene from the Opt^S SMG is represented as a doubly shaded arrow where the gray color indicates sequence divergence either from *S. pneumoniae* or *S. mitis* genes. (B) Partial genomic map of the DNA regions flanking the *S. mitis*^{T} 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 previously sequenced (23), and corresponds to the *S. mitis*/*S. oralis* scheme shown in A. Genes are named according to their *S. pneumoniae* R6 homologues. Yellow and pink arrows indicate genes that are translocated as 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 solid arrows represent transposase genes or genes lacking any significant similarity with those included in the databases, respectively. T, B, and R indicate the location of transposase/IS-like elements, and BOX or RUP repeats, respectively, as annotated in the *S. pneumoniae* R6 genome sequence (17).

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

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

[a] BAA, bronchoalveolar aspirate; LRT, lower respiratory tract

[b] Susceptibility categorizations for OPT were: resistant, MICs ≥6 μg/ml or zone size diameter <10 mm; intermediate, MICs of 3 to 6 μg/ml or zone size diameter of 10 to 14 mm; susceptible, MICs ≤ 1.5 μg/ml or zone size diameter >14 mm.