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Genetic Characterization of Optochin-susceptible viridans streptococci

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Running title: A RECOMBINANT OPTOCHIN-SUSCEPTIBLE VS STRAIN

ABSTRACT

Two clinical isolates of viridans streptococci (VS), with different degrees of susceptibility to optochin (OPT) i.e., the fully OPT-susceptible (Opt^S) VS 1162/99 (MIC equal to that of *Streptococcus pneumoniae*, 0.75 μg/ml) and the intermediate Opt^S VS 1174/97 (MIC of 6 μg/ml) were studied. Besides being Opt^S, they showed typical VS characteristics such as bile insolubility, lack of reaction with pneumococcal capsular antibodies and lack of hybridization with rRNA (AccuProbe), *lytA*, and *pnl* specific pneumococcal probes. However, these VS Opt^S strains, and VS type strains hybridized with *ant*, a gene not present in *S. pneumoniae*. A detailed characterization of the genes encoding the 16S rRNA and SodA classified isolates 1162/99 and 1174/97 as *S. mitis*. Analysis of the *atpCAB* region that encodes the *c*, *a* and *b* subunits of the F_0F_1 H^+-ATPase, the target of optochin, revealed high similarity between *S. mitis* 1162/99 and *S. pneumoniae* in *atpC*, *atpA* and the N-terminus of *atpB*. Moreover, amino acid identity between *S. mitis* 1174/97 and *S. pneumoniae* was found in α-helix 5 of the *a* subunit. The organization of the chromosomal region containing the *atp*-operon of the two Opt^S VS and VS type strains was *spr1284-atpC*, *spr1284* being located at 296-556-bp from *atpC* whereas in *S. pneumoniae* this distance is longer than 68 kb. In addition, gene order in *S. pneumoniae* was IS*1239*-74 bp-*atpC*. Results suggested that the full OPT-susceptibility of *S. mitis* 1162/99 is due to the acquisition of *atpC*, *atpA* and part of *atpB* from *S. pneumoniae* and that the intermediate OPT-susceptibility of *S. mitis* 1174/97 correlates with the amino acid composition of its *a* subunit.

Streptococcus pneumoniae (the pneumococcus) remains a major etiological agent of community-acquired pneumonia, meningitis and acute otitis media (6). Four phenotypic characteristics are classically used in the diagnostic laboratory for the identification of the pneumococcus: colony morphology on blood agar plates, optochin (OPT) susceptibility, bile solubility, and immunological reaction with type-specific antisera (23). Although their colony morphology can be very similar, the alpha-hemolytic oral streptococci (known as viridans streptococci, VS), notably *Streptococcus mitis* and *Streptococcus oralis*, are classically OPT-resistant (Opt^R) and bile insoluble (23).

Genetic and biochemical evidences support that the typical OPT-susceptibility of the pneumococcus resides in the characteristics of the F_0 complex of its F_0F_1 H^+-ATPase, an enzyme that is essential for the viability of this organism (10). 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 (25) as in other related bacteria (18). However, in bacteria having a respiratory chain, the role of the F_0F_1 H^+-ATPase is the synthesis of ATP from the proton gradient of the respiratory chain. Hydrolysis of ATP on the cytoplasmic F_1 sector (formed by the α, β, δ, ξ, and γ subunits) drives proton transport through the F_0 cytoplasmic membrane sector (formed by the *a*, *b*, and *c* subunits) by long-range conformational changes. Conformational changes in the F_1 β subunits drive hydrolysis of ATP (1), which generates rotation of the attached γ and ξ subunits. This rotation, in turn, causes the rotation of an oligomeric ring of *c* subunits (33, 38) and the pumping of protons across the membrane through F_0. The activity of the F_0F_1 ATPase of *S. pneumoniae* is pH-inducible and is regulated at the level of initiation of transcription (25).

Pneumococcal strains resistant to amino-alcohol antimalarial drugs such as OPT, quinine (QIN), and mefloquine (MFL) have point mutations that change amino acid residues

located in one of the two transmembrane α-helices of the *c* subunit or in one of the two last α-helices of the *a* subunit (9, 24, 28, 31) suggesting that those α-helices of the *c* and *a* subunits interact and that the mutated residues are important for the structure of the F_0 complex and proton translocation. Although OPT was used at the beginning of the 20th century for the treatment of pneumococcal infections (26), its use is nowadays restricted to diagnostic purposes due to its high toxicity. However, new less toxic, mefloquine-related compounds that also target the F_0 complex of the F_0F_1 ATPase (24) have been synthesized (22).

Although several *S. pneumoniae* Opt^R isolates have been reported (21, 27, 30, 31, 40), to the best of our knowledge, there is a single report of VS with an Opt^S phenotype (5). In this work we describe the genetic characterization of two Opt^S VS clinical isolates.

MATERIALS AND METHODS

Antimicrobial susceptibility testing. OPT sensitivity tests were performed by placing 5-μg OPT disks (Becton Dickinson Microbiology Systems) onto Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood streaked with the bacteria being tested. After overnight incubation at 37ºC in a CO_2 atmosphere, inhibition zones around the disk were measured, and isolates with zones ≥14 mm were considered as sensitive. MICs were determined by the microdilution method, using cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood as recommended by the National Committee for Clinical Laboratory Standards (29). The inoculum was prepared by suspension of several colonies from an overnight blood agar culture in Mueller-Hinton broth and adjusting the turbidity to the 0.5 McFarland standard (ca. 10^8 CFU/ml). The suspension was further diluted to provide a final bacterial concentration of 10^4 CFU/ml in each well of the microdilution trays. Plates were covered with plastic tapes and incubated in ambient atmosphere at 37ºC for 20-24 h. MIC was defined as the lowest concentration of drug that inhibited visible growth. *S.*

pneumoniae ATCC strains 6303 and 49619, *S. mitis* NCTC 12261^T (Smi^T), and *S. oralis* NCTC 11427^T (Sor^T) and ATCC 10557 were used for the quality control. OPT and QIN were purchased from Sigma Chemical, St. Louis, Mo. MFL (Ro 21-5998-000) was provided by Roche Laboratories, Basel, Switzerland.

PCR amplification and DNA sequence determination. Chromosomal DNA was prepared as described previously (12) from the laboratory *S. pneumoniae* R6 strain, Smi^T, Sor^T, and the clinical isolates. Gel electrophoresis of PCR products was carried out in agarose gels as described previously (35). PCR amplifications were performed using 0.5–1 units of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 0.1 μg of chromosomal DNA, 0.4–1 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 min denaturation at 94ºC, 25–30 cycles of 30–60 seconds of denaturation at 94ºC, 90 seconds of annealing at 55ºC, and 1–2 min of polymerase extension at 72ºC, with a final 8 min extension at 72ºC and slow cooling at 4ºC. Oligonucleotides used in PCR amplifications are described in Table 1. To amplify the *atpC* and *atpA* genes, two pairs of oligonucleotides were used: atp660 (28) and atpB56, or atpWO and atpB56 (24). For the sequencing of the *atpCA* regions of the various strains used, several internal oligonucleotides were synthesized. Oligonucleotides atpc18RSpn, atpc18RSmi, atpc18RSor and UPatp3 were used for sequencing of the *atpC* upstream regions. We also used primers 16SDNAF1 and 16SDNAR1 to amplify the 16sRNA genes, and these same primers and the internal oligonucleotides 16SDNAF2 and 16SDNAR2 for sequencing. Primers SOD–UP and SOD–DOWN were used to amplify and sequence the *sodA* gene (20). 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.

Southern blot identification of strains and determination of the genetic structures of the chromosomal regions located upstream of *atpC*. 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 % blood agar. *lytA* and *pnl* DNA probes were prepared as described (11). A 798-bp probe derived from *S. pneumoniae* 3870 (11), a strain with a recombinational origin that had acquired the *ant* gene from a VS, encoding amino acid residue positions 26 to 290 of the 290-amino-acid-residue Ant protein (3) was obtained by PCR amplification with oligonucleotides antUP and antDOWN. The 936-bp spr1284 probe was obtained by amplification of *S. pneumoniae* R6 with oligonucleotides pepti101 and pepti413R. The AtpCA probe was obtained by amplification of *S. pneumoniae* R6 with oligonucleotides atpWO and atpA107R. DNA inserts and PCR fragments 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 GenBank under accession numbers AY172982 (VS isolate 1162/99), AY172983 (VS isolate 1174/97), AY172984 (*S. mitis* NCTC 12261), AY172985 (*S. pneumoniae* ATCC 6303) and AY172986 (*S. pneumoniae* ATCC 49619). The *sodA* fragments have been assigned accession numbers AY314979 and AY314980.

RESULTS

Isolation and identification at the species level of strains 1162/99 and 1174/97. Clinical isolates are received at the Pneumococcal Reference Laboratory, Madrid, Spain, for typing purposes and antibiotic resistance surveillance. Isolates are routinely confirmed to be *S. pneumoniae* by colony morphology on blood agar, OPT-susceptibility, and sodium

deoxycholate solubility. Two of these isolates, which were isolated from valuable sputum samples from patients with pneumonia and identified at the hospital laboratory as *S. pneumoniae* for being Opt^S (optochin disk inhibition zones of 14 mm for isolate 1174/97 and 20 mm for isolate 1162/99), drew our attention since they were insoluble in sodium deoxycholate. In addition, their DNAs did not show hybridization with the *pnl*, *lytA* and ribosomal DNA (AccuProbe) pneumococcal-specific probes (data not shown). On the other hand, phenotypic characterization of the Opt^S isolates 1174/97 and 1162/99 by the API 32 Strep system classified them as *S. oralis*.

In a recent work carried out in our laboratory we detected an open reading frame, *ant* that is not present in *S. pneumoniae* strains but that it is found in *S. mitis* and *S. oralis* (3). A probe containing this gene was used in Southern blot experiments using chromosomal DNAs from isolates 1162/99 and 1174/99. Both Opt^S VS isolates, Smi^T and Sor^T showed hybridization with the *ant* probe, whereas *S. pneumoniae* R6 did not (Fig. 1A). These experiments suggested that, in spite of being Opt^S, isolates 1162/99 and 1174/97 were not pneumococcal strains. To determine the phylogenetic position of strains 1162/99 and 1174/97 among VS, we amplified and sequenced a 1,138-nt internal fragment of the genes encoding their 16S rRNA (data not shown). Sequence comparison revealed more than 99% identity with the 16S rRNA genes of *S. pneumoniae*, *S. mitis*, and *S. oralis*, whereas lower similarities (98.2 to 97.6) were found with other species (*Streptococcus gordonii*, *Streptococcus sanguis* and *Streptococcus parasanguis*) of the mitis group, indicating that isolates 1162/99 and 1174/97 belong to the mitis group of VS (19). Recent works (20, 32) have shown that sequence analysis of *sodA* is a faithful method for identification of species within the mitis group. The sequences of an internal *sodA* portion (366 bp) of the 1162/99 and 1174/97 strains were determined and compared with those of the laboratory R6 strain, *S. pneumoniae* $NCTC7465^T$, Smi^T, Sor^T, *S. gordonii* $ATCC10558^T$, *S. sanguis* $ATCC10556^T$ and *S.*

parasanguis ATCC15910^T. The *sodA* sequences of 1162/99 and 1174/97 were very similar (98.1 % identical) and showed high similarities (higher than 96%) with those of Smi^T and *S. pneumoniae* strains (Fig. 1B). Lower similarities were found with other species of the *S. mitis* group: lower than 93 %, 83%, 82% and 88% with Sor^T, *S. gordonii*, *S. sanguis* and *S. parasanguis*, respectively.

Taken together, these results classified the 1162/99 and 1174/97 isolates as *S. mitis*.

Susceptibilities of strains to amino alcohol antimalarial drugs and sequence of the *atpC* and *atpA* genes. The characterization of pneumococcal strains carrying point mutations in *atpC* or *atpA* (24) provided a criteria to categorize the susceptibility to OPT, QIN and MFL. Strains were considered susceptible when their OPT MICs were ≤1.5 μg/ml, their QIN MICs were ≤50 μg/ml, or their MFL MICs were ≤1.25 μg/ml. MICs for intermediate susceptible strains being in the 3 to 6 μg/ml OPT range, in the 100 to 200 μg/ml QIN range, and in the 0.31 to 0.62 μg/ml MFL range. Strains were considered resistant when their OPT MICs were >6 μg/ml, their QIN MICs were >200 μg/ml, or their MFL MICs were >0.62 μg/ml. By these criteria, isolate 1162/99 was susceptible to OPT and QIN, and intermediate susceptible to MFL (Table 2). However, isolate 1174/97 showed intermediate susceptibility to OPT and resistance to QIN and MFL (Table 2). Since the mutations involved in OPT resistance map in the *atpC* and *atpA* genes, the nucleotide sequences of these genes from the two Opt^S VS clinical isolates, together with that of Smi^T, *S. pneumoniae* ATCC 6303 and *S. pneumoniae* ATCC 49619 were determined and compared to the published sequences of *S. pneumoniae* R6 and Sor^T (9). PCR fragments of about 1 kb were obtained by amplification of *S. pneumoniae* ATCC 6303 and *S. pneumoniae* ATCC 49619 strains (by using oligonucleotides atp660 and atpB56) and Smi^T, *S. mitis* 1162/99 and *S. mitis* 1174/97 (by using oligonucleotides atpWO and atpB56). Those fragments were sequenced with the same primers used in the PCR amplifications as well as with internal primers. The nucleotide

sequence of the *atpC* and *atpA* genes from isolate 1162/99 showed almost complete identity with that of *S. pneumoniae* strains (Fig. 2A). However, the nucleotide sequence from isolate 1174/97 showed higher identities with that of Smi^T (83.07 %) and Sor^T (80.99%) than with *S. pneumoniae* (79.51%). These nucleotide changes yielded, when compared with *S. pneumoniae* R6, a single amino acid change (*c*L6F) in the *c* subunit of isolate 1162/99, 8 changes in isolate 1174/97 (Fig. 2B), but no changes in *S. pneumoniae* ATCC 6303 and *S. pneumoniae* ATCC 49619. Two changes in the *a* subunit were observed in *S. pneumoniae* ATCC 6303 (*a*E104G and *a*D171F) and *S. pneumoniae* ATCC 49619 (*a*L99I and *a*E104G), 52 changes in 1174/99, but no changes in 1162/99 (Fig. 2B).

Chromosomal organization of the *atp* operon region. The virtual identity of the *atpC* and *atpA* sequences of *S. mitis* 1162/99 and *S. pneumoniae* led to the hypothesis that *S. mitis* 1162/99 have acquired this genome region from *S. pneumoniae via* horizontal transfer. To test this hypothesis, the chromosomal region located 5' of *atpC* was sequenced using chromosomal DNAs from the various strains, and oligonucleotides corresponding to the complementary strand of the primer coding for AtpC residues 11-18 (atpc18RSpn, atpc18RSmi or atpc18RSor). The comparisons of these sequences showed the presence in Smi^T, Sor^T and in the 1162/99 and 1174/97 isolates of a gene with high similarity (>87%) to *spr1284* of *S. pneumoniae* R6 (16), or *sp1429* of *S. pneumoniae* TIGR4 (39), encoding a putative peptidase. The *spr1284* gene was contiguous to *atpC* in VS strains but is located in the R6 genome at 68,630 bp upstream *atpC*, and at 73,606 bp in the TIGR4 genome. Given the high similarity among the sequences of *spr1284* of *S. pneumoniae* R6 and of the VS, an oligonucleotide (pepti101) was designed based on the *spr1284* R6 sequence. Confirmation of the *spr*1284-*atpCA* gene organization in VS was obtained by PCR experiments. Amplifications with oligonucleotide pepti101, located in *spr1284*, and two oligonucleotides located either at *atpC* (atpC18RSmi) or *atpB* (atpB56 Fig. 3A) rendered DNA fragments of

1.4 and 2.4 kb, respectively, in Smi^T, *S. mitis* 1162/99, and *S. mitis* 1174/97. Amplification of Sor^T with oligonucleotides pepti101 and atpC18RSor yielded a 1.6-kb fragment instead the 1.4-kb fragment observed in Smi^T, as expected from the larger size of the *spr1284-atpC* intergenic region of Sor^T, but no amplification with the pair peti101 and atpB56 was observed, probably due to low similarity between *S. pneumoniae* R6 and Sor^T at the *atpB* sequence. On the other hand, no amplification was observed in *S. pneumoniae* R6 with pepti101 and either atpC18RSpn or atpB56. However, PCR amplifications with an oligonucleotide located into IS*1239* (upatp3), and atpC18RSpn yielded a 0.6 kb fragment in R6, and none in the VS (data not shown). The PCR products resulting from these amplifications were sequenced with the same oligonucleotides used for the amplifications as well as with internal primers. A comparison of the sequences of *S. pneumoniae* R6 and *S. mitis* 1162/99 showed almost complete identity in a 50-nt region located 5' of *atpC* (positions 1 to −50 of the sequence of Fig. 3B) that includes the −10 region of the *atp* promoter . From position −50 to −115, the sequence of *S. mitis* 1162/99 showed higher identity with Smi^T (93.8%) that with *S. pneumoniae* R6 (78.5%). In addition, the size of the *spr*1284-*atpC* intergenic region in Sor^T DNA was 242−260 nt longer than those of the *S. mitis* clinical isolates and Smi^T (data not shown).

An additional testing of the genetic structure of the *atpC* upstream chromosomal region in *S. pneumoniae* and VS was carried out by Southern blot hybridization using two probes derived from *S. pneumoniae* R6, one coding for Spr1284 amino acid residue positions 101 to 413 (spr1284 probe) and another encoding AtpC and the first 113 residues of AtpA (atpCA probe). The spr1284 probe, hybridized with the DNAs of all strains tested, indicating a high sequence similarity among *spr1284* genes (Fig. 3C). However, the atpCA probe hybridized only with *S. pneumoniae* R6 and *S. mitis* 1162/97 DNAs, as was expected from the low similarities among the sequences of the *atpCA* genes of *S. pneumoniae* R6 and those of

the rest of the VS strains (Fig. 2A). Hybridization of the spr1284 probe with *S. pneumoniae* R6 DNA showed two bands of 4.7 and 4.2-kb (*Nhe*I + *Hin*dIII) and a single band of 6.5-kb (*Nhe*I + *Eco*RV), whereas hybridization with the atpCA probe was at bands of 1.1-kb (*Nhe*I +*Hin*dIII) and 2.9-kb (*Nhe*I +*Eco*RV), suggesting that *spr1284* and *atpCA* were located at different chromosomal positions, accordingly with the restriction map for those enzymes in the R6 genome. However, hybridization of the spr1284 probe or the atpCA probe with *S. mitis* 1162/99 showed bands of identical sizes (11-kb for *Nhe*I +*Hin*dIII and 5.2-kb for *Nhe*I + *Eco*RV digestions, Fig. 3C), indicating that *spr1284* was located near to *atpC* and *atpA* in the *S. mitis* 1162/99 chromosome.

DISCUSSION

Natural genetic transformation is essential for the genetic plasticity of *S. pneumoniae* (7). Interspecies recombination events between *S. pneumoniae* and the genetically related VS *S. oralis* and *S. mitis* have been described for the penicillin-binding-protein encoding genes (13, 37) and the fluoroquinolone target genes (4, 11). In these cases, interchanges have been detected in the presence of intense selective pressure, but this does not imply that other interchanges, both inter- and intraspecific, in genes not involved in antimicrobial resistance, are being occurring, as is the case for the genes encoding the capsular types (8), the *comCDE* loci required for competence (14) and for the genes of the F_0 complex of the F_0F_1 H^+ –ATPase of *S. mitis* 1162/99 showed in this work. A comparison of the chromosomal organization of the *atp* operon regions in *S. pneumoniae*, VS strains and *S. mitis* Opt^S strains (Fig. 2), together with that of the nucleotide sequences of the *atpC-atpA-atpB* regions, strongly suggest a recombinational origin for strain 1162/99. That recombination resulted in the acquisition of a region of about 1.3 kb that includes the complete *atpC* and *atpA* genes, and of 300-bp of *atpB* from *S. pneumoniae*. That interchange is responsible for the Opt^S phenotype of *S. mitis*

1162/99 (Table 1) since the F_0 (*c*, *a*, and *b* subunits) complex of the F_0F_1 H^+ -ATPase is responsible for that phenotype. Although the mutations conferring Opt^R map in *atpC* and *atpA* (9, 24, 28, 31), it is well know that the *b*-subunit is required for H^+ translocation (36). The structure of the *Escherichia coli* subunit-*b* dimer is that of a single 33-amino acid residues transmembrane helix at its N-terminal region than interacts with F_0 subunit-*a* and a 123-residue-long cytoplasmic domain that binds to the δ-subunit and to one of the α-subunits of F_1 (2, 15, 34). This structure is shared by the *b*-subunits of *S. pneumoniae*, Smi^T, Sor^T, *S. mitis* 1162/99 and *S. mitis* 1174/97 (data not shown). The N-terminal part of *atpB* of pneumococcal origin that is present in *S. mitis* 1162/99 includes the coding region of the first 100-amino acid residues of the *b*-subunit in which the transmembrane helix (residues 6 to 26) that interacts with the *a*-subunit is located. The same occurred in an Opt^R recombinant laboratory strain (*S. pneumoniae* M222) that we constructed by genetic transformation of *S. pneumoniae* R6 with DNA from Sor^T (9). Comparisons of the *atp* sequences of these strains and that of *S. mitis* 1162/99 showed that in the interchanges yielding the Opt^R *S. pneumoniae* strain M222 and the Opt^S *S. mitis* 1162/99 isolate, the same region of the *atp* operon was involved: the complete *atpC* and *atpA* genes, and 300-bp of the 5'-end of *atpB*. These results suggest that the organization of a functional F_0F_1 ATPase in both recombinant strains requires a *b*-subunit with a N-terminus compatible with the *a*-subunit and of a C-terminus compatible with the δ-subunit. In this way, in *S. mitis* 1162/99, the N-terminus of the *b*-, *a*- and *c*-subunits are of pneumococcal origin, whereas the C-terminus of the *b*- and δ-subunits are of VS origin. Likewise, *S. pneumoniae* M222 had *c*-, *a*- and the 5'-end of *b*-subunits from Sor^T, whereas the δ-subunit (and the rest of F_1 subunits) came from *S. pneumoniae* R6. The simultaneous interchange of the *c*- and *a*-subunits seems to be also a requisite for proper functioning of the F_0 complex. The 5´ point of recombination in *S. mitis* 1162/99 would be at a 31-bp sequence located upstream *atpC* that is identical in *S. pneumoniae* and in all VS strains studied, with the

exception of a single change in *S. pneumoniae* strains (Fig. 3B). The 3´ point of recombination in both *S. mitis* 1162/99 and *S. pneumoniae* M222 would be at a 20-bp sequence in *atpB* that is almost identical in *S. pneumoniae* and in the VS strains studied (Fig. 3B=). The sizes of these recombination regions are close to the minimal efficient processing segment for recombination in *S. pneumoniae* that is of about 30-bp (17).

On the other hand, the intermediate Opt^S isolate 1174/97 has an *atpCA* sequence compatible with a VS species, and had several amino acid changes in their *c*- and *a*-subunits when compared with *S. pneumoniae*, Smi^T and Sor^T (Fig. 2B). Among the amino acid residues of the *c*- and *a*-subunits know to be involved in the Opt^R/Opt^S phenotype of *S. pneumoniae* (9, 24, 28, 31), there is only one residue that changed in Sor^T and Smi^T but that is conserved in *S. pneumoniae* and *S. mitis* 1174/97: residue L186 of the *a*-subunit. A L186P change has been shown to be responsible for the intermediate Opt^S phenotype (MIC of 6 μg/ml, the same observed for strain 1174/97) of the *S. pneumoniae* MJM21 strain (24). That residue is located in α-helix 5 of the *a*-subunit that includes residues 172 to 191 (24). Identity was observed among *S. pneumoniae* and *S. mitis* 1174/97 in that α-helix 5 while several changes are present in Smi^T and in Sor^T with respect *S. pneumoniae*. The identity found between α-helix 5 of *S. mitis* 1174/97 and *S. pneumoniae* could be responsible for the intermediate Opt^S phenotype of *S. mitis* 1174/97.

Currently most clinical laboratories depend on the OPT susceptibility test for *S. pneumoniae* identification. Accordingly, the two Opt^S VS described in this work, the fully Opt^S and the intermediate Opt^S VS, were identified as *S. pneumoniae* by the clinical laboratories that had sent them to the reference laboratory. Since misidentification of Opt^S VS 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 is strongly recommended.

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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. **Aris, J. P., and R. D. Simoni.** 1983. Cross-linking and labeling of the *Escherichia coli* F_1F_0-ATP synthase reveal a compact hydrophylic portion of F_0 close to an F_1 catalytic subunit. J. Biol. Chem. **258:**14599-14609.
3. **Balsalobre, L., M. J. Ferrandiz, J. Liñares, F. Tubau, and A. G. de la Campa.** 2003. Viridans group streptococci are donors in the horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **47**(7).
4. **Bast, D. J., J. C. S. de Azevedo, T. Y. Tam, L. Kilburn, C. Duncan, L. A. Mandell, R. J. Davidson, and D. E. Low.** 2001. Interspecies recombination contributes minimally to fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **45:**2631-2634.
5. **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.

6. **Centers for Diseases Control and Prevention.** 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb. Mortal. Wkly Rep. **46:**1-24.

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. **Coffey, T. J., M. C. Enright, M. Daniels, J. K. Morona, R. Morona, W. Hryniewicz, J. C. Paton, and B. G. Spratt.** 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus leading to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. Mol. Microbiol. **27:**73-83.

9. **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.

10. **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. **10514:**1-6.

11. **Ferrandiz, 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.

12. **González, I., M. Georgiou, F. Alcaide, D. Balas, J. Liñares, and A. G. de la Campa.** 1998. Fluoroquinolone resistance mutations in the *parC, parE*, and *gyrA*

genes of clinical isolates of viridans group streptococci. Antimicrob. Agents Chemother. **42:**2792-2798.

13. **Hakenbeck, R., K. Kaminski, A. Köning, M. Van der Linden, J. Paik, P. Reichmann, and D. Zähner.** 2000. Penicillin-binding proteins in β-lactam-resistant *Streptococcus pneumoniae*. *In* A. Tomasz (ed.), *Streptococcus pneumoniae*: molecular biology and mechanism of disease. Mary Ann Liebert, Inc., Larchmont, N. Y.

14. **Håvarstein, L. S., R. Hakenbeck, and P. Gaustad.** 1997. Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges. J. Bacteriol. **179:**6589-6594.

15. **Hermolin, J., J. Gallant, and R. H. Fillingame.** 1983. Topology, organization, and function of the psi subunit in the F_0 sector of the H^+-ATPase of *Escherichia coli*. J. Biol. Chem. **258:**14550-14555.

16. **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.

17. **Humbert, O., M. Prudhomme, R. Hakenbeck, C. G. Dowson, and J.-P. Claverys.** 1995. Homologous recombination and mismatch repair during transformation in *Streptococcus pneumoniae*: saturation of the Hex mismatch repair system. Proc. Natl. Acad. Sci. USA **92:**9052-9056.

18. **Kakinuma, Y.** 1998. Inorganic cation transport and energy transduction in *Enterococcus hirae* and other streptococci. Microbiol. Mol. Biol. Rev. **62:**1021-1045.

19. **Kawamura, Y., X.-G. Hou, F. Sultana, H. Miura, and T. Ezaki.** 1995. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int. J. Syst. Bacteriol. **45:**406-408.

20. **Kawamura, Y., R. A. Whiley, S.-E. Shu, T. Ezaki, and H. J. M.** 1999. Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. Microbiology **145:**2605-2613.

21. **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.

22. **Kunin, C. M., and W. Y. Ellis.** 2000. Antimicrobial activities of mefloquine and a series of related compounds. Antimicrob. Agents Chemother. **44:**848-852.

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

24. **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.

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

26. **Moore, H. F., and A. M. Chesney.** 1917. A study of ethylhydrocuprein (optochin) in the treatment of acute lobar pneumonia. Arch. Intern. Med. **19:**611-682.

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 Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; 5th ed. Publication. National Committee for Clinical Laboratory Standards, Wayne, Pa.

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

31. **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.

32. **Poyart, C., G. Quesne, S. Coulon, P. Berche, and P. Trieu-Cuot.** 1998. Identification of Streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. J. Clin. Microbiol. **36:**41-47.

33. **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.

34. **Rodgers, A. J., S. Wilkens, R. Aggeler, M. B. Morris, S. M. Howitt, and R. A. Capaldi.** 1997. The subunit δ-subunit b domain of the *Escherichia coli* F_1F_0 ATPase. The β subunits interact with F_1 as a dimer and through the δ subunit. J. Biol. Chem. **272:**31058-31064.

35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor laboratory Press, Cold Spring Harbor, N. Y.

36. **Schneider, E., and K. Altendorf.** 1984. Subunit b of the membrane moiety (F_0) of ATP synthase (F_1F_0) from *Escherichia coli* is indispensable for H^+ translocation and binding of the water-soluble F_1 moiety. Proc. Natl. Acad. Sci. USA. **81:**7279-7283.

37. **Spratt, B. G.** 1994. Resistance to antibiotics mediated by target alterations. Science **264:**388-393.

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

39. **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.

40. **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.

TABLE 1. Oligonucleotides used in this work

Name	Sequence (5'-3')[a]	Nucleotide (amino acid) positions[b]
atp660	ggtcggaaTTCCAATAGCGGTTAAAAGTTG	–83 to –62 of *atpC*
antDOWN	TCATGAGTCTTCTCCTCTCGC	Complementary to 853 to 873 of *ant* (^{285}ARGEDS^{290})
antUP	GCTGTCGCCATGTCTGGTTCACG	76 to 98 of *ant* (^{26}AVAMSGSR^{33})
atpA 107R	GCGGTTGGCGAACTCCACCAG	Complementary to 318 to 338 of *atpA* (^{107}WWSSPTA^{113})
atpB56	GACGGGCTTCTTCAGCTCTGTC	Complementary to 169 to 147 of *atpB* (^{50}DRAEEAR^{56})
atpc18RSmi	CCAAGAGACACACCCATACAGGC	Complementary to 31 to 53 of *atpC* (^{11}ACMGVSVG^{18})[c]
atpc18RSor	GCAAGAGATACACCCATACAGGC	Complementary to 31 to 53 of *atpC* (^{11}ACMGVSVG^{18})[d]
atpc18RSpn	CCGACAGATACGCCCATACAGGC	Complementary o 31 to 53 of *atpC* (^{11}ACMGVSVG^{18})
atpWO	gcgcatgcTTAAAGGAGAATTTGTTATGAA	–15 to 5 of *atpC* (^{1}MN^{2})
pepti101	GCAGTTATCGTATCTGACCCAGCC	304 to 327 of spr1284 (^{102}AVIVSDPA^{109})
pepti413R	CGAACCATGTCTCCTGATTGAACGGG	Complementary to 1213 to 1238 of spr1284 (^{405}PVQSGDMVR^{413})
UPatp3	tcggaagcttAGGAAAAGCGCTTAAGAACA	–651 to–631 of *atpC*
16SDNAF1	GAGTTGCGAACGGGTGAGT	86 to 104 of 16S rRNA

16SDNAF2	GTGGCGAAAGCGGCTCTCTGG	719 to 739 of 16S rRNA
16SDNAR1	AGCGATTCCGACTTCAT	Complementary to 1326 to 1342 of 16S rRNA
16sDNAR2	CCAGAGAGCCGCTTTCGCCAC	Complementary to 719 to 739 of 16S rRNA

[a] The 5' end of upatp3 and atpWO contained a sequence including a *Hin*dIII or *Eco*RI restriction sites, respectively, which are shown underlined. Lower case letters indicate bases not present in the nucleotide sequence of *S. pneumoniae* R6.

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

[c] The nucleotide and amino acid numbering refers to the numbering for *atpC* gene and protein of the *S. mitis* NCTC 12261^{T}, with the first nucleotide or amino acid being at position 1.

[d] The nucleotide and amino acid numbering refers to the numbering for *atpC* gene and protein of the *S. oralis* NCTC 11427^{T}, with the first nucleotide or amino acid being at position 1.

TABLE 2 . Susceptibilities of *S. pneumoniae* and VS strains to amino alcohol antimalarials

Strain	MIC (μg/ml)[a]		
	OPT	QIN	MFL
S. pneumoniae R6	1.5	50	0.15
S. pneumoniae ATCC 6303	0.75	25	0.15
S. pneumoniae ATCC 49619	1.5	50	0.15
S. mitis NCTC 12261	96	800	40
S. oralis ATCC 10557	48	400	40
S. oralis NCTC 11427	48	400	20
S. mitis 1162/99	0.75	50	0.30
S. mitis 1174/97	6	800	10

[a] Susceptibility categorizations for OPT, QIN and MFL: resistant, MIC >6 μg/ml, MICs >200 μg/ml, and MICs >0.62 μg/ml, respectively; intermediate, MICs 3 to 6 μg/ml, MICs 100 to 200 μg/ml, and MICs 0.3 to 0.6 μg/ml, respectively; susceptible, MIC ≤1.5 μg/ml, MIC ≤50 μg/ml, and MIC ≤1.25 μg/ml, respectively.

FIG. 1. Identification of VS by hybridization with a specific DNA probe (A) and identification as *S. mitis* by sequencing *sodA* (B). (A) Chromosomal DNAs from *S. pneumoniae* R6 (SPN), *S. mitis* NCTC 12261^T (SMI), *S. oralis* NCTC 11427^T (SOR), and isolates 1162/99 and 1174/97 were cleaved with *Nhe*I plus *Hin*dII (lanes NH) or *Nhe*I plus *Eco*RV (lanes NE), and the fragments were separated in 1% agarose gels. Mw, biotinylated DNA ladder. The gel was blotted and the blot was probed with a 798-bp biotinylated fragment containing most of the *ant* gene. (B) UPGMA tree of 365-bp *sodA* fragments. (B) The sequences of strains 1162/99 and 1174/97 were compared with that of the type strains of *S. mitis* (NCTC 12261), *S. pneumoniae* (NCTC 7465), *S. oralis* (NCTC 11427), *S. parasanguis* (ATCC 15912), *S. gordonii* (ATCC 10558), and *S. sanguis* (ATCC 10556). Phylogenetic analyses were conducted with the MEGA program (version 2.1) by the UPGMA method. Only bootstrap confidence intervals exceeding 90% are shown.

FIG. 2. Nucleotide sequence variations in the *atpC* and *atpA* genes (A) and comparisons of the amino acid sequences and secondary structures of the *c* and *a* subunits (B) of *S. pneumoniae* and VS strains. (A) The nucleotides present at each polymorphic site are shown for strain R6 , but for the other strains, only nucleotides that differ from those in 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. Triangles indicate gaps. (B) The predicted α-helices (H1 to H6) are shown above the sequence of SPN R6. Residues mutated in Opt^R strains (24) are shown in boldface and double underlined. Residues involved in proton translocation are boxed. *S. pneumoniae* R6 (SPN R6, accession number Z25851), *S. pneumoniae* ATCC 6303 (SPN 6303), *S. pneumoniae* ATCC 49619 (SPN 49619), VS 1162/99 (1162), VS 1174/97 (1174), *S. mitis* NCTC 12261 (SMI), and *S. oralis* NCTC 11427 (SOR, accession numbers Z26852 and Z26853).

FIG 3. Genetic structure of the *atp* region of *S. pneumoniae* and VS (A), Nucleotide sequence of the region surrounding the recombination point in strain 1162/99 (B), and Southern blot hybridizations with two different probes (C). (B) The putative recombination regions are boxed. (C) Symbols are as in Fig. 1, except that H means digestion with *Nhe*I plus *Hin*dII and E means digestion with *Nhe*I plus *Eco*RV. The gel was blotted and the blot probed with the spr1284 probe. After stripping, the blot was reprobed with the atpCA probe.