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# Identification of Streptococci to Species Level by Sequencing the Gene Encoding the Manganese-Dependent Superoxide Dismutase

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**We have used a PCR assay based on the use of degenerate primers in order to characterize an internal fragment ($sodA_{int}$) representing approximately 85% of the genes encoding the manganese-dependent superoxide dismutase in various streptococcal type strains (*S. acidominimus*, *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. bovis*, *S. constellatus*, *S. canis*, *S. cricetus*, *S. downei*, *S. dysgalactiae*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. equinus*, *S. gordonii*, *S. iniae*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguis*, *S. pneumoniae*, *S. porcinus*, *S. pyogenes*, *S. salivarius*, *S. sanguis*, *S. sobrinus*, *S. suis*, *S. thermophilus*, and *S. vestibularis*). Phylogenetic analysis of these $sodA_{int}$ fragments yields an evolutionary tree having a topology similar to that of the tree constructed with the 16S rRNA sequences. We have shown that clinical isolates could be identified by determining the positions of their $sodA_{int}$ fragments on the phylogenetic tree of the $sodA_{int}$ fragments of the type species. We propose this method for the characterization of strains that cannot be assigned to a species on the basis of their conventional phenotypic reactions.**

The genus *Streptococcus* could be taxonomically divided into six major clusters which included at least 31 species (4, 8, 17, 18, 32, 34–36). These are (i) the pyogenic group, which includes *S. agalactiae*, *S. canis*, *S. dysgalactiae*, *S. equi*, *S. iniae*, *S. porcinus*, and *S. pyogenes*; (ii) the bovis group, which includes *S. bovis*, *S. equinus*, and *S. alactolyticus*; (iii) the salivarius group, which includes *S. salivarius*, *S. thermophilus*, and *S. vestibularis*; (iv) the mutans group, which includes *S. cricetus*, *S. downei*, *S. mutans*, and *S. sobrinus*; (v) the anginosus group (also referred to as the milleri group), which includes *S. anginosus*, *S. constellatus*, and *S. intermedius*; and (vi) the mitis group, which includes *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguis*, *S. parasanguis*, and *S. gordonii*. No single system of classification suffices for the differentiation of this heterogeneous group of organisms. Instead, classification depends on a combination of features including patterns of hemolysis observed on blood agar plates, antigenic composition, growth characteristics, biochemical reactions, and more recently, genetic analysis (3, 14, 18, 28).

In clinical laboratories, the current means of identification of streptococci rely on phenotypic tests such as those developed for the API ID 32 Strep system. However, the potential problems inherent to the use of phenotypic tests are that not all strains within a given species may be positive for a common trait (3, 18) and that the same strain may exhibit biochemical variability (15, 30). Moreover, small alterations in the realization of a test may give false results. Consequently, the routine technique based on phenotypic tests do not allow for an unequivocal identification of certain streptococcal species, in particular, those belonging to the milleri, the mutans, and the mitis groups (2, 3, 10, 18, 19). Nucleic acid-based technologies such as DNA hybridization (1, 16, 29) or amplification of selected targets (25, 27, 33) have been developed in recent years to complement and improve the identification of streptococci. We previously described a PCR assay based on the use of degenerate primers which enabled amplification of an internal fragment representing approximately 85% of the *sodA* gene encoding a manganese-dependent enzyme (manganese-dependent superoxide dismutase [Mn-SOD]) in various gram-positive bacteria including streptococci and enterococci (24). This gene has been identified as a target for the identification of mycobacteria at the species level by PCR (37), and we investigated in this study the sequencing of the *sodA* PCR product as an approach to the genotypic identification of 29 different streptococcal species including those constituting the milleri, mitis, and mutans groups.

(A report of this work was presented at the XIIIth Lancefield International Symposium [16 to 20 September 1996, Paris, France].)

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The main characteristics of the streptococcal strains used in this study, including the type strains, are listed in Tables 1 and 2. All strains were grown at 37°C on Columbia horse blood agar (bioMérieux, Marcy l'Etoile, France) in an anaerobic atmosphere. Phenotypic identifications were performed with the rapid ID 32 Strep System (API-bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The API profiles were interpreted from the computer database for identification.

**DNA manipulations.** Rapid extraction of bacterial genomic DNA was performed as described previously (6), and primers *d1* (5′-CCITAYICITAYGAYG CIYTIGARCC-3′) and *d2* (5′-ARRTARTAIGCRTGYTCCCAIACRTC-3′) were used to amplify an internal fragment representing approximately 85% of the *sodA* genes of the bacterial strains. PCRs were performed with a Gene Amp System 9600 instrument (Perkin-Elmer Cetus, Roissy, France) in a final volume of 50 μl containing 250 ng of DNA as template, 0.25 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase in a 1× amplification buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). The PCR mixtures were denatured (3 min at 95°C) and were then subjected to 35 cycles of amplification (90 s of annealing at 37°C, 90 s of elongation at 72°C, and 30 s of denaturation at 95°C) and to a final elongation cycle of 72°C for 10

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TABLE 1. Streptococcal type strains used in this study

| Strain[a] | Other designation[b] | Relevant characteristics | $sodA_{int}$ accession no. |
|---|---|---|---|
| *S. acidominimus* CIP 82.4$^T$ | NCDO 2025 | Type strain | Z95892 |
| *S. agalactiae* CIP 103227$^T$ | ATCC 13813 | Type strain | Z95893 |
| *S. alactolyticus* CIP 103244$^T$ | ATCC 43077 | Type strain | Z95894 |
| *S. anginosus* CIP 102921$^T$ | ATCC 33397 | Type strain | Z95895 |
| *S. bovis* CIP 102302$^T$ | ATCC 33317 | Type strain | Z95896 |
| *S. canis* CIP 103223$^T$ | ATCC43496 | Type strain | Z99175 |
| *S. constellatus* CIP 103247$^T$ | ATCC 27823 | Type strain | Z95897 |
| *S. cricetus* CIP 102510$^T$ | ATCC 19642 | Type strain | Z95898 |
| *S. downei* CIP 103222$^T$ | ATCC 33748 | Type strain | Z95899 |
| *S. dysgalactiae* CIP 102914$^T$ | ATCC 43078 | Type strain | Z95900 |
| *S. equinus* CIP 102504$^T$ | ATCC 9812 | Type strain | Z95903 |
| *S. equi* subsp. *equi* CIP 102910$^T$ | ATCC 33398 | Type strain | Z95901 |
| *S. equi* subsp. *zooepidemicus* CIP 103228$^T$ | ATCC 43079 | Type strain | Z95902 |
| *S. gordonii* CIP 105258$^T$ | ATCC 10558 | Type strain | Z95905 |
| *S. iniae* CIP 102508$^T$ | ATCC 29178 | Type strain | Z99176 |
| *S. intermedius* CIP 103248$^T$ | ATCC 27335 | Type strain | Z95908 |
| *S. mitis* CIP 103335$^T$ | NCTC 12261 | Type strain | Z95909 |
| *S. mutans* CIP 103694 | ATCC 35668 | Quality control strain for API product | Z95910 |
| *S. oralis* CIP 10922$^T$ | ATCC 35037 | Type strain | Z95911 |
| *S. parasanguis* CIP 104372$^T$ | ATCC 15910 | Type strain | Z95913 |
| *S. pneumoniae* CIP 102911$^T$ | ATCC 33400 | Type strain | Z95914 |
| *S. porcinus* CIP 103218$^T$ | ATCC 43138 | Type strain | Z99177 |
| *S. pyogenes* CIP 56.41$^T$ | ATCC 12344 | Type strain | Z95915 |
| *S. salivarius* CIP 102509$^T$ | ATCC 19645 | Type strain | Z95916 |
| *S. sanguis* CIP 55.1328$^T$ | ATCC 10556 | Type strain | Z95918 |
| *S. sobrinus* CIP 103230$^T$ | ATCC 33478 | Type strain | Z95919 |
| *S. suis* CIP 103217$^T$ | ATCC 43765 | Type strain | Z95920 |
| *S. thermophilus* CIP 102303$^T$ | ATCC 19258 | Type strain | Z95921 |
| *S. vestibularis* CIP 103363$^T$ | ATCC 49124 | Type strain | Z95922 |

[a] CIP, Collection de l'Institut Pasteur.
[b] NCDO, National Collection of Dairy Organisms; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

min. The PCR products were resolved by electrophoresis on a 1% agarose gel stained with ethidium bromide.

**Cloning and sequencing.** Amplification products were purified on a Sephadex S-200 column (Pharmacia, Uppsala, Sweden) and were ligated into the pUC18-*Sma*I dephosphorylated vector by using the Sure-clone ligation kit (Pharmacia, Uppsala, Sweden). Recombinant plasmids were analyzed by colony-PCR as follows. Twelve randomly chosen clones were amplified by using the universal −21 (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-AACAGCTATGAC CATG-3′) primers in a final volume of 50 μl containing $10^3$ bacteria, 0.1 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase in a 1× amplification buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). The PCR mixtures were denatured (10 min at 95°C) and were then subjected to 30 cycles of amplification (90 s of annealing at 45°C, 1 min of elongation at 72°C, and 1 min of denaturation at 95°C). Colony-PCR products were directly sequenced after purification on a Sephadex S-400 column (Pharmacia). The entire nucleotide sequences of both strands of two cloned amplicons obtained from independent PCRs were determined by using the dideoxy chain termination method of Sanger with the dye primer cycle sequencing ready reaction kit on a Genetic ABI PRISM 310 Sequencer Analyzer (Perkin-Elmer, Applied Biosystem Division, Roissy, France).

Direct sequencing of the $sodA_{int}$ PCR products with either of the degenerate oligonucleotides *d1* and *d2* was performed with the dRhodamine dye terminator sequencing kit (Perkin-Elmer, Applied Biosystem Division), as follows. After purification on a Centricon-100 Concentrator column, 200 ng of the PCR product was mixed with 8 μl of terminator reaction mixture and 10 pmol of primer in a final volume of 20 μl, and the mixture was subjected to the following thermal cycling: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min (which was repeated for 25 cycles).

**Sequence analysis.** The nucleotide sequences were analyzed with Perkin-Elmer software (Sequence Analysis, Sequence Navigator, and Autoassembler). Multiple alignment of *sod* genes was carried out by the CLUSTAL X program (31). The construction of the unrooted phylogenetic tree was performed by the neighbor-joining method (26).

**Nucleotide sequence accession numbers.** The sequences were submitted to the EMBL gene bank and were assigned the accession numbers listed in Tables 1 and 2.

## RESULTS AND DISCUSSION

**Amplification and sequencing of $sodA_{int}$ from various streptococcal type strains.** By using the primers *d1* and *d2* in a PCR

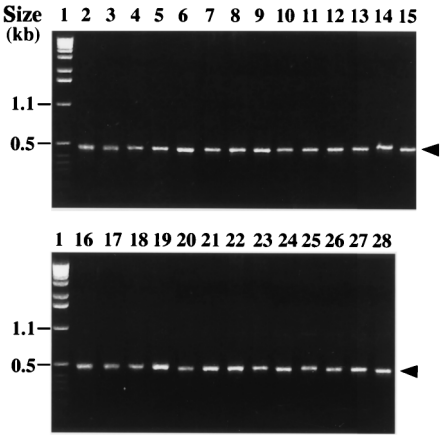


FIG. 1. Amplification of streptococcal type strains with the primers *d1* and *d2* and separation of the amplicons by 1% agarose gel electrophoresis. Lanes: 1, 1-kb ladder (Gibco, BRL); 2, *S. acidominimus*; 3, *S. agalactiae*; 4, *S. alactolyticus*; 5, *S. anginosus*; 6, *S. bovis*; 7, *S. constellatus*; 8, *S. cricetus*; 9, *S. downei*; 10, *S. dysgalactiae*; 11, *S. equi* subsp. *equi*; 12, *S. equi* subsp. *zooepidemicus*; 13, *S. equinus*; 14, *S. gordonii*; 15, *S. intermedius*; 16, *S. mitis*; 17, *S. mutans*; 18, *S. oralis*; 19, *S. parasanguis*; 20, *S. pneumoniae*; 21, *S. porcinus*; 22, *S. pyogenes*; 23, *S. salivarius*; 24, *S. sanguis*; 25, *S. sobrinus*; 26, *S. thermophilus*; 27, *S. suis*; 28, *S. vestibularis*. Arrowheads, 480-bp amplicon.

TABLE 2. Comparative identification of various streptococcal strains

| Strain[a] | Relevant characteristics[b] | Species identification | | $sodA_{int}$ accession no. |
|---|---|---|---|---|
| | | ID 32 Strep[c] | $sodA_{int}$[d] | |
| *S. agalactiae* | | | | |
| CIP 82.45 | Lancefield group B (ATCC 12403) | ND | *S. agalactiae* (98.6) | Z99178 |
| NEM318 | Lancefield group B | ND | *S. agalactiae* (97.9) | Z99179 |
| NEM1317 | Lancefield group B | ND | *S. agalactiae* (98.4) | Z99180 |
| Milleri group | | | | |
| NEM1164 | Nontypeable, β-hemolytic | *S. constellatus* (99.6) | *S. anginosus* (97) | Z99181 |
| NEM1166 | Nontypeable, β-hemolytic | *S. anginosus* (99.8) | *S. anginosus* (97.4) | Z99182 |
| NEM1124 | Lancefield group F, β-hemolytic | *S. constellatus* (99.9) | *S. constellatus* (98.1) | Z99183 |
| NEM1162 | Lancefield group F, β-hemolytic | *S. constellatus* (99.6) | *S. constellatus* (99.1) | Z99184 |
| NEM1165 | Nontypeable | *S. constellatus* (93.1) | *S. constellatus* (98.9) | Z99185 |
| NEM1275 | Nontypeable | *S. constellatus* (63.9) | *S. constellatus* (99.1) | Z99186 |
| MG19 | Nontypeable | Milleri group | *S. constellatus* (99.1) | Z99187 |
| Mitis group | | | | |
| CIP 103221 | ATCC 33399 | *S. gordonii* (?) | *S. gordonii* (95.9) | Z99189 |
| BM120 | Strain Challis | *S. gordonii* (99.9) | *S. gordonii* (98.2) | Z99188 |
| NEM666 | Nontypeable | ? | *S. gordonii* (95.9) | Z99190 |
| NEM1222 | Nontypeable | *S. mitis* (95.6) | *S. mitis* (97.8) | Z99192 |
| NEM1126 | Lancefield group C | *S. mitis* (59.5) | *S. mitis* (97.8) | Z99191 |
| *S. mutans* | | | | |
| GS-5 | | ND | *S. mutans* (99.8) | D01037 |
| NEM1163 | Nontypeable | *S. mutans* (99.9) | *S. mutans* (99.5) | Z99193 |
| *S. oralis* | | | | |
| CIP 103216 | ATCC 10557 | | *S. oralis* (93.7) | Z99194 |
| NEM1121 | Lancefield group C | *S. salivarius* (99.8) | *S. oralis* (96.1) | Z99195 |
| NEM895 | Nontypeable | ? | *S. parasanguis* (97.8) | Z99196 |
| *S. pneumoniae* | | | | |
| NEM667 | Serotype 11 | ND | *S. pneumoniae* (100) | Z99246 |
| NEM1278 | Serotype 23F | ND | *S. pneumoniae* (99.8) | Z99204 |
| NEM1279 | Serotype 23F, Pen$^r$ | ND | *S. pneumoniae* (100) | Z99205 |
| NEM1280 | Serotype 23F, Pen$^r$ | ND | *S. pneumoniae* (100) | Z99206 |
| NEM1251 | Serotype 16 | ND | *S. pneumoniae* (100) | Z99201 |
| NEM1252 | Serotype 18 | ND | *S. pneumoniae* (100) | Z99202 |
| NEM1253 | Serotype 6 | ND | *S. pneumoniae* (100) | Z99203 |
| NEM1122 | Serotype 23F, Opt$^r$ | ? | *S. pneumoniae* (99.8) | Z99200 |
| *S. pyogenes* | | | | |
| BM105 | Lancefield group A | ND | *S. pyogenes* (100) | Z49247 |
| HSC5 | Lancefield group A | ND | *S. pyogenes* (100) | U43776 |
| *S. salivarius* | | | | |
| CIP 102505 | ATCC 13419 | *S. salivarius* (?) | *S. salivarius* (96.3) | Z99197 |
| NEM1250 | Lancefield group D | *S. salivarius* (99.9) | *S. salivarius* (98.6) | Z99198 |
| NEM1257 | Nontypeable | *S. salivarius* (99.5) | *S. salivarius* (96.3) | Z99199 |

[a] Data for all strains except *S. agalactiae* CIP 82.45 (24), *S. agalactiae* NEM318 (11), MG19 (5), *S. gordonii* BM120 (20), *S. mutans* GS-5 (21), *S. pneumoniae* NEM667 (24), *S. pyogenes* BM105 (24), and *S. pyogenes* HSC5 (13) are from this work.

[b] Opto$^r$ and Pen$^r$, resistance to optochin and penicillin, respectively.

[c] The rapid ID 32 Strep system (API-bio-Mérieux) was used according to the manufacturer's instructions to identify isolates to the species level. The API profiles were interpreted from the computer database for identification. The numbers in parentheses indicate the API identification percentage. ND, not determined; ?, undetermined.

[d] The species identification was based on the phylogenic position of the $sodA_{int}$ fragment of the strain studied relative to those of the type strains, as indicated in Fig. 1. The numbers in parentheses indicate the percent identity of the $sodA_{int}$ fragment with that of the corresponding type strain.

assay, we amplified an internal fragment representing approximately 85% of the *sodA* gene encoding a manganese-dependent enzyme (Mn-SOD) in 29 type strains of streptococci (*S. acidominimus*, *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. bovis*, *S. canis*, *S. constellatus*, *S. cricetus*, *S. downei*, *S. dysgalactiae*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. equinus*, *S. gordonii*, *S. iniae*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguis*, *S. pneumoniae*, *S. porcinus*, *S. pyogenes*, *S. salivarius*, *S. sanguis*, *S. sobrinus*, *S. suis*, *S. thermophilus*, and *S. vestibularis*). A single amplification product having the expected size of 480 bp was observed with all streptococcal species (Fig. 1 shows the results of part of this analysis). The nucleotide sequences of the $sodA_{int}$ fragments from these type strains were determined following cloning into pUC18 (Table 1). Analysis of the corresponding deduced amino acid sequences (data not shown) revealed that they all possessed three histidyl residues and one aspartyl residue that supposedly serve as metal ligands at positions characteristic of Mn- or

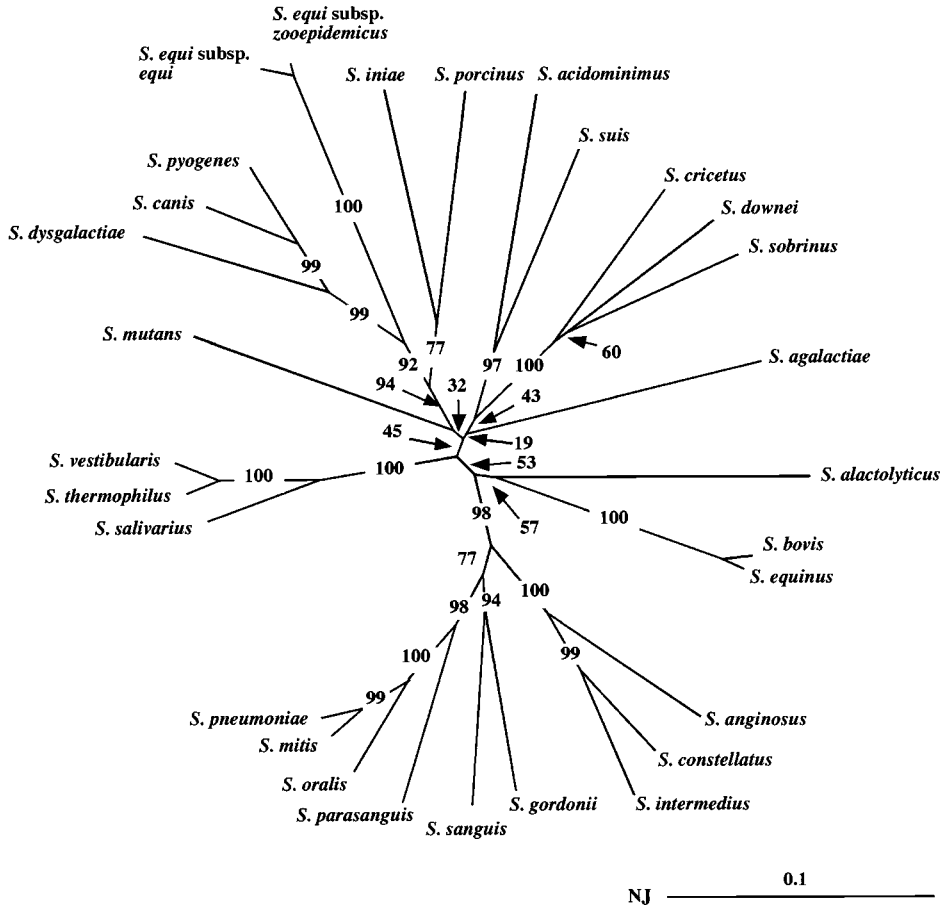


FIG. 2. Phylogenetic unrooted tree showing relationships among the $sodA_{int}$ fragments from various streptococcal type strains. The tree was established from an analysis of the sequences listed in Table 1 by using the neighbor-joining method (26). The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by bootstrap analysis. The scale bar (neighbor-joining [NJ] distance) represents 10% differences in nucleotide sequences.

Fe-SODs (22, 23). Moreover, they all contain in the vicinity of the active site four other residues characteristic of Mn-SODs, which suggests that the corresponding enzymes are activated by Mn ions (23). We therefore concluded that the PCR products cloned and sequenced were actual $sodA_{int}$ fragments. Multiple alignment of the streptococcal $sodA_{int}$ sequences was carried out by the CLUSTAL X program (31), and an unrooted phylogenetic tree was constructed by the neighbor-joining method (26). Domains I and II corresponding to the degenerate oligonucleotides *d1* and *d2*, respectively, and alignment gaps were not taken into consideration for calculations. The topology of the phylogenetic tree obtained (Fig. 2) was evaluated by bootstrap analyses to give the degree of confidence intervals for each node on the phylogenetic tree. The confidence values are given at the branches, which show possibly monophyletic clades of related organisms separated at each node. It is generally accepted that the monophyly of a clade can be accepted if the clade occurs in more than 95% of the bootstrapped trees (9). If this critical value is used, the $sodA_{int}$-based phylogenetic positions of the streptococcal species studied here were in general agreement with those inferred from an analysis of their 16S rRNA sequences (4, 17), with the following remarkable exceptions: *S. agalactiae* did not cluster with the species constituting the pyogenic group, and *S. mutans* was genetically separate from *S. cricetus*, *S. downei*, and *S. sobrinus*. Pairwise comparison of two given streptococcal species always revealed a lower percentage of sequence identity between their $sodA_{int}$ fragments than between their 16S RNAs (Table 3 and data not shown). For example, the sequence identities of the 16S RNAs of type strains of *S. mitis*, *S. oralis*, and *S. pneumoniae* are greater than 99% (17), whereas those of their $sodA_{int}$ fragments vary from 92% (*S. mitis* versus *S. oralis* and *S. oralis* versus *S. pneumoniae*) to 96.6% (*S. mitis* versus *S. pneumoniae*) (Table 3). These results indicate that the *sodA* gene might constitute a more discriminative target sequence than the 16S RNA for differentiating closely related bacterial species. On the other hand, it is worth noting that the close structural relationship (98.9% identity) observed between the $sodA_{int}$ fragments of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* is consistent with their association in a single species (7).

**Species identification of streptococcal clinical isolates by sequencing the $sodA_{int}$ gene.** The design of species-specific oligonucleotides enabling the amplification of a given target DNA constitutes an interesting molecular approach for the identification of bacterial species by PCR (12, 37). The two major problems inherent to these techniques are that (i) species-specific oligonucleotides often cannot be designed for closely related species, and (ii) the number of PCRs necessary for the identification of one isolate is proportional to the num-

TABLE 3. Identity matrix based pairwise comparisons of *sodA*$_{int}$ fragments of streptococcal type strains

| Strain[a] | % Identity with the following strain[b]: | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
| 1. *S. acidominimus* | 71.3 | 67.8 | 71.7 | 68.0 | 69.9 | 69.9 | 70.8 | 73.3 | 67.6 | 65.3 | 66.0 | 69.9 | 72.6 | 70.8 | 68.5 | 69.7 | 72.6 | 70.8 | 66.9 | 69.0 | 70.3 | 68.0 | 72.0 | 69.7 | 70.1 | 79.5 | 73.3 | 73.3 |
| 2. *S. agalactiae* | | 69.0 | 72.0 | 72.6 | 71.7 | 73.1 | 71.3 | 71.5 | 71.5 | 66.7 | 66.7 | 74.0 | 73.3 | 71.7 | 71.0 | 69.7 | 74.5 | 69.9 | 72.4 | 70.1 | 71.3 | 70.8 | 74.5 | 73.1 | 74.0 | 74.7 | 72.4 | 72.6 |
| 3. *S. alactolyticus* | | | 73.1 | 74.9 | 69.7 | 76.1 | 66.4 | 69.4 | 69.7 | 66.2 | 65.7 | 75.2 | 72.0 | 66.9 | 73.1 | 70.8 | 70.6 | 70.6 | 71.0 | 71.0 | 69.7 | 70.3 | 74.5 | 72.2 | 68.3 | 68.3 | 72.2 | 71.3 |
| 4. *S. anginosus* | | | | 71.7 | 67.1 | 86.4 | 69.4 | 68.5 | 65.5 | 66.9 | 66.7 | 72.2 | 80.5 | 65.3 | 83.4 | 75.9 | 69.7 | 74.3 | 75.9 | 75.2 | 66.9 | 66.7 | 69.2 | 76.6 | 66.7 | 72.4 | 70.8 | 70.3 |
| 5. *S. bovis* | | | | | 72.0 | 70.3 | 72.9 | 70.6 | 68.7 | 66.4 | 66.4 | 97.9 | 70.6 | 68.0 | 69.9 | 72.9 | 74.0 | 71.7 | 73.3 | 72.2 | 68.5 | 70.6 | 77.5 | 69.4 | 69.7 | 70.3 | 75.9 | 76.3 |
| 6. *S. canis* | | | | | | 64.1 | 73.1 | 73.1 | 85.1 | 78.9 | 79.5 | 72.4 | 65.5 | 78.9 | 63.0 | 67.8 | 76.8 | 64.6 | 66.7 | 67.6 | 74.0 | 92.2 | 74.0 | 64.6 | 72.9 | 67.8 | 72.0 | 71.3 |
| 7. *S. constellatus* | | | | | | | 65.5 | 66.4 | 66.0 | 62.3 | 62.1 | 71.5 | 81.8 | 66.9 | 90.1 | 76.8 | 68.7 | 75.6 | 78.2 | 76.1 | 69.2 | 66.7 | 70.3 | 79.8 | 65.3 | 71.5 | 71.5 | 70.6 |
| 8. *S. cricetus* | | | | | | | | 84.1 | 70.3 | 68.0 | 68.7 | 73.3 | 70.1 | 72.2 | 64.8 | 71.0 | 75.6 | 70.3 | 70.6 | 70.8 | 69.2 | 72.0 | 75.9 | 69.0 | 84.1 | 74.7 | 73.6 | 73.3 |
| 9. *S. downei* | | | | | | | | | 70.1 | 71.0 | 70.8 | 70.1 | 65.7 | 73.1 | 63.9 | 68.3 | 74.0 | 66.9 | 66.2 | 68.7 | 69.7 | 71.3 | 73.8 | 68.7 | 85.5 | 76.8 | 73.1 | 73.1 |
| 10. *S. dysgalactiae* | | | | | | | | | | 74.9 | 74.9 | 69.0 | 62.8 | 77.5 | 64.6 | 66.9 | 69.9 | 66.4 | 67.6 | 67.1 | 72.4 | 86.9 | 72.0 | 65.3 | 73.1 | 65.7 | 73.1 | 71.5 |
| 11. *S. equi* subsp. *equi* | | | | | | | | | | | 98.9 | 65.7 | 63.0 | 71.3 | 63.2 | 63.7 | 70.1 | 63.7 | 64.1 | 63.7 | 68.0 | 76.1 | 69.7 | 63.4 | 68.7 | 66.9 | 66.7 | 66.4 |
| 12. *S. equi* subsp. *zooepidemicus* | | | | | | | | | | | | 65.7 | 63.0 | 71.3 | 63.0 | 64.1 | 69.7 | 64.1 | 64.8 | 64.1 | 68.3 | 77.0 | 69.9 | 62.5 | 69.0 | 66.2 | 66.7 | 66.4 |
| 13. *S. equinus* | | | | | | | | | | | | | 71.7 | 68.5 | 71.7 | 72.6 | 74.7 | 72.2 | 73.3 | 71.7 | 69.2 | 70.3 | 77.2 | 70.1 | 69.2 | 69.9 | 75.9 | 76.8 |
| 14. *S. gordonii* | | | | | | | | | | | | | | 64.1 | 81.1 | 80.5 | 70.3 | 79.8 | 80.9 | 80.2 | 66.7 | 63.9 | 73.3 | 85.7 | 68.7 | 72.9 | 73.3 | 73.1 |
| 15. *S. iniae* | | | | | | | | | | | | | | | 63.9 | 66.0 | 74.0 | 66.4 | 65.7 | 66.9 | 77.0 | 79.3 | 72.4 | 65.3 | 70.3 | 71.0 | 70.8 | 70.1 |
| 16. *S. intermedius* | | | | | | | | | | | | | | | | 75.6 | 67.4 | 76.1 | 77.2 | 74.3 | 68.5 | 63.2 | 68.5 | 77.0 | 62.3 | 70.1 | 70.6 | 70.1 |
| 17. *S. mitis* | | | | | | | | | | | | | | | | | 68.5 | 91.7 | 85.7 | 96.6 | 67.1 | 66.7 | 76.3 | 78.9 | 70.8 | 73.3 | 75.6 | 75.2 |
| 18. *S. mutans* | | | | | | | | | | | | | | | | | | 67.4 | 67.6 | 68.5 | 74.3 | 75.2 | 78.4 | 68.7 | 72.0 | 74.5 | 74.7 | 75.6 |
| 19. *S. oralis* | | | | | | | | | | | | | | | | | | | 85.3 | 92.0 | 66.9 | 63.0 | 73.6 | 79.5 | 69.4 | 73.3 | 74.0 | 73.6 |
| 20. *S. parasanguis* | | | | | | | | | | | | | | | | | | | | 85.7 | 65.3 | 64.8 | 70.8 | 79.3 | 68.5 | 72.2 | 70.6 | 70.8 |
| 21. *S. pneumoniae* | | | | | | | | | | | | | | | | | | | | | 66.4 | 66.7 | 75.4 | 78.2 | 71.3 | 73.6 | 75.2 | 74.9 |
| 22. *S. porcinus* | | | | | | | | | | | | | | | | | | | | | | 74.9 | 71.0 | 66.4 | 69.4 | 71.3 | 73.1 | 72.0 |
| 23. *S. pyogenes* | | | | | | | | | | | | | | | | | | | | | | | 74.5 | 63.7 | 72.6 | 66.7 | 71.5 | 70.3 |
| 24. *S. salivarius* | | | | | | | | | | | | | | | | | | | | | | | | 72.2 | 75.6 | 73.3 | 89.7 | 90.1 |
| 25. *S. sanguis* | | | | | | | | | | | | | | | | | | | | | | | | | 67.4 | 72.0 | 72.4 | 71.7 |
| 26. *S. sobrinus* | | | | | | | | | | | | | | | | | | | | | | | | | | 75.4 | 77.0 | 75.6 |
| 27. *S. suis* | | | | | | | | | | | | | | | | | | | | | | | | | | | 72.0 | 72.9 |
| 28. *S. thermophilus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | 96.8 |
| 29. *S. vestibularis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

[a] The main characteristics of the strains are listed in Table 1.
[b] The strain numbers correspond to the strains identified by the numbers on the left.

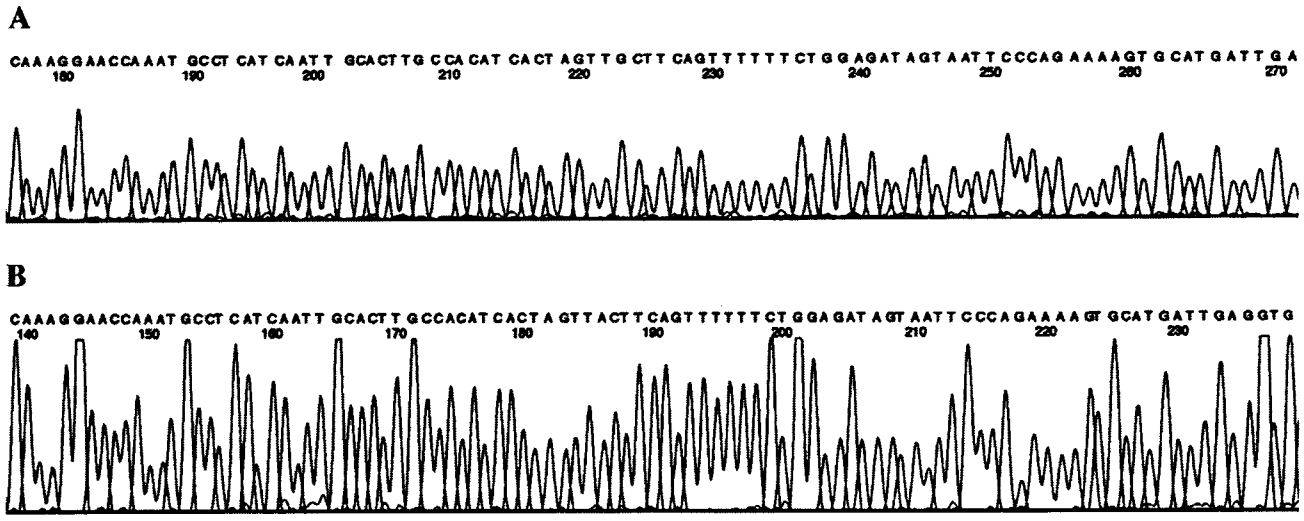


FIG. 3. Electropherograms showing part of the nucleotide sequence of $sodA_{int}$ from *S. porcinus*. Sequence reactions were carried out with a pUC18Ω$sodA_{int}$ recombinant plasmid with the −21 dye primer sequencing kit (A) and $sodA_{int}$ PCR product with the degenerate oligonucleotide *d2* and the dRhodamine dye terminator sequencing kit (B).

ber of bacterial type species that should be considered. Taking into consideration the fact that cloning and sequencing techniques are increasingly used as routine techniques in medical microbiology laboratories, we propose the sequencing of the $sodA_{int}$ fragment as a molecular approach to the identification of streptococcal species. In this system, the identification of a clinical isolate is determined by the position of its $sodA_{int}$ fragment on the phylogenetic tree of the $sodA_{int}$ fragments of the type species (Fig. 2). To test the functionality of this approach, various typeable and nontypeable streptococcal isolates were identified by using the ID 32 Strep and/or the $sodA_{int}$ systems (Table 2). We also include in this study the sequences of $sodA_{int}$ fragments of known streptococcal species present in the databases. The results obtained with the $sodA_{int}$ system indicated that, as expected, the two group A and the three group B streptococcal strains studied were identified as *S. pyogenes* and *S. agalactiae*, respectively. When the streptococcal strains that were unambiguously identified to the species level by the phenotypic method (API identification percentage, ≥99.9), the $sodA_{int}$-based identification method gave the same results (Table 2). Some discrepancies were observed for the strains the species of which were determined with an API identification percentage of less than 99.9. This was the case for NEM1164 and NEM1121, which were identified with the ID 32 Strep system as *S. constellatus* and *S. salivarius*, respectively, but which were identified with the $sodA_{int}$ system as *S. anginosus* and *S. oralis*, respectively (Table 2). The sequence of the $sodA_{int}$ fragment of NEM1164 displays 97 and 86% identity with the sequences of the type strains of *S. anginosus* and *S. constellatus*, respectively. The sequence of the $sodA_{int}$ fragment of NEM1121 displays 96.1 and 73.6% identity with the sequences of the type strains of *S. oralis* and *S. anginosus*, respectively. On the basis of these sequence distances, we considered the $sodA_{int}$-based identification of NEM1164 (*S. anginosus*) and NEM1121 (*S. oralis*) to be more reliable than the ID 32 Strep system-based identification. Interestingly, certain strains (NEM1275, MG19, NEM666, NEM1126, and NEM895) were identified to the species level with the $sodA_{int}$ system but not with ID 32 Strep system (Table 2). Finally, it is important that the intraspecies divergence between the $sodA_{int}$ fragments may vary greatly depending upon the species considered, conceivably because of differences in sequence divergence rates. Consequently, it is not possible to delineate streptococcal species on the basis of the level of DNA homology. In the case of *S. oralis*, the levels of intraspecies divergence of the $sodA_{int}$ fragments can exceed those observed between the $sodA_{int}$ fragments of the type strains of *S. oralis*, *S. mitis*, and *S. pneumoniae*. These results might suggest that the species *S. oralis*, as defined, is genomically heterogeneous. Surprisingly, the $sodA_{int}$ fragments from unrelated *S. pneumoniae* strains were found to display the highest level of intraspecies sequence identity (>99%), which suggests that transformation is not a source of sequence heterogeneity for the *sodA* gene, at least in pneumococci.

**Concluding remarks.** We have described a method that enables the reliable identification to the species level of groupable and nongroupable streptococci. It consists of (i) a PCR carried out with a single pair of degenerate oligonucleotides for amplification of a streptococcal $sodA_{int}$ fragment, (ii) molecular cloning of the resulting amplicon into an *Escherichia coli* vector, and (iii) sequencing of the insert on both DNA strands. Sequencing of a streptococcal $sodA_{int}$ fragment by using this procedure necessitates 72 h following receipt of the bacterial strain; however, we anticipate further simplification and/or automation of various steps of this method. For example, based on the observation that a single abundant PCR product was obtained by using the degenerate *sod* primers, whatever the species tested (Fig. 1), we successfully tried to sequence directly both strands of the amplified DNA with either of the degenerate PCR primers (Fig. 3 shows part of the results of that analysis). Removal of the cloning step greatly enhances the applicability of the procedure and reduces the delay required for bacterial identification to 24 h. This method might be useful in reference laboratories for the characterization strains that could not be assigned to a species on the basis of their conventional phenotypic reaction. It provides a convenient alternative to the DNA-DNA hybridization method, which constitutes the reference technique for the identification of strains to the species level. We are expanding the applicability of this approach by determining the nucleotide sequence

of $sodA_{int}$ fragments from other species of streptococci as well as from other related genera such as *Abiotrophia*, *Enterococcus*, *Gemella*, *Leuconostoc*, and *Pediococcus*.

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