

rpoB Gene Sequence-Based Identification of *Staphylococcus* Species

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The complete sequence of *rpoB*, the gene encoding the beta subunit of RNA polymerase was determined for *Staphylococcus saccharolyticus*, *Staphylococcus lugdunensis*, *S. taphylococcus caprae*, and *Staphylococcus intermedius* and partial sequences were obtained for an additional 27 *Staphylococcus* species. The complete *rpoB* sequences varied in length from 3,452 to 3,845 bp and had a 36.8 to 39.2% GC content. The partial sequences had 71.6 to 93.6% interspecies homology and exhibited a 0.08 to 0.8% intraspecific divergence. With a few exceptions, the phylogenetic relationships inferred from the partial *rpoB* sequences were in agreement with those previously derived from DNA-DNA hybridization studies and analyses of 16S ribosomal DNA gene sequences and partial HSP60 gene sequences. The staphylococcal *rpoB* sequence database we established enabled us to develop a molecular method for identifying *Staphylococcus* isolates by PCR followed by direct sequencing of the 751-bp amplicon. In blind tests, this method correctly identified 10 *Staphylococcus* isolates, and no positive results were obtained with 10 non-*Staphylococcus* gram-positive and gram-negative bacterial isolates. We propose partial sequencing of the *rpoB* gene as a new tool for the accurate identification of *Staphylococcus* isolates.

The genus *Staphylococcus* comprises 36 species of gram-positive, catalase-positive cocciform bacteria, nine of which also contain subdivisions with subspecies designations (11, 25). Only a few species are coagulase positive, namely, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus delphini*, *Staphylococcus schleiferi* subsp. *coagulans*, and some strains of *Staphylococcus hyicus* (22). These species are readily isolated, and cultures may be obtained from environmental, animal, and human clinical samples (24). *S. aureus* is well documented as an opportunistic pathogen in humans and is responsible for staphylococcal toxin-mediated food poisoning, toxic shock syndrome, and pyogenic infections characterized by frequent metastatic spread (22). Also, nosocomial infections due to methicillin-resistant *S. aureus* have emerged as major clinical and epidemiological problems (22). The coagulase-negative staphylococcal species constitute a major component of the normal microflora of humans. Their role in nosocomial infections has been well documented and has increased dramatically within the past 20 years (21). Precise delineation of the clinical disease produced by this group of bacteria is based on accurate species identification (21), aiding clinicians in the choice of appropriate antibiotic (13). Therefore, there is a pressing need for the rapid and accurate identification of isolates of clinically important coagulase-negative staphylococci. Such a goal remains difficult.

Currently, conventional benchtop phenotypic characterization is used for the identification of *Staphylococcus* species. For the reference identification of staphylococci the Centers for Disease Control and Prevention has developed a numerical code system based on a panel of 18 selected conventional biochemical reactions. Several biochemical-based commercial

kits and automated systems (23) are also available for this purpose. In clinical studies, however, various degrees of accuracy have been achieved with these different phenotypic formats (12, 33, 35). For example, commercial identification systems have been shown to commonly misidentify *Staphylococcus hominis* and *Staphylococcus warneri* with error rates as high as 36 and 27%, respectively (16, 17). *S. schleiferi* may also be misidentified by automated systems (5). Phenotypic characterizations thus have limitations that are partly due to variable expression of characters and ambiguity in the interpretation of end point reactions (3).

The use of nucleic acid targets with their high sensitivity and specificity may provide an alternative means of accurately identifying *Staphylococcus* species. Several molecular targets have been exploited for the molecular identification of *Staphylococcus* species, including the 16S rRNA gene (2, 8), the tRNA gene intergenic spacer (27), the heat shock protein 60 (HSP60) gene (14, 15, 26), and the *femA* gene (44). These targets, however, have been exploited through the technology of molecular probe hybridization, and therefore, they are useful only in laboratories that have the complete panel of probes and then only for identifying recognized *Staphylococcus* species. Further molecular targets that have been identified include the *nuc* gene, which occurs only in *S. aureus* (4), and a chromosomal DNA fragment specific for *Staphylococcus epidermidis* (29). There is, therefore, still a need for a simple and reliable molecular test for the identification of all *Staphylococcus* species (19).

rpoB, the gene encoding the highly conserved β subunit of the bacterial RNA polymerase, has previously been demonstrated to be a suitable target on which to base the identification of enteric bacteria (31), spirochetes (37), bartonellas (36), and rickettsias (10). The gene has been shown to be more discriminative than the 16S ribosomal DNA (rDNA) gene (31), which has also been used for identifying enteric bacteria. In this report we describe the molecular identification of

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TABLE 1. List of 31 *Staphylococcus* species investigated by *rpoB* sequencing

Species or subspecies	GenBank accession no.	Strain ^a
<i>S. caprae</i>	AF325868	CIP 104000 ^T
<i>S. gallinarum</i>	AF325890	CIP 103504 ^T
<i>S. aureus</i> subsp. <i>anaerobius</i>	AF325894	CIP 103780 ^T
<i>S. aureus</i> subsp. <i>aureus</i>	X64172	CIP 103428
<i>S. epidermidis</i>	AF325872	CIP 81.55 ^T
<i>S. haemolyticus</i>	AF325888	CIP 81.56 ^T
<i>S. intermedius</i>	AF325869	CIP 81.60 ^T
<i>S. lugdunensis</i>	AF325870	CIP 103642 ^T
<i>S. saccharolyticus</i>	AF325871	CIP 103275 ^T
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	AF325886	CIP 103643 ^T
<i>S. xylosum</i>	AF325883	CIP 81.66 ^T
<i>S. capitis</i> subsp. <i>capitis</i>	AF325885	ATCC 27840 ^T
<i>S. arlettae</i>	AF325874	ATCC 43957 ^T
<i>S. warneri</i>	AF325887	ATCC 27836 ^T
<i>S. hominis</i>	AF325875	ATCC 27844 ^T
<i>S. simulans</i>	AF325877	ATCC 27848 ^T
<i>S. saprophyticus</i>	AF325873	ATCC 15305 ^T
<i>S. equorum</i>	AF325882	ATCC 43958 ^T
<i>S. cohnii</i> subsp. <i>cohnii</i>	AF325893	ATCC 29974 ^T
<i>S. auricularis</i>	AF325889	ATCC 33753 ^T
<i>S. carnosus</i> subsp. <i>carnosus</i>	AF325880	ATCC 51365 ^T
<i>S. kloosii</i>	AF325891	ATCC 43959 ^T
<i>S. chromogenes</i>	AF325892	ATCC 43764 ^T
<i>S. hyicus</i> subsp. <i>hyicus</i>	AF325876	ATCC 11249 ^T
<i>S. pulvereri</i>	AF325879	CCUG 33938 ^T
<i>S. felis</i>	AF325878	CIP 103366 ^T
<i>S. lentus</i>	AF 036973	ATCC 49574
<i>S. muscae</i>	AF325884	CIP 1035641 ^T
<i>S. sciuri</i>	AF325881	ATCC 29062 ^T

^a ATCC, American Type Culture Collection; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; CIP, Institut Pasteur Collection, Paris, France.

Staphylococcus isolates using a PCR-sequencing approach based on the sequence of staphylococcal *rpoB*.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. They were grown on blood agar at 37°C under aerobic conditions except for *Staphylococcus aureus* subsp. *anaerobius* and *Staphylococcus saccharolyticus*, which were grown under anaerobic conditions. All isolates were streaked on blood agar plates to determine the purity of each of the cultures by macroscopic examination of colonies and microscopic examination of gram-stained preparations. Colonies were scraped from the plates and boiled for 15 min in Chelex 100 (39) and 1% sodium dodecyl sulfate solution (Bio-Rad Laboratories, Hercules, Calif.) before genomic DNA was extracted and purified with QIAamp DNA minikits (Qiagen GmbH, Hilden, Germany).

Construction of a *Staphylococcus rpoB* database. Consensus *rpoB* PCR primers were designed after the alignment of *rpoB* genes of *S. aureus* (GenBank accession number X64172) and *Bacillus subtilis* (GenBank accession number L43593) and numbered on the basis of the *S. aureus rpoB* sequence. Primer pair 1302F-2309R was used to amplify a 1,007-bp fragment in *S. saccharolyticus*, *Staphylococcus lugdunensis*, *Staphylococcus caprae*, and *S. intermedius*. These species were chosen as representatives of the major phyla from the 16S rDNA phylogenetic tree. Additional oligonucleotides were selected on the basis of data obtained from ongoing base sequence determinations (Table 2). The alignment of the *rpoB* sequences of *S. aureus*, *S. lugdunensis*, *S. intermedius*, *S. saccharolyticus*, and *S. caprae* enabled us to design consensus PCR primers 2491F (5'-AACCAATTC CGTATIGGTTT-3'; base positions 2491 to 2511) and 3554R (5'-CCGTCCCA AGTCATGAAAC-3'; base positions 3554 to 3573) to amplify a 1,081-bp variable fragment in 27 additional *Staphylococcus* species under investigation. All PCR mixtures contained 2.5 × 10⁻² U of *Taq* polymerase per μl, 1× *Taq* buffer, 1.8 mM MgCl₂ (Gibco BRL, Life Technologies, Cergy Pontoise, France), 200 μM concentrations of dATP, dTTP, dGTP, and dCTP (Boehringer Mannheim GmbH, Hilden, Germany), and 0.2 μM concentrations of each primer (Eurogen-

TABLE 2. Primers used in the sequencing of the entire *rpoB* gene in *S. lugdunensis*, *S. intermedius*, *S. saccharolyticus*, and *S. caprae*

Primer name	Primer sequence (5'-3')	Position ^a	T _m (°C)
30F	GGTTTAGGATAAAAGATGC	30-50	41
192F	GAAGAAGTTGGAGCTACTG	192-211	44
806F	AATAAGAGCAGGGAAAGAAAC	806-827	43
920F	AAAGAAAAGAATGAATGAACTT	920-942	39
1165F	TATGCTTATGGTATTTAGCTA	1165-1186	39
1302F	AACTTAATAGAAATTCAAAATAAA	1302-1327	58
1450F	GTTCAAACGATAAATAGAGAA	1450-1471	39
1741F	GAAACAGATGCTAAAGATGT	1741-1761	41
1850F	CCATATACTGCGAGTGGGAA	1850-1870	47
2245F	TAGAAATTCAATCAATTAAGTATATG	2245-2271	62
2309F	TTGGTAATGCTTTACCAGAT	2309-2329	41
2334F	TGCATTACACCAGCAGATATCATTG	2334-2359	70
2412F	GATGATATTGACCATTTAGG	2412-243	41
2534F	TGAAAAGAAATGTCAATTCAAAGA	2534-2555	39
2663F	AAACCCATTAGCTGAGTT	2663-268	38
2995F	TGGTCGTTTTTCATGGATGTGAAGTTG	2995-3119	74
2924F	AAGATAGCTATGTTGTAGCA	2924-2944	41
3200F	CTTAGAGAACGATGACTCTAA	3200-3221	43
3498F	TAGTTGGTTTTTCATGACTTGGGA	3498-3520	46
3550F	TTGAAAAGTCCAACAAAGCAA	3550-3570	38
3843F	GGTAAAGTAACGCCTAAAGGT	3843-3864	45
4494F	TGGAGGTATGGGCACTTGAA	4494-4514	47
1759R	ACATCTTTMGCATCTGTTTC	1779-1759	48
1460R	ATCGTTTGAACGCCACTCTT	1480-1460	45
1910R	TCATAGTAAGTTTGCGCCAT	1930-1910	43
2309R	ATCTGGTAAAGCATTACCAA	2329-2309	41
2334R	CAATGATATCTGCTGGTGAATGCA	2354-2334	68
2432R	CCTAAATGGTCAATATCATC	2452-2432	41
2573R	CGAATATTAATTAATTGTTG	2593-2573	34
2892R	GTGATAGCATGTGTATCTAAATCA	2912-2892	64
2915R	TAACATATCTTCTTCATCAG	2935-2915	41
2924R	TGCTACAACATAGCTATCTT	2944-2924	41
2995R	CAACTTCATCATCCATGAAACGACCA	3015-2995	74
Cm32b	ATGCAACGTCAGGCCGTTCCG	3211-3191	64
3321R	AGACGACGAACAGAATTTCA	3341-3321	56
3610R	GCTCGAATGATAACGTGATT	3630-3610	43
4139R	ACTTGTCCATGTTCCATACG	4159-4139	44
4502R	CATATGCTTCAAGTGCCATA	4523-4502	45
4508R	CCAAGTGGTTGTTGTGTAAC	4428-4508	45
4871R	TTTAGAGCTTTCCTGTTTG	4891-4871	41
5000R	CACCATATGACCAAGAACGAA	5021-5000	45
5018R	CAATCAAGGAGCCTACCTCCTT	5040-5018	50
5030R	GAAATATTTACATCAATCAA	5051-5030	36
5041R	TAACATATCTTCTTCATCAGC	5061-5041	41
5085R	CCCAGTCTTTGTAGGTCCG	5105-5085	49
5188R	CCCATTCTTTCACGACGTAC	5208-5188	47

^a Position relative to the *S. aureus rpoB* sequence.

tec, Seraing, Belgium). PCR mixtures were subjected to 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and de novo DNA extension at 72°C for 60 s. Every amplification program began with a denaturation step of 95°C for 2 min and ended with a final elongation step of 72°C for 5 min. Amplicons were purified for sequencing using a QIAquick spin PCR purification kit (Qiagen) following the protocol of the supplier. Partial reverse and forward (bidirectional) sequencing of a 598-bp fragment was obtained by using internal primers at positions 2643 (2643F: 5'-CAA TTC ATG GAC CAA GC-3') and 3241 (3241R: 5'-GCI ACI TGI TCC ATA CCT GT-3'). Sequencing reactions were carried out with the reagents of the ABI Prism dRhodamine dye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions and with the following program: 30 cycles of denaturation at 94°C for 10 s, primer annealing at 50°C for 10 s, and extension at 60°C for 2 min. Products of sequencing reactions were separated by electrophoresis on a 0.2-mm 6% polyacrylamide denaturing gel and recorded with an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems) following the standard protocol of the supplier. The results were pro-

cessed into sequence data with Sequence Analysis software (Applied Biosystems), and partial sequences were combined into a single consensus sequence with Sequence Assembler software (Applied Biosystems). The *rpoB* sequences were aligned by using the multisequence alignment program of Sequence Assembler software (Applied Biosystems). Pairwise sequence comparisons for nucleic acid or peptidic sequence homology were determined with PC Gene software (Intelligenetics, Mountain View, Calif.).

Phylogenetic comparisons. Phylogenetic trees were constructed based on *rpoB* sequences and compared with the phylogenetic tree based on 16S rDNA sequence data. The nucleotide sequences were aligned using the ClustalW program (42) supported in the Bisanse workstation (9). Phylogenetic trees were inferred with the PHYLIP software package (12) supported in the Bisanse workstation. Evolutionary matrices, generated by DNADIST, were constructed by the method of Kimura (18). The matrices were used to infer dendrograms using the neighbor-joining method (38). Bootstrap values were obtained for a consensus based on 100 randomly generated trees using Seqboot and Consense in the PHYLIP package. Tree figures were generated with the Tree View program, version 1.61 (32). Sequence homology comparisons were made with the GAP program of the Genetics Computer Group (Madison, Wis.) package, again supported in the Bisanse workstation.

rpoB sequence-based identification blind testing. The *rpoB*-based system we developed to identify staphylococci was first applied to a collection of clinical isolates in order to assess intraspecific sequence variation. This collection comprised five *S. aureus* isolates, five *S. epidermidis* isolates, three *S. saprophyticus* isolates, and three *S. haemolyticus* isolates. Blind tests were then performed on a collection of 20 bacterial isolates, including rifampin-susceptible *S. aureus*, rifampin-resistant *S. aureus*, *S. epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *S. intermedius*, *Staphylococcus equorum*, *S. schleiferi*, *S. lugdunensis*, *Staphylococcus gallinarum*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Corynebacterium amycolatum*, *Gemella morbillorum*, *Acinetobacter anitratus*, *Micrococcus luteus*, and *Propionibacterium acnes*. After the isolates were coded, extraction of bacterial DNA and PCR incorporating primer pair 2491F-3241R were performed as described above. The presence of 751-bp amplicons was revealed by 1% agarose gel electrophoresis, and amplicons were sequenced with sequencing primers 2643F and 3241F as described above. Sequences were aligned by using FASTA with the *Staphylococcus rpoB* database in Infobiogen (http://www.infobiogen.fr/services/analyse/cgi-bin/fasta_in.pl), and identification was assessed by >97% similarity with one of the database sequences.

Nucleotide sequence accession numbers. The GenBank accession numbers of the staphylococcal *rpoB* sequences determined in this study are AF325870 for the *S. lugdunensis* complete *rpoB* sequence, AF325869 for the *S. intermedius* complete *rpoB* sequence, AF325871 for the *S. saccharolyticus* complete *rpoB* sequence, AF325868 for the *S. caprae* complete *rpoB* sequence, AF325875 for the *S. hominis* partial *rpoB* sequence, AF325876 for the *Staphylococcus hyicus* subsp. *hyicus* partial *rpoB* sequence, AF325880 for the *Staphylococcus carnosus* partial *rpoB* sequence, AF325881 for the *Staphylococcus sciuri* partial *rpoB* sequence, AF325885 for the *Staphylococcus capitis* partial *rpoB* sequence, AF325890 for the *S. gallinarum* partial *rpoB* sequence, AF325894 for the *S. aureus* subsp. *anaerobius* partial *rpoB* sequence, X64172 for the *Staphylococcus aureus* subsp. *aureus* partial *rpoB* sequence, AF325872 for the *S. epidermidis* partial *rpoB* sequence, AF325888 for the *S. haemolyticus* partial *rpoB* sequence, AF325886 for the *Staphylococcus schleiferi* subsp. *schleiferi* partial *rpoB* sequence, AF325883 for the *Staphylococcus xylosum* partial *rpoB* sequence, AF325885 for the *Staphylococcus capitis* subsp. *capitis* partial *rpoB* sequence, AF325874 for the *S. arlettae* partial *rpoB* sequence, AF325887 for the *S. warneri* partial *rpoB* sequence, AF325877 for the *Staphylococcus simulans* partial *rpoB* sequence, AF036973 for the *Staphylococcus lentus* partial *rpoB* sequence, AF325873 for the *S. saprophyticus* partial *rpoB* sequence, AF325882 for the *S. equorum* partial *rpoB* sequence, AF325893 for the *Staphylococcus cohnii* subsp. *cohnii* partial *rpoB* sequence, AF325889 for the *Staphylococcus auricularis* partial *rpoB* sequence, AF325880 for the *Staphylococcus carnosus* subsp. *carnosus* partial *rpoB* sequence, AF325891 for the *Staphylococcus kloosii* partial *rpoB* sequence, AF325892 for the *Staphylococcus chromogenes* partial *rpoB* sequence, AF325879 for the *Staphylococcus pulvereri* partial *rpoB* sequence, AF325878 for the *Staphylococcus felis* partial *rpoB* sequence, and AF325884 for the *Staphylococcus muscae* partial *rpoB* sequence.

RESULTS

Determination of *rpoB* sequences in *Staphylococcus* species. Consensus primers 1302F and 2309R enabled the amplification of a 1,107-bp *rpoB* fragment and consensus primers 3843F

and 5041R enabled the amplification of a 1,198-bp *rpoB* fragment in *S. intermedius*. Alignment of these two fragment sequences along with that of *S. aureus rpoB* enabled us to design the additional primers 2245F, 4139F and 4139R, 4508F and 4502R, and 4494F and 1751R, which allowed us to determine the complete sequence of *rpoB* of *S. intermedius*. The complete sequence for *S. caprae* was obtained with the same primers, whereas the additional primers 2924F, 2924R, 3200F, and 3050R were required for *S. lugdunensis* and the additional primers 5000R, 2915R, and 2663F were required for *S. saccharolyticus*. The *rpoB* sequence varied in length, being 3,845 bp in *S. intermedius*, 3,461 bp in *S. caprae*, 3,452 bp in *S. lugdunensis*, and 3,461 bp in *S. saccharolyticus*. The GC contents were 39.2, 37.4, 38.4, and 36.8% in the above species, respectively. Based on the alignment of these four sequences with that of *S. aureus rpoB* in GenBank, the most variable region flanked by conserved regions was chosen as a target for PCR-sequencing-based molecular identification of staphylococci. Consensus PCR primers 2491F and 3241R were designed for the amplification of the region, and one additional primer, 2643F, was designed for sequencing. An *rpoB* partial sequence database was done for an additional 27 *Staphylococcus* species under investigation.

Phylogenetic trees. The *rpoB* sequence similarity matrix tabulated after ClustalW alignment of 29 staphylococcal *rpoB* partial sequences showed interspecific similarity values of 71.6 to 93.6%, the most closely related sequences being those of *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius*. The phylogenetic tree (Fig. 1) inferred from neighbor joining showed nine clusters which were characterized by a sequence similarity level of >85% within the cluster. The nine clusters were the *S. aureus* group, comprising *S. aureus* subsp. *aureus*, *S. aureus* subsp. *anaerobius*, and *S. cohnii*; the *S. saprophyticus* group, comprising *S. saprophyticus*, *S. xylosum*, *S. kloosii*, *S. equorum*, *S. gallinarum*, and *S. arlettae*; the *S. haemolyticus* group, comprising *S. haemolyticus*, *S. capitis*, and *S. caprae*; the *S. sciuri* group, comprising *S. sciuri*, *S. pulvereri*, and *S. lentus*; the *S. epidermidis* group, comprising *S. epidermidis*, *S. saccharolyticus*, and *S. lugdunensis*; the *S. simulans* group, comprising *S. simulans* and *S. carnosus*; and the *S. hyicus* group, comprising *S. hyicus*, *S. schleiferi*, and *S. chromogenes*. Bootstrap values of >70% were, however, noted only for five nodes, namely, the *S. aureus* group, the *S. hyicus* group, the *S. intermedius* group, the *S. simulans* group and the *S. sciuri* group.

Results of blind identification testing. A 751-bp amplicon was obtained in 16 of 16 *Staphylococcus* sp. isolates previously identified at the species level and belonging to four different species. In this collection, intraspecific sequence divergence was 0.5% among five *S. aureus* isolates, 0.8% among five *S. epidermidis* isolates, and 0.08% among three *S. saprophyticus* isolates as well as among three *S. haemolyticus* isolates. Sequence analysis assigned every one of these 16 isolates to the correct species. A 751-bp amplicon was further obtained for 10 of 20 bacterial isolates tested after PCR with the primer pair 2491F-3241R. The sequences of these 10 amplicons had >99% homology with our reference staphylococcal sequences, and an exact identification was obtained for all 10. None of the other species used in the trial were amplified in the PCRs.

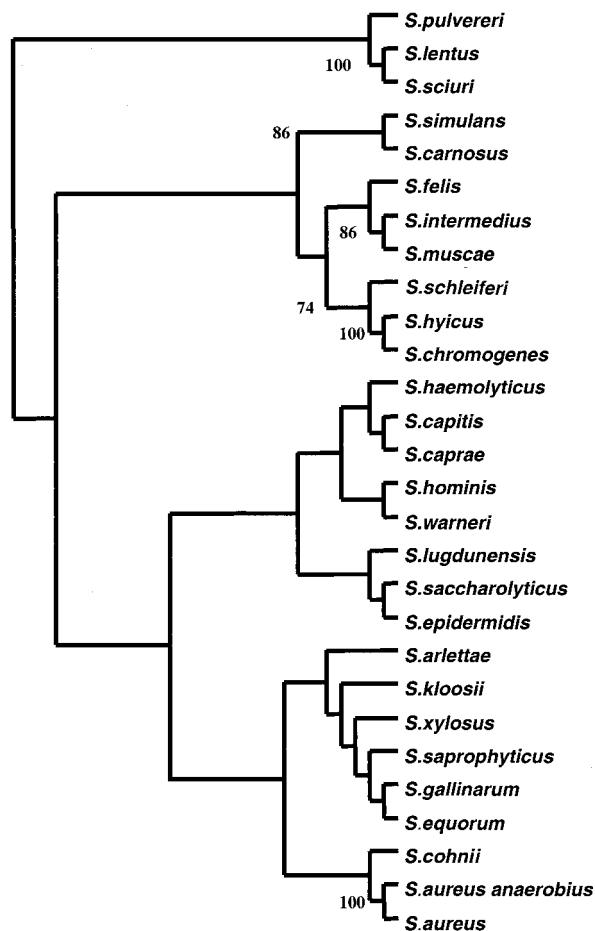


FIG. 1. Unrooted neighbor-joining tree based on partial *rpoB* gene sequences showing the phylogenetic relationships among 29 species of the genus *Staphylococcus*. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis; only bootstraps values of >70% are indicated. The scale bar indicates the evolutionary distance between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms (0.01 substitution/site).

DISCUSSION

The data we present show that PCR amplification of a 751-bp *rpoB* gene fragment using primer pair 2491F-3241R followed by sequence analysis is a suitable molecular approach for the identification of *Staphylococcus* isolates at the species level. Moreover, these primer pairs were shown to be specific for *Staphylococcus*, since no amplification products were obtained from species in 10 other bacterial genera.

To date, the primary method for the molecular identification of *Staphylococcus* species has been the analysis of genomic DNA restriction fragment length patterns on Southern blots probed with labeled rRNA genes (2, 6–8, 43, 45). PCR and sequencing of rRNA genes, however, have been found to have limited discriminating power for *Staphylococcus* species, since the 16S rDNA sequence similarity has been shown to be very high, 90 to 99%, in 29 *Staphylococcus* species (26). By contrast,

the sequence similarity of the *hsp60* gene was 74 to 93% in 23 *Staphylococcus* species (26), and for the partial *rpoB* gene sequence in our study the similarity was even less, 71.6 to 93.6% in 29 *Staphylococcus* species. The *rpoB* gene, then, clearly has the most discriminative power, and while *S. caprae* and *S. capitis* cannot be distinguished by their 16S rDNA gene sequences (41), the partial *rpoB* sequences of these two species have only 84.5% homology. Similarly, some *Staphylococcus* taxa have the same 16S rDNA gene sequences in variable regions V1, V3, V7, and V9 (34) with identical sequences occurring in *S. pulvereri* and *Staphylococcus vitulinus*; in *S. saccharolyticus*, *S. capitis* subsp. *urealyticus*, and *S. caprae*; in the two subspecies of *S. aureus*; and in the two subspecies of *S. cohnii* (41). The *rpoB* partial sequence similarity, however, was only 87.3% between the two subspecies of *S. aureus* and 84.5 to 89% in the *S. saccharolyticus*, *S. capitis*, and *S. caprae* group. Also, we determined that intraspecific sequence variation was 0.08 to 0.5%, further indicating that this *rpoB* fragment was an efficient tool for species identification.

The gene encoding chaperonin 60 has also been proposed as a suitable target for the molecular identification of *Staphylococcus* species (14, 15). A unique pair of degenerate PCR primers which amplify a 600-bp region of *hsp60* have been described, and the sequences of this region have been reported for *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus*, *S. schleiferi* (14), and *S. intermedius*, *S. delphini*, and four *S. hycicus* strains (15). It should be noted, however, that the cloned partial HSP60 gene DNA sequences of nine isolates of *S. aureus* showed a mean variability of 2% (40). Also, cross-hybridization occurred between cloned partial HSP60 gene DNAs from the two *S. schleiferi* subspecies and between the DNAs from *S. intermedius* and *S. delphini* (15). Also, the DNA from isolates identified as *S. hycicus* failed to hybridize. Other suitable targets for the molecular identification of *Staphylococcus* species that have been proposed include the *femA* gene, which was used in a multiplex PCR-reverse hybridization approach to identify 55 clinical isolates (44). These, however, included only five *Staphylococcus* species, namely *S. aureus*, *S. epidermidis*, *S. hominis*, *S. saprophyticus*, and *S. simulans*. Discrepant results were found in the identification of *S. aureus* using tRNA gene intergenic spacer length polymorphism (27), but restriction fragment length polymorphism analysis of the PCR-amplified 16S-23S rDNA intergenic spacer proved to be effective in the identification of a collection of 221 isolates from 31 *Staphylococcus* species (30). Some intraspecific heterogeneity was shown for *Staphylococcus saprophyticus* subsp. *saprophyticus* and for *S. aureus*, with five fragment length patterns being observed in eight isolates. Also, PCR-RFLP analysis of the *gla* gene has been carried out, but only with 12 species of *Staphylococcus*, with some degree of intraspecific heterogeneity being observed in three species (47). Finally, molecular identification methods for the identification of one or only a few *Staphylococcus* species have been reported for *S. saprophyticus* (28), *S. aureus* (1), and *S. epidermidis* (29, 46). In our study, we used PCR sequencing of the *rpoB* gene to effectively identify staphylococcal species. In contrast to the probe hybridization technique and the RFLP approach, sequencing enables any isolate to be characterized, including new species by their phylogenetic relationships.

The phylogenetic relationships among *Staphylococcus* spe-

cies we derived in our study of *rpoB* sequences were limited, as some species and subspecies (11) were not available for study and only partial *rpoB* gene sequences were analyzed. The *rpoB* sequence-based relationships we found, however, were in accordance with those published previously. Earlier studies on the taxonomy of *Staphylococcus* species based on DNA-DNA reassociation indicated that in the genus there were nine distinct species groups, represented by *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. intermedius*, *S. hyicus*, *S. sciuri*, *S. auricularis*, *S. aureus*, and *Staphylococcus caseolyticus* (23, 24). The same groups were identified in a study using *hsp60* gene sequence analysis (26). The 16S rDNA sequence-derived trees (40, 41) gave different results, however, with 12 genogroups being identified. In fact, it has been shown that *S. caseolyticus* should be reclassified as *Macrococcus caseolyticus* (20), thus limiting the number of 16S rDNA gene sequence-derived genogroups.

In our *rpoB*-derived data, we found that *S. lugdunensis* is related to *S. epidermidis*, as was also found by DNA hybridization. The position of the organism should be regarded as uncertain, however, as low bootstrap values ($\leq 50\%$) were obtained in studies of the bacteria using 16S rDNA (41) and *hsp60* (20) sequence data. The *rpoB*-derived data indicating that *S. cohnii* is outside the *S. saprophyticus* group and closely related to *S. aureus* are in disagreement with previously published data and may be incorrect. Similarly, the positions of *S. caprae* and *S. capitis* within the *S. haemolyticus* group and outside the *S. epidermidis* group appear to be incorrect. Complete *rpoB* sequence analysis of these species may be helpful in resolving these questions.

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