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Genetic Classification and Distinguishing of *Staphylococcus* Species Based on Different Partial *gap*, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* Gene Sequences▽

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The analysis of 16S rRNA gene sequences has been the technique generally used to study the evolution and taxonomy of staphylococci. However, the results of this method do not correspond to the results of polyphasic taxonomy, and the related species cannot always be distinguished from each other. Thus, new phylogenetic markers for *Staphylococcus* spp. are needed. We partially sequenced the *gap* gene (~931 bp), which encodes the glyceraldehyde-3-phosphate dehydrogenase, for 27 *Staphylococcus* species. The partial sequences had 24.3 to 96% interspecies homology and were useful in the identification of staphylococcal species (F. Layer, B. Ghebremedhin, W. König, and B. König, J. Microbiol. Methods 70:542–549, 2007). The DNA sequence similarities of the partial staphylococcal *gap* sequences were found to be lower than those of 16S rRNA (~97%), *rpoB* (~86%), *hsp60* (~82%), and *sodA* (~78%). Phylogenetically derived trees revealed four statistically supported groups: *S. hyicus*/*S. intermedius*, *S. sciuri*, *S. haemolyticus*/*S. simulans*, and *S. aureus*/*epidermidis*. The branching of *S. auricularis*, *S. cohnii* subsp. *cohnii*, and the heterogeneous *S. saprophyticus* group, comprising *S. saprophyticus* subsp. *saprophyticus* and *S. equorum* subsp. *equorum*, was not reliable. Thus, the phylogenetic analysis based on the *gap* gene sequences revealed similarities between the dendrograms based on other gene sequences (e.g., the *S. hyicus*/*S. intermedius* and *S. sciuri* groups) as well as differences, e.g., the grouping of *S. arlettae* and *S. kloosii* in the *gap*-based tree. From our results, we propose the partial sequencing of the *gap* gene as an alternative molecular tool for the taxonomical analysis of *Staphylococcus* species and for decreasing the possibility of misidentification.

The genus *Staphylococcus* comprises 42 validly described species and subspecies of gram-positive, catalase-positive cocci (1, 21, 30), 10 of which contain subdivisions with subspecies designations (6, 10, 27, 30). Staphylococci, including *S. aureus*, generally are opportunistic pathogens or commensals on host skin. However, they may act as pathogens if they gain entry into the host tissue through a trauma to the cutaneous barrier, inoculation by needles, the implantation of medical devices, or in cases in which the microbial community is disturbed or in immunocompromised individuals (17–19). Thus, the accurate species identification of *S. aureus* as well as that of the other staphylococcal species in microbial communities is highly desirable to permit a more precise determination of the host-pathogen relationships of staphylococci (13, 15). The precise identification of these bacteria to the species level is quite laborious. Various molecular DNA-based methods for the identification of *Staphylococcus* species have been developed. These methods typically require the use of several species-specific PCR primers, hybridization probes, or multiple restriction enzymes and usually are not designed to differentiate all known species simultaneously. 16S rRNA gene sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis have been described for *Staphylococcus* species identification (2–4), but these methods do not differentiate between *Staphylococcus lentus* and *Staphylococcus sciuri*. PCR-RFLP analysis of the 23S rRNA gene with two restriction enzymes is able to discriminate between *Staphylococcus* species (23), but the interpretation of the results is complicated by intervening sequences (9). More recently, amplified fragment length polymorphism fingerprinting has proven to be useful for *Staphylococcus* species identification, but the method is time-consuming and expensive (34). Whole-genome DNA-DNA hybridization analysis (31) allows species identification, but the method is not suitable for routine use.

The use of nucleic acid targets, with their high sensitivity and specificity, provides an alternative technique for the accurate identification and classification of *Staphylococcus* species. Besides the 16S rRNA gene (2–4), the 16S-23S rRNA intergenic spacer region (23), and the heat shock protein 60 (*hsp60*) gene (11, 12), other gene sequences have been used in genetic studies: the *femA* gene (35), the *sodA* gene (28), the *tuf* gene (24), the *rpoB* gene (5, 26), and the *gap* gene (36, 37).

In our study, we assessed the usefulness of the ~931-bp partial sequence of *gap* for the studied staphylococci ($n = 27$) in species differentiation and for interfering interspecies phylogenetic relationships. These are among the most commonly occurring species of greater clinical significance and are preferentially novobiocin-sensitive staphylococci: e.g., *S. aureus*, *S. epidermidis*, *S. warneri*, *S. haemolyticus*, and *S. lugdunensis*. The other 15 species that were not subjects of this study are rarely associated with infections in humans; e.g., *S. pasteuri*, *S. vitulinus*, and *S. saccharolyticus*. The *gap* gene encodes a 42-kDa transferrin-binding protein (Tpn) located within the cell wall of

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TABLE 1. Primers used for sequencing the *gap* gene

Primer	Sequence (5′→3′)	Position[a]
Gap1-for[b]	ATGGTTTTGGTAGAATTGGTCGTTTA	22–47
Gap3-rev[c]	G(ACT)TTT(AGCT)A(CT)TTCTT(AGT)(AT)CC(AG)TT(ACT)AC(AGT)C	220–196
Gap4-for[c]	GA(CT)GT(AGCT)GT(AGCT)(CT)T(AT)GAATGTAC(AT)GG	270–292
Gap5-rev[c]	GTT(AT)GT(AT)GTACA(AGT)GA(ACT)GCACC(AT)G	462–440
Gap6-for[c]	GAAGG(ACT)(CT)T(ACT)ATGAC(AGT)AC(AT)AT(CT)CA(CT)G	511–535
Gap7-rev[c]	GAACC(AT)GT(AT)GC(AT)AC(AT)GG(ACT)ACACGTTG	723–698
Gap8-for[c]	GAA(ACT)CATT(CT)GGTTACA(AC)(ACT)GA(AT)GA(CT)G	809–834
Gap2-rev[b]	GACATTTCGTTATCATACCAAGCTG	956–932

[a] Position relative to the *S. aureus gap* sequence.
[b] Primer sequences from Yugueros et al. (36).
[c] Designed primer sequences from this study.

the staphylococci. Tpn is a member of the newly emerging family of multifunctional cell wall-associated glyceraldehyde-3-phosphate dehydrogenases, which is well known for its glycolytic function of converting D-glyceraldeyde-3-phosphate to 1,3-bisphosphoglycerate. *gap* commonly has been considered a constitutive housekeeping gene (8, 25).

Yugueros and coworkers published the sequences of the *gap* genes of 12 staphylococcal species relevant for humans (36). We extended these studies and sequenced the ~931-bp sequence encoding a partial region of the *gap* gene from a total of 27 different staphylococcal species (22). We consider these species to be among the clinically significant species, as do other groups (2–5, 12, 28).

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the staphylococcal strains were grown on blood agar and incubated at 37°C for 18 to 24 h. Reference strains were selected from the German Collection of Microorganisms and Cell Cultures (DSMZ), the Czech Collection of Microorganisms (CCM), and the American Type Culture Collection (ATCC), and they included the following: *Staphylococcus arlettae* DSM 20672^T, *S. aureus* ATCC 29213^T, *S. carnosus* subsp. *carnosus* DSM 20501^T, *S. cohnii* subsp. *cohnii* DSM 20260^T, *S. delphini* DSM 20771^T, *S. epidermidis* DSM 20044^T (CCM 2124^T), *S. equorum* subsp. *equorum* DSM 20674^T, *S. hyicus* DSM 20459^T, *S. intermedius* DSM 20373^T (CCM 5739^T), *S. kloosii* DSM20676^T, *S. lugdunensis* DSM 4804^T (ATCC 43809^T), *S. warneri* DSM 20316 (CCM 2730^T), *S. capitis* subsp. *capitis* CCM 2734^T, *S. caprae* CCM 3573^T, *S. chromogenes* CCM 3387^T, *S. gallinarum* CCM 3572^T, *S. haemolyticus* CCM 1798^T, *S. hominis* subsp. *hominis* CCM 2732^T, *S. lentus* CCM 3472^T, *S. muscae* CCM 4175^T, *S. saprophyticus* subsp. *saprophyticus* CCM 883^T, *S. sciuri* CCM 3473^T, *S. simulans* CCM 2705^T, *S. xylosus* CCM 2725^T, *S. auricularis* ATCC 33753^T, *S. felis* ATCC 49168^T, and *S. schleiferi* subsp. *schleiferi* ATCC 43808^T.

Isolation of genomic DNA. Chromosomal DNA was isolated from overnight cultures grown on blood agar at 37°C. Genomic DNA was extracted by using the Qiagen DNA extraction kit according to the manufacturer's suggestions (Hilden, Germany), with the modification that 20 μl of lysostaphin (1 mg/ml; Sigma) and 20 μl lysozyme (100 mg/ml; Qiagen) were added at the cell lysis step. The concentration of the DNA was assessed spectrophotometrically.

DNA sequencing. Consensus *gap* PCR primers (Table 1) were used as previously described (22). Gap1-for and Gap2-rev were used to amplify the ~931-bp fragment as described before (37), and the PCR products were purified with the Qiagen gel extraction kit (Hilden, Germany). Partial reverse and forward sequencing of the ~931-bp fragment was obtained by using the consensus primers at 3.25 pmol (Table 1). Sequencing reactions were carried out with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions and using the previously described sequencing protocols (22). The results were processed into sequence data with sequence analysis software (Applied Biosystems), and partial sequences were combined into a single consensus sequence with assembler software (Applied Biosystems) (22). The gene sequences other than that of *gap* were obtained from GenBank (Table 2).

Phylogenetic analysis. Sequences were aligned manually in Sequencher 3.0 (Gene Codes Corporation) to edit the sequences, if necessary, and to note which regions were to be excluded for the phylogenetic analysis. Multiple-sequence alignments and topology predictions were done with DNASISMAX, version 2.0.5 (2003) (Hitachi Software Engineering, Japan). Phylogenetic trees were generated with the neighbor-joining algorithm by using DNASISMAX. All trees were resampled with 1,000 bootstrap replications to ensure the robustness of the data (7). The phylogenetic analyses were displayed with the TreeView drawtree program, version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The DNA sequence similarity analysis was performed with BioEdit, version 7.0.1 (14).

RESULTS

PCR, sequencing of the *gap* gene, and sequence similarity for the staphylococcal species. The amplification of the partial *gap* gene for all of the *Staphylococcus* species yielded a single product of nearly 931 bp. The GenBank accession numbers are DQ321674 to DQ321700.

The sequence similarity of the *gap* sequences ranged from 24.3 to 96% (Table 3). The species *S. lentus* and *S. sciuri* revealed a sequence similarity of 93% according to the *gap* gene sequences, whereas the other gene-based similarities ranged between 88 and 98% (88% for *hsp60*, 88.9% for *sodA*, and 98% for 16S rRNA). The species *S. capitis* and *S. caprae* revealed a sequence similarity of 27% according to the *gap* gene sequences, whereas the other gene-based similarities ranged between 94 and 99% (94% for *sodA*, 95% for 16S rRNA, and 99% for *hsp60*). The species *S. carnosus* and *S. simulans* revealed a sequence similarity of 95% according to the *gap* gene sequences, whereas the other gene-based similarities ranged between 86 and 98% (87 to 88% for *rpoB* and 96% for 16S rRNA).

The species *S. chromogenes* and *S. hyicus* revealed a sequence similarity of 91% according to the *gap* gene sequences, whereas the other gene-based similarities ranged between 77.6 and 98% (77.6% for *sodA* and 98% for 16S rRNA).

***Staphylococcus* phylogeny derived from *gap* sequences.** The *Staphylococcus* species were divided into three clades (Fig. 1) with significant bootstrap values (>90%); the first contained the *S. hyicus*/*S. intermedius* group, comprising *S. hyicus*, *S. chromogenes*, *S. delphini*, and *S. intermedius*. The second clade contained two groups, the *S. sciuri* group, comprising *S. sciuri* and *S. lentus*, and the *S. haemolyticus*/*S. simulans* group, comprising *S. haemolyticus*, *S. xylosus*, *S. muscae*, *S. simulans*, *S. schleiferi* subsp. *schleiferi*, *S. carnosus* subsp. *carnosus*, *S. caprae*, and *S. felis.* The third clade contained only one group, the *S. aureus*/*S. epidermidis* group, comprising *S. aureus*, *S. hominis* subsp. *hominis*, *S. warneri*, *S. epidermidis*, *S. capitis* subsp. *capitis*, and *S. lugdunensis.* The branching of *S. auricularis*, *S. cohnii*, and the heterogeneous *S. saprophyticus* group, comprising *S. saprophyticus* subsp. *saprophyticus*, *S. equorum* subsp. *equorum*, *S. gallinarum*, *S. arlettae*, and *S. kloosii*, was not reliable (bootstrap values of 75, 75, and 29%, respectively).

For the *gap* gene comparison between *S. sciuri* and *S. lentus*, a sequence similarity value of 82% (Table 3) was determined, as the position of these species in the phylogenetic tree is supported by a bootstrap value of 100% (see below).

TABLE 2. Partial gene sequences from GenBank used for phylogenetic tree

Staphylococcus strain	GenBank sequence for:				
	16S rRNA	*hsp60*	*rpoB*	*sodA*	*tuf*
S. arlettae	AY688029	AF053580	AF325874	AJ343894	
S. aureus	AY688034	AF060191	AF325894	AY485191	AF274739
S. auricularis	AY688030	AF242278	AF325889	AJ343937	AF298797
S. capitis subsp. *capitis*	AY688039	AF036322	AF325885	AJ343940	AF298798
S. caprae	AY688036	AF053574	AF325896	AJ343898	
S. carnosus subsp. *carnosus*	AY688041	AF242279	AF325880	AJ343899	
S. chromogenes	AY688044	AF242280	AF325892	AJ343945	
S. cohnii subsp. *cohnii*	AY688046	AF053582	AF325893	AJ343902	
S. delphini	AY688050	AF019774		AJ343905	
S. epidermidis	AY688053	AF029245	AF325872	AJ343906	AF298800
S. equorum subsp. *equorum*	AY688054	AF242281	AF325882	AY878697	
S. felis	AY688057	AF242282	AF325878	AJ343908	
S. gallinarum	AY688059	AF053579	AF325890	AJ343909	
S. haemolyticus	AY688062	U92809	AF325888	AJ343910	AF298801
S. hominis subsp. *hominis*	AY688064	AF053572	AF325875	AJ343911	
S. hyicus	AY688066	AF019778	AF325876	AJ343913	
S. intermedius	AY688070	AY123723	AF325869	AJ343914	
S. kloosii	AY688072	AF053575	AF325891	AJ343915	
S. lentus	AY688073	AF053586	AY036973	AY485195	
S. lugdunensis	AY688076	AF053570	AF325870	AJ343917	AF298803
S. muscae	AY688079	AF242285	AF325884	AJ343919	
S. saprophyticus subsp. *saprophyticus*	AY688089	AF053578	AF325873	AJ343954	AF298804
S. schleiferi subsp. *schleiferi*	AY688093	AF053585	AF325886	AJ343955	
S. sciuri	AB212276	AY820255	AY820256	AY820257	AY763434
S. simulans	AY688101	AF053584	AF325877	AJ343956	AF298805
S. warneri	AY688106	AF053569	AF325887	AJ343958	AF298806
S. xylosus	AY688107	AF053573	AF325883	AJ343959	AY763438

Comparative phylogeny based on various gene sequence-derived trees. The *gap*-derived sequence similarity analysis for the staphylococcal species is given in Table 3. According to the staphylococcal gene sequences of *gap*, *rpoB*, and *sodA*, the species *S. felis* and *S. muscae* grouped within the same cluster, whereas in the 16S rRNA- and *hsp60*-derived trees these two species did not show a close relationship.

The species *S. hyicus* and *S. chromogenes* clustered together according to the 16S rRNA, *hsp60*, and *rpoB* sequence analysis, but the clustering by *sodA* analysis was less close. The grouping of *S. hyicus* and *S. chromogenes* was more affirmed by the *gap* gene than by the other genes as well.

The grouping of *S. arlettae* and *S. kloosii* in the *gap*-based tree (with bootstrap values of 98%) was fairly supported by *hsp60*-, *rpoB*-, *sodA*-, and 16S rRNA-derived trees (bootstrap values of <43%).

S. hominis subsp. *hominis* and *S. lugdunensis* clustered into the same group according to *gap* (bootstrap value of 95%), *tuf* (bootstrap value of 44%), and *hsp60* (bootstrap value of 29%) analyses, whereas this was not observed for 16S rRNA, *rpoB*, and *sodA* analyses.

The grouping of *S. delphini* and *S. intermedius* was supported by *gap*, 16S rRNA, *hsp60*, and *sodA* analyses. The phylogenetic relationship of *S. chromogenes* and *S. hyicus* in the *gap*- and *rpoB*-derived trees (for each bootstrap value of 100%) was supported by low bootstrap values in *hsp60*-, 16S rRNA-, and *sodA*-derived trees. The close relationship of *S. carnosus* subsp. *carnosus* and *S. simulans* in *gap* (bootstrap value of 100%), *hsp60*, *sodA* (for each bootstrap value of 98%), and *rpoB* (bootstrap value of 79%) analyses was confirmed as well. *S. delphini* and *S. intermedius* clustered together according to *gap*, 16S rRNA, *sodA*, and *hsp60* analyses, with bootstrap values of 100% each.

DISCUSSION

Several molecular targets have been exploited for the molecular identification of *Staphylococcus* species. Because a large amount of *rrs* sequence data is available in a public database, it is not surprising that the 16S rRNA gene has been an obvious choice. Gene sequence-based identification of bacteria at the species level may require resolving the whole gene, yet in some cases, phylogenetically closely related bacterial species cannot be differentiated from each other. Although the comparison of the 16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in studies at the species level. In this regard, the 16S rRNA sequence similarity has been shown to be very high, 90 to 99%, in 29 *Staphylococcus* species (20). *S. caprae* and *S. capitis* cannot be distinguished by their 16S rRNA gene sequences (34). Similarly, some *Staphylococcus* taxa have the same 16S rRNA gene sequences in variable regions V1, V3, V7, and V9, with identical sequences occurring in, e.g., *S. vitulinus*, *S. saccharolyticus*, *S. capitis* subsp. *urealyticus*, *S. caprae*, the two subspecies of *S. aureus*, and the two subspecies of *S. cohnii* (34). Other gene sequences have been used empirically in attempts to classify the *Staphylococcus* species, namely, the *hsp60* gene, the *sodA* gene, the *rpoB* gene, and the *tuf* gene. With regard to the *hsp60* gene, it should be noted that the cloned partial *hsp60* gene DNA sequences of nine isolates

TABLE 3. DNA sequence identity matrix based on comparisons of the *gap* gene sequences of the *Staphylococcus* species

Taxon (*gap* gene no.)	% Identity with *gap* gene no.[a]:																										
	1	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
S. arlettae (1)	ID																										
S. aureus (2)	75	ID																									
S. auricularis (3)	85	74	ID																								
S. capitis (4)	75	90	75	ID																							
S. caprae (5)	24	27	24	27	ID																						
S. carnosus (6)	29	27	27	27	45	ID																					
S. chromogenes (7)	84	74	83	72	24	29	ID																				
S. cohnii (8)	88	75	87	75	26	28	83	ID																			
S. delphini (9)	85	74	84	72	25	29	88	85	ID																		
S. epidermidis (10)	74	89	75	91	27	27	72	76	74	ID																	
S. equorum (11)	90	74	86	74	25	28	84	89	84	74	ID																
S. felis (12)	47	46	47	46	25	27	47	48	48	47	46	ID															
S. gallinarum (13)	89	74	87	74	25	28	84	88	84	74	89	47	ID														
S. haemolyticus (14)	30	28	28	28	44	85	30	29	31	28	29	27	29	ID													
S. hominis (15)	86	75	83	75	25	28	85	85	84	75	85	46	85	29	ID												
S. hyicus (16)	84	74	84	72	24	27	91	84	88	72	84	48	84	29	83	ID											
S. intermedius (17)	86	74	84	73	25	29	88	85	96	74	84	49	84	31	84	87	ID										
S. kloosii (18)	92	75	86	75	25	28	84	89	85	74	89	48	90	30	85	85	85	ID									
S. lentus (19)	81	70	80	71	26	27	81	81	82	71	83	48	83	28	80	81	83	82	ID								
S. lugdunensis (20)	74	88	75	88	26	26	72	74	73	87	73	46	74	27	77	72	73	74	69	ID							
S. muscae (21)	29	27	28	27	44	83	30	29	30	27	28	27	28	85	28	28	30	29	28	26	ID						
S. saprophyticus (22)	89	74	86	73	24	28	85	90	84	73	91	47	90	29	85	84	84	89	82	72	29	ID					
S. schleiferi (23)	30	27	28	27	43	83	29	29	30	28	29	26	28	86	29	28	29	30	27	27	88	29	ID				
S. sciuri (24)	82	69	80	70	26	27	80	81	82	70	83	48	83	29	81	80	83	82	93	69	28	82	27	ID			
S. simulans (25)	29	27	27	27	44	95	29	28	29	27	28	28	28	86	28	27	29	28	28	26	84	28	83	28	ID		
S. warneri (26)	87	77	85	78	24	27	84	86	84	76	88	47	89	29	87	83	85	89	83	75	28	86	29	83	28	ID	
S. xylosus (27)	29	27	29	26	41	81	30	28	30	27	28	27	28	84	29	28	30	29	27	25	80	29	80	27	82	27	ID

[a] ID, identical.

of *S. aureus* showed a mean variability of only 2% (33). Also, cross-hybridization occurred in cloned partial *hsp*60 genes between the DNAs from *S. intermedius* and *S. delphini* (12). The *gap* sequences were less conserved compared to the above-mentioned genes (sequence similarities, 24 to 96%). Therefore, the *gap* gene is rather more discriminative, as shown for *S. caprae* and *S. capitis*, which were clearly distinguished from each other, in contrast to results from 16S rRNA gene analyses. For the *sodA* gene, a pairwise comparison of these sequences revealed a mean sequence similarity of 81.5%, which was lower than that calculated for the *rrs* sequences of staphylococci (98%). The *rpoB*, *hsp60*, and *tuf* partial sequences showed interspecific similarity values of 71.6 to 93.6, 74 to 93, and 86 to 97%, respectively.

The *gap*-based tree indicates the divergence of the selected staphylococcal species, which was well supported for most of the strains studied. We compared the *gap*-derived tree (Fig. 1) to those inferred from sequences of the 16S rRNA, *rpoB*, *sodA*, *hsp60*, and *tuf* genes of *Staphylococcus* species available from GenBank (Table 2). Earlier studies on the taxonomy of *Staphylococcus* species based on DNA-DNA reassociation indicated that in this genus there were eight distinct species groups, represented by *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. intermedius*, *S. hyicus*, *S. sciuri*, *S. auricularis*, and *S. aureus* (17, 18). The same groups were identified in a study using *hsp60* (20) and the *sodA* gene sequence analysis (28). The phylogenetic tree generated from *rpoB* sequences revealed nine clusters, including an additional *S. haemolyticus* group. The 16S rRNA sequence-derived trees with 38 taxa of the genus *Staphylococcus* identified 11 genogroups (*S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. carnosus*, *S. hyicus*/*S. intermedius*, *S. sciuri*, *S. auricularis*, *S. warneri*, *S. haemolyticus*, *S. lugdunensis*, and *S. aureus*) (32, 33). However, the bootstrap values for most of the nodes of the distinct clusters were low. With the *gap* sequences and a bootstrap value of >90%, the *Staphylococcus* species were divided into four well-supported clusters: the *S. sciuri* group, the *S. hyicus*/*S. intermedius* group, the *S. haemolyticus*/*S. simulans* group, and the *S. aureus*/*S. epidermidis* group.

In *hsp60*-, 16S rRNA-, *sodA*-, and *rpoB*-generated trees, the *S. sciuri* group was formed by the two members *S. sciuri* and *S. lentus* with bootstrap values of >97%. All of the species from the *S. sciuri* group, including *S. vitulinus* (not included in this study), differ from the other *Staphylococcus* species in several remarkable features. They are novobiocin resistant and oxidase positive, and they all share the same characteristic pattern of amino acid substitution in their *hsp60* proteins (12, 20). The close relationship between *S. sciuri* and *S. lentus* was reproduced in the results from our tree analysis based on *gap* sequences. Thus, members of the *S. sciuri* group form a constant cluster in *hsp60*, 16S rRNA, *sodA*, *rpoB*, and *gap* gene trees.

The *S. hyicus*/*intermedius* group, as defined by 16S rRNA sequence analysis and confirmed by *rpoB* and *hsp60* sequence analysis, includes *S. hyicus*, *S. chromogenes*, *S. muscae*, *S. intermedius*, *S. delphini*, *S. schleiferi* subsp. *schleiferi*, and *S. felis*. The *S. intermedius* group, as defined by *sodA* sequence analysis, consisted of *S. intermedius* and *S. delphini* (bootstrap value of 100%). *S. schleiferi* subsp. *schleiferi* and *S. felis* were not included in this cluster. Moreover, the related species *S. hyicus*, *S. muscae*, and *S. chromogenes* did not cluster to form an *S.*

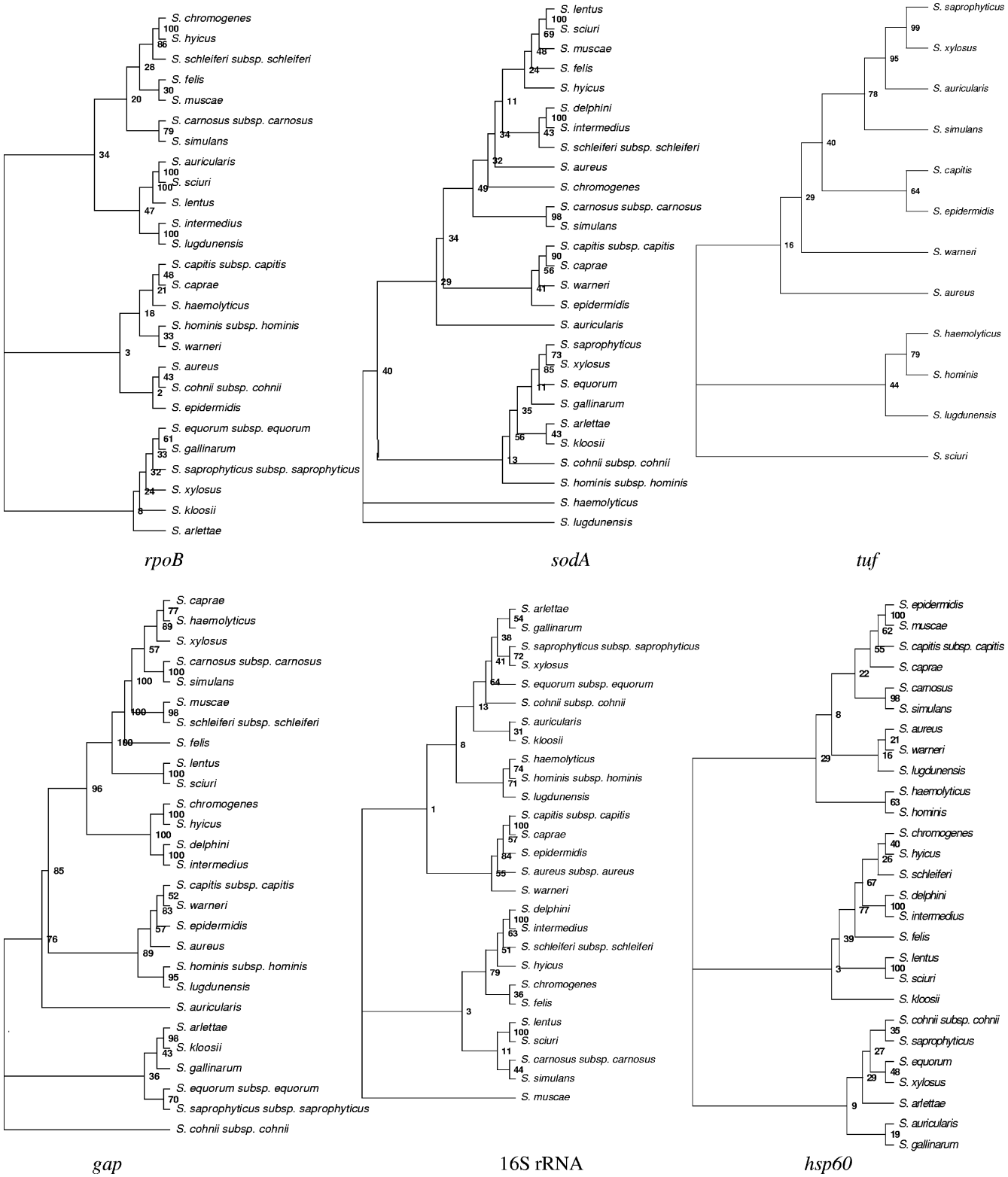

FIG. 1. Neighbor-joining tree based on the 931- to 933-bp *gap* sequences and 16S rRNA, *rpoB*, *sodA*, and *tuf* gene sequences showing the phylogenetic relationships among the staphylococcal species selected for this study. The value on each branch is the percent occurrence of the branching order in bootstrapped trees (7).

hyicus species subgroup. The *gap*-derived data support the idea that *S. chromogenes* and the non-*S. aureus* coagulase-positive staphylococci, such as *S. intermedius*, *S. delphini*, and *S. hyicus*, belong to the *S. hyicus*/*S. intermedius* species group. In our study, *S. schleiferi* subsp. *schleiferi* and *S. muscae* were outside of the *S. hyicus*/*S. intermedius* group as well. Based on the *gap*-derived data, *S. schleiferi* subsp. *schleiferi* and *S. muscae* could be grouped into the *S. haemolyticus*/*S. simulans* group. According to the 16S rRNA gene sequence, the phylogenetic classification of *S. felis* fell into the clade of *S. hyicus*/*S. intermedius*. However, based on DNA-DNA reassociation studies, *S. felis* was thought to be related to members of the *S. simulans* group (17, 18). In our *gap*-derived data, *S. felis* is outside of the *S. hyicus*/*S. intermedius* group and is closely related to members of the *S. simulans* group.

In contrast to the 16S rRNA sequence-based phylogenetic tree but in accordance with the *sodA*, *hsp60*, and *rpoB* gene sequences, we did not divide *S. simulans* and *S. carnosus* into two closely related groups by using the *gap* gene-derived sequences.

The *S. saprophyticus* group, as defined by 16S rRNA sequence analysis, includes the novobiocin-resistant and oxidase-negative species *S. saprophyticus* subsp. *saprophyticus*, *S. arlettae*, *S. kloosii*, *S. cohnii*, *S. gallinarum*, *S. equorum* subsp. *equorum*, and *S. xylosus*. The *rpoB*-derived data indicated that *S. cohnii* is outside of the *S. saprophyticus* group. From the *sodA*-derived data, one could conclude that the monophyly of this clade is uncertain, since it is associated with a bootstrap value of only 68%. In our study, *S. cohnii* and *S. xylosus* are clearly outside of the *S. saprophyticus* group. *S. cohnii* belongs to the *S. saprophyticus* group according to the 16S rRNA and *hsp60* trees. Based on *gap*-derived sequences, the branching of *S. auricularis*, *S. cohnii*, and the heterogeneous *S. saprophyticus* group, comprising *S. saprophyticus* subsp. *saprophyticus*, *S. equorum* subsp. *equorum*, *S. gallinarum*, *S. arlettae*, and *S. kloosii*, was not reliable (98, 70, 43, and 36%, respectively).

Based on the 16S rRNA data, the *S. epidermidis* species group was divided into five cluster groups, as described by Kloos (16): *S. lugdunensis*, *S. haemolyticus*, *S. warneri*, *S. epidermidis*, and *S. aureus* (33). The *S. epidermidis* cluster, composed of *S. epidermidis*, *S. capitis*, *S. caprae*, and *S. saccharolyticus* (not included in our study), constitutes a monophyletic clade supported by a high bootstrap value (97%) on the basis of 16S rRNA sequence analysis (20). In the *rpoB* study, *S. caprae* and *S. capitis* appeared to be in the *S. haemolyticus* group. Similarly to the *S. saprophyticus* group, the *S. epidermidis* group did not form a clearly distinct lineage in the *sodA*-based study (bootstrap value of 38.9%). Similar results were obtained in our study using *gap*-based sequences. Moreover, in our study, *S. caprae* showed no close relationship to *S. epidermidis* or *S. capitis*. On the other hand, the association of *S. warneri* with the *S. epidermidis* group was inferred from our data as well as from the *rpoB* tree analysis.

Obviously, the determination of the sequences of several genes is an important tool for pathogen identification and phylogenetic studies. Although each gene-derived tree will differ from the others and will have different levels of statistical support, it has been found that groupings obtained with two different sequences with bootstrap values of >90% are stable and reliable (29). The data we present show that the sequence analysis of a 931-bp *gap* gene fragment is a suitable molecular method for the identification of *Staphylococcus* isolates at the species level.

The *gap* gene sequence-based relationships of the staphylococcal species obtained were in accordance with phylogenetic trees published previously (37). In support of the *gap*-derived tree results, the *tuf* gene sequence-derived tree indicates that *S. warneri* is associated with the *S. epidermidis* group, which includes *S. capitis*. This is in agreement with results described earlier for sequencing assays targeting the *sodA* gene (28). The *tuf* gene-derived data often showed more intraspecies sequence divergence than the 16S rRNA-derived data. Apparently, the 16S rRNA gene is more highly conserved than the *tuf* gene. A pairwise comparison of the *tuf* gene sequences revealed that their mean identity (92.6%) is lower than the mean identity (95.9%) of 16S rRNA gene sequences. These results indicate that the *tuf* gene constitutes a more discriminatory target gene than the 16S rRNA gene to differentiate closely related *Staphylococcus* species.

The phylogenetic analysis of the staphylococcal *gap* sequences yields an evolutionary tree having a topology similar to that of the tree constructed with the 16S rRNA sequences, although minor differences were observed (Fig. 1).

We have determined the *gap* sequences of 27 *Staphylococcus* type strains and demonstrated the usefulness of the *gap* GenBank database for distinguishing the staphylococcal species and giving an approach for interpreting the phylogenetic relationship of the staphylococci. This method consists of a PCR carried out with a single pair of degenerate oligonucleotides for the amplification of a staphylococcal partial *gap* gene sequence and the direct sequencing of the amplicon. The sequencing also can be performed with the two respective PCR primers instead of the eight primers, as was done in this study, since the sequencer is adjusted for sequence analysis. In our case, the sequencer also was used for terminal RFLP analysis, so that we had troubles with the sequencing of fragments of >800 bp to obtain a confident sequence properly. The methodology might be useful in reference laboratories for the characterization of strains that could not be assigned to a species on the basis of their conventional phenotypic reaction and can stand on its own more effectively than 16S rRNA analysis, as this is a highly conserved gene and has limited discriminatory power compared to that of the *gap* gene, especially in closely related staphylococcal species. Shortening the region of interest within the *gap* gene sequence was not possible due to the scattering of the divergent regions throughout the whole sequence. However, *gap* sequencing did not allow the detection of intraspecies polymorphism among the studied *Staphylococcus* species; e.g., for *Staphylococcus epidermidis*, the sequence similarity among different isolates of this species was more than 99%. This also was shown by the *gap*-based terminal RFLP analysis of *S. epidermidis* isolates in our previous publication (22).

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