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Rapid and Accurate Identification of *Staphylococcus* Species by tRNA Intergenic Spacer Length Polymorphism Analysis

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PCR-amplified tRNA gene (tDNA) intergenic spacer length polymorphism (tDNA-ILP) was analyzed for its ability to identify to the species level type strains ($n = 18$) and clinical isolates ($n = 163$) of staphylococci. Amplified products obtained by PCR with outwardly directed consensus tDNA primers were separated by agarose and polyacrylamide gel electrophoreses. The results were compared with those obtained by biochemical identification and ribotyping. Each type strain presented a specific tDNA-ILP pattern. PCR with fluorescent primers allowed for the detection of labelled DNA fragments on polyacrylamide gels by using an automated laser fluorescence sequencer and provided enhanced pattern resolution in comparison with that by analysis on agarose gels. tDNA patterns indistinguishable from those of the type strains were produced by clinical isolates of all tested species except for some isolates of *S. aureus* ($n = 3$) and *S. haemolyticus* ($n = 1$), which showed variant patterns. Strains of *S. saprophyticus* and *S. xylosus* showed very closely related profiles, and *S. cohnii* subspecies were indistinguishable. The identities obtained by tDNA-ILP analysis agreed with those obtained by the biochemical method to the species level for 99% (162 of 163) of the strains tested and to the subspecies level for 96% (156 of 163) of the strains tested. These results indicate that fluorescence-labelled PCR analysis of tDNA-ILP provides an accurate and rapid molecular method for the identification of human staphylococci.

Whereas the pathogenicity of *Staphylococcus aureus* has long been established, coagulase-negative staphylococci (CoNS) are increasingly recognized as etiologic agents of infections of soft tissue, indwelling foreign bodies, and the bloodstream in humans (10, 11, 14, 23). The use of implanted medical devices such as intravascular catheters and the growing number of immunocompromised patients contribute to the increasing importance of CoNS as causes of nosocomial infections (4, 16). Particular species of CoNS are associated with distinct types of infections and patterns of antimicrobial susceptibility (10, 22). Therefore, identification of clinical isolates to the species level is increasingly of clinical and epidemiological interest.

Among the 32 species currently recognized in the genus *Staphylococcus* (7), isolates of coagulase-negative species that are commonly recovered from clinical specimens include *S. epidermidis*, followed by *S. haemolyticus* and *S. hominis. S. saprophyticus* is commonly associated with community-acquired urinary tract infections (10). Isolates of more recently described species, including *S. warneri*, *S. lugdunensis*, *S. capitis*, and *S. schleiferi*, are occasionally recovered from clinical specimens (7, 9, 10, 22). Among these species, *S. lugdunensis* appears to be of particular virulence, notably as a cause of destructive endocarditis (23). CoNS are conventionally identified in the clinical laboratory by using biochemical identification systems which have accuracies ranging between 60 and 98% (1, 7, 9). Molecular biology-based methods that enable characterization of staphylococci to the species level include total genomic DNA-DNA hybridization, ribotyping analysis (24), and hybridization of genomic DNA to labelled probes specific for hypervariable sequences of the gene encoding the heat shock protein (3). Another strategy for bacterial identification which shows considerable promise is PCR analysis of length polymorphism of the intergenic spacers lying between tRNA genes (12, 26, 27). Because tRNA genes contain sequence motifs that are highly conserved among the eubacteria, consensus tRNA gene primers can be designed to explore the amount of tRNA gene intergenic length polymorphism (tDNA-ILP) between species (26).

The objective of this study was to evaluate the performance of PCR analysis of tRNA intergenic spacer polymorphism for the differentiation of staphylococcal type strains as well as for the identification of clinical isolates of diverse origins belonging to 15 species reported as human commensals and pathogens.

MATERIALS AND METHODS

Bacterial strains. The type strains of the genus *Staphylococcus* ($n = 18$) used in this study are listed in Table 1. The strains were kindly provided by the Centre National de Référence des Staphylocoques (Lyon, France).

Clinical isolates collected from blood, urine, and wound specimens in four Belgian hospitals during the period from 1988 to 1995 included *S. epidermidis* ($n = 32$) and *S. saprophyticus* ($n = 15$), with each isolate showing different major *Sma*I pulsed-field gel electrophoresis types; *S. haemolyticus* ($n = 13$); *S. hominis* ($n = 23$); *S. lugdunensis* ($n = 11$); *S. schleiferi* ($n = 6$); *S. capitis* subsp. *ureolyticus* ($n = 15$); *S. warneri* ($n = 7$); and *S. simulans* ($n = 3$). Methicillin-resistant ($n = 15$) and -susceptible ($n = 23$) *S. aureus* clinical isolates showing various *Sma*I pulsed-field gel electrophoresis types were selected from an international collection and included isolates from four European countries including Belgium (19) and from a previous collection (20) (1988 to 1993).

Phenotypic characterization. Clinical isolates of CoNS ($n = 125$) were identified by the ID 32 Staph system (BioMérieux, Marcy l'Etoile, France), which was used according to the manufacturer's instructions, and by the use of the APILAB ID 32 software (version 2.2.7, 1994). Coagulase activity was detected with human plasma after 4 and 24 h to identify *S. aureus* isolates. The identification of *S. saprophyticus* isolates was confirmed by xylose fermentation and the novobiocin (Rosco Diatabs, Taastrup, Denmark) susceptibility test.

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TABLE 1. tDNA-ILP patterns of 181 strains belonging to 15 species and 6 subspecies of human staphylococci

Species	Origin of strains	tDNA-ILP pattern	No. of strains
S. aureus	Type strain CCM 885	A	1
S. aureus	Clinical strains	A	35
S. aureus	Clinical strains	A1	2
S. aureus	Clinical strain	A2	1
S. epidermidis	Type strain CCM 2124	F	1
S. epidermidis	Clinical strains	F	32
S. haemolyticus	Type strain CCM 2737	G	1
S. haemolyticus	Clinical strain	G	12
S. Haemolyticus	Clinical strain	G2	1
S. hominis	Type strain DSM 20328	H	1
S. hominis	Clinical strains	H	22
S. hominis	Clinical strain	Unrelated	1
S. auricularis	Type strain ATCC 33753	B	1
S. capitis subsp. *capitis*	Type strain CCM 2734	C	1
S. capitis subsp. *ureolyticus*	Type strain ATCC 49326	C1	1
S. capitis subsp. *ureolyticus*	Clinical strains	C1	15
S. caprae	Type strain CCM 3573	D	1
S. cohnii subsp. *cohnii*	Type strain CCM 2736	E	1
S. cohnii subsp. *urealyticum*	Type strain ATCC 49330	E	1
S. lugdunensis	Type strain ATCC 43809	I	1
S. lugdunensis	Clinical strains	I	11
S. pasteuri	Type strain ATCC 51129	J	1
S. saprophyticus	Type strain CCM 883	K	1
S. saprophyticus	Clinical strains	K	15
S. schleiferi subsp. *coagulans*	Type strain GA 211	L	1
S. schleiferi subsp. *schleiferi*	Type strain ATCC 43808	M	1
S. schleiferi subsp. *schleiferi*	Clinical strains	L	6
S. simulans	Type strain ATCC 27848	N	1
S. simulans	Clinical strains	N	3
S. warneri	Type strain CCM 2730	J1	1
S. warneri	Clinical strains	J1	7
S. xylosus	Type strain ATCC 29971	K1	1

Oxacillin susceptibility. Oxacillin agar screening (Becton Dickinson, Sparks, Md.) was performed according to National Committee for Clinical Laboratory Standards guidelines.

Ribotyping. Ribotyping was performed by using *Eco*RI endonuclease, as described previously (24).

tDNA-ILP PCR. Genomic DNA was prepared by the rapid lysis procedure described by Ünal et al. (21). PCR was carried out with outwardly directed tRNA gene (tDNA) consensus primers T5A (5′-GTCCGGTGCTCTAACCAACT GAG) and T3B (5′-AGGTCGCGGGTTCGAATCC) described by Welsh and McClelland (27). Fifty microliters of PCR solution contained 1.25 U of *Taq* polymerase (Cetus Corp., Emeryville, Calif.), 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM $MgCl_2$, 0.5 μM (each) primer, 0.2 mM each of the four deoxynucleoside triphosphates, and 5 μl of the bacterial lysate as the DNA template. The reaction mixture was overlaid with 1 drop of mineral oil. Amplification conditions were as described previously (27).

Analysis of tDNA-ILP by agarose gel electrophoresis. The products of amplification were separated by electrophoresis for 3 h and 30 min at 160 V in a 4% Metaphor agarose gel (FMC BioProducts, Zellik, Belgium) supplemented with ethidium bromide (0.25 mg/ml) in Tris-Borate-EDTA buffer (pH 8.3). The size marker pBR322 *Hae*III digest (Sigma, Bornem, Belgium) contains DNA fragments of between 8 and 587 bp. The patterns of the amplified products were visually compared and codified by using a capital letter with arabic numerals. The arabic numerals indicate the number of different fragments in comparison with the of fragments of the reference strains.

Analysis of tDNA-ILP by using the ALFexpress (Automated Laser Fluorescent) DNA sequencer. tDNA-ILP was analyzed by using the ALFexpress DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands). Primer T5B was 5′ end labelled with Cy5 during the synthesis (Pharmacia). The amplification products were separated by electrophoresis through 6% (wt/vol) acrylamide-bisacrylamide–7 M urea denaturating gels (ReadyMix Gel, A.L.F. grade; Pharmacia) in 1× TBE (Tris-borate-EDTA) buffer. A fluorescein-labelled molecular size marker (Cy5 Sizer 50-500; Pharmacia), including 10 fragments ranging in size from 50 to 500 bp, was used as an external size ladder. The samples, including 2 μl of the PCR products, 5 μl of gel loading solution (Pharmacia), and 0.4 μl of each of the internal reference standards of 100 and 1,064 bp (5), were denatured at 95°C for 2 min and loaded onto the gel. Electrophoretic separation was done at 1,500 V, 60 mA, and 15 W for 4 h at 40°C. Internal and external markers were compared by using the Software Fragment Manager (Pharmacia) to normalize the gel. The fluorescence densitograms were visually compared, and digitized fluorescence data were exported to and analyzed with the GelCompar software (Applied Maths, Kortrijk, Belgium) by using Pearson correlation coefficients.

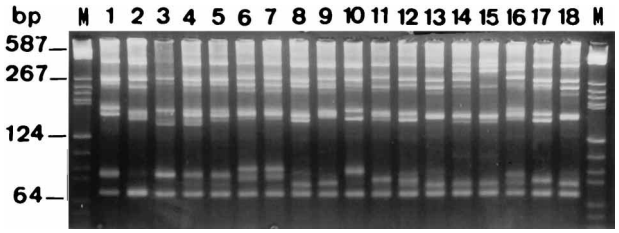

FIG. 1. tDNA-ILP patterns of 18 staphylococcal species type strains by electrophoresis in a 4% Metaphor agarose gel. Lanes M, molecular size marker (pBR322 *Hae*II digest); lane 1, *S. aureus* CCM 885; lane 2, *S. auricularis* ATCC 33753; lane 3, *S. capitis* subsp. *capitis* CCM 2734; lane 4, *S. capitis* subsp. *ureolyticus* ATCC 49326; lane 5, *S. caprae* CCM 3573; lane 6, *S. cohnii* subsp. *cohnii* CCM 2736; lane 7, *S. cohnii* subsp. *urealyticum* ATCC 49330; lane 8, *S. epidermidis* CCM 2124; lane 9, *S. haemolyticus* CCM 2737; lane 10, *S. hominis* DSM 20328; lane 11, *S. lugdunensis* ATCC 43809; lane 12, *S. pasteuri* ATCC 51129; lane 13, *S. saprophyticus* CCM 883; lane 14, *S. schleiferi* subsp. *coagulans* GA 211; lane 15, *S. schleiferi* subsp. *schleiferi* ATCC 43808; lane 16, *S. simulans* ATCC 27848; lane 17, *S. warneri* CCM 2730; lane 18, *S. xylosus* ATCC 29971.

RESULTS

The tDNA-ILP patterns of the type strains of 15 *Staphylococcus* species and 4 (among 6) subspecies showed different banding patterns by agarose electrophoresis (Fig. 1). Each pattern consisted of 8 to 11 DNA fragments ranging in size from approximately 60 to 600 bp. The tDNA-ILP patterns distinguished most of the species by a number of specific DNA fragments. The patterns of *S. saprophyticus* and *S. xylosus* differed only by a single fragment that showed a small difference in size (Fig. 1, lanes 13 and 18). The type strains of the two subspecies of *S. cohnii* were indistinguishable (Fig. 1, lanes 6 and 7), whereas the subspecies of *S. capitis* and *S. schleiferi* showed distinctive patterns (Fig. 1, lanes 4, 5, 15, and 16).

The tDNA-ILP pattern, evaluated with duplicate bacterial lysates of each type strain coamplified in the same PCR exper-

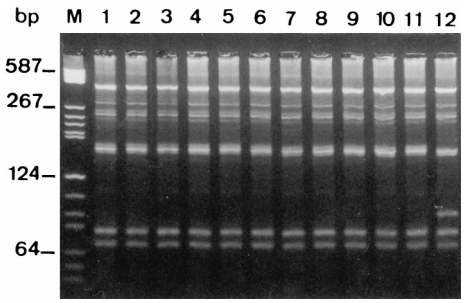

FIG. 2. tDNA-ILP patterns of clinical isolates and type strain of *S. haemolyticus* by electrophoresis in a 4% Metaphor agarose gel. Lane M, molecular size marker (pBR322 *Hae*III digest); lane 1: *S. haemolyticus* CCM 2737; lanes 2 to 12, clinical isolates of *S. haemolyticus.*

TABLE 2. Comparison of results of biochemical identification and identification by tDNA-ILP analysis and by ribotyping for 25 clinical isolates biochemically not identifiable to the species level or showing discrepant results between biochemical and tDNA-ILP analysis-based identification methods

Species identification (no. of strains) by the following method:		
ID 32 Staph	tDNA-ILP analysis	Ribotyping (*Eco*RI)
Not identified[a] (21)	*S. epidermidis* (4) *S. hominis* (5) *S. lugdunensis* (1) *S. schleiferi* subsp. *coagulans* (2) *S. haemolyticus* (1) *S. capitis* subsp. *ureolyticus* (4) *S. warneri* (3) *S. simulans* (1)	*S. epidermidis* (4) *S. hominis* (5) *S. lugdunensis* (1) *S. schleiferi* subsp. *schleiferi* (2) *S. haemolyticus* (1) *S. capitis* subsp. *ureolyticus* (4) *S. warneri* (3) *S. simulans* (1)
S. haemolyticus (1)	*S. haemolyticus*-like (1)[b]	*S. haemolyticus* (1)
S. warneri (1)	*S. capitis* subsp. *ureolyticus* (1)	*S. capitis* subsp. *ureolyticus* (1)
S. epidermidis (1)	*S. hominis* (1)	*S. hominis* (1)
S. hominis (1)	Unknown pattern (1)	Unknown pattern (1)

[a] Not identified to the species level (two species possible).
[b] Atypical pattern compared with the pattern of the type strain.

iment as well as in separate PCR experiments and analyzed on the same gel, showed complete reproducibility.

As indicated in Table 1, all clinical strains of *S. epidermidis*, *S. capitis* subsp. *ureolyticus*, *S. lugdunensis*, *S. saprophyticus*, *S. schleiferi*, *S. simulans*, and *S. warneri* showed tDNA-ILP patterns that were identical to those of the type strain. Thirty-five of the 38 clinical isolates of *S. aureus* had a banding pattern identical to that of the *S. aureus* type strain. Three *S. aureus* isolates showed patterns with one fragment ($n = 2$) or two fragments ($n = 1$) in addition to the fragments in the core pattern of *S. aureus* CCM 885, but the patterns were clearly different from the patterns exhibited by other species. The clinical strains of *S. haemolyticus* ($n = 13$) were assigned to the same tDNA-ILP type as *S. haemolyticus* CCM 2737, but one strain had a pattern that was different by two fragments from the pattern of the type strain, but the pattern was also different from those of all the other type strains by several fragments (Fig. 2, lane 12).

Some discrepancies between the results of phenotypic identification and those of tDNA-ILP analysis-based identification were observed. To resolve these discrepancies, identification by means of ribotyping (24) was done for 25 clinical isolates for which no definite identification at the species level could be obtained ($n = 21$) or for which biochemical identification and tDNA-ILP analysis-based identification were in disagreement ($n = 4$). For two isolates discrepancies between identities by tDNA-ILP analysis and with the ID 32 Staph system were found (Table 2). These two isolates, identified by the ID 32 Staph system as *S. warneri* and *S. epidermidis*, respectively, exhibited ribotype patterns and tDNA-ILP patterns identical to those of *S. capitis* subsp. *ureolyticus* and *S. hominis*, respectively. Except for subspecies characterization of *S. schleiferi*, results from tDNA-ILP analysis and ribotyping were in complete agreement (Table 2). The single *S. hominis* isolate that showed an unknown tDNA-ILP fingerprint also could not be classified by ribotyping when its ribotype pattern was compared to the ribotype patterns of 32 described species. Overall, the results of tDNA-ILP analysis were in agreement with those of biochemical identification complemented with ribotyping analysis to the species level for 99% (162 of 163) of the clinical isolates of 10 species tested and to the subspecies level for 96% (156 of 163) of the subspecies tested.

ALFexpress analysis of tDNA-ILP patterns was performed with all type strains and 85 clinical isolates belonging to 10 species. The densitometric curves generated by amplified products from clinical isolates appeared highly conserved within a species (Fig. 3 and 4). Visual comparison of the densitometric curves provided by the ALFexpress system was considerably easier than visual of the patterns exhibited on agarose gels (Fig. 1, 3, and 4). Figure 5 illustrates the relatedness between the normalized DNA patterns produced by PCR of each type strain by clustering by the unweighted pair group method with averages of Pearson correlation coefficients. At the species level, all type strains were differentiated. Although *S. xylosus* and *S. saprophyticus* type strains exhibited visually indistinguishable fluorescence densitograms, the degree of similarity between these patterns measured with the Pearson correlation coefficient was 82%, indicating quantitative variation in peak intensities. The minimum similarity values within a species, based on the patterns of 10 clinical isolates and the type strain,

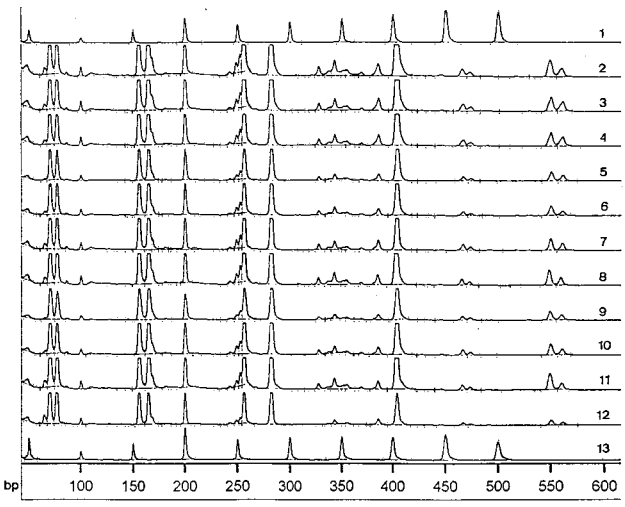

FIG. 3. Fluorescence densitogram of type strain and clinical isolates of *S. epidermidis*. Rows 1 and 13, 50- to 500-bp external Cy5-labelled marker (Pharmacia); row 2, *S. epidermidis* CCM 2124; rows 3 to 12, clinical isolates of *S. epidermidis.*

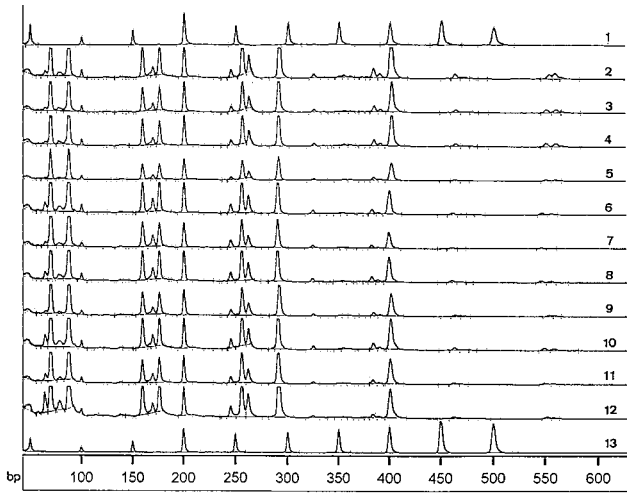

FIG. 4. Fluorescence densitogram of type strain and clinical isolates of *S. hominis*. Rows 1 and 13, 50- to 500-bp external Cy5-labelled marker (Pharmacia); row 2, *S. hominis* DSM 20328; rows 3 to 12, clinical isolates of *S. hominis.*

were 92 and 84% for *S. epidermidis* and *S. hominis*, respectively.

DISCUSSION

Differentiating clinically significant isolates of CoNS from the contaminating flora is a daily issue in clinical bacteriology. Identification to the species level of isolates from independent specimens is helpful in achieving this aim. Phenotypic methods often do not allow for the correct identification of all clinical isolates of CoNS (7, 8, 13, 15, 18). For example, *S. hominis*, one of the most common species after *S. epidermidis* and *S. haemolyticus*, was identified with low accuracy by the API ID 32 Staph system (Biomérieux) (7) and the Staph-Ident system (Biomérieux) (1). Recently, a number of molecular biology-based approaches to the identification of species of clinically important bacteria have been described. Widjojoatmodjo et al. (28) proposed a general approach based on single-strand conformation polymorphism analysis of PCR-amplified 16S rRNA gene fragments. A disadvantage of this method is the use in the PCR amplification of conserved primers that can be hampered by contamination of reagents with exogenous bacterial DNA. Another method, called amplified ribosomal DNA restriction analysis, has been reported to discriminate between bacteria in different genera to the species level (25). However, compared to tDNA-ILP analysis, several restrictions may be needed to obtain a final discrimination between species. Also, restriction fragment length polymorphism analysis of the amplified 16S rRNA gene is unable to differentiate between most *Staphylococcus* species (24a).

PCR analysis of the length polymorphism of the intergenic spacers lying between 16S and 23S rRNA genes (6) or between tRNA genes (2, 27) is useful for identifying microorganisms to the species level. The initial description of tRNA-ILP analysis was applied to a limited number of species of staphylococci (27). We evaluated more extensively the usefulness of tDNA-PCR for the identification of these microorganisms in the study described in this report. Except for *S. aureus* and *S. haemolyticus*, which both exhibited minor intraspecific variations, a remarkable level of conservation of tDNA-ILPs was seen within each species. Because the clinical isolates were selected as being epidemiologically nonrelated, these findings indicated a high degree of intraspecies stability of the organization of tRNA genes in staphylococci, thereby supporting the concept that the organization of tRNA genes changes very slowly. On the other hand, the extensive interspecies diversity reported here indicates that tDNA-ILP analysis can be used with a high degree of specificity as a tool for the rapid identification of human staphylococci. The usefulness of the method was confirmed by comparison of the results with those of ribotyping in cases in which biochemical identification either proved inconclusive or the pattern by biochemical identification was at variance with the tDNA-ILP pattern. Overall, the accuracy of the tDNA-ILP analysis method was 99% for the identification of clinical isolates belonging to 10 staphylococcal species.

Accurate and reproducible identification by this novel molecular biology-based method critically depends on correct analysis of the sizes of the PCR products. Analysis of fragment patterns by electrophoresis at 160 V for 3 h and 30 min in a 4% Metaphor agarose gel showed the best resolving power for products of ≤1 kb. Although pattern reproducibility was good, the resolution of the electrophoretic profiles was occasionally affected by minor variations in the porosity of the agarose. The

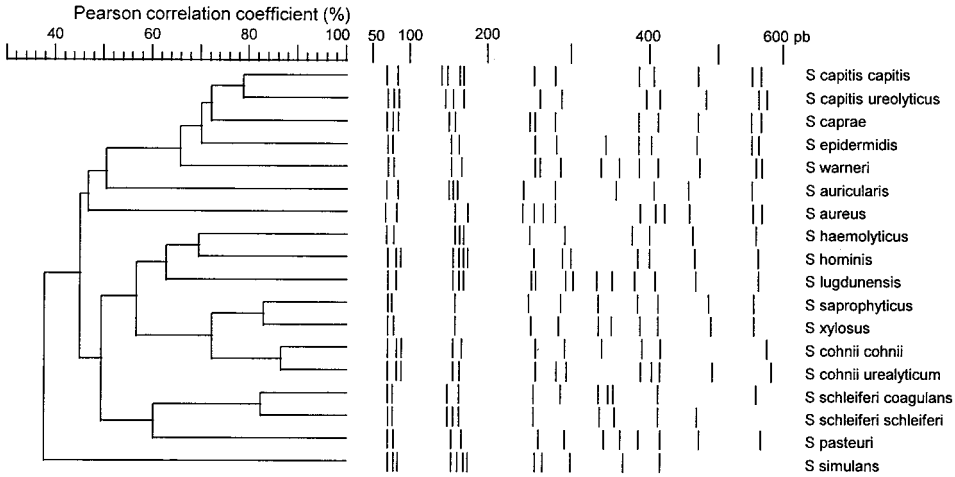

FIG. 5. Dendrogram of tDNA-ILP pattern relatedness of 18 type strains of staphylococcal species and subspecies constructed by the unweighted pair group method with averages. Amplified DNA products were separated and analyzed with the ALFexpress automated sequencer (see Materials and Methods).

use of a more accurate method of post-PCR analysis with an automated laser fluorescence sequencer (ALFexpress; Pharmacia) showed excellent intraspecies reproducibility of the tDNA-ILP patterns and discrimination between the 15 species of staphylococci by computer-assisted analysis (Fig. 5).

An interesting concordance was found between the clustering based on tDNA-ILP analysis and that based on DNA-DNA homology classification (17). A first group comprised *S. hominis* and *S. haemolyticus* (70% related by tDNA-ILP analysis and 45% related by DNA-DNA hybridization). A second group included the species *S. capitis*, *S. caprae*, *S. epidermidis*, and *S. warneri* (more than 65% related by tDNA-ILP analysis and about 40% related by DNA-DNA hybridization). *S. xylosus*, *S. saprophyticus*, and *S. cohnii* formed a third cluster (more than 70% related by tDNA-ILP analysis and about 40% related by DNA-DNA hybridization). This suggests that tDNA-ILP analysis reflects phylogenetic taxonomy in a manner comparable to that of DNA homology determination.

In summary, tDNA-ILP analysis exhibited 99% accuracy for the identification of staphylococcal isolates encountered in a clinical laboratory within 9 h of obtaining a pure culture. Fluorescent DNA fragment analysis provided with higher-resolution software-based pattern interpretation allowed for the rapid and accurate comparison of patterns and species identification. Further clinical evaluation should define the optimal use of tDNA-ILP analysis either as a confirmatory test or as a first-line identification method in the laboratory.

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