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Identification of a Variety of *Staphylococcus* Species by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry▽

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Whole-cell fingerprinting by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) in combination with a dedicated bioinformatic software tool (MALDI Biotyper 2.0) was used to identify 152 staphylococcal strains corresponding to 22 staphylococcal species. Spectra of the 152 isolates, previously identified at the species level using a *sodA* gene-based oligonucleotide array, were analyzed against the main spectra of 3,030 microorganisms. A total of 151 strains out of 152 (99.3%) were correctly identified at the species level; only one strain was identified at the genus level. The MALDI-TOF MS method revealed different clonal lineages of *Staphylococcus epidermidis* that were of either human or environmental origin, which suggests that the MALDI-TOF MS method could be useful in the profiling of staphylococcal strains. The topology of the dendrogram generated by the MALDI Biotyper 2.0 software from the spectra of 120 *Staphylococcus* reference strains (representing 36 species) was in general agreement with that inferred from the 16S rRNA gene-based analysis. Our findings indicate that the MALDI-TOF MS technology, associated with a broad-spectrum reference database, is an effective tool for the swift and reliable identification of *Staphylococci*.

Most staphylococci are harmless and reside normally on the skin and mucous membranes of humans and other organisms (16, 22, 34). Staphylococcal strains are isolated from various food products in which they are involved in fermentation (18, 29). *Staphylococcus* species can cause a wide variety of diseases in humans and other animals (2, 22, 30–32, 35). *S. aureus* is a major pathogen in human infections (31). Several other *Staphylococcus* species have also been implicated in human infections, notably *S. saprophyticus*, *S. epidermidis*, *S. lugdunensis*, and *S. schleiferi* (4, 16, 31, 34). Coagulase-negative staphylococci (CoNS) have emerged as predominant pathogens in hospital-acquired infections (4, 16, 31, 34). One of the major challenges of daily diagnostic work is therefore to identify *Staphylococcus* species.

Several manual and automated methods based on phenotypic characteristics have been developed for the identification of *Staphylococci* (12, 24). Unfortunately, these systems have their limitations, mostly due to phenotypic differences between strains from the same species (6, 10, 19, 21). Over the last 10 years, many genotypic methods based on the analysis of selected DNA targets have been designed for species-level identification of most common isolated CoNS (20, 26, 33). The sequence polymorphism of the *sodA* gene has significant discriminatory power (20) and allows the development of assays based on DNA chip technologies ("Staph array") (8). Recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using protein "fingerprints" was used for the identification of microorganisms (1, 3, 5, 9, 11, 14, 25, 36). In the present study, we assessed the ability of the MALDI Biotyper system (Bruker Daltonique, Wissembourg, France) to identify *Staphylococcus* species of clinical and environmental origins previously identified by *sodA* gene-based oligonucleotide array (8).

MATERIALS AND METHODS

Bacterial strains. A total of 152 blind-coded *Staphylococcus* strains from our collection, isolated from clinical ($n = 60$) and food and plant samples ($n = 92$) and representing 22 species, were studied (Table 1). All strains were previously identified as belonging to a specific species by the oligonucleotide "Staph array" (8). This system associates PCR amplification of the *sodA* gene with an oligonucleotide-based array to efficiently discriminate between the *Staphylococcus* species. Identifications found with this array were identical to those obtained by sequencing the internal fragment *sodA* ($sodA_{int}$). In addition, the Vitek 2 system has been used with the Gram-positive (GP) identification card (bioMérieux, Marcy l'Etoile, France) to identify *Staphylococcus* strains. If the GP card indicated the possibility of two or three species, supplementary tests (pigmentation, hemolysis, or novobiocin resistance) were then performed to determine the identification.

Sample preparation. Sample preparation for MALDI-TOF MS was performed as previously described (14). Briefly, a few colonies of a fresh overnight culture grown on Columbia blood agar at 37°C under aerobic conditions were suspended in 300 μl distilled water to which 900 μl absolute ethanol was added. The mixture was centrifuged at 12,000 × *g* for 2 min, and the supernatant was discarded. Ten microliters of formic acid (70%) was added to the pellet and mixed thoroughly by pipetting before the addition of 10 μl of acetonitrile to the mixture. The mixture was centrifuged at 12,000 × *g* for 2 min. One microliter of the supernatant was placed onto a steel target plate and air dried at room temperature. Each sample was overlaid with 2 μl saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid and air dried at room temperature. MALDI-TOF MS measurements were performed with a Bruker Ultraflex II MALDI-TOF/TOF (tandem TOF) instrument equipped with 200-Hz Smartbeam laser technology. Spectra were recorded in the

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TABLE 1. Clinical and environmental staphylococcal strains used in this study

Species ("Staph array" identification)	No. of isolates		
	Clinical	Environmental	Total
S. arlettae	0	1	1
S. aureus	6	1	7
S. capitis	5	2	7
S. caprae	1	0	1
S. carnosus	0	7	7
S. cohnii subsp. *ureal*	2	2	4
S. delphini	1	0	1
S. epidermidis	11	8	19
S. equorum	0	13	13
S. fleurettii	0	2	2
S. haemolyticus	5	1	6
S. hominis	5	1	6
S. lugdunensis	5	0	5
S. pasteuri	0	8	8
S. saprophyticus	6	10	16
S. schleiferi	2	0	2
S. sciuri	1	2	3
S. simulans	4	0	4
S. succinus	0	9	9
S. vitulinus	0	5	5
S. warneri	6	6	12
S. xylosus	0	14	14
Total	60	92	152

positive linear mode within a mass range of 2,000 to 20,000 Da. Five hundred laser shots were recorded for each spectrum.

Data analysis. Raw spectra of the *Staphylococcus* strains were analyzed by MALDI Biotyper 2.0 software (Bruker Daltonique) with default settings. The whole process from MALDI-TOF MS measurement to identification was performed automatically without any user intervention. The peak lists generated were used for matches against the reference library directly using the integrated pattern-matching algorithm of the software. The software provides a log score, and the cutoff log score of 2 was used to validate identification at the species level, as recommended by the manufacturer. For strain classification, the creation of the dendrogram is based on cross-wise minimum spanning tree (MSP) matching. Similar MSPs result in a high matching score value. Each MSP is matched against all MSPs of the analyzed set. The list of score values is used to calculate normalized distance values between the analyzed species, resulting in a matrix of matching scores. The visualization of the respective relationship between the MSPs is displayed in a dendrogram using the standard settings of the MALDI Biotyper 2.0 software. Species with distance levels under 500 have been described as reliably classified (25).

RESULTS AND DISCUSSION

Classification of staphylococcal reference strains. A score-oriented dendrogram was generated on the basis of the 120 reference strains in the Bruker MALDI Biotyper 2.0 database (Fig. 1). This dendrogram revealed, with the default critical distance level of 500, 4 of the 11 cluster groups defined by Poyart et al. from the phylogenetic analysis based on the *sodA* gene (20): *S. sciuri*, *S. warneri*, *S. lugdunensis*, and *S. auricularis* (Fig. 1). These cluster groups were also in accordance with those established by the phylogenetic analysis of the 16S rRNA gene (28). With a critical distance level of 750, the dendrogram revealed overall the cluster groups of 16S rRNA genes, with some variations (Fig. 1) (17, 28). The 16S rRNA gene-based cluster group *S. haemolyticus* including *S. haemolyticus* and *S. hominis* was divided into branches which linked at a distance level of 850. Unlike in other phylogenetic analyses, in which *S. kloosii* belonged to *S. saprophyticus* cluster group, this species was clustered within the *S. simulans* group in the MALDI-TOF MS-based dendrogram (13, 20, 28). Inside the *S. saprophyticus* cluster group, the two strains *S. equorum* subsp. *equorum* DSM 20675 and DSM 20674 belonged to two distinct clusters. It is noteworthy that the *S. lutrae* and *S. schleiferi* species each constituted a well-defined clade inside the *S. hyicus-S. intermedius* cluster group, like *S. succinus*, *S. xylosus*, *S. cohnii*, and *S. arlettae* in the *S. saprophyticus* cluster group and *S. capitis* in the *S. epidermidis* cluster group.

Although some differences were observed, the topology of the MALDI-TOF MS-based phylogenetic tree was in more general agreement with that inferred from the analysis of the 16S rRNA gene sequences than that based on the *sodA* gene (20, 28). The proteins analyzed by MALDI Biotyper correspond to predominant proteins, such as ribosomal proteins. The probable coevolution of the ribosomal proteins and rRNAs may explain the similarities between 16S rRNA gene and MALDI-TOF-based dendrograms (23).

MALDI-TOF MS could thus be a valuable tool in phyloproteomics. It might serve as a technique for protein profiling, which is recommended as an additional test for the description of new staphylococcal species (7).

Species identification of staphylococcal isolates. Our dendrogram showed that the 36 reference *Staphylococcus* species were correctly distinguished. *S. vitulinus* and *S. pulvereri*, which have recently been shown to be one single species (27), were not differentiated by the MALDI-TOF MS technique. This technique seemed to be suitable for the differentiation of *Staphylococcus* isolates at the species level.

MALDI-TOF MS spectra were then obtained for all clinical and environmental staphylococcal isolates. A total of 151 strains out of 152 (99.3%) were identified at the species level by the MALDI Biotyper. One *S. saprophyticus* strain out of 16 was identified as *S. xylosus*, with a log score of 1.995. It was therefore identified at the genus level. The system identified both clinical (14 different species) and environmental (17 different species) strains without any complementary tests.

Carbonnelle et al. used the MALDI-TOF technology to identify a clinical collection of *Staphylococcus* strains constituted by the four major clinically relevant *Staphylococcus* species (*S. aureus*, *S. epidermidis*, *S. warneri*, and *S. haemolyticus*) (3). Their reference database contained 22 staphylococci reference strains, including 17 staphylococcal species. In our study, we identified 22 *Staphylococcus* species from the MALDI Biotyper database, which comprised the spectra of 3,030 microorganisms, corresponding to Gram-negative and Gram-positive bacteria, whose 120 staphylococcal strains represented 36 staphylococcus species. *A fortiori*, the MALDI Biotyper did not require any initial assessment such as Gram staining or a catalase test, unlike the technology developed by Carbonnelle et al., which requires a presumptive identification of *Staphylococcus* at the genus level.

The MALDI Biotyper database comprised 36 staphylococcal species. The *Staphylococcus* genus comprises 39 validly described species (J. P. Euzéby, List of bacterial names with standing in nomenclature, 5 November 2008, posting date; http://www.bacterio.cict.fr/). The three species *S. pettenkoferi*, *S. pseudintermedius*, and *S. gallinarum* were absent from the MALDI Biotyper database. The MALDI Biotyper database

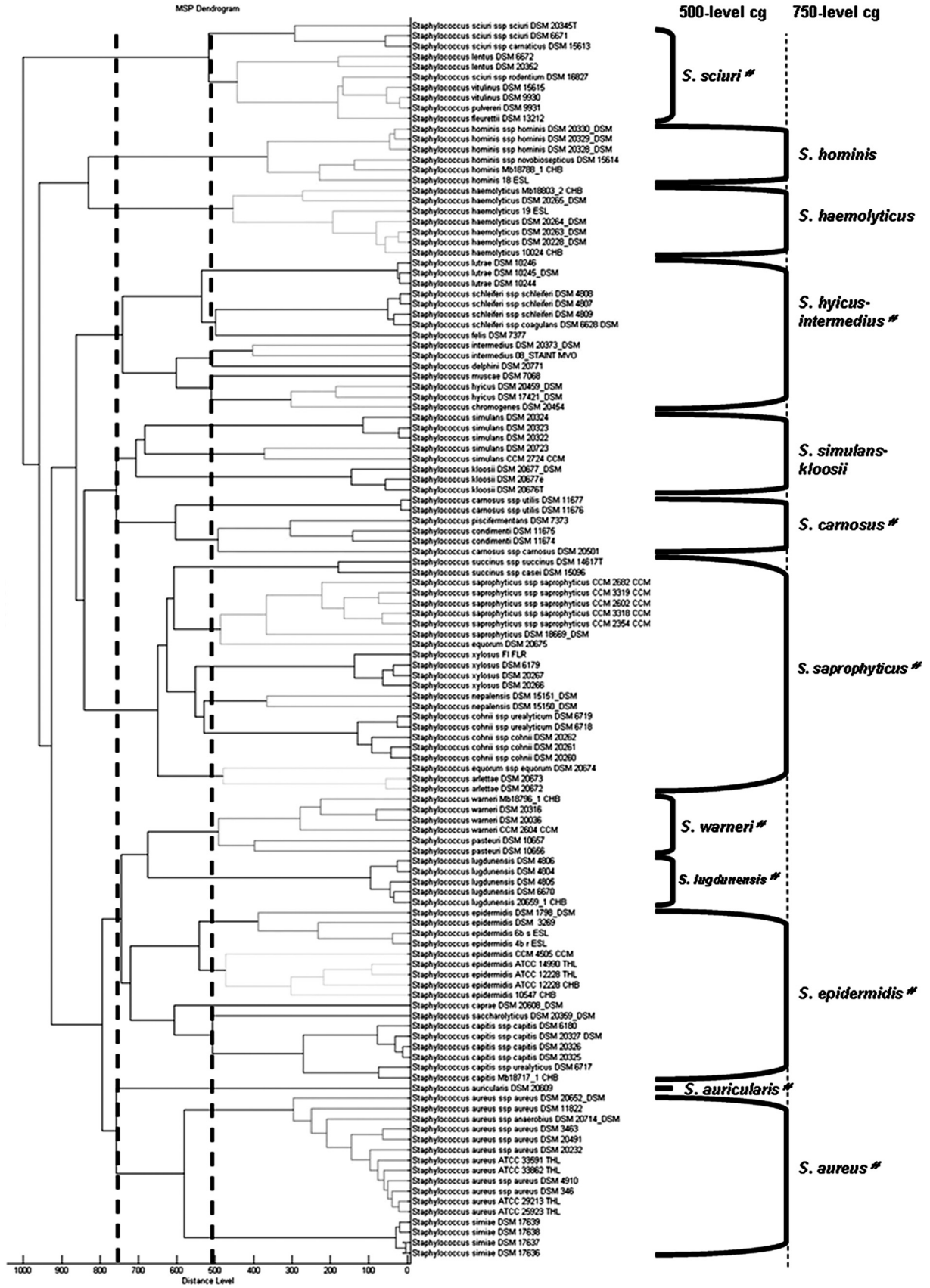

FIG. 1. Classification of staphyloccal reference strains. Shown is a score-oriented dendrogram of staphylococcal reference strains included in the database. The terms "500-level cg" and "750-level cg" define cluster groups (cg) based on the branching pattern using critical distance levels of 500 and 750, respectively. #, cluster groups as defined by the phylogenetic analysis of 16S rRNA genes.

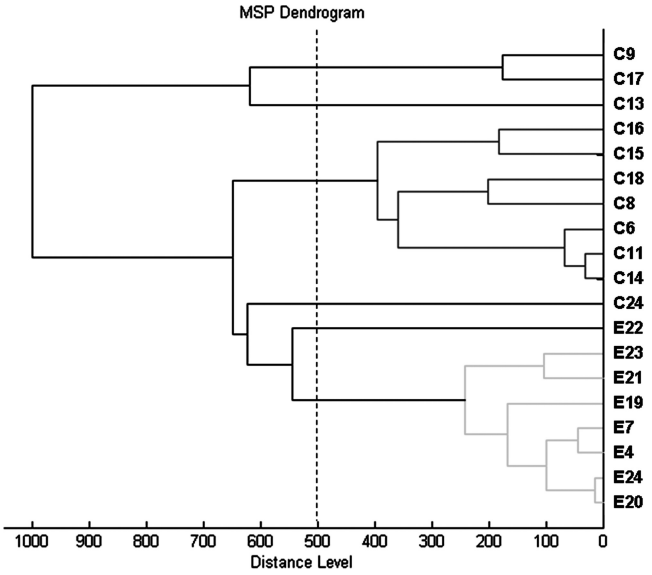

FIG. 2. Score-oriented dendrogram of clinical (C) and environmental (E) *S. epidermidis* isolates.

contains four species (*S. pasteuri*, *S. delphini*, *S. fleurettii*, and *S. succinus*) absent from the Vitek 2 Gram-positive (GP) card database (bioMérieux, La-Balme les Grottes, France). Moreover, according to the previously published data from our strain collection, the Vitek 2 system had misidentified or not identified 1 out of 7 *S. capitis*, 2 out of 7 *S. carnosus*, 11 out of 13 *S. equorum*, 1 out of 4 *S. simulans*, and 1 out of 5 *S. vitulinus* strains (6). This suggests that the MALDI-TOF MS technique is better fitted to the identification of staphylococcal strains that are rarely isolated in clinical bacteriology than automated biochemical methods. In addition, sample preparation and analysis with the MALDI-TOF MS technique are easier and more time-saving (approximately 20 min for one identification) than with genotypical methods, and result acquisition is faster than with biochemical methods.

Biodiversity of *S. epidermidis* species. The dendrogram of Fig. 2 shows protein "fingerprint" heterogeneity of clinical and environmental (salt meat originated) *S. epidermidis* strains. Clinical isolates are gathered within two cluster groups and two isolated strains, whereas meat origin isolates displayed one distinct cluster group and one isolated strain. Diversity within the *S. epidermidis* species has been observed for human origin *S. epidermidis* and animal origin *S. epidermidis* (15). The MALDI-TOF MS method might have enough discriminatory power to group isolates below the species level.

Conclusion. MALDI-TOF MS associated with MALDI Biotyper software appears a reliable and accurate tool for the identification of *Staphylococcus* species. However, further studies are required to test this technology with a large collection of staphylococci of diverse origins. The speed and the simplicity of sample preparation and result acquisition associated with minimal consumable costs make this method well suited for routine and high-throughput use. Hence, it is an excellent alternative to traditional methods in food processing and medical care laboratories. It may also be used for the analysis of clonal and/or taxonomic relationships.

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