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Rapid Identification and Typing of *Listeria* Species by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry▽†

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Listeria monocytogenes **is a food-borne pathogen that is the causative agent of human listeriosis, an opportunistic infection that primarily infects pregnant women and immunologically compromised individuals. Rapid, accurate discrimination between *Listeria* strains is essential for appropriate therapeutic management and timely intervention for infection control. A rapid method involving matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) that shows promise for identification of *Listeria* species and typing and even allows for differentiation at the level of clonal lineages among pathogenic strains of *L. monocytogenes* is presented. A total of 146 strains of different *Listeria* species and serotypes as well as clinical isolates were analyzed. The method was compared with the pulsed-field gel electrophoresis analysis of 48 *Listeria* strains comprising *L. monocytogenes* strains isolated from food-borne epidemics and sporadic cases, isolates representing different serotypes, and a number of *Listeria* strains whose genomes have been completely sequenced. Following a short inactivation/extraction procedure, cell material from a bacterial colony was deposited on a sample target, dried, overlaid with a matrix necessary for the MALDI process, and analyzed by MALDI-TOF MS. This technique examines the chemistry of major proteins, yielding profile spectra consisting of a series of peaks, a characteristic "fingerprint" mainly derived from ribosomal proteins. Specimens can be prepared in a few minutes from plate or liquid cultures, and a spectrum can be obtained within 1 minute. Mass spectra derived from *Listeria* isolates showed characteristic peaks, conserved at both the species and lineage levels. MALDI-TOF MS fingerprinting may have potential for *Listeria* identification and subtyping and may improve infection control measures.**

The genus *Listeria* consists of a group of gram-positive bacteria of low G+C content with six species, *Listeria monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*. However, only *L. monocytogenes* and *L. ivanovii* exhibit pathogenic features, human cases of *L. ivanovii* infection being quite rare (8). *L. monocytogenes* is a food-borne pathogen that is the causative agent of human listeriosis, an opportunistic infection that primarily infects pregnant women and immunologically compromised individuals. Approximately 2,500 human listeriosis cases occur annually in the United States, resulting in 500 deaths (28). Meat, poultry, dairy, and vegetable products have all been implicated as vehicles of listeriosis (21). Since food-borne listeriosis was first reported in 1981 (34), food-borne outbreaks of *L. monocytogenes* have been documented worldwide (20).

Listeria monocytogenes has a strongly clonal population structure (5). *L. monocytogenes* strains are serotyped according to variation in the somatic (O) and flagellar (H) antigens (35). By using various genetic typing techniques, *L. monocytogenes* can be further classified into three lineages, of which lineage I encompasses serotypes 1/2b, 3b, 4b, 4d, and 4e; lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c; and lineage III comprises serotypes 4a and 4c (29, 30, 42, 43). Although more than 12 serotypes of *L. monocytogenes* have been described (15), only 3 serotypes (1/2a, 1/2b, and 4b) cause the vast majority of clinical cases. Because of the importance of *L. monocytogenes* to human health, a number of discriminatory subtyping and identification methods for studying the epidemiology of this organism have been described (9, 14, 26, 42). Currently pulsed-field gel electrophoresis (PFGE) is the most commonly employed subtyping technique, but new technologies are constantly introduced and tested in hopes of increasing the resolution, speed, and reproducibility of *L. monocytogenes* subtyping; these include multilocus sequence subtyping (33), ribotyping (43), and microarray genomic analysis (3). Most of the current techniques for the epidemiological identification of microorganisms are laborious and time-consuming. Although rapid methods to detect *Listeria* spp. based on PCR have been developed, some problems remain, such as the identification of atypical strains.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has become a valuable tool for the analysis of microorganisms (22, 32). The accuracy and speed of data acquisition by MALDI-TOF MS make this a potentially important tool for biological public health hazards, food processing, blood screening, and disease diagnoses. Other MS techniques have utilized pyrolysis gas chromatography/MS (13), fast atom bombardment MS (16, 17), and elec-

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trospray ionization MS (36) to obtain bacterial profiles. But all these techniques primarily focus on the low-molecular-weight components, e.g., lipids, of the cell. The MALDI technique couples high sensitivity with accuracy and is ideal for the detection of high- and low-molecular-weight proteins. MALDI-TOF MS has the potential for rapidly distinguishing between pathogenic and nonpathogenic species of bacteria (22, 23). In addition, mass spectra obtained from unknown bacterial samples can be used for comparison with reference libraries of known organisms for a correct identification (19). This concept of using a reference database with which unknown bacterial samples can be identified on the basis of comparisons with reference spectra currently is the most promising approach for microorganism identification based on MALDI-TOF MS. The goal is the elucidation of a reproducible genus-, species-, and strain-specific "fingerprint" for any given organism. Various attempts to apply MALDI-TOF MS to the identification and typing of bacteria have been described (2, 4). Early protocols involved solvent extraction and chemical modification before analysis, but some methods use simple sample preparation and direct analysis via appropriate ionization methods (37). Further work in this field was focused on investigations of the reproducibility of these spectra (41) and the possibility of coupling MALDI measurements with database analysis (1, 10).

In this study, the objective was to develop a robust method to analyze the extracts of *Listeria* species and strains by MALDI-TOF MS and to evaluate the reproducibility of this method. An additional objective was to establish a library of mass spectral fingerprints from reference strains of *Listeria* spp.

MATERIALS AND METHODS

Bacterial strains. Sources and other information on the *Listeria* strains examined are given in Table S1 in the supplemental material. All strains were either type, reference, or other well-characterized isolates that have been previously identified as belonging to a specific species.

The following reference strains were used in this study for MALDI-TOF MS analyses: *Listeria monocytogenes* DSM20600, *L. ivanovii* subsp. *londoniensis* DSM12491, *L. ivanovii* subsp. *ivanovii* DSM20750, *L. innocua* DSM20649, *L. seeligeri* DSM20751, *L. welshimeri* DSM20650, *L. grayi* DSM20601, and *L. grayi* DSM20596.

MALDI-TOF MS. *Listeria* strains were grown overnight at 37°C on brain heart infusion agar. Cells of a whole colony were transferred from the plate to a 2.0-ml conical screw-cap extraction tube (Eppendorf, Germany) with a disposable inoculating loop and mixed thoroughly in 300 μl water to resuspend the bacterial cells. Later, 900 μl of absolute ethanol was added and mixed with the cell suspension, the mixture was centrifuged at 10,000 × *g* (5415D; Eppendorf) for 2 min, and the supernatant was discarded. Subsequently, 10 μl of formic acid (70%) was added to the pellet and mixed thoroughly by pipetting before the addition of 10 μl acetonitrile to the mixture. The mixture was centrifuged again at maximum speed for 2 min. One microliter of the supernatant was placed onto a spot of a steel target plate and air dried at room temperature. Each sample was overlaid with 2 μl of matrix solution (saturated solution of α-cyno-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid) and air dried at room temperature. MALDI-TOF MS was performed on a Microflex LT instrument (Bruker Daltonik GmbH, Leipzig, Germany). The spectra were recorded in the linear, positive mode at a laser frequency of 20 Hz within a mass range from 2,000 to 20,000 Da. The acceleration voltage was 20 kV, the IS2 voltage was maintained at 18.60 kV, and the extraction delay time was 200 ns.

For each spectrum, 500 laser shots were collected and analyzed (10 times 50 laser shots from different positions of the target spot). The spectra were externally calibrated using the standard calibrant mixture (*Escherichia coli* extract including the additional proteins RNase A and myoglobin). Calibration masses were as follows: RL36, 4,364.3 Da; RS22, 5,095.8 Da; RL34, 5,380.4 Da; RL33meth, 6,254.4 Da; RL32, 6,315.2 Da; RL29, 7,273.5 Da; RS19, 10,299.1 Da; RNase A, 13,682.2 Da; myoglobin, 16,952.5 Da).

Data analysis. Manual/visual estimation of the mass spectra was performed using FlexAnalysis 2.4 software (Bruker Daltonik GmbH, Germany). For automated data analysis, raw spectra were processed using the MALDI BioTyper 1.1 software (Bruker Daltonik GmbH, Germany) with default settings. The software performs smoothing, normalization, baseline subtraction, and peak picking, thereby creating a list of the most significant peaks of a spectrum (*m*/*z* values with a given intensity, with the threshold set to a minimum of 1% of the highest peak and a maximum of 100 peaks). To identify unknown bacteria, the generated peak lists were used for matches against reference libraries directly using the integrated pattern matching algorithm of the software. After import of a spectrum into the BioTyper software, the whole process from processing to identification was performed automatically, without any user intervention.

For reference library construction, 20 independent spectra were recorded for each bacterial isolate (five independent measurements at four different spots each). To create peak lists of the spectra, the BioTyper software was used as described above. The 20 independent peak lists of a strain were used for automated "main spectrum" generation with default settings of the BioTyper software. Thereby, for each library entry a reference peak list (main spectrum) which contains information about averaged masses, averaged intensities, and relative abundances in the 20 measurements for all characteristic peaks of a given strain was created.

For "weighted" pattern matching analysis, the five closely related species (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*) were used for recalculation of the peak ratings. Peaks which are present in all species were removed from the score calculation. Peaks occurring is one to four of the five different species were used for the weighted score calculations. The more specific such a peak is, the higher its weighted score contribution is set. For example, a peak occurring in only one species has the greatest impact in the weighted score calculation. Based on the remaining species-discriminating peaks, the "weighted pattern matching libraries" were created.

PFGE. PFGE was performed according to the PulseNet standardized protocol (14). Briefly, genomic DNA was prepared by mixing 240 μl of a standardized cell suspension and 60 μl of a 10-mg/ml lysozyme solution (Sigma, St. Louis, MO), followed by incubation at 37°C for 10 min. An equal volume of molten 1.2% agarose, 1% sodium dodecyl sulfate, and 0.2 mg/ml proteinase K prepared in sterile distilled water and maintained at 53 to 56°C was added to the cell suspension, and the mixture was mixed by gently pipetting it up and down several times. The mixture (600 μl) was dispensed into forms of a sample reusable plug mold and allowed to cool for 5 min. The agarose plugs were transferred to tubes containing 4 ml of lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0 [TE buffer], 1% sodium lauryl sarcosine, 0.15 mg/ml proteinase K), incubated for 2 h at 50 to 54°C in an orbital water bath shaker, and shaken at 200 rpm. After proteolysis, the lysis buffer solution was removed and the plugs were washed twice with 15 ml of preheated (50 to 54°C) sterile distilled water for 10 min, followed by four washes with 15 ml of preheated (50 to 54°C) TE buffer for 15 min in the orbital water bath shaker at 50 to 54°C and 200 rpm. After the final TE wash, the plugs were stored in 1.5 ml TE at 48°C until ready for restriction. Sample plugs were digested with 25 U of AscI (New England Biolabs, Beverly, MA) at 37°C for 3 h or 160 to 200 U of ApaI (New England Biolabs) at 30°C for 5 h. Plugs were then loaded on 1% agarose gel in 0.5× TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) buffer and electrophoresed on a CHEF-DR II apparatus (Bio-Rad) using the following parameters: initial switch time, 4 s; final switch time, 40 s; run time, 22 h; angle, 120°; gradient, 6 V/cm; temperature, 14°C; and ramping factor, linear. Gels were stained with ethidium bromide and visualized by a UV transilluminator. The generated PFGE patterns were analyzed using the Gel Compare II (Applied Maths) software.

RESULTS AND DISCUSSION

MALDI-TOF MS spectra were obtained for 146 strains of different *Listeria* species. Initial MALDI-TOF MS studies were performed with well-characterized reference *Listeria* strains. MALDI-TOF MS was further used for identification of *L. monocytogenes* (86), *L. innocua* (9), *L. ivanovii* (20), *L. seeligeri* (15), *L. welshimeri* (4) and *L. grayi* (4) isolates. *L. monocytogenes* strains from outbreaks and clinical cases of human listeriosis were also included. All the *Listeria* strains were correctly identified at the species level. The identification result of

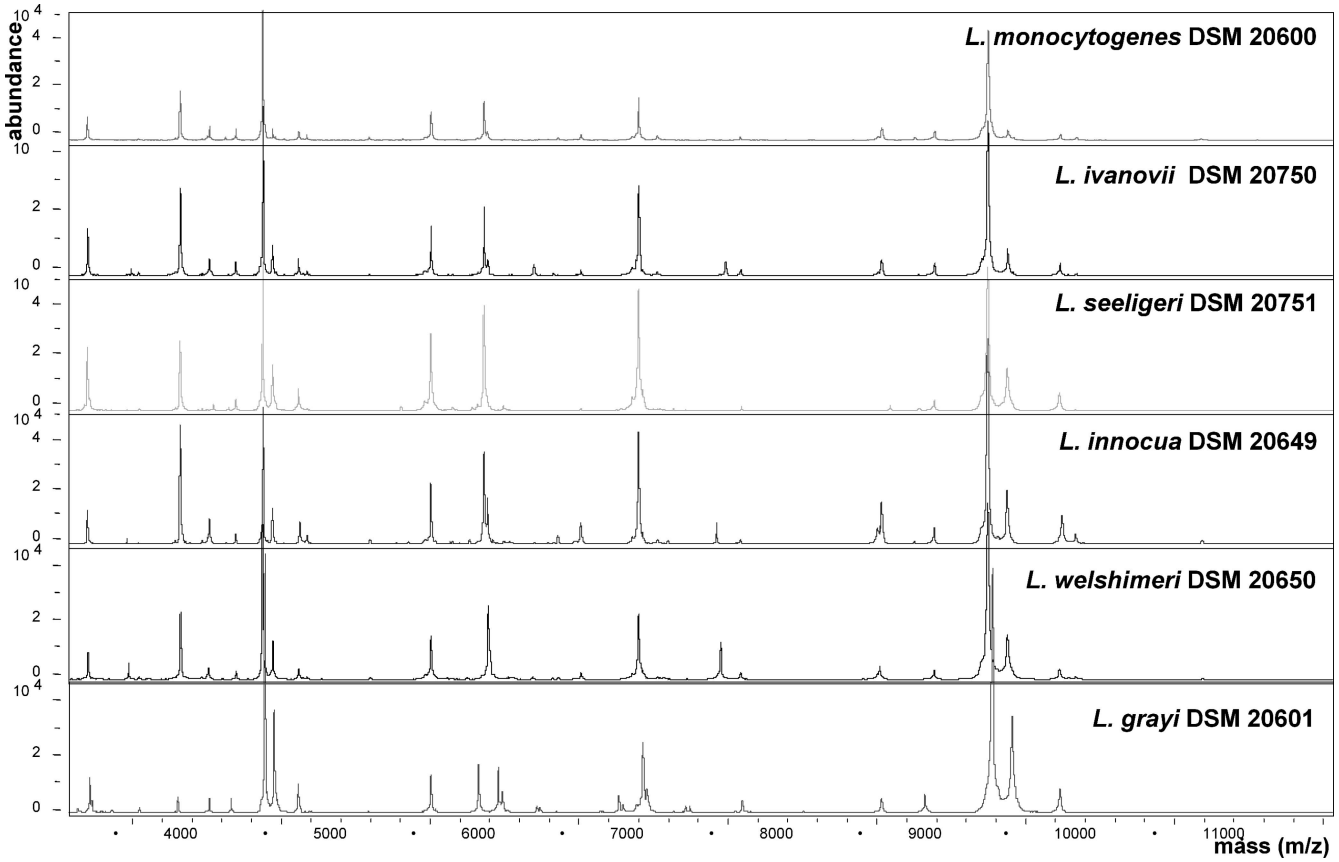

FIG. 1. MALDI-TOF MS spectra of whole-cell extracts of *Listeria* reference strains. Spectra representative of the MS profile were obtained from the German Collection of Microorganisms and Cell Cultures. The absolute intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis. The *m*/*z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein.

MALDI-TOF MS was confirmed in each case. Secure identification of *L. grayi* was possible by direct pattern matching against all of the patterns present in our library, representing diverse microbial species. For exact identification of the nearly related *Listeria* species, the weighted pattern matching approach leads in all cases to a correct identification result (see Table S2 in the supplemental material).

In a typical analysis of *Listeria* strains by MALDI-TOF MS, 20 to 50 prominent ion peaks were noted in the spectra in the region between 2,000 and 20,000 Da, with the highest-intensity peaks consistently in the range of 4,000 to 12,000 Da. Comparisons of spectra for multiple strains within a species revealed that multiple ions corresponded to a characteristic "fingerprint." Samples analyzed at different times over the course of several hours after calibration yielded results that were reproducible, indicating that the precision of the method was sufficient for a rapid determination of *Listeria* species. Mass abundance was reflected by the height of a peak. Masses illustrated in Fig. 1 for *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, and *L. grayi* are the values determined from an individual experiment. The method described here is based on detection of mainly ribosomal protein fractions of bacteria, which are the most abundant and conserved (31, 38, 39). The molecular weights of putative "species-identifying" biomarker ions were determined by averaging the results for multiple strains tested in multiple experiments using the BioTyper 1.1 software (Fig. 2). Different mass spectrometric methods for analyzing whole bacterial cells for intact proteins, including the identification of protein biomarker ions, have been reported previously (6, 18, 23). *Campylobacter* and *Helicobacter* strains were analyzed by MALDI-TOF MS, and biomarker ions in the 10- to 20-kDa range (presumably proteins) were reported to be the most discriminatory of those observed (44).

PFGE is currently the method of choice for investigating food-borne outbreaks of listeriosis and for tracing the outbreak-causing strain to the source of contamination. We therefore performed PFGE analysis of the *Listeria* species and *L. monocytogenes* serotypes in this study. PFGE is highly discriminatory (see Fig. S1 in the supplemental material) and permitted the grouping of the various serotypes in three lineages after ApaI digestion. Analysis of patterns generated by PFGE showed three lineages (29), lineage I (serotypes 1/2b, 3b, 4b, 4ab, 4d, 4e, 7), lineage II (serotypes 1/2a, 1/2c, 3a, 3c), and lineage III (serotypes 4a and 4c) (Fig. 3).

The MALDI-TOF MS method is robust and less laborious. The cost of instrumentation is comparable to that of a medium-throughput capillary-based DNA sequencing machine, but running costs and consumables are considerably lower than those for other identification methods. The sample processing is easier than that for PFGE. The method can separate the *L.*

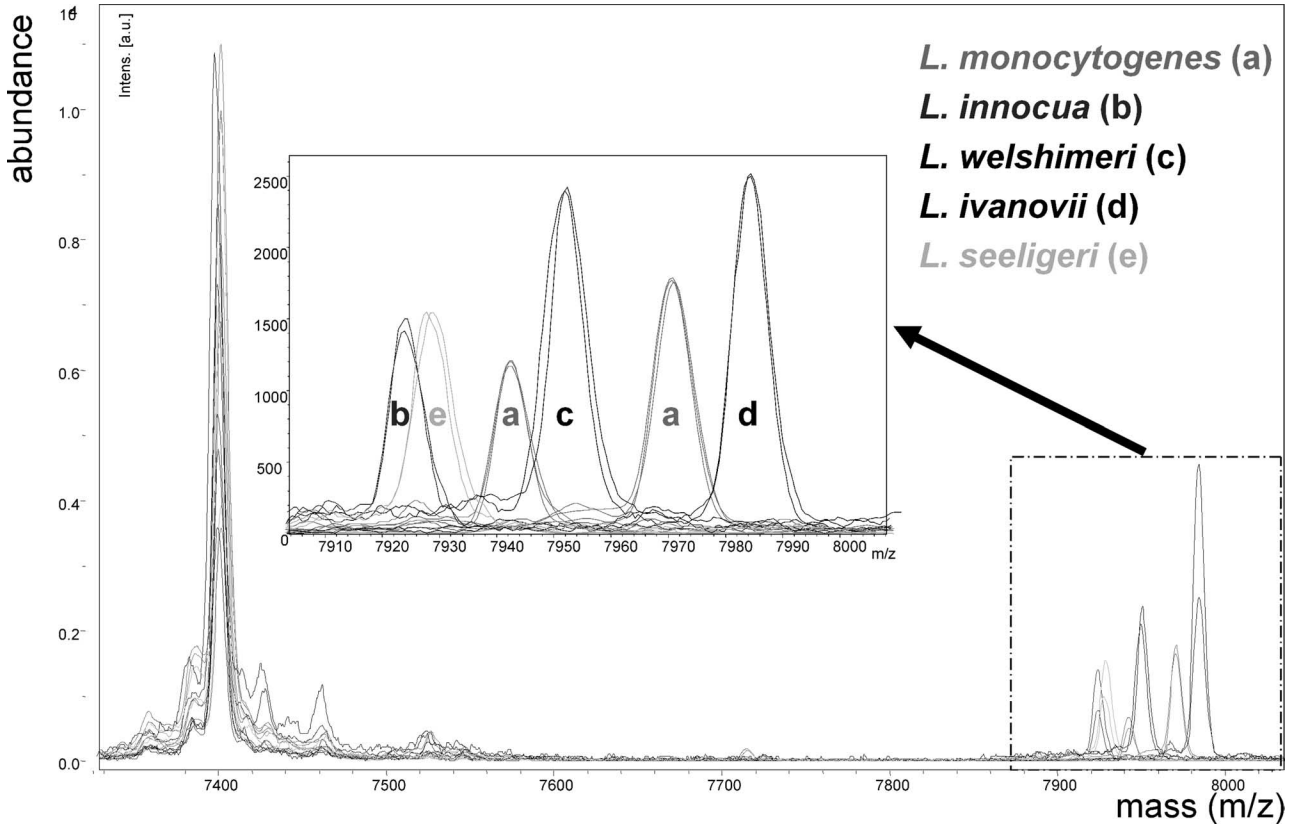

FIG. 2. Discriminating peaks of *Listeria* strains analyzed by MALDI-TOF MS. The absolute intensities of the ions are shown on the *y* axis, and the masses (in Da) of the ions are shown on the *x* axis. The *m*/*z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein.

monocytogenes isolates up to the level of clonal lineages. Two different peak pairs (5,590/11,179 Da and 5,597/11,193 Da) lead to a separation of lineages. A stable variation at 7,970 Da can differentiate *L. monocytogenes* serotypes 4a and 4c from other serotypes. Several *L. monocytogenes* serotype 4a and 4c strains also showed a peak at 5,590/11,179 Da. The PFGE lineages were in complete agreement with the MALDI-based groupings (Table 1; see Table S1 in the supplemental material).

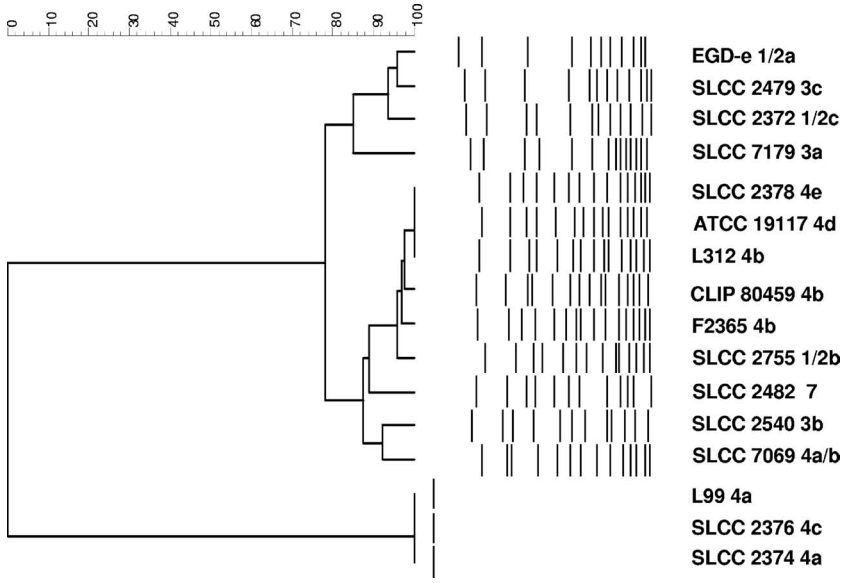

FIG. 3. Dendrogram derived from a PFGE profile of ApaI macrorestriction showing restriction patterns among the *L. monocytogenes* serotypes. Isolate identification numbers and serotype are indicated to the right.

TABLE 1. Comparative analysis of *L. monocytogenes* serotypes by MALDI-TOF MS and PFGE

Serotype	Source	Lineage by:	
		MALDI-TOF MS	PFGE (ApaI digestion)
1/2a	EGD-e	II	II
1/2b	SLCC 2755	I	I
1/2c	SLCC 2372	II	II
3a	SLCC 7179	II	II
3b	SLCC 2540	I	I
3c	SLCC 2479	II	II
4a	SLCC 2374	III	III
4a/b	SLCC 7069	I	I
4b	L312	I	I
4c	SLCC 2376	III	III
4d	ATCC 19117	I	I
4e	SLCC 2378	I	I
7	SLCC 2482	I	I
4a	L99	III	III
4b	F2365	I	I
4b	CLIP 80459	I	I

Ten discrepancies between the MALDI-based data and the original assignments given in the strain collection were found. We reanalyzed all of these strains using 16S rRNA gene sequencing for species differentiation and phylogenetically based PCR analysis for clonal-lineage determination (data not shown). In every case, the MALDI-based assignments were accurate and correctly predicted concerning species and clonal lineage (see Table S1 in the supplemental material).

A reference database employing the main spectra generated by analysis of *Listeria* strains by MALDI-TOF MS, which can easily be transferred among different laboratories using the identification system, has been generated. The database can serve as a ready source for identification of the *Listeria* strains in future analyses. A dendrogram generated using the *m/z* signatures of all the species tested indicated that the mass spectral data contained sufficient information to distinguish between species of *Listeria*.

Reproducibility, speed, and sensitivity of analysis are major advantages of the MALDI-TOF MS method. Extended periods of *Listeria* growth on agar plates, i.e., for several days, had only minor effects and did not affect the identification result. Even after 4 days of cultivation, the spectra revealed peaks sufficient for correct species identification. Reproducibility tests were also performed by cultivation of bacteria on brain heart infusion agars purchased from different manufacturers, but also with media not commonly used for the cultivation of *Listeria*. Identification to the species level was shown for *L. monocytogenes* for all kinds of media (see Table S3 in the supplemental material). Also, the interlaboratory reproducibility with different instruments was investigated using three different Bruker MALDI-TOF MS systems (Microflex LT, Autoflex II, and Ultraflex III) in two different Bruker laboratories, located in Bremen and Leipzig, Germany. No significant influence among the different laboratories and different MALDI-TOF MS systems regarding spectrum quality and identification results was observed.

Spectrum quality, as judged from the width of the diagnostic mass range and from the number of diagnostic ions generated, is largely determined by the matrix-solvent system, i.e., by MALDI sample preparation, whereas the reproducibility of spectra depends largely on bacterial-sample handling techniques. Earlier methods for various pathogens used different protocols for sample preparations and were based on cell surface proteins or intact cells. More recently, whole-cell MALDI-TOF MS has been used to rapidly differentiate microorganisms (24). The MALDI-TOF MS approach generates unique *m/z* signatures for different microorganisms based on inherent differences in the cellular proteins expressed by the microbiological species. Earlier studies of the detection of *Aeromonas* spp. (12) and *Campylobacter* species (27) by MALDI-TOF MS used whole cells for generation of mass spectra. Species from the genera *Escherichia* (7), *Helicobacter* (11), *Bacillus* (23, 40), *Salmonella* (25), and *Pseudomonas* (40) have been characterized by MALDI-TOF MS. To our knowledge, this is the first study employing the MALDI-TOF fingerprinting technique for the in-depth analysis of *Listeria*. We applied a method which inactivates the bacteria and extracts the cell content, leading to excellent and reproducible mass spectra and accurate species identification. In addition, the inactivated bacteria can be stored in ethanol for months in advance of cell extraction. Even the bacterial extracts can be stored at −20°C for weeks without significant loss in spectrum quality (data not shown).

The major advantages of using MALDI-TOF MS-based protein mass spectral fingerprinting for the identification of microorganisms as well as for their differentiation at the subspecies level are ease and speed of analysis combined with relatively simple sample preparation techniques, the potential for the identification of mixed cultures, and rapid identification of candidate biomarkers, even when minimal genetic data are available. The accuracy and speed of data acquisition make MALDI-TOF MS a powerful tool especially suited for environmental monitoring and detection of biological hazards in food processing plants and in the hospital. Additionally, no initial assessment of samples like Gram staining, oxidase testing, or microscopy is necessary.

We have developed an expanded MALDI-TOF MS method for analyzing multiple *Listeria* species, applied this method to determine species and lineages, and validated the method using *Listeria* strains isolated from a variety of sources. MALDI-TOF MS is shown to be a rapid and powerful tool for the fast and simple discrimination of *Listeria* species, which could be useful for food processing establishments and for tracking epidemic outbreaks.

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