APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2006, p. 1180–1189 Vol. 72, No. 2
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.2.1180–1189.2006

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for the Discrimination of Food-Borne Microorganisms

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Received 19 September 2005/Accepted 9 November 2005

A methodology based on matrix-assisted laser desorption ionization–time of flight mass spectrometry of intact bacterial cells was used for rapid discrimination of 24 bacterial species, and detailed analyses to identify *Escherichia coli* O157:H7 were carried out. Highly specific mass spectrometric profiles of pathogenic and nonpathogenic bacteria that are well-known major food contaminants were obtained, uploaded in a specific database, and made available on the Web. In order to standardize the analytical protocol, several experimental, sample preparation, and mass spectrometry parameters that can affect the reproducibility and accuracy of data were evaluated. Our results confirm the conclusion that this strategy is a powerful tool for rapid and accurate identification of bacterial species and that mass spectrometric methodologies could play an essential role in polyphasic approaches to the identification of pathogenic bacteria.

Rapid and reliable identification of microorganisms is a required and routine task that is carried out in various types of studies, including medical, food safety, and environmental studies.

Traditional methods for bacterial identification and classification, which are based on analysis of morphological, physiological, and biochemical features or genetic approaches (DNA-DNA or RNA-DNA hybridization, determination of G+C contents of DNAs,), are now complemented by sequence analysis of small-subunit rRNAs by PCR methods (3, 10, 20, 36, 45). Notwithstanding these recent developments, alternative complementary analytical approaches that ensure minimal time consumption, low cost, high accuracy, and sensitivity are still an area for investigation.

Mass spectrometric methodologies have been used for bacterial characterization since 1975 (1), and the first results were obtained by pyrolysis mass spectrometry (MS) (23, 25, 28, 42), fast atom bombardment mass spectrometry (7, 27), gas chromatography-mass spectrometry (19), and electrospray ionization mass spectrometry (17, 24, 56). However, significant progress in this field was marked by the introduction of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (29, 33). Briefly, in MALDI-TOF MS samples are mixed with an appropriate matrix material, and upon laser irradiation, sample molecules are ionized and desorbed as gaseous ions. These ions are accelerated and transferred to an analyzer, where they are separated and detected based on the ratio of molecular weight to charge (m/z). The advantages of this technique, such as the remarkable tolerance for contaminants and the ability to simultaneously detect different components in a broad m/z range present in complex mixtures, made it possible to analyze intact bacterial cells by mass spectrometry (ICMS) for the first time. Due to experimental conditions used during the MS analysis, particular surface components and/or proteins released from bacterial cells after partial cellular lysis are ionized, giving rise to signals at different m/z values (18, 41). Therefore, the mass spectra can be considered specific fingerprints or molecular profiles of the bacteria analyzed. Since the introduction of the strategy in 1996 (5, 30), researchers have realized that this method could become a rapid method for bacterial discrimination that involves minimal sample handling. In the last 10 years, several protocols for ICMS analysis have been proposed. In fact, the reproducibility and accuracy of mass spectra can be affected by different experimental conditions, such as the culture medium, the growth time, the bacterial concentration, sample preparation, and the MALDI matrices (13, 14, 22, 40, 41, 43, 49, 51, 57). At the same time, bioinformatic tools were developed in order to use mass spectrometric data for bacterial identification by means of searches either in protein databases available on the Web or in mass spectrum reference libraries constructed in house. The whole body of publications led to the conclusion that highly reproducible and accurate mass spectral profiles having characteristic peaks in the m/z range up to 15,000 Da can be obtained by ICMS, thus allowing rapid identification and/or discrimination of bacteria belonging to different genera and species (2, 4, 11, 12, 32, 34, 48, 58, 61).

Here, we describe an extensive ICMS study carried out with the most common food-borne pathogens. In particular, a systematic study of several species belonging to the genera *Escherichia*, *Yersinia*, *Proteus*, *Morganella*, and *Salmonella*, reported in detail here for the first time, resulted in a more comprehensive picture of the family *Enterobacteriaceae*. Moreover, as an example of a useful application, 25 *Escherichia coli* strains were analyzed, which resulted in unambiguous identification of *E. coli* O157:H7. Finally, the results obtained for five species

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TABLE 1. Bacterial strains analyzed

Taxon	No. of strains	Strain(s) and/or source
Escherichia hermannii	1	DSMZ 4560
Escherichia fergusonii	1	DSMZ 13698
Escherichia vulneris	1	DSMZ 4564
Escherichia blattae	1	DSMZ 4481
Escherichia coli	16	ATCC 25922, DSMZ 301, DSMZ 5802, DSMZ 8695, DSMZ 8696, DSMZ 8700, DSMZ 8711, DSMZ 9024, DSMZ 9029, DSMZ 9030, DSMZ 9031, DSMZ 9033, DSMZ 10722, DSMZ 11752, DSMZ 30083, University of Udine
Escherichia coli O157:H7	9	ATCC 43894, University of Udine
Yersinia enterocolitis	2	NCTC 9499, NCTC 11503
Yersinia kristensenii	1	NCTC 11471
Yersinia frederiksenii	1	NCTC 11470
Yersinia intermedia	1	NCTC 11469
Salmonella enterica serovar Enteritidis	2	University of Udine
Salmonella enterica serovar Typhimurium	2	ATCC 6994, University of Udine
Pseudomonas fluorescens	1	University of Udine
Pseudomonas putida	1	University of Udine
Proteus mirabilis	1	University of Udine
Proteus vulgaris	1	University of Udine
Morganella morganii	1	University of Udine
Staphylococcus aureus	3	ATCC 6538, ATCC 14458, ATCC 25923
Micrococcus spp.	1	University of Udine
Lactococcus lactis	1	University of Udine
Leuconostoc mesenteroides	1	University of Udine
Listeria monocytogenes	3	ATCC 9525, ATCC 15313, ATCC 19114
Listeria innocua	1	University of Udine
Listeria ivanovii	1	University of Udine
Listeria seeligeri	1	University of Udine
Listeria welshimeri	1	University of Udine

belonging to the genus *Listeria* are reported below as a clear example of ICMS analysis of gram-positive bacteria.

Mass spectrometric data were processed by using original data processing software, and bacterial molecular profiles truly representative of the species analyzed were obtained. Molecular profiles of all the bacteria analyzed were collected in order to set up a database to identify food contaminant microorganisms that cause human diseases (http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm). At present, this database contains information on about 50 bacterial strains belonging to 24 species and, among the databases that are freely available, it is the most complete database for the genera analyzed in this study.

In the near future, analytical tools developed in the present study could become a reliable approach for rapid screening of bacterial species to better direct microbiological investigations toward the detection of specific pathogens.

MATERIALS AND METHODS

Cytochrome *c*, sinapinic acid, and α-cyano-4-hydroxycynnamic acid were purchased from Sigma (St. Louis, Mo.). Trifluoroacetic acid (TFA) and all the organic solvents used were the highest purity available from Carlo Erba (Milan, Italy). Nutrient broth (NB), tryptone soya agar, tryptone soya broth (TSB), tryptone, and yeast extract were purchased from Oxoid (Basingstoke, Hampshire, United Kingdom). A third culture medium, tryptone yeast broth (TYB), was prepared using 5 g liter^{-1} tryptone and 2.5 g liter^{-1} yeast extract at pH 7.0.

Bacterial cultures. Bacterial cultures (Table 1) were obtained from the American Type Culture Collection (Manassas, Va.), from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), from the National Collection of Type Cultures (PHLS Central Public Health Laboratory, London, United Kingdom), and from the Department of Food Science of the University of Udine, Udine, Italy.

Growth of bacterial samples. Bacterial samples were prepared by using a two-step procedure. Bacteria from slants were first inoculated into culture broth and incubated for 24 h at 37°C, and then fresh broth was inoculated with 1% of the resulting bacterial cultures and incubated under the same growth conditions. To standardize the sample preparation protocol for MALDI-TOF MS analysis, three culture media (NB, TSB, and TYB) and three growth times (18 h, 24 h, and 48 h) were tested using *E. coli* ATCC 25922 as a model system.

Bacterial growth was determined both by plate counting on tryptone soya agar and by measuring the optical density at 600 nm. Frozen samples were prepared by storing fresh bacterial cultures in 10% dimethyl sulfoxide (DMSO) or 20% glycerol at −80°C.

Finally, three bacterial cultures were produced for each strain using the optimized protocol.

Sample preparation and loading on MALDI target plate. One-milliliter aliquots of a bacterial culture containing 1×10^9 cells, as determined by optical density, were centrifuged at 13,000 rpm for 5 min to collect the cells, and the supernatants were discarded. For sample preparation for MALDI-TOF MS analysis, we used a method based on the method described by Dai and coworkers (9). Cellular pellets were washed once in 1 ml of water and twice in 1 ml of 0.2% TFA. After each step samples were centrifuged at 13,000 rpm for 5 min. The pellets were dissolved by vortexing for 2 min in an appropriate volume of 0.2% TFA to obtain cell concentrations ranging from 10^3 to 10^8 cells μl^{-1}.

Aliquots (0.5 μl) of a bacterial suspension were deposited on a MALDI target plate, mixed with 0.5 μl of matrix (10 mg ml^{-1} α-cyano-4-hydroxycynnamic acid in 50% acetonitrile containing 1 pmol μl^{-1} of cytochrome *c* as an internal standard), and dried under ambient conditions. Then 0.5 μl of matrix solution was added to each well and dried under ambient conditions. Frozen and lyophilized samples were prepared and analyzed by using the protocol described below.

Data acquisition and processing. All mass spectra were acquired with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, Calif.) operating in the linear, delay extraction, positive-ion mode. The laser intensity (N_2, 337 nm) was set just above the ion generation threshold. The low mass gate was set to *m/z* 2,400, the delay time was 550 ns, the accelerating voltage was 25,000 V, and the grid voltage and guide wire were set to 95% and 0.3% of the accelerating voltage, respectively. From the three cultures produced for each bacterial strain, triplicate mass spectra were acquired by accumulating 100 laser shots in the *m/z* range from 2,500 to 15,000. Spectra were calibrated using as internal standards the double and singly charged peaks that originated from cytochrome *c* (*m/z* 6,181 and 12,361, respectively) so that the error for the

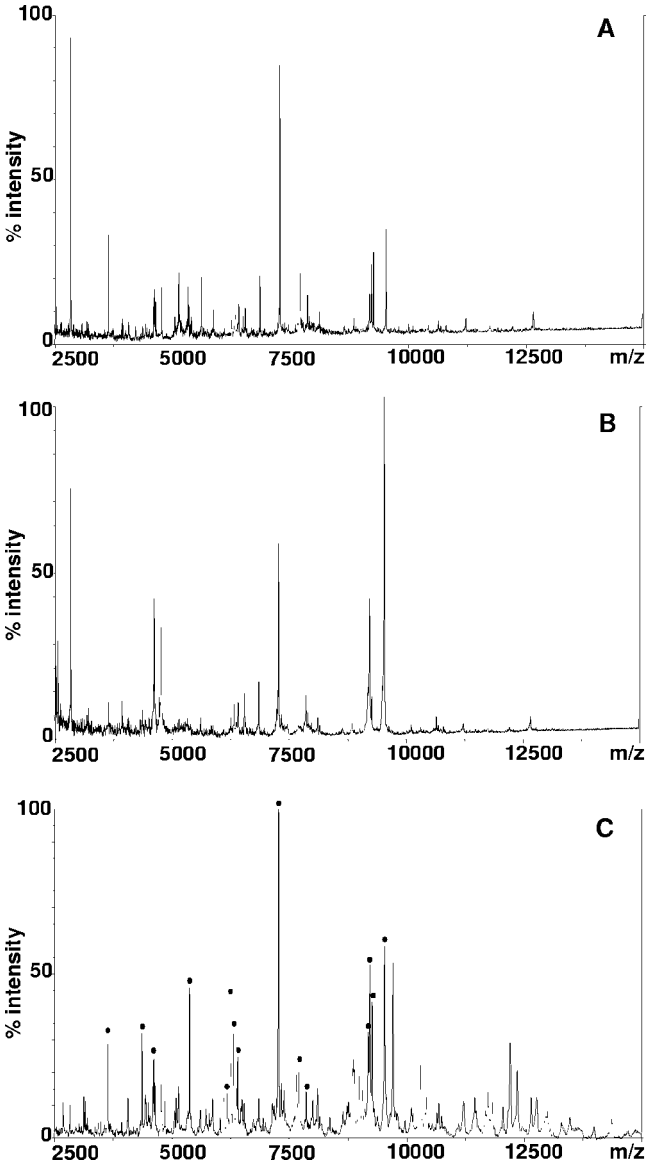

FIG. 1. Mass spectrometric profiles obtained from the analysis of *E. coli* ATCC 25922 grown in different culture media, including TYB (A), NB (B), and TSB (C). Signals that the profiles have in common are indicated by dots in panel C.

experimental mass measurements was less than 5 Da. All mass values were reported as average masses. The background was subtracted from the mass spectra, and the spectra were smoothed with Gaussian smoothing with the filter width set to 11 points. Data lists containing *m/z* values and corresponding peak intensities were extracted from mass spectral data with the specific software of the instrument (Data Explorer, version 4.0.0.0) by means of a macro in the Visual Basic language provided by the producer. Signals with intensities greater than 5% were included in the lists. Data lists were further processed with original data processing software developed in the Visual Basic language as a Microsoft Excel application. The program selected *m/z* values present in all nine spectra acquired for each bacterial strain, taking into account a tolerance error of ±7 Da, and it calculated arithmetic means and standard deviations both for *m/z* values and for the corresponding intensities. The accuracy of mass measurements was such that the standard deviation was less than ±3 Da. The specific peak lists generated by this program constituted the bacterial molecular profiles. Species-specific biomarkers were extracted by comparison of the peak lists obtained.

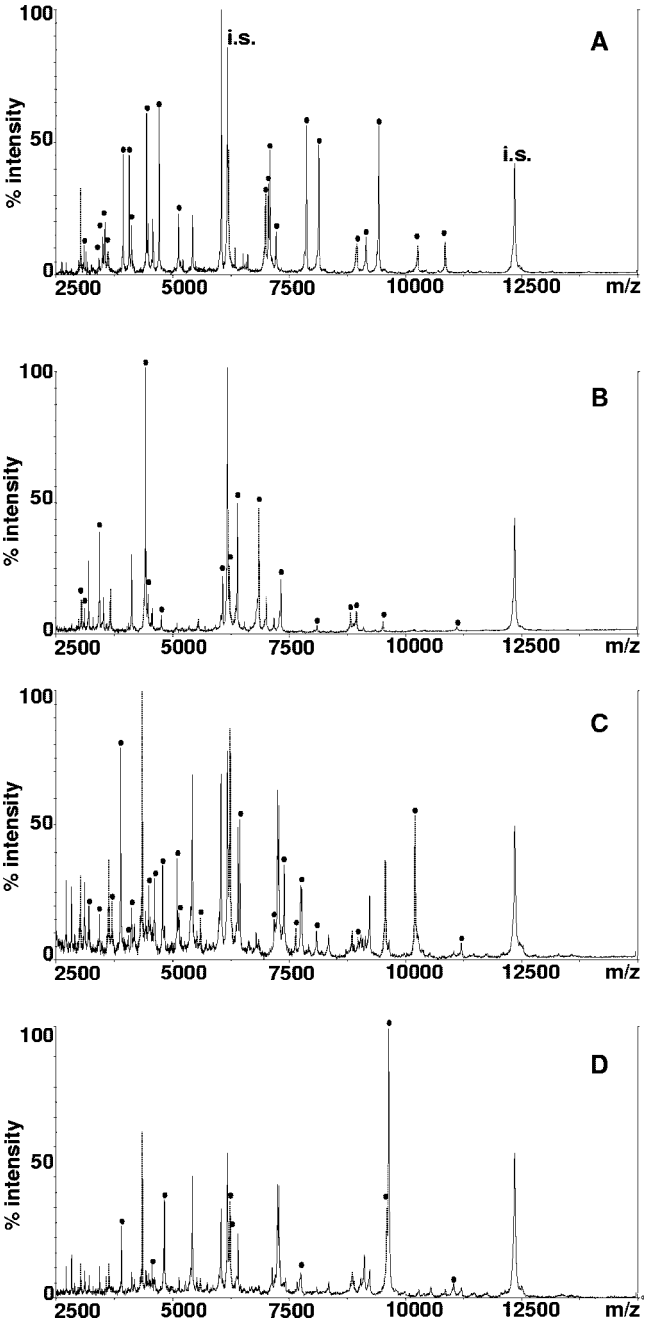

FIG. 2. Mass spectrometric profiles obtained from the analysis of *Yersinia* species, including *Y. enterocolitica* (A), *Y. intermedia* (B), *Y. frederiksenii* (C), and *Y. kristensenii* (D). Species-specific biomarkers are indicated by dots.

RESULTS

Standardization of sample preparation protocol for MALDI-TOF MS analysis and creation of the database for food-borne bacteria. To standardize a sample preparation protocol for MALDI-TOF MS analysis, several experimental parameters were evaluated using *E. coli* ATCC 25922 as a model microorganism.

In order to test the reproducibility and accuracy of the ICMS analysis, three bacterial cultures were produced in TSB on

TABLE 2. Peaks obtained from the analysis of four *Yersinia* species[a]

Yersinia frederiksenii NCTC 11470		*Yersinia kristensenii* NCTC 11471		*Yersinia intermedia* NCTC 11469		*Yersinia enterocolitis* NCTC 11503	
Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity
2,715.10	22.41					2,715.90	12.59
2,828.06	22.01					2,828.61	12.83
		3,026.89	50.79			3,025.23	13.42
				3,040.24	**18.36**		
				3,113.28	**14.56**		
3,122.25	21.17						
3,209.04	**16.52**						
		3,145.09	**16.24**				
				3,429.40	**36.67**		
		3,503.55	**23.63**				
		3,536.78	**24.25**				
		3,551.77	**28.03**				
		3,619.93	**18.50**				
3,632.53	34.64					3,632.88	15.53
				3,665.55	**19.93**		
				4,428.40	**100.00**		
3,701.72	**20.92**						
3,892.18	**55.00**						
						3,906.69	**17.57**
		3,943.93	**36.99**				
		4,072.44	**52.92**				
4,350.45	96.86					4,350.73	38.84
4,432.50	**18.61**						
		4,451.19	**58.70**				
		4,481.83	25.33	4,476.33	24.95		
4,496.77	**23.36**						
						4,563.78	**13.68**
				4,572.90	**14.78**		
		4,583.04	**18.74**				
4,619.06	**24.50**						
		4,716.96	**64.65**				
				4,763.07	**28.34**		
4,788.63	**31.00**						
						4,804.71	**25.88**
						4,826.47	**58.62**
5,106.54	**33.50**						
		5,136.18	**24.43**				
5,149.85	**14.78**						
5,426.82	68.90	5,432.57	26.99			5,427.33	34.51
5,606.83	**13.64**						
6,044.67	64.07	6,050.27	90.63			6,044.96	28.24
				6,078.83	**25.60**		
				6,223.89	**48.04**		
6,240.29	77.58					6,240.30	31.93
				6,398.85	**27.90**		
6,414.04	46.51					6,414.61	16.16
6,454.11	**46.69**						
				6,857.04	**46.41**		
		7,070.40	**31.53**				
		7,100.91	**42.72**				
7,187.21	**17.58**						
		7,232.20	**19.24**				
7,261.26	78.32					7,260.93	30.58
7,286.38	62.31					7,286.67	25.67
						7,315.39	**24.01**
				7,329.47	**27.07**		
7,399.70	**43.07**						
7,652.76	**14.16**						
						7,688.11	**7.79**
						7,745.12	**20.73**
7,761.11	**26.53**						
7,779.84	**24.96**						
		7,843.03	**10.35**				
		7,884.96	**35.55**				
8,097.96	**13.56**						

Continued on following page

TABLE 2—*Continued*

Yersinia frederiksenii NCTC 11470		*Yersinia kristensenii* NCTC 11471		*Yersinia intermedia* NCTC 11469		*Yersinia enterocolitis* NCTC 11503	
Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity	Avg *m/z*	Avg intensity
				8,105.07	**5.79**		
		8,142.43	**43.67**				
				8,824.14	**10.44**		
8,861.84	13.64					8,865.21	8.78
				8,887.75	**6.36**		
				8,952.56	**14.42**		
		8,960.98	**13.18**				
9,051.63	**14.07**						
						9,125.20	**14.08**
9,145.59	**12.75**						
		9,163.08	**11.88**				
9,235.73	26.72					9,236.15	9.34
		9,390.28	**9.88**				
		9,431.36	**46.30**				
				9,524.91	**20.31**		
9,574.62	**37.64**						
						9,606.90	**33.59**
						9,650.71	**100.00**
10,211.63	**59.32**						
		10,270.03	**10.62**				
		10,863.60	**12.31**				
						11,052.01	**5.30**
				11,115.54	**5.61**		

[a] Species-specific biomarkers are indicated by boldface type.

different days; each sample was loaded into three wells of a MALDI target plate, and mass spectra were acquired in triplicate. Electronic processing (as described in Materials and Methods) of the mass spectrometric data obtained from the analysis of a single culture allowed us to obtain a peak list containing around 50 peaks, and, considering all 27 mass spectra acquired, a lower number of constant signals (34 peaks) was obtained. This confirmed that there was a certain variability in the mass spectra obtained from different cultures. Therefore, all the experiments described below were carried out with three different bacterial cultures grown over a period of 3 months, and triplicate mass spectra were acquired from each bacterial culture.

To test the effects of different culture media and growth times, *E. coli* ATCC 25922 was grown in NB, TYB, and TSB, and 1-ml aliquots of each broth culture were taken at 18 h, 24 h, and 48 h. Different growth times did not affect the bacterial molecular profiles significantly (data not shown), while some differences were discerned in the mass spectra obtained from the analysis of *E. coli* grown in different culture media. However, it was possible to extrapolate a set of at least 15 signals present in all the mass spectra obtained from the analysis of bacteria grown in different culture media, while there were major differences in the peak intensities (Fig. 1; see Table SA in the supplemental material). High-quality mass spectra were obtained by analyzing *E. coli* samples grown in TSB, since they had the greatest number of signals and the highest signal-to-noise ratio. To minimize the influence of medium composition on mass spectra, TSB was used for all subsequent experiments.

Analyses were also carried out with broth cultures frozen in 10% DMSO or 20% glycerol or with lyophilized cellular pellets. The quality and reproducibility of mass spectra acquired from cell samples subjected to freezing or lyophilization were low, and there was increased background noise and decreased mass signal intensity, probably due to uncontrolled breakage of bacterial cells. In particular, the peak lists obtained with lyophilized samples contained a lower number of constant peaks (19 values) compared with peak lists obtained with fresh bacterial cultures (34 values). Similarly, only 20 peaks were present in the lists obtained with samples frozen either in glycerol or in DMSO. Therefore, all the experiments were carried out with fresh broth cultures. Different cell concentrations (1×10^3 to 1×10^8 cells μl^{-1}) spotted on MALDI target plates were also tested. Mass spectra were obtained only when samples containing about 1×10^5 to 10^6 cells were analyzed, while either lower or higher cell concentrations produced no useful spectra.

In conclusion, the best experimental conditions for obtaining good-quality and highly reproducible spectra were as follows: bacterial cells grown in TSB at 37°C for 24 h and analyzed directly by spotting 1×10^6 cells in each well.

Bacterial strains listed in Table 1 were analyzed using the optimized protocol, and mass spectrometric data were processed by means of the electronic processing program. Peak lists that contained the most reproducible and significant signals that originated from the mass spectrometric analysis constituted unambiguous molecular profiles of the bacterial species analyzed. These molecular profiles and representative mass spectra were used to create a specific database, designated "FoodBIMS," which is freely available on the website http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm.

Results obtained for species belonging to the family *Enterobacteriaceae* and the genus *Listeria* are described in detail below. Results obtained for the other species have been uploaded in the database due to the great amount of experimental data.

Definition of molecular profiles of species belonging to the family *Enterobacteriaceae* and the genus *Listeria*. For the genus *Yersinia*, four species that are widely found as food contaminants were analyzed, *Yersinia enterocolitis*, *Yersinia kristensenii*, *Yersinia frederiksenii*, and *Yersinia intermedia*,. The ICMS analysis gave mass fingerprints containing a different number of peaks for each species, and the number of peaks ranged from 20 to 35 (Fig. 2 and Table 2). The four species could be easily discriminated by means of a direct comparison of the mass spectra. In fact, by using electronic processing, we found that the most intense signals corresponded to the species-specific biomarkers and no genus-specific biomarkers could be defined.

Similarly, the most common *Escherichia* species were analyzed (*E. coli*, *Escherichia hermannii*, *Escherichia vulneris*, *Escherichia blattae*, and *Escherichia fergusonii*), and the number of *m*/*z* values on the peak lists ranged from 30 to 40. The ICMS analysis revealed the presence of several species-specific biomarkers among the most intense signals present in the spectra. Interestingly, two genus-specific biomarkers were also detected. Although there was a high level of similarity between the peak lists of *E. coli* and *E. fergusonii* (80% of the peaks were present in both lists), it was possible to easily discriminate between these organisms by identifying a few significant biomarkers (Fig. 3; see Table SB in the supplemental material).

Not surprisingly, the analysis of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis resulted in fingerprints that were more similar to the fingerprints obtained from the analysis of *E. coli* than to the fingerprints obtained for all the other *Enterobacteriaceae* species. Nevertheless, these taxa could be discriminated on the basis of specific biomarkers. A direct comparison of the peak lists obtained by analyzing the two serovars indicated that they could be differentiated on the basis of two signals (see Table SC in the supplemental material).

Finally, the two *Proteus* species, *Proteus vulgaris* and *Proteus mirabilis*, and *Morganella morganii* had very different molecular profiles and could be easily discriminated (see Table SD in the supplemental material).

Gram-positive bacteria produced mass spectra that had a lower number of strong signals than the spectra of gram-negative bacteria, as revealed by the analysis of *Staphylococcus aureus*, *Listeria* spp., *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Micrococcus* spp., and *Sarcina flava* (see the website http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm). In particular, the five *Listeria* species analyzed in the present study (*Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria welshimeri*, and *Listeria seeligeri*) exhibited very similar mass fingerprints. Six genus-specific biomarkers were detected together with species-specific biomarkers (Fig. 4; see Table SE in the supplemental material). Interestingly, *L. welshimeri* had very peculiar mass spectra; in fact, as many as 13 biomarkers were present, and the most intense signal was at *m*/*z* 6,392 instead of at *m*/*z* 6,362, as detected for all the other species. These signals could have originated from the same protein having a slightly different amino acid sequence in *L. welshimeri*. The mass spectra of *L. monocytogenes* and *L. innocua* had 76% peaks in common, and these species could be discriminated on the basis of a few biomarkers.

Identification of *E. coli* O157:H7 by ICMS. To investigate the ability of the ICMS approach to rapidly identify *E. coli*

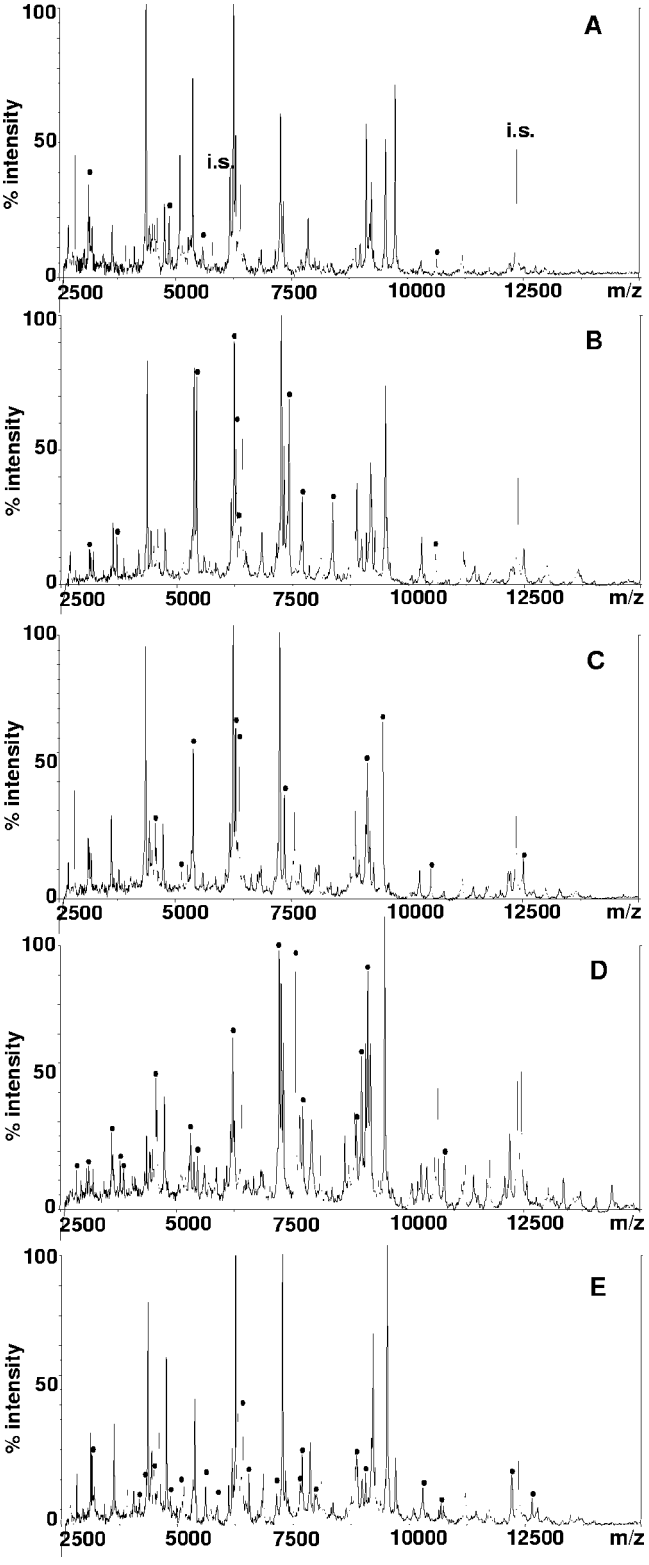

FIG. 3. Mass spectrometric profiles obtained from the analysis of *Escherichia* species, including *E. fergusonii* (A), *E. hermannii* (B), *E. vulneris* (C), *E. blattae* (D), and *E. coli* (E). Species-specific biomarkers are indicated by dots.

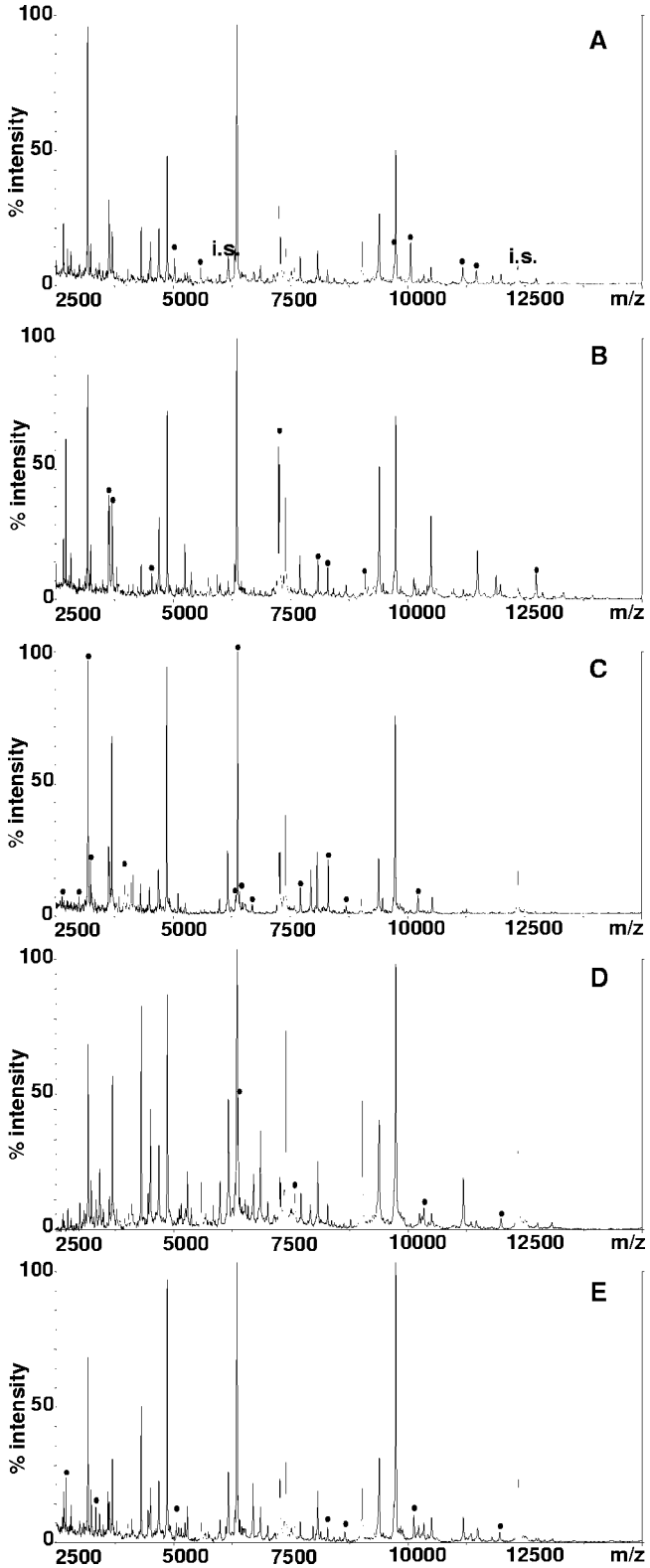

FIG. 4. Mass spectrometric profiles obtained from the analysis of *Listeria* species, including *L. monocytogenes* (A), *L. seeligeri* (B), *L. welshimeri* (C), *L. innocua* (D), and *L. ivanovii* (E). Species-specific biomarkers are indicated by dots.

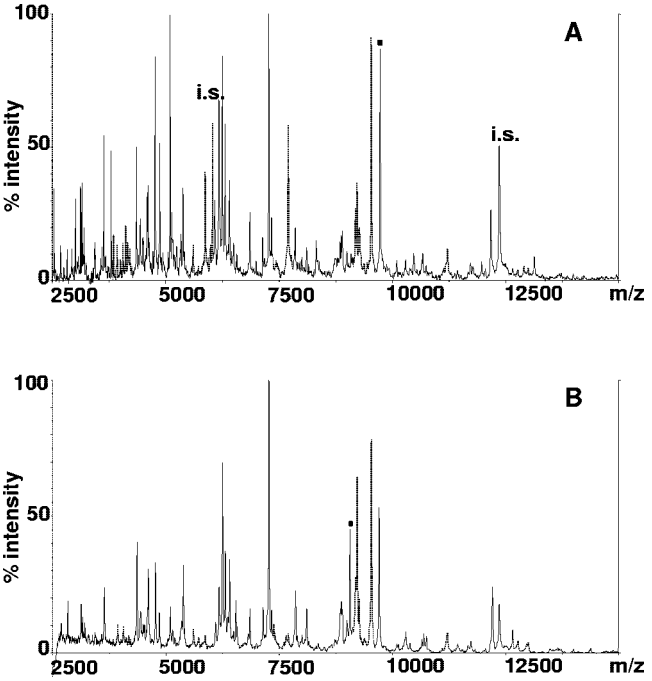

FIG. 5. Mass spectrometric profiles obtained from the analysis of *E. coli* O157:H7 (A) and non-O157:H7 *E. coli* (B). The signal at *m/z* 9,740 is indicated by a dot in panel A, and the signal at *m/z* 9,060 is indicated by a dot in panel B.

O157:H7, nine serotype O157:H7 strains and 16 *E. coli* strains that were different from O157:H7 were analyzed.

The mass spectra obtained from the analysis of the *E. coli* strains were very similar. In the mass spectral profiles of all the *E. coli* O157:H7 strains the most intense signal was at *m/z* 9,740, whereas a signal at *m/z* 9,060, which was present in the mass spectra obtained for non-O157:H7 *E. coli*, was never found (Fig. 5). These features made the mass spectral profiles of *E. coli* O157:H7 unique and allowed us to unambiguously identify this pathogen.

DISCUSSION

A description of morphological and physiological features is still the fundamental requirement for assigning an isolate to a genus or species of bacteria or for including new taxa in *Bergey's Manual* (46). Today, most microbiologists recommend a polyphasic approach which includes traditional taxonomic and bacteriological methods and nucleic acid analyses for successful bacterial classification and identification (52, 53). This is particularly important in the analysis of strains that exhibit a high level of sequence homology in the 16S rRNA gene but less than 50% DNA similarity as determined by DNA-DNA reassociation assays (6, 21, 26, 47, 52, 53, 59, 60). In view of this, ICMS analysis could be considered an accurate and reliable complementary tool for screening species with a minimal time requirement and reduced costs and handling of samples.

The present study was performed to analyze common foodborne microorganisms and to develop an additional analytical tool for rapid detection of pathogens. The capacity of ICMS to discriminate bacteria was ascertained by analyzing closely re-

lated species belonging to the genera *Escherichia*, *Yersinia*, *Salmonella*, *Proteus*, and *Morganella* representing the family *Enterobacteriaceae* and five *Listeria* species representing the gram-positive bacteria; the latter taxa have not been described in detail so far.

As reported previously, the reproducibility and soundness of the data acquired with regard to different experimental parameters are fundamental (18). Therefore, we optimized a protocol for preparation of bacteria and ICMS analysis which was strictly followed throughout the present study in order to improve the reproducibility of mass spectra.

The use of a single culture medium for all the bacterial species analyzed did not affect the results obtained; moreover, it prevented the presence of nonspecific signals which could be due to culture media. In fact, our results showed that a representative pattern of signals is always present in the mass spectra regardless of the medium used for bacterial growth, in agreement with the results reported by Valentine and coworkers (57).

Our study resulted in highly reliable and reproducible lists of peaks that were specific molecular profiles suitable for discriminating the 24 bacterial species examined.

Interestingly, in the family *Enterobacteriaceae*, no genus-specific biomarker could be found for the genus *Yersinia*, and only two signals are shared by all the *Escherichia* species. On the other hand, *E. coli* and *Salmonella* have a great number of signals in common, and a few biomarkers that were significant allowed us to discriminate between *E. coli* and *E. fergusonii*. This confirms that DNA-DNA relatedness can show up in mass spectrum profiles; in fact, *E. coli* and *Salmonella*, as well as *E. coli* and *E. fergusonii*, are very closely related (15, 38). Moreover, *M. morganii*, previously classified in the genus *Proteus*, does not have any signal in common with the two *Proteus* species according to the low level of DNA-DNA relatedness (<20%) (44).

The *Listeria* species selected for this study are very closely related to each other, belonging to the same subgroup (50); in fact, six genus-specific biomarkers could be defined. In addition, *L. monocytogenes* and *L. innocua*, which are discriminated with difficulty by traditional and genetic microbiological approaches, had very similar molecular profiles with a few species-specific biomarkers that resulted in unambiguous identification by ICMS analysis, while the other species could be differentiated more easily.

A thorough study of *E. coli* species showed that defining strain-specific biomarkers is difficult when a good number of strains are analyzed (25 strains were analyzed in our study) because it is more likely that signals are shared by different strains. However, our results led to unambiguous and clear identification of *E. coli* O157:H7, as also reported by Bright and coworkers, who used a different approach for processing mass spectrometric data (4). Computational processing of mass spectrometric data showed that bacterial strains with the O157:H7 antigen had very peculiar features in their MALDI spectra; more specifically, a very strong signal at *m/z* 9,740 was consistently present, and there was no signal at *m/z* 9,060, which was present in the mass spectra of all the other *E. coli* strains. In *E. coli* 1090, a non-O157:H7 strain, these signals were correlated with the mature forms of acid-resistant proteins HdeA and HdeB, respectively, after proteolytic cleavage of the peptide signal by the peptidase Lep (31). However, the length of the signal sequence of the HdeB protein in *E. coli* O157:H7 has not been reported yet, and the definition of a consensus sequence for peptidase Lep is still being studied (35). It could be hypothesized that *E. coli* O157:H7 expresses a slightly different mature form of HdeB, thus explaining the absence of the corresponding signal (*m/z* 9,060) in the mass spectra. For the signal at *m/z* 9,740, which was present only in seven of the non-O157:H7 *E. coli* strains analyzed in this study, it is not possible to rule out the possibility that in *E. coli* O157:H7 the signal could also originate from other proteins peculiar to this microorganism, such as those involved in pathogenicity.

TABLE 3. Numbers of protein entries in databases

Species	No. of entries	
	Swiss-Prot/TrEMBL	NCBInr
E. coli	22,675	93,585
E. blattae	3	20
E. vulneris	9	17
E. fergusonii	64	87
E. hermanii	12	18
Y. enterocolitica	644	1,285
Y. kristensenii	41	44
Y. frederiksenii	44	47
Y. intermedia	49	57
L. monocytogenes	7,062	30,047
L. seeligeri	51	92
L. welshimeri	24	37
L. ivanovii	84	105
Salmonella spp.	17,181	59,208
P. mirabilis	215	352
P. vulgaris	367	800
M. morganii	65	14

Analysis of *E. coli* O157:H7 by bacteriological methods still requires a great number of tests in order to obtain conclusive identification (16, 54). The results of recent methodologies based on PCR or biosensor technology suggest that methods for rapid and reliable detection of *E. coli* O157:H7 still need further development (8, 39). In this regard, ICMS analysis, which requires just a few minutes for unambiguous identification of this pathogen, is worth considering. Additionally, the ICMS approach could discriminate between *Y. enterocolitica* and *Y. enterocolitica*-like species more efficiently than other methods that have been described (55). ICMS analysis also led to successful discrimination of two *Salmonella* serovars, although a wider study of different serovars is needed to definitively asses the capability of the methodology for this task.

The applicability of ICMS to bacterial identification relies strictly on the availability of specific bioinformatic tools. Several workers have focused on the construction of reference libraries of mass spectra (2, 4, 34) and/or on the development of peculiar algorithms to use mass spectrometric data to identify bacteria by means of searches in protein databases available on the Web (11, 12). To the best of our knowledge, so far neither mass fingerprint databases nor mass spectrum reference libraries are available at no cost online. On the other hand, a problem in the identification of bacteria using lists of *m/z* values for searches in protein databases based on Swiss-Prot/TrEMBL or NCBInr data arises from the low number of

protein entries for bacteria whose genomes have been only partially sequenced. In fact, while there are about 23,000 and 93,000 protein entries for *E. coli* in the Swiss-Prot/TrEMBL and NCBInr databases, respectively, and 7,062 and 30,047 protein entries for *L. monocytogenes*, the numbers are drastically lower for other *Escherichia*, *Yersinia*, *Proteus*, *Morganella*, and *Listeria* species, as shown in Table 3. Additionally, postbiosynthetic processing of cellular proteins can often make it difficult to match signals present in mass spectra to the corresponding protein entries in the databases (37).

Therefore, bacterial identification based on searches in a database of lists containing *m*/*z* values could provide a more flexible approach. Data acquired with different instruments could be used for searches (62), and identification would not be hampered by the drawbacks described above.

At this time, our database is one of the most complete collections of bacterial molecular profiles determined by ICMS analysis, and we believe that this approach, which complements the well-established methods, could become a key tool in food safety control and in clinical microbiology.

ACKNOWLEDGMENTS

This work was supported by "Ministero dell'Università e della Ricerca Scientifica e Tecnologica" (LEGGE 449/97-99), project "Trattamento di prodotti freschi altamente deperibili per garantirne qualità, sicurezza e salubrità—PROFSICURI."

We thank Marie De Vito for revising the manuscript.

This article is especially dedicated, with respect and affection, to the memory of Arturo Leone, Head of the Institute of Food Science and Technology of the CNR, who died on 30 December 2005.

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