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Review

Identification of Pathogens by Mass Spectrometry

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BACKGROUND: Mass spectrometry (MS) is a suitable technology for microorganism identification and characterization.

CONTENT: This review summarizes the MS-based methods currently used for the analyses of pathogens. Direct analysis of whole pathogenic microbial cells using MS without sample fractionation reveals specific biomarkers for taxonomy and provides rapid and high-throughput capabilities. MS coupled with various chromatography- and affinity-based techniques simplifies the complexity of the signals of the microbial biomarkers and provides more accurate results. Affinity-based methods, including those employing nanotechnology, can be used to concentrate traces of target microorganisms from sample solutions and, thereby, improve detection limits. Approaches combining amplification of nucleic acid targets from pathogens with MS-based detection are alternatives to biomarker analyses. Many data analysis methods, including multivariate analysis and bioinformatics approaches, have been developed for microbial identification. The review concludes with some current clinical applications of MS in the identification and typing of infectious microorganisms, as well as some perspectives.

SUMMARY: Advances in instrumentation (separation and mass analysis), ionization techniques, and biological methodologies will all enhance the capabilities of MS for the analysis of pathogens.

Rapid and reliable identification of microorganisms without extensive manipulation is a major goal in environmental and clinical microbiology. Analysis of biological samples will always be challenging because of their great diversity and complexity *(1)*. The accurate identification of microorganisms using classic techniques is time-consuming and complicated. Early detection and characterization of microorganisms can help minimize health hazards and avoid the spread of microbe-related diseases. The requirements of modern methods suitable for microorganism identification and characterization are robustness, simple handling, low cost, rapidity, and high-throughput capability. Because it satisfies most of these requirements, mass spectrometry (MS)[2] is emerging as a technology capable of identifying microorganisms *(1)*.

Fig. 1 presents a flow chart of the microbial enrichment and analysis strategies used to identify microorganisms. In general, a sufficient number of microbial cells are collected, using culture or nonculture methods, before MS-based analysis. Nonculture approaches often use affinity techniques based on biochemical, chemical, and physical interactions with target cells. After collection of the cells, MS analysis of the samples is then performed. Direct sampling techniques such as bioaerosol mass spectrometry have been developed to analyze individual aerosolized microbial particles in real time. Coupling of nucleic acid amplification to MS combines the high amplification power of the former and the high mass-resolving power of the latter. To comprehensively investigate potential biomarkers, extraction of biomarkers from microbial cells followed by fractionation steps is conducted before mass spectrometric analysis. In the flow chart, direct analysis means that microbial cells are directly analyzed without using biomarker extraction and separation steps.

In biological systems, biomolecule-conjugated nanoparticles have been used to understand and manipulate biomolecular interactions, recognize molecular targets, and perform medical diagnoses. In addition, nanoparticles play important roles in the MS-based identification of microorganisms *(2, 3)*. Using functionalized magnetic nanoparticles to detect bacteria at ultralow concentrations requires less time and

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[2] Nonstandard abbreviations: MS, mass spectrometry; ESI, electrospray ionization; GC, gas chromatography; LC, liquid chromatography; NP, nanoparticle; POA, pigeon ovalbumin; DESI, desorption electrospray ionization; MS/MS, tandem MS; BAMS, bioaerosol mass spectrometry; CE, capillary electrophoresis; SPA, selective proteotypic-peptide analysis; BHP, bacteriohopanepolyol; HBV, hepatitis B virus; HCV, hepatitis C virus; SNP, single nucleotide polymorphism; TIGER, triangulation identification for the genetic evaluation of risks; SARS, severe acute respiratory syndrome; MVA, multivariate analysis; ANN, artificial neural network; PCA, principal component analysis; MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; ESBL, extended-spectrum β-lactamase.

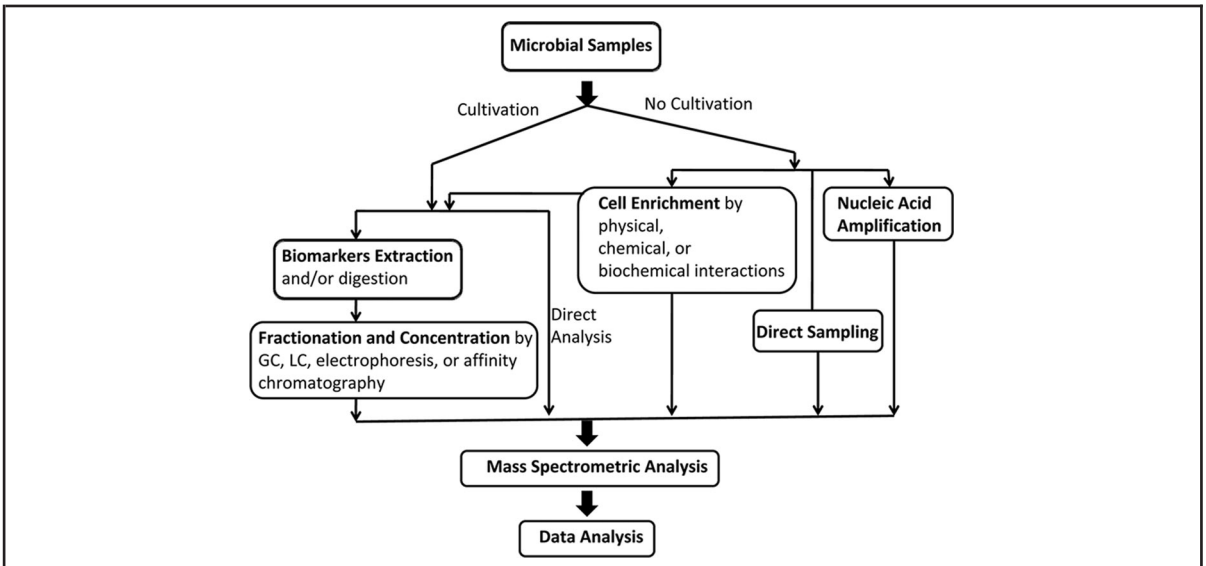

Fig. 1. Flow chart of microbial enrichment and analysis strategies for the identification of microorganisms.

offers obvious benefits and advantages in clinical research.

The development of 2 so-called soft ionization techniques—MALDI and electrospray ionization (ESI)—has provided practitioners of MS with the means to generate gas-phase ions from moderate to high molecular weight thermally labile compounds. Microorganism identification using MALDI- and ESI-MS techniques is an excellent alternative to traditional laboratory methods used in medicinal and clinical microbiology. Recently, advanced techniques such as desorption electrospray ionization have been applied to identification of bacteria. Direct identification of bacteria using MALDI, ESI, and bioaerosol mass spectrometry provides several advantages, such as simplicity and rapidity.

Sample pretreatment is an important issue in the analysis of microorganisms. Careful sample pretreatment is required to achieve sufficient detection capability and selectivity, but the time taken should be kept to a minimum to ensure high throughput. The complexity of microbial biomarkers may be reduced through various chromatography-based methods. This review describes the MS-based analyses of various classes of biomolecules and the applications for each class achieved through chromatographic techniques, including gas chromatography (GC), capillary electrophoresis, liquid chromatography (LC), and affinity methods, such as SELDI.

MS approaches toward the identification of microorganisms may take advantage of variations in microbial genomic sequences resulting from measurable differences in the molecular masses of PCR products. Although current PCR assays may be specific, rapid, and analytically sensitive, they cannot be used directly for classification, especially in the case of unknown microbial samples. Methods combining PCR and MS draw on the strengths of each technique and, in some cases, provide additional information not supplied by either technique alone *(4)*. This review also summarizes the methods of data analysis, including library searches and statistical approaches, for the differentiation of pathogens, and concludes with selected examples of clinical applications of microbial analysis and some perspectives.

Affinity-Based Techniques for Cell Concentration

In general, the presence of proteins or metabolites in biological fluids will suppress the signals of the biomarkers generated from the microorganism cells during MS analysis. Therefore, the development of specific affinity probes for target bacteria is an approach that can effectively minimize interference from sample matrices and concentrate the microorganisms of interest. Afonso and Fenselau *(5)* described the detection of bacteria from complex biological mixtures using affinity capture coupled with MALDI-MS; they used an affinity method—using a lectin-immobilized substrate—to trap traces of bacterial cells from complex biological mixtures.

Immunomagnetic separation is a well-known technique that involves application of antibody-coated magnetic particles to separate pathogenic microorgan-

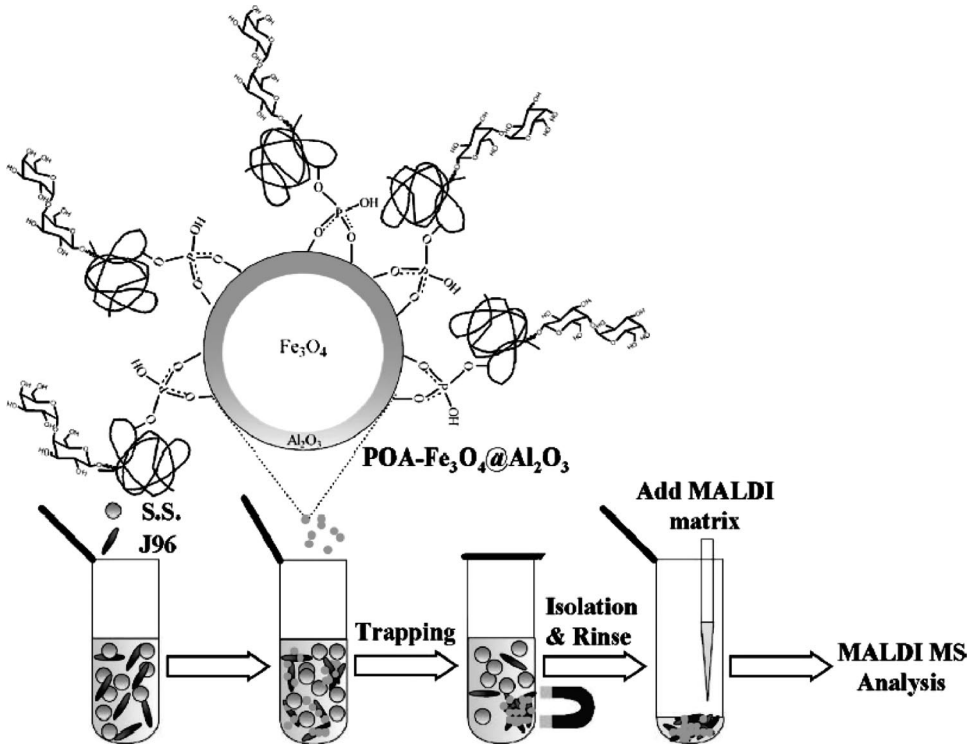

Fig. 2. Representation of the process for using POA-bound Fe_3O_4@Al_2O_3 magnetic nanoparticles to trap target bacteria.

The particles were vortex-mixed with a bacterial sample for 1 h before isolation. The isolated conjugates were mixed with sinapinic acid and subjected to MALDI analysis. S.S., *S. saprophyticus*. Reprinted, with permission, from Liu et al. *(3)*, © 2009 (American Chemical Society).

isms and biological cells from clinical, food, and environmental samples. Madonna et al. *(6)* developed a rapid method for identifying target bacteria from complex biological sample solutions by coupling immunomagnetic separation with MALDI-MS. This method requires only microliter volumes of materials; for example, a minimum of 1.0×10^7 bacteria cells/mL could be detected from a physiological buffer in an analysis time of <1 h. In addition, nanoparticles (NPs) have become interesting probes for the separation of analytes from sample solutions because of their large surface-to-volume ratios. Covalent binding between NPs and targets is readily achieved by self-assembling thiolated molecules onto NP surfaces. Lin et al. *(7)* used mannose-encapsulated gold NPs as nanoscale probes to recognize type 1 pili in *Escherichia coli*. Gu and colleagues *(2, 8)* proposed that vancomycin-modified Fe–Pt NPs (biofunctional magnetic NPs) could be used as affinity probes to trap vancomycin-resistant bacteria; they used S–Pt bonding to capture the pathogens at concentrations of ca. 10^1 colony-forming units (CFU)/mL within 1 h *(2)*.

In a similar study, Chen and colleagues *(9)* used vancomycin-bound magnetic NPs to trap *Staphylococcus saprophyticus* and *Staphylococcus aureus* selectively from urine samples (3 mL) containing bacteria at a concentration of ca. 7×10^4 CFU/mL. Although most of these affinity methods have used MALDI-MS for sample analysis, LC-ESI-MS should, in principle, be equally applicable. Recently, Liu et al. *(3)* used pigeon ovalbumin (POA)–bound Fe_3O_4@Al_2O_3 magnetic NPs to capture uropathogenic *E. coli*. Fig. 2 provides a representation of this process. The POA-bound magnetic particles were mixed with *S. saprophyticus* and *E. coli* J96. *E. coli* J96 was selectively trapped, isolated through magnetic separation, and characterized by direct MS analysis of the NP–bacterium conjugates.

Direct MS-Based Analysis of Microorganisms

MALDI-MS

Direct analysis of microorganisms using MALDI-MS provides a number of advantages, such as rapidity, low detection limits, simplified mass spectra (featuring the

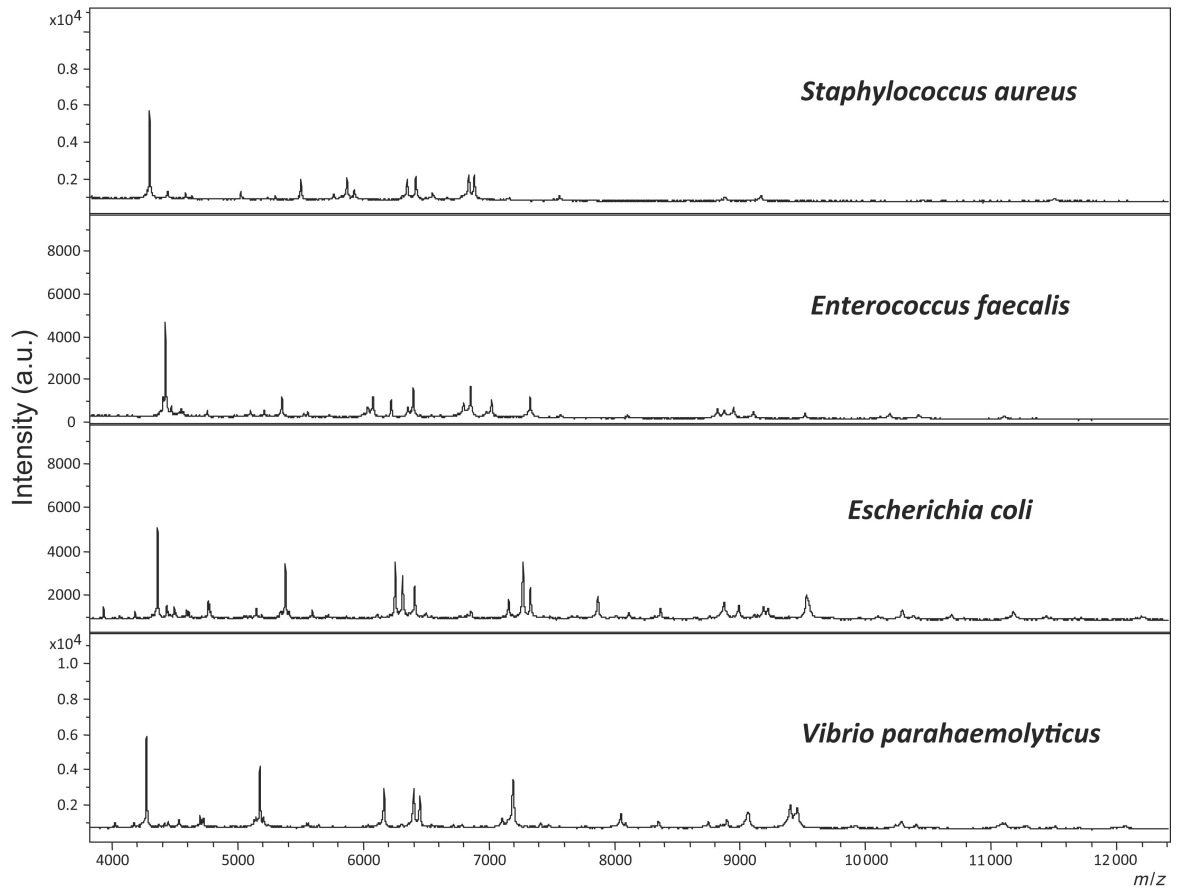

Fig. 3. MALDI spectra obtained from direct analysis of intact bacterial cells of *S. aureus*, *E. faecalis*, *E. coli*, and *V. parahaemolyticus*.

Spectra were acquired in the linear mode using a time-of-flight mass spectrometer. Each sample contained 10^7 CFU bacteria. a.u., arbitrary units.

signals of predominantly singly charged ions), and tolerance to contaminants. The protein profiles in whole-cell MALDI mass spectra have taxonomically characteristic features that can be used to differentiate bacteria at the genus, species, and strain levels, although only a small portion of the bacterial proteome is detected. Direct analyses of intact bacterial cells through MALDI-TOF MS have been used to differentiate bacterial species and subspecies in many clinical microbiology laboratories *(10)*. Fig. 3 presents MALDI mass spectra obtained from the direct analyses of intact bacterial cells of 4 pure cultures including *S. aureus*, *Enterococcus faecalis*, *E. coli*, and *Vibrio parahaemolyticus*. Because the protein profiles clearly differ, they can be used to differentiate these 4 bacteria. Identification and typing of microorganisms using MALDI-MS has been very successful and reviewed by many authors *(1, 11–14)*. We do not attempt to cover this area here.

ESI-MS

Although ESI-MS has been used less frequently for direct microbial identification, Xiang et al. *(15)* characterized microorganisms by performing global ESI–tandem mass spectrometry (MS/MS) analyses of cell lysates. Vaidyanathan et al. *(16)* reported the direct ESI-MS analyses of whole bacterial cells without prior separation. The newly developed technique of desorption electrospray ionization (DESI) MS allows the direct analysis of condensed phase samples by spraying them with electrosprayed solvent droplets. This approach has been used to differentiate several bacteria species—including *E. coli*, *S. aureus*, *Enterococcus* sp., *Bordetella bronchiseptica*,

Bacillus thuringiensis, *Bacillus subtilis*, and *Salmonella typhimurium*—based on their DESI mass spectral profiles. Distinguishable DESI mass spectra, in the mass range 50–500 Da, have been obtained from whole bacteria using both positive and negative ion modes; the analysis time can be <2 min *(17)*.

BIOAEROSOL MASS SPECTROMETRY

Bioaerosol mass spectrometry (BAMS) has been developed to analyze and identify individual aerosolized microbial particles in real time *(14)*. BAMS has the potential to instantaneously detect species-level differences between single cells without the need for sample workup or preconcentration; it offers a high degree of detection specificity and analysis times in the millisecond range. It has been applied to the differentiation of the *Bacillus* spore species *B. thuringiensis* and *B. atrophaeus (18)* and to the detection of airborne *Mycobacterium tuberculosis* bacteria *(19)*. The reagentless BAMS reflectron mass spectra of *Bacillus* spores and vegetative cells have been limited to signals having masses of less than *m*/*z* 300. Although BAMS has provided some success at species-level differentiation, it has been hindered by low sensitivity at high mass *(18)*. Improved high-mass sensitivity will extend the fingerprint mass range and increase the probability of detecting more robust species-specific biomarkers.

The high-mass analyses of airborne pathogens can be improved by combining aerosol time-of-flight MS with MALDI *(20)*. In general, the aerosol and matrix vapor are mixed and then transported into a cooling chamber, where the matrix is condensed onto the aerosol. The coated particles are then analyzed using a time-of-flight mass spectrometer. Van Wuijckhuijse et al. *(20)* developed an online system for analyzing proteins and other biological material present in single aerosol particles. Protein aerosol particles having masses of up to 20 000 Da can be detected; this mass range encompasses those of useful biomarkers for bacteria including *Bacillus* spores *(20)*.

Fractionation Methods Coupled with Mass Spectrometry

GC-MS AND PYROLYSIS–GC-MS

GC-MS has been applied successfully to metabolite profiling for the characterization of microorganisms *(21)*. The maximum molecular weight of compounds that can be analyzed by GC-MS is <1000 Da. For nonvolatile analytes, conversion of samples to volatile compounds through derivatization or degradation is often required before GS-MS analysis. Ishida et al. *(22)* applied thermally assisted hydrolysis and methylation-GC (in the presence of tetramethylammonium hydroxide) with MALDI-MS/on-probe sample pretreatment as a complementary means of directly analyzing the bacterial phospholipids in whole cells (*E. coli K-12*), without requiring any tedious sample pretreatment steps.

Mas et al. *(23)* studied the potential of GC-MS and direct-infusion MS methods to produce specific and discriminant metabolite profiles from different yeast mutants (*Saccharomyces cerevisiae* strains). The combination of GC-MS with LC-MS can provide greater coverage of the metabolome *(24)*. Smilde et al. *(24)* detected a very high number (93%) of commercially available metabolites of the in silico metabolomes of *B. subtilis* and *E. coli*. Similar coverage (95%–97%) for the same microorganisms and *S. cerevisiae* required the application of 6 different analytical methods *(25)*. In total, 905 different metabolites were present in these 3 in silico metabolomes, with 410 metabolites being unique to 1 of each of these 3 microorganisms.

GC-MS/MS can improve the detection limit and specificity of the analysis of carbohydrate biomarkers (e.g., muramic acid) in complex environmental and clinical samples. Specifically, this technique involves monitoring the specific transitions of precursor ions to fragment ions in the multiple reaction monitoring mode. With this approach, Wunschel et al. *(21)* studied the feasibility of using 2 variants of the alditol acetate procedure to analyze agar markers associated with spores of *Bacillus anthracis*. These methods could detect the agar background component, 3,6-anhydro-α-l-galactose, in the presence of spores. Pyrolysis-MS is often used to obtain a pyrolysate fingerprint to classify and identify bacteria. Pyrolysis is a process in which chemical or biochemical compounds are decomposed by heating. Pyrolysis products derived from many classes of compounds including lipids *(26)* and proteins *(27)* have all been used for bacterial discrimination.

CAPILLARY ELECTROPHORESIS–MS

Capillary electrophoresis (CE)-MS is a well-accepted multidimensional analytical approach that is complementary and/or competitive to classic MS-coupled separation techniques *(28)*. CE in combination with ESI-MS has the ability to perform the rapid, automated, miniaturized, and highly efficient separation of complex mixtures. CE-MS is a useful technology for the discovery of biomarkers from pathogens *(28)*. Hu et al. *(29)* identified bacterial species-specific proteins in a sample by performing CE-MS analysis on the proteolytic digest of the cell extract and monitoring only the selected marker peptide masses using MS/MS. Through database searching, they identified peptides that matched proteins associated with *Pseudomonas aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis*

and selected these peptides as marker ions for bacterial identification of the mixtures. The method was later termed selective proteotypic-peptide analysis (SPA). The SPA method has also been used to identify the pathogens present in a saliva sample spiked with bacterial mixtures. Minor bacterial species present at even 1% relative abundance were identified with high confidence. Furthermore, the rate of data analysis was greatly improved because only selected markers, rather than whole protein digests, were analyzed. They also reported the identification of bacterial species using CE-MS and a database search algorithm with an empirical scoring function *(30)*.

LC-MS

Liquid chromatography coupled on- or off-line with MS is currently the most widely used analytical method for the separation and analysis of such biological samples as proteins, peptides, nucleotides, and metabolites. It is a separation technique presumably best suited for clinical applications in microbial analysis because of its chromatographic separation ability and flexibility of coupling multiple separation columns. The effects of the sample preparation methods on the detection of bacterial proteins obtained from *E. coli* by LC-ESI-MS have been investigated *(31)*. Furthermore, the effect of variations in the protein patterns on bacterial identification using a database search approach has been examined. Lo et al. *(32)* used an LC-selective proteotypic-peptide analysis method to identify multiple bacterial species in a single MS analysis. The corresponding bacterial species could be identified if the selected peptides eluted during the preset time window and could be identified correctly from the database. This method has been applied to identify up to 8 pathogens present in bacterial mixtures in a single LC-selective proteotypic-peptide analysis experiment *(32)*.

A multidimensional LC-MS method has been used for the rapid determination and identification of bacterial proteins. By combining affinity purification with multidimensional protein identification technology, Krishnamurthy et al. *(33)* reported the separation and characterization of a highly specific protein biomarker (antigenic protein EA1) from sporulated *B. anthracis* cells.

Although the lipid profiles may depend on the growth conditions, phospholipids have proved to be useful biomarkers for some microorganisms *(34)*. Phospholipids can be ionized as intact entities for MS and MS/MS analyses. Individual fatty acids present in bacterial cells can be identified from product ion (MS/MS) spectra acquired in the negative ion mode. Dramatic improvements in the detection limits for phospholipid analysis have been achieved using ESI-MS and MS/MS *(35)*. Bacteriohopanepolyols (BHPs) are membrane lipids produced by a wide range of eubacteria. Talbot et al. *(36)* described the detailed LC-MS analyses of a number of less commonly reported, but highly useful, BHP markers derived from bacterial cultures.

SELDI-MS

SELDI-MS is based on the retention of analytes on a target plate before MALDI-MS analysis. Using affinity chromatography, proteins are captured on a chemically or biochemically modified protein chip surface. An advantage of the SELDI technique is that target proteins can be characterized after they have been retained and purified on an affinity surface uniquely designed according to the specific properties of the proteins *(37)*.

A protein profiling approach based on SELDI-MS has been used for phenotypic discrimination of 4 closely related subspecies of *Francisella tularensis (38)*. The method is based on the differential binding of protein subsets to chemically modified surfaces. The ability of the selective array surfaces to retain subsets of the proteome and to wash and desalt proteins on the array surface allows the direct analysis of bacterial lysates or other protein-containing samples, thereby minimizing sample losses and accelerating sample preparation. Lancashire et al. *(39)* used SELDI-MS to identify potential biological markers for well-characterized strains of *Neisseria meningitides* and created a model to distinguish them from closely related species. Shah et al. *(40)* analyzed intact cells of human pathogens (strains of *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Prevotella nigrescens*) using MALDI- and SELDI-MS to obtain specific spectral profiles that could be used to delineate bacterial species. One key challenge for profiling methods is to achieve reproducible results. Standardized sample preparation protocols are always required to improve spectral reproducibility.

Nucleic Acid–Based Approaches

Methods for microbial identification can be classified into phenotyping and genotyping categories. Genotypic techniques are based on the genetic conservation of a species and genetic variability among different species. Phenotypic testing, which relies on physiological or biochemical characteristics, can provide information about specific metabolic pathways or antibiotic resistance. Whereas traditional biochemical analysis remains a major approach used in clinical laboratories, nucleic acid–based methods are gaining increasing popularity in clinical applications.

The rapid analyses of PCR products through ESI-MS and MALDI-MS have been applied success-

fully to the identification of pathogens. For instance, Mayr et al. *(41)* reported the identification of bacteria using a combination of PCR and LC-MS; the specificity of this method in a human background was successfully tested by amplifying and analyzing 1000–10 000 genome equivalents of *S. aureus* spiked into human plasma. They also analyzed DNA fragments amplified from the 16S rRNA region that were amenable to the ESI-MS–based genotyping of 10 different bacterial species generally found in clinical environments.

To achieve a more accurate distinction among species, RFLPs can be used after PCR to identify species based on the 16S rRNA fragment patterns. The fragments are obtained from digestion of the PCR products with restriction enzymes. The decreased size of the digested PCR products also results in more favorable ionization and mass spectroscopic analysis. The RFLP method has been combined with MALDI-MS to detect hepatitis B and C viruses (HBV and HCV) in human serum samples *(42, 43)*. This method allowed the detection of as few as 100 copies of the HBV gene per milliliter of serum and the differentiation of wild-type and variant viruses *(43)*. In all the reports discussed above, the microbial species were distinguished based on size determination of the PCR-amplified specific marker genes. Using these approaches for accurate microbial identification is limited, however, because of length heterogeneities of specific marker genes. von Wintzingerode et al. *(44)* developed a method for 16S rRNA gene-based bacterial identification that combines base-specific fragmentation of PCR products with MALDI-MS. Amplification of 16S rDNA signature sequences in the presence of dUTP is followed by uracil-DNA-glycosylase–mediated fragmentation at T-specific sites. MS analysis of the base-specific fragment patterns has allowed unambiguous discrimination of several cultured *Bordetella* species and as-yet-uncultured bacteria.

Because of the difficulties associated with MALDI-MS–based analyses of DNA, several research groups have analyzed the more stable RNA transcribed from DNA. PCR amplification of the16S rDNA was performed using RNA promoter–tagged forward primers, and the in vitro transcription was followed using a T7 RNA polymerase. The base (G)-specific fragments were produced by RNAase T1 cleavage of the RNA transcript and analyzed using MALDI-MS *(45)*.

Single nucleotide polymorphisms (SNPs) arise from single base changes at specific positions in a genome. SNPs are the most abundant form of genetic variation and can be used for pathogen identification. Many methods are available for determination of the alleles of a SNP. In addition to the conventional RFLP approach mentioned above, primer extension, oligonucleotide ligation, and hybridization have all been combined with MS for genotyping *(46)*. Ilina et al. *(47)* used primer extension and MALDI-MS to genotype HCV from HCV-positive blood sera and plasma. They designed 3 oligonucleotide primers to detect 2 sets of genotype-specific SNPs. The primer extension reaction was performed using modified thermostable DNA polymerase in the presence of dideoxynucleosides; the reaction products were analyzed using MALDI-MS. We note that the diversity of microbial strains and the occurrence of novel mutations limit the use of genotypic methods based on single nucleotide differences between strains. Genotypic identification assays using base-specific cleavage strategies, however, allow not only the differentiation of known sequence strains but also the detection of new strains.

Triangulation identification for the genetic evaluation of risks (TIGER) is a promising approach for the detection of a broad spectrum of microorganisms *(48)*. It uses high-performance ESI-MS to detect the base compositions of PCR products. The essential aspect of this approach is the use of "intelligent PCR primers" that target broadly conserved regions of microbial genomes that flank variable regions. The PCR products in the 80- to 140-bp size range can be measured using high-resolution Fourier transform ion cyclotron resonance or time-of-flight MS in a high-throughput mode. Accurate mass measurements allow determination of the base compositions obtained from multiple primer pairs. These base compositions were used to "triangulate" the identities of the pathogens. The approach is equally well suited to the detection of bacteria, viruses, fungi, and protozoa. Fig. 4 presents ESI-MS mass spectra of PCR amplicons derived from both strands of the *tkt* genes for 6 *Campylobacteria jejuni* strains *(49)*.

The same approach has been applied to the rapid identification and strain-typing of respiratory pathogens *(50)*. Broad-range PCR primers targeted highly conserved regions of 16S and 23S DNA genes. Additional primers targeted to highly variable regions of specific bacterial genomes have been used for high-resolution genotyping of specific species. Identification of 14 isolates of 9 diverse coronavirus spp., including the severe acute respiratory syndrome (SARS)–associated coronavirus, has been demonstrated using the TIGER approach, with a detection limit of 1 PFU per milliliter of human serum.

Data Analysis Methods

Data analysis methods for the differentiation of pathogens can be based on the most straightforward profiling of intact proteins or on the complicated statistical treatment of MS data. MALDI-MS analysis of whole intact cells has been used for protein profiling of water-

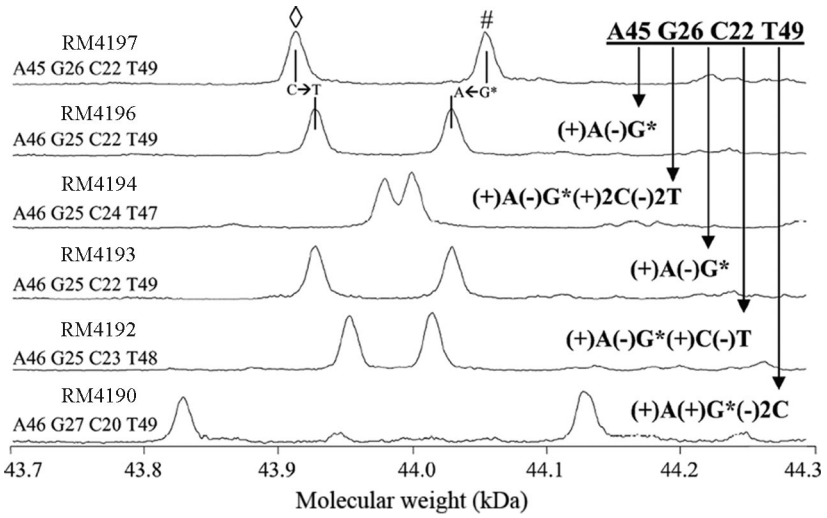

Fig. 4. Deconvoluted ESI mass spectra of both strands of the *tkt* gene amplicons from 6 *C. jejuni* strains.
Both the forward (◇) and the reverse (#) strands of the PCR amplicons from each strain are clearly observed. The base compositions are listed to the left of the spectra. Reprinted, with permission, from Hannis et al. *(49)*, © 2009 (American Society for Microbiology).

borne pathogen *Aeromonas (51)*. A library of mass signatures was established for 17 species of *Aeromonas*; the abundances of the ions were not taken into account—only their masses were used to compile the library. This approach has several advantageous features: the ability to detect intact biomarkers (e.g., proteins), simple sample preparation, and high throughput. The challenge facing this technique is spectral reproducibility, which is markedly influenced by variations in culture conditions and analytical parameters.

Because of the complexity of mass spectral data, many statistical algorithms have been developed to match the analyzed spectra to reference spectra or to generate biomarker spectra for microbial differentiation. A biomarker-selective algorithm has been used to extract key signals from spectra; the constructed biomarker library has been used to identify bacterial mixtures *(52)*. The identification process involved comparison of the sample spectra with the library spectra using an algorithm similar to a statistical test of significance. A MALDI mass spectral database including >100 genera and 350 species has been compiled to characterize bacteria implicated in human infectious diseases *(53)*. The authors noted that species- or subspecies-specific markers in the spectra are sometimes difficult to identify because the number of overlapping signal ions increases along with the number of strains registered in the database.

The 2 mathematical methods used most often for the classification of spectral data are multivariate analysis (MVA) and the artificial neural network (ANN). MVA is based on multivariate statistics, which involves observation and analysis of more than 1 statistical variable (in this case, *m*/*z*) at a time. Many MVA techniques—including principal component analysis and cluster analysis—have been applied to the differentiation of microorganisms. The ANN is an algorithm for machine learning. This type of artificial intelligence allows the identification of biomarkers that are capable of distinguishing microbial species.

Multivariate statistics is often used to analyze pyrolysis mass spectra of bacterial samples. Principal component analysis (PCA) has been applied to pattern recognition of fatty acid methyl esters profiles obtained from *B. anthracis*, *Yersinia pestis*, *Vibrio cholerae*, *Brucella melitensis*, and *F. tularensis (54)*. PCA is the most commonly used multivariate data analysis tool. PCA is a technique for forming new variables (i.e., principle components) which are linear combinations of the original variables (i.e., *m*/*z* values in mass spectra). The principle components account for the differences among spectra. Ilina et al. *(55)* reported the direct bacterial profiling of 2 human pathogens, *N. meningitidis* and *Neisseria gonorrhoeae*, using MALDI-MS. Cluster analysis grouped mass spectra into clusters by measuring similarities between spectra. The method successfully separated the mass spectra collected from 3 groups that corresponded to the pathogenic and nonpathogenic *Neisseria* isolates. Because the protein chip technique SELDI may yield thousands of data points

per bacterial sample, computer algorithms must be used to identify useful biomarkers for bacterial identification. Seibold et al. *(56)* differentiated single strains within the subspecies *F. tularensis* by combining SELDI-MS with cluster analysis and PCA. ANN combined with SELDI-MS has been used for the identification of *N. meningitides (39)* and *S. aureus (57)*. The relative abundance for each *m*/*z* value was used in the input layer of the ANN model. The output layer consisted of nodes representing target bacterial categories. The model was trained using bacterial spectra to generate good correlation between input and output layers. The model was further tested and validated using different sets of spectra.

Demirev et al. *(58)* developed an approach for microorganism identification that combines MS with protein database searches. The protein masses in mass spectra are compared with those in the proteome databases. Probable organism sources are assigned to each mass, with the best match (i.e., that having the most matched proteins), in theory, leading to identification of the correct microorganism. When using this approach, spectral reproducibility is not critical as long as the observed masses match the protein masses in the database. To avoid the limited mass accuracy of protein mass measurements, the sequence information of the peptides from virus and bacterial samples has been used to identify the proteins and deduce the microorganism's source *(59)*. The sequence information can be derived from fragmentation of intact proteins or proteolytic peptides of the intact proteins by searching fragment ion (MS/MS) mass spectra against proteome databases. Top-down proteomics methodology identifies intact proteins without the need for biomarker digestion, separation, and cleanup. This methodology has been used successfully for microbial proteomics in the analysis of bacterial species, either pure or in a mixture *(60, 61)*. Microbial identification has been demonstrated using experimentally derived databases of virus-specific tryptic peptide masses *(62)* and bacterial protein masses *(63)*.

Data analysis using protein database search algorithms should be very accurate and useful as long as the protein databases are available for the pathogens of interest. Analysis of spectra using statistical methods is complicated. Further, identification results may be inaccurate when the spectra are not reproducible. However, as mentioned above, various statistical methods have been employed for differentiation among species or subspecies and proved to be useful. PCA has advantages because it is better to reveal differences between biomarker profiles than cluster analysis. ANN holds especially great promise for high throughput because it may require less computer time.

Clinical Applications

MS-based identification of microorganisms currently plays a crucial role in clinical research because mass analyzers provide low detection limits, universality (yet with potential for selectivity and specificity), and amenability to qualitative and quantitative analyses over a wide dynamic range. Cell culturing is by far the most used sample preparation technique for purifying and enriching microbial cells before mass spectrometric analysis. Although enrichment of pathogens using functionalized magnetic particles has not been applied to clinical samples, the approach has proved successful for bacteria-spiked biological samples and should play an important role in the near future. Clinical specimens, obtained from pus, wound, sputum, and urine samples, have all been analyzed using selective CE-MS/MS methods *(64)*. The analyses of bacteria-specific marker peptides provided additional advantages of excellent selectivity and high confidence in microbial identification. The bacteria in these clinical samples were cultivated directly, without prior isolation of a pure colony, before performing the selective MS/MS analyses. Many pathogen species (Table 1) were identified, including *P. aeruginosa*, *S. aureus*, and *Streptococcus agalactiae*. The rapid and accurate discrimination between methicillin-resistant and methicillin-sensitive microbial strains could lead to major improvements in the treatment strategies of infected patients. Du et al. *(65)* used MALDI-MS to identify clinical isolates of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). The comparative proteomic analyses of *S. aureus* strains exhibiting different resistances toward the cell wall–targeting antibiotic vancomycin have been performed, with significant changes in the abundance of 65 proteins, as determined using MALDI-MS/MS and LC-MS/MS *(66)*.

Intact-cell MS has been used for the discrimination and identification of *Burkholderia cepacia* complex species, including strains of the novel Taxon K, recovered from cystic fibrosis patients *(67)*. Carbonnelle et al. *(68)* reported the rapid MALDI-MS–based identification of intact staphylococci isolated in clinical microbiology laboratories; they identified 23 clinically relevant bacterial species or subspecies belonging to the micrococcaceae family.

Real-time PCR is at present the leading nonculture–based technique for the determination of infection *(69)*. PCR-MS is currently conducted as a reference laboratory technique; it has been used successfully to determine nucleotide compositions for strain typing in epidemiological studies of outbreaks of respiratory infections with *Streptococcus*, *Hemophilus*, or *Neisseria (50)*. Extended-spectrum β-lactamases (ESBLs) are

Table 1. Identified pathogens by the selective proteotypic-peptide analysis of each clinical specimen [based on the results from Hu et al. *(64)*].

Bacterium	Gram stain	Specimen	Number of samples
Enterococcus faecalis	Positive	Pus	1
Escherichia coli	Negative	Pus	2
Escherichia coli	Negative	Urine	1
Pseudomonas aeruginosa	Negative	Sputum	5
Salmonella spp.	Negative	Pus	1
Staphylococcus aureus	Positive	Sputum	6
Staphylococcus epidermidis	Positive	Wound	2
Streptococcus agalactiae	Positive	Wound	1
Streptococcus agalactiae	Positive	Pus	1
Streptococcus agalactiae	Positive	Urine	1
Streptococcus pneumoniae	Positive	Sputum	5
Streptococcus pyogenes	Positive	Pus	3
S. aureus + *P. aeruginosa*	Positive + negative	Pus	1
E. coli + *P. aeruginosa*	Negative + negative	Urine	1

among the most clinically significant β-lactamases because they confer resistance to all penicillins, cephalosporins, and aztreonam. Ikryannikova et al. *(70)* applied a MALDI-MS–based minisequencing method to the rapid detection of TEM-type ESBLs in clinical strains of the enterobacteriaceae family. Hujer et al. *(71)* demonstrated that ESI-MS analysis of PCR amplification products could be used to identify quinolone resistance mediated by mutations in the quinolone resistance–determining regions of 2 essential housekeeping genes. The analysis provides critical information for therapeutic decision-making in the treatment of *Acinetobacter* sp. infections.

Conclusions and Perspectives

The tools for determining the identity of a microbial sample include genetic and nongenetic methods and informatics. MS has proven to be a useful method for the identification of biomarkers from microbial samples. One of its major challenges, however, is the detection of candidate biomarkers from complex samples. Methods for sample analyses without pretreatment or after extensive biochemical and chromatographic fractionations have been developed to overcome the issues associated with sample complexity. Direct MS-based analyses of microorganisms have a number of advantages, including rapidity and ease of operation. The MALDI interface is best suited for use in field-portable mass spectrometers deployed on battlefields or in our surroundings to detect microbial threats. Microbial analyses usually use a pure culture of microorganisms to avoid interference from the background—unless the interferents are absent (or removed) or data analysis algorithms are implemented. The complexity of microbial biomarkers may be simplified through the use of various chromatography-based methods (e.g., HPLC and CE); although the rate of identification is sacrificed to some extent, the selectivity, accuracy, and dynamic range are improved substantially.

Since the US anthrax mailing incidents in 2001, means of dealing with microbial threats have gained tremendous attention. Several recent outbreaks, including the SARS outbreak caused by coronavirus and the H1N1 flu outbreak caused by a novel influenza A virus, have further triggered the development of more rapid, sensitive, and accurate real-time detection methods. Improvement of the detection limits for microbial cells will continue to be a major task in the years to come. To this end, cell enrichment through affinity techniques will play an increasingly important role. Top-down protein analysis and selective biomarker analysis coupled with affinity techniques will boost the detection capability and accuracy of microbial identifications. PCR amplification of nucleic acids and MS detection might be an alternative approach toward improving sensitivity, especially for the analyses of nonculturable microorganisms. In addition to MALDI, several new ionization techniques, including desorption electrospray ionization *(72)*, direct analysis in real time *(73)*, and ESI-assisted laser desorption ionization *(74)*, hold great potential for use in the real-time direct MS-based analyses of pathogens. Furthermore, advances in other MS-related technologies will provide more powerful tools for characterization of pathogens; these technologies include high-speed and high-resolution LC, ion mobility spectrometry, and high-capacity linear ion trap and various high-resolution mass analyzers, such as Fourier transform ion cyclotron resonance, Fourier transform-Orbitrap, and time-of-flight mass detectors. The combination of these methodologies and instrumentation will certainly enhance the capabilities of MS for the analysis of pathogens.

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acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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