Systematic and Applied Microbiology 34 (2011) 2–11

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Minireview

Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology

Martin Welker[a,b,*], Edward R.B. Moore[c,d]

[a] Anagnostec GmbH, Am Mühlenberg 11, 14476 Potsdam, Germany
[b] Technische Universität Berlin, FG Umweltmikrobiologie, Franklinstraße 29, 10587 Berlin, Germany
[c] Culture Collection University of Gothenburg (CCUG), Department of Clinical Bacteriology, Sahlgrenska University Hospital, PO Box 7193, 40234 Göteborg, Sweden
[d] Department of Infectious Disease, Sahlgrenska Academy of the University of Gothenburg, Guldhedsgatan 10A, 41346 Göteborg, Sweden

ARTICLE INFO

Article history:
Received 16 November 2010

Keywords:
MALDI-TOF MS
Mass spectrometry
Microbial identification
Microbial typing
Prokaryote
Bacteria

ABSTRACT

In the last few years matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly studied and applied for the identification and typing of microorganisms. Very recently, MALDI-TOF MS has been introduced in clinical routine microbiological diagnostics with marked success, which is remarkable considering that not long ago the technology was generally seen as being far from practical application. The identification of microbial isolates by whole-cell mass spectrometry (WC-MS) is being recognized as one of the latest tools forging a revolution in microbial diagnostics, with the potential of bringing to an end many of the time-consuming and man-power-intensive identification procedures that have been used for decades. Apart from applications of WC-MS in clinical diagnostics, other fields of microbiology also have adopted the technology with success. In this article, an over-view of the principles of MALDI-TOF MS and WC-MS is presented, highlighting the characteristics of the technology that allow its utilization for systematic microbiology.

© 2010 Elsevier GmbH. All rights reserved.

Microbial identification

The abilities to identify microbial isolates and diagnose relevant taxa have been essential for medicine since microorganisms were appreciated to be causative agents of infection and disease. Microbial typing has been used in the production of foods and in agriculture for millennia. Recognizing and placing a name onto a strain imparts implications and assumptions associated with that organism. Microbial identifications allow clinical diagnoses, comprehension of their impacts on environmental activities and insights into biotechnological applications. Importantly, microbial identification should not be confused with microbial typing. The terms ‘typing’ or ‘type’ are often used in microbiology with different connotation. Literally, ‘type’ refers to any category, distinct from others, that can be defined by a given criterion or methods of analyses. In microbiology, this literal definition may be applied, e.g., phenotype, serotype, pathotype, ecotype, genotype, etc. However, microbiologists also refer to ‘typing’ or ‘type’ with respect to sub-specific delineations of microorganisms. In this review, ‘typing’ or ‘type’ is used particularly with the later understanding in mind. Typing of microorganisms is the characterization and registration of discriminating features of strains, generally of the same species, with the focus on defining differences. Identification relies on the characterization of strains, with the focus on recognizing phenotypic and genotypic similarities, allowing the assignment of strains to given species. Identification is necessarily dependent upon an established taxonomy and stable nomenclature (see Fig. 1).

Microbial taxonomy may be regarded as the study of the organization and prioritization of microbial diversity [74] based on natural relationships. Without a functional taxonomy, identifications of isolates would not be possible. The species comprises the basic unit of microbial taxonomy, even though the concept and definitions of a prokaryotic ‘species’ continue to be debated. Innovations and developments in new methodologies are essential for improving the sensitivity and level of resolution and the reliability of results, as well as the practical issues of speed and cost of analyses for microbial typing and identification.

MALDI-TOF MS for microbial identification – a history of success

Since its invention in the late 1980s, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been adopted for applications in many fields of the life sciences [29]. The principle of soft ionization and, hence, the

* Corresponding author. Present address: BioMérieux – R&D Microbiology, 3 route de Port Michaud, La Balme les Grottes 38390, France. Tel.: +33 6 12764121; fax: +33 4 74952632.
E-mail addresses: martin.welker@biomerieux.com, m.welker@anagnostec.com (M. Welker).

0723-2020/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2010.11.013

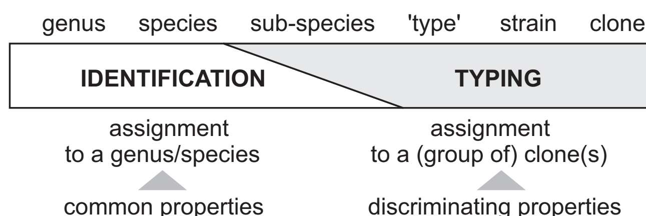

Fig. 1. An overview of the distinction between identification and typing of microorganisms; 'type' refers to different categories commonly used for sub-specific delineation, for example: observable physical properties (phenotype); cell and colony morphology (morphotype), chemical composition and metabolite production (chemotype); antigenic reaction (serotype); host pathogenicity (pathotype); adaptation to ecosystem (ecotype); genetic composition (genotype); etc.

possibility to detect unfragmented large molecules and molecule complexes pushed the door open for protein analysis as was not possible before. The most immediate adaption of MALDI-TOF MS has been in the field of proteomics, where the high throughput analysis of digested proteins allows the identification of very small amounts of proteins eluted from electrophoresis gels, in combination with the availability of increasing numbers of genome sequences.

The applications of chemical analyses of microorganisms have been explored for many years. Early attempts in using mass spectrometry were applied to the characterization of polar and non-polar lipids, such as quinones and long-chain fatty acids [1]. While these analyses provided useful characterizations at the genus and species levels, typing of bacterial isolates was also attempted, using pyrolysis mass spectrometry [61]. However, mass spectral approaches were limited by the ability to analyse mass ranges only up to 1,500 Da [28]; larger molecules could be characterized only through laborious techniques, such as plasma desorption mass spectrometry [69]. For the rapid analyses of biomolecules, biopolymers and macromolecule complexes, i.e., proteins and protein complexes, the application of a crystallizing, light-adsorbing matrix compound proved to be the essential trick for the preservation of the integrity of the molecules [40] during the analytical procedure. Matrix crystals absorb photonic energy from a nitrogen laser, effecting the desorption and 'soft' ionization of large, intact biomolecules [18].

Since the first commercial apparatus became available in the early 1990s (PerSeptive Biosystems – Vestec) [29], MALDI-TOF MS has received increasing attention by microbiologists, who recognized the immense potential of the technology for the analysis of microorganisms. Early studies revealed that 'intact-' or 'whole-cell' mass spectrometry (IC-MS or WC-MS) could elucidate mass spectra of total cellular components by analysing microbial cells without laborious extraction procedures [7,31,43]. The term 'intact-cell', however, proved to be not strictly accurate since, after a simple extraction step, by which whole cells are suspended in the matrix solution, the integrity of the cell wall is destroyed and the cells no longer remain intact. It was also recognized that the mass spectra of different bacterial species were distinct from one another, exhibiting the potential to be a species-discriminating characteristic, analogous to genetic fingerprinting techniques such as AFLP or RAPD. Despite the potential and early successes of WC-MS as a technique for microbial identification, more than a decade passed before the first MALDI-TOF MS-based identification systems would be commercialized.

The development of WC-MS for microbiological diagnostics followed a typical hype cycle: after the technological 'trigger', i.e., the invention of MALDI-TOF MS and applications for WC-MS, promises and expectations were overly high. Within a few years, several studies claimed to identify bacteria by WC-MS, although, in fact, most studies were carried out with only distantly related species, producing markedly different peak patterns [7]. Inflated expectations peaked with studies that claimed to be able to differentiate antibiotic resistance types within minutes [19,20]. Such analyses normally require several hours to days to be completed. However, with increasing numbers of studies, it became evident that the analysis is not as straight-forward as initially postulated. One reason WC-MS did not meet the initial high expectations was that, at the turn of the millennium, no comprehensive database existed that would allow reliable mass-based identifications. Despite a number of studies on selected prokaryotic and fungal taxa (for a recent review see [39]), large gaps existed in the databases of reference species, initially devoted to the clinically relevant taxa and their close relatives, as well as only a limited understanding of the cellular compounds actually detected by WC-MS. The latter issue raised suspicions about the stability and consistency of the mass spectral patterns of different strains of a species. Questions of reproducibility and accuracy were addressed by several studies demonstrating that mass spectra are reproducible in different laboratories, as well as under varying cultivation conditions [75,91,92]. Nevertheless, it became more clear that a functional and reliable mass spectra-based identification system requires the acquisition of reference mass spectra of tens of thousands of strains and the development of appropriate software tools to handle the data [34,35,38].

Finally, WC-MS applied to microbial identification reached a plateau of productivity with the first systems placed in clinical diagnostic laboratories, where the method replaced the biochemical identification systems that had been predominant for decades [63]. The acceptance of WC-MS as an identification tool of high accuracy and reproducibility was also supported by parallel advances in genomic studies, i.e., with the increasing number of available microbial genome sequences, the peaks in mass spectra became more readily identifiable. It is now recognized that a large percentage of the recorded peaks in whole-cell mass spectra, i.e., in the mass range of a typical WC-MS of m/z 2–20 kDa, are comprised of ribosomal proteins [15,16,70,71] (Fig. 2) which may explain the marked stability of spectra for given species, even under varying cultivation conditions.

Although differing cultivation conditions will result in some degree of variation in the mass spectra for a strain, in general, the peak patterns derived by WC-MS are stable, even when strains are grown on different media or are analysed at different ages of cultivation (Fig. 3).

However, not all mass peaks are from ribosomal proteins. Maximally, 50 individual ribosomal proteins (including variants and fragments) can be identified in the mass range from 3,000 to 20,000 Da among the total number of peaks, ranging between 70 and 200 generally recorded in the mass spectra of most microbial samples. In the spectra shown in Fig. 2, for example, a number of peaks could not be identified by *in silico* searches for corresponding proteins. Besides the ribosomal proteins, other identifiable proteins in a typical WC-MS are mostly "structural" proteins, i.e., those without catalytic function but which are a constitutive part of the cell structure and function, such as ribosome modulation factors, carbon storage regulators, cold-shock proteins, DNA-binding proteins and RNA chaperones [15,16]. Finally, considerable allocations of proteins that can be assigned to peaks in mass spectra are labelled with 'putative' identifications or are 'uncharacterized' in the protein databases. Despite a lack of comprehensive and unambiguous protein assignment to all mass signals in a microbial mass spectrum, it has been suggested to compare mass spectral patterns directly to *in silico* protein profiles, to overcome the necessity for a database of reference mass spectra [12,93]. With the rapidly increasing number of genome sequences and protein and enzyme identifications, as well as programmes targeting genome sequence determinations for all validly published microbial species [26], the database derived from such an approach would be expanded exponentially.

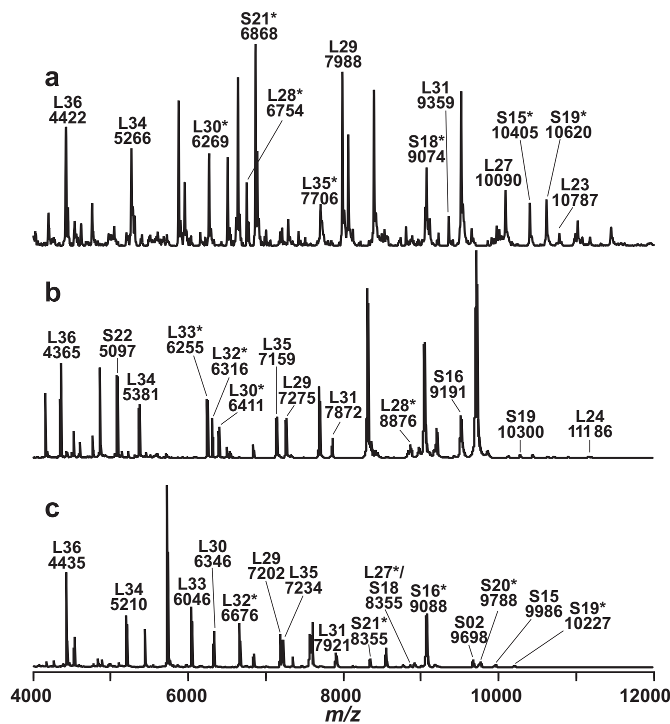

Fig. 2. Whole cell MALDI-TOF mass spectra of: (a) *Streptococcus pneumoniae* DSM 20566T; (b) *Escherichia coli* DSM 1576; and (c) *Pseudomonas aeruginosa* DSM 50071T; with the peaks corresponding to proteins of the ribosomal large (L) and the small (S) subunits, based on annotated theoretical *m*/*z* values. Theoretical protein mass data were obtained by searching multiple genomes for proteins in the mass range of 3000–20,000 Da (EMBL-EBI, http://www.ebi.ac.uk/). The asterisk refers to a probable methionine cleavage of the respective proteins.

The fact that, indeed, ribosomal and other cell structure and regulatory ('house-keeping') proteins represent the predominant number of peaks in the mass spectra of microbial cells has notable consequences:

- mass spectral patterns are stable because conserved ribosomal and other house-keeping proteins are integral, ubiquitous, and abundant components of all living cells;
- mass signals of house-keeping proteins can be analysed as phylogenetic markers, comparable to multi-locus sequence analyses of house-keeping genes;
- the more similar the mass spectral patterns are, the closer are the phylogenetic relationships of the microorganisms.

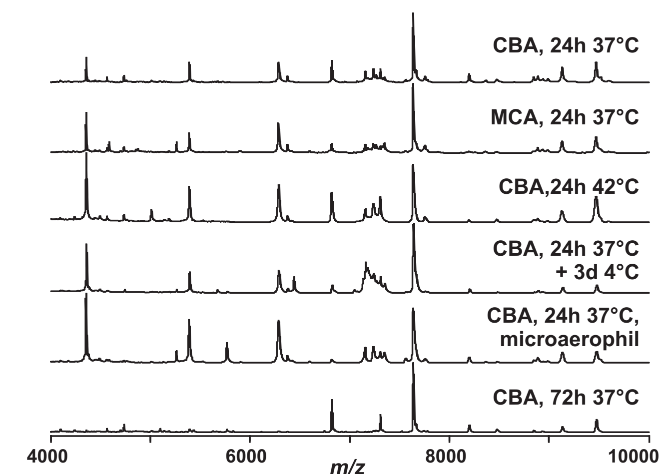

Fig. 3. Whole cell MALDI-TOF mass spectra of *Enterobacter aerogenes* DSM 20478 incubated under different cultivation conditions as indicated. CBA: Columbia blood agar, MCA: MacConkey agar. When not indicted differently, cultures were incubated at atmospheric oxygen partial pressure. All spectra were acquired by WC-MS, using the direct smear method with on-target extraction, using DHB matrix solution.

There are, thus, excellent precedents for the application of MALDI-TOF MS for taxonomic studies, as well as for routine diagnostics. When mass spectral patterns of closely related species are compared, e.g., those for species of the family *Enterobacteriaceae*, a number of peaks can be recognized to be found in all or the majority of the spectra [85]. The varying specificity of individual mass signals for different taxonomic levels (e.g., genus, species, strain, etc.) can be exploited for taxonomic studies.

MALDI-principles and application in microbiology

MALDI-TOF MS applications in microbiology, are important for proteomic and natural product analyses [51,86,94]. In this introductory chapter, the focus is on the application of WC-MS for taxonomic characterization for microbial systematics, i.e., identification and typing. The procedure of MS-based identification of microorganisms is illustrated schematically in Fig. 4 and details of the various steps have been described by Freiwald and Sauer [24], for example.

Generally, microorganisms are analysed from cultures on solid media commonly used in microbiology (for other kinds of samples, see below). Major advantages of WC-MS are the convenience, ease and speed with which samples are prepared and analysed. This is enabled by the samples being introduced into the mass spectrometer in solid state on a sample support plate, commonly referred to as the 'target', without the necessity for a fractionation process, as in liquid chromatography–mass spectrometry (LC–MS) applications. To obtain mass spectra with the number of well defined protein peaks in the range of 70–200, it is generally sufficient to place a small amount of fresh cell biomass (10^5–10^6 cells) on the sample spot of the target and extract the cells, using a 'matrix solution'. For some microorganisms, such as yeasts or mycobacteria, a preceding extraction step may be necessary [2], either in a separate tube or, conveniently, directly on the target. More laborious extraction steps are generally not required, although more elaborate protocols have been suggested [47].

The matrix solution comprises a mixture of solvents, i.e., commonly, combinations of water, ethanol, methanol, acetonitrile and a strong acid, such as trifluoroacetic acid (TFA), in which the matrix compound is dissolved. The solvents of the matrix solution penetrate the cell wall and make intracellular proteins accessible for analysis. When the solvents evaporate from the cell suspension, matrix crystals begin to form, within which the protein molecules and other cellular compounds are embedded, i.e., a process of 'co-crystallization' [32]. A number of different matrix compounds have been used, most of which are organic, small molecular weight, aromatic acids, such as derivatives of benzoic or cinnamic acid. Two matrix compounds are used for most applications today: 2,5-dihydroxy benzoic acid (DHB) and α-cyano-4-hydroxy cinnamic acid (CHCA) and, occasionally, sinapinic acid or ferulic acid; or mixtures of matrix compounds [41]. These matrix compounds have proven to be suitable in most cases of analysing microorganisms and for a wide range of analyte compounds, i.e., besides proteins, target molecules can be small peptides, lipids, carbohydrates and others. For particular applications, new matrix compounds surely will be developed that will further expand the fields of application of MALDI-TOF MS [36,65].

The actual MS measurement is generally performed in an automated mode by scanning the sample spot with the laser beam,

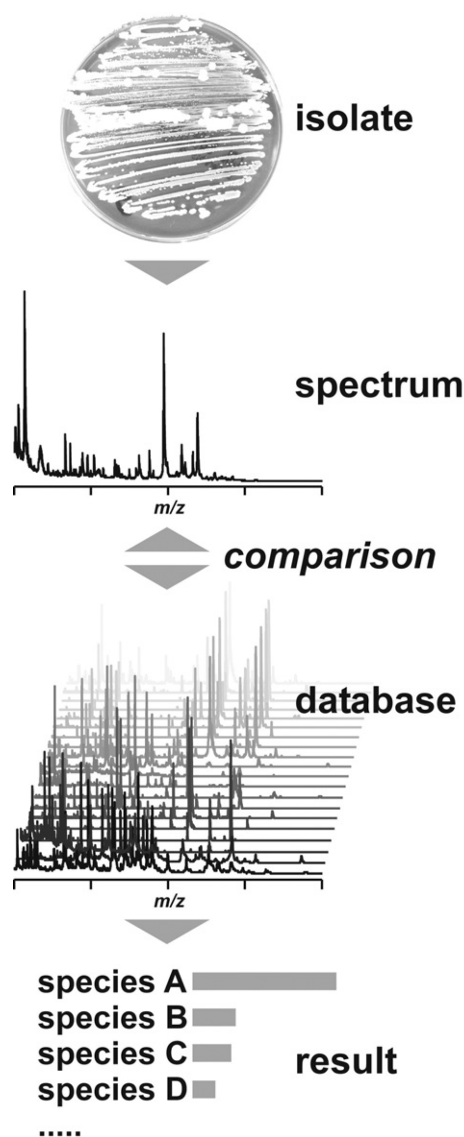

Fig. 4. General scheme of MALDI-TOF MS-based identification of microorganisms, using microbial biomass, generating whole-cell mass spectra to derive a composite strain-specific mass spectrum and comparing the mass spectrum to a reference database.

following a preset pattern. With modern instruments equipped with a laser emitting single pulses at frequencies of 50 Hz or greater, a single sample is analysed in less than 30 s, i.e., a batch of 100 samples can be analysed in less than 1 h. After signal acquisition, the raw mass spectra, generally accumulated from 300 to 1,000 laser pulse cycles, are processed automatically by smoothing, baseline correction and peak recognition. The essential information used for microbial identification is contained in a peak list containing m/z values and intensities, i.e., the so-called 'mass fingerprint' of a sample. This mass fingerprint is then analysed by comparison to a database containing reference mass fingerprints of relevant species.

The essential prerequisite for accurate identification of microbial samples, necessarily, is the inclusion of reliable reference data for a comprehensive listing of species in the database. In the case of MALDI-TOF MS databases, reference data can be individual mass fingerprints, mass fingerprints computed from repeated analyses of a single isolate or mass fingerprints computed from analyses of multiple con-specific isolates. Regardless of the particular analytical approach, for reliable identifications, a comprehensive database is essential. For clinical purposes, the database should contain reference spectra of all relevant pathogenic bacteria and fungi, as well as the respective closely related species. Since microbial species generally show a degree of intra-specific variation, with respect to nucleotide sequences, biochemical properties, as well as mass spectral patterns, a given species should be represented by multiple strains, i.e., to cover the natural diversity of the species [46]. This insight, of course, is not new. Sneath [66] proposed that characterizations of 25, preferably, but not less than 10, strains are necessary for describing a (new) species. Furthermore, the species assignment of isolates used for establishing the database should be validated, through the use of well-characterized reference strains, i.e., type strains and other reference strains used for quality control in laboratories, as well as other clinical and environmental isolates that have been thoroughly 'vetted'. Due to the never-ending developments in technology for analysing microorganisms and the perpetually unsettled nature of microbial taxonomy, strains often have been assigned to species that subsequently undergo changes in nomenclature, which may cause eventual confusion (Hinse et al. this issue [30]). It also should be mentioned that the designated type strains occasionally are not 'typical' representatives of a species and may not be appropriate as the sole reference for identifying unknown isolates.

As a result of the mass spectral comparison of a microbial strain, a list of species matches is generated with a value for the quality of the mass spectra similarities, analogous to BLAST or FASTA analyses of nucleotide sequences. From this list, the sample's taxonomic designation may be derived. The two commercial systems available to date generate a 'score value' (MALDI Biotyper, Bruker) or a 'confidence value' (SARAMIS [Spectral Archive and Microbial Identification system] Anagnostec/BioMérieux). The species-level identification thresholds are empirical values, derived from comparisons of strains and correlated with other phenotypic and genotypic characterizations. The species-level threshold will vary for different microbial taxa, i.e., as is also the case for nucleotide sequence similarities that are used for microbial characterizations. The actual mass spectra comparison can be based on different criteria. For example, the presence or absence of peaks in mass spectra can be the sole criteria or peak height can be taken into account. Different mass ranges can be used and should be defined for analyses; in most cases of microbial identification, 2–20 kDa has been observed to be the most applicable. Mass peaks can be filtered further, in order to select for taxon-specific biomarker peaks or to reduce spectral noise and background similarity [58]. This strategy is consequently followed in SARAMIS where 'SuperSpectra' are applied for identification of isolates. SuperSpectra are composite spectra computed from the reference spectra of multiple strains or clones of a given species by, firstly, identifying stable mass signals by computing a consensus spectrum and, secondly, identifying species-specific patterns of mass signals, by comparing the consensus spectrum to 100,000+ reference spectra in the developing database representing more than 3,000 microbial species [39]. Remarkably, different statistical algorithms for analysing a set of mass data spectra of prokaryotic isolates generally lead to similar results, underlining the basic robustness of the method De Bruyne et al. [10], [80], Wittwer et al. this issue [90].

MALDI-TOF MS applications in microbial systematics

Microbial systematics and taxonomy can be said to be made up of three components: (1) characterization; (2) classification; and (3) nomenclature. In turn, microbial systematics provides the basis for identification. Identification of a microbial strain (i.e., a microbial "population", derived from a clonal variant, obtained from a given specimen) may follow two basic modes of analyses, i.e.,

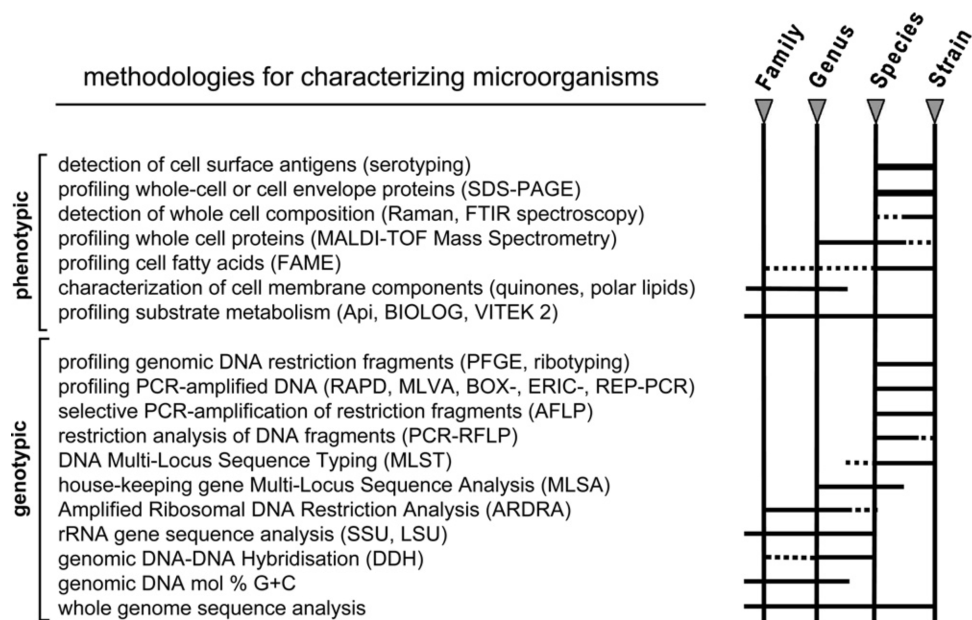

Fig. 5. An overview of different methods commonly used for microbial typing, with estimates of the levels of expected resolution for each method. The horizontal bars indicate the level of resolution covered by the given methodology. MALDI-TOF MS can be expected to differentiate micro-organisms at the species or sub-species levels. Modified from [78,49].

characterization of phenotypic traits and characterization of genotypic traits. Phenotypic traits are the observable characteristics that result from expression of the genes of an organism. Genotypic traits of an organism are those encoded within its genetic material, the genome. The *Ad Hoc* Committee for the Re-evaluation of the Species Definition in Bacteriology [67] and, more recently, Tindall et al. [72] have comprehensively described the methods and rationale that should be used for the characterization of prokaryotes.

Phenotypic characterizations have been the traditional methods for microbial identification. However, the last 50 years have witnessed a shift in emphasis, from phenotyping to genotyping, for characterizing prokaryotes and fungi. Wayne et al. [84] proposed that phylogeny should determine taxonomy (of prokaryotes) and that the complete DNA sequence would be the reference standard for phylogeny. Thus, while genome sequence analyses of microorganisms will, presumably, provide the ultimate basis for establishing and defining microbial species, the working criteria of laboratories for the identification of microorganisms will rely on methods that are sensitive, rapid, cost-effective and, ideally, correlate with the genomic sequence data and phylogenies. To this end, systematists will continue to develop methods exploiting biomarkers that are able to elucidate microbial species delineations (Fig. 5).

The species is the basic unit in biological taxonomy, and the concept of what this unit means in microbial taxonomy has been formulated as "a category which is a (preferably) genetically related group of individual isolates/strains which includes a large degree of agreement, in independent characteristics which have been tested in comparative terms under highly standardized conditions" [56]. This 'species concept' applies to the idea that a microbial species is comprised of an aggregation of strains that bear a common evolutionary history (i.e., a monophyletic group) and are coherent genotypically and phenotypically, clearly discriminated from the closest relatives [56]. This idea or concept seems to be universally applicable. However, the way that species are circumscribed, or defined, is recognized to be, often, subjective or arbitrary.

In the case of bacteria, DNA–DNA hybridisation (DDH) of genomic DNA [37] is recognized to be the genotypic "gold standard", with a genomic DDH similarity of 70% serving as the recommended boundary 'guideline' for defining bacterial species [84]. However, DDH protocols are recognized to be complicated, with inherently large degrees of error; the necessity for alternative methods has been recognized [67]. DNA sequence analyses have become the 'methods of choice' among bacterial systematists, as those that offer the highest levels of resolution and differentiation. With the application of targeted PCR-amplification and sequencing of ribosomal RNA (rRNA) genes [95], phylogenetic relationships of bacteria are able to be estimated rapidly, reliably and with reproducibility in different laboratories. More recently, multi-locus sequencing, targeting selected house-keeping genes (i.e., genes of conserved enzymes and proteins essential for cellular function), as well as combinations of gene targets, offer opportunities to exploit the varying degrees of conservation contained within genomes for elucidating bacterial taxa at the highest potential level of resolution, i.e., the single nucleotide [3,48]. The best possibility, to date, for an alternative method to replace DDH as the genotypic 'gold standard' for defining prokaryotic species, is the Average Nucleotide Index (ANI) [42] of fully or partially sequenced (at least 20%) genomes [55].

MALDI-TOF MS is increasingly applied to taxonomic issues of microbiology, for example, to rapidly reveal cryptic species in large batches of related isolates [8,44], Munoz et al. this issue [50]. However, at present, it is improbable to define a concrete similarity value for mass spectral fingerprints of con-specific isolates, i.e., a threshold that separates species. However, this is not entirely surprising because the same rules for defining microbial species do not apply to all taxa. Furthermore, there exist, as well, historical biases that have defined microbial species according to criteria that are not necessarily correlated with current views of how microbial species should be delineated. When mass spectra fingerprints of multiple, well characterized strains of closely related species are analysed, e.g., by cluster analysis, separation of distinct groups is usually evident. The studies reported in this special issue consistently demonstrate the ability of MALDI-TOF MS to delineate strains at species-levels for several genera, including *Acinetobacter* (Šedo et al. this issue [62]), *Legionella* (Gaia et al. this issue [25]), *Leuconostoc* (De Bruyne et al. this issue [10]), *Staphylococcus* (Decristophoris et al. this issue [11]), *Stenotrophomonas* (Vasileuskaya-Schulz et al. this issue [81]), *Streptococcus* (Hinse et al. this issue [30]), *Yersinia*

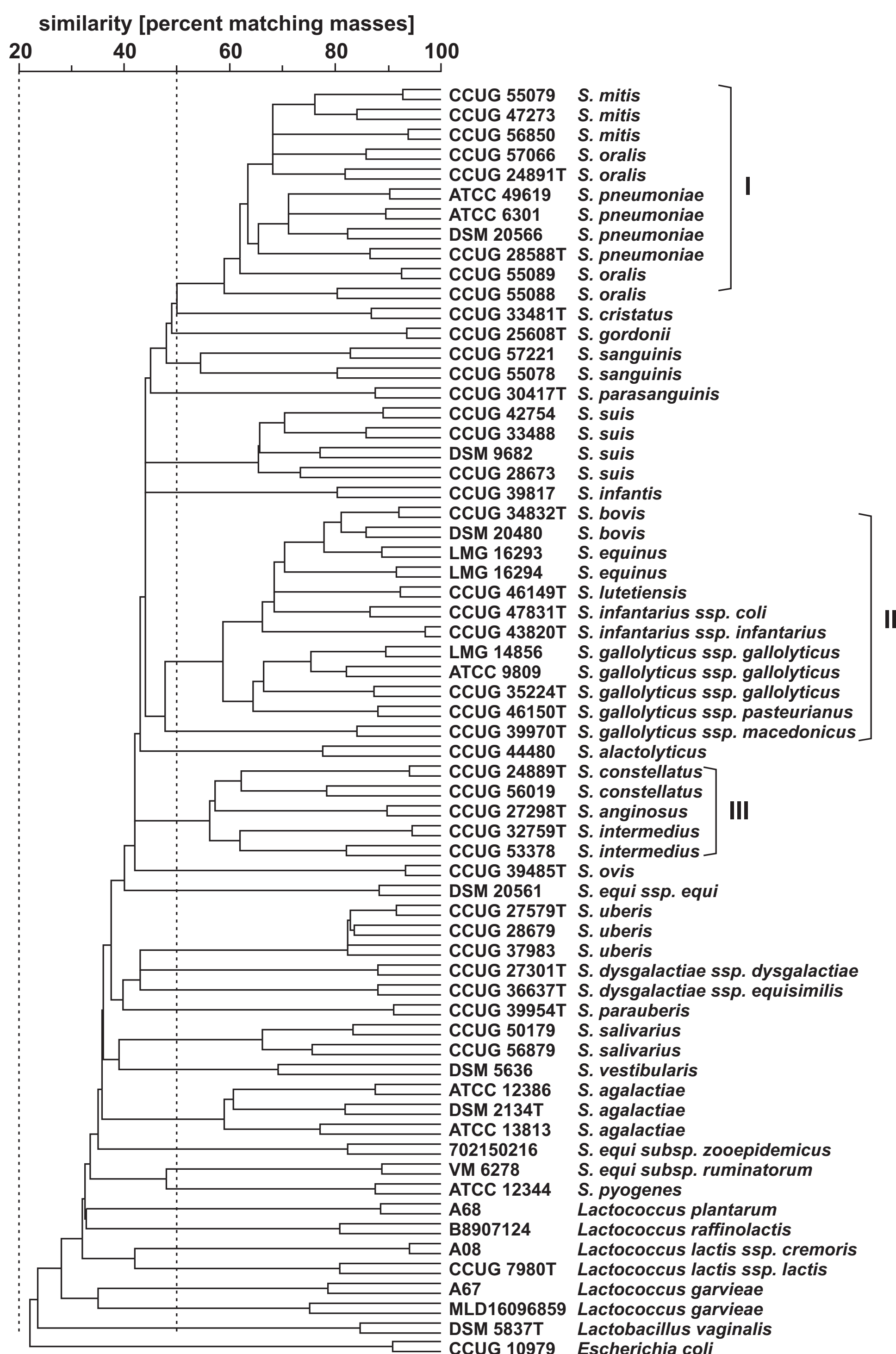

Fig. 6. Dendrogram constructed by single linkage agglomerative clustering from a similarity matrix, computed from whole cell mass spectra of strains of *Streptococcus* and *Lactococcus* spp., using SARAMIS. All strains have been measured in duplicate, in linear ion extraction mode on an AXIMA-Confidence (Shimadzu) instrument after direct smear preparation and on-target extraction with CHCA matrix solution. The scale refers to the relative similarity, in percent of matching mass signals.

(Wittwer et al. this issue [90]), From a study on Gram-positive anaerobic cocci, for example, it is evident that strains that have been assigned preliminarily to a single species, *Peptoniphilus ivorii*, based on 16S rRNA gene sequence similarities (>95% similarity), in fact, comprise, at least, three different, novel species (Wildeboer-Veloo et al. [88]). In this case, a comparatively low number of well-characterized reference strains are available, to date, and the number of validly published species certainly does not reflect the full diversity of this group.

In consequence, thresholds of mass spectral fingerprint similarity for discriminating species must, therefore, be set expressly for the particular taxa. As with DNA sequence data, e.g., 16S rRNA gene sequences, species assignment is not always straight-forward. One major reason is the intra-specific variation that exists for microbial taxa. While a set of strains of one species could share more than 75% of mass signals, this number can be lower than 50% for strains of other species. In Fig. 6, a dendrogram computed from the mass spectral

patterns of various *Streptococcus* and *Lactococcus* species are shown.

Most species are seen to be separated by less than 50% peak pattern similarity, while forming consistent groups of multiple con-specific strains (e.g., *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus suis*), whereas several species cluster in groups with approximately 60% peak pattern similarity (groups denoted by roman numbers). These groups may be recognized as 'species complexes' by other methods, with the respective species being extremely difficult to differentiate, sometimes based on only a few or a single reaction. In the *Streptococcus mitis/pneumoniae/oralis* complex ([13] group I), a clear distinction of the three individual species could not be achieved, based on mass spectral patterns; particularly, strains identified as *Streptococcus oralis* appear to be 'paraphyletic'. The same applies to the other groups (*Streptococcus equinus/bovis* complex [30,59]; *Streptococcus anginosus* [*Streptococcus milleri*] [79]). Mass spectra-based identifications of individual strains of these groups may be hampered by the background similarity and could allow assignment only to the level of a species complex. On the other hand, mass spectra similarity of approximately 20% has to be considered to be non-specific, as is demonstrated by the *Escherichia coli* out-group. With only slightly higher mass spectra similarities, all strains of *Lactococcus* species are clearly separated from *Streptococcus*, the genus from which they have been separated taxonomically [60], although they do not form a genus-specific cluster.

When examining the duplicate analyses of individual strains in a dendrogram, a particular spectrum-to-spectrum variation is evident, with mass spectra similarities as low as 70% observed between replicate measurements in some cases. The variation between individual replicate spectra can be reduced, to a large degree, by standardizing the preparation procedures, although a residual 'haziness' intrinsic to the MALDI-TOF spectral analysis itself cannot be completely eliminated. In fact, replicate mass spectra with 100% similarities are practically not achievable.

While MALDI-TOF MS and WC-MS has been shown to be applicable for species-level differentiation and identification, attempts to apply the methodology to higher-resolution differentiation has met with mixed success. The difficulties in conclusively differentiating closely related species of species complexes in various taxa has been mentioned. Thus, the potential for elucidating sub-specific taxa, currently, is certainly limited. However, in this issue, MALDI-TOF MS differentiation is described for new sub-species proposed for *Bifidobacterium longum* (Sato et al. this issue [57]) and nitrogen-fixing strains of *Frankia* spp. (Hahn et al. this issue [27]). Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) are differentiated from methicillin-sensitive strains (MSSA) (Shah et al. this issue [64]). Thus, it can be concluded that advances in sample preparation and new approaches in data analyses provide the potential for increasing the level of resolution at which MALDI-TOF MS analyses may be applicable. The advantages in the speed and cost of analyses provide a strong incentive for further developments and improvements for extending the range of applications.

While strains of a particular species generally produce similar mass spectral patterns, using WC-MS, similarities at the genus- or family-levels are rarely useful. At taxonomic levels above families, WC-MS generates only limited resolution, because the mass fingerprints generally are so dissimilar that conclusions about taxonomic relationships are problematic and unreliable. Based on mass spectrometry alone, an isolate cannot be assigned to an order or a class, for example, when no reference data are available. Accordingly, delineation of Gram-positive and Gram-negative bacteria is not possible. With improvements in reference databases, however, it may become increasingly possible to identify new isolates from a wide range of habitats.

Despite these limitations, mass spectrometry is increasingly applied in microbial taxonomy as a novel technique for characterizing and differentiating strains [52,73]. For culture collections, the rapid screening of large batches of strains can reveal the necessity to reassess strain identifications [8].

MALDI-TOF MS applications in clinical microbiology

In cases of clinical diagnostics, the identification of microorganisms in patient specimens in a rapid, reliable and cost-effective way may be crucial for initiating appropriate treatment, as well as defining associated risks that impact the patient and, potentially, other patients and health-care personnel that come into contact with the source. Since WC-MS, to date, is applicable primarily for analysing clonal isolates (with some exceptions, see below), cultivation of the microorganisms is necessary. This cultivation step is, necessarily, more time-consuming than alternative cultivation-independent approaches, such as PCR- or hybridisation probe-based protocols. However, currently, many clinical diagnoses are not possible by cultivation-independent methods and WC-MS provides the potential to obtain microbial identification of isolates within minutes of obtaining the isolate culture, rather than hours to days that are typically required for traditional phenotypic and genotypic characterizations. Identification systems based on mass spectrometry are being introduced in clinical laboratories to replace, at least in part, the biochemical and metabolic profile-based identification systems [6,63,76]. This is fostered by the, now, wide acceptance and appreciation of the power of WC-MS for microbial identification, as well as by the fact that official accreditations and certifications have been granted [4]. Mass spectra-based identification protocols have been validated in numerous laboratories.

The number of samples that can be analysed by WC-MS at a given time depends largely on instrumental parameters, such as target design and laser frequency. As mentioned above, batches of 100 samples can be analysed in less then an hour; the analysis of more than 1,000 samples per day with a single instrument is realistic, even when maintenance and stand-by times are considered. Through-put could be enhanced further by efficient automation of the sample preparation process [45]. MALDI-TOF and WC-MS-based identification can be integrated into existing laboratory routines. The results delivered by WC-MS will, most likely, effect significant changes to the current clinical microbiological practice [85]. On the one hand, the volume of samples analysed and the number of isolates identified can be effectively increased due to the speed and ease with which multiple colonies on a single agar plate are processed. The resulting increase in information will likely improve the understanding of microbial infection and pathogen diversity. On the other hand, microorganisms that are usually combined according to phenotypic criteria may be reconsidered. For example, the phenotypes commonly referred to as 'coagulase negative staphylococci' or 'Gram-negative non-fermenters', etc., cannot be identified as such by MALDI-TOF MS. This may encourage a reassessment of basic principles in how clinical laboratories will operate.

MALDI-TOF MS applications in environmental microbiology

MALDI-TOF MS shows particular potential for applications in environmental microbiology, e.g., to rapidly reveal cryptic species in large batches of related isolates [8,44], Munoz et al. this issue [50]. However, the present limits of mass spectral approaches for characterization and identification in environmental microbiology are evident. Compared to estimates of a few millions to hundreds of millions of prokaryotic species populating the earth, the number of isolated, characterized, described and validly published prokary-

otic species of approximately 10,500 (at the end of year 2010) [22] is relatively low, equating prokaryotic taxonomy to the 'black hole' of biological systematics [89]. Somewhat less dramatic, perhaps, is the situation confronting the fungi, where estimates of the number of potential species go as high as 1.5 million, with approximately 70,000 species described (albeit, Mycology does not have in place a validation system, as does Bacteriology, for publishing new names and species). Nevertheless, compared to the estimates of numbers of potential microbial species, only a minor fraction is presently contained in mass spectra databases. Consequently, the probability that an environmental isolate will represent a 'new' species is high, whereas the probability that the isolate is represented in a mass spectra database and identifiable is, currently, relatively low. In combination with the acknowledgement that reliable taxonomic classification of microorganisms above the family level is not realistic, using mass spectral analyses, and the current low number of reference mass spectra in databases, MALDI-TOF MS should be appreciated to present relatively limited capability for the identification of environmental isolates. However, WC-MS represents an efficient tool for rapidly characterizing and comparing environmental isolates that originate from given ecosystems. With WC-MS, a large number of isolates can be screened rapidly and at relatively low cost [14,33]. Analyses of mass spectra allow groupings of individual isolates, consequently offering the possibility of reducing the redundancy in the number of isolates to be analysed further for identification, e.g., by DNA sequencing. Once isolates have been identified by other methods, i.e., genotypic approaches, the mass spectral fingerprints can be added to the database for future identifications.

Future applications and possible limitations of MALDI-TOF MS

Although applications of MALDI-TOF MS are today not fully exhausted, certain limitations are already evident. These include issues such as analyses of uncultivable microorganisms, analyses of samples of mixed strains and differentiation of very closely related taxa. With further advances in technology, as well as more refined methods of sample preparation, some of the current constraints likely will be overcome.

A number of studies postulate the possibility of using MALDI-TOF MS to differentiate antibiotic resistant strains from susceptible ones of the same species, as has been done for MRSA vs. MSSA [19,20], Shah et al. [64]. As many or most genes responsible for antibiotic resistance are encoded in mobile genetic elements with a high potential of horizontal gene transfer, resistance factors are not necessarily linked to the phylogenetic relationships of strains [9,21]. However, an indirect approach assigns isolates to taxonomic lineages for which the resistance patterns are known [54,87]. An additional approach, focused on the detection of enzymes responsible for antibiotic resistance, e.g., β-lactamase, has been reported [5], although it has not yet been validated that the presence or absence of respective peaks in mass spectra is evidence for susceptibility and is not due merely to a lack or low expression of the enzyme.

Several studies have evaluated the possibility to use MALDI-TOF MS for analysing mixed samples to correctly identify the individual components [82,83]. Although two or three bacterial species can generally be separated and identified when present at similar amounts in the sample, the limit of MALDI-TOF MS is evident when one species in a mixture strongly predominates. Since MALDI-TOF MS is a chemical analysis, the abundance of particular analytes, in a sample, i.e., protein biomarkers, determines directly their ability to be detected. Consequently, the protein signals of a microorganism with only a minor share, e.g., less than 10% of the total biomass (unpublished data), in a mixed sample are prone to be lost in the background noise. Nevertheless, uncultivable microorganisms or microbial consortia can be analysed, analogous to clonal isolates. The interpretation of the resulting mass spectral fingerprints and the identification of individual species, however, are possible only for species existing in a sample in significant dominance. This can be the case, for example, with urinary tract infections, wherein a single species generally predominates within a urine sample [23]. In other clinical samples, for example respiratory and stool samples, the background of commensal bacteria and degradation products hinders the detection of potential pathogens that may be present in comparatively low abundances. While the identification of pathogens directly from patient blood samples is problematic, the identification of pathogens from positive blood cultures has recently been established, significantly reducing the time-to-result [17]. Especially in cases of blood stream infections, a rapid and efficient onset of therapy is of high relevance. More complex mixtures of natural microbial consortia, such as biofilms, generally contain dozens or hundreds of different species and the straightforward identification of individual species by mass spectrometry is improbable. However, mass spectra could, conceivably, be useful for monitoring changes in communities in response to changing environmental conditions.

The taxonomic resolution of MALDI-TOF-MS is currently considered comparable or superior to comparative 16S rRNA gene sequence analysis. Future studies will most likely increase taxonomic resolution, at least for particular taxa, by extending the mass ranges, effective filtering of background or uninformative peak signals, improved algorithms for mass spectral comparison, etc. However, despite impending technical advances that may be developed to improve the capabilities of MALDI-TOF MS for identification of microorganisms, difficulties in differentiating particular closely related taxa may persist, such as the situation of *Shigella* species and *Escherichia coli*, where a distinction may be considered to be clinically relevant but which may not be justified under taxonomic criteria [53].

Future applications of MALDI-TOF MS in microbiology will depend on the expansion of databases for areas such as veterinary medicine, plant pathology, food safety, industrial microbiology, drinking water production, and pharmacology, to name a few. To achieve reliable identifications of microorganisms in these fields of study, the relevant reference species and strains have to be analysed and included into the databases. Apart from database expansion, technological progress will allow increased automation and throughput, as well as a minimization of the required sample amount. The latter has been achieved, for example, in aerosol MALDI-TOF mass spectrometers that allow single bacterial cells and spores to be analysed and identified [68,77]. Advancements in the MALDI-TOF MS technology, itself, will improve not only the speed of data acquisition but, most likely, the quality of mass spectra and, thus, the information content of a mass fingerprint. This will be increasingly explored for sub-specific typing and epidemiological studies. Whatever future applications of MALDI-TOF MS in microbiology that emerge, it is very likely that this technology will occupy a foremost position for analyses in diverse fields of microbiology.

Acknowledgements

We acknowledge the helpful comments of anonymous reviewers on an earlier draft of the manuscript. We are indebted to many friends and colleagues who shared thoughts and ideas on numerous occasions. ERBM was supported by funding of the FoU-Västra Götaland Region Projects VGFOUREG-30781 and 83080, by ALF-medel för forskning Västra Götaland Region project ALFGBG-11574 and by SIDA project VR 254405107.

References

[1] Anhalt, J.P., Fenselau, C. (1975) Identification of bacteria using mass spectrometry. Anal. Chem. 47, 219–225.
[2] Arniri-Eliasi, B., Fenselau, C. (2001) Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. Anal. Chem. 73, 5228–5231.
[3] Bishop, C.J., Aanensen, D.M., Jordan, G.E., Kilian, M., Hanage, W.P., Spratt, B.G. (2008) Assigning strains to bacterial species via the Internet. BMC Biol. 7, 3.
[4] Burak, S., Engels-Schwarzlose, S., Erhard, M., Welker, M., Gehrt, A. (2010) Official accreditation of a MALDI-TOF MS based identification system for diagnostic microbiology. Int. J. Med. Microbiol. 299, 9.
[5] Camara, J.E., Hays, F.A. (2007) Discrimination between wild-type and ampicillin-resistant *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Bioanal. Chem. 389, 1633–1638.
[6] Cherkaoui, A., Hibbs, J., Emonet, S., Tangomo, M., Girard, M., Francois, P., Schrenzel, J. (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J. Clin. Microbiol. 48, 1169–1175.
[7] Claydon, M.A., Davey, S.N., Edwards-Jones, V., Gordon, D.B. (1996) The rapid identification of intact microorganisms using mass spectrometry. Nat. Biotechnol. 14, 1584–1586.
[8] Clermont, D., Diard, S., Motreff, L., Vivier, C., Bimet, F., Bouchier, C., Welker, M., Kallow, W., Bizet, C. (2009) Description of *Microbacterium binotii* sp. nov., isolated from human blood. Int. J. Syst. Evol. Microbiol. 59, 1016–1022.
[9] Davies, J., Davies, D. (2010) Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417–433.
[10] De Bruyne, K., Slabbinck, B., Waegemann, W., Vauterin, P., De Baets, B., Vandamme, P. Bacterial species identification from MALDI-TOF MS spectra through data analysis and machine learning. Syst. Appl. Microbiol., this issue.
[11] Decristophoris, P., Fasola, A., Benagli, C., Tonolla, M., Petrini, O. Identification of *Staphylococcus intermedius* group by MALDI-TOF MS. Syst. Appl. Microbiol., this issue.
[12] Demirev, P.A., Lin, J.S., Pineda, F.J., Fenselau, C. (2001) Bioinformatics and mass spectrometry for microorganism identification: proteome-wide post-translational modifications and database search algorithms for characterization of intact *H. pylori*. Anal. Chem. 73, 4566–4573.
[13] Denapaite, D., Bruckner, R., Nuhn, M., Reichmann, P., Henrich, B., Maurer, P., Schahle, Y., Selbmann, P., Zimmermann, W., Wambutt, R., Hakenbeck, R. (2010) The genome of *Streptococcus mitis* B6 – what is a commensal? PLoS One 5, e9426.
[14] Dieckmann, R., Graeber, I., Kaesler, I., Szewzyk, U., von Döhren, H. (2005) Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS). Appl. Microbiol. Biotechnol. 67, 539–548.
[15] Dieckmann, R., Helmuth, R., Erhard, M., Malorny, B. (2008) Rapid classification and identification of Salmonellae at the species and subspecies level by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. 74, 7767–7778.
[16] Dieckmann, R., Strauch, E., Alter, T. (2010) Rapid identification and characterization of *Vibrio* species using whole-cell MALDI-TOF mass spectrometry. J. Appl. Microbiol. 109, 199–211.
[17] Drancourt, M. (2010) Detection of microorganisms in blood specimens using matrix-assisted laser desorption ionization time-of-flight mass spectrometry: a review. Clin. Microbiol. Infect. 16, 1620–1625.
[18] Dreisewerd, K. (2003) The desorption process in MALDI. Chem. Rev. 103, 395–425.
[19] Du, Z., Yang, R., Guo, Z., Song, Y., Wang, J. (2002) Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 74, 5487–5491.
[20] Edwards-Jones, V., Claydon, M.A., Evason, D.J., Walker, J., Fox, A.J., Gordon, D.B. (2000) Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J. Med. Microbiol. 49, 295–300.
[21] Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H., Spratt, B.G. (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. U.S.A. 99, 7687–7692.
[22] Euzéby, J.P. (1997) List of prokaryotic names with standing in nomenclature. Int. J. Syst. Bacteriol. 47, 590–592, Available from: <http://www.bacterio.net>.
[23] Ferreira, L., Sanchez-Juanes, F., Gonzalez-Avila, M., Cembrero-Fucinos, D., Herrero-Hernandez, A., Gonzalez-Buitrago, J.M., Munoz-Bellido, J.L. (2010) Direct identification of urinary tract pathogens from urine samples by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry. J. Clin. Microbiol. 48, 2110–2115.
[24] Freiwald, A., Sauer, S. (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. Nat. Prot. 4, 732–742.
[25] Gaia, V., Casatim, S., Tonolla, M. Rapid identification of *Legionella* spp. by MALDI-TOF MS based protein mass fingerprinting. Syst. Appl. Microbiol., this issue.
[26] Göker, M., Klenk, H.-P. (2010) En route to a genome-based taxonomy of *Archaea* and *Bacteria*? Syst. Appl. Microbiol. 33, 175–182.
[27] Hahn, D.R., Mirza, B., Benagli, C., Vogel, G., Tonolla, M. Typing of nitrogen-fixing *Frankia* strains by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Syst. Appl. Microbiol., this issue.
[28] Heller, D.N., Cotter, R.J., Fenselau, C., Uy, O.M. (1987) Profiling of bacteria by fast atom bombardment mass spectrometry. Anal. Chem. 59, 2806–2809.
[29] Hillenkamp, F., Karas, M. (2000) Matrix-assisted laser desorption/ionization, an experience. Int. J. Mass Spectrom. 200, 71–77.
[30] Hinse, D., Vollmer, T., Erhard, M., Welker, M., Moore, E.R.B., Kleesiek, K., Dreier, J. Differentiation of *Streptococcus bovis/equinus*-complex isolates by MALDI TOF mass spectrometry in comparison to sequencing methods. Syst. Appl. Microbiol., this issue.
[31] Holland, R.D., Wolkes, J.G., Rafii, F., Sutherland, J.B., Persons, C.C., Voorhees, K.J., Lay, J.O. (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 10, 1227–1232.
[32] Horneffer, V., Forsman, A., Strupat, K., Hillenkamp, F., Kubitscheck, U. (2001) Localization of analyte molecules in MALDI preparations by confocal laser scanning microscopy. Anal. Chem. 73, 1016–1022.
[33] Ichiki, Y., Ishizawa, N., Tamura, H., Teramoto, K., Sato, H., Yoshikawa, H. (2008) Environmental distribution and novel high-throughput screening of APEO-degrading bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS). J. Pestic. Sci. 33, 122–127.
[34] Jarman, K.H., Daly, D.S., Anderson, K.K., Wahl, K.L. (2003) A new approach to automated peak detection. Chemometr. Intell. Lab. Syst. 69, 61–76.
[35] Jarman, K.H., Wahl, K.L. (2006) Development of spectral pattern-matching approaches to matrix assisted laser desorption/ionization time-of-flight mass spectrometry for bacterial identification. In: Wilkins, C.L., Lay, J.O. (Eds.), Identification of Microorganisms by Mass Spectrometry, John Wiley & Sons, Hoboken, NJ, pp. 153–160.
[36] Jaskolla, T.W., Lehmann, W.D., Karas, M. (2009) 4-Chloro-a-cyanocinnamic acid is an advanced, rationally designed MALDI matrix. Proc. Natl. Acad. Sci. U.S.A. 105, 12200–12205.
[37] Johnson, J.L., Ordal, E.J. (1968) Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. J. Bacteriol. 95, 893–900.
[38] Kallow, W., Dieckmann, R., Kleinkauf, N., Erhard, M., Neuhof, T. (2000) Method of identifying microorganisms using MALDI-TOF-MS. European Patent.
[39] Kallow, W., Erhard, M., Shah, H.N., Raptakis, E., Welker, M. (2010) MALDI-TOF MS and microbial identification: years of experimental development to an established protocol. In: Shah, H.N., Gharbia, S.E., Encheva, V. (Eds.), Mass Spectrometry for Microbial Proteomics, Wiley, Chichester, pp. 255–276.
[40] Karas, M., Bachmann, D., Bahr, U., Hillenkamp, F. (1987) Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int. J. Mass Spectrom. Ion Processes 78, 53–68.
[41] Kemptner, J., Marchetti-Deschmann, M., Mach, R., Druzhinina, I.S., Kubicek, C.P., Allmaier, G. (2009) Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 23, 877–884.
[42] Konstantinidis, K.T., Tiedje, J.M. (2005) Genomic insights that advance the species definition for prokaryotes. Proc. Natl. Acad. Sci. U.S.A. 102, 2567–2572.
[43] Krishnamurthy, T., Ross, P.L., Rajamani, U. (1996) Detection of pathogenic and non-pathogenic bacteria by matrix assisted laser desorption/ionization time of flight mass spectrometry. Rapid Commun. Mass Spectrom. 10, 883–888.
[44] Kroppenstedt, R.M., Mayilraj, S., Wink, J.M., Kallow, W., Schumann, P., Secondini, C., Stackebrandt, E. (2005) Eight new species of the genus *Micromonospora*, *Micromonospora citrea* sp. nov., *Micromonospora echinaurantiaca* sp. nov., *Micromonospora echinofusca* sp. nov., *Micromonospora fulviviridis* sp. nov., *Micromonospora inyonensis* sp. nov., *Micromonospora peucetia* sp. nov., *Micromonospora sagamiensis* sp. nov., and *Micromonospora viridifasciens* sp. nov. Syst. Appl. Microbiol. 28, 328–339.
[45] Lange, O., Erhard, M., Teutsch, C., Sander, J. (2008) MIROB: automatic rapid identification of micro-organisms in high through-put. Ind. Robot – Int. J. 35, 311–315.
[46] Lartigue, M.F., Hery-Arnaud, G., Haguenoer, E., Domelier, A.S., Schmit, P.O., van der Mee-Marquet, N., Lanotte, P., Mereghetti, L., Kostrzewa, M., Quentin, R. (2009) Identification of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 47, 2284–2287.
[47] Liu, H., Du, Z., Wang, J., Yang, R. (2007) Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Appl. Environ. Microbiol. 73, 1899–1907.
[48] Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russel, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U.S.A. 95, 3140–3145.
[49] Moore, E.R.B., Mihaylova, S.A., Vandamme, P., Krichevsky, M.I., Dijkshoorn, L. (2010) Microbial systematics and taxonomy: relevance for a microbial commons. Res. Microbiol. 161, 430–438.
[50] Munoz, R., López-López, A., Urdiain, M., Antón, J., Moore, E.R.B., Rosselló-Móra, R. Evaluation of MALDI-TOF whole cell profiles to assess the culturable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. Syst. Appl. Microbiol., this issue.
[51] Neuhof, T., Dieckmann, R., Druzhinina, I.S., Kubicek, C.P., von Döhren, H. (2007) Intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma/Hypocrea*: can molecular phylogeny of species predict peptaibol structures? Microbiology 153, 3417–3437.
[52] Pukall, R., Schumann, P., Clermont, D., Bizet, C. (2008) Bacillus aeolius DSM 15084(T) (=CIP 107628(T)) is a strain of Bacillus licheniformis. Int. J. Syst. Evol. Microbiol. 58, 1268–1270.

[53] Pupo, G.M., Lan, R., Reeves, P.R. (2000) Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc. Natl. Acad. Sci. U.S.A. 97, 10567–10572.
[54] Rezzonico, F., Vogel, G., Duffy, B., Tonolla, M. (2010) Whole cell MALDI-TOF mass spectrometry application for rapid identification and clustering analysis of *Pantoea* species. Appl. Environ. Microbiol. 76, 4497–4509.
[55] Richter, M., Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U.S.A. 106, 19126–19131.
[56] Rosselló-Móra, R., Amann, R.I. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. 25, 39–67.
[57] Sato, H., Teramoto, K., Ishii, Y., Watanabe, K., Benno, Y. Phylogenetic analysis of *Bifidobacterium longum* strains based on ribosomal protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Syst. Appl. Microbiol., this issue.
[58] Sauer, S., Freiwald, A., Maier, T., Kube, M., Reinhardt, R., Kostrzewa, M., Geider, K. (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS One 3, e2843.
[59] Schlegel, L., Grimont, F., Ageron, E., Grimont, P.A.D., Bouvet, A. (2003) Reappraisal of the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov. Int. J. Syst. Evol. Microbiol. 53, 631–645.
[60] Schleifer, K.-H., Kraus, J., Dvorak, C., Klipper-Bälz, R., Collins, M.D., Fischer, W. (1986) Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. Syst. Appl. Microbiol. 36, 354–356.
[61] Schulten, H.R., Beckey, H.D., Meuzelaar, H.L.C., Boerboom, A.J.H. (1973) High resolution field ionization mass spectrometry of bacterial pyrolysis products. Anal. Chem. 45, 191–195.
[62] Šedo, O., Vorác, A., Zdráhal, Z. Optimization of mass spectral features in MALDI-TOF MS profiling of *Acinetobacter* species. Syst. Appl. Microbiol., this issue.
[63] Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.E., Rolain, J.M., Raoult, D. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Infect. Dis. 49, 543–551.
[64] Shah, H.N., Rajakaruna, L., Ball, G., Misra, R., Al-Shahib, A., Fang, M., Gharbia, S.E. Tracing the transition of methicillin resistance in sub-populations of *Staphylococcus aureus*, using SELDI-TOF mass spectrometry and artificial neural network analysis. Syst. Appl. Microbiol., this issue.
[65] Shroff, R., Rulisek, L., Doubsky, J., Svatos, A. (2009) Acid–base-driven matrix-assisted mass spectrometry for targeted metabolomics. Proc. Natl. Acad. Sci. U.S.A. 106, 10092–10096.
[66] Sneath, P.H.A. (1977) The maintenance of large numbers of strains of microorganisms, and the implications for culture collections. FEMS Microbiol. Lett. 1, 333–334.
[67] Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden, M.C.J., Nesme, X., Rosselló-Móra, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C., Whiotman, W.B. (2002) Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52, 1043–1047.
[68] Stowers, M.A., van Wuijckhuijse, A.L., Marijnissen, J.C.M., Scarlett, B., van Baar, B.L.M., Kientz, C.E. (2000) Application of matrix-assisted laser desorption/ionization to on-line aerosol time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 14, 829–833.
[69] Sundqvist, B., Hedin, A., Hakansson, P., Kamensky, I., Salehpour, M., Säwe, G. (1985) Plasma desorption mass spectrometry (PDMS): limitations and possibilities. Int. J. Mass Spectrom. Ion Processes 65, 69–89.
[70] Teramoto, K., Kitagawa, W., Sato, H., Torimura, M., Tamura, T., Tao, H. (2009) Phylogenetic analysis of *Rhodococcus erythropolis* based on the variation of ribosomal proteins as observed by matrix-assisted laser desorption ionization-mass spectrometry without using genome information. J. Biosci. Bioeng. 108, 348–353.
[71] Teramoto, K., Sato, H., Sun, L., Torimura, M., Tao, H., Yoshikawa, H., Hotta, Y., Hosoda, A., Tamura, H. (2007) Phylogenetic classification of *Pseudomonas putida* strains by MALDI-MS using ribosomal subunit proteins as biomarkers. Anal. Chem. 79, 8712–8719.
[72] Tindall, B.J., Rosselló-Móra, R., Busse, H.J., Ludwig, W., Kämpfer, P. (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int. J. Syst. Evol. Microbiol. 60, 249–266.
[73] Toth, E.M., Schumann, P., Borsodi, A.K., Keki, Z., Kovacs, A.L., Marialigeti, K. (2008) *Wohlfahrtiimonas chitiniclastica* gen. nov., sp nov., a new gammaproteobacterium isolated from Wohlfahrtia magnifica (Diptera: Sarcophagidae). Int. J. Syst. Evol. Microbiol. 58, 976–981.
[74] Trüper, H.G., Schleifer, K.-H. (1991) Prokaryote characterization and identification. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes, Springer, New York, pp. 126–148.
[75] Valentine, N.B., Wunschel, S.C., Wunschel, D.S., Petersen, C.E., Wahl, K.L. (2005) Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. Appl. Environ. Microbiol. 71, 58–64.
[76] van Veen, S.Q., Claas, E.C., Kuijper, E.J. (2010) High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J. Clin. Microbiol. 48, 900–907.
[77] van Wuijckhuijse, A.L., Stowers, M.A., Kleefsman, W.A., van Baar, B.L.M., Kientz, C.E., Marijnissen, J.C.M. (2005) Matrix-assisted laser desorption/ionisation aerosol time-of-flight mass spectrometry for the analysis of bioaerosols: development of a fast detector for airborne biological pathogens. J. Aerosol Sci. 36, 677–687.
[78] Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60, 407–438.
[79] Vandamme, P., Torck, U., Falsen, E., Pot, B., Goossens, H., Kersters, K. (1998) Whole-cell protein electrophoretic analysis of viridans streptococci: evidence fro heterogeneity among *Streptococcus mitis* biovars. Int. J. Syst. Bacteriol. 48, 117–125.
[80] Vanlaere, E., Sergeant, K., Dawyndt, P., Kallow, W., Erhard, M., Sutton, H., Dare, D., Samyn, B., Devreese, B., Vandamme, P. (2008) Matrix assisted laser desorption ionization-time of flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex species. J. Microbiol. Methods 75, 279–286.
[81] Vasileuskaya-Schulz, Z., Kaiser, S., Maier, T., Kostrzewa, M., Jonas, D. Delineation of *Stenotrophomonas* spp. by multi-locus sequence analysis and MALDI-TOF mass spectrometry. Syst. Appl. Microbiol., this issue.
[82] Wahl, K.L., Wunschel, S.C., Jarman, K.H., Valentine, N.B., Petersen, C.E., Kingsley, M.T., Zartolas, K.A., Saenz, A.J. (2002) Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 74, 6191–6199.
[83] Warscheid, B., Fenselau, C. (2004) A targeted proteomics approach to the rapid identification of bacterial cell mixtures by matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 4, 2877–2892.
[84] Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Trüper, H.G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37, 463–464.
[85] Welker, M. (2010) MALDI-TOF MS for identification of microorganisms: a new era in clinical microbiological research and diagnosis. In: Hays, J.P., van Leeuwen, W.B. (Eds.), The Role of New Technologies in Medical Microbiological Research and Diagnosis, Bentham Science Publishers, Bussum, NL (eBook) in press.
[86] Welker, M., Marsalek, B., Sejnohova, L., von Döhren, H. (2006) Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: toward an understanding of metabolic diversity. Peptides 27, 2090–2103.
[87] Wilcox, S.K., Cavey, G.S., Pearson, J.D. (2001) Single ribosomal protein mutations in antibiotic-resistant bacteria analyzed by mass spectrometry. Antimicrob. Agents Chemother. 45, 3046–3055.
[88] Wildeboer-Veloo, A.C.M., Erhard, M., Welker, M., Welling, G.W., Degener, J.E. Identification of Gram-positive anaerobic cocci by AXIMA@SARAMIS MALDI-TOF mass spectrometry. Syst. Appl. Microbiol., this issue.
[89] Wilson, E.O. (2003) The encyclopedia of life. Trends Ecol. Evol. 18, 77–80.
[90] Wittwer, M., Heim, J., Schär, M., Dewarrat, G., Schürch, N. Tapping the potential of intact cell mass spectrometry with a combined data analytical approach. Syst. Appl. Microbiol., this issue.
[91] Wunschel, D.S., Hill, E.A., McLean, J.S., Jarman, K.H., Gorby, Y.A., Valentine, N.B., Wahl, K.L. (2005) Effects of varied pH, growth rate and temperature using controlled fermentation and batch culture on matrix assisted laser desorption/ionization whole cell protein fingerprints. J. Microbiol. Methods 62, 259–271.
[92] Wunschel, S.C., Jarman, K.H., Petersen, C.E., Valentine, N.B., Wahl, K.L., Schauki, D., Jackman, J., Nelson, C.P., White, E. (2005) Bacterial analysis by MALDI-TOF mass spectrometry: an inter-laboratory comparison. J. Am. Soc. Mass Spectrom. 16, 456–462.
[93] Wynne, C., Fenselau, C., Demirev, P.A., Edwards, N. (2009) Top-down identification of protein biomarkers in bacteria with unsequenced genomes. Anal. Chem. 81, 9633–9642.
[94] Xu, C.X., Lin, X.M., Ren, H.X., Zhang, Y.L., Wang, S.Y., Peng, X.X. (2006) Analysis of outer membrane proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline. Proteomics 6, 462–473.
[95] Yarza, P., Richter, M., Peplies, J., Euzéby, J.P., Amann, R.I., Schleifer, K.-H., Ludwig, W., Glöckner, F.O., Rosselló-Móra, R. (2008) The All-Species Living Tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst. Appl. Microbiol. 31, 241–250.