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Mini-review

Rapid dereplication of microbial isolates using matrix-assisted laser desorption ionization time-of-flight mass spectrometry: A mini-review

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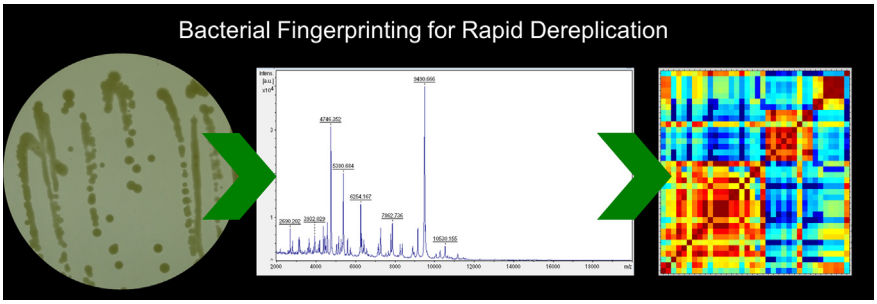
HIGHLIGHTS

- MALDI-TOF MS is applicable as high-resolution and high-throughput tool.
- The classification and characterization of cultivable microorganisms is targeted.
- Advantageous are its simple sample preparation and short measurement time.
- It accelerates the dereplication of isolates from large-scale screening campaigns.
- Applications for studying microbial diversity and future trends are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has become one of the most popular methods for the rapid, cost-effective and accurate classification and characterization of cultivable microorganisms. Due to its simple sample preparation and short measurement time, MALDI-TOF MS is an excellent choice for the high-throughput study of microbial isolates from rhizospheres or plants grown under diverse environmental conditions. While clinical isolates have a higher identification rate than environmental isolates due to the focus of commercial mass spectral libraries on the former, no identification is necessary in the dereplication step of large environmental studies. The grouping of large sets of isolates according to their intact protein profiles can be performed without knowledge of their taxonomy. Thus, this method is easily applicable to environmental samples containing microorganisms from yet undescribed phylogenetic origins. The main strategies applied to achieve effective dereplication are, first, expanding existing mass spectral libraries and, second, using an additional statistical analysis step to group measured mass spectra and identify unique isolates. In this review, these aspects are addressed. It closes with a prospective view on how MALDI-TOF MS-based microbial characterisation can accelerate the exploitation of plant-associated microbiota.
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Introduction to MALDI-TOF MS-based microbial characterisation

Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) is an advanced tool for the fast and high-resolution characterization of microorganisms [1]. The

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method is based on measurement of the molecular mass of ions generated from the most abundant proteins of a sample culture and uses the mass spectral information as a fingerprint for a particular organism (exemplified in Fig. 1). A typical workflow containing MALDI-TOF MS starts with the isolation of microorganisms from a chosen sample and their cultivation on nutrient medium to obtain a pure axenic culture [2–4]. Microbial cultures in their exponential phase are grown under standardized conditions and then subjected to sample processing in two different ways: a direct method or a solvent extraction method. The first is a fast technique where a smear of microbial cells is applied directly to the MALDI target plate. This approach usually leads to low-quality spectra due to overloading or the presence of compounds disturbing the ionization process, but can be recommended for routine assessments. For acquisition of high-quality spectra, cell walls are lysed in a suitable way, and proteins are extracted with (usually) formic acid using the solvent extraction method (Fig. 2). The samples spotted onto the MALDI target plate are then overlaid with matrix, and spectra are acquired from intact proteins in the range of m/z 2000 – 20,000 [5]. These spectra are then matched to a reference library to determine the identity of the microorganism. There are several vendors on the market providing instrumental and software solutions as well as commercial spectral libraries for MALDI-TOF MS-based biotyping [6]. As an example, the Bruker MALDI Biotyper library contains spectra of 7014 bacterial and 1300 filamentous fungi isolates (as on February 1st, 2019).

The popularity of MALDI-TOF MS for microbial biotyping is based on its speed, simplicity and cost efficiency. Due to these advances, MALDI-TOF MS diagnosis has been successfully adapted to clinical microbiology in the past 20 years to accelerate patient diagnosis and therapy [7] and plays a vital role in the characterisation of human gut microbiota [8]. Constant enhancements in instrumental platforms, sample processing methods and extent of spectral libraries accelerated the establishment of MS-based diagnosis in clinical laboratories and readers are referred to comprehensive reviews regarding its clinical application [1,9]. In some cases, acquired MALDI-TOF spectra are used to create dendrograms and establish pseudo-phylogenetic groupings based on the similarity of mass spectra. However, because MS spectra, having a limited number of peaks, lack the evolutionary relatedness of small-subunit rRNA sequences or other genomic information, a determination of relatedness of unknown isolates is difficult but can be facilitated by combined analysis of additional biomarkers [10,11].

The method is applicable for a wide range of microbial isolates, including those of bacteria [12–14], fungi [15] and archaea [16], and extends to many other cultivable organisms, such as microalgae [17], protozoa [18] or viruses [19]. Although MALDI-TOF MS is successfully applied in the identification of clinical isolates [2,20], characterization of isolates from plant-associated samples is hampered by a lack of reference spectra in available databases. Nevertheless, MALDI-TOF MS as a powerful tool for the rapid grouping of bacterial isolates, i.e., dereplication, in large-scale screening campaigns. In this review, the applicability of this method as a high-resolution tool for studying microbial diversity is discussed.

MS-based exploration of plant-associated microbial communities

With an increased understanding of the diversity of plant-bacterial associations, future biotechnological applications for stable crop production, conservation of biodiversity and sustain-

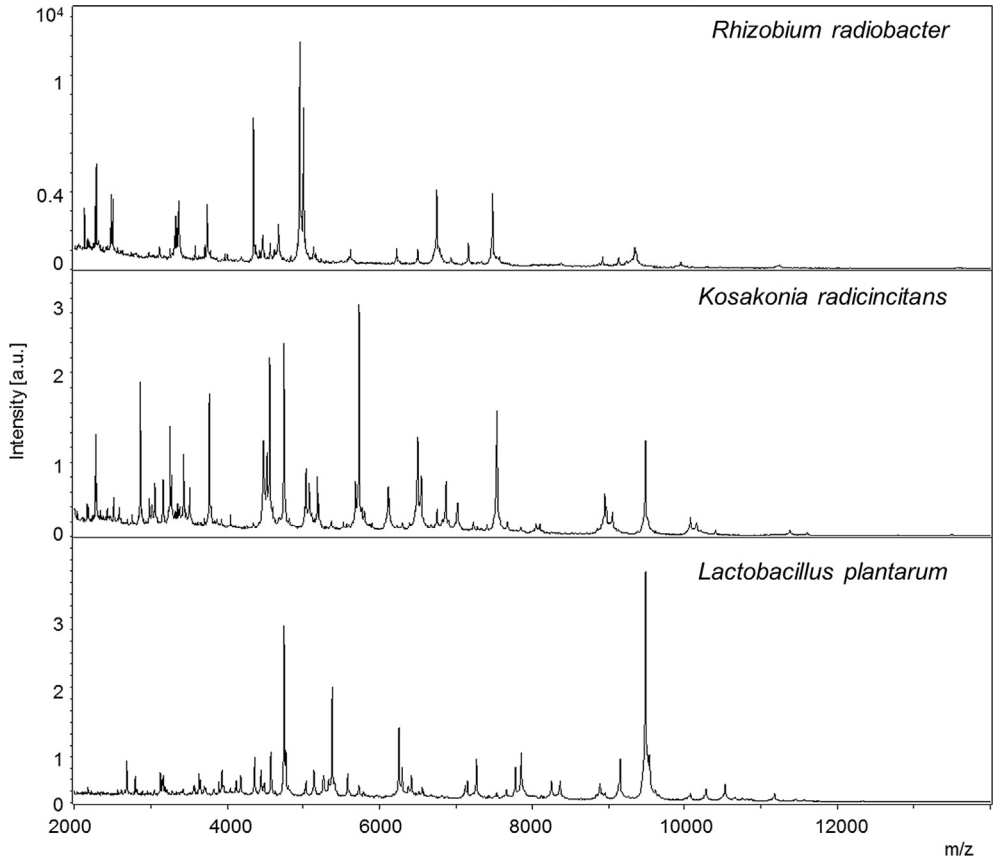

Fig. 1. Exemplary MALDI-TOF MS profiles of three plant-associated bacterial species showing the heterogeneity of protein profiles. The sequence and intensity of mass peaks, representing ionized intact proteins, forming a characteristic microorganisms' profile is called protein fingerprint and this is used for similarity searches of reference spectra.

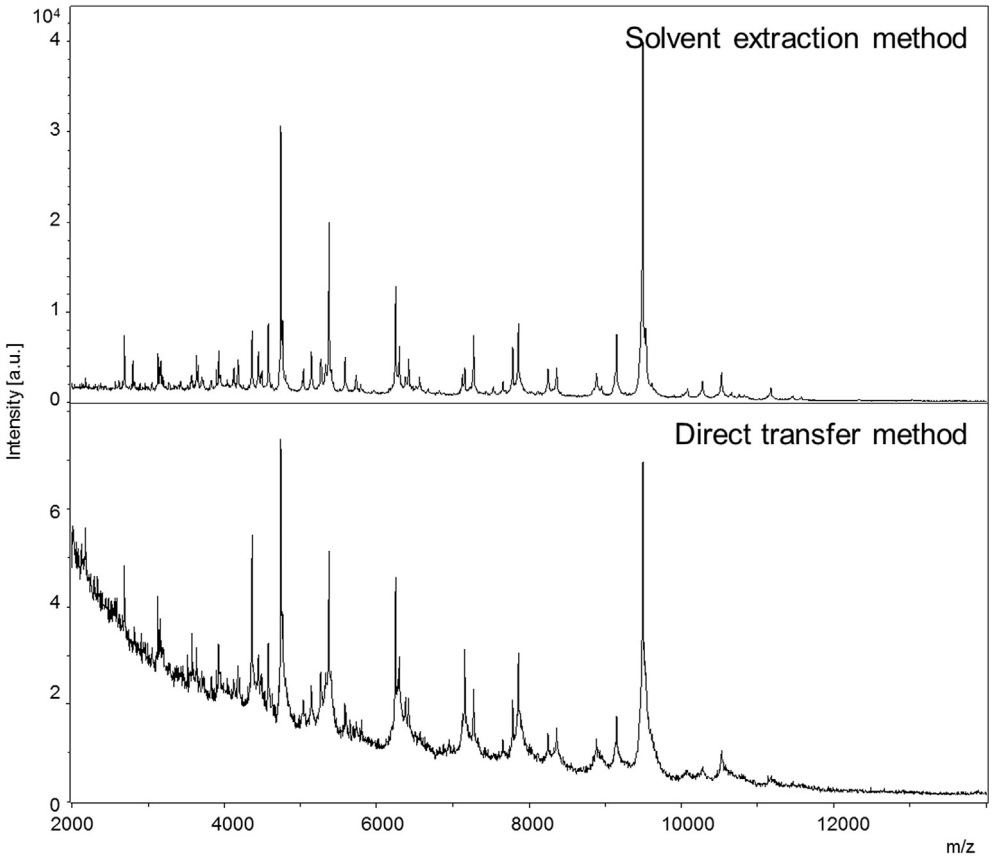

Fig. 2. The effect of sample processing on quality of mass spectra. Application of a protein extraction method (upper panel) results in higher number of detected peaks and better signal-to-noise ratios as compared to the direct transfer method (lower panel). The direct transfer of bacterial cells to the MALDI target gives higher background signals, but the quality of spectra might be sufficient for routine analysis.

able agro-ecosystems are foreseeable. Hence, there is a high demand for high-throughput methods for the classification and characterization of cultivable microorganisms isolated from soils, rhizospheres or plants grown under diverse environmental conditions. There is a growing awareness of the complex interplay between plants, soil and their microbial communities, and current research efforts aim at understanding how microbiota present in rhizospheres and endospheres of crops account for plant health and productivity [21–23]. Up to the present, microbial communities were described often by shotgun sequencing approaches, which left their functionalities and activities aside. More recently, in order to close this gap, microbes have been isolated from their respective environments in extensive culture experiments and assessed for their physiological properties [24]. Novel nutrient media are developed to allow the isolation of niche microorganisms [25,26]. A long-term goal is to manage and engineer soil microorganisms by agricultural practice, to select proper plant genotypes or to apply microbial inoculants with a distinct function, such as biocontrol, growth promotion or abiotic stress alleviation [27].

A common strategy in studying cultivable microorganisms is plating the chosen disrupted tissue or sample on culture medium and assessing the growth of developing colonies. Then, morphologically different colonies are selected for further analysis such as 16S rRNA sequencing or biochemical testing [28,29–32]. This approach usually leads to a bias in assessing the diversity of a habitat since morphological similar species, that may have different metabolic capabilities, are excluded from downstream investigations. Another way of conducting such ecological experiments is to process all isolated microbes for nucleotide analysis without preselection, which results in a considerable sample load and high expense [33–35]. The application of MALDI-TOF MS for fast and inexpensive dereplication of recurrent isolated microorganisms would be of particular advantage in large microbial community studies since the grouping of large sets of isolates according to their intact protein profiles can be performed without knowledge on their taxonomic identification.

Previous studies demonstrated the applicability of MALDI-TOF MS for high-throughput dereplication and its applicability for unbiased studies of the cultivable microbial community [36–39]. The discrimination power of MALDI-TOF MS by combining MS data from both intact proteins and specialized metabolites was recently demonstrated and allowed the characterisation of isolates based on their identity and potential environmental function [10]. In the following, an overview of the bioinformatic background of the dereplication principle is provided.

Bioinformatic means for dereplication of microbial isolates

Identification of plant-associated isolates is less successful as compared to clinical microorganisms due to an underrepresentation of environmental reference strains in commercial mass spectral databases [40,41]. Two main bioinformatic strategies are commonly used to improve dereplication when using whole-cell or simple acidic protein extracts for MALDI-TOF MS. The first strategy is to expand the commercial databases by including additional plant-associated reference strains. This approach has the advantage of still profiting from the simplicity of sample preparation and rapidness in measurement and identification of the MALDI biotyping as no additional statistical analysis is required, but

achieves better identification rates [16,42]. Most platforms have options for researchers to customize the mass spectral libraries with user-selected reference strains and provide training or protocols to create a personalized database. Suitable reference strains can be (1) cultivated environmental samples that have not been identified, (2) purchased, cultivated and measured known reference strains and (3) strains whose mass spectra have been received from other institutes. While some attempts were made to create open-access repositories, they are still very limited in scope [15]. Often, commercial libraries allow only genus-level identification for plant-associated samples. The accuracy of identification can generally be improved by including in-house reference spectra, as they are measured with the same techniques, technicians and machines. In the context of expanding a spectral library, it is crucial to realize that confident species or strain identification can be achieved only when several reference strains of one species are available in the database. Usually, only one strain per species, with the exception of the most common clinical bacteria, is present in the commercial databases. To improve identification, it is advised by commercial library vendors that three to six strains for one species that take into account biological variations should be included for common environmental microorganisms. However, these library expansions need continuous work, and their maintenance can be time consuming.

In large cohort microbial studies, identifying the number of unique species or strains can also be achieved without identifying a microorganism. For the dereplication step, grouping isolates from the same taxon rapidly to determine and reduce the number of isolates for further analysis is sufficient [36]. Therefore, the second strategy involves using statistical analysis to group mass spectra. A first step can be to use available opportunities for visualisation provided by commercial software to create for instance dendrograms or composite correlation matrices [43] to determine similar isolates from all the isolates of one study (Fig. 3). These methods are performed by clustering the obtained peak mass lists or the whole mass spectra of different isolates. However, it can be difficult to visually decide whether individual clusters in a dendrogram represent isolates from the same species or what level of correlation between mass spectra represents isolates from the same species. Using additional statistical analysis steps or software with further options can therefore improve the approach in creating a nonredundant set of isolates. Several studies have successfully implemented MALDI-TOF MS-based biotyping to classify mass spectra for dereplication [36–39,44]. Generally, highly similar clusters were used to identify identical isolates and select representatives of these clusters for further validation, e.g., via partial 16S rRNA gene sequence analysis, ITS region sequencing or repetitive element-based PCR. The evaluation of appropriate cut-off values for cluster delineation was based on threshold values established by the additional validation steps and/or a minimum number of mass peaks shared between isolates. It was shown that a similarity-based MALDI-TOF MS approach can be used for dereplication without additional, costly DNA-based methods [38]. New approaches also include machine learning algorithms such as Random Forest models to automate the identification of isolates in environmental studies [45,46].

Current issues and implications for using MS-based biotyping

The quality of mass spectra is important for successful MALDI-TOF MS-based analysis of microorganisms. For example, that identification can be improved in various ways other than adding missing or rare species to the database or optimizing pre-analytical settings (e.g., sample preparation, growth conditions, and matrix use [5,47–49]). A quality check of the acquired mass spectra and of the pre-set parameters for automated data acquisition is essential, especially when using automated measuring tools. Including low-quality spectra can lead to false positive identification or no identification. Common problems include suppressed peaks, low peak intensities, and matrix background signals. Quality checks need to be included, especially when reference mass spectra for libraries are created. It was suggested that a good spectrum should have a minimum of 70 peaks for bacteria and 30–40 peaks for fungi and an average peak intensity of 10^3 arbitrary units or higher [41].

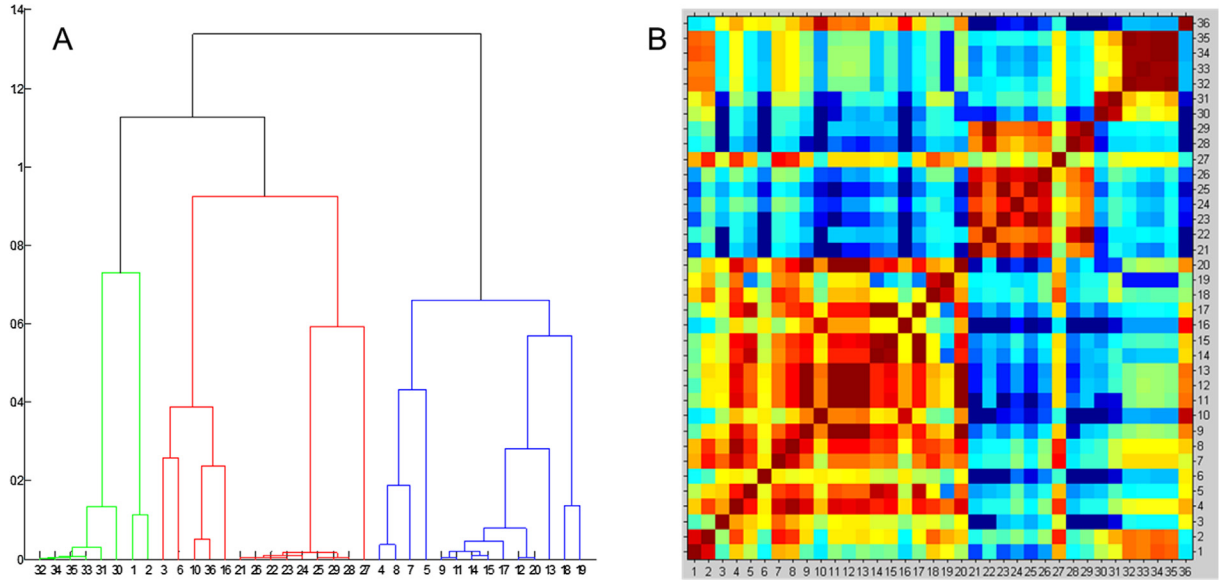

Fig. 3. Approaches for visualizing the relationship between mass spectra derived from microbial samples. Protein extracts from 36 bacterial isolates, originated from parsley phyllosphere, were analysed by MALDI-TOF MS. Recurrent isolated microorganisms can be identified by cluster analysis of protein patterns, where the height of each node is proportional to the dissimilarity value (A). In a composite correlation index matrix, the degrees of mass spectrum correlation are indicated by colour coding (dark red = closely related, dark blue = not closely related) and scoring from 0 to 1, where 1 is an exact match (B). Experimental sample set was kindly provided by Dr. Silke Ruppel, Leibniz Institute of Vegetable and Ornamental Crops, Germany.

To reduce variations caused by technicians and instruments, samples should be spotted in triplicate, and each spot should be analysed at least three times. In a comparison of manual and automatic mass spectrum measurements, it was found that while automatic measurement tended to increase the base peak resolution, other measures of spectrum quality such as signal-to-noise ratio, data richness and reproducibility were reduced [50]. Looking at the same issue of the low reproducibility of automated spectrum acquisition, another study reported optimized user threshold values of several parameters (peak selection mass range, signal-to-noise ratio, threshold peak intensity, threshold minimum resolution, and number of shots summed) and improved reproducibility [51].

A further aspect to consider is that MALDI-TOF MS requires a monoculture to perform identification. However, morphologically similar samples do not need to contain the same species. In environmental dereplication studies, a re-cultivating step from a single colony to control for a pure culture may not be performed. Therefore, some unidentified spectra may be bacterial mixtures that can be hard to identify with algorithms developed to identify single microorganisms. The resulting mass spectra often have signals with suppressed intensity. However, the main abundant peaks of all the species should be present but at much lower intensity than peaks from samples from pure cultures. Further statistical analysis could be used to identify these spectra as well. The large variation in peaks and intensities occurring with mixtures is an issue. Only a few studies have attempted to address this challenge by applying different biomarker identification strategies [52–54]. However, this methodology is yet to be standardized and mixtures of microorganisms are more frequently analysed via tandem MS strategies [55].

Conclusions and future perspectives

MALDI-TOF MS is the method of choice for the grouping of plant-associated microbial isolates due to its fast, simple and cost-effective measurement of a large number of samples. Recent developments in automation of colony picking and deposition on the MALDI target as well as matrix deposition should further decrease consumable costs and preparation time [56]. An increased use of MALDI-TOF MS in large-scale screening campaigns to collect microorganisms from rhizospheres or plants is going to lead to the detection of novel species that could bear a potential use to sustainably increase crop production [57]. Future improvements in dereplication, either by expanding commercial mass spectral libraries and/or by implementing an additional data analysis step to group mass spectra, are expected to further exploit the capacity of MALDI-TOF MS in microbial studies.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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