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RESEARCH ARTICLE

MALDI-TOF mass spectrometry: An efficient tool for rapid and reliable identification of bacterial strains isolated from forest soil

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Abstract

Soil contains the highest diversity level of bacterial communities. The rapidly developing field of soil microbiology seeks reliable and rapid methods for the identification of bacterial strains. This work compared three methods for taxonomical identification of bacterial strains obtained from forest soil samples of Algeria. The methods compared were: morphological, physiological and biochemical tests, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and the sequencing of 16S rRNA gene.

Use of morphological, physiological and biochemical tests, showed a limited application. The MALDI-TOF MS inferred existence of nine different bacterial species among the nine isolates (100% of the isolates were correctly identified to the species level with a high score ≥2). All the isolates were identified using the 16S rRNA gene sequencing method which also proved the existence of the same nine different taxonomical units. This work showed a high potential of MALDI-TOF MS as an efficient tool for reliable identification and rapid discrimination of different bacterial strains isolated from forest soil with minimal time demand, reduced costs and simple handling of the samples.

Introduction:

Research has shown the complexity of microbial populations in environmental samples (Uhlik et al., 2009). 10 g of soil can contain up to 10^7 different bacterial species (Hall, 2007), it has also been estimated that about 5000 bacterial species have been described in soil (Pace, 1997, 1999). Although conserved protein-coding genes can be used to identify bacterial lineages (Santos and Ochman, 2004), the most commonly used phylogenetic markers are genes for ribosomal RNA, most frequently the small subunit RNA gene (16S rRNA gene) (van Elsas et al., 1998).

Until now, bacterial identification has always been a major challenge in all microbiological fields including soil microbiology. The identification of bacterial strains isolated from soil is currently mainly based on the morphological, physiological and biochemical tests. Altogether, these tests allow the identification of most bacterial isolates with great accuracy, but they are costly and time-consuming. The matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) method was first used in microbiology for the identification of small molecules released from cells (Anhalt and Fenselau, 1975). Over time, this method has become an important tool for bacterial identification. It can be used to generate protein fingerprint signatures from whole bacterial cells (Fenselau and Demirev, 2001) and it allows identification of both Gram-positive (Smole et al., 2002; Friedrichs et al., 2007) and Gram-negative bacteria (Conway et al., 2001; Mellmann et al., 2008) to the species level in a few minutes by measuring the molecular masses of proteins and other bacterial components obtained from whole bacterial extracts. By comparing these fingerprints to a database of reference spectra by the use of various algorithms, bacteria can be rapidly identified (Fenselau and Demirev, 2001). The advantage of this technique include a fast sample preparation time, which allows the researcher to perform the analysis in

approximately 10 min (Krishnamurthy and Ross, 1996), rapid results, and very low reagent costs. Although upcoming spectral evaluation does not have to be so explicit, MALDI-TOF MS has a great potential for massive identification screening in clinical microbiology as well as environmental microbiology (Ruelle et al., 2004).

In this study we tested MALDI-TOF MS as an efficient tool for soil microbiology research especially since it has become a routine identification method in clinical laboratories. More specifically, we verified the reliability of the identification of bacterial strains isolated from forest soil samples.

Materials and methods:

Soil sampling:

A soil sample was collected from El Meridj forest, located in El Khroub municipality, south-east of Constantine - east of Algeria- (coordinates: 36°20'37.04"N, 6°41'21.36"E). The sample was sieved (2 mm) to remove organic debris and larger inorganic fragments. The soil was extracted from one site, at 5 cm depth. Samples were divided and used in triplicate for microbiology experiences.

Bacterial isolation and presumptive identification:

Bacteria isolated from the sampling site (described above), were grown on nutrient agar at 37°C for 18 to 24 hours. Each colony was the subject of the following tests: morphological, physiological and biochemical tests. Then our results are compared to the known characteristics of bacteria in Bergey's Manual of Systematic Bacteriology (Holt and Williams, 1989).

Whole-cell MALDI-TOF mass spectrometry analysis:

The MALDI-TOF mass spectrometry protein analysis was carried-out as previously described (Seng et al., 2009; Bizzini et al., 2010). The identification of the isolates by MALDI-TOF MS was performed on a Microflex LT instrument (Bruker Daltonics GmbH, Leipzig, Germany) with FlexControl (version 3.0) software (Bruker Daltonics) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa, according to the instructions of the manufacturer. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 2.0) software (Bruker Daltonics); it makes use of a large database containing reference spectra for more than 3200 reference strains (Nagy et al., 2009). The score value is defined by three components, the matches of the unknown spectrum against the main spectrum, the matches of the main spectrum peaks against the unknown spectrum, and the correlation of intensities of the matched peaks. This leads to a first score, from 0 (no match) to 1,000 (perfect identity), which is converted into a log score from 0 to 3. When the score is greater than 2.0 is considered to indicate good species-level identification and scores above 2.3 correspond to excellent species-level identification. Values between 1.7 and 2.0 correspond to reliable genus-level identification and values below 1.7 indicate no identification (no significant similarity) (Lartigue et al., 2009; Cherkaoui et al., 2010). The identification was first performed by touching the surface of the investigated colony with a sterile pipette tip and directly applying the small amount of sample on polished or ground steel MSP 96 target plates (Bruker Daltonics). The deposited bacteria were overlaid with 1µl of HCCA matrix (a saturated solution of -cyano-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid) and air dried at room tem- perature to allow cocrystallization with the experimental sample. The spectra were then acquired by the mass spectrometer and were compared by using the BioTyper software. Briefly, colony samples were resuspended in a 1.5 ml polypropylene tube (Eppendorf, Hamburg, Germany) containing 1 ml of a water-ethanol (1:2) solution. The cell suspension was centrifuged at 12,500 X g for 2 min, and the supernatant was discarded. The pellet was suspended in 25 µl of 70% formic acid in water and 25 µl of 100% acetoni- trile. A final centrifugation was performed at 12,500 X g for 2 min. Then, 1 µl of supernatant was spotted on the MSP 96 target plate and allowed to dry at room temperature before it was overlaid with 1 µl of the HCCA matrix and analyzed as described above.

PCR amplification of the 16S rRNA gene and sequencing:

For all strains, a nearly full-length 16S rRNA gene (1450 bp) was amplified with the universal primers 16S_FD1 and 16S_rP2 (Weisburg et al., 1991). Each PCR was carried out in a final volume of 50 µl. Reaction mixtures contained 1×PCR buffer with 5 µl template, 50 mM KCl, 1.5 mM MgCl2, deoxynucleoside triphosphate solution (200 µM each dATP, dCTP,dGTP and dTTP), 0.2 µM each oligonucleotide primers and 0.5 units of Taq DNA polymerase. The thermal cycle consisted of an initial 5 min denaturation at 95°C followed by 35 cycles of 30s denaturation at 95°C, primer hybridization at 52°C for 30s and elongation at 72°C for 5 min and a final 5 min elongation step at 72°C. PCR reactions were examined by electrophoresing. Purified PCR products were sequenced with the use of a BigDye® Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Courtabeuf, France), the Bvd1, 5X Sequencing Buffer and the primers 357F, 357R, 536F, 536R, 800F, 800R, 1050F and 1050R (table 1). Sequencing was then performed in ABI3700 automated capillary sequencer (Applied

Biosystems, Foster City, California, United States). The nucleotide sequences were edited using ChromasPro 1.34 software (Copyright (c) 2003-2006 by Technelysium Pty Ltd) and were submitted in GenBank under accession numbers HG421009-HG421017.

Table 1: Primers used in the amplification and the sequencing of the 16S rRNA gene (Adékambi and Drancourt, 2004).

	Primers	Sequence (5′–3′)	Tm* (°C)
Amplification	FD1	AGAGTTTGATCATGGCTCAG	56
	rP2	ACGGCTACCTTGTTACGACTT	62
Sequencing	357F	TACGGGAGGCAGCAG	50
	357R	CTGCTGCCTCCCGTA	50
	536F	CAGCAGCCGCGGTAATAC	58
	536R	GTATTACCGCGGCTGCTG	58
	800F	ATTAGATACCCTGGTAG	48
	800R	CTACCAGGGTATCTAAT	48
	1050F	TGTCGTCAGCTCGTG	48
	1050R	CACGAGCTGACGACA	48

***:** Tm values were determined using the formula: Tm=(2×AT + 4×GC) – 4.

Results:

We obtained nine bacterial isolates from forest soil; these strains were designated S1- S9 and were characterized by their morphological, physiological and biochemical profiles. Three strains of the nine (S1, S7 and S9) belong to the genus Enterobacter. Whereas the other bacterial strains belong to different genera as mentioned in the table 2.

All bacterial strains were subjected to whole-cell MALDI-TOF MS analysis. This method discriminates bacteria on the basis of screening of characteristic peaks observed as biomarkers for bacterial identification. Large numbers of peaks are retained for each reference strain, constituting a spectrum typical of the species concerned. This strategy is improved by the use of several reference strains for each species, which must be included in the database. In our experiment, we used the software MALDI Biotyper (Bruker Daltonics) to compare the collected spectra of our nine bacterial strains with the reference database and to generate a numerical value (score) based on the similarities between the observed and stored datasets. As shown in table 3, all the nine spectra aligned with the MALDI BioTyper database were correctly identified to the species level (scores ≥2).

The 16S rRNA gene sequencing supported the results from the mass spectrometry; we obtained the same bacterial species. The entire identification process is summarized in table 4

Table 2: Morphological, physiological and biochemical characteristics of the different bacterial strains.

Strains	Identification	Tests																	
		Morphological		Physiological			Biochemical												
		Gram	Mob[a]	ONPG[b]	OX[c]	Cat[d]	Lac[e]	Saccharose	Mannose	H_2S	Gaz	Urease	Ind[f]	Cit[g]	RM[h]	VP[i]	ODC[j]	LDC[k]	ADH[l]
S1	*Enterobacter sp.*	-	+	+	-	+	+	+	+	-	+	+	-	+	-	+	+	-	+
S2	*Pantoea sp.*	-	+	+	-	+	+	+	+	-	-	-	-	+	-	+	-	-	-
S3	*Staphylococcus sp.*	+	-	-	-	+	-	-	-	-	+	+	+		+	+	+	+	+
S4	*Bacillus sp.*	+	+	+		+	+	-	-	-	+	+	+		-	+	+	+	+
S5	*Lysinibacillus sp.*	+	+	+	+	+	+	-	-	-	+	-	+		+	-	+	+	+
S6	*Pseudomonas sp.*	-	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+
S7	*Enterobacter sp.*	-	+	+	-	+	+	+	+	-	+	-	-	+	-	+	+	+	-
S8	*Stenotrophomonas sp.*	-	+	+	+	+	+	-	_	-	-	+	-	+	-	+	+	-	+
S9	*Enterobacter sp.*	-	+	+	-	+	+	+	+	-	+	-	-	+	-	+	+	-	+

(+): Strains positive; (-): Strains negative.
([a]): Mobility; ([b]): Ortho-Nitrophenyl-β-galactoside; ([c]): Oxidase; ([d]): Catalase; ([e]): Lactose; ([f]): Indole production; ([g]): Citrate; ([h]): Methyl red; ([i]): Voges-Proskauer;
([j]): Ornithine decarboxylase; ([k]): Lysine decarboxylase; ([l]): Anti-Diuretic hormone.

Table 3: Identification of the nine bacterial strains by MALDI-TOF MS.

Strains	Identification according to MALDI Biotyper	Scores
S1	*Enterobacter hormaechei*	2.340
S2	*Pantoea vagans*	2.240
S3	*Staphylococcus caprae*	2.160
S4	*Bacillus cereus*	2.120
S5	*Lysinibacillus fusiformis*	2.115
S6	*Pseudomonas putida*	2.380
S7	*Enterobacter aerogenes*	2.300
S8	*Stenotrophomonas rhizophila*	2.202
S9	*Enterobacter cloacae*	2.320

Table 4: Identification of bacterial strains by 16S rRNA gene sequencing.

Strains	Identification	Accession numbers
S1	*Enterobacter hormaechei*	HG421009
S2	*Pantoea vagans*	HG421010
S3	*Staphylococcus caprae*	HG421011
S4	*Bacillus cereus*	HG421012
S5	*Lysinibacillus fusiformis*	HG421013
S6	*Pseudomonas putida*	HG421014
S7	*Enterobacter aerogenes*	HG421015
S8	*Stenotrophomonas rhizophila*	HG421016
S9	*Enterobacter cloacae*	HG421017

Discussion:

The morphological, physiological and biochemical tests represent one of the oldest methods used for bacterial identification and characterization in soil microbiology. But as seen in table 2, all the nine bacterial strains were identified to genus level only, which is not sufficient for a complete and accurate identification of our strains. These limitations due to the difficulties in dislodging bacteria from soil particles, selecting suitable growth media (Tabacchioni et al., 2000), provision of specific growth conditions (temperature, pH, light), inability to culture a large number of bacterial species using techniques available at present and the potential for inhibition or spreading of colonies other than that of interest (Trevors, 1998). However, the morphological, physiological and biochemical

tests should not be underestimated; they still have an irreplaceable role in obtaining and characterizing new strains. Their important advantages include low cost and easy performance.

The main task of this study was to evaluate the applicability of MALDI-TOF MS and the MALDI Biotyper database in soil microbiology for the identification of bacterial strains in forest soil. Our results show that MALDI-TOF MS and MALDI Biotyper can be used for these purposes for several reasons. First, it is well recognized that MALDI-TOF MS exhibit the potential to be a species discriminating technique (Welker and Moore, 2011); it has successfully been used for the identification of a wide array of bacterial species (van Baar, 2000; Conway et al., 2001; Fenselau et al.,2001; Lay, 2001; Kumar et al.,2004), especially in such a diverse field as soil microbiology. Nevertheless, the results should be approached with a certain caution. From our data we can see that Gram-positive bacteria (S3, S4 and S5) provided fewer biomarkers than Gram-negative ones (S1, S2, S6, S7, S8 and S9). This phenomenon has also been described by other research groups, and Smole et al. (2002) suggested treatment of such samples with lysozyme as a method of dealing with this limitation. However, Grosse-Herrenthey et al. (2008) used, for their identification of various *Clostridium* strains, only extra treatment with trifluoroacetic acid and acetonitrile, a method that has also been suggested by Vargha et al. (2006) for *Arthrobacter* strains. Second, MALDI-TOF MS is able to identify bacteria on the level of species (Maier et al., 2006), which has been illustrated with our strains (Table 3). The use of MALDI-TOF MS has been shown to be able to identify different strains (Hettick et al., 2006; Dubois et al., 2010). Put the other way around, employing this technique can allow for the determination of which strains are the same strain and thereby reduce the financial costs associated with further analyses of isolates, as only one representative can be chosen and worked with further. Third, due to its rapidity, easy sample preparation and relatively low sample size, MALDI-TOF MS is a very suitable method for the screening of a larger number of bacterial soil (Krishnamurthy et al., 1996); the whole process consists only of growing the culture on a medium, transferring a colony onto the MALDI target, mixing it with a matrix, and performing MS analysis (which takes about 30 s per spot). Fourth, regarding speed of identification of bacteria, Seng et al. (2009) previously estimated a mean time to result for samples identified by MALDI-TOF-MS of 6 min, whereas the morphological, physiological and biochemical tests would yield the same identifications in 5–48 h. Cherkaoui et al. (2010) demonstrated that, the analysis of 9 isolates in parallel can be accomplished in <15 min with limiting working time by MALDI-TOF-MS, which is the case of our results. Fifth, the cost of bacterial identification by MALDI-TOF-MS was estimated to represent only 17–32% of the costs of identification using the morphological, physiological and biochemical tests in the study performed by Seng et al. (2009), which is supported by two other prospective studies by Bizzini and Greub (2010) and Cherkaoui et al. (2010). Sixth, it is important to highlight that the available databases that serve as references for bacterial identification need to be progressively supplemented with spectra of newly isolated bacteria. Our results suggest that all the nine bacterial strains are included in the MALDI Biotyper database. Finally, on the other hand, to fully ensure the reliability of the identification of our strains by MALDI-TOF MS, we used an alternative method based on the 16S rRNA gene sequencing; we obtained the same bacterial species compared to MALDI-TOF MS results as we see clearly in table 4. Among the reasons of choosing the 16S rRNA gene sequencing method, first, it is the most useful technique used to bacterial identification in soil (Ward et al,. 1992) and according to Song et al. (2003), the 16S rRNA gene is regarded as the most widely accepted gene for bacterial identification. Second, over time, it has become a reliable tool for identifying and classifying bacteria, it has shown functional consistency with a relatively good clocklike behaviour (Chanama, 1999) and the 16S rRNA gene length of approximately 1,500 bp is sufficient for bioinformatic analysis and species identification (Janda and Abbott, 2007). Third, this method has been so widely adopted that DNA sequence databases are flooded with sequences of the 16S rRNA gene. According to Barghoutti (2011), when pure PCR products of the 16S gene are obtained, sequenced, and aligned against bacterial DNA data base, then the bacterium can be identified. Finally, the distinctive approach when identifying bacterial species using this method is to perform high-throughout sequencing of 16S rRNA genes, which are then taxonomically classified based on their similarity to known sequences in existing databases (Mizrahi-Man et al., 2013).

Conclusion:

The data prospectively gathered in the present study demonstrated that MALDI-TOF MS identification is an efficient, cost-effective method for the rapid and routine identification of bacterial strains in the soil microbiology laboratory. It can be used as the first-line method of bacterial identification, before Gram staining and any morphological, physiological and biochemical tests. On the other hand, MALDI-TOF MS has the potential to reduce the need for molecular identification methods such as 16S rRNA gene sequencing and might replace this time-consuming and expensive method. As well, further expansion of the MALDI Biotyper database of the instrument will undoubtedly increase the accuracy of identification by the MALDI-TOF MS and the diversity of species that might be efficiently identified by this promising approach.

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