Chapter 11

In Situ Analysis of Bacterial Lipopeptide Antibiotics by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) is a technique developed in the late 1990s enabling the two-dimensional mapping of a broad variety of biomolecules present at the surface of a sample. In many applications including pharmaceutical studies or biomarker discovery, the distribution of proteins, lipids or drugs, and metabolites may be visualized within tissue sections. More recently, MALDI MSI has become increasingly applied in microbiology where the versatility of the technique is perfectly suited to monitor the metabolic dynamics of bacterial colonies. The work described here is focused on the application of MALDI MSI to map secondary metabolites produced by *Bacilli*, especially lipopeptides, produced by bacterial cells during their interaction with their environment (bacteria, fungi, plant roots, etc.). This chapter addresses the advantages and challenges that the implementation of MALDI MSI to microbiological samples entails, including detailed protocols on sample preparation (from both microbiologist and mass spectrometrist points of view), matrix deposition, and data acquisition and interpretation. Lipopeptide images recorded from confrontation plates are also presented.

Key words Matrix-assisted laser desorption/ionization mass spectrometry imaging, Lipopeptides, Bacteria

1 Introduction

Antimicrobial compounds represent a broad range of molecules, probably not completely discovered nor explored. Antibiotics may be produced by different kinds of microorganisms, which display very different lifestyles and evolve in various environments. Some of these microorganisms live in the rhizosphere, the zone of soil that surrounds and is influenced by the roots of plants [1]. This complex ecosystem gathers diverse types of microbes, such as fungi, nematodes, and others, including bacteria [2]. The interactions existing between the plants and these microorganisms may be positive or negative (or even neutral); bacteria having a beneficial

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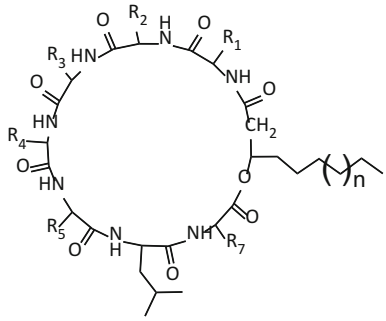
effect on plants are called plant growth-promoting rhizobacteria (PGPR) [3]. Some isolates of the bacterial genera *Bacillus* and *Paenibacillus* may be found in soil and are considered powerful biocontrol agents, owing to their beneficial behavior toward plants [4]. How do they do it to protect their host? Multiple mechanisms are involved in that process but the best characterized and understood is the direct antagonism against phytopathogens. To that end, soil *Bacilli* and *Paenibacilli* produce antibiotics, mobilizing up to 8 % of their genome to antibiotic synthesis [5–7]. Among these antimicrobial compounds are found cyclic lipopeptides (cLP) which are synthesized by nonribosomal peptide synthetases (NRPS) or hybrid polyketide synthases/nonribosomal peptide synthetases (PKS/NRPS) [8]. Their particular chemical structure, made of a cyclic peptide linked to a fatty acid chain, leads to a wide structural variety. Especially, modifications to the amino acids' structure and/or sequence explain the occurrence of different variants within a family (fusaricidins A and C; fengycins A, B, C, and S; or iturins A and C). Also, the diversity in length and isomery (linear, iso, or anteiso) of the acyl chain generates, for each variant, different homologues (Fig. 1) [9–11].

Actually, these compounds are well characterized and have been studied for decades [12]; however, the producing strains are cultivated in "lab" conditions which are not representative of what bacteria face in their natural habitat where nutrients are limited and the growth rates are slowed down. Besides, bacteria evolve in biofilm-related structures on the surface of plant roots [13]. The role of cLPs in the biocontrol activity of their producers has already been investigated, and surfactins were proven to be elicitors of the immune-related responses in the host plant (ISR, induced systemic resistance) [14–16]. Nevertheless, adequate models and innovative analytical methods are still needed to determine if these compounds are actually produced and accumulate in the microenvironment in a biologically relevant way.

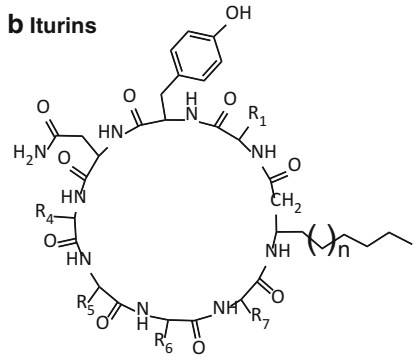
One available analytical technique for the analysis of secondary metabolites originating from microbes is matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). MALDI MS relies on the use of a laser (most of the time, a UV laser), to desorb and ionize the molecules of interest present in the sample. The energy of the laser beam is transferred to the analyte through a MALDI matrix. Usually, the MALDI matrix is a small organic molecule, exhibiting an acidic (to work in positive ion mode) or a basic (to work in negative ion mode) character and absorbing at the wavelength of the laser. Different chemical reactions take place in the ionization source, such as proton transfer from the ionized matrix to the analyte, leading to protonated ions, noted $[M+H]^+$. In the case of biological samples analyzed without purification steps, $[M+Na]^+$ or $[M+K]^+$ ions are usually detected. MALDI MS

a **Surfactins**



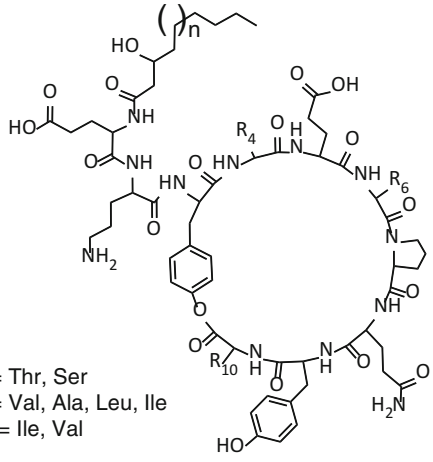
R_1 = Glu, Gln; R_2 = Val, Leu, Ile; R_3 = Met, Leu;
R_4 = Val, Ala, Leu, Ile; R_5 = Asp, Pro; R_7 = Val, Leu, Ile

b **Iturins**



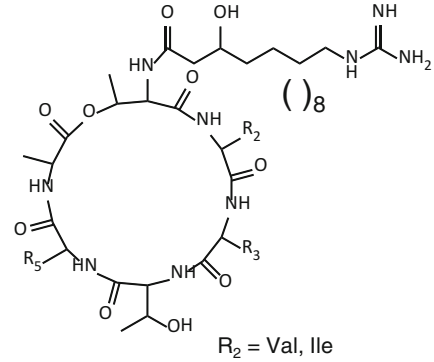
R_1 = Asn, Asp; R_4 = Pro, Gln, Ser; R_5 = Glu, Pro; R_6 = Ser, Asn; R_7 = Thr, Ser, Asn

c **Fengcycins**



R_4 = Thr, Ser
R_6 = Val, Ala, Leu, Ile
R_{10} = Ile, Val

d **Fusaricidins and closely-related LI-F-type LP**



R_2 = Val, Ile
R_3 = Val, Tyr, Ile, Phe
R_5 = Asn, Gln

	Surfactins	**Iturins**	**Fengycins**	**Fusaricidins**
Number of amino acids	7	7	10	6
Number of variants	4	7	4	12
Chain lengths	C_{12} to C_{15}	C_{14} to C_{17}	C_{14} to C_{18}	C_{15}
Isomeries	Linear, iso, anteiso	Linear, iso, anteiso	Linear, iso, anteiso	-

Fig. 1 Examples of structures of lipopeptides from *Bacillus* and *Paenibacillus*. General chemical structures are represented for (**a**) surfactins, (**b**) iturins, (**c**) fengycins, and (**d**) fusaricidins. The table summarizes the structural heterogeneity of each family

is nowadays routinely used in microbiology labs as a tool for bacterial identification, based on the detection of a species-characteristic pattern of ribosomal proteins [17]. Applications of this method include the detection of environmental toxins in water by analyzing peptides and polyketides from cyanobacteria [18] or the establishment of protein profiles from intact fungal spores harvested from the surface of fruits, enabling the identification of unknown fungi [19]. More recently, MALDI MS applied to microorganisms has known new developments in the implementation of MALDI MS imaging (MALDI MSI) to microbial samples. Over the last 15 years, MALDI MSI has become a very powerful tool allowing the localization of potentially hundreds to thousands of compounds (peptide, proteins, lipids, drugs, metabolites, etc.) simultaneously. The principle of MALDI MS imaging relies on the acquisition of an array of coordinate-specific mass spectra, the information of which is integrated to get the spatial distribution of any detected compound. Usually dedicated to the analysis of tissues in proteomic, metabolomic, or lipidomic studies [20], MALDI MSI has gained interest in the microbiological field. Recently, articles have been published, exploiting the unique features of MALDI MSI, to map metabolites, including notably the surfactin-type cLP, secreted during bacterial competition [21, 22], even in 3D [23]. Even more recently, high-resolution MALDI MSI was used to map the secondary metabolites (triterpenoids) of two basidiomycetes *Cyathus striatus* and *Hericium erinaceus* [24]. For our part, we developed a method in which MALDI MSI allows localizing and identifying cLPs produced by bacteria when confronted by a phytopathogen [25] or during the interaction with plant roots [26]. This last methodology uses an *in planta* culture model in which the different partners are grown together on semisolid agar-based medium in a Petri dish.

In this chapter, we describe the methodology and the step-by-step protocols used from the preparation of confrontation plates to MALDI images acquisition.

2 Materials

2.1 Confrontation Plate Preparation

1. Experiments described here were conducted with the *Paenibacillus polymyxa* strain Pp56, isolated from field soil in Ohio and kindly provided by Dr. B. McSpadden-Gardener from Ohio State University, USA. The bacterial strain was confronted by a phytopathogen, *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Forl).
2. Agar.
3. $Ca(NO_3)_2$; KNO_3; $MgSO_4$; KH_2PO_4.

4. H_3BO_3, $MnCl_2{\cdot}4H_2O$; $ZnSO_4{\cdot}7H_2O$; $CuSO_4{\cdot}5H_2O$; $NaMoO_4{\cdot}2H_2O$; EDTA·2Na, $FeSO_4{\cdot}7H_2O$.
5. Petri dishes (10 cm diameter).
6. 1 L boxes.
7. Incubator.
8. Autoclave.
9. ITO (indium tin oxide)-coated glass slide, beforehand sterilized.

2.2 Sample Preparation for MALDI MS Imaging

1. Scalpel.
2. Vacuum desiccator.
3. Pump.

2.3 MALDI Matrix Deposition

1. HPLC grade acetonitrile (ACN).
2. Trifluoroacetic acid (TFA).
3. HPLC grade methanol (MeOH).
4. α-Cyano-4-hydroxycinnamic acid (α-CHCA).
5. Milli-Q water.
6. Flatbed scanner.
7. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.4 Data Acquisition and Processing

1. MALDI Slide Adapter II (target plate).
2. MALDI mass spectrometer (MALDI TOF/TOF Ultraflex II or FTMS Solarix 9.4T, both from Bruker Daltonics, Bremen, Germany).
3. For image acquisition and visualization: FlexImaging (Bruker Daltonics, Bremen, Germany).
4. For data processing: Flex Analysis (for data recorded with Ultraflex II mass spectrometer).
5. For data processing: Data Analysis (for data recorded with Solarix mass spectrometer).

3 Methods

In this example, the antibiotics secreted by *Paenibacillus polymyxa* Pp56 were studied in the context of the interaction of the bacteria with a phytopathogen. The aim was thus to study the secretome in real conditions, which are very different from lab conditions, notably according to nutrients scarcity. Consequently, the first step consisted in producing plant exudates in order to supplement the culture medium with these naturally occurring nutriments.

Table 1
Concentrations of salts in the agar medium for the preparation of culture medium used in confrontation plates

$Ca(NO_3)_2$	5 mM
KNO_3	5 mM
$MgSO_4$	2 mM
KH_2PO_4	1 mM
H_3BO_3	1.4 mg/L
$MnCl_2{\cdot}H_2O$	0.9 mg/L
$ZnSO_4{\cdot}7H_2O$	0.1 mg/L
$CuSO_4{\cdot}5H_2O$	0.05 mg/L
$NaMoO_4{\cdot}2H_2O$	0.02 mg/L
EDTA, 2Na	5.2 mg/L
$FeSO_4{\cdot}7H_2O$	3.9 mg/L

3.1 Production of Plant Exudates

1. Prepare an agar solution at a concentration of 15 g/L.
2. Dissolve the salts in the agar solution, in order to get the concentrations specified in Table 1.
3. Pour this nutrient medium into Petri dish.
4. Deposit tomato seeds on this gelified medium and let it germinate for 5 days at room temperature in the dark.
5. Transfer germinated seeds to 1 L boxes filled with 500 mL of nutrient solution (four plants per box).
6. Grow plants for 4 weeks with a 16 h photoperiod alternating sunlight and fluorescent light.
7. Collect the hydroponic liquid and centrifuge to remove debris.

3.2 Preparation of Confrontation Plates

1. Dilute the exudate solution to fivefold volume and supplement with agar to reach a concentration of 15 g/L.
2. Sterilize in an autoclave.
3. Insert a sterilized ITO-coated glass slide into a 10 cm diameter Petri dish. Add gently the nutrient medium in order to cover it with 2–3 mm of gelified medium.
4. Prepare a fresh bacterial suspension from overnight pre-culture in order to obtain a final concentration of 5×10^7 cells/mL.
5. Streak the bacterial suspension over the width of the glass slide.
6. Inoculate the fungus at one extremity of the plate.
7. Leave the Petri dish to incubate the required duration at 25 °C.

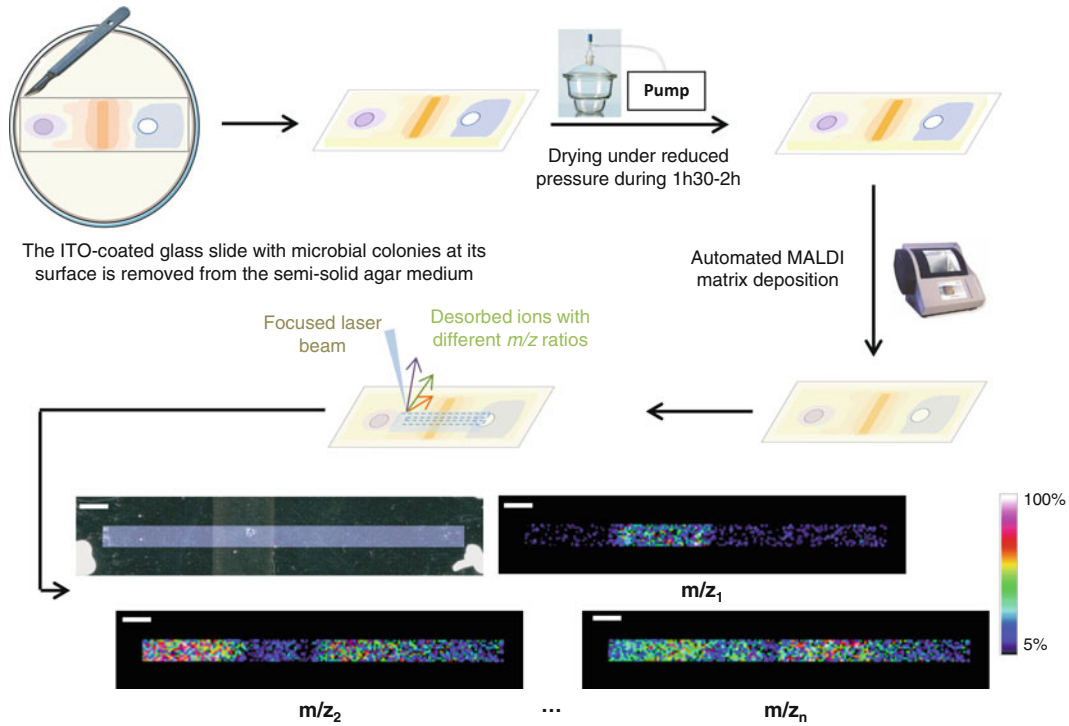


Fig. 2 General workflow of a MALDI mass spectrometry imaging experiment

3.3 Sample Preparation for MALDI MS Imaging (Figs. 2 and 3)

The general principle of sample preparation for MALDI MS imaging is given in Fig. 2. Figure 3 shows pictures of the sample, at different steps of the preparation.

1. With a scalpel, cut the agar around the glass slide in order to remove it from the Petri dish (Fig. 3b) (*see* **Note 1**).
2. Discard the useless agar (Fig. 3c).
3. Place the glass slide into the vacuum desiccator.
4. Dry the agar under reduced pressure until complete dryness (*see* **Notes 2** and **3**). The time needed for drying completion depends on the quantity of agar present on the slide; less agar, faster drying. In the case of Fig. 3d, the drying took 2 h at a reduced pressure of 300 mbar.
5. Use a white pen to mark off the area to be analyzed from underneath.
6. Take a picture of this area with the scanner.

3.4 MALDI Matrix Deposition

1. Prepare a fresh CHCA solution at 5 mg/mL in ACN/0.2 % TFA 70:30 vol/vol. Sonicate the solution for 10 min to ensure a perfect dissolution (*see* **Notes 4** and **5**).
2. If working with a MALDI TOF/TOF instrument, spot 1 µL of a calibration mixture right to the area of interest (on top of the white marks) (*see* **Note 6**).

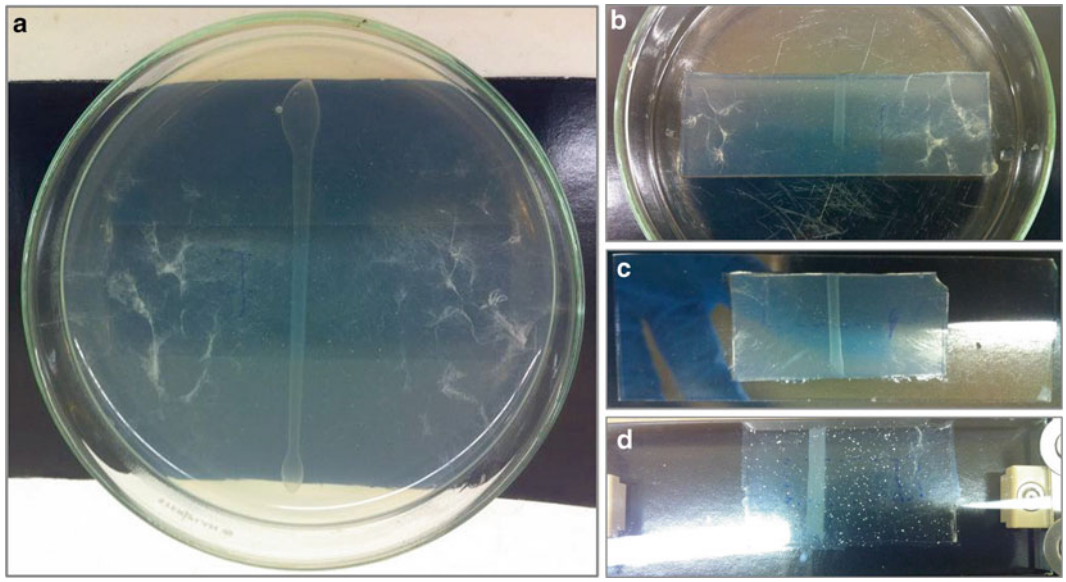


Fig. 3 The different steps of the MALDI MSI sample preparation workflow: (**a**) the sample before any processing, (**b**) the sample after cutting the agar around the ITO-coated glass slide, (**c**) the sample after discarding the useless agar, and (**d**) the sample after drying

3. Coat the slide with the ImagePrep device (*see* **Note 7**). The method used was optimized for these particular samples and was adapted from the manufacturer's standard protocol. The complete parameters of the method are given in Table 2.
4. At the end of preparation, check for the quality of the coating with a microscope. If not homogeneous or if the layer of matrix is too weak, run the last phase of the method again (*see* **Note 8**).

3.5 MALDI MS Imaging Acquisition

1. Introduce the ITO slide with the matrix-coated agar film into the MALDI adapter plate.
2. Insert the adapter plate into the ion source of the MALDI mass spectrometer.
3. Test the quality of signal. (Is the signal-to-noise ratio high? Do you detect matrix adduct peaks?)
4. Calibrate the instrument. Either externally with the calibration mixture (for TOF/TOF instrument) or internally with matrix adduct peaks (for FTMS instrument) (*see* **Note 9**).
5. Design the sequence file in FlexImaging (import the optical image of the sample, teach the sample, define the area of analysis and the raster width) (*see* **Note 10**).
6. Start automated image acquisition (*see* **Note 11**).
7. At the end of acquisition, protect the slide from dust and store it at room temperature.

Table 2
Detailed parameters of the ImagePrep method for the coating with the MALDI matrix

Phase	1	2	3	4
Final ddp	–	0.3 V	0.4 V	0.5 V
Number of cycles	12	6–18	12–40	24–60
Spray power	25 ± 25 %	27 ± 27 %	28 ± 28 %	30 ± 30 %
Spray duration	2.1 s			
Voltage drop per spray	–	0.16 V	0.3 V	0.35 V
Incubation duration	35 s	30 ± 30 s	30 ± 30 s	30 ± 30 s
Drying duration	20 s			
Residual humidity	–	20 ± 60 %	20 ± 60 %	20 ± 40 %
Complete drying every…	1 cycle	2 cycles	4 cycles	6 cycles
Safe dry duration	–	20 s	30 s	30 s

3.6 MALDI Images Visualization

1. In case of TOF data, perform data processing using batch mode in FlexAnalysis (*see* **Note 12**).
2. Load the sequence file in FlexImaging.
3. If wanted, perform a normalization of data.
4. Select the compounds of interest and display ion image.

Images of Fig. 4 display the distribution of some of the detected compounds in the average mass spectrum (Fig. 4b). All ion images show a lower signal in the bacterial colony, meaning that antibiotics diffuse into the medium right after their production by the bacterial cells. However, ion images also show that these compounds don't diffuse the same way. The ion at *m/z* 911.5954 (mixture of different cLPs sharing the same mass) has an intense signal on the left side of the colony (Fig. 4d), where the mycelium of the pathogen was the closest, demonstrating a likely involvement of this compound in the growth inhibition. A similar behavior is observed for the ion at *m/z* 925.6113 (LIF 08b peptide, Fig. 4e), albeit the compound stays closer to the bacterial cells than the previous one, suggesting a weaker involvement in the antagonism against the fungus. At last, the ion at *m/z* 905.5495 (unidentified, Fig. 4f) has the most intense signal far from the colony (on the right of the image), meaning it gets a higher power of diffusion into the medium, compared to the other compounds. The meaningfulness of this observation may be that this compound is probably not involved in the defense mechanisms of the bacteria but may be useful to the colonization process, as a wetting agent, for example.

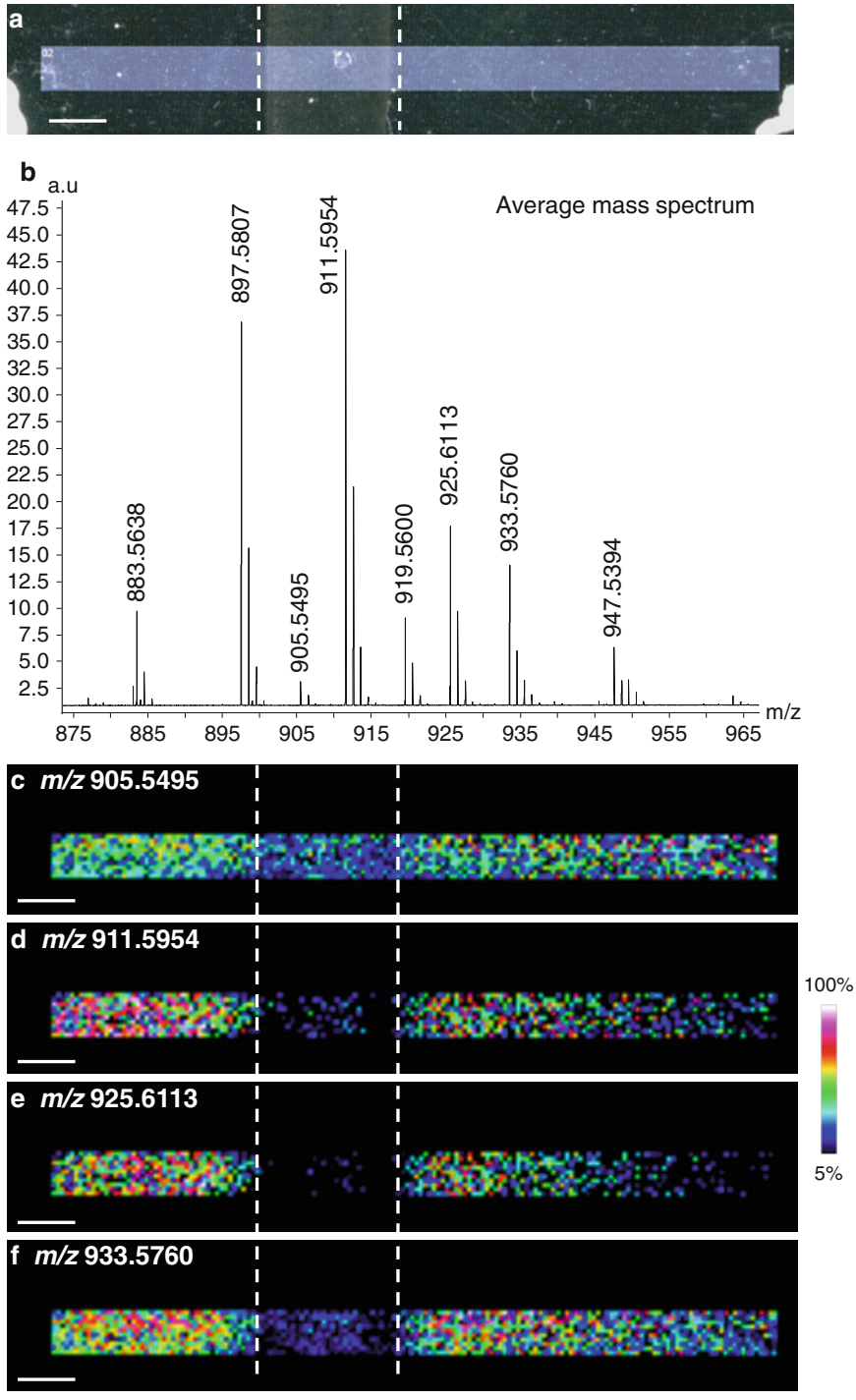


Fig. 4 (**a**) Picture of the sample, showing the bacterial colony, represented by the *white dotted lines* and the *blue square* represents the analyzed area, (**b**) MALDI average mass spectrum recorded on the area (zoom on the 875–965 *m/z* range), (**c**) MALDI image of the ion at *m/z* 905. 5495, (**d**) MALDI image of the ion at *m/z* 911.5954, (**e**) MALDI image of the ion at *m/z* 925.6113, and (**f**) MALDI image of the ion at *m/z* 933.5760. Scale bar: 2 mm. Color scale represents relative intensity for each signal: 5–100 %

3.7 Compound Identification

The identification of a compound is achieved thanks to exact mass measurements, requiring an instrument with a high mass accuracy, and on tandem mass spectrometry experiments. Exact mass measurements allow for elemental composition searches and MS/MS spectra give information on the chemical structure of the molecule. In our case, both kinds of experiments were performed using the FTMS instrument.

1. In Data Analysis, open a mass spectrum corresponding to a pixel in which the compound of interest is highly abundant.
2. Internally recalibrate the mass spectrum using matrix adduct peaks in order to reach an average error less than 0.2 ppm.
3. Use the SmartFormula tool included in Data Analysis to perform elemental composition searches.
4. To perform MS/MS experiments, reinsert the plate into the mass spectrometer.
5. Record a MS/MS spectrum in a region where the unknown compound is highly abundant.

4 Notes

1. During the cutting of agar around the glass slide, the movement of the scalpel blade may displace the bacterial colony or the fungal mycelium. Rather than a sliding motion, prefer a lever movement.
2. The pressure has to be carefully set. Beyond 5–10 mbar, the water contained in the agar gel freezes. Once dried, the resulting agar film is very fragile and does not support the vacuum of the MALDI ionization source. It cracks and comes off the glass slide.
3. This drying procedure is suited for poor culture medium without sugars or peptone-like compounds. The presence of these molecules in the agar-based medium leads to "caramel," which will never completely dry. Introducing such a sample into the MALDI ionization source prevents establishing a good vacuum and the MS analysis cannot be performed.
4. The choice of CHCA was done according to the results of an optimization process concerning the nature of the MALDI matrix, the concentration of the solution, and the solvent mixture. For other kinds of molecules, a novel optimization process should be undertaken.
5. A perfect dissolution of CHCA crystals in solvent is necessary to avoid clogging of the aluminum foil of the ImagePrep.
6. The calibration mixture may ideally be a mixture of commercial lipopeptides. Otherwise, a mixture of compounds whose molecular masses cover the targeted mass range may also be used.

7. The placement of the slide covered with the agar must not prevent the light-scattering sensor to work. Consequently, it is preferable to put on top of the sensor a part of the slide without agar and to add a coverslip, according to the manufacturer's recommendations.
8. If needed, the slide may be kept overnight at room temperature and atmospheric pressure.
9. Acceptable average error values are 5 ppm on the TOF/TOF instrument and 0.5 ppm for the FTMS instrument.
10. The smaller the pixel size, the longer the acquisition and the larger the dataset (especially true for FTMS data).
11. If multiple areas are to be analyzed on the sample carrier, a batch acquisition should be considered if working with Ultraflex mass spectrometer. This option is not yet available on the Solarix mass spectrometer.
12. For TOF data, it may be necessary to submit the whole dataset to a processing method including smoothing, baseline correction, and external calibration, using if possible a lock mass.

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