Chapter 22

Characterization of Microorganisms by MALDI Mass Spectrometry

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Summary

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) for characterization and analysis of microorganisms, specifically bacteria, is described here as a rapid screening tool. The objective of this technique is not comprehensive protein analysis of a microorganism but rather a rapid screening of the organism and the accessible protein pattern for characterization and distinction. This method is based on the ionization of the readily accessible and easily ionizable portion of the protein profile of an organism that is often characteristic of different bacterial species. The utility of this screening approach is yet to reach its full potential but could be applied to food safety, disease outbreak monitoring in hospitals, culture stock integrity and verification, microbial forensics, or homeland security applications.

Key words: Bacteria, Spores, Microorganisms, Mass spectrometry, MALDI, Proteins, Fingerprinting.

1. Introduction

There is an ever-increasing need for the consistent and rapid identification of intact microorganisms. Mass spectrometry is a powerful analytical tool that can also be used for screening microorganisms rapidly. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been used to identify microorganisms based on expressed protein profiles. MALDI-TOF MS provides rapid analysis time (< 1 min/ sample analysis), has low sample volume requirements (<1 µL of fluid), and yields very specific and unbiased analysis based on the molecular weights of true components of the sample. The high sensitivity and high tolerance toward contaminants have made

MALDI-TOF MS a viable technique for the analysis of complex biological samples.

MALDI-TOF MS has established itself as an analytical technique having the ability to identify bacteria to the species level in pure cultures and simple mixtures of bacteria *(1)*. It can be used as a rapid screening tool in distinguishing between pathogenic and nonpathogenic species, which potentially makes it a powerful tool in countering terrorism. MALDI-TOF MS has also been successfully extended to the identification of sporulated varieties of Gram-positive strains of *Bacillus* with modification to the sample preparation techniques *(2)*.

The screening of bacterial samples by mass spectrometry for quick identification can be accomplished with direct analysis of a subset of the protein profile by MALDI-TOF MS analysis. While MALDI-TOF MS is known to have a wide dynamic range of analysis it is also a competitive ionization method and therefore may not yield a profile representative of all components present in the sample. The chemical complexity in most vegetative bacterial samples along with the many orders of magnitude difference in concentration often results in only a minor number of cell components observed by direct MALDI-TOF MS analysis of the entire cell. However, this only presents a significant challenge if the desired result is the complete profiling of the cell contents. A reproducible pattern of putative proteins is readily observed from simple sample preparation and analysis of bacterial cultures *(3, 4)*. The sample preparation and analysis method described here was developed as a rapid screening tool for identification of microorganisms without individual protein identification *(5, 6)*. While relatively straightforward, there are a few steps that are helpful to follow in order to successfully obtain data from microorganisms directly by MALDI-TOF MS. The purpose of this chapter is to provide some guidance on one possible approach to analysis of microorganisms by MALDI-TOF MS. This is not the only method available but is similar to many other published methods and approaches *(7, 8)* and commercial protocols (e.g., Micromass, Bruker) as well. Two review articles *(7, 8)* provide a good overview of this research field as well as a book devoted to this subject of microorganisms analysis by mass spectrometry that provides additional information and approaches to the analysis of these complex biological samples *(9)*.

Several steps are important for successful MALDI-TOF MS analysis of microorganisms (specifically bacteria). The first step is obtaining relevant microorganism samples for analysis. While only small volumes (µL) of sample are required for MALDI-TOF MS analysis, currently at least 10^3 cells are necessary. Analyses of approximately 10^6 cells per microliter are used more routinely for this direct MALDI-MS analysis. The relative concentrations

of matrix and analyte are critical for successful analysis and will be discussed in more detail later. Removal of the growth media from the microorganisms is also important for successful mass spectrometric analysis. A proven and effective way to clean the bacterial samples harvested from liquid growth media is to pellet the cells and wash with water or appropriate volatile solution, such as 2% ammonium chloride, repeatedly. Once adequate bacterial samples are prepared/obtained, the second step is to spot the microbial sample (*see* **Note 1**) onto a MALDI sample plate along with a MALDI matrix compound, which is used to aid in desorption and ionization of intact protein and nonvolatile components within the bacterial cells. There are numerous matrix recipes and spotting procedures in the literature for successful MALDI-MS analysis that are for the most part applicable to analysis of components of microorganisms. The research community currently performing bacterial analysis does not consistently use the same sample matrix and spotting procedures. Provided here is the method we have optimized in our laboratory and have successfully applied to analysis and characterization of vegetative and sporulated bacteria. We have also analyzed fungi with the use of double-stick tape for sampling directly from a fungal colony and applying to the MALDI sample plate *(10)*. After the sample is effectively spotted onto a commercial MALDI sample plate, the sample is analyzed with straightforward instrument parameters. Note that the instrument needs to be well calibrated prior to data collection as with any analysis. The final step is data processing and analysis. PNNL has developed algorithms for justified comparison of sample data with a collected database of MALDI-TOF MS spectra for comparative identification. There are other commercial products available that are designed for use with specific vendor instrumentation and other published approaches to data analysis *(9)*.

Mass spectra can be obtained from unknown bacterial samples and compared with reference spectra to provide information for a correct identification with a computed degree of confidence. Well-defined sample preparation procedures and calibration routines must be adhered to during the collection of replicates for reference spectra. Protein profiles, or fingerprints, obtained from microorganisms by means of MALDI-TOF MS can vary in connection with the choice of solvent system for the matrices *(11)*. Sufficient replication is also necessary to capture minor variations in growth or handling procedures (*see* **Notes 2 and 3**). Thereafter, automated statistics-based data analysis algorithms can be used to compile these replicates into a reference spectrum for inclusion into a database *(6)*. This process has also been successfully extended for the identification of microorganisms grown under different growth conditions making it a viable tool in terms of attribution in forensics *(12)*.

Matrix-assisted laser desorption ionization as its name suggests depends on the cocrystallization of the analyte material and matrix molecules *(13, 14)*. A variety of matrices are compatible for use in instruments equipped with a nitrogen laser (337 nm). The ionization process is well suited for mass spectrometric analysis of large biomolecules. The analyte substance is imbedded in the crystallized matrix and irradiated by sufficient laser power to assist in ionization of intact molecules but does not result in fragmentation. Thus, MALDI is often referred to as a *soft* ionization technique. MALDI matrices have conjugated ring structures and typically differ only in their attached moieties. Structures for three of the most commonly used matrices for vegetative and sporulated bacteria are shown in **Fig. 1**.

Each matrix has a unique initial velocity when exposed to a pulsed laser beam under vacuum and as such can provide advantages when interrogating different mass ranges of interest. For example, alpha-cyano-4-hydroxy cinnamic acid (ACHC), often referred to as a hot matrix, has a high initial velocity, and is a preferred matrix for smaller molecules. Sinapinic acid (SA) or 3,5-dimethyoxy-4-hydroxy cinnamic acid and 3-methoxy-4-hydroxy cinnamic acid, commonly referred to as ferulic acid (FA), are more suited for use with most intact vegetative bacterial cells as well as bacterial spore preparations. Typical mass ranges for the ions observed by direct MALDI-MS analysis of the bacterial cells with the protocols described here are 2–20 kDa. A schematic of the preparation of the MALDI sample spot and a scanning electron microscopy image of a portion of the sample spot is provided in **Fig. 2**.

Once the matrix has been selected, an appropriate solvent system must also be determined. Matrices will be dissolved, usually to the point of saturation, in some ratio of H_2O and organic material. The solvent system is a crucial component of sample preparation and has a major impact both on crystal structure and the degree to which the analytes are incorporated into them.

For the analysis of most readily accessible and ionizable components from intact bacterial cells or spores it is desirable to have the solvent system at a low pH, which is accomplished
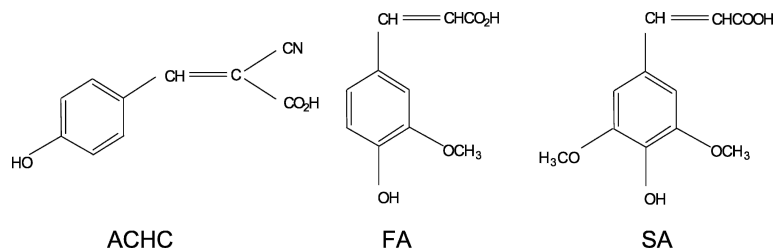

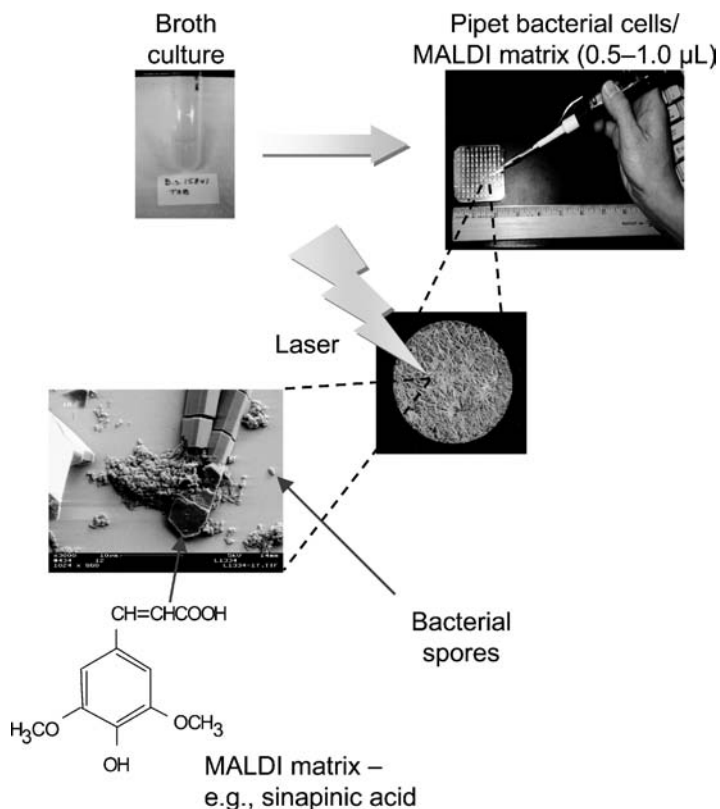Fig. 1. Structures of common MALDI matrices.

Fig. 2. Schematic of MALDI sample preparation.

through the addition of an acid such as trifluoroacetic acid (TFA) or formic acid (*see* **Note 49**). Small acid-soluble proteins (SASPs) are key components of bacterial cells that are readily accessible and ionizable *(15)*.

2. Materials

2.1. Reagents

1. Alpha cyano-4-hydroxycinnamic acid (ACHC), and sinapinic acid (SA) (Bruker Daltonics, Bremen, Germany).
2. Ferulic acid (Aldrich, Milwaukee, WI).
3. Protein mix 1 and 3 (Bruker Daltonics, Bremen, Germany).
4. Horse heart cytochrome *c* and angiotensin I (Sigma, St. Louis, MO).
5. TFA (Aldrich, Milwaukee, WI). Note: Trifluoroacetic acid is corrosive and causes severe burns. Suitable protection, including gloves, laboratory coat, and eye and face protection should be donned when working with concentrated solutions.

6. Vegetative bacteria (American Type Culture Collection, (ATCC®) Manassas, VA).

7. Bacto Luria Bertani (LB), Broth Miller (Difco), and Bacto tryptic soy broth (TSB) without dextrose (Difco), and Bacto nutrient broth (Difco) (Becton Dickinson, Sparks, MD).

8. Lab Lemco Medium: Lab Lemco 23 g and 1.0 L of E-Pure® distilled deionized water.

9. *Nutrient sporulation medium (16)*. 3.0-g tryptone, 3.0-g yeast extract, 2.0-g Bacto agar, 23-g LL agar, and 1.0-µL 1% $MnCl_2 \cdot 4H_2O$ in 1.0 L of E-Pure® distilled deionized water.

10. Acetonitrile and ammonium chloride (J.T. Baker, Phillipsburg, NJ).

11. Teflon coated 96 × 2 stainless steel MALDI plate (Applied Biosystems, Foster City, CA).

12. Stainless steel insert plate (Bruker Daltonics, Bremen, Germany).

2.2. Matrix Recipes

1. ACHC: 2-mg ACHC, 140-µL acetonitrile, and 60-µL 0.1% TFA.

2. SA: 2-mg sinapinic acid, 140-µL acetonitrile, and 60-µL 0.1% TFA.

3. FA (recipe #1): 2-mg ferulic acid, 140-µL acetonitrile, and 60-µL 0.1% TFA.

4. FA (recipe #2) *(17)*: 7-mg ferulic acid, 123-µL 65% formic acid, 155-µL acetonitrile, and 192-µL H_2O (*see* **Note 4**).

2.3. Instrumen-tation

Two different commercial MALDI-TOF MS instruments were used during the development of microorganism analysis in our laboratory due to the instrumentation available at different stages of the research. Any commercial MALDI mass spectrometer should be capable of obtaining data as discussed here.

1. An Applied Biosystems Voyager-DE RP MALDI-TOF MS operated in linear, delayed extraction, positive mode was used primarily for data collection of the vegetative cells.

2. A Bruker Autoflex II MALDI TOF/TOF MS instrument operated in reflector, positive mode was used for data collection of the spores.

2.4. Software

Instrument control and data collection is performed using the instrument control software as provided with the commercial instrument. The majority of the data collected in our laboratory for microorganism analysis has been done with manual data collection. However, automated data collection routines can be used.

1. *Applied Biosystems Grams.* Standard instrument control software supplied with the Voyager DE RP MALDI-TOF MS instrument.

2. *Bruker Daltonics Flex Control and Analysis Programs.* Standard instrument control software supplied with the Bruker Autoflex TOF/TOF MS instrument.

3. *Algoworks.* Algorithms developed at PNNL were used for data analysis. This includes the use of a novel patented peak detection algorithm incorporated with algorithms to account for the variability in the MALDI spectra due primarily to the variability in the microbial samples. Briefly these algorithms statistically compute a weighted average of sample replicate analyses and provide a means for statistically comparing an unknown sample to a reference library of MALDI spectra for different microorganisms *(6, 18, 19)*.

3. Methods

Several different MALDI matrices have been used for analysis of the microorganisms and the recipes are provided. As previously discussed, there is a difference in the mass spectra of a given sample obtained with the use of different MALDI matrices.

3.1. Vegetative Bacterial Sample Preparation

Bacterial samples can be cultured in a variety of culture media and culture conditions. Typical culture conditions for each organism of interest were used initially to get some reference data of several different types of organisms. *Bacillus subtilis (Bs), Bacillus thuringiensis (Bt), Bacillus atrophaeus, Bacillus cereus (Bc), Serratia marcescens*, and the "unknown control," *Bacillus sphaericus*, were cultured in TSB and incubated overnight in a shaker incubator at 30°C, 120 rpm. *Pseudomonas stutzeri, Pantoea agglomerans*, and *Pseudomonas putida* were cultured in TSB and incubated at 37°C, 130 rpm. *Escherichia coli* (*E. coli*) was cultured in Luria Berani (LB) broth and incubated at 37°C, 130 rpm. The cells were centrifuged at 14,000 rpm for 2 min, decanted, and washed twice with 1 μL of 2% ammonium chloride to remove the majority of the culture medium and cell debris. In order to establish the effect of biological variability on the MALDI mass spectra, it is important to culture triplicates of each sample at a minimum to capture the biological variability.

3.2. Bacterial Spore Preparation

Spore cultures can be prepared in many different medium types. For research purposes to develop mass spectrometric methods several sporulation recipes were chosen to ensure sufficient sample for development purposes. *Bacillus subtilis* spores can be prepared in media such as nutrient sporulating media (NSM) or Lab Lemco (LL).

1. The agar plates are inoculated with 200 µL of starter culture grown in TSB at 30°C overnight (~14 h).

2. The plates are inverted and incubated in a 37°C incubator for 5–7 days.

3. The spores are harvested from the medium using sterile E-Pure® water and a sterile loop.

4. To produce clean spores the sample can be pelleted and washed by centrifugation 5–7 times in 20–30 µL of sterile E-Pure® water. The centrifugation is performed as follows: 1,000 rpm for 1–2 min, 5,000 rpm for 1–2 min, 11,000 rpm for 6–8 min. The sample is then decanted and a clean aliquot of water is added for the next wash. A clean spore preparation is approximately 95–98% phase bright spores as determined by phase contrast microscope evaluation. Spore counts can be determined by plate counts and are typically in the 10^8 CFU/mL range or higher for a clean spore preparation.

3.3. Estimation of Bacterial Spore Mass Used for MALDI-MS Analysis

Fergenson et al. *(20)* estimate the mass of one *Bacillus anthracis* spore being approximately 1×10^{-12} g or 1 pg. Based on this estimation and the use of approximately 0.5-µL sample deposited onto the MALDI sample plate, MALDI-MS analysis uses at most ~50 µg of bacterial spores per analysis. The majority of this sample remains after MALDI-MS analysis and can be retrieved from the MALDI sample plate for further analysis or archiving.

3.4. MALDI Vegetative Cell Sample Preparation

1. Ferulic acid (recipe #1), with two internal standards, cytochrome *c* and angiotensin I (at 5 and 2.5 mg/µL, respectively), was prepared.

2. A layering method was used for the bacterial analysis in which 1 µL of the bacterial sample was applied to the sample plate and allowed to air-dry. The concentration of cells was ~10^5 cells/µL when deposited onto the MALDI sample plate. Then 1 µL of the ferulic acid matrix solution was applied to the bacterial sample spot and allowed to air-dry (*see* **Note 5**).

3. Each spectrum was obtained averaging 128 laser shots. Each spectrum was internally calibrated (*see* **Note 6**) with the monomer ion of cytochrome *c* (*m/z* 12,361) and the monomer of angiotensin I (*m/z* 1,297). Regular stainless steel plates manufactured by Applied Biosystems were used.

4. The data files were then transferred to the data analyst for automated peak extraction and analysis using Algoworks algorithms.

5. Library reference fingerprints were created using ten replicate MALDI-TOF MS spectra/organism collected from replicate cultures on each of 3 days, for a total of 60 spectra/bacterium.

3.5. Matrix-to-Analyte Ratio Consi-derations

The ratio of matrix to cell concentration is important for successful MALDI mass spectral analysis. When possible, concentration of the sample with either cell counts or absorbance measurement is useful. However, if an unknown sample with no concentration information is to be analyzed, it might be helpful to prepare several different ratios of analyte to matrix. Shown in **Fig. 3** is a representation of the type of spectra obtained when the sample is too concentrated relative to the matrix added, too dilute relative to the matrix used, and appropriate ratio of analyte to matrix. In this case the use of an internal standard is helpful to determine whether an unknown sample is too dilute or too concentrated when no useful MALDI-MS data is collected *(21) (see* **Note 6**). The internal standard (cytochrome *c*) ions are not observed when the sample is too concentrated for the amount of matrix used. However, when the sample concentration is too dilute, the internal standard ions are observed.

3.6. MALDI Spore Sample Preparation

Without some pretreatment, bacterial spore samples do not yield as much MALDI mass spectral data compared with vegetative cells. One method for obtaining additional protein data from bacterial spore samples is to subject them to a simple pretreatment procedure.

1. A Petri dish, 10 cm in diameter, was used to accommodate an Applied Biosystems Teflon-coated 96 × 2 stainless steel MALDI plate (*see* **Note 7**).

2. Several layers of paper towels were cut into the shape of a circle slightly smaller than the Petri dish.

3. The layers of paper towels were placed into the bottom of the dish and wetted with distilled water, and then the Petri dish was covered.
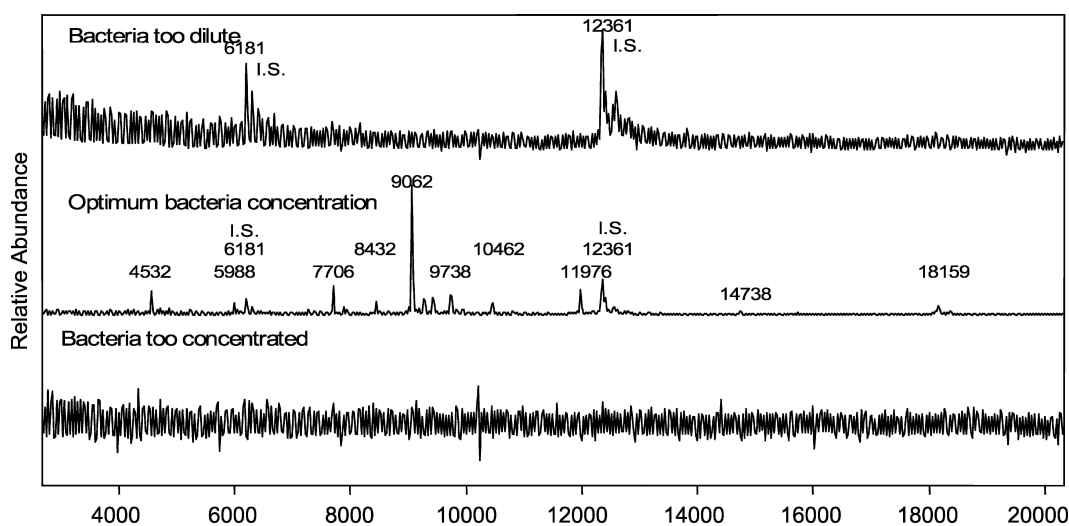

Fig. 3. Use of internal standard to determine appropriate relative ratio of sample to matrix.

4. The Petri dish was then placed into a previously heated 37°C oven to create a moisture chamber (*see* **Note 8**).

5. A 2% solution of TFA was prepared. Using a mechanical pipettor a 1-µL aliquot of 2% TFA was placed onto the MALDI spots in an *every other spot arrangement.*

6. Spore samples were vortexed slightly to resuspend and homogenize.

7. A 0.5-µL aliquot of the spores was pipetted onto each of the droplets of 2% TFA.

8. The prepared MALDI plate with the spots still wet was then placed into the warmed Petri dish with moist towels and covered.

9. The covered Petri dish was then placed back into the 37°C oven for 30 min.

10. After 30 min the Petri dish and MALDI plate were removed from the oven and allowed to dry.

11. Once the spots were dried 0.5-µL aliquots of an appropriate matrix were applied on top of the spore spots.

12. The spots were then analyzed after the instrument was properly calibrated (quadratic fit) using peptide 1 mix and protein 1 mix commercially available from Bruker. One of three different Bruker reflectron methods with parameters optimized for the three different matrices (ACHC, FA recipe #2, or SA) was loaded and used for data collection. Five 100-shot spectra were added together using the sum buffer to generate one saved spectrum.

3.7. MALDI Mass Spectral Data

Representative samples of the types of mass spectral data that can be obtained from direct analysis of vegetative bacterial cells and spores are presented here. **Figures 4 and 5** demonstrate
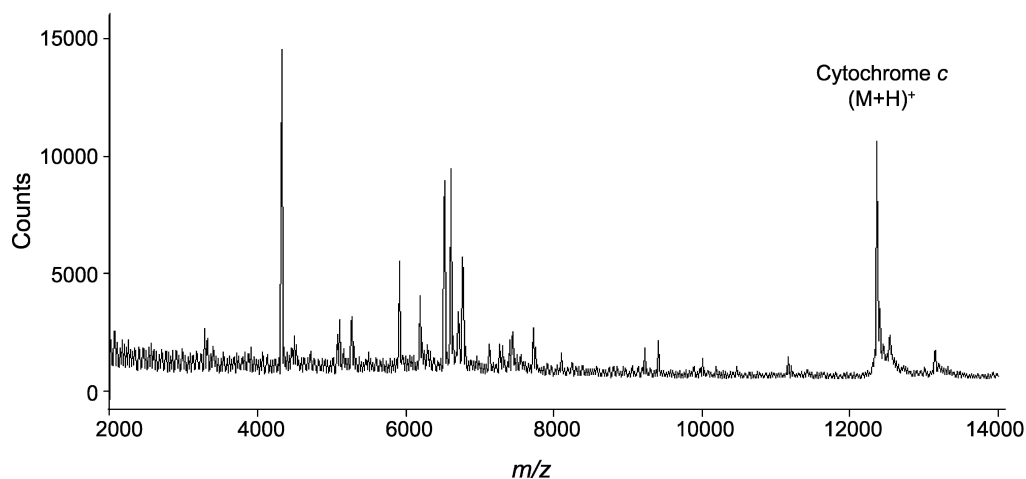

Fig. 4. Representative MALDI-MS spectrum of vegetative *Bacillus subtilis* 15841 cultured in tryptic soy broth with internal standard added prior to analysis.
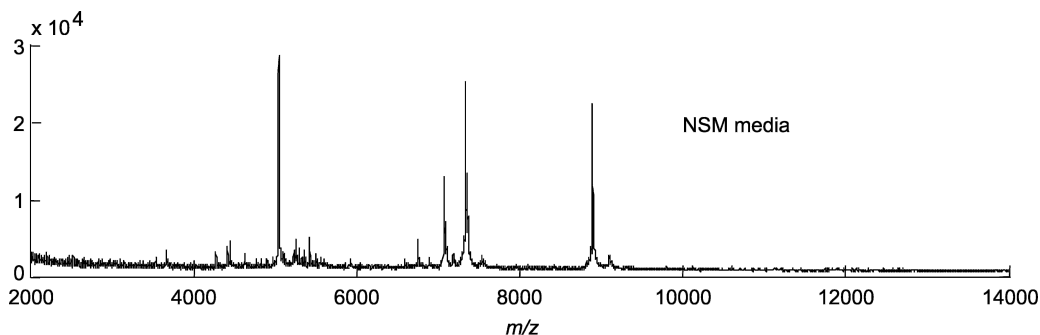
Fig. 5. Representative MALDI-MS of sporulated *Bacillus subtilis* 49760 in nutrient sporulating media agar plates without internal standard.

the species-specific differential biosignatures that emerge when vegetative cells are grown in different media. Contrasted are *Bacillus subtilis* grown in TSB and NSM agar. The effect of adding an internal standard to the sample spot prior to analysis is underscored as well.

4. Notes

1. If automation is to be employed in data collection, the best level of homogeneity obtainable across the MALDI spot is desired. When spotting MALDI plates it is best to allow wicking action to *pull* the aliquot of sample or matrix from the pipettor rather than touching the surface with the pipette tip.

2. Toward the goal of improving reproducibility when manually collecting data, it is important to capture as much variability across the MALDI spot as possible. Average as many laser shots from as many different locations across the spot as possible to achieve the desired level of reproducibility. The bacterial samples are more heterogeneous than protein solutions/standards, and therefore the MALDI sample spot containing bacterial cells is often more heterogeneous than for other samples.

3. Microorganisms are inherently heterogeneous variable samples compared with protein solutions and standards, and therefore some variability in MALDI spectra of intact bacteria and other microorganisms is expected. We have found the variability from the sample (biology) to be much more significant than the variability in the analytical method *(5)*.

4. To minimize the formylation of sample components, the formic acid content in prepared matrices should be at or below 17%.

5. While it is true that MALDI, as an ionization technique, is known for its tolerance toward impurities, there are concentration levels, beyond which it is nearly impossible to obtain useful signal. If you suspect that detergents or extraneous salts are interfering with data collection a simple spot washing step after cocrystalization of the sample and matrix may be all that is necessary. Simply apply a microliter of Milli-Q water to the spot and allow it to stand for several seconds. The water can then be removed with a pipette tip or with a corner of a Kimwipe. This step may need to be repeated for challenging samples/spots. As an alternative, pipette tips with packed beds such as C18 material may be used to prefractionate proteins and peptides from such unwanted components (e.g., ZipTips from Millipore).

6. The choice of internal standard vs. external standard for mass axis calibration is always a good question. The internal standard is useful for most accurate mass determinations but also leads to potential issues with competitive ionization in MALDI and suppression of some sample peaks. However, as discussed previously when having difficulty in getting useful signal from a true unknown sample, the use of internal standard can sometimes help determine if a negative MALDI-MS result is due to the sample being too concentrated (and no ions are observed) or too dilute (only ions from the internal standard are observed). It might be useful in the early stages of method development to spot samples both with and without internal standards to determine the variability in the *m/z* values detected as well as the appropriate matrix to analyte ratio for optimal MALDI-MS analysis.

7. Successful transfer of this method to the commercial Bruker sample plates was not completed in our laboratory due to project priorities.

8. To enhance reproducibility, within a sporulated sample set, always prewarm the moisture chamber to the desired temperature necessary, prior to insertion of the spotted MALDI plate.

References

1. Wahl, K., S. Wunschel, K. Jarman, N. Valentine, C. Petersen, M. Kingsley, K. Zartolas, and A. Saenz, (2002) Analysis of Microbial Mixtures by MALDI Time-of-Flight Mass Spectrometry. *Anal. Chem.* 74, 6191–6199

2. Hathout, Y., P.A. Demirev, Y.-P. Ho, J.L. Bundy, V. Rhyzov, L. Sapp, J. Stutler, J. Jackman, and C. Fenselau, (1999) Identification of *Bacillus* Spores by Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry. *Appl. Environ. Microbiol.* 65, 4313–4319

3. Arnold, R.J. and J.P. Reilly, (1999) Observation of *Escherichia coli* Ribosomal Proteins and Their Posttranslational Modifications by Mass Spectrometry. *Anal Biochem.* 269, 105–112

4. Warscheid, B. and C. Fenselau, (2003) Characterization of *Bacillus* Spore Species and Their Mixtures Using Postsource Decay with a Curved-Field Reflectron. *Anal Chem.* 75, 5618–5627

5. Saenz, A.J., C.E. Petersen, N.B. Valentine, S.L. Gantt, K.H. Jarman, M.T. Kingsley, and

K.L. Wahl, (1999) Reproducibility of Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry for Replicate Bacterial Culture Analysis. *Rapid Commun. Mass Spectrom.* 13, 1580–1585

6. Jarman, K.H., S.T. Cebula, A.J. Saenz, C.E. Petersen, N.B. Valentine, M.T. Kingsley, and K.L. Wahl, (2000) An Algorithm for Automated Bacterial Identification Using Matrix-assisted Laser Desorption/Ionization Mass Spectrometry. *Anal. Chem.* 72, 1217–1223

7. Lay, J., (2001) MALDI-TOF Mass Spectro-metry of Bacteria. *Mass Spectrom.* 20, 172–194

8. Fenselau, C. and P. Demirev, (2001) Characterization of Intact Microorganisms by MALDI Mass Spectrometry. *Mass Spectrom.* 20, 157–171

9. Wilkins, C. and J.O. Lay, eds., (2005) Identification of Microorganisms by Mass Spectrometry. Wiley Interscience, NJ

10. Valentine, N.B., J.H. Wahl, M.T. Kingsley, and K.L. Wahl, (2002) Direct Surface Analysis of Fungal Species by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 16, 1352–1357

11. Wang, Z., L. Russon, L. Li, D.C. Roser, and S.R. Long, (1998) Investigation of Spectral Reproducibility in Direct Analysis of Bacteria Proteins by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 12, 456–464

12. Valentine, N., S. Wunschel, D. Wunschel, C. Petersen, and K. Wahl, (2005) Effect of Culture Conditions on Microorganism Identification by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Appl. Environ. Microbiol.* 71, 58–64

13. Karas, M., D. Bachmann, U. Bahr, and F. Hillenkamp, (1987) Matrix-Assisted Ultraviolet Laser Desorption of Non-Volatile Compounds. *Int. J. Mass Spectrom.* 78, 53–68

14. Karas, M. and U. Bahr, (1991) Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Mass Spectrom. Rev.* 10, 335–357

15. Hathout, Y., B. Setlow, R.-M. Cabrera-Martinez, C. Fenselau, and P. Setlow, (2003) Small, Acid-Soluble Proteins as Biomarkers in Mass Spectrometry Analysis of *Bacillus* Spores. *Appl. Environ. Microbiol.* 69, 1100–1107

16. Nicholson, W.L. and P. Setlow, (1990) Sporulation, Germination and Outgrowth, in Molecular Biological Methods for *Bacillus*, C.R. Harwood and S.M. Cutting (eds). Wiley, Chichester, England

17. J. Madonna, A., F. Basile, I. Ferrer, M.A. Meetani, J.C. Rees, and K.J. Voorhees, (2000) On-Probe Sample Pretreatment for the Detection of Proteins Above 15 KDa from Whole Cell Bacteria by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 14, 2220–2229

18. Jarman, K.H., D.S. Daly, C.E. Petersen, A.J. Saenz, N.B. Valentine, and K.L. Wahl, (1999) Extracting and Visualizing Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectral Fingerprints. *Rapid Commun. Mass Spectrom.* 13, 1586–1594

19. Jarman, K.H., D.S. Daly, K.K. Anderson, and K.L. Wahl, (2003) A New Approach to Peak Detection. *Chemometrics Intell. Lab. Syst.* 69, 61–76

20. Fergenson, D.P., M.E. Pitesky, H.J. Tobias, P.T. Steele, G.A. Czerwieniec, S.C. Russell, C.B. Lebrilla et al., (2004) Reagentless Detection and Classification of Individual Bioaerosol Particles in Seconds. *Anal Chem.* 76, 373–378

21. Gantt, S.L., N.B. Valentine, A.J. Saene, M.T. Kingsley, and K.L. Wahi, (1999) Use of an Internal Control for Matrix-assisted Laser Desorption /Ionization Time-of-Flight Mass Spectrometry Analysis of Bacteria. *J. Am. Soc Mass Spectrom.* 10, 1131–1137.