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APPLICATION NOTE

Utilizing a Robotic Sprayer for High Lateral and Mass Resolution MALDI FT-ICR MSI of Microbial Cultures

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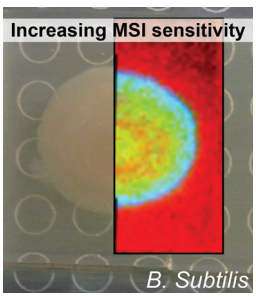

Abstract. The ability to visualize biochemical interactions between microbial communities using MALDI MSI has provided tremendous insights into a variety of biological fields. Matrix application using a sieve proved to be incredibly useful, but it has many limitations that include uneven matrix coverage and limitation in the types of matrices that could be employed in studies. Recently, there has been a concerted effort to improve matrix application for studying agar plated microbial cultures, many of which utilized automated matrix sprayers. Here, we describe the usefulness of using a robotic sprayer for matrix application. The robotic sprayer has two-dimensional control over where matrix is applied, and a heated capillary that allows for rapid drying of the applied matrix. This method provided a significant increase in MALDI sensitivity over the sieve method, as demonstrated by FT-ICR MS analysis, facilitating the ability to gain higher lateral resolution MS images of *Bacillus subtilis* than previously reported. This method also allowed for the use of different matrices to be applied to the culture surfaces.

Key words: Microbial interactions, MALDI, FT-ICR, MSI, Matrix application, *Bacillus subtilis*

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Introduction

Visualizing microbial interactions with mass spectrometry imaging (MSI) has provided insights into many important biological processes, where it has captured molecular snapshots of metabolic exchange, antibiotic resistance, microbial competition, and more [1–7]. There are a number of different MSI methods reported for chemical mapping of microbial systems [3, 4, 6]. Among these, MALDI MSI has been the most utilized for attaining direct spatial and molecular information of microbial samples. Typically, sample preparation for these MALDI MSI experiments is performed by culturing microbial systems on thin agar, extraction and transfer of the agar plated sample, sieve application of dry matrix, and, lastly, dehydration of the sample [6].

The sieve matrix application method is inexpensive with minimal setup and requires expertise [6, 8]. However, within the microbial imaging scope, it has been limited in the type of matrix that can be used for analysis, where CHCA, from Universal MALDI matrix (1:1 CHCA: DHB), forms crystals with the surface analytes [6]. Furthermore, matrix surface coverage is particularly heterogeneous using this route, where practical lateral resolution of these measurements is typically a few hundred microns. Recently, Vergeiner reported an alternative sample preparation route that produced more homogenous matrix coverage on agar samples and expanded the type of matrices that can be utilized in microbial MALDI MSI measurements [9]. Also, Hoffmann and Dorrestein expanded on another group's work [1], detailing the benefits of using an automated nebulizing sprayer for matrix application, which lead to improved S/N and an increase in the number of analytes detected in MALDI analysis of microbial cultures [10].

Here, we describe a new sample preparation protocol using a robotic sprayer for MALDI MSI of microbial samples. We utilize the well-characterized model system of wild type *Bacillus subtilis* to demonstrate the benefits of this sample preparation method [2, 6]. This robotic sprayer offers two-dimensional control of matrix application, providing an even more uniform matrix surface coverage and sublimation-like crystal sizes [11]. It also demonstrates the potential to exploit the full arsenal of available matrices to be applied to the analysis of microbial communities.

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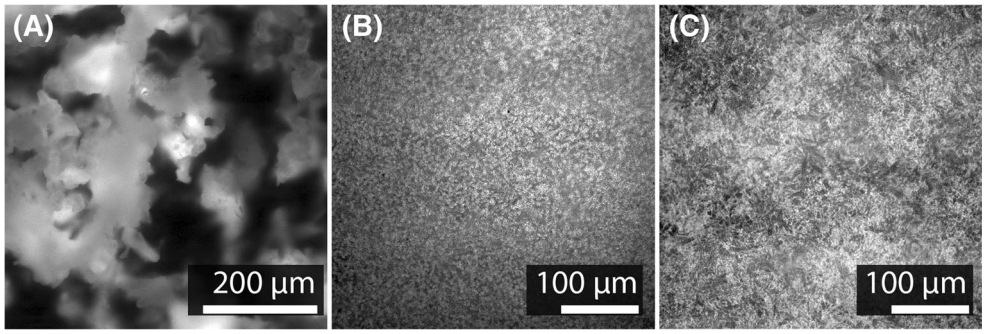

Figure 1. Optical microscope images of the agar sample surfaces after matrix has been applied, using (**A**) a 53 µm sieve or (B and C) the automated sprayer. The matrix at the surface of (**A**) and (**B**) is CHCA, whereas the matrix used in (**C**) is DHB. All samples are dry. Photographs of prepared samples can be seen in Figure S2

Experimental

Matrix Application with a Sieve

A 1:1 mixture of Universal MALDI matrix was applied to mounted samples with a 53 µm stainless steel sieve (Hogentogler and Co., Inc., Columbia, MD, USA) as described previously [6, 8]. Samples were left to dry at 37 °C overnight and stored in a vacuum desiccator until imaged.

Matrix Application with an Automated Sprayer

DHB was applied to mounted samples using a dedicated stainless steel sieve (described earlier). This facilitated sticking of the agar to the grounded MALDI plates, as discussed [6]. Coated samples were then left overnight at 37 °C to dry. We found other agars like LB [12] and AY [13], did not need to be coated with DHB to remain fixed to the stainless steel grounded MALDI plates after drying (Figure S1), and others have reported similar findings using potato dextrose agar [1]. We assume, that DHB diffuses through the ISP2 agar because no MALDI signal can be seen after drying, as described previously [6].

Matrix was applied to dried samples via the HTX TM-Sprayer (HTX Technologies, Chapel Hill, NC, USA). For CHCA, 10 mg/mL in 70% MeOH was used and sprayed with four passes at 240 µL/min and at 65 °C, with a spray spacing of 3 mm. For DHB, 40 mg/mL in 70% MeOH was used and eight passes were sprayed at 100 µL/min and at 65 °C, with a spray spacing of 2 mm. Spray pressure was 10 psi (N_2), a spray velocity of 1200 mm/min, and a 40 mm sprayer nozzle distance from the sample was maintained for all samples. We found that recrystallizing matrices, even commercially "ultrapure" ones, provided the best MALDI signal.

MALDI MSI

Imaging was performed on a 15T MALDI FTICR MS (Bruker Daltonics). The instrument was modified with an extra pumping stage to reduce the pressure in the analyzer cell (Infinity cell [14]). It is equipped with the commercially available Bruker SmartBeam II laser source (355 nm). Laser conditions can be found in the Supplemental Information for all images. Data was collected from *m/z* 200 to 2000, with transients of 0.472 s, which resulted in a resolving power of ~60,000 at *m/z* 1074. External

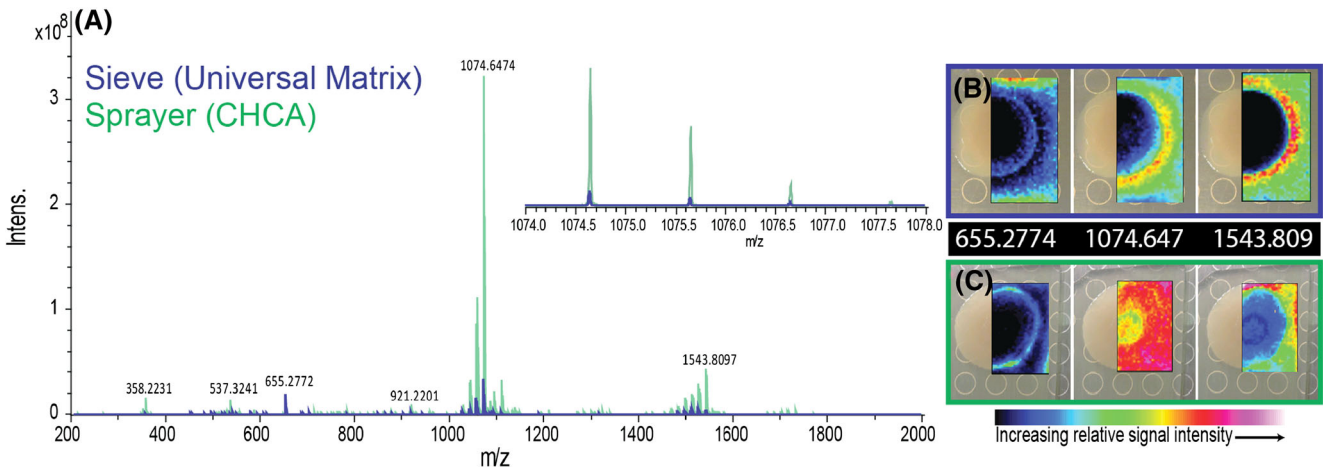

Figure 2. Spectral and ion image comparison of MALDI MSI analysis of *B. subtilis* cultures using the sieve method (blue) and the sprayer method (green). (**A**) Average mass spectrum of the entire image for both methods. (**B**) Ion images from sieve method, and (**C**) ion images from sprayer method, where *m/z* of 655.2774 is coproporphyrin I or III $[M + H]^+$, 1074.647 is surfactin $[M + K]^+$, and 1543.809 is plipastatin-C17-Val $[M + K]^+$. Molecular identities based on gnps.ucsd.edu. All ion images are normalized to the total ion current. More ion images can be seen in Figure S3

calibration was performed using PepMix II (Bruker Daltonics), resulting in mass measurement accuracies typically within 1 ppm for singly charged molecules across the entire *m/z* range. Peak picking was done using in-house software. Image data was processed using SCiLS Lab (GmbH, Bremen, Germany), and data visualization was performed using FlexImaging (v 4.1, Bruker Daltonics). All images were normalized to the total ion current, and peaks were identified using the Global Natural Produces Social Molecular Networking Spectral Library (gnps.ucsd.edu).

Results and Discussion

Comparison of Matrix Applications

The common sieve method is very easy to use, is a one step application process, and is affordable to set up. The hydrated agar assists in matrix-analyte crystallization during the drying process. This process is extremely robust as is evident by the number of researchers independently performing and achieving similar results [15]. However, this method is limited in the types of matrices that can be used for analysis (i.e., CHCA). For example, DHB will diffuse through the agar during the drying process and, therefore, no DHB-analyte crystallization occurs at the sample surface [6]. A method using a dispersed solid sprayer for applying matrix to agar surfaces prior to drying has been reported, where they demonstrated the ability to spray DHB, as well as CHCA, to their agar surfaces [9]. However, all of the sample prep methods reported thus far still illustrate fairly heterogeneous crystalline surface. We observe such results with the sieve method as well (Figure 1A), where crystals are consistently considerably larger than 53 µm (sieve size) and have significant topography. This can be seen in the inability to focus through the plane in Figure 1A.

We find that applying matrix with the robotic sprayer after agar samples have dried provided highly homogenous crystallize surfaces (Figure 1B and C). We were able to apply both CHCA and DHB with this approach. The heated spray tip provided very fine matrix-solvent droplets to the agar surface, which prevented excessive lateral dissociation of surface analytes, but provided ample analyte extraction into the matrix crystals. Moreover, crystal sizes were much smaller than laser beam sizes we utilized in this study.

Comparison of MALDI MSI Results

Not only did the robotic sprayer create more uniform films than the sieve method but it also helped improve sensitivity of the

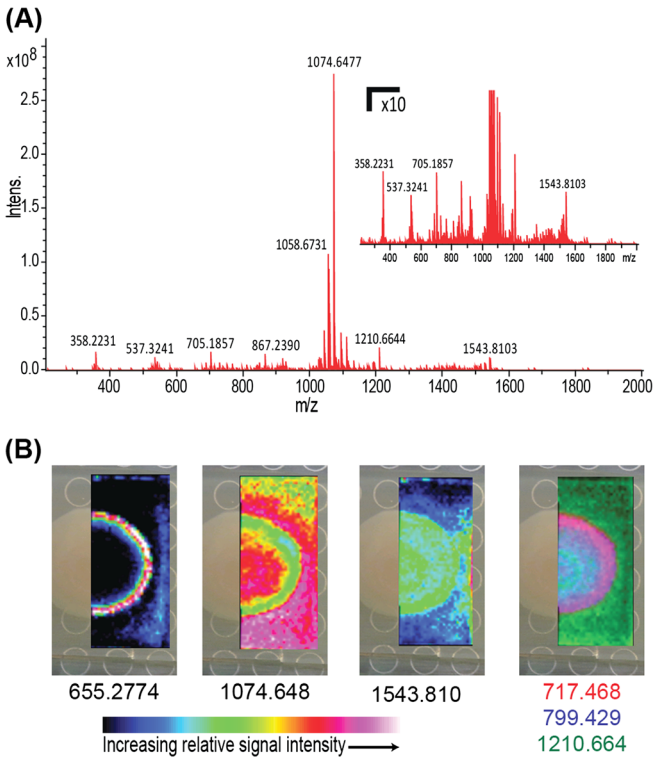

Figure 3. (**A**) Average spectrum and (**B**) ion images of MALDI MSI analysis of a *B. subtilis* culture where DHB was applied using the sprayer method. (**B**) Ion images of *m/z* of 655.2774 (coproporphyrin I or III; $[M + H]^+$), 1074.648 (surfactin; $[M + K]^+$), 1543.810 (plipastatin-C17-Val; $[M + K]^+$), and an overlay of 717.468 (PG 30:0; $[M + H]^+$), 799.429 (phakellistatin 13; $[M + H]^+$), and 1210.664 (unknown) ion images. All ion images are normalized to the total ion current. Molecular identities based on gnps.ucsd.edu. Figure S4 contains addition ion images

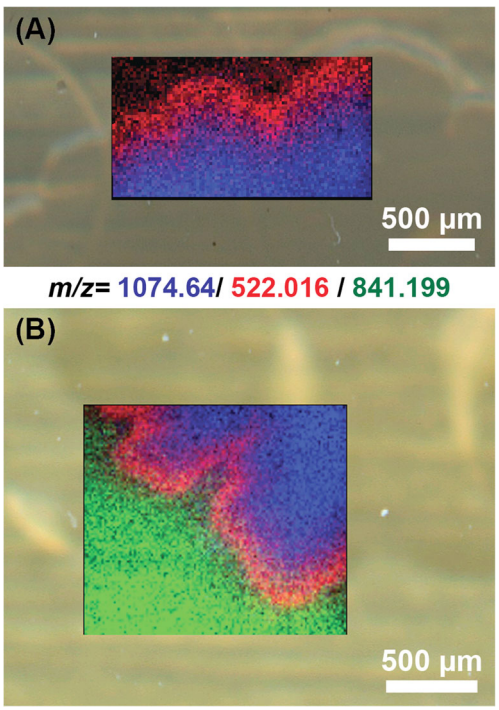

Figure 4. Ion images around the boarder of the *B. subtilis* using the sprayer method and applying either (**A**) CHCA or (**B**) DHB. A 30 µm and a 20 µm step size was used in (**A**) and (**B**), respectively. Overlay of *m/z* 1074.64 ± 0.01 (surfactin; $[M + K]^+$; blue), 522.016 ± 0.003 (unknown; red), and 841.199 ± 0.004 (unknown; green); *m/z* 841.199 was only detected within the colony (Figure S5) using CHCA; very little MALDI signal was seen outside the colony in general with these imaging conditions. Additional high-lateral resolution images of the DHB sample can be seen in Figure S6

MALDI measurements. Figure 2A illustrates the increase in signal achieved by applying CHCA via the robotic sprayer compared with the sieve method, where nearly a 10-fold increase in absolute intensity of the surfactin- and plipastatin-related peaks is observed. After removing agar-related peaks, we detected 2356 *B. subtilis*-related peaks using the sprayer method versus the 737 *B. subtilis*-related peaks using the sieve method. From the ion images, a sharper band of coproporphyrin (I or III; *m/z* 655.2774) around the *B. subtilis* colony is observed using the sprayer method compared with the sieve method (Figure 2C and B, respectively). Also, more surfactin and plipastanin (*m/z* 1074.647 and 1543.809, respectively) are also detected within the colony using the sprayer method, whereas we primarily only detect these molecules outside the colony with the sieve method.

Using the robotic sprayer to apply DHB to the culture resulted in the detection of even more *B. subtilis*-related peaks (2876 in total) with MALDI MSI (Figure 3). Most of *B. subtilis*-related peaks detected by spraying DHB match the peaks detected in the MALDI MSI where CHCA was applied with the sprayer. We observe far more peaks in the *m/z* range of 600 to 1000, where lipids are often detected, using the DHB method rather than the CHCA methods. We also see a dominant *m/z* 1210.664 peak (Figure 3B), which is an unknown and not observed in the sieve method analysis.

With the increase in sensitivity and uniformity of the matrix crystalline surface using the sprayer over the sieve method, we were able to take high-lateral resolution images of *B. subtilis* cultures on agar. These cultures were less mature than the ones shown in Figures 2 and 3. By reducing the beam size, beam power, and number of shots, fewer peaks were detected. However, we could achieve 30 and 20 μm lateral resolution of the colonies using the sprayer method and applying CHCA and DHB, respectively, the highest lateral resolution reported to date of microbial cultures. A high-lateral resolution MS image around the boarder of the *B. subtilis* colony using CHCA can be seen in Figure 4A, and around the boarder of a different *B. subtilis* colony using DHB can be seen in Figure 4B. We observed more species outside of the culture area using DHB than CHCA with these imaging conditions.

Conclusions

The ability to observe microbial interaction though molecular mapping by MSI has provided invaluable insights into many biological processes. However, further optimization of the matrix application process was needed to achieve higher sensitivity and to increase the lateral resolution of MALDI MSI of agar-plated cultures. Here, we demonstrated the use of a new sample preparation method, which uses a robotic sprayer, for analysis of WT *B. subtilis* cultures. This method allows the use of different matrices, while creating matrix coatings more homogenous than the sieve-based method. Using the sprayer, we observed a significant increase in sensitivity and were able to take higher lateral resolution measurements than reported previously.

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