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Imaging with Mass Spectrometry of Bacteria on the Exoskeleton of Fungus-Growing Ants

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Abstract

Mass spectrometry imaging is a powerful analytical technique for detecting and determining spatial distributions of molecules within a sample. Typically, mass spectrometry imaging is limited to the analysis of thin tissue sections taken from the middle of a sample. In this work, we present a mass spectrometry imaging method for the detection of compounds produced by bacteria on the outside surface of ant exoskeletons in response to pathogen exposure. Fungus-growing ants have a specialized mutualism with *Pseudonocardia*, a bacterium that lives on the ants' exoskeletons and helps protect their fungal garden food source from harmful pathogens. The developed method allows for visualization of bacterial-derived compounds on the ant exoskeleton. This method demonstrates the capability to detect compounds that are specifically localized to the bacterial patch on ant exoskeletons, shows good reproducibility across individual ants, and achieves accurate mass measurements within 5 ppm error when using a high-resolution, accurate-mass mass spectrometer.

Graphical Abstract

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Microbial organisms produce many secondary metabolite compounds that have been extensively studied as potential drug leads in natural product research. Much of this research examines the production of these small molecules when microbes are grown in isolation. These compounds may act in chemical communication between microbes,^{1–3} as such, recent work examined pairwise interactions between microbes and detected induction of differential compounds.^{4–7} While this is an effective tool, particularly for natural product discovery, it still overlooks the diversity and complexity of species interactions that occur within a microbial niche. Improved approaches to examine small molecules produced by microbes *in situ* will greatly benefit microbial ecology and have great implications for natural product research. Here, we developed a method using mass spectrometry imaging to examine *in situ* chemical interactions in the leaf-cutter ant symbiosis, an established, multipartite symbiosis.

Leaf-cutter ants participate in a multispecies symbiosis. These ants provide leaf substrate to a fungus that they cultivate as their sole food source.^{8,9} This association is an obligate mutualism and the ants are under strong selective pressure to protect their food source. To do this, the ants maintain a mutualism with Actinobacteria in the genus *Pseudonocardia*. The *Pseudonocardia* symbionts live on the ants' propleural plates (the plate on the ventral side of the ant thorax directly posterior to the head, see photograph in Figure 1) and provide antimicrobial protection against pathogens, including the system-specific pathogen *Escovopsis*.^{10–13} The quadripartite symbiosis between the ant host, the fungal cultivar, antibiotic-producing *Pseudonocardia*, and garden pathogen *Escovopsis* is particularly well-suited for ecological studies as each player can be isolated from the system for individual study and the intact association can be manipulated *in vivo*. Developing an approach to study secondary metabolites *in vivo* could reveal functionally and ecologically relevant chemical interactions between microbial species as well as unlock a vast array of natural products.

In this study, matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) was used to examine *in situ* species interactions between *Pseudonocardia* and *Escovopsis*. MSI has rapidly emerged as a powerful analytical technique for understanding the spatial distributions of molecules within a variety of biological samples,^{14–20} and more recently, scientists have taken advantage of the capabilities of MSI and applied this technique toward the discovery of new natural products.^{21–23} Although LAESI-MSI techniques have been used to characterize compounds on nonflat sample surfaces,²⁴ typically MALDI-MSI is limited to the analysis of thin tissue sections or bacterial colonies

in culture. However, this study aimed to analyze metabolites produced by *Pseudonocardia* in its native ecological niche on the organism's outer surface. There have been few reports of studies using ultraviolet (UV)-LDI-MS and MALDI-MSI to analyze other insects, such as flies.^{25,26} A previous study used MALDI-MSI to examine whole ants but did not analyze bacteria specifically localized to the ants' propleural plates.²⁷ Thus, a unique sample preparation method was developed to analyze chemical compounds produced by bacteria localized to the ant propleural plate in an accurate and reproducible manner *via* MALDI-MSI. The uniqueness in the sample preparation comes from using modified glass slides in which three-dimensional (3D) samples (ants) can be inlaid so that the outer surface of the organism can be analyzed, in comparison to traditional MALDI-MSI sample preparation in which thin sections are sliced from the middle of a 3D sample and the inside of the organism is analyzed. We take advantage of the high-resolution, accurate-mass capabilities of a MALDI-LTQ Orbitrap mass spectrometer which maintains <5 ppm mass accuracy even with slight differences in the sample height, unlike the more common MALDI-time-of-flight (TOF) systems. This approach will increase our understanding of this host/microbe symbiosis and has the capability to provide ecological insights into chemical interactions between microbial species in a unique biological system.²⁸

MSI Method.

We developed a method for MSI of *Pseudonocardia* on the surface of the ants' propleural plates, as described in the Materials and Methods section and shown in Figure 1. A photograph of the ants and glass slides coated with DHB matrix is shown in Figure S1. As proof-of-principle, ants were raised without *Pseudonocardia* and ProteoMass MALDI-MS calibration mix (Sigma-Aldrich) was spiked onto ant propleural plates laid into the modified glass slide in different ways (see Supporting Information for more details) and directly onto the glass slide. Images were obtained for six calibration standards spiked onto ant propleural plates, and the glass slide in the mass range of 300–1800 m/z and the mass spectra collected for each imaged area were averaged to report the average m/z value detected of each standard. Table S1 details the calibrant m/z values measured and the calculated mass errors. The method displayed a ppm mass error of 2.1 ± 0.1 ppm for the calibrants spiked onto the ants, which is comparable to the 1.9 ppm mass error observed for the calibration mix that was measured directly from the glass slide. The absolute ppm error measurements varied but could be minimized with instrument calibration. These results suggest that any nonplanarity of the ant samples does not greatly affect the mass accuracy of the experiment, highlighting the unique advantage of using an Orbitrap-based mass analyzer compared to a time-of-flight mass analyzer, where even the difference of 100 μm in sample height can change the detected mass by up to 0.1 Da. We further examined the necessity of the groove in the glass slide by spiking a metabolite standard of ergothioneine (m/z 230.096) on ant propleural plates. The spiked propleural plates were then laid in the groove of the modified slides or laid onto standard glass slides and secured with double-sided tape. The ant thoraxes were either secured with a lateral tilt (tilted to the right or left), a longitudinal tilt (tilted up or down), or left planar. The results, shown in Figure S2, show that ergothioneine was not able to be detected on several of the ant thoraxes placed on the standard glass slides and tilted either laterally or longitudinally, likely due to being out of the plane of the MALDI laser.

This issue was not observed when the ant thoraxes were placed in the groove of the slide. Placing the ant thoraxes in the groove of the glass slide minimized the impact of any tilt of the sample, or nonplanarity, when compared to ant thoraxes placed on standard glass slides. The mass accuracy was not affected by the tilt of the ants. Furthermore, laying the ants into the groove of the glass slide keeps the samples in a sturdy, fixed position for the entire analysis time which ranged from 2 to 7 days depending on the number of ants being imaged and the spatial resolution selected.

Three ants from three different subcolonies (nine ants in total) were imaged over the course of several months and the representative images are displayed in Figure 2. The images show that many of the same m/z values could be detected on multiple biological replicates. Furthermore, multiple groups of ants were analyzed at different times, and still many of the same m/z values were detected across individual and groups of biological replicates, thus demonstrating overall strong reproducibility of the method. Some biological variation, mainly in the spatial distributions of the detected compounds, was expectedly observed as the localization of the bacteria varies slightly from ant to ant.

MSI of Ant/ *Pseudonocardia*/ *Escovopsis* Interactions.

Representative MS images of compounds produced by *Pseudonocardia* on the ant propleural plate are displayed in Figure 3, showing that this method can clearly and confidently distinguish bacterial-derived compounds (localized to the propleural plate) from ant or environment derived compounds (no specific localizations). These data show that a lack of *Pseudonocardia* results in fewer detectable compounds on the ant propleural plate. Furthermore, many compounds were observed to be produced only in response to *Escovopsis* exposure (such as m/z 390.098, 490.084, 533.166, and 620.009 shown in Figure 3). The results showing that *Pseudonocardia* only synthesizes these compounds in response to pathogen exposure led to the hypothesis that these molecules could be natural products or drug leads with antimicrobial properties. Compound identifications were attempted by searching collected MS/MS data via several metabolomics and natural products databases. The preliminary database searches either resulted in tens of potential identifications or no search results for a given mass, which could indicate the detection of unique compounds. Future work will focus on additional compound elucidation and identifications. The MSI data have been made publically available on METASPACE (<http://annotate.metaspaces2020.eu>)²⁹

Interestingly, the compound detected at m/z 230.096 (shown in Figure 3) is only observed when *Pseudonocardia* is present on the ant propleural plates. This compound was identified as ergothioneine. Ergothioneine is a unique compound that is only known to be specifically synthesized by certain species of Cyanobacteria, Mycobacteria, Actino-bacteria, and certain fungi such as *Ascomycetes* and *Basidiomycetes*.^{30,31} Currently, the physiological role of ergothioneine is unknown; however, *in vitro* studies have shown antioxidant properties of this compound.³¹ The ergothioneine identification was confirmed by comparing the sample to a purchased ergothioneine standard (Sigma-Aldrich). The retention time was identical to that of the ergothioneine standard, and the MS/MS spectra were also nearly identical. Differences in the MS/MS spectra are due to co-isolation of a similar-mass metabolite

because of a wide isolation width (3 Da). A mirror plot of the MS/MS spectra for the experimental sample and the ergothioneine standard is shown in Figure 4. These results indicate that ergothioneine is indeed being produced by *Pseudonocardia* and not by the fungal pathogen, *Escovopsis*, because this compound is detected when *Pseudonocardia* is present (regardless of *Escovopsis* exposure) but is absent when the ants without *Pseudonocardia* were exposed to *Escovopsis*. A followup analysis of the *Pseudonocardia* genome showed that *Pseudonocardia* do indeed possess the genes required for ergothioneine synthesis.

From a technological standpoint, the work presented here demonstrates a method for MALDI-MSI of metabolites from a host/microbe system *in situ*. It was essential to use MSI for this study in order to be truly confident that the detected compounds were indeed being produced by *Pseudonocardia* and not from the ants or the environment. This protocol can be used as a tool for chemical biologists and adapted in the future for MALDI-MS imaging of the surface of other organisms with three-dimensional shapes, rather than thin tissue sections, in order to tackle difficult biological questions. We demonstrated the usefulness of this protocol by studying the ant/ *Pseudonocardia* symbiosis. The approach developed here can provide important insights into metabolites that mediate the microbial interactions within the fungus-growing ant symbiosis and provide insights into the expression of cryptic secondary metabolite clusters.

MATERIALS AND METHODS

Sample Preparation for MALDI-MSI.

Prior to sample preparation, grooves were cut into glass slides, and double-sided tape was applied to the back of the slide. The double-sided tape allows for a flexible backing in which ants of different sizes can still be positioned so that their propleural plate is parallel to the top of the slide.

Ants were randomly assigned to one of three treatments: *Pseudonocardia*/pathogen treatment, no-*Pseudonocardia*/pathogen treatment, or *Pseudonocardia*/control treatment (see Supporting Information for ant colony preparation and pathogen treatment details). The ants were dissected by removing the head, abdomen, and appendages from the thorax with a razor blade. The thorax was placed in the groove of the slide with additional tape over the bottom portion of the thorax (posterior to the *Pseudonocardia* patch on the propleural plate) to secure into place. Ant thoraxes were then inlaid into the groove of the slide with the propleural plate facing outward and parallel with the top of the slide. Additional dissection methods were evaluated (see Supporting Information).

A matrix (40 mg mL⁻¹ DHB in 50:50 water/methanol) was applied to the ants using a TM-Sprayer (HTX Technologies, LLC). DHB was purchased from Sigma-Aldrich. The TM-Sprayer method for applying DHB to the ants was as follows: 80 °C, 0.2 mL/min flow rate, eight passes–rotate/offset, 3 mm spacing, 30 s dry time between passes, velocity of 950 mm/min.

MALDI-Orbitrap MSI.

A MALDI- LTQ Orbitrap mass spectrometer (Thermo Scientific) equipped with an N₂ laser (spot diameter of 75 μ m) was used in positive ion mode for MSI. Multiple ants of each treatment type ($n = 3-4$) were imaged with a step size (pixel size) of 75 μ m using a mass range of m/z 100–1700 and a mass resolution of 60 000. The region to be imaged and the raster step size were controlled using the LTQ software (Thermo Scientific) and the instrument methods were created using Xcalibur (Thermo Scientific). MALDI-MSI data were processed using MSiReader³² (see Supporting Information for more information).

LC-ESI-MS and MS/MS.

Metabolites were extracted from the propleural plates of ants that were and were not exposed to *Escovopsis* (see Supporting Information for additional extraction procedure details). MS and MS/MS data of the ant extracts were acquired on a quadrupole-orbital trapping instrument (Q-Exactive Orbitrap, Thermo Scientific) equipped with an ESI source operated in positive ion mode. The MS scan range was m/z 135–1300. The MS/MS scan range was adjusted depending on the parent mass and high-energy collisional dissociation (HCD) was used for fragmentation with collision energy of 35 eV and an isolation width of 3 Da. See the Supporting Information for LC parameters and links to publically available LC-MS and LC-MS/MS data sets.

Metabolite Identifications.

Online databases were used to search the accurate mass and MS/MS data for potential metabolite identifications. Ergothioneine was identified by searching the accurate mass and MS/MS data against reference MS/MS spectra in the METLIN database³³ and then comparing the LC retention time and MS/MS spectra to a purchased ergothioneine standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Workflow for MS imaging of *Pseudonocardia* on the ant propleural plate. a) Photograph and b) cartoon of *Pseudonocardia* on the ant exoskeleton. c) Grooves were cut into glass slides and double-sided tape was applied to the back of the slide. The ant thoraxes were removed and positioned into the groove of the slide with the propleural plate facing outward and even with the top of the slide. An additional thin strip of double-sided tape was applied below the propleural plate to stabilize and secure the thorax into place. d) Matrix was applied to the slide using an automatic sprayer. e) A laser was fired at the sample to ionize compounds of interest and introduce them into the mass spectrometer. f) An array of mass spectra was acquired using a MALDI-LTQ Orbitrap and g) compiled into MS images. Photo courtesy of Alex Wild.

Figure 2.

Representative MS images. The images demonstrate the reproducibility of the developed method by comparing ant propleural plates from nine technical replicates. The color scale represents low (blue) to high (red) relative intensity (0–100%). Scale bar = 500 μm . All images are the m/z value ± 5 ppm.

Figure 3.

Representative MS images comparing ant propleural plates (a) without the presence of their native *Pseudonocardia* and also exposed to the pathogen, (b) *Escovopsis*, with the presence of *Pseudonocardia*/ no pathogen exposure, and (c) with *Pseudonocardia* and exposed to *Escovopsis*. The color scale represents low (blue) to high (red) relative intensity (0–100%). Scale bar = 500 μm . All images are the m/z value ± 5 ppm.

Ergothioneine
m/z 230.09558 **Figure 4.**

Mirror plot of the MS/MS spectrum of the identified metabolite, ergothioneine (m/z 230.09558), compared to the MS/MS spectrum of a purchased ergothioneine standard. Differences in the MS/MS spectra are due to coisolation of a similar-mass metabolite because of a wide isolation width (3 Da). The spectrum is annotated with structures corresponding to fragment ions that match fragments reported in the literature spectra in the MELTIN database.