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CAPTURING BACTERIAL METABOLIC EXCHANGE USING THIN FILM DESI-IMAGING

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Abstract

Over 60% of current pharmaceutical drugs have origins in natural products. To expand on current methods allowing one to characterize natural products directly from bacterial culture, herein we describe the use of desorption electrospray ionization (DESI) imaging mass spectrometry in monitoring the exchange of secondary metabolites between *Bacillus subtilis* and *Streptomyces coelicolor* using a simple imprinting technique.

The importance of studying bacterial secondary metabolites can be assessed by a quick look at the current pharmaceutical landscape. With over 60% of all therapeutic agents having origins in natural products, the need for novel methods to identify and characterize these compounds remains incredibly high¹. Even some of the most studied producers of secondary metabolites, such as *Bacillus subtilis* and *Streptomyces coelicolor*, continue to yield novel compounds as methods for studying these systems continue to advance^{2,3}. It is in this arena that imaging mass spectrometry (IMS) is becoming a useful tool for many natural product researchers owing to its ability to identify compounds based on their mass to charge ratio (m/z) and show the spatial distribution of a compound of interest throughout the sample.

Recently, our laboratory has reported a novel method for capturing chemical exchange between neighboring bacterial populations using matrix assisted laser desorption ionization (MALDI) imaging mass spectrometry³. Using this technique we were able to capture the exchange of secondary metabolites between neighboring populations of *Bacillus subtilis* and *Streptomyces coelicolor* on nutrient agar. When monitored over time, the production of various secreted secondary metabolites from *B. subtilis* were shown to both inhibit and elicit secondary metabolite production in *S. coelicolor*. In agreement with published biochemical studies^{4–6}, known interactions between these two species such as the inhibition of prodigiosin production in *S. coelicolor* by the hybrid PKS-NRPS peptide bacillaene from *B. subtilis* and the inhibition of aerial hyphae formation in *S. coelicolor* by the lipopeptide antibiotic surfactin produced by *B. subtilis* were confirmed. It was also possible to build upon these known interactions by finding a number of unknown ions from *S. coelicolor* whose production was repressed by bacillaene and that surfactin was able to actively inhibit production of calcium-dependent antibiotic (CDA) and SapB, a compound necessary for

aerial hyphae formation, in *S. coelicolor*³. Herein we describe an alternative, complimentary method for imaging metabolic exchange in bacterial populations using the ambient ionization technique desorption electrospray ionization (DESI).

The relatively recent invention of the DESI source⁷ has allowed for atmospheric analysis of a wide variety of compounds ranging from lipids⁸ to pharmaceutical compounds⁹ to proteins¹⁰ under atmospheric temperature and pressure with little to no sample preparation. When applied to the imaging of biological samples, DESI becomes an attractive method of choice due to its potential to analyze the system in its native state. However, despite being commercially available for over 5 years, there exists only a single report of its use in imaging secondary metabolites from a biological sample¹¹. This surprising lack of precedent can possibly be attributed to the fact that DESI signal is highly dependent on many experimental parameters¹² such as the spray solvent, spray angle, spray voltage, DESI emitter height, mass spectrometer inlet height, DESI emitter to mass spectrometer inlet distance, nebulizing gas pressure, solvent flow rate and sample surface can significantly decrease the applicability of the tool to biological samples.

The mechanism of ionization employed by DESI relies on the ability of a pneumatically assisted electrosprayed solvent to form secondary droplets upon impact of the sample surface before entering the mass spectrometer¹³. This momentum-transfer based mechanism for desorption of the analyte from the sample surface relies on a hard, uniform and non-conductive sampling surface in order maintain consistent signal during rastering therefore limiting the types of surfaces that are available for DESI imaging. These requirements have thus far prevented the direct analysis of metabolites from bacterial culture on nutrient agar by DESI due to the absorbent, deformable and conductive nature of the agar sample surface¹⁴. We therefore reasoned that better ion signal, and therefore better images, could be obtained by imaging bacterial colonies from a hard, uniform surface. A similar problem was encountered by Debois *et al* in their attempts to image surfactin from *Bacillus subtilis* using secondary ion mass spectrometry (SIMS)¹⁵. Analogous to DESI, SIMS utilizes a similar ionization mechanism and requires a hard and flat surface in order to maintain consistent signal while imaging. Their solution to this problem was to make an imprint of the culture using a silicon wafer, which was then used for the subsequent imaging process. Similar to this solution, we screened a variety of surfaces and discovered that mixed cellulose ester filter membranes work effectively as a complimentary surface that can be used to make an imprint of bacterial culture from solid agar and that this filter imprint can be directly imaged by DESI-MS with no additional sample preparation.

To demonstrate the utility of this method, we investigated interaction between *Bacillus subtilis* 3610 and *Streptomyces coelicolor* A3(2). Both of these bacterial systems produce a large number of known natural products whose rate of production can be attenuated based on the strain of *B. subtilis* used in the study^{3,5}. The arsenal of secondary metabolites available to *B. subtilis* can actively silence production of defensive secondary metabolites as well as aerial hyphae formation in *S. coelicolor*^{3-5,16}. Mutant strains of *B. subtilis* lacking the ability to synthesize certain natural products results in different phenotypes for *S. coelicolor* due to changes in metabolic output from both bacterial populations. Capturing

these changes in metabolic output between genotypic variants by DESI imaging mass spectrometry provided a means of validation for the method.

The experiment was carried out by inoculating 1μL of *Streptomyces coelicolor* A3(2) on ISP-2 nutrient agar. Following a 20 hour incubation period at 28°C, 0.2μL of *Bacillus subtilis* 3610 or PY79 laboratory domesticated strain¹⁷, which is impaired in the ability to produce molecules via polyketide or non-ribosomal peptide synthetase biosynthetic paradigms (e.g. bacillaene, surfactin and plipastatin), were inoculated approximately 2mm away from the *S. coelicolor* colony and incubated at 28°C for 48 hours. Imprinting was done by placing the imprinting surface (0.45μm 47mm Millipore HA filter membrane; Part No. HAWP4700) on top of the bacterial culture and applying light uniform pressure for 30 seconds after which the imprinting surface was removed and dried in a vacuum dessicator overnight. Imaging was done using a ProSolia DESI source (Model OS-3201) coupled to a Thermo-Finnigan LTQ-FT-ICR MS. DESI parameters used for the images include an emitter height of 2.5mm, mass spectrometer inlet height of 0.2mm, inlet to emitter distance of 4mm, 58° spray angle, 2.0kV spray voltage, -125V tube lens voltage, 2μL/min flow rate, 140psi ultra-pure nitrogen nebulizing gas pressure, and a spray solvent of Methanol : Water : Ammonium Hydroxide (50:50:0.1) (v:v:v). Images were collected from m/z 150–2000 with a step size of 250μm, a scan rate of 100μm/sec and a 2000ms ion trap time resulting in a pixel size of 200μm × 250μm. These parameters are not optimal for one individual ion but rather were optimized to capture a wide scope of molecular species ranging from polyketides to fatty acids to peptides. Collected data was converted into image files using Firefly data conversion software (version 1.2.0.1) and viewed using BioMAP software (version 3.7.5.6).

By utilizing bacterial imprinting to monitor metabolic exchange between *B. subtilis* and *S. coelicolor* 57 unique signals spatially localized to the bacterial colonies were observed (Fig. 1A). Among these signals, the identities of actinorhodin, surfactin and plipastatin were confirmed by either interpretation of CID fragmentation (surfactin and plipastatin) or by comparing fragmentation patterns with purified compounds (actinorhodin) as seen in Figure 1C–E. The tandem mass spectrometry spectrum for surfactin is also nearly identical to a recent non-imaging DESI report on *B. subtilis* strain 168 (sfp⁺) by Cooks *et al.*⁶. The full characterization of some of the more interesting ions such as m/z 1076 are ongoing (note that the observed difference in the distribution of the 1076 ion between the two strains is due to the transfer of the analyte to the filter membrane being shielded by the formation of the white aerial hyphae on the surface of the *S. coelicolor* colony when co-inoculated with *B. subtilis* PY79).

As expected, wild type *B. subtilis* 3610 produced considerably more secondary metabolites than the PY79 domesticated lab strain including multiple forms of the lipopeptide antibiotics surfactin and plipastatin exhibiting variations in the length of their lipid tails. The interaction with the PY79 strain allowed for increased metabolite production, including actinorhodin production, as well as the formation of white aerial hyphae in *S. coelicolor*. The wild type and PY79 *B. subtilis* strains produced a total of 23 and 6 unique mass spectrometric signals, respectively, with an additional 3 signals common between the two samples (Fig. 1B). *Streptomyces coelicolor*, when cultured with either wild type or PY79 *B. subtilis*, produced a

total of 3 and 13 unique signals, respectively, with 9 additional signals common to both samples (Fig. 1B). This data clearly demonstrates the ability of wild type *B. subtilis* to inhibit the production of certain secondary metabolites in *S. coelicolor* and the ability of DESI imaging to characterize such phenotypes.

Further analysis of the data provided additional confirmation of the fidelity of the method. Within the wild type *B. subtilis* 3610 strain we can clearly identify multiple forms of both surfactin and plipastatin being secreted from the culture while these signals are completely absent from the PY79 domesticated lab strain which lacks the ability to make both antibiotics. Additionally, the antibiotic actinorhodin¹⁸ can be seen localized to the area of purple pigment in *S. coelicolor* cultured with *B. subtilis* PY79 while this signal is not observed when *S. coelicolor* is cultured with *B. subtilis* wild type. This data illustrates the ability of *B. subtilis* to silence antibiotic production in *S. coelicolor* allowing *B. subtilis* to gain a fitness advantage over competing bacteria.

Upon comparison with similar data collected from MALDI imaging, the complementarity of the two methods becomes evident. With the ionization efficiency of DESI being greater in negative ion mode (at least during our studies), DESI is able to capture compounds not easily seen in positive ion mode, such as actinorhodin, while MALDI imaging is able to capture compounds that do not readily form negative ions, such as prodiginines. A notable difference between the two methods can be seen in the distribution of ion intensity of surfactin within the bacterial colony. When imaged by MALDI, the concentration of surfactin appears to be greatest outside the colony³ while the DESI image shows ion intensity greatest within the bacterial colony. We attribute this difference in intensity to the imprinting method. While imprinting, only exposed, secreted natural products are absorbed onto the membrane therefore natural products embedded in the growth media are not readily observed whereas the MALDI laser is able to penetrate the surrounding media and ionize the secreted metabolites. It should be noted that signal during DESI imaging is drastically decreased when imaging over areas of the filter membrane where the bacterial colony has adhered to the filter, which is the cause of the circular absence in signal in the wild type *B. subtilis* colony in Figure 1a. The dried cellular material tends to absorb the spray solvent preventing the solvent from desorbing from the membrane and entering the mass spectrometer for analysis.

Due to all areas of biology employing multiplexed metabolic exchange, it is important to develop methods that can capture the interdependence of these molecules to further elucidate their role in the communication dynamic. A better understanding of metabolic exchange may lead to the discovery of new therapeutics and therapeutic paradigms. Herein we have shown that the use of DESI imaging of imprints of bacterial colonies is another tool that can be effectively used to monitor multiplexed metabolic exchange between microbial species.

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Figure 1.

Monitoring secondary metabolite production by DESI imaging mass spectrometry (IMS). **A)** IMS of Millipore HA filter imprints of *Bacillus subtilis* 3610 wild type (WT) and PY79 mutant strain cultured with *Streptomyces coelicolor* A3(2). Signals corresponding to surfactin (m/z 1035) and plipastatin (m/z 1504) can be seen localized to the *B. subtilis* 3610 wild type colony while signals corresponding to the purple pigmented antibiotic actinorhodin (m/z 629) and an unknown ion (m/z 1076) can be seen localized to the *S. coelicolor* colony. **B)** Distribution of unique ion signals between *B. subtilis* 3610 wild type and PY79 mutant strains and between *S. coelicolor* colonies cultured with *B. subtilis* 3610 and PY79. For surfactin and plipastatin, only ions corresponding to the $[M-H]^-$ ions were included with other charge states excluded from the count. **C)** Identification of the signal at m/z 629 as actinorhodin by comparing tandem MS fragmentation patterns for HPLC purified actinorhodin extracts (top) and data from the HA filter imprint of the bacterial colony (bottom). **D–E)** Tandem MS confirmation of surfactin (**D**) and plipastatin (**E**) from *B. subtilis* 3610 wild type directly from HA filter imprint of the bacterial colony.