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Ion mobility mass spectrometry enables the efficient detection and identification of halogenated natural products from cyanobacteria with minimal sample preparation

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ABSTRACT

Direct observation of halogenated natural products produced by different strains of marine cyanobacteria was accomplished by electrospray ionization and matrix assisted laser desorption ionization and gas phase separation via ion mobility mass spectrometry of extracts as well as intact organisms.

1. Introduction

Natural product and natural product inspired compounds account for some 70% of all current pharmaceutical products on the market.[1] Although the pharmaceutical industry has wavered in its dependence on natural products as leads during the last 40 years, there has been a resurgence of interest recently. This is likely the result of a failure of combinatorial chemistry to deliver very many clinically useful drugs, the rise of genomics which is giving a resurgence of interest into natural products, and the development of user-friendly but powerful mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques to identify and dereplicate natural products from a variety of organisms.[2,3] These bioactive compounds, usually characterized as secondary metabolites, vary widely in chemical structure and physiological targets and span a range of potential clinical uses.[1,4]

Bacteria and plants have traditionally accounted for the majority of bioactive metabolites, however, marine organisms, such as sponges, tunicates, cyanobacteria and assorted microorganisms, have emerged as exciting and relatively unexplored sources of novel chemistries, likely a product of evolving within a very different set of environmental conditions.[5] A number of marine derived natural products have entered clinical and preclinical trials and hold promise for a variety of diseases, with a particular emphasis on cancer chemotherapeutics.[6,7]

Although the field of marine natural products drug discovery holds significant attractions, it is not without its limitations. The producing organisms are frequently found in close association with one another, forming complex assemblages that result in challenges in the identification and isolation of the producing organism.[3] It is estimated that cyanobacteria and bacteria account for nearly 80% of reported bioactive marine natural products that have advanced past the pre-clinical phase.[8] During the discovery process, these assemblages of poorly understood organisms often result in very complex mixtures of metabolites that represent significant analytical challenges to the natural products chemist.[3]

It has recently been suggested that there is an urgent need for the development of new more powerful and high-throughput methods for the structural analysis of natural products.[9] In this regard, ion mobility mass spectrometry has the potential to accelerate the characterization of natural products. Traveling wave ion mobility mass spectrometry, which is often paired with matrix

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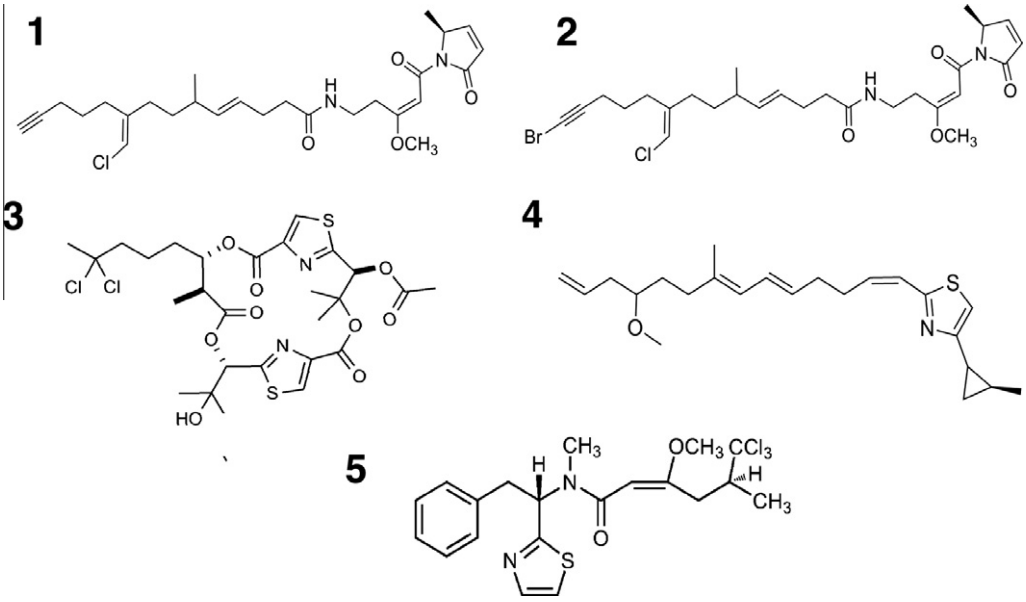
assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) sources, essentially provides an additional separation step in the gas phase without the loss of sensitivity.[10] This enables the separation of ions that have similar mass to charge ratios (*m/z*) but which possess differing collisional cross-sections. The results are displayed as trend-lines defined by both *m/z* value and drift time through the ion mobility chamber containing an inert gas (nitrogen, argon, etc.). Ions with the smallest masses and tightest configurations or most aerodynamic shape emerge first, and are assigned lower drift times. Larger and spatially bulky molecules emerge later, and thus have longer drift times. Overall, this enables the separation of matrix signals (from the MALDI preparation) as well as different classes of molecules from one another.[10,11] This is an especially advantageous addition in the study of small molecules because most natural products, quorum sensing signals, pheromones, and other bioactive natural products involved in cell-to-cell communication and adaptive metabolism are observed in the region below *m/z* 2000.

This report describes proof-of-principle experiments characterizing halogenated metabolites to demonstrate the utility of ion mobility separation of three species of *Lyngbya*, a genus of mainly tropical marine cyanobacteria and prolific producer of potently bioactive metabolites.[12] These natural products can be observed not only in crude extracts but also from intact cyanobacterial filaments using ion mobility instrumentation (Waters Synapt G1 Mass Spectrometers equipped with ESI or MALDI sources). The ESI-ion mobility experiments described herein resulted in a clear separation of multiply halogenated metabolites from other metabolites and assorted small molecules. This capability was especially useful in allowing overlapping isotopic signals to be more clearly resolved into their true isotopic ratios. The imaging experiments on the MALDI instrument allowed the highest spatial resolution images yet reported for the distribution of specific natural products in a marine organism.[13,14] Ion mobility data were also gathered on the same organisms, giving a powerful insight into the composition and distribution of these interesting metabolites. Indeed, natural product imaging results have been powerful approaches for addressing the origin of biosynthesis of several previously characterized compounds, and provide further evidence of their distribution along cyanobacterial filaments.[14] The addition of ion mobility allowed us to separate matrix interference and *m/z* signals stemming from pigments and assorted small molecules from halogenated and other natural products in the gas phase without the need for chromatography.

2. Results and discussion



Crude extracts of two strains of the cyanobacterium *Lyngbya majuscula*, JHB and 3L, were run on an ESI ion-mobility instrument (Fig. 1). In the JHB extract, we judged empirically that four trend-lines were resolved (highlighted in Fig. 1A), indicating ion mobility separation of various metabolites. There is no specific trend in this data to suggest that there are predictable effects of halogenation but it is notable that many of the multiply halogenated natural product fall into a different drift space that the rest of the molecules. The spectra for these trend-lines are shown in Fig. 1B and contain some exciting observations; trend-line 1 gives rise to unknown metabolites, possibly nitrogen-containing linear molecules that have slower mobility. Trend-line 2 contains peptides and metabolites that have a similar charge to hydrodynamic radii to peptides, moving more quickly relative to their mass than the ions in trend-line 1, and include the halogenated compounds jamaicamide B (**1**) and jamaicamide A (**2**). However, trend-line 3 contained the halogenated and cyclic compound, hectochlorin (**3**), while trend-line 4 contained doubly charged ions. Similar results were obtained from the crude extract of *L. majuscula* 3L, however in this case the approach yielded three clear trend-lines (Fig. 1C). Trend-line 1 contains the majority of the metabolites observed, including the linear, non-halogenated compound curacin A (**4**), while trend-line 2 contained barbamide (**5**), a highly halogenated compound containing three chlorine atoms, as well as several putative undescribed analogs of the molecule. Trend-line 3 contained doubly charged ions (Fig. 1D). Thus, it is possible to separate out compounds with varying levels of halogenations as well as those that are cyclic or linear in overall construction.

Live intact filaments of three different strains of cyanobacteria, *L. majuscula* JHB, *L. majuscula* 3L and a recently collected *L. majuscula* from Papua New Guinea (PNG), were removed from culture, washed in water, airbrushed with universal matrix on the target plate, and then subjected to MALDI imaging with the Synapt system from Waters Co. The resulting data was imported into BioMap

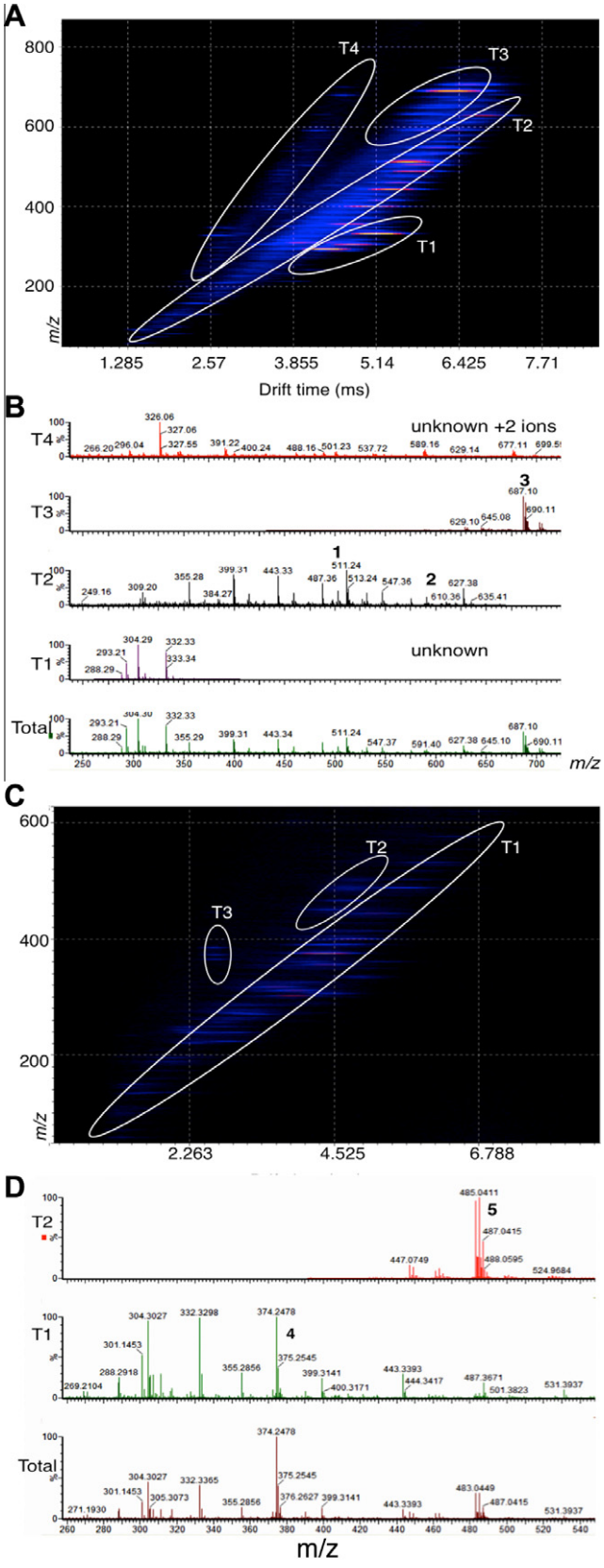


Figure 1. Ion mobility separation of known natural products from crude extracts of *Lyngbya majuscula*. Panels A and C show the main trend-lines (T3) associated with classes of molecules containing similar ion-mobility behavior in *L. majuscula* JHB and 3L, respectively. T4 and T3 contain doubly charged ions. (B) Spectra associated with the trend-lines in panel A (T1–T3)-T1 likely contains unidentified linear, nitrogen-containing hydrocarbons. T2 contains the linear halogenated natural products, jamaicamide B (**1**) and A (**2**) as well as the majority of small peptides and metabolites associated with this strain. T3 contains the cyclic, halogenated compound hectochlorin (**3**), and possibly related analogs. (D) Contains the spectra associated with panel C (T1 and T2), with trend-line 1 containing the linear natural product curacin A (**4**) as well as the majority of small peptides and metabolites associated with this strain. T2 contains the highly halogenated natural product barbamide and related analogs.

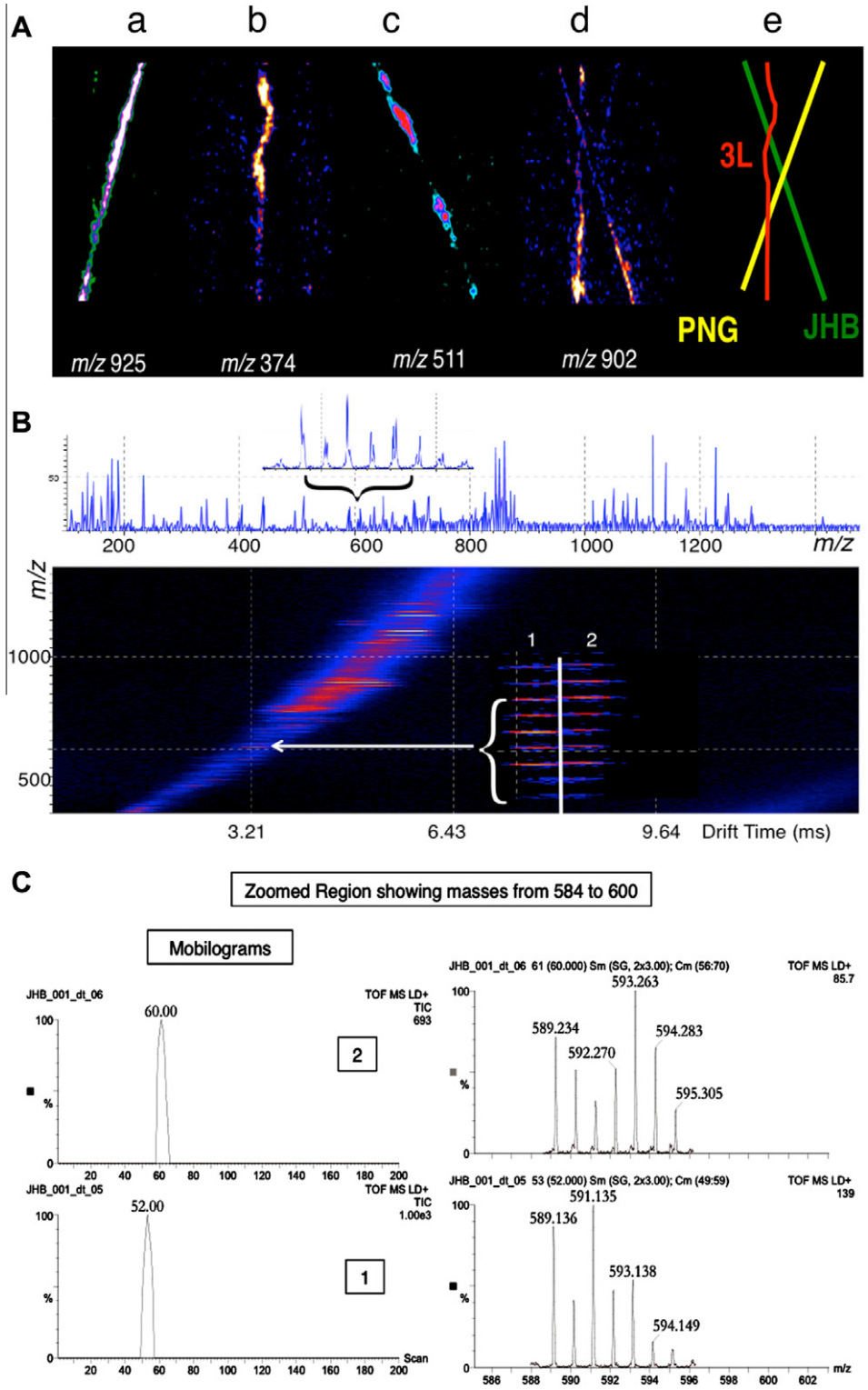


Figure 2. MALDI imaging of filaments of *L. majuscula* paired with ion-mobility. (A) Three filaments from different strains were overlaid and imaged by MALDI, arrangement is shown in (e). The localization of natural products associated with specific strains is confirmed in a–c, with an unknown metabolite in PNG (a), curacin A (**4**) in 3L (b), and jamaicamide A in (**1**) JHB (c). Part d shows an unknown compound, possibly a chlorophyll-derived metabolite common to all three strains. (B) Top, MALDI spectra from the same culture as the imaged filament, inset contains the region containing the isotopic profile for (**2**) overlapping another ion cluster. Bottom, ion mobility drift time analysis for this sample, inset shows the same jamaicamide A region and the two different ion mobility trends associated with the overlapping isotopic clusters. (C) Analysis of the same two mobilograms (drift times) associated with trend-lines 1 and 2 in the panel above. The peak at 60 (top left) is associated with the spectra on the top right, and is probably derived from matrix and pheobarbide. The peak at 52 (bottom left) is associated with the spectra on the bottom right, and matches the mass and isotopic profile for the halogenated compound jamaicamide A.

(Novartis) software and analyzed. Over a dozen metabolites associated with these filaments were observed; some of the *m*/*z* values were present in all three species while others were only found in specific strains. In Fig. 2A, the *m*/*z* 511 ion, which corresponded to the sodium adduct of the neurotoxin jamaicamide B (**1**) (expected 511.242 $[M+Na]^+$, observed 511.240), was found associated with the JHB strain, while the *m*/*z* 374 signal corresponded to the anticancer agent curacin A (**4**) previously isolated from the 3L strain (expected 374.252 $[M+H]^+$, observed 374.248). Finally, an unknown ion with a mass at *m*/*z* 925 corresponded to the less well investigated PNG strain. Each of these data was localized to the single producing organism and showed very few background signals. In addition, the spatial resolution of the image was generated by an oversampled rastering of the laser, resulting in 50 μm resolution, the highest spatially resolved MALDI images yet obtained for any cyanobacterium. Therefore, imaging via ion mobility MS provides a very rich data set from both a data quality and resolution perspective. Additionally, the depth of the data set obtained by ion mobility is an extra dimension of these experiments.

The above MALDI imaging experiments were run in conjunction with ion-mobility using whole-cell preparations; thus, it was possible to generate ion mobility trend-lines for the JHB experiment (Fig. 2B). Because the carrier gas was nitrogen in this experiment, the separation wasn't as robust as in the ESI based experiments which used argon. Nonetheless, this experiment yielded separation of ions with similar masses. In whole cell and MALDI imaging preparations on this instrument, the *m*/*z* region between 587 and 593 in *L. majuscula* JHB is composed of overlapping isotope clusters from a breakdown product of chlorophyll (pheobarbide *a*, *m*/*z* 592.1 $[M+H]^+$) and other unknown, possibly matrix-associated signals, as well as that of jamaicamide A (**2**) (*m*/*z* 589.152 $[M+Na]^+$), a neurotoxic and ichthyotoxic metabolite containing both a chlorine and a bromine atom. While the average *m*/*z* signal (Fig. 2B top) shows overlapping isotopes for these compounds, a closer inspection of the ion mobility component (inset, Fig. 2B bottom) reveals a robust separation of these isobaric molecules. In other words it was possible to visualize two molecules, one halogenated and one non-halogenated, something impossible to do without ion mobility separation. When these ion mobility signals (labeled **1** and **2**) are visualized independently (Fig. 2C), the distinct isotopic signals for all three compounds are clearly resolved, with signal **1** containing the multiply halogenated jamaicamide A and signal **2** containing the pheobarbide and the unknown compound, the isotopic distributions are highly consistent with the expected ratios for the known molecules. These results further highlight the ability of this technology to simplify and expedite the analysis and dereplication of complex natural product samples.

In conclusion, the identification and dereplication of natural products, especially from crude extracts deriving from complex marine assemblages, is a difficult endeavor but one which can be effectively investigated using modern soft-ionization mass spectrometry techniques. However, in most cases, the complexity of these samples remains challenging because spectral signals can overlap, increasing the effort required to identify and dereplicate the underlying compounds. In the proof-of-principle experiments reported here, the addition of ion-mobility to modern ESI and MALDI approaches greatly simplified the parsing of crude extracts into different structural classes of molecules. Halogenated compounds are widely known to have enhanced bioactivity and lipid solubility and are thus attractive as lead compounds.[16] Similarly, complex cyclic compounds are indicative of specialized and highly specific three dimensional topologies that derive from interesting biosynthetic mechanisms. The ion-mobility mass spectrometry experiments reported were consistently able to separate out different structural classes of metabolites, including known halogenated and possibly cyclic ones, as well as identifying unknown analogs or new compounds, from both whole cell and crude extracts of different strains of the natural product rich marine organism *L. majuscula*. That halogenated compounds can be separated by ion mobility has been shown with model compounds[17,18] but never before from extracts of organisms and therefore demonstrate the enormous capacity of ion mobility in natural product research. Thus, we conclude that this new method in mass spectrometry could greatly simplify the discovery and de-replication of new bioactive lead compounds in natural product screening and drug discovery programs.

3. Experimental section

3.1. Strain collection and cultures

L. majuscula strain JHB was originally collected in Hector's Bay, Jamaica in 2002. *L. majuscula* 3L was collected in Curacao, Netherlands Antilles in 1996, and *L. majuscula* PNG was collected in Milne Bay Papua New Guinea in 2005. Pan and Erlenmeyer flask cultures of each strain were maintained at Scripps Institution of Oceanography, UCSD in SW BG-11 media[15] at 29 °C, under 16 h light/8 h dark cycles at approximately 5 $\mu E\ m^{-2}\ s^{-1}$.

3.2. Preparation of cyanobacterial extracts for ion mobility

Approximately 2.5 g (wet weight) of fresh biomass, for both *L. majuscula* JHB and 3L was placed in a 100 μl beaker and soaked 20 ml of 2:1 DCM/MeOH for 30 min on hot plate (low setting). The biomass and liquid were then filtered with cheesecloth and filter paper. The biomass was returned to the beaker and the process was repeated five times, with the resulting liquid extraction combined after each step.

The resulting extract was placed in a rotary evaporator until dry. The sample was redissolved in ACN and to concentrate the sample, the extract was loaded onto a C-18 Sep-Pak column pretreated with ACN. Using a round bottom flask as a collection vessel, the extract was eluted with ACN, followed by methanol until clear. This concentrated extract, free of cellular debris, was placed in a rotary evaporator until dry. The dried extract was transferred to a glass vial using ether, dried under nitrogen, and placed in a hi-vacuum overnight.

3.3. Analysis of the extract using ion mobility mass spectrometry

Samples were dissolved in 50:50 H_2O:MeOH 0.1% formic acid and infused using nanospray needles. The samples were run on the Waters ESI SYNAPT ™HDMS™. The instrument was calibrated externally. Calibration was performed with NaI from *m*/*z* 50 to 2000 The data was acquired in mobility mode. Capillary voltage = 1.0 V cone voltage = 10–25 V with the IMS wave height ramped from 5–10 V. The spectra were visualized with Masslynx, the software on the Synapt instrument.

3.4. MALDI ion mobility imaging and dried-droplet experiments

Tp perform an imaging mass spectrometry experiment, 1 g wet weight cultures of each of the three strains were removed from the parent culture, placed in 50 ml culture flasks and flown to the Waters facility in Milford Massachusetts for MALDI imaging. For imaging, single filaments of each of three *L. majuscula* strains (JHB, 3L, and PNG) were rinsed in a drop of deionized water and laid down on the glass microscope slide so that a cross was made

(Fig. 1). A MALDI matrix solution (70 mg/ml Universal MALDI matrix from Sigma) in 80% acetonitrile, 19.8 % MilliQ H_20, and 0.2 % μl TFA) was prepared and the sample was coated with this matrix using an airbrush. Fifty passes of five cycles were applied such that an uniform coating was deposited on the slide. The sample was allowed to dry between in each pass in order to avoid delocalization of the ions.

3.5. Preparation of microbial samples for dried droplet profiling

For each strain ~1 μg of biomass was rinsed in deionized water and placed in an 1.5 ml eppendorf tube using ethanol-cleaned tweezers. MALDI matrix solution (as described above) was added to the wells at 1.0 μl per 0.1 μg of biomass. After 20–30 s, 1 μl of this matrix extract was deposited on a 96 well target plate and allowed to dry prior to running.

3.6. Instrument parameters for imaging and dried droplet profiling

Both the imaging and profiling experiments were performed on a Waters® MALDI SYNAPT ™HDMS™. During the experiments the following general instrument parameters were used. Ionization mode: positive ion laser type: Nd:YAG repetition rate: 200 Hz acquisition range: varied depending on sample being analyzed, from *m*/*z* 200 to 2000 Da. Collision energy: trap CE = 6.0 eV. *Instrument calibration*: calibration mixture of lPEG oligomers (PEG 200, 400, 600, 800, 1000, 2000, and 3000) were prepared at concentrations of 10 mg/ml in water. Sodium iodide solution (NaI) was prepared at 2 mg/ml in 50:50 acetonitrile:water. A mixture was prepared by mixing 10 μl of each PEG oligomer with 10 μl of the NaI solution. A 10-fold dilution was made of this mixture into water and this was then mixed 1:1 with matrix and 1 μl spotted. Calibration data was obtained from 100 to 2000 Da in IMS mode. The imaging data was visualized using BioMap (http://www.maldi-msi.org/)

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