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Data-independent Microbial Metabolomics with Ambient Ionization Mass Spectrometry

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Abstract

Atmospheric ionization methods are ideally suited for prolonged MS/MS analysis. Data-independent MS/MS is a complementary technique for analysis of biological samples as compared to data-dependent analysis. Here, we pair data-independent MS/MS with the ambient ionization method nanospray desorption electrospray ionization (nanoDESI) for untargeted analysis of bacterial metabolites. Proof-of-principle data and analysis are illustrated by sampling *Bacillus subtilis* and *Pseudomonas aeruginosa* directly from Petri dishes. We found that this technique enables facile comparisons between strains via MS and MS/MS plots which can be translated to chemically informative molecular maps through MS/MS networking. The development of novel techniques to characterize microbial metabolites allows rapid and efficient analysis of metabolic exchange factors. This is motivated by our desire to develop novel techniques to explore the role of interspecies interactions in the environment, health, and disease. This is a contribution to honor Professor Catherine C. Fenselau in receiving the prestigious ASMS Award for a Distinguished Contribution in Mass Spectrometry for her pioneering work on microbial mass spectrometry.

Keywords

Ambient mass spectrometry; data-independent MS/MS; FTICR-MS; MALDI-TOF IMS; metabolomics; microbiology; MS/MS molecular networking; nanoDESI

Introduction

We are part of a complex multi-species community, as illustrated in the recently published human microbiome project [1]. Nature whether soil, water, or air is saturated with complex microbial communities. Microbes produce large numbers of metabolic exchange factors, molecules that are optimized to be biologically active, to shape their ecological niches, thus exhibiting multicellular behavior in both microbes and host or environment. These exchange factors also have significant impact on our food supplies and healthcare. Two examples of such factors that originate from microbes are penicillin and the chemotherapeutic ET-743 [2]. However our understanding of the molecular transactions that define these systems is limited, in particular because previous studies of microorganisms have too often been

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focused on identifying a single “key” factor as being responsible for a given biological phenomenon, due in part to limits in technologies which restrict the number of analytes that can easily be monitored at once [3].

Nature is unaware that the scientists who study glycans, lipids, metabolites, natural products, and proteins consider their fields to be quite separate. We believe that monitoring as many signals as possible in an unbiased manner with appropriate informatics may be the key to understanding complex biological phenomena. An excellent example of such systems would *S. aureus*, where redundant signaling, resistance, and pathogenic factors all work together to effect virulence [4]. Key challenges we face in the field of biological mass spectrometry include decoding the molecular transactions within and/or between cells [5]. Further challenges exist in the field of bioinformatics as it is difficult to intuitively analyze systemic change. Here we forward a workflow that enables the systematic evaluation of microbial metabolic exchange factors using data-independent acquisition methods with atmospheric ionization mass spectrometry.

Inspired by proteomic data-independent approaches for untargeted analysis [7-9], we combined ambient ionization with data-independent MS/MS analysis for untargeted analysis of microbes. The data-independent analysis using nanoDESI (Figure 1A-B) in a series of m/z 3 isolation windows in the range m/z 50-2,000 (Figure 1E) and molecular networking (Figure 1C) contrasts to data-dependent approaches, which only sample abundant precursors (Figure 1D). While the lack of chromatographic separation may limit the total number of analytes detected due to ion suppression and more abundant isomers masking less abundant ones, we still can detect hundreds of unique metabolites from each sample. A benefit of data-independent MS/MS analysis is that each dataset forms a single ion map (Figure 1E) which can be directly compared across samples by superimposition. For example, different strains, spatial locations within a sample, or time-points can be compared in this manner. The analogous data from a data-dependent MS/MS experiment will not contain the same spectra, and thus direct comparisons of raw data are not straightforward. We conceive of our MS/MS datasets in two views: 1) a raw mass spectral view of the intensity (**Z-coordinate**), versus MS/MS spectra (**Y-coordinate**) and isolation window (**X-coordinate**), and 2) processed MS/MS networks, representing spectral similarity as our proxy for chemical similarity (Figure 1C, E).

An unexpected consequence of implementing this method in our laboratory was that we were generating MS/MS datasets that were difficult to process with conventional metabolomics and proteomic algorithms. With the reduced extraction bias of data-independent MS/MS acquisition, we were simultaneously observing diverse molecules including lipids, peptides, and sugars. Therefore, we developed MS/MS molecular networking, a global data analysis and visualization approach to organize and explore our MS/MS results [6]. In MS/MS molecular networking, MS/MS spectra are compared, computationally processed, and assembled into a molecular MS/MS network based on spectral similarity (Figure 1C) within the free software Cytoscape [10]. Spectra are illustrated as a series of nodes with associated metadata such as parent mass, source organism, and scan number. Spectral similarity is depicted as edges connecting the nodes, thus delineating a putative chemical similarity. Such organization of molecules enables facile compound identification, the slow/challenging step in metabolome analysis, to be propagated to related chemical species [11].

Mass spectrometry has a significant capacity to differentiate between different microorganisms—indeed such has been a key focus of Professor Fenselau who is honored in this issue [12]. The technique of MALDI-TOF profiling with library searching has proven to be a viable approach for identifying microbes in the clinical microbiology laboratory [13].

High-throughput profiling techniques are thought to primarily derive signals from abundant, ionizable low molecular weight proteins. However, these approaches may not be able to differentiate the same microbes present under different environmental stresses or growth conditions. Herein, we first examine the possibility that profiling metabolic exchange factors enables the differentiation of both phylogenetically similar and phylogenetically distant microbial strains in addition to the microbial metabolic exchange factors produced by these strains [14]. Microbial colonies of two strains of the ubiquitous Gram negative opportunistic pathogen *Pseudomonas aeruginosa* and two strains of Gram positive *Bacillus subtilis*, each of which produces a unique array of signature metabolic exchange factors (Figure 2, (1-4) and (5-7)), were selected as analytes for the ambient ionization data-independent approach.

For *P. aeruginosa* we investigated the classic laboratory strain PA01 originally isolated from a wound in Melbourne Australia PA01 [17], as well as the more pathogenic model PA14 selected from an isolate library for plant and animal virulence [18]. For *B. subtilis* we investigated the “domestic” strain PY79 [19] and the natural wild-type strain 3610 (Bacillus Genetic Stock Center Collection). All four of these strains have been investigated previously in our laboratory and are model gram negative/positive organisms used throughout laboratories in the world [15, 20]. Secondly, we utilized spectral networking to analyze microbial metabolite production over time. This technique also complements microbial imaging mass spectrometry (IMS)—where microbial colonies can be imaged by *m/z* directly from agar, revealing interspecies interactions and secreted metabolites. This work highlights the emerging strategy of trying to consider the sum total of data from a single sample—as inspired by the field of systems biology. Such strategies may offer new insight into the role of microbial communities in agriculture, diagnostics, and therapeutic discovery sciences.

Experimental

Materials

All solvents used in this study were LC-MS grade from Fisher. Authentic standards were purchased from Sigma, Fisher, or isolated from the native producer and fully characterized by 1D and 2D NMR.

Microbiology

Strains of *Pseudomonas aeruginosa* PA01 [17], *Pseudomonas aeruginosa* PA14 [18], *Bacillus subtilis* PY79 [19], and *Bacillus subtilis* 3610 (Bacillus Genetic Stock Center Collection) were first streaked on LB agar plates from frozen glycerol stocks. Specific *Bacillus* strains described were generously provided by the laboratory of K. Pogliano at UCSD. *Pseudomonas* strains described were generously provided by the laboratory of D. Hung at Harvard Medical School. As *Pseudomonas* is an opportunistic human pathogen, all procedures were conducted with Biosafety Level 2 precautions. Overnight cultures from single colonies were incubated in LB liquid medium at 37 °C, and 1 µL of inoculum was applied to ISP2 agar plates. ISP2 plates were poured from a single batch of medium, and were identical to plates used for the following experiments (Figures 3-5, S1-5). ISP2 agar plates were incubated at 37 °C, and withdrawn for nanoDESI MS/MS analysis at 12, 24, 36, and 48 hours. The colony was sampled midway between the colony center and colony perimeter. Briefly, samples subjected to nanoDESI analysis were analyzed with the majority of the Petri dish cover in place, permitting the capillaries access to the colonies, while protecting the environment from exposure. Samples are not volatilized during surface desorption, as solvent is eluted from the primary capillary at a low flow (1-10 µL/ min). Microbial colonies at the capillary junction immediately face 50% acetonitrile with 0.1% formic acid, which extracts and eventually saturates the contact surface. The organic and acid treated extract is then nano-sprayed from the capillary. Organic solvents mixed with

acid are bactericidal—indeed colonies will not grow on the portion of the plate analyzed after reincubation. However we recommend caution when performing nanoDESI, and never analyze sporulating pathogens such as *Aspergillus*. Matched plates were prepared for MALDI-TOF IMS.

NanoDESI FTICR-MS/MS

NanoDESI was performed on a Thermo LTQ-FT 6.4 T instrument using a modified Prosolia Omnispray DESI source with 2D automated stage [6]. 150 μm ID, 360 μm OD fused silica capillary tubing was used for the primary capillary and 75 μm ID, 150 μm OD fused silica capillary tubing secondary capillaries and solvent (60% acetonitrile/40% water/0.1% formic acid) was infused at 1.5 $\mu\text{L}/\text{min}$ with an applied voltage of 2 kV. Gas settings were all set to 0 arbitrary units with an inlet temperature of 250 $^{\circ}\text{C}$. The instrument scan cycle consisted of two segments. The first segment had a duration of 3 min during which three profile mode MS scans were cycled: m/z 100-350 at 25,000 resolution in the FT cell with three microscans and a max inject time of 2 s, m/z 300-1,000 at 50,000 resolution in the FT cell with three microscans and a max inject time of 2 s, and one full MS scan in the iontrap (IT) with 5 microscans from m/z 100-2,000 with 1 s max fill time. The second MS/MS segment was allowed to run for 397 scans (7-27 minutes), with a m/z 3 isolation window swept through the mass range by m/z 2 units (e.g 101, 103, 105, ... 889, 891). These scans consisted of 3 microscans with a maximum injection time of 2,000 ms, 25% normalized collision energy, 0.25 activation Q, and 0.05 s activation time. Two additional acquisitions were performed to cover the entire precursor mass range from m/z 50-2,000 for a total acquisition time of 30-90 minutes per sample. This can be substantially shortened if desired by reducing microscans or max inject time, at the expense of sensitivity for low abundance metabolites. MS/MS spectra were acquired from m/z 50-2,000, with the lower mass limit increased as required by the 1/3 rule and all were acquired in the IT detector. The instrument was calibrated as described by the manufacturer. Automatic gain control was utilized for all experiments.

MALDI-TOF IMS

Imaging mass spectrometry experiments were performed on a Bruker Autoflex Speed TOF/TOF instrument with the Bruker Daltonics FlexImaging 3.0 software suite. Mass calibration and tuning was performed with a Pepmix II standard solution (Bruker Daltonics) in the quadratic mode. For reflectron positive mode analysis the following settings were used: laser size medium, laser offset 45%, laser range 40%, laser power 70%, laser frequency 200 Hz, laser summed shots 128 total at 32 shots in 4 groups on random walk, mass range m/z 0 to 4,000, deflectron off, gain enhanced 14.3x 4GS/s, ionsource 1 19 kV, ionsource 2 16.7 kV, lens 8.2 kV, reflectron 1 21 kV, reflectron 2 9.65 kV, delay 150 nS. Imaging mass spectrometry sample preparation is described in detailed tutorial manner, and these procedures were followed in our experiments [21] This publication includes a video demonstration, as well as other useful data such as troubleshooting and potential challenges. Briefly, microbial colonies and surrounding agar were excised from the Petri dish with a metal spatula, and then transferred to a ground stainless steel MALDI target. Photographs were taken, and then samples were coated with Universal MALDI matrix using a 50 μm sieve with the “salt-shaker” method. Samples were then dried at 37 $^{\circ}\text{C}$ for 4-8 hours. Excess matrix was removed from the target with a damp tissue, and the loose matrix dust was removed with compressed air. Calibration mixture was spotted onto a piece of blank agar on the target.

Data analysis

Manual IMS data analysis was performed in FlexImaging 3.0 and nanoDESI and ESI analysis was performed in Thermo QualBrowser 2.0. The nanoDESI MS/MS plots were

generated using custom made scripts in Matlab by creating a table of MS/MS spectra from data-independent MS/MS datasets. When performing comparison or overlay of MS/MS plots, the MS/MS m/z values were considered with the nearest neighbor interpolation exploited. Data shown represent the peaks greater than the 95% quartile and are not normalized. Final figures containing results from Matlab and MS vendor software were assembled in Adobe Creative Suite 5.

Spectral networks computational analysis was performed on all MS/MS data collected from nanoDESI, including analysis of blank agar-only samples, as described with the following settings: PAIRS_MATCH_MODE=cosine, PAIRS_MIN_COSINE=0.7, MAX_MOD_MASS=300, MIN_RATIO=0.4, MIN_MATCHED_PEAKE=4 [6]. The networks were analyzed in the free and open-source Cytoscape suite (<http://cytoscape.org/>) as previously described [6]. Briefly, nodes and edges were loaded into Cytoscape from a text file with the “import network from table” function. Then metadata such as node parent mass, node class, node file source, were added with the “import node attribute from table” function. We then removed all network nodes corresponding to blank samples, such as solvent and agar (as in Figures 4, S3). The network layout was organized with the FM3 function, and node colors were mapped based on bacterial species. In all networks except agar control (Figure S1) nodes from solvent and agar controls were subtracted. The mass spectral data was explored in the Cytoscape browser, and correlations between spectra of interest were then validated based on manual inspection in QualBrowser.

Results and discussion

Data-independent vs. data-dependent MS approaches

Data-independent MS/MS methods are often deployed for the orthogonal goal of targeted analysis, as in pharmacokinetic studies [22] in which either a single MS/MS transition is detected (selected reaction monitoring) or series of transitions are cycled through (multiple reaction monitoring), or further scheduled over a chromatographic elution window over the course of a run (scheduled multiple reaction monitoring) where only a smaller subset of the entire mixture may be of interest. These MS/MS methods offer advantages in terms of increased reproducibility, but at the cost of reduced analyte capacity. Data-dependent MS/MS methods, often utilized in untargeted analysis of complex mixtures such as proteomics or metabolomics, use algorithms to select targets for fragmentation on the fly based on parameters such as peak intensity, charge-state, inclusion lists, or exclusion lists. Because precursor selection is a stochastic process, the resulting analysis cannot be accomplished in a systematic fashion.

The paradigm of data-dependent MS/MS is only used for untargeted analysis, and data-independent MS/MS for targeted analysis has recently been challenged with the development and applications of data-independent MS/MS methods for untargeted proteomic analysis. One option is to fragment many ions at the same time through “broad-band” CID [23-25]. This has been described in both the source, collision cell, and vendor-specific implementations are termed MS^e. Alternative data-independent MS/MS strategies scan through multiple target windows in repeated acquisitions to cover the entire mass range, increasing analyte depth at the cost of instrument time [7, 8]. A recent data-independent approach for untargeted LC-MS/MS proteomic analysis is termed SWATH-MS [9], during which a m/z 25 isolation window is swept through 32 possible masses covering a broad range of m/z 400-1200 on a chromatographic time-scale. Dereplication of mixed chimeric spectra from multiple precursor ions is facilitated by high-mass accuracy/resolution MS/MS. Advantages are reported in terms of specificity, reproducibility, and dynamic range as compared to data-dependent MS/MS when applied in proteomics. Because there is no

pre-separation of the molecules to be analyzed, and thus signal is constant over time, atmospheric ionization could benefit from data-independent analysis.

Ambient mass spectrometry techniques, such as DESI [26], DART [27], and others [28], are performed at atmospheric pressure with no sample preparation, and are changing the way the molecular details of biological surfaces are investigated. “Touch and go” atmospheric methods typically result in minimal sample perturbation and reduced extraction bias, and sometimes enable repeated analysis of living systems [28, 29]. The ambient ionization method nanospray desorption electrospray ionization (nanoDESI) combines a surface extraction using a liquid micro-junction with ESI ionization occurring at the secondary capillary distal to the droplet, and can be used for monitoring fragile surfaces of tissue sections and, in particular, microbial colonies grown on surfaces containing up to 95% water,[6, 30, 31] such as agar-based bacterial growth media (Figure 1A-B). Ambient ionization may be a desirable alternative to other techniques such as MALDI or ESI for microbial analysis primarily due to two factors: 1) ease of use, and 2) reduced extraction bias. In terms of ease of use, microbes can be analyzed directly from the Petri dish—this advantage may be underappreciated by experienced analytical chemists, but for microbiologists and other scientists becoming interested in mass spectrometry, simplicity is paramount. Reduced extraction bias is also preferred to detect glycans, lipids, metabolites, natural products, and proteins in a single sample. No desalting or chromatographic method can be expected to reasonably perform with this many molecular classes. Even MALDI, where untreated “salty” microbial analytes can be examined, typically requires different matrices for optimal performance for specific molecular classes. When analysis of diverse molecular classes is desired, ambient ionization techniques which require no desalting or sample preparation are worth investigating.

Evaluating day-to-day variability of data-independent MS/MS with spectral networking

To fulfill our aim to monitor changes in metabolic exchange profiles of microbial colonies, it was important to verify the reproducibility of data-independent nanoDESI over the time frame of the planned experiments. Fully understanding the degree of variation among replicate samples is necessary prior to evaluating samples where larger differences are expected. Agar media is a complex mixture containing complex polysaccharides (1), peptides, vitamins, and other undefined components. Our initial study evaluated identical ISP2 agar Petri dishes over four days, the proposed time frame we wanted to investigate our microbial samples, to assess performance of our analytical and computational workflow (Figure S2). Over 90% of MS/MS spectra were matched or linked by edges between all four time points highlighting the reproducibility and performance of our analytical and computational method as currently implemented (Supplemental, Figure S1). Four cases of networking were examined as case studies for the 10% of MS/MS spectra that were not matched as identical in molecular networking. Now that the rules are established for when the comparative analysis breaks down in our current workflow implantation, we investigated the results of data-independent nanoDESI MS/MS of two dissimilar microbes from different phyla: *B. subtilis* 3610 and *P. aeruginosa* PA01 (Figures 3-4).

The well-studied model organisms *B. subtilis* 3610 and *P. aeruginosa* PA01 were directly analyzed from solid media by atmospheric ionization with data-independent MS/MS. The FTICR-MS spectra of our two taxonomically distant bacteria reveals them, as expected, to be quite distinct at the molecular levels (Figure 3A). The MS/MS profiling data of the two organisms, as in the MS/MS ion maps (Figure 3B), are substantially different especially as compared to the agar control replicates (Figure S1). Indeed the profiling results are also supported when distinguishable features are targeted such as prominent metabolic exchange factors including phenazines (2), quinolones (3), and rhamnolipids (4) in PA01, as well as

surfactins (5) and SKF (6) in 3610. Clearly, data-independent MS/MS of microbes by ambient ionization allows for facile discrimination of different bacteria. The strength of this approach is further evident when molecular networking is applied to organize spectra based on MS/MS spectral similarity (Figure 4).

As captured by molecular networking, the organism profiles are even more clearly differentiated based on MS/MS analysis with <2% of nodes shared between samples, and almost complete segregation between species-specific clusters (Figure 4A). When examined in more detail for characterized compounds (2-6), it is revealed that precursor isolation windows are largely non-chimeric and that MS/MS spectra are distinct between species (Figure 4B-F). All *Pseudomonas* compounds (2-4) indicate a single predominant monoisotopic peak with a mass within 10 ppm of theoretical, and experimental MS/MS spectra that agree well with literature data [15]. Matching isolation window data from 3610 do not share similar MS or MS/MS as compared to the identified PA01 metabolites. Likewise, for 3610 surfactin (4) and SKF (5) MS and MS/MS data are shown with the spectra from PA01 at the same isolation window. Here the data are within 10 ppm of expected by MS the 1⁺ or 3⁺ charge state, with MS/MS spectra that agree well with literature [6]. These data confirm that data-independent nanoDESI MS/MS profiling of microbes provides distinct signatures for these different species at both the level of metabolite profiling as well as for specific identified metabolites of interest. By using a nanoDESI approach, we were able to rapidly characterize these differences with no sample preparation. The data-independent MS/MS allowed us to clearly compare between the two species at every MS/MS window. These encouraging results led us to test if this methodology could be used to visualize differences in metabolic exchange factors of related strains (Figure S3).

P. aeruginosa PA01 and PA14 were chosen as part of our test set since 92% of PA14 genome is present in PA01, and 96% of PA01 genome is present in PA14 [32]. Thus they represent highly similar organisms at the genetic level, although many of the key variable differences are due to regulatory and virulence associated genes. Similarly, the domestic strain *B. subtilis* PY79, which originates from a 3610 lineage, behaves differently than the non-domesticated 3610 strain [33] PY79 contains a frame shift in the *sfp* gene, resulting in a truncated transcript, which affects phosphopantetheinylation of biosynthetic proteins that produce metabolic exchange factors such as surfactin and plipastatin.

Despite the genetic similarity, *P. aeruginosa* PA01 and PA14 provide substantially different nanoDESI profiles (Figure S3). This is reflected in both MS (Figure S3A) and MS/MS analysis (Figure S3C). Even though the strains have the same genetic origin, differences are also observed between the strains of *B. subtilis* 3610 and PY79 (Figures S3B and S3D). When MS/MS data from the four species were mapped together, the vast majority of all nodes were different between organisms irrespective of the species. Therefore, this data suggests that data-independent MS/MS may be a useful technique for profiling even closely related microbial species.

The molecular segregation between these related strains is remarkable, given that the two pairs each share >90% similarity at the genetic level. Distinct separated nodes can be seen between the four species when four datasets are networked together (Figure S3E), with large overlaps between PA01 and PA14 as well as 3610 and PY79, but not across phyla. Indeed, previously identified compounds (2-6) could be identified again. Again, MS/MS molecular networking is a viable approach for visualizing and organizing large datasets.

To further explore the discriminatory powers of MS/MS networking of nanoDESI data-independent MS/MS, *B. subtilis* 3610 was analyzed over a 4-day time course for (Figures 5,

S4). It is known that 3610 produces a wide variety of metabolites in different environmental conditions [6, 16] In MS/MS mode, metabolites (**5-7**) can be consistently identified over time, with increasing or decreasing signal correlating with that observed by FTICR-MS (Figures S4B). We chose to further evaluate changes in plipastatin A (**7**) over time to illustrate the ability of nanoDESI with data-independent MS/MS to elucidate fine changes in molecular output for low-intensity precursors that may not be selected in data-dependent mode.

By FTICR-MS, we see a relatively low signal for the 2^+ ion that shifts over time from the monoisotopic peak at m/z 757 to the +2 Da monoisotopic peak at m/z 758. It is possible that this peak may not be selected for data-dependent acquisition, especially in all cases over 4/4 days. Further evaluation of the MS/MS data, which contain easily comparable spectra over the course of four days due to the data-independent acquisition mode, reveals more insight (Figure 5). In particular, for the $[M + H + K]^{2+}$ ion, the first loss of K^+ to provide the $[M + H]^+$ ion at m/z 1474 shifts to m/z 1476. This reflects a change in the side chain component of plipastatin, as these are molecules are known to exist in a variety of alkyl chain lengths, branch points, and oxidation states [34]. By observing this data over the identical and defined set of MS/MS windows collected over 4 days, we were able to clearly monitor this shift in forms of (**7**) even though chimeric MS/MS spectra were collected. To better understand the changes in metabolic output over time for compounds **5-7**, we utilized imaging mass spectrometry to see if localization might be responsible for the changes observed in metabolites over time (Figure S5).

MALDI-TOF IMS complements the compound identification and relative abundances determined by nanoDESI by providing a view of metabolite spatial localization (Figure S5). Interestingly, 3610 metabolic exchange factors display differential localization, which may partially account for the changes seen over time by nanoDESI. For example, surfactin (**5**) is dispersed over the entirety of the field imaged, SKF (**6**) is localized to the colony, and plipastatin (**7**) is excreted from the colony. The plipastatin (**7**) IMS result correlates well with the nanoDESI data, as plipastatin is an abundant metabolite produced by 3610. However, low levels of plipastatin were detected by nanoDESI (Figure S4), which can be rationalized by the molecule being secreted away from the colony where nanoDESI sampling occurred. One challenge of microbial MALDI-TOF IMS is that it is hard to compare samples run at different times due to variability in matrix addition and sample drying. This can be addressed by “staggering” experiment start times—however this is not always appropriate for a given experiment. However, with nanoDESI, due to the lack of sample preparation as compared to MALDI, we achieve very reproducible levels of overall signal intensity for samples over the course of four days (Figures S1, S4). Nonetheless, the opportunity to characterize microbes in both time and space could be a very useful strategy for understanding microbes and the complex environments they inhabit through the combined technologies of nanoDESI data-independent MS/MS and MALDI-TOF IMS. Indeed, by combining extended MS/MS analysis of chemical space over time with nanoDESI with IMS analysis, we can move closer towards our goal of holistically understanding biological systems—spatial systems (micro)biology.

Conclusions

In conclusion, we show that ambient ionization is ideally suited for data-independent acquisition methods, providing increased discriminatory power of the types of molecules that are present. Our experimental and computational methods provided reproducible performance of replicate samples based on manual qualitative inspection of the presence/absence of peaks and intensities from day-to-day. Proof of principle applications of this approach were explored, including the differentiation of *B. subtilis* and *P. aeruginosa* strains

based on their metabolic exchange factor profiles. This analytical method also proved useful studying subtle metabolite variation over time, elucidating key differences that are difficult to capture. This method of “in-time” analysis also complimented the orthogonal “in-space” technique of MALDI-TOF IMS. Fundamentally, organized acquisitions such as these will move mass spectrometry experiments towards those in the field of genomics and proteomics, where raw data can be easily searched against standardized databases for homology.

There are many future avenues for the investigation of microbial metabolic exchange factors with data-independent MS/MS. As ambient ionization gains utility, interfacing with data-independent methods will become more useful. Although this study has been focused on qualitative analysis as tool for organizing and analyzing entire datasets, it is possible to develop this technique as a tool for quantitative analysis. Other instrument types, such as Q-TOF MS/MS may allow for semi-quantitative or quantitative analysis which is more challenging in a trapping instrument with gain control. Other ambient ionization methods such as DART [27], LAESI [35], and DESI MS/MS [26], to name a few [28], may also benefit from data-independent approaches. The depth of analyte analysis could be expanded, particularly for structurally dissimilar isobars, by applying FAIMS as a front-end gas-phase filter [36].

Once analysis is quantitative, this approach can be employed as a discriminatory classifier or identification strategy similar to MALDI Biotyper (Bruker Daltonics). MS/MS datasets can be evaluated with hierarchical clustering to generate dendrograms, analogous to 16S rRNA phylogeny trees. However, interpretation of these results has proven to be much more challenging. Perhaps once a greater range of species and growth conditions are tested, this approach can be used to determine virulent strains or resistance mechanisms based on an altered profile of secreted and/or retained metabolites [37].

We are currently developing our molecular networking approach as a publically available remote web service to facilitate access by other laboratories. The real applicability of the combination of ambient ionization and data-independent analysis may come from direct analysis of uncultured microbes from biofilms, soil or other environmental sources and will find uses in agricultural, diagnostic, and therapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Data-independent nanoDESI MS/MS is a complementary technique for analysis of microbes as compared to data-dependent analysis

A. NanoDESI FTICR-MS of microbial colonies. **B.** Zoom illustrating nanoDESI contact with microbial colony surface through a liquid micro-junction. **C.** Spectral networking representing spectral similarity as a proxy for chemical similarity. MS/MS datasets are collected, scored pair-wise for spectral similarity, and then spectral networks are generated illustrating MS/MS spectral similarity [6]. Each node represents one consensus MS/MS spectrum per precursor ion, and edges indicate spectral similarity. **D.** Data-dependent MS/MS mock sample dataset. The MS spectrum is presented as intensity versus spectra in m/z . The MS/MS ion map is presented as intensity versus MS/MS spectra in m/z versus MS/MS isolation window in m/z . In this example ten ions pass the criteria for data-dependent MS/MS acquisition. **E.** Data-independent MS/MS mock sample dataset. The MS spectrum is presented as intensity versus spectra in m/z . The MS/MS ion map is presented as intensity versus MS/MS spectra in m/z versus MS/MS isolation window in m/z . In this example, the

range is split into 23 isolation windows and ions are selected regardless of precursor ion characteristics. These data can be easily overlaid for facile visual or bioinformatics comparisons of entire datasets. Comparisons of different datasets are facilitated by data-independent MS/MS acquisition, as the set of precursor masses selected will be identical.

Figure 2. Matrix and microbial metabolic exchange factors described in this paper

All compounds were identified by accurate precursor mass, MS/MS with manual validation, and comparison to reference data (1-4) [15] (5) [16], and (6-7) [6]. Stereochemistry is not displayed as mass spectrometry does not capture this information in a MS/MS spectrum.

Figure 3. Data-independent nanoDESI MS/MS of two different bacteria

Pseudomonas aeruginosa PA01 is orange, *Bacillus subtilis* 3610 is red, and overlap is in black. Vertical lines represent compounds **A.** NanoDESI FTICR-MS. Intensity is displayed on the Y-axis, versus mass spectra from m/z 100-2,000. **B.** NanoDESI data-independent IT-MS/MS. MS/MS absolute intensity is displayed as color saturation, MS/MS spectra from m/z 100-2,000 are displayed on the Y-axis, and MS precursor isolation window is displayed on the X-axis for m/z 100-2,000.

Figure 4. Data-independent nanoDESI MS/MS of two different bacteria
Pseudomonas aeruginosa PA01 is orange, *Bacillus subtilis* 3610 is red, and overlap is in black. **A.** Networking of MS/MS spectra of both bacteria considered jointly. **B-F.** MS/MS spectra of signature metabolites. These spectra display the vertical lines in Figure 3. Compound names, detected ion, and FTICR-MS precursor mass error are noted. NanoDESI FTICR-MS spectra are presented over the width of the isolation window as absolute intensity on the Y-axis versus m/z on the X-axis. NanoDESI IT-MS/MS spectra are in absolute intensity on the Y-axis versus m/z on the X-axis.

Figure 5. Data-independent nanoDESI MS/MS time-course analysis and comparison with imaging mass spectrometry

Bacillus subtilis 3610 is displayed with day 1 in red, day 2 is orange, day 3 in green, day 4 in blue. **A.** NanoDESI FTICR-MS of (7). Intensity is displayed versus MS spectra in m/z over the selected ionization window. Notice the species at m/z 757 (7A) and m/z 758 (7B) are in the 2^+ charge state, corresponding to m/z 2 difference. **B.** NanoDESI data-independent MS/MS of (7). Intensity is displayed versus MS/MS m/z with selected peaks labeled. Changes of $m/z + 2$ are apparent for selected fragment ions in the 1^+ charge state over time.