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Mass spectrometry tools and workflows for revealing microbial chemistry

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Abstract

Since the time Van Leeuwenhoek was able to observe microbes through a microscope, an innovation that led to the birth of the field of microbiology, we have aimed to understand how microorganisms function, interact and communicate. The exciting progress in the development of analytical technologies and workflows has demonstrated mass spectrometry as very powerful technique for the interrogation of microbiology at the molecular level. In this review, we aim to highlight the available and emerging tools in mass spectrometry for microbial analysis by overviewing the methods and workflow advances for taxonomic identification, microbial interaction, dereplication and drug discovery. We emphasize their potential for future development, point out unsolved problems and future directions that would aid in the analysis of the chemistry produced by microbes.

1. Introduction

The study of microbes as the basic form of life and their evolution holds key answers to some of the most fascinating questions of our being, where the chemistry acts as the building blocks of our existence. For the past few decades, mass spectrometry (MS) has evolved tremendously and widely enhanced our understanding of microbial chemistry^{1, 2}. MS based analytical methods have been integrated in microbiology research from the identification of microorganisms in environmental or clinical samples; the study of their interactions and communication with their surroundings; to the pioneering field of biologically active small molecule discovery that defines the current era of medicine.

The adaptation of MS based analytical methods to routine lab use has opened new horizons to the diverse scientific microbiology communities. The scientific literature demonstrating the useful capacity of MS in microbiology had increased from a few publications in the late 60's - early 70's to over 1100 publications per year in the years 2012 – 2014, (PubMed search with “mass spectrometry microbiology”). The use of these terms together in the literature has grown exponentially, and we believe will continue over the next decade (Fig 1).

In general, mass spectrometers consist of three functional units: 1) an ionization source, which ionizes and transfers analyte ions into the gas phase; 2) a mass analyzer, which separates ions by their mass-to-charge ratio (m/z); 3) and detection device that observes and

captures ions^{3, 4}. The analysis relies on the “weight” of ionized molecules (m/z). High resolution instruments enable the elemental composition to be determined; some MS instruments can measure the mass of small molecules to within a mass of a single electron^{1, 5}. The output of a mass spectrometer gives a specific fingerprint that can be defined as a chemotype for particular sample or microbial taxa. Further fragmentation of the ions, known as MS/MS or tandem MS, can give a comprehensive amount of information on the chemical structure and may also be used to suggest the relevant biosynthetic pathways for novel natural products (NPs). The progress in mass resolution and accuracy combined with development of user friendly instruments offers applications for diverse arenas of microbial chemical analysis^{1, 5}.

With the 100 years anniversary for the development of MS, many publications reviewed the advances and different methods of MS^{1, 6–14}. Therefore, in this review we aim to highlight the utility and future potential of MS in the field of microbiology. It should be noted that this review will not be comprehensive in terms of all the methods that have been described for microbial mass spectrometric analysis, rather it will focus on the applications that enable the analysis of specialized metabolites¹⁵ involved in metabolic exchange and that have general utility for medical and other biotechnological applications. The reader is strongly encouraged to read the primary papers on any of the methods and applications described in this paper if they are interested in the details of such methods.

2. Mass spectrometry based microbial classification and identification

Microbial classification and identification are required in many different aspects of everyday life such as food analysis, agriculture and ecological research. Rapid and reliable microbe identification, especially for pathogens, is crucial in medicine and clinical diagnosis for prescribing the most efficient treatment. The traditional methods for microbial identification are based on isolation, analysis of morphological characteristics, biochemical assays and molecular analysis such as sequencing that are often time consuming and laborious¹⁶. Mostly, methods such as enzyme-linked immunosorbent assay (ELISA) are often only designed for specific bacterial species¹⁷. These have stimulated interest in the development of alternative microbial identification methods^{18–21}. Chemotaxonomy offers an alternative approach, in which classification of microbial strains is based upon the analytical measurement of chemical constituents, either as unique biomarkers or as contributors to distinctive ratios of chemical components e.g. specialized metabolites, proteins, carbohydrates, lipids, and nucleic acids^{20, 22–24}. Mass spectrometry can be used as a chemotaxonomic measure by utilizing MS data from known microbes as reference data. For such analysis MS libraries are generated at the genus, species and strain levels (Fig 2, 3). Different mass spectrometry platforms that can be used to generate MS based chemotaxonomic libraries are covered hereafter (see below).

GC/MS

Over 300 fatty acids and related molecules are found in bacteria²⁵. The wealth of information contained in these compounds is both in the qualitative differences (usually at genus level) and relative abundances (commonly at species level) where the composition can

be impacted by culturing conditions²⁶. As a result, fatty acid inventories have become a well-established method for bacterial chemotaxonomic identification. Analysis of modified bacterial fatty acids derivatized to Fatty Acids Methyl Esters using a Gas Chromatograph mass spectrometer (GC-FAME) enables the classification and identification of bacteria at the genus and specie level^{20, 27}. It has been suggested that branched chain fatty acids are more common in many Gram-positive bacteria, while Gram-negative bacteria are composed of predominately straight chain fatty acids. Such an intriguing observation is currently remains to be supported by large scale experiments, but is an observation that could influence a physician's choice of recommended course of antibiotic treatment^{25, 28}

Identification of FAME with this system is accomplished with measurements of retention time, which is the time for a specific molecule to pass through the GC column to give a unique pattern of fatty acid for specific microbes. The analysis software (e.g. Sherlock® Analysis Software, MIDI Systems, Inc.) uses a pattern recognition algorithm to match the unknown FAME profile to the stored library entries for identification based on similarity index (SI) score of matched spectra. The commercial Sherlock libraries are comprised of nearly 2,000 microbial species, including 700 environmental aerobic species, 620 anaerobic species, and 200 species of yeasts²⁸. Although this system requires microbial culturing followed by a standard extraction procedure and chemical derivatization, the analysis itself only takes a few minutes^{3, 20, 29, 30}.

MALDI-TOF-MS

Although FAME is a powerful technique, matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) has become the prominent method for microbial identification owing to its speed, simplicity and recent FDA clinical diagnostic approval^{3, 20, 31}. The differentiation of bacteria by MALDI-TOF is largely based on abundant protein patterns. Such protein profiling methods were initially reported by measuring abundant proteins from bacterial cell lysates³². In 1996, it was shown that this soft ionization technique could be performed directly on whole intact bacteria^{23, 32}. Using direct analysis, the majority of proteins that can be detected are ribosomal proteins. Such proteins are detected with relative ease due to their smaller size and abundance. Since the amino acids sequences of ribosomal proteins are sufficiently conserved at the genus and sometimes even specie level, slight variations in the sequence gives rise to a unique pattern that can -at times- distinguish microbes at the strain level^{17, 33, 34}. Although slight variations to the workflow may exist, a common method that is utilized is to take a small amount of intact microbes grown on an agar surface of a Petri dish and transfer these cells to a stainless steel target plate. Once a bacterial sample is transferred, a solution with matrix is added (such as alpha-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid) and left to air dry. The addition of the organic matrix to the biomolecules results in co-crystallization on the target MALDI plate. The matrix selected has great impact on the intensities of the detected molecules and therefore standardization of the protocol for clinical use is very important. The sample is then analyzed by MALDI-TOF where a laser desorbs the analytes present in the matrix and then subjected to MS analysis. Each spectrum desorbed from the sample surface corresponds to a specific molecular fingerprint of the analyzed microbe (Fig 2.),^{3, 16, 19, 23, 29, 33}.

MALDI-TOF-MS can detect a large number of proteins simultaneously and is able to discriminate between closely related bacterial genera and oftentimes even species^{3, 19, 35, 36}. Recent studies also confirm that MALDI-TOF-MS used in clinical medicine is capable of identifying various fungal genera as well as black yeasts, which are relatively more difficult to identify, down to the species level^{37, 38}. Spectra obtained from whole cells/spores are reproducible under defined growth conditions. Because the patterns are reproducible, it became possible to compare unknown samples with a database via data analysis algorithms that enable the automatic detection, indexing and statistical comparisons of the matching patterns. Currently, several MALDI based identification platforms are available: BioTyper (Bruker Daltonics), Vitex and SARAMIS (bioMérieux), and Andromas (Andromas SAS) with success rates ranging 94–100% identification for routine lab isolates^{3, 19, 20, 39, 40}. The Bruker and bioMérieux platforms are approved for clinical use in both Europe and the US³¹. The benefits of this approach include straightforward and low cost sample preparation, fast analysis times and affordable requirements. A diagnostic lab or hospital can recover the instrument's cost in about 50,000 runs. Its ease-of-use and higher-throughput compared to sequence based methods makes this approach a preeminent method for microbial diagnostic work. The time reported for sample analysis by MALDI MS ranges between 5–8.5 minutes per sample, and the cost is 10–20 times cheaper than other conventional automated platforms^{20, 39, 41}. It can be anticipated that additional clinically approved identification platforms will be developed over the next decade.

REIMS and Paper Spray

An emerging method for prompt microbial identification is the Rapid Evaporative Ionization Mass Spectrometry (REIMS). It was recently introduced to discriminate a small number of clinical bacterial isolates based on their MS fingerprints at the family, genus and species levels. In REIMS, standard electrosurgical methods convert sample components into gas-phase ionic species amenable to MS analysis⁴². Pair of forceps-like electrodes with an opening at the tip is used as the sampling probe. The forceps are connected with a transfer tube to embed the fluid line of the electrodes, and the inlet capillary pulls the aerosols to the adjacent mass spectrometer via the instrument's vacuum system⁴³, or in some cases aerosols are aspirated by the air jet pump⁴⁴. The sample is analyzed directly from the agar plate without any sample preparation by collecting the biomass with the forceps electrode, while closing an electric circuit. A small number of microbes previously identified by MALDI-TOF, were tested and subjected to microbial classification with REIMS. By focusing on the mass range 500–1900 m/z yielded the highest percentage of correct identification (96.7–100%) in a total time of 2–3 seconds per sample of intact bacteria, with phospholipids being a major source of the differentiation of the organisms in agreement to the observations for FAME. The data collected is further converted and analyzed by spectral profile alignment and principal component analysis (PCA). This easy-to-use method was recently tested on surgical samples and holds great potential in identification of virulent and antibiotic resistant bacterial strains in pathological samples^{43, 44}. The approach can discriminate the very diverse but small set of organisms tested. If the fidelity of identification continues to be valid and can be correlated with the thousands of 16S sequenced organisms, then this approach could likely become a viable alternative to the MALDI-TOF based identification system.

Other new MS applications aimed at rapid microbial characterization are continuously being developed. A proof-of-principle application using Paper Spray Mass Spectrometry (PS-MS) for microbial identification has been recently reported⁴⁵. Sample of bacteria colony is basically smeared onto a precut filter paper, wetted with solvent to allow the formation of droplets then subjected to MS analysis. The data is further analyzed by PCA, with early reports showing success rate of 98% for Gram-positive and 87% for Gram-negative bacteria at the specie level⁴⁵.

Implementation of the above tools offers exciting prospects in everyday clinical diagnostic labs and perhaps can find a wider utility in our fundamental understanding of microbial ecology and early phases of NPs based drug discovery. Nevertheless, these methods are not without limitations, the culturing is still the rate limiting step and differences in sample growth media can significantly affect the chemical composition of microbes^{26, 46}, although culturing effects can be managed by using standard protocols³⁹. Additionally, many microbes cannot be identified because they have not been adequately described and integrated into modern classifications. Currently, mostly fast growing bacteria are amenable to analysis. The databases usually do not include slow-growing or yet-to-be cultured microbes, and other microorganisms such as viruses, protozoans, algae and fungi have been largely ignored. In such cases, complimentary diagnostic methods (e.g. 16S/18S rDNA or viral sequencing) may still be required and such cases present unexplored opportunities in microbial mass spectrometry. Some approaches are trying to address such issues. An example is the PLEX-ID and PCR amplification of pathogen DNA, including viruses, with electrospray ionization mass spectrometry (PCR/ESI-MS) are designed to achieve rapid diagnosis from clinical samples. Such tools can eliminate the culturing step, where results may be acquired within a few hours. These methods are very sensitive and create a challenge in determining “true and false positives”, therefore still undergoing improvements to complement the current technologies⁴⁷. The strength of PLEX-ID is that it can be used to detect, in principle, any RNA or DNA from any organism through amplification. Abbot launched the technology and then quickly discontinued the product. The reason for removal from the market has been ascribed due to the high cost and that the machine is the size of a large motorcycle and therefore not compatible with clinical laboratories. However in its current implementation the PLEX-ID system is also not compatible with the amount of information modern RNA and DNA sequencing technologies provide. However, the excitement this tool generated when Abbott introduced it to the market shows that we are in need of technologies that can detect a wide range of microbes directly from biological samples, without having to culture first. Therefore, it is important to invest in the development of approaches and workflows that enable culture-independent microbial identification so that adequate therapeutic intervention can be provided in a timelier manner. When these technologies can be deployed, we will hear about the next generation PLEX-ID type systems.

3. The use of mass spectrometry to study microbial interactions

Microbial interactions exist everywhere in nature and play an essential role in maintaining the diversity by symbiotic, mutualistic, and pathogenic relationships^{48–55}. Microbes communicate and interact via dozens to hundreds of secreted specialized metabolites that

govern the behavior, survival, morphology, nutrient adaptation and differentiation of members of the community in a process called metabolic exchange^{15, 52, 56, 57}. Microbes utilize metabolic exchange to convey messages, share nutrients and sense the environment. Examples of metabolic exchange include quorum sensing, metal chelation (siderophores), biofilm formation and secretion of antibiotics. These interactions define the chemical ecology in intraspecies and interspecies of microbial communities^{52, 57–59}. The discovery of penicillin by Alexander Fleming in 1928 is a well-known example of an antagonistic form of metabolic exchange between the fungus *Penicillium notatum* and *Staphylococcus aureus*. This discovery changed the world of medicine and initiated the interest into these significant interactions and the roles specialized metabolites play in nature^{15, 60}. The traditional methods for studying such interactions relied on indirect measurements with tedious bioactivity-directed fractionation assays to reach that single target molecule. On top of that, microbial communities rarely exist as a monoculture and contain high degree of complexity, employing many different classes of molecules necessary for their interactions^{58, 61}. Today's MS tools are beginning to enable the analysis of multiple molecules (chemotype) and correlate the observed chemistries to the relevant phenomenon or biological question (phenotype) from a complex sample^{56, 57, 62, 63}.

Revealing a hidden molecular world through MALDI IMS

While interactions have been of interest to the microbial community, the true nature of metabolic exchange remains largely hidden from the experimentalists view. MALDI imaging mass spectrometry (IMS) was introduced to visualize fundamental disease signatures from biological samples by detecting proteins and peptides directly from tissue sections^{64, 65}. In 2008, IMS was utilized in microbial associated chemistry and since, has been broadly used for various studies on microbial interactions to decipher the chemistry of interactions^{56, 63, 66–94}. Table 1 highlights the microbial interactions and short description of key conclusions that were uncovered using MALDI IMS. IMS techniques allow the two and three-dimensional (2D/3D) visualization of the spatial dispersal of molecules (e.g. proteins, peptides, sugars and other metabolites such as lipids etc.) in a wide molecular range (~300–5000 m/z) directly from microbial community of a complex biological sample^{68, 95, 96}. The workflow for performing microbial IMS was extensively described in a primer with step by step instructions and what to pay attention to when performing these experiments, as well as key challenges of the workflow⁹⁷. Alternative approaches for sample preparation continue to be developed and these continued developments will greatly enhance the depth and scope of microbial interactions that can be characterized^{77, 98}. There are five general steps involved in performing microbial imaging mass spectrometry: 1) culturing microbes on thin agar and excising it with colonies of interest onto a conductive support; 2) matrix application, uniform dry coat to saturate the sample (for ionization of molecules and sample adherence to the target plate); 3) dehydration of the samples; 4) data acquisition and 5) data analysis and interpretation⁹⁷. The molecular information is collected by MS and in order to give insight into the chemistries in relationships to phenotypes; this data is superimposed onto optical or other images. In a typical experiment using IMS, the total area of sample is subdivided into pixels (x and y raster spots) that are individually sampled by a laser. A single mass spectrum or the average of many mass spectra is collected for each pixel and is compiled together with the spatial coordinates so that the location of sampling is retained. The distribution of

specific ions can be organized visually as an image by adding a false color gradient to represent the intensity of particular ions^{2, 4, 63}. The end result of IMS is a 2D snapshot of the density and distribution of chemical substances present in a sample, without the need for labels, probes or pigments^{4, 99}. MALDI IMS had shown great potential not only for microbe-microbe interactions, but also interactions with higher host organisms such as plants, insects and animals^{2, 85, 87, 93, 99, 100}.

Although 2D MALDI IMS is capable of revealing a vast range of chemical signals in a spatial manner from the sample surface, the natural environment of a living organism has 3D architecture^{67, 101}. By applying 3D MALDI IMS, one can detect differences in metabolite distributions or discover media composition alterations that are not observed using 2D IMS. This capability to observe the diffusion of metabolites within the 3D spatial environment, as opposed to just on the surface, becomes important when studying microbial systems that develop sub-surface structures (e.g. vegetative hyphae)^{2, 67}.

While microbial IMS is a valuable tool and provides an unprecedented molecular vantage point for microbiology studies, opportunities to improve and expand these applications still exist: There are challenges in sample preparation that can damage the sample or instrument (i.e. flaking)⁹⁷; the need of matrix and the effect of homogeneity of the coating on sensitivity and spatial resolution⁷⁷; dehydration of the sample limits analysis to a single time point; data acquired is currently semi-quantitative⁷¹; the spatial resolution is about 20 μm , while many bacterial cells are 0.5–3 μm in size^{57, 95, 97, 98}. If needed, the spatial resolution can even be improved by laser beam focusing which was recently reported¹⁰², however has not yet applied for microbial IMS. Resolution may also be improved by using complementary methods such as Secondary Ion Mass Spectrometry (SIMS) imaging for high spatial resolution or even single cell analysis. The challenge is that SIMS requires a flat sample, which is less common for microbial interaction assays^{1, 95}.

Altogether, IMS techniques complement liquid chromatography (LC) MS based metabolomic approaches by enabling the preservation of the information with respect to the location of the molecules, yielding insight into the underlying biology and for determining the origin of observed molecules. It serves as a tool for generation of functional hypotheses based on visual phenotype of the chemistry behind biological processes^{62, 97}. A large share of microbial chemistry is triggered upon specific signals from their environment, meaning that some molecules can only be detected under distinct conditions or co-culture interaction of complex microbial assemblages. IMS allows the screening for such interactive chemistries by assisting in understanding this micro-language and the discovery of new bioactive chemicals. When appropriate, scaling up such interaction will enable the extraction and isolation of compounds for further structure elucidation by nuclear magnetic resonance (NMR) and other analytical techniques^{69, 78}.

Analysis of living microbial samples by ambient MS

Most MS ionization techniques employ vacuum for creating and maintaining ions, while ambient MS generates ions at atmospheric pressure. Therefore one can analyze microbes in open air usually with no sample preparation^{1, 104–106}. The Cooks lab has set the stage for the vision of what atmospheric ionization can accomplish when they launched Desorption

Electrospray Ionization (DESI) as an ionization source. The introduction of this technique is a major step along the road of being able to analyze any sample under atmospheric conditions, a goal that the Cook lab and many other labs are pushing towards. They continue to develop MS based approaches towards more accessible, portable and affordable MS technologies, aiming to analyze samples from their native natural environment with minimum intervention^{104, 107–109}. Several ambient methods have developed for diverse applications including: Direct Analysis in Real Time (DART), Desorption Atmospheric Pressure Chemical Ionization (DAPCI), Electrospray-associated Laser Desorption Ionization (ELDI), Laser-Ablation Electrospray Ionization (LAESI) and Liquid Extraction Surface Analysis (LESA)^{110, 111}. DESI, nanospray DESI (nanoDESI) and liquid microinjection surface sampling probe (LMJ-SSP) are some of the atmospheric ionization methods that have been used for microbial analysis and are further discussed^{95, 104, 111–114}.

DESI/nanoDESI/LMJ-SSP/REIMS

The ability to analyze the chemistry directly from microbial colonies has been a holy grail in microbiology¹¹⁵. Desorption Electrospray Ionization (DESI) was initially introduced in 2004 and started a revolution towards the development of atmospheric ionization that continues today. The key aspect of the development is that ambient ionization methods require no sample preparation, such as extractions, adding matrix etc. This is an exciting prospect for scientists to be able to directly monitor the chemistry of microorganisms. DESI generates charged droplets that are projected with gas jet onto a sample placed on a surface¹⁰⁵. The extracted analytes, such as proteins, peptides and small molecules, are directed into the adjacent mass spectrometer, enabling the analysis of native surface at the microbial community level^{95, 105, 116, 117}. DESI's spraying mechanism, however, causes secondary droplets to splash onto the surrounding area, while too high back pressure results in the creation of the holes in the agar^{118, 119}. This inspired the development of alternative approach to confine the liquid flow through probe capillaries, for example: nanoDESI, liquid microinjection surface sampling probe (LMJ-SSP) and such, transferring the analytes directly to the mass spectrometer^{120–123}.

In 2010, the Laskin lab reported a complementing method to DESI, called nanospray-DESI (nanoDESI)^{118, 124}. NanoDESI, eliminates the splashing and the use of nebulizing gas that is destructive of soft surfaces such as agar which is commonly used for microbial growth¹¹³. In nanoDESI, the analytes are desorbed by a solvent flowing from a primary capillary to self-aspirating secondary capillary and nano-electrosprayed into the mass spectrometer¹¹⁸. The small liquid bridge formed between the two capillaries gently extracts the analytes and enables spatially resolved analysis with less damage to a soft surface^{118, 124}. NanoDESI has now been reported for analyzing living microbial colonies and demonstrated to have 2D imaging capabilities for spatiotemporal metabolomics profile directly from a Petri dish^{113, 118, 124–126}. Because the advance of being able to monitor chemistries directly from microbial colonies under atmospheric conditions, many new methods are being adopted and introduced each year. Examples include the continuous flowprobe, that is based on the coaxial tube geometry liquid microinjection surface sampling probe (LMJ-SSP)^{121, 123}. LMJ-SSP overcomes some of the challenges of nanoDESI (e.g. capillary clogging). In the preliminary assessment of LMJ-SSP, 34 microorganisms were profiled on strains collected

worldwide including bacteria, filamentous fungi and yeasts¹²¹. Future advances are expected to evolve, improving the data acquisition that may further be displayed spatially based on x-y stages that correlate the coordinates to the 2D image similarly to MALDI IMS (Fig 4)^{67, 121}. Finally integration of microscopy with nanoDESI MS promises to become a valuable tool to map and understand microbial chemistry^{95, 113, 121, 127}.

Rapid Evaporative Ionization (REIMS), developed by the Takats lab, is another ambient mass spectrometry technique that has been mentioned in microbial classification and identification section above. This promising technique enables microbial analysis in real time with and with no sample preparation. A major advantage of this technique is that it requires no solvent flow. Since an analyte evaporates along with the excess of water molecules from the culturing media, the method avoids clogging problems. Aside from being able to identify mass spectral fingerprints that are specific to bacterial species⁴³, REIMS is capable of detecting NP's produced by living microbes, (Fig 5) which is demonstrated in the supplementary video (supplementary video). In order to show REIMS ability to detect natural products we tested the system in our own laboratory. Even with limited optimization, REIMS readily detected the bromoalterochromide NPs directly from a living *Pseudoalteromonas* spp. colony grown on a Petri dish¹²⁸.

Overall, ambient MS techniques open up new vistas for the studies of the chemistry associated with microbes. The absence-of-vacuum conditions in sample analysis enables the authenticity of *in vivo* study and the unveiling of new specialized metabolites and functional substances such as quorum sensing signaling molecules, small redox compounds, cell differentiation signals, colony communication factors and the discovery of new antibiotics and potential drugs.

4. Studying the microbial metabolome with mass spectrometry as a tool for natural product discovery

One of the key applications for microbial MS is the analysis and discovery of NPs. Microbial NPs are specialized metabolites that are genetically encoded and selected for by evolutionary pressures. They have been among the most successful sources for drugs and drug leads^{129–131}. Penicillin, vancomycin, rapamycin and lovastatin are examples of microbial NPs that are commonly used in the clinic. The recent advances in MS and IMS technologies described in this review, facilitate the study of microbial NPs by: 1) prioritizing and targeting compounds within a biological context or differential expression; 2) detection at trace concentrations; 3) rapid and high-throughput screening of the microbial metabolome; 4) providing elemental composition and structural information. These capabilities have assisted in expediting the discovery of new NPs with potential therapeutics, have helped microbial NPs regain their prominence among other sources of chemical structural diversity in drug discovery^{60, 129, 132–137}. In pursuit for new chemistries, microbes continue to be isolated from diverse and exotic terrestrial niches, as well as marine environments^{138–140}. The accentuation on microorganisms revealed a wealth of new chemistries, as well as the realization that many compounds previously isolated from macro-organisms, are in fact metabolic products of associated microbes instead^{138, 141, 142}. While

IMS methods, as discussed previously, have the advantage in visualizing the chemistry associated with microbial isolates and accentuating the focus towards target compounds, for complete structural elucidation additional analytical techniques are essential. Although NMR is generally required for final structure determination and stereochemical analysis, MS has an important advantage over NMR in sensitivity for instance that makes it the method of choice for first-pass compound detection and identification in medium- to high-throughput screening applications^{1, 2, 6, 9, 108, 143–145}. For any structural information, a parent ion scan (MS¹) is not sufficient and cannot provide information beyond the compound mass and molecular formula. To obtain structural information, the ion is fragmented (i.e. tandem MS) with fragmentation methods that include methods such as collision-induced dissociation (CID), electron capture dissociation, and electron transfer dissociation (ETD) to provide insights into the structure^{143, 144, 146}. The way molecules fragment in the gas phase is defined by the fundamental principles of chemical behavior. High mass accuracy is also quite informative, enabling the assignment of possible structures and functional groups when analyzing the fragmentation output and mass shifts (e.g. amino acids, sugars etc.). In the past fast atom bombardment (FAB) instruments were the high resolution mass spectrometers of choice to obtain high resolution data⁹. Today, the common instruments for obtaining high resolution separation and mass accuracy detection are Fourier- transform ion-cyclotron resonance (FT-ICR), Orbitrap Fourier-transform (Orbitrap) time-of-flight MS (TOF) and quadrupole TOF (QTOF)^{24, 147, 148}.

Identifying microbial metabolites and new NPs

In the workflow of exploring the microbial metabolome, the elucidation of a complete structure is usually the expensive and time consuming step. The costs for one structure elucidation, under the ideal conditions may in some cases exceed \$15,000 (this includes personnel time, solvents, NMR/MS costs, media, columns, etc.) and can go much beyond that¹. Under optimal circumstances, the structure elucidation would involve one day of purification and then one week to solve the structure after all the required data has been collected. One can, however, reasonably expect that if the procedure diverges from these ideal circumstances that the cost for the structure elucidation of a relatively simple new molecule runs in the hundreds of thousands with some of them well into the millions of dollars and decades of research. Therefore, in comparison, MS analysis is quite inexpensive and therefore can be harnessed to make NPs analysis more cost effective. The spectra generated can be used for identifying known molecules by fragmentation pattern comparisons and retention-time analysis compared to known commercial standards or to available MS/MS libraries. It may also assist in the characterization of unknown compounds by the mass losses of the fragments in a given spectrum. However, while some metabolomes (e.g. the human metabolome) have been widely characterized and are accessible in databases such as: Metlin¹⁴⁹, Massbank¹⁵⁰, LipidBlast¹⁵¹, HMDB¹⁵², NIST¹⁵³, ReSpect^{104, 1, 154, 155} (Table 2), complementary microbial metabolome databases are now emerging for a few model species^{156, 157}. But the vast majority of microbial metabolomes are missing. Lack of data is a direct result of the high diversity of organisms and species, while the data collected from different samples have not been accessible to the wider community. Therefore the study of microbial metabolomes offers many opportunities for future developments.

Identifying known compounds efficiently reduces the cost of further analysis and may expedite the discovery of new NPs and potential drug-leads. Hence, it is crucial to discriminate between known and unknown compounds at the earliest stage as part of the workflow - in a process termed “Dereplication”^{138, 158, 159}. This is an important step in prioritizing and avoiding the expensive and time consuming re-analysis of the known compounds.^{135, 144, 158}. In most NPs labs, as a part of traditional workflow, dereplication is based on the UV signature, the retention time and the molecular mass, which are then compared to *in-house* or external databases^{24, 145}. Rarely MS/MS data is used for dereplication^{1, 158}. Nowadays, with the advance of MS technologies, the dereplication workflows are leaning towards LC systems equipped with MS/MS detectors, combined with informative databases of NPs that contain molecular information (e.g., SciFinder, AntiMarin, MarinLit, ChemSpider, Antibase) and MS/MS libraries as mentioned above (Table 2)¹⁶⁰. The quality of these NPs libraries is determined by the diversity of the microbial sources and the tools used to exploit their biosynthetic potential, and are updated yearly^{132, 138, 161, 162}. However, as databases and datasets continue to grow, manual interpretation of the resulting data is becoming way too time-consuming and in some cases nearly impossible (i.e. “Big Data” - datasets too big for manual organization, analysis and interpretation). Analysis still remains the major bottleneck and rate-limiting factor in dereplication of microbial metabolites and metabolomics as a whole; consequently methods for automated analysis are being developed, leading to exciting new era of NPs mass spectrometry informatics^{126, 146, 159, 163, 164}.

Mass spectrometry computational tools

As science becomes more and more multi-disciplinary, the scientific community is beginning to bridge the gaps between the different research arenas, leading to fantastic opportunities and exciting developments. In today’s science, it is nearly impossible to preform “omics” type research without computational tools, statistical analysis and web-based interfaces. Yet the NPs communities, including microbial metabolomics have not embraced such computational tool development, until recently. The progress of useful tools requires an interdisciplinary approach in the way of collaboration. These synergistic opportunities between biologists/chemists and mathematicians/statisticians/computer scientists is not only profoundly influencing the way microbial metabolomes are characterized currently, but also how they will be characterized in the future. Naturally, as computational power is taking a central role in research, the amount of data that may be analyzed efficiently is increasing, enabling interesting correlations among multiple datasets and perhaps ask new questions that could not be addressed prior. A recent example for multi- disciplinary science had demonstrated the correlation between the spatial distribution of microbes on the human skin and specific human, environmental and microbial metabolites¹⁶⁵. Such synergistic collaborations drive the development of computational tools that benefits the greater scientific community.

Most recent developments in “Big Data” metabolomics are based on mathematical algorithms for organizing and visualizing MS data (Table 2). For example, molecular networking is an organizing principle for visualizing tandem MS data from an experiment¹²⁶. Multiple publications have demonstrated the potential of molecular

networking in microbial metabolomics and NPs identification/dereplication studies, with capacity of analyzing large scale datasets^{68, 74, 83, 84, 113, 126, 155, 159, 165–171}. The rationale behind molecular networking analysis is that molecular fragmentation is not a random event resulting in similar fragmentation patterns, but highly conserved bond breakage^{126, 159, 172}. The assumption is made that analogous molecular entities behave in similar manner in gas phase of MS instrument which give rise to similar fragmentation patterns or MS/MS spectrum. Molecular networking is applied in a few basic steps: 1) MS/MS data collection; 2) similarity calculation of spectra using cosine scores by the special algorithm to generate a molecular network; 3) visualizing the network using the systems biology tool Cytoscape¹⁷³ for displaying the mass spectrometry detectable chemical space from the entire data set or project and its associated metadata (Fig 6). The data is visualized by chemical similarity where one node represents one consensus MS/MS spectrum labeled by parent mass and edge thickness represents the cosine similarity score, creating clusters of molecular families¹⁵⁹. Such visualization enables the display of additional attributes for the analysis of complex samples to emphasize different origins of samples, intensities and seeding of known molecules for dereplication processes (Fig 6)^{74, 155, 159, 169, 174}. In effect molecular networking is an organizational and visualization tool¹²⁶. This approach has been compared as the molecular equivalent of metagenomic sequencing and is expected to have a significant impact on not only NPs discovery but also on the analysis of microbial communities.¹¹⁵

Further metabolomics computational methods for dereplication, based on MS fragmentation that were recently reported are described in Table 2, such as “MetFusion”¹⁴⁶. An algorithm that combines the search of the query’s spectrum to molecular structure databases (e.g. KEGG or PubChem) and other spectral libraries, attempts to match the different peaks by scoring them with matching candidate structures. The idea is that, for the correct compound, high spectral similarity score should correlate with high chemical similarity¹⁷⁵. Another recently developed tool is the “Fragmentation Tree Basic Local Alignment Search Tool” (FT-BLAST)¹⁷⁶. The algorithm organizes tandem MS data in fragmentation cascades, where the nodes are labeled with molecular formulas of the compound and its fragments and edges correspond to the mass losses. Similarly to molecular networking, the idea is based on that the fragmentation pattern similarities are correlated with the chemical resemblance of the corresponding compound^{163, 176}.

Mass spectrometry based microbial genome mining and biosynthesis

The increasing number of available sequences of microbial genomes in public databases has confirmed the potential and genetic diversity that are involved in the biosynthesis of NPs^{184, 185}. Genome mining is a process in which molecules are discovered by predicting what compound will be biosynthesized based on the sequences of gene clusters. Interpretation of NP-encoding genes and their organization can predict new pathways for discovery of silent or ‘cryptic’ biosynthetic gene clusters^{128, 132, 186–188}. Combining the developments in tandem MS methods enable one to correlate the production of NPs with the genomic capacity of the microorganism in query or in an interactive complex sample. New approaches such as peptidogenomics and glycogenomics have demonstrated the potential of MS genome mining to connect MS/MS sequences to predicted genomic-based structures following biosynthetic logic of ribosomal peptides (RiPPs), nonribosomal peptides (NRP)

and glycosylated NPs^{170, 182, 189, 190}. Recently several tools were introduced for the automation of MS based microbial genome mining such as “Pep2Path”¹⁸¹ for peptidic NPs, “RiPPquest”¹⁸² for RiPPs and “NRPquest” for NRPs¹⁸³ (Table 2). Furthermore, following MS genome mining approach, molecular networking has been used to characterize novel molecular families of unsequenced microbes by using previously discovered NP’s from sequenced organisms¹²⁸. It is expected that advances in these approaches will enable the mining of diverse classes of small-molecules, such as isoprenoids, polyketides, oligosaccharides, glycolipids and lipids generated by other gene clusters and biosynthetic pathways^{128, 185}.

While sequencing and genome mining have opened a new era in studying of biosynthetic pathways and prediction of natural product outcome, confirmation of these pathways is acquired by introducing of isotopic labeled precursors. This technique is largely studied and applied in primary metabolism research; consequently it is widely utilized in the studies of microbial NPs. Specifically in cases involving enzymatic tailoring the use of isotopic labeling often leads to discovery of novel NP’s. Recent examples of isotopic labeling studies demonstrate the discovery of new fungal yanuthones from *Aspergillus niger* and structure elucidation of unprecedented spirobicyclic warhead of colibactin produced by *Escherichia coli*^{191–193}.

5. Unsolved problems and future directions

As MS becomes more accessible, affordable and widely used in microbiology studies, the major challenges ahead are the development of new bioinformatics platforms for “Big Data” to mine and interpret. Such platforms are available for genomic and proteomic data of model organisms (e.g. Genbank, BLAST), but remain undeveloped in the field of microbial metabolomics. Our laboratory, together with Nuno Bandeira, the director of the UCSD center for computational MS, is building a platform for sharing, storing and mining data, as a key resource for future collaborative science. Investment in such resources holds great potential, especially as the scientific community continues to feed it with new identified chemistries¹. The social aspect of such interface will have the prospective of social-curation, similar to the “Wikipedia”; nowadays it is the only way to manage and learn from “Big Data” and lay the foundations for predictive computer based analysis. Such initiatives are highly encouraged by the Metabolomics Society aiming to facilitate the identification of microbially derived molecules with high fidelity of annotation and dereplication providing a stage for collaborations and inter-laboratory synergy¹⁹⁴. As MS technology advances and miniaturizes, it is expected that data acquisition will become more and more simplified in terms of sample preparation and size, enabling the analysis of microbes directly from their natural habitat in the field and in real time. Excitingly, we are fairly confident that within the next two decades these tools will emerge and enable to perform “Google-type” searches to “find” the important microbial correlations at the molecular level. The prospects for informatics developments combining metabolomics with metadata and other “Omics”, light the future in microbial chemical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.
Evolution of the number of publications found under the keywords search of “mass spectrometry microbiology” (PubMed).

Fig 2. MALDI-TOF pattern based analysis. (a) Sample covered with matrix is desorbed and ionized by laser to generate a specific fingerprint; (b) Fingerprint pattern is searched against a microbial standard library for most matching spectra; (c) Output shows the top scored hits from the database.

Fig 3.

Mass spectrometry workflows for microbial classification and identification. Gas chromatography fatty acid methyl esters (GC-FAME); matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS); rapid evaporative ionization mass spectrometry (REIMS); paper spray mass spectrometry (PS-MS).

Fig 4.

Mass spectrometry based methods commonly used in microbial studies (e.g. microbial interactions on a Petri dish). (a) an identified phenotype of interest. Matrix-assisted laser desorption/ionization - imaging mass spectrometry (MALDI-IMS) workflow: (b) transferring sample to a target plate; (c) sample is covered with matrix; (d) dehydrated sample is ionized by laser. Ambient pressure methods: (e) Desorption electrospray ionization (DESI), (f) Nanospray DESI (nanoDESI), (g) Liquid microinjection surface sampling probe (LMJ-SSP).

Fig 5. REIMS method for identification of Natural Products. (a,b) Photographs of REIMS setup with bipolar forceps and PTFE tubing attached for smoke evacuation and aspiration system for MS analysis of aerosol. (c) Summarized MS trace of direct sampled *Pseudoalteromonas* spp with circled ions corresponding to sodium adducts of known Bromoalterochromides. (d) Structures of identified Bromoalterochromides A on the top and B on the bottom.

Fig 6.

Molecular networking of mass spectrometry data obtained from a microbial sample as an organizing and visualization approach. MS/MS data is collected from microbial sample. Spectra are aligned and scored according to fragmentation similarity and neutral losses. Each node represents a consensus spectrum and is labeled by parent mass, edge width indicate the cosine similarity score, creating a cluster of nodes that share structural similarities as molecular families within the entire network.

Table 1

Studies and recent discoveries by MALDI IMS on the chemistry behind microbial interactions

Microorganism	Microorganism name	Key findings	References
Cyanobacteria	<i>Lyngbya majuscula</i> <i>Lyngbya bouillonii</i> <i>Oscillatoria nigro-viridis</i>	Identification of secreted natural products from intact marine cyanobacteria and imaging of metabolites in a heterogeneous assemblage with the <i>Dysidea herbacea</i> sponge	Esquenazi 2008 ⁶⁶ Simmons 2008 ⁷⁵
	<i>Lyngbya majuscula</i>	Discovery of the depsipeptide palmyranide A in the cyanobacterial/red algal extract and shown by MALDI IMS to be produced by <i>Lyngbya majuscula</i> .	Taniguchi 2010 ⁷⁶
	<i>Lyngbya majuscula</i> 3L <i>Lyngbya bouillonii</i> <i>Lyngbya majuscula</i> JHB	Monitor the biosynthetic timing of marine NPs, by 15N feeding assay imaging.	Esquenazi 2011 ⁷⁰
Bacteria	<i>Bacillus subtilis</i> vs <i>Streptomyces coelicolor</i>	Bacillaene produced by <i>B. subtilis</i> silences the defensive arsenal of <i>S. coelicolor</i> , demonstrating the chemical dynamics involved in the interaction between these microbes in a co-culture.	Yang 2009 ⁶³
	<i>Streptomyces roseosporus</i>	The discovery of three ions related to the anti-infective agent arylomycin in <i>S. roseosporus</i>	Liu 2011 ⁷³
	<i>Bacillus subtilis</i>	Two candidate metabolites active in a <i>B. subtilis</i> cannibalism siblings system, sporulation killing factor (SKF) and sporulation delaying protein (SDP).	Liu 2010 ⁶⁹
	<i>Bacillus amyloliquefaciens</i>	GA40, an antifungal from the iturin family, produced by <i>B. amyloliquefaciens</i> against various terrestrial and marine fungal strains.	Moree 2013 ⁷⁴
	<i>Bacillus subtilis</i> <i>Staphylococcus aureus</i>	<i>B. subtilis</i> inhibits <i>S. aureus</i> growth and virulence factor expression, through elaboration of its natural cyclic lipopeptide antibiotics.	Gonzalez 2011 ⁷¹
	<i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>	Novel extracellular antimicrobial peptide from community-associated methicillin-resistant <i>S. aureus</i> (CA-MRSA) with homology to the phenol- soluble modulins (PSMs) from <i>S. epidermidis</i> .	Gonzalez 2012 ⁸⁰
	<i>Streptomyces</i> sp. Mg1 <i>Bacillus subtilis</i>	<i>Streptomyces</i> sp. Mg1 produces the macrolide antibiotic chalcomycin A, causes cellular lysis of <i>B. subtilis</i> colonies.	Barger 2012 ⁷⁸
	<i>Streptomyces</i> sp. Mg1 <i>Bacillus subtilis</i>	<i>Streptomyces</i> sp. Mg1 hydrolyses surfactin produced by <i>B. subtilis</i> as an inhibition resistance mechanism.	Hoefler 2012 ⁸²
	<i>Bacillus subtilis</i> <i>Promicromonosporaceae</i>	Promicroferrioxamine, a hydroxamate siderophore, produced by <i>Promicromonosporaceae</i> strain SIO-11 in the presence of <i>B. subtilis</i> SIO-1.	Yang 2011 ⁵⁶
	<i>Paenibacillus polymyxa</i>	Antibiotic type compounds such as fusaricidins A, B and C, found in the interaction between <i>P. polymyxa</i> with <i>Fusarium oxysporum</i> .	Debios 2013 ⁷⁹
	<i>Streptomyces</i> sp.	Valinomycin, an antibiotic from a microbial symbionts of leaf-cutting <i>Acromyrmex echinator</i> workers ants, localized directly on the integument	Schoenian 2011 ⁸⁵

Microorganism	Microorganism name	Key findings	References
	<i>Bacillus subtilis</i> <i>Streptomyces coelicolor</i> <i>Pseudomonas aeruginosa</i>	afford protection against fungal pathogens. Three-dimensional (3D) models of microbial interspecies interactions chemistry. Increase in rhamnolipid production by <i>P. aeruginosa</i> , is inhibiting hyphal growth produced beneath <i>C. albicans</i> .	Watrous 2013 ⁶⁷
	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i> isolated from octocoral produce light-dependent antifungal polyketide alteramides at higher levels in the dark than in the light.	Moree 2014 ⁸³
	<i>Propionibacterium acnes</i>	Investigation of the microbial community signals within sarcoidosis granulomas specimens.	Oswald-Richter 2012 ⁹²
	<i>Streptomyces coelicolor</i>	A global chemical view of the model bacterium <i>S. coelicolor</i> interacting with five other actinomycetes. At least 12 different versions of a siderophore molecule desferrioxamine, used by the bacteria to gather iron.	Traxler 2013 ⁶⁸
	<i>Janthinobacterium agaricidamnosum</i>	A new antifungal NRPs jagaricin produced by <i>J. agaricidamnosum</i> in the infection of the mushroom <i>Agaricus bisporus</i> .	Graupner 2012 ⁸⁸
	<i>Chromobacterium violaceum</i>	Inhibition of quorum sensing signals produced by the plant pathogen <i>C. violaceum</i> visualized by of spatial distribution of the quorum sensing molecules.	Kusari 2014 ⁹⁰
	<i>Bacillus amyloliquefaciens</i>	New variant of surfacins produced by <i>B. amyloliquefaciens</i> antibiome in plant-microbe interaction in the rhizosphere.	Debios 2014 ⁸⁷
	<i>Streptomyces</i> sp.	Nine antibiotic substances produced by symbiotic <i>Streptomyces</i> from beewolf cocoons and their <i>in situ</i> spatial distribution.	Kroiss 2010 ⁸⁹
	<i>Bifidobacterium longum</i> <i>Bacteroides thetaiotaomicron</i>	Interaction between the host mouse model and gut microbiota.	Rath 2012 ⁹³
	<i>Shewanella oneidensis</i> MR1 <i>Pseudomonas stutzeri</i> RCH2	Detection of small molecules spatial pattern by acoustic printing of bacteria with replica- extraction-transfer (REX) nanostructure-initiator mass spectrometry (NIMS).	Louie 2013 ⁹¹
	<i>Mycoplasma</i> spp. <i>Vibrio</i> spp.	The sacoglossan <i>Elysia rufescens</i> mucus contains the antifungal kahalalide F, originates from symbiotic bacteria.	Davis 2013 ⁸⁶
Fungi	<i>Mycena metata</i>	Screening for new natural products in fruiting bodies of <i>M. metata</i> indicating that 6 - hydroxymetatacarboline D is present only in the upper surface of the fruiting body.	Jaeger 2013 ⁷²
	<i>Aspergillus fumigatus</i>	Increasing signal sensitivity for the siderophore desferri-triacetylfusarinine C (dfTafC), a virulence determinant secreted during iron starvation by the opportunistic pathogenic mold <i>A. fumigatus</i> .	Vergeiner 2014 ⁷⁷
Inter-kingdom interactions	<i>Pseudomonas aeruginosa</i> <i>Aspergillus fumigatus</i>	Inter-kingdom interaction between <i>P. aeruginosa</i> and <i>A. fumigatus</i> . <i>P. aeruginosa</i> phenazine metabolites	Moree 2012 ⁸⁴

Microorganism	Microorganism name	Key findings	References
		converted by <i>A. fumigatus</i> in a co-culture. (inter-domain interaction)	
	<i>Putterlickia verrucosa</i> <i>Putterlickia retrospinosa</i> Bacterial endophytes	Maytansine is biosynthesized by the endophytic bacterial community within the roots of <i>P. verrucosa</i> and <i>P. retrospinosa</i> plants and is accumulated in the root cortex. (inter-domain interaction)	Kusari 2014 ¹⁰³
	<i>Sinorhizobium meliloti</i> <i>Medicago truncatula</i>	Metabolite distribution in roots and root nodules of <i>M. truncatula</i> during nitrogen fixation. (Inter-domain interaction)	Ye 2013 ⁹⁴
	<i>Bacillus subtilis</i> <i>Bacillus pumilus</i> <i>Streptomyces coelicolor</i> Actinomycete <i>Salinispora</i> sp. <i>Pseudomonas aeruginosa</i> <i>Lysobacter enzymogenes</i> <i>Staphylococcus aureus</i> <i>Streptovorticillium griseovorticillatum</i> <i>Beauveria bassiana</i> ATCC 7159 <i>Chaetomium chiversii</i> CS-36-62 <i>Fusarium</i> sp. <i>Thermofungus</i> strains	Metabolic exchange patterns of a diverse array of microbes, including fungi, cyanobacteria, marine and terrestrial actinobacteria and pathogenic bacteria.	Gonzalez, 2012 ⁸¹

Table 2

Databases and computational tools for the identification and dereplication of NPs

Utility	Appellation	Description	Link
MS libraries	Metlin ¹⁴⁹	Human primary metabolites	http://metlin.scripps.edu/index.php
	Massbank ¹⁵⁰	Primary metabolites	http://www.massbank.jp/
	HMDB ¹⁵²	Human metabolites	http://www.hmdb.ca/spectra/ms_ms/search
	NIST ¹⁵³	Metabolites, drugs, peptides, sugars etc.	http://chemdata.nist.gov/mass-spc/msms-search/
	ReSpect ¹⁰⁴	Phytochemicals	http://spectra.psc.riken.jp/
	GNPS	Global Natural Product Social Molecular Networking	http://gnps.ucsd.edu
Databases with microbial metabolites	SciFinder	Diverse chemical structures	https://scifinder.cas.org/
	AntiMarin	MS/NMR for NPs	
	Antibase	Microorganisms NPs	
	MarinLit	Marine NPs	http://pubs.rsc.org/marinlit/
	ChemSpider	Metabolites, NPs, NRPs, drugs etc.	http://www.chemspider.com/
	PubChem	Diverse chemical structures	https://pubchem.ncbi.nlm.nih.gov/
	Dictionary of NPs	NPs	http://dnp.chemnetbase.com/tour/index.jsp
	Norine	NRPs	http://bioinfo.lifl.fr/norine/
	ECMDB ¹⁵⁶	<i>E. coli</i> Metabolome Database	http://www.ecmdb.ca/
MS based dereplication tools	GNPS	Global Natural Product Social Molecular Networking	http://gnps.ucsd.edu
	MetFusion ¹⁴⁶	Combine fragmentation data with databases for identification	http://msbi.ipb-halle.de/MetFusion/
	FT-BLAST ¹⁷⁶	Fragmentation Tree Basic Local Alignment Search Tool	Contact: sebastian.boecker@uni-jena.de
	LipidBlast ¹⁵¹	Tandem MS lipids identification	http://fiehnlab.ucdavis.edu/projects/LipidBlast
	MS2Analyzer ¹⁷⁷	Small molecule substructure annotations from accurate tandem MS	http://fiehnlab.ucdavis.edu/projects/MS2Analyzer/
	iSNAP ¹⁷⁸	NRPs dereplication and discovery	http://www-novo.cs.uwaterloo.ca:8180/isnap/
	HSEE ¹⁷⁹	Hypothetical Structure Enumeration and Evaluation for RiPPs	
	CMF ¹⁸⁰	Competitive Fragmentation Modeling for metabolite identification	http://cfmid.wishartlab.com/
MS based Genome mining tools	Pep2Path ¹⁸¹	Automated MS-guided genome mining of peptidic NPs	http://pep2path.sourceforge.net/
	RiPPquest ¹⁸²	Graphic interface for MS/Genome-based RiPP discovery	http://cyclo.ucsd.edu:4567/ms_ripper_full
	NRPquest ¹⁸³	Coupling MS/Genome mining for NRPs	http://cyclo.ucsd.edu:4568/nrpquest_full