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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Molecular Architecture of Lichen Polymicrobial Community

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

Yi Zeng

Committee in charge:

Professor Pieter Dorrestein, Chair
Professor Nuno Bandeira
Professor Neal Devaraj

2014

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Chair

University of California, San Diego

2014

DEDICATION

I would like to dedicate this work, and the effort in completing this, to my parents for their constant of love and all financially and mental supports during this process. To my mentor, Pieter Dorrestein, for his patient on guiding me through the path of science and his being encouraging on every little progress that I made on my project, which ultimately come together as a piece of fine scientific work. Also, to my girl friend, Fan Mo, for being with me and supporting me.

TABLE OF CONTENTS

Signature Page…… iii

Dedication…… iv

Table of Contents…… v

List of Figures……. viii

List of Tables…….. xi

Acknowledgements…… xii

Abstract of the Thesis…… xvi

Chapter 1: General Introduction……. 1

Chapter 1.1 - Abstract ……. 2

Chapter 1.2 - General Introduction of Lichen…….. 2

Chapter 1.3 - Mass Spectrometry Guided 3D Map …….. 7

Chapter 1.4 - Introduction to Mass Spectrometry …… 9

Chapter 1.5 - Reference …… 14

Chapter 2: Mass Spectrometry Guided 3D Molecular Map…….. 21

Chapter 2.1 - Abstract …… 22

Chapter 2.2 - Introduction to Three Dimensional Molecular Map ..…. 22

Chapter 2.3 - Results and Discussion ……. 29

Chapter 2.4 - Conclusion …….. 42

Chapter 2.5 - Method …… 43

Chapter 2.6 - Reference …… 46

Chapter 2.7 - Supplementary Figures …… 49

Chapter 3: Future Directions …… 59

Chapter 3.1 - Abstract …… 60

Chapter 3.2 - Research Goal …… 60

LIST OF FIGURES

Figure 2.1: General Introduction of Sample ……. 25

Figure 2.2: Constructing the *Peltigera sp*. 3D Molecular Maps …….. 27

Figure 2.3: Demonstration of *Peltigera sp.* Lichen Molecular Distribution…. 30

Figure 2.4: Molecular Networking of Lichen Extract vs. Lichen Isolates..…... 33

Figure 2S1: Fungal Phosphotidylcholine Lipid …….. 49

Figure 2S2: Chlorophyll Family ……. 50

Figure 2S3: Polyacetylated Sugar Family …… 51

Figure 2S4: Uridine Diphosphate N-acetylglucosamine ……. 52

Figure 2S5: Hexose Alcohol ……..…… 53

Figure 2S6: Hexose-base Polysaccharide Family ……..... 54

Figure 2S7: Unknown Bacterial Derived Molecule …… 55

Figure 2S8: Bacterial Derived Sterol-like Molecule ……..…… 56

Figure 2S9: Polypeptide Family ……..…. 57

Figure 2S10: Novobiocin ……..……. 58

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Abstract of the Thesis

The Molecular Architecture of *Peltigera sp*. Polymicrobial Community

by

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Master of Science in Chemistry

University of California, San Diego, 2014

Professor Pieter Dorrestein

Previously, scientist studied metabolites of polymicrobial communities by direct chemical extraction or microbial isolation. While the old day method does not provide a lot of information from a polymicrobial community, in this thesis dissertation, I have introduced and shown a novel method to investigate the metabolites and the metabolites distribution in three-dimensional space on a polymicrobial community by guidance of advanced technique of mass spectrometry such as MALDI-IMS & LC-MS. Specifically, I have developed a methodology to construct a three-dimensional metabolites map of a *Peltigera sp.* lichen to reveal the spatial metabolites distribution on lichen, hypothesis the possible function of a specific metabolites in the community and identify some of the specific metabolites. As a consequence

of this new 3D metabolites map methodology, wide ranges of molecules were observed in this *Peltigera sp*. 3D model such as polypeptides, single amino acid, photosensitive molecules, polysaccharide and some lipid molecules. In addition, the 3D model clearly shows that the metabolites distribution inside *Peltigera sp*. lichen, and distributions of chlorophylls as well as a lipid from lichen fungi are correlated with previous reported literature. Some of these molecules are able to identify and derive its function in this biological system. These results demonstrated that the 3D metabolites map of *Peltigera sp.* lichen provides a means to direct visualize the spatial architecture of metabolites inside this polymicrobial community as well as study them from different angle, and hence useful for studying the metabolites and biological system of polymicrobial communities.

Chapter 1:

General Introduction

1. CHAPTER 1 – General Introduction

1.1 Abstract

In this chapter, I will be giving a general introduction of lichen polymicrobial community, mass spectrometry based techniques for generation of 3D models to investigate the molecular architecture of *Peltigera sp.* lichen polymicrobial community. These tools and techniques, including 3D modeling based on the xyz coordinates of the sampling site, glass slide imaging mass spectrometry, tandem mass spectrometry, and molecular networking, were developed as proof-of-principle concept to allow researchers to construct three dimensional molecular map of polymicrobial communities with, *Peltigera sp.* lichen as an example in this thesis. Direct visualization of the molecular information inside the polymicrobial community through 3D molecular maps will allow researchers to create relevant hypothesis for in-depth analysis of roles of various molecules in shaping the growth and development of the polymicrobial community.

1.2 General Introduction of lichen, lichen polymicrobial community and techniques of studying lichen polymicrobial community.

What is lichen and why they are important and important to study?

Lichen are seen in various habitats such as grass, deadwood, rock, desert, and snowfield[1-3]. There are a lot of definitions of lichen. The earliest definition that I could find is “a fungus which lives during all or part of its life in parasitic relation with the algal host and also sustains a relation with an

organic or an inorganic host" by Fink[4]. This definition has roughly described lichen as a multi- species habitat in the nature[5]. As researchers accrued further information on different lichen species, it became clear that the fungi play the role of symbionts rather than parasites and encouraged the use of symbiosis to describe the interactions between the fungus and the other species comprising the lichen[1, 5]. Further, studies provided evidence showing that the co-inhabitance of mycobiont and photobiont is the underlying mechanism for formation of lichen in nature[5-7]. Under this symbiotic relationship, the photobiont in lichen, i.e. the algae and/or cyanobacteria[8], is the carbohydrate nutrition provider of the whole lichen biomass[3, 8], and the mycobiont, i.e. the lichenized fungi, utilizes the energy source provided by the photobiont to generate biomass and to build up defense system providing protection against the competitors[3, 4]. As a result, mycobiont in many lichen reside as exhabitant while photobiont will reside as inhabitant of lichen[9]. This physical protection mechanism of lichen is similar to chicken eggshell and the egg, and it strengthens the vitality of lichen and prolongs its living period in nature.

The life of lichen is important to the nature and us in many ways. Ecologically, lichen serve as food source for a lot of animals, for example, animals in cold habitats eat lichen as a source of proteins and vitamins in winter[10, 11]. Also, lichen prefers to grow in clean air and thus, can potentially serve as indicators of clean environment[12-14]. Some lichens,

can purify the air by absorbing pollutants like SO_2[15]. Since lichen grow in extreme conditions, they represent an excellent model system to study microbial surviving under extreme conditions[16]. Lastly, in academic and medical drug research, lichen consist of a tremendous source of unique secondary metabolites[17-21] that could be exploited for drug discovery to develop new drug candidates for curing diseases[22-27] such as cancer[28]. This particular application of lichen is discussed in further detail later in this chapter.

Peltigera is one of the lichenized fungi that belong to the lichen family *Peltigeraceae*[3, 9]. It's usually found in lichens that grow in soil, but also appear on rock and dead wood[29]. In this thesis, we have constructed both 3D average surface metabolites map and 3D internal metabolites map for a piece of *Peltigera sp.* polymicrobial community. For this study, we showed that the three dimensional molecular map enabled direct visualization of the real-time molecular profile of the *Peltigera sp.* polymicrobial community and extrapolation of this information to derive biological hypothesis. The details of 3D modeling are provided in section 1.2.

What is lichen polymicrobial community and why we do want to study them?

Polymicrobial community could be defined as "community that is marked by the presence of several microorganisms." In nature, most microbes are not living alone. Instead, various microorganisms live together

and develop their territories together, and such a community is called polymicrobial community[1, 30, 31]. Lichen, as combination of mycobiont and photobiont, is a polymicrobial community, exhibiting tremendous microbial communications making inter-specie interactions a hallmark of the mutualistic relationship displayed by this polymicrobial community[5]. While the mechanisms of formation of lichen polymicrobial community is not exactly clear, the reasons of formation could be generally described as the mutualistic symbiotic interactions inside the polymicrobial community such as passive defensive, nutrient sharing[1, 5].

There is a tremendous interest in studying lichen polymicrobial communities due to the complex interplay between different microbes inhabiting lichen giving it a unique ecological and pharmaceutical edge. The importance of lichen polymicrobial community covers ecological, academic and pharmaceutical realms. In ecology, lichen is an important form of life in nature and it is very meaningful to ecological system as described above. In academic research, due to the lichen's polymicrobial nature, lichen is an excellent model to study polymicrobial interactions[32] and various behaviors of microbial interactions such as microbial inhibition[22] and gene activation[33]. Pharmaceutically, lichen is a promising source of secondary metabolites since the presence of these metabolites is inherent to the development of polymicrobial interactions between different microbes

inhabiting the lichen[34]. There are a lot of bioactive secondary metabolites such as usnic acid that are only produced by certain species of lichen[6].

Current techniques of investigation lichen metabolites and its limitations?

People study lichen through biology and chemistry methods. Scientist has never stopped parsing out information from lichen polymicrobial community. In biology, scientist isolated the microbes from lichen and studied the type of organisms present in that particular lichen[35]. Later, scientists utilized microscopy to explore the biology components of thin slice of appropriately stained lichen placed on a glass slide in a more direct way under microscope[36-38]. With the further advancement in microscopy techniques, the 3D spatial microbial distribution has been revealed by combinations of co-focal microscope and situ-hybridization[32]. In lichen polymicrobial community, metabolites were found on both the surface as well as trapped in the intra-microbial matrix. Direct solvent extraction is an extremely useful method to isolate the metabolites from lichen. Different type of solvents will extract out different metabolites from the lichen polymicrobial community, and then these metabolites can be identified by different detection methods such as from liquid chromatograph mass spectrometry (LC-MS) and NMR[39-41]. In addition, metagenome sequencing of the same lichen will enable linking the identified metabolites to their biosynthetic gene cluster[42]. Although isolation of metabolites followed by their identification and characterization of the biosynthetic routes reveals some of the molecular

composition of the lichen, it is completely lacking in the ecological role of those metabolites and their spatial distribution in the lichen. For example, for native lichen, it still remains to be explored where the metabolites such as polypeptides, photosensitive molecules, lipid molecules and sugar molecules are located. Also, how these molecules might affect the growth of lichen itself and protect it from the environment? Such molecular distributions and their ecological roles is an important aspect of the polymicrobial community of lichen that to investigate the nature of lichen, and the mechanisms by which lichen polymicrobial community is formed.

1.3 Utilizing 3D model to investigate lichen polymicrobial community and constructing mass spectrometry guided molecular map.

What is 3D models and how a 3D scanner works.

A model of a subject could be interpreted as a system to imitate the actually object. A 3D model refers to a digital representation of particular object in three dimensional space. Current technology allows us to construct a 3D model of an object with a high-resolution 3D scanner. One type of 3D scanner combines a laser and a HD camera to construct a 3D object[43]. There are various 3D scanners available in the market that utilize similar theoretical concept for creating 3D models. The 3D canner used in this study (in fig) is from NextEngine Inc[44, 45]. The general theory of this 3D scanner is that a fixed high definition camera will take high definition 2D pictures of the object, which has been fixed on a rotatable stage, while a laser scanner, just

above the HD camera, will scan through the whole object recording the distance of object and the laser. The stage could rotate 360 degree and tilt to an angel from -45 degree to 45 degree maximum. At each rotational and tilted angle, the HD camera and laser detector could record one set of data, and same process could take place when the rotational stage rotate to another position. All collected data from camera and laser scanner will then be merged together through computational calculations generating a digital 3D model[44].

3D model editing software.

Sometimes, depending on the scanning object, the digital 3D model does not contain all features that the original object had[46]. For example, there might be small gaps, fake scanned pieces and over scanned pieces that are not eliminated by the computational calculation. A further step to polish the 3D digital model involves the use of a 3D model editing software. There are tools such as “fill holes”, “polish edge” or “delete pixels” in most 3D editing software[47]. There also is a tool that is used to extrapolate xyz coordinates of certain points of interests on the digital 3D model. All tools introduced above will be described in Chapter 2 for further details.

1.4 Introduction to Mass Spectrometry

What is mass spectrometry?

Mass spectrometry is a molecular analytical instrument that is capable to separate and detect an ion as mass to charge ratio, and those detected ions will be transformed and printed as peaks on a profile, called mass spectrum, which usually has mass to charge ratio as x axis and intensity as y axis. Mass spectrometry has been applied to many fields of study because of its ability to accurately identify most chemicals from complicated chemical mixtures. It has become necessary instrument for industry and academic research to analyze complex biological systems[48, 49].

In mass spectrometry, all sample molecules will go through the whole equipment as gas phase, and all manipulations are done by applying and changing strength of electrical field on charged gas phase samples. All mass spectrometer contain three components, which could be defined as an ionizer, a separator, and a detector. Samples of molecules will be ionized at the ionizer compartment once it was injected into the mass spectrometry. Ionized ions will fly through a vacuum space that contain single or multiple electric field filters for ion selection. The most simple vacuum space filter is called time of flight (TOF), which applies electric field to both sides of a tube shape vacuum space for separating ions that comes into the vacuum space at the same time. Some other ion filters are included ion trap, orbitrap, quadropole, triple quadropole, hexapole. Detector was the final station for all

traveling ions. As charged molecule, ions detected will be converted to mass to charge ratio signal and recorded on a spectrum profile. More details of the ionizer, filter and detector are presented in the following paragraph in this section.

There are multiple ionization methods, for example electrospray ionization (ESI), matrix assist laser absorption ionization (MALDI), chemical ionization, and electronic ionization. ESI is the most popular ionization technique typically for liquid samples[49, 50]. Herein, certain amount of single or mixed chemical samples is dissolved into a mixture of certain ratio of water and organic solvents. In order to increase the ionizability of molecules, a volatile acid or base such as trifluoroaccetic acid, formic acid, and ammonium hydroxide is added into the organic solvents. Liquid mixture of sample, solvent, and volatile acid/base is transferred to the source by a float of nitrogen gas. The source contained a nebulizer which applies a high voltage potential, and the liquid mixture will break down into fine droplets carrying charge. Then, all droplets will flow into the mass spectrometry glass tube inlet, which is typically about 200 to 250 °C, for evaporating the remaining organic solvents on the droplets. ESI-MS coupled to HPLC increases the efficiency of ionization because a mixture of chemical samples might reduce the ability of some molecules to be ionized. Also, for a purified peptide, protein, enzyme, etc, connecting HPLC compartment to ESI-MS could reduce

loss of sample. So, HPLC-ESI-MS is a decent tool for separating and relative quantification of components of a mixture of chemical samples[48, 49, 51].

Another ionization method is Matrix Assist Laser Desorption Ionization (MALDI). In this method, chemical molecules are ionized by matrix assistance[48, 49, 52]. Matrix used in MALDI is typically organic acid molecules that easily form crystals. Crystals absorb the laser energy and the energy is transferred to experimental sample, trapped in the matrix. Here common matrixes are dehydroxyl benzoic acid (DHB), alpha-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid and universal matrix (DHB + CHCA). These matrixes are dissolved in water and organic solvents mixture, where organic solvents are higher in percentage by volume. A volatile organic acid/base is added to the matrix mixture depending on the mode (either positive or negative) in which mass spectrum data is being collected. Typically, adding acid helps ionize molecules that are detected in the positive mode while adding base will facilitate ionization in negative mode. The dissolved chemical samples are mixed with equal amount of acidified/basified matrix solution, and a drop of the total mixture is placed on the sample carrier, MALDI plate, which is a metal plate that could conduct electricity. MALDI ionization is commonly followed with time of flight (TOF) detector. MALDI-TOF MS is widely used in medical research because of the ease of sample preparation and short detection time though the accuracy of mass detected is low[48, 49].

Tandem mass spectrum and collision induced dissociation

One of the greatest capabilities of mass spectrometry is the ability to fragment a targeted molecule and detect fragment ions generated in this process. This process is named as tandem mass spectrometry[53]. A full scan of non-targeted molecular profile on mass spectrometry is called MS1, and if a molecule is selected and send to fragmentation in the mass spectrometry for one time, the collected profile is called MS/MS or MS2 of this particular molecule. Various fragmentation techniques can be employed to MS2 profile. In this study collision induced dissociation (CID) was used for collecting MS2 data. Basically, the CID fragmentation method uses nitrogen gas with high kinetic energy as collision source to collide incoming flying analytes. As the kinetic energy is transferred from the nitrogen to the analytes, some relatively unstable chemical bonds will break and form fragments. Because mass spectrometry is detecting charged ions, only the charged part of fragmented molecule will be detected. Preliminary molecular structure could be revealed by the MS2 mass spectrum profile because the fragments of most molecules are the relatively stable molecular moieties such as single amino acids for polypeptides and dehydrated saccharides for polysaccharides.

Some mass spectrometry instruments, for example ion trap and orbit trap, are capable to do multiple mass fragmentations. The fragmented ions from parent mass will be sent to CID cell for fragmenting the second time. As

the times of fragmentation take place[48], we will call the process MSn, where n+1 is the times of fragmentation take place. This extent of fragmentation is extremely helpful in investigating the preliminary structure of molecules or identified molecules by matching the experimental MS2 fragmentation result with the published MS2 profile of the same molecules.

Imaging mass spectrometry (IMS)

Imaging mass spectrometry is a mass spectrometry technique based on MALDI-TOF mass spectrometry. This technique is mainly used for visualizing metabolite distributions on samples surface[54], and has been successfully applied on biological samples in order to study the relationship of microbial behaviors and its secreted metabolites[31]. In principle, area of interest will be fixed on the MALDI target plate and be covered with a thin layer of MALDI matrix. The source of MALDI-TOF MS contained a movable rotor that moves the laser beam parallel against the MALDI plate. Therefore, MALDI data is collected on over the surface of interest of the sample[48].

There are two major ways of preparing microbial samples for imaging mass spectrometry (IMS). One is glass slide imaging mass spectrometry; the other is agar-based imaging mass spectrometry. Glass slide imaging mass spectrometry is mostly used for mammalian tissue imaging[55-60]. Traditionally, the tissue will be frozen and sliced by a cryotome machine. Some of samples are not easy to cut or natively fragile, so embedding media

such as gelatin is needed for a better slicing. Sliced out piece will be mounted on a Tin-Indium coded glass slide, and deliver into MALDI TOF MS for data collection. The other method is agar-based mass spectrometry imaging. Main target of the agar-based imaging mass spectrometry is microbial samples cultured on agar media. A detailed methodology for agar-based imaging mass spectrometry is published in a review paper written by our previous member, Jane Yang[61]. Herein, I will focus on the glass slide imaging mass spectrometry technique used for imaging of lichen in this study.

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Chapter 2:

Mass Spectrometry Guided 3D Molecular Map

CHAPTER 2 – Mass Spectrometry Guided 3D Molecular Map

2.1 Abstract

In this Chapter, I will show a novel method of studying polymicrobial community. A three dimensional molecular map of a piece of *Peltigera sp.* lichen is generated by utilizing techniques of 3D modeling and mass spectrometry. The three molecular molecular map show a large range of molecule distributions of *Peltigera sp.* lichen sample, and it indicated that chlorophyll, polysaccharide, polypeptide and lipid molecules distribute in different area inside this lichen polymicrobial community. All these distribution shown on three dimensional molecular map are also shown correlations to their biological distribution according to previous literatures report. The distribution of chlorophyll is tightly associated with cyanobacteria distribution inside *Peltigera sp.* lichen, and it showed a sequential decreasing of intensity from the sun exposed layer to the sun shaded layer, which is novel observation to the lichen polymicrobial community. Also, the amount of chlorophylls and bacterial derived molecules are affected by intensity of light source exposure according to the 3D metabolites map. In short words, the three dimensional molecular map is a great tool to visualize the molecular features and study similar polymicrobial community.

2.2 Introduction to three-dimensional molecular map.

Short summarize of current technique on study lichen molecular and its disadvantages

Lichen is a polymicrobial community in nature. Depends on the environmental differences, the composition of microbes inside a lichen might be completely different[1-3]. Due to this biological dynamics of lichen polymicrobial, lots of unique metabolites are being produced[4, 5], and this draw scientist interests on investing lichen polymicrobial community. Chemists are able to apply direct extraction to lichen sample with different organic solvents, and then extracted lichen analytes are subjected to HPLC or LC/MS for full scan profile. If any analytes are needed to be further structural elucidated, HPLC purification following by NMR could be applied[6, 7].

Above chemistry method could get most of the chemical profile of lichen polymicrobial community. However, for investigation purposes, pure extraction method will not give you much information except full molecular profile of the lichen because direct extraction is not a targeted method. For instance, for investigating bioactive molecules in a piece of lichen species, a series of HPLC bioactive tests are needed before perusing to next step analysis, for example, large quantity purification of tested bioactive molecules from crude extract. In addition, for ecology and academic purposes, direct extraction method is not capable to give much useful messages of formation of lichen polymicrobial community and microbial interactions, and less is

known to the lichen polymicrobial community including the formation mechanisms of lichen. In contrast, biologists, with currently advanced sequencing, microscopy and computational technologies, are able to parse out the biological information of lichen such as types microbial species existed and their relatively quantity in lichen, some microbial spatial location inside lichen[8] and the metagenome information of lichen[9]. However, without a deep understanding of chemistry information, sometimes it's hard to interpret some biological phenomenon. Therefore, new tools of investigating lichen polymicrobial community are needed.

The idea of 3D metabolites map

As advanced of mass spectrometry techniques developed, imaging mass spectrometry (IMS) technique has become a promising tool to visualize and hypothesize the potential bioactive molecules from agar microbial inoculation on a p-tri dish. Most agar co-inoculation microbial interaction could be observed by 2D imaging mass spectrometry[10] even though there's evidence to indicate that there were large validated microbial interaction information embedded under the agar surface[11]. However, lichen is a 3D object and its microbial interactions are also expected to be three dimensional. It's hard to extrapolate the microbial interaction information from lichen with current 2D imaging mass spectrometry techniques. Therefore, a three dimensional imaging mass spectrometry tool is needed for further studying the lichen polymicrobial community.

A good starting point for overcoming this difficulty would be utilizing the current 3D modeling technique as well as advanced mass spectrometry tools

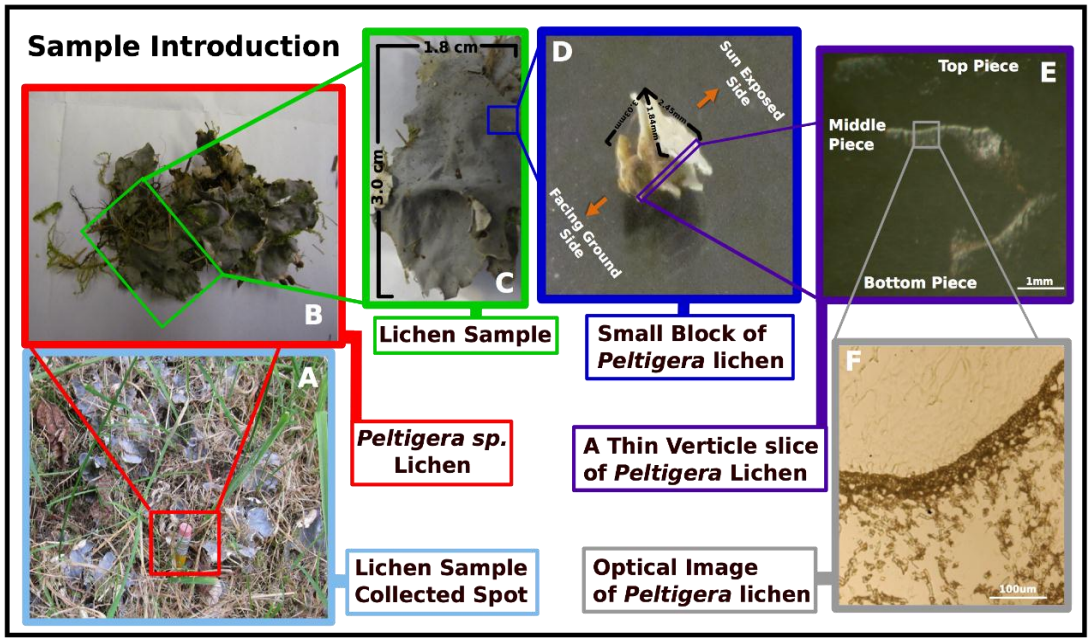


Figure 2.1: General Introduction of Sample.
A) is indicating the collection spot of entire piece of Peltigera sp. lichen at UBC, Vancouver, Canada. **B)** is the collected lichen sample collected from A. **C)** is a part of the Peltigera sp. lichen sample that used for constructing the three dimensional molecular map. **D)** is a small block of Peltigera lichen taken out from lichen sample in C. **E)** showed an optical image one of the section slices (thickness: 10um) from Peltigera lichen sample in D. **F)** is a high resolution optical image of partial sample E.

to simulate a three-dimensional molecular distribution map of lichen. In general, a three-dimensional molecular distribution map is a 3D model of the object printed with the object's molecular information, such as mass, as well as their spatial distribution on both surface and internal. To represent the concentration of molecules, color code could be applied, such as light color represented low concentration while deep color represent high concentration.

The overall map could make us scan through the molecular distribution from inside to outside of lichen. Here, we used a piece of *Peltigera sp*. lichen as template for constructing a three-dimensional molecular distribution map. This lichen sample is collected on grass around UBC, Vancouver, Canada. The advantages of constructing a molecular three-dimensional map of lichen polymicrobial community are multiple. One of the most advantages is that the three dimensional lichen metabolites map provides an easy and direct way to explore the metabolites of lichen. For instance, molecules distributed on the top surface or bottom surface should be expected quite different due to different growing environment. The 3D molecular map should also help to explore internal molecular architecture of lichen because same species microbial tend to stay and grow together in lichen polymicrobial community, and their secreted metabolites are able to be detected by mass spectrometry as well as localized in the 3D molecular map. Another advantage is that the molecular three-dimensional map could be a supportive reference of some biological behaviors of lichen such as the formation and growth of lichen.

Strategies of constructing the Peltigera sp. 3D molecular maps

With current mass spectrometry and 3D modeling techniques, it is realistic to construct a three-dimensional metabolites map of Peltigera sp. polymicrobial community. The first step could be generating a simulated three dimensional digital model of Peltigera sp. lichen sample. A precise three dimensional digital model of the object will greatly help on constructing the

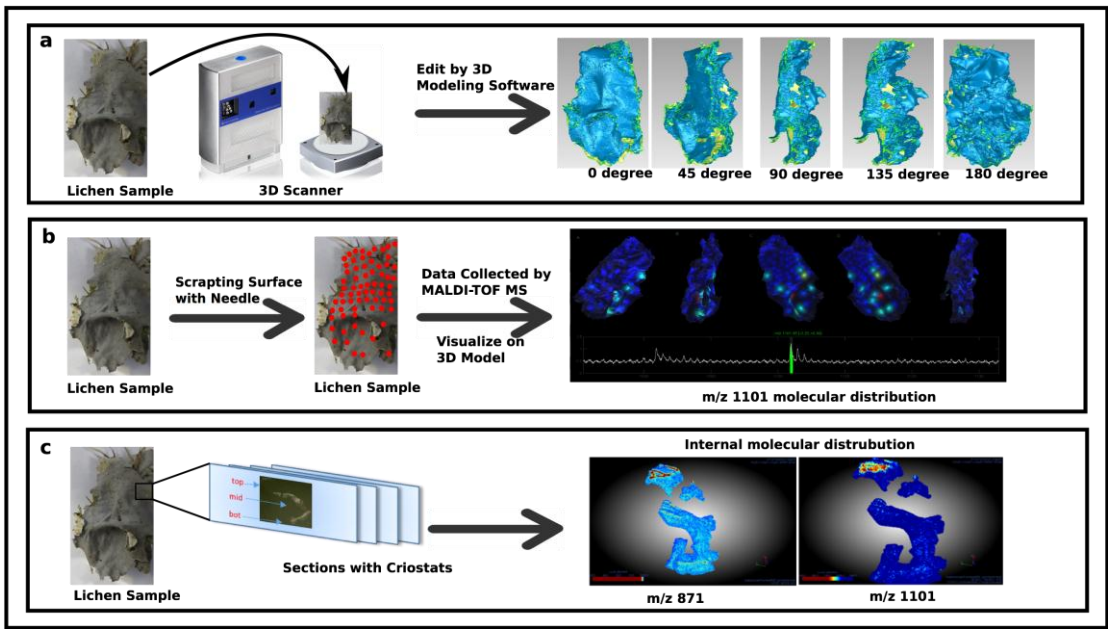


Figure 2.2: Constructing the *Peltigera sp*. 3D Molecular Maps.
A) Shown a piece of *Peltigera sp.* lichen sample and steps of constructing three dimensional digital model with NextEngine 3D HD scanner and post-editing step by 3D modeling software of Geomagic 2012(Trial Version). Right hand side of the arrow has shown simulated *Peltigera sp.* lichen 3D digital model with 0°, 45°, 90°, 135°, 180°. **B)** Shown a strategy of constructing average surface 3D metabolites map of *Peltigera sp*. lichen. Red dot shown in middle indicates the scrapped sampling position. Right side of the figure showed simulated 1101 m/z distribution on the 3D *Peltigera sp.* lichen model with 0°, 45°, 90°, 135°, 180°. **C)** Shown a process of constructing internal 3D metabolites map of *Peltigera sp.* A small block of lichen, black square in figure, was subjected to cryostat section and glass slide imaging mass spectrometry. Middle of figure has shown a demo of several consecutive sectioning lichen slides. Right hand showed simulated 1101 m/z distribution on the internal 3D *Peltigera sp.* lichen model.

three dimensional molecular map because later on the all molecular information are going to map on the three dimensional digital map as simulating their actual positions on the real Peltigera sp. lichen. Essentially, the dimensional digital model will be obtained by first scanning with a high-resolution 3D scanner following by 3D software editing. The second step could be generating a three dimensional average-surface molecular map of *Peltigera sp.* lichen surface. With the three dimensional average-surface molecular map, we can direct visualize the complete molecular profile of the

Peltigera sp. lichen surface and fast scan any molecules localized or co-localized on the surface of the Peltigera sp. sample. Specifically, it could be accomplished by systematically collecting average surface materials from the *Peltigera sp.* polymicrobial sample, direct extracting metabolites from the collected materials, fast detecting metabolites information by MALDI-TOF MS method and putting the molecular information onto the same location of the same pre-scan digital polymicrobial lichen 3D model. Last step, other than average surface information of the polymicrobial lichen, the internal information of polymicrobial community information is also significant for studying the polymicrobial community. It's also reported that some polymicrobial lichen community could have different biological layers that each layer composed a specific groups of microbial species. For instance, a photobiont biological layer was observed beneath the top microbial biological layer in *Peltigera sp.* lichen under microscopic image; since the microbial content in each layer is different from other layers, it is expected that some unique molecular distribution inside the lichen community would be distributed along the biological layers. For further visualizing the molecular distribution inside the *Peltigera sp.* polymicrobial community and studying molecular information of this lichen, an internal three dimensional molecular map of polymicrobial lichen will be helpful. This internal three dimensional molecular map could be achieved by aligning numbers of consecutive 2D MALDI-IMS images by computational techniques. 2D MALDI-IMS is first developed for mammalian tissue metabolites MALDI imaging. A histological sectioned tissue

was collected on a piece of conductive glass slide, and then the sample loaded glass slide was covered with matrix; at last, matrix coated glass slide sample will be delivered into the MALDI-TOF MS, and MS data of area of interest will be collected through systematically shooting spots on the glass slide sample. Similar technique could be apply to histological sectioned lichen polymicrobial sample, and then align all collected MALDI IMS images to generate a 3D internal metabolites map of lichen polymicrobial community. This internal 3D molecular map could help us to direct visualize the molecular architectural structure of lichen polymicrobial community and derive the relationship between molecular spatial distribution and the biological layer inside the lichen polymicrobial community.

2.3 Results and Discussion

The ability of studying lichen polymicrobial in nature has been started since it is possible to isolated microbes from the polymicrobial community[12]. Biologically, confocal microscopy and situ hybridization could give us a clean 2D or 3D information embedded inside lichen. In sequencing, novel metagenomic sequencing could extract out polymicrobial’s gene cluster information and give explanation on some metabolites produced in the polymicrobial[9]. However, in chemistry, old technique such as direct extraction and purification are still major techniques for studying lichen polymicrobial community while no much information could be generated from these old techniques. In order to investigate more in depth molecular

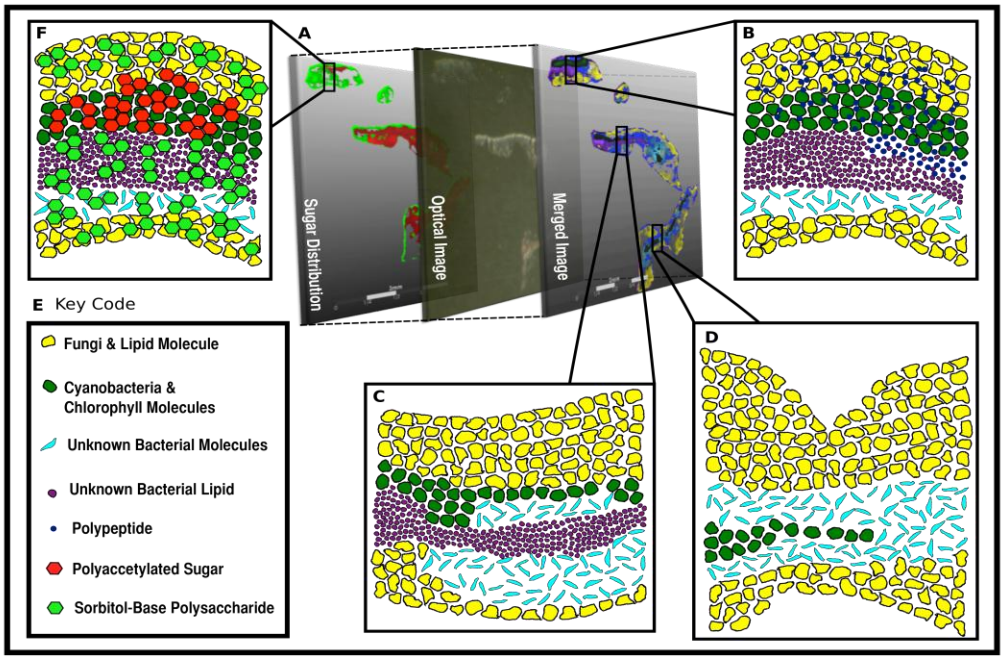


Figure 2.3: Demonstration of *Peltigera sp.* Lichen Molecular Distribution. **A)** Three figures are shown in this figure. Middle one is an optical image of one of the *Peltigera* lichen vertical section slice. Left hand side showed a distribution of multiple acetylated sugar (red hexagon) and hexose alcohol (green hexagon). Right hand side showed a merged distribution 2D figure of several varied molecules. **B, C & D)** These three figures showed a cartoon demonstration of the internal molecular architecture from top piece to bottom piece of *Peltigera sp.* lichen. Top piece is exposed to sunlight while bottom piece is adhered to ground. By comparing the distribution pattern of B, C & D, noticeable molecules intensity changed for chlorophylls (deep green cell), two unknown bacterial derived molecules (light blue and purple cell) and a polypeptide molecule (deep blue circle). **F)** This figure showed a distribution of two sugar in the top piece of *Peltigera sp.* Combining with A, multiple acetylated sugar (red hexagon) mainly distributed at the middle and bottom piece, and sorbitol-base polysaccharide mainly distributed on the surface of each piece of *Peltigera sp.* lichen community.

information from lichen polymicrobial *Peltigera sp.* lichen is a common lichen populated high altitude area[2]. Its appearance continent has been identified as very similar from species to species while the molecular information is relatively dynamics. The *Peltigera sp.* lichen in this research is a *Peltigera hymeninna*. Figure 2.1 (A-F) showed a general process of samples collection to a fine detail microscopic optical image of *Peltigera sp*. lichen. The sample

in figure 2.1 (C) was used to constructed average surface molecular three dimensional map. Figure 2.1 (D) indicated a small block of *Peltigera sp*. lichen was taken for cryosectioning, and an optical image was shown in 2.1 (F).

Instead of just showing the 3D model, for better demonstration on molecular architecture of *Peltigera sp*. lichen, several molecule distribution have been consolidated into *Peltigera sp*. section slice and performed as a cartoon model shown in Figure 2.3. This figure summarized a big picture of chemistry architecture of *Peltigera sp.* lichen according to the three dimensional molecular map. Left hand side of figure 2.3(A) showed an optical image of one of the lichen section slices obtaining from technique shown in figure 2.2(C), and right hand side of figure 2.3(A) has shown some molecular distribution on this lichen section slide. Figure 2.3(B), (C) and (D) showed conceptual cartoon of molecular architecture of *Peltigera sp*. lichen according to the molecules distributions of lichen shown on the 3D map. Figure 2.3(B), (C) and (D) are three small districts from top, middle and bottom piece of *Peltigera sp.* lichen, respectively. The color code of the cartoon diagram indicated different m/z observed in the mass spectrometry and its corresponding distribution on one vertical section of *Peltigera sp*. lichen. From figure 2.3(B) to (D), several distinct chemo layer of *Peltigera sp.* lichen was shown in each figure, but not all of them had same chemo layer distribution, which could also imply that there is a gradient change of molecule distributed intensity throughout the top piece of lichen to the bottom piece of lichen.

Fungi species are one of the major component in forming the lichen polymicrobial community. In *Peltigera sp.* fungi, it is expected to observe some fungi secreted molecules that will fall around the location where fungi species resided. Also, fungi have been reported to as physically protecting cyanobacteria by residing exterior of lichen. Indeed, some fungi associated molecules are detected in the mass spectrometry and reveal on the three dimensional metabolites maps. A phosphocholine like lipid, shown in figure 2S1, was detected and found in one of the *Peltigera sp.* lichen fungi isolates. The MS2 pattern of this molecule showed a peak of 184 m/z (Figure 2S1), which correspond to the mass of protonated phosphocholine residue. The distribution pattern of this phosphocholine lipid molecule is mainly distributed on the exterior region of every piece of *Peltigera sp.* lichen, and this revealed that, in *Peltigera sp.*, there were some fungi species reside on the exterior area for protecting the polymicrobial community. From the molecular networking, it has shown several other types of phosophcholine lipid that clustered together, and these molecules are distributed on the similar location. However, only one of those phosphotidylcholine is produced by one of the *Peltigera sp.* fungi isolates.

Another important player in *Peltigera sp* would the massive cyanobacteria content. The ability of being able to do photosynthesis make cyanobacteria fit in the role of energy station in the polymicrobial

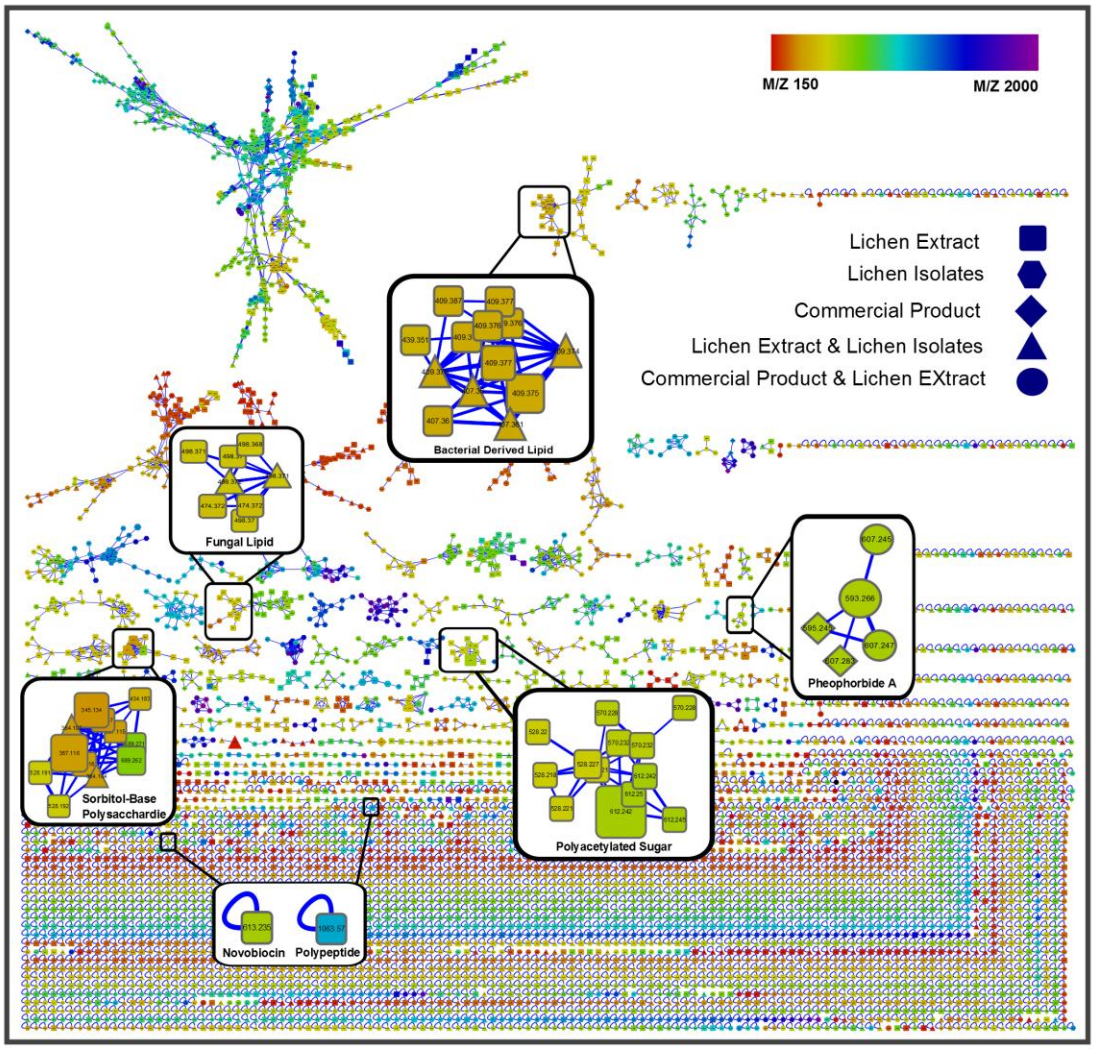


Figure 2.4: Molecular Networking of Lichen Extract vs. Lichen Isolates. The figure showed a molecular networking figure generated from lichen extracted, lichen isolates and some commercial standards. Negative controls were put into generating the networking file above and were eliminated from the network. This figure has highlight some molecules shown in the network that correlated with figure 2.2. Top right color scale indicating color of nodes, which are nodes color are based on their parent mass, and right hand side showed node shapes and their representations in the network.

community[3]. A key finding on this study would be the distribution of chlorophyll produced by cyanobacteria. Because cyanobacteria are producing glucose by photosynthesis process, and it is believed that this is the major

sugar source to build up and support the growth of lichen polymicrobial community[13, 14]. In figure 2.2(B), a clear chlorophyll distributed area was colored with deep green color in the figure, and it was located beneath the top fungal lipid layer, which is exposed directly to the sun. The chlorophyll colored in green cartoon diagram is representing distribution of one of the chlorophyll molecules, pheophrobide A. On the same exact location, the distribution of chlorophyll A has been found as well. As supplemental material shown in figure 2S2, the MS/MS pattern of detected pheophorbide A and chlorophyll A molecules from lichen extract has been comparing with their commercial product on the market. The perfect match between these molecules and their commercial products has firmly proven the existing distribution of chlorophyll molecules. Comparing figure 2.2 (B), (C) and (D), it's noticeable that the density distribution of chlorophyll molecules are obviously decreasing. This distribution indicated that the chlorophyll contents density inside each piece of *Peltigera sp.* lichen would be varied according to their abilities exposed to sunlight. It's reasonable to interpret that the necessary of cyanobacteria in lichen could be depending on its ability of producing glucose and support the community. On other aspect, once the cyanobacteria do not have enough sunlight to process photosynthesis and make their food, they will not have enough resource for building their self-defense. Other microbes around that could live without sunlight support will invasively eliminate these cyanobacteria content due to food competition. The sequential decrease chlorophyll distribution from sunlight expose surface to face ground surface

has clearly indicated the amount of surviving cyanobacteria are reducing due to less accessible to sunlight resource at the bottom piece comparing to the top piece in this *Peltigera sp.* lichen. This novel phenomenon has reflected the self-adaptable ability of *Peltigera sp.* polymicrobial community.

Another important reported function of cyanobacteria in the lichen polymicrobial community would produce source of carbon. In particular case, saccharide and polysaccharide derived from cyanobacteria are the original carbon source of the whole *Peltigera sp.* community, but it was transferred and delivered to the other organisms such as fungi and bacteria in the polymicrobial community[15, 16]. Researcher has been noticed that the lichenized fungi could actually turn the glucose source secreted by cyanobacteria into sugar alcohol unit. In such, cyanobacteria are not able to reutilize the glucose again[17-19], and the carbon source are transferred to another member in the polymicrobial community[18]. In figure 2S5 showed a commercial mass spectrum for sorbitol, 183.083 Da [M+H], and a high concentrated molecule from lichen extract. The MS2 patterns for these two molecules are completely identical, which is suggesting there is sorbitol like molecules distributed in lichen. According to previous research, cyanobacteria are not able to produce sorbitol, but lichenized fungi species are able to convert glucose source to sorbitol for its own usage[18]. In figure 2S5 has shown a MS2 spectrum of commercial purchased mannitol, 183.083 Da [M+H], and a MS2 spectrum of detected sorbitol from *Peltigera sp.* lichen

extract. The distribution of this molecule is, unfortunately, not clear because the collected the MALDI-TOF-IMS is from m/z 200 to m/z 2000. However, a putative distribution of sodiumnated add-up sorbitol, m/z 205, is shown in figure 2S5. The putative sugar distribution fall on fungal distributed area in the *Peltigera sp*. lichen. This co-localization of sorbitol and fungal distribution has suggesting the sorbitol molecules are actually converted and utilized by the fungi species inside *Peltigera sp*. lichen. In figure 2S6, the remaining figures are polysaccharide distribution, and, interestingly, the distribution intensity of all this polysaccharide molecules, except m/z 345, showed strong ion intensity around cyanobacteria area and weaker intensity as they radiate out. This distributed actually suggested that cyanobacteria might be able to polymerase these polysaccharides even though they are not able to use them.

As cyanobacteria are the prominent sugar producer inside lichen polymicrobial community, there is another type of unknown polysaccharides observed in large amount inside the *Peltigera sp*. polymicrobial community. In figure 2.3(F), red hexagons are represented molecular distribution of polyaccetylated sugar molecules while green hexagons are represented the distribution of sorbitol based polysaccharide. Cartoon model of figure 2.3(F) has only shown a partial distribution of these two types of sugars, where polyacetylated sugar is in the center while sorbitol base polysaccharide are surrounded it at the outside. On left hand side of figure 2.3(A), a general

distribution profile of these two molecules are shown. The polyacetylated sugar, which majorly distributed at the middle and bottom pieces of *Peltigera sp*. lichen, are polysaccharide that has been multiple addition of acetylation starting from 528 m/z to 654 m/z shown in figure 2S3. These polyacetylted sugars are all involving structurally similar according to the MS2 pattern, and result of molecular networking also could show these molecules are connected to each other as cluster in figure 2S3. In all MS2 mass spectrometry in figure 2S3, some mass shift such as 176 Da, 42 Da and 162 Da are annotated, and the candidates of these mass shift are methylated dehydrated glucose, acetyl group and dehydrated respectively shown in the chart of figure 2S3. According to the structure and distribution of this molecule, the function of these polyacetylated molecules inside *Peltigera* lichen are not clearly known. It might be for energy storage purpose or for the base construction of lichen polymicrobial as more concentration shown at the middle and bottom piece of Peltigera sp. lichen. Another molecule distribution shown in figure 2S4 has the same location as these polyacetylated sugars, and it was uridine diphosphate N-acetylglucosamine, which was a starting head group for elongating polysaccharide. The co-localized distribution of polyacetylated sugar and the uridine diphosphate N-acetylateglucosamine are actually suggesting that there are polysaccharides synthesizing processing happening in this area. More study need to be carried for further understanding the process of this polyacetylation and its function.

Bacteria species was discovered in lichen species, but the function of these residential bacteria inside lichen are no much known[13, 20]. Some scientists have pointed out that some bacteria could fix nitrogen with their nitrogenase and constantly supplied nitrogen source to the community[13, 21]. However, that does not happen in all a lot of lichen because a lot of photobiont in lichen are capable to fix the nitrogen and provide nitrogen source to the polymicrobial community. In the three dimensional molecular map, there are at least two bacteria derived molecules showed a significant distribution in interior of *Peltigera sp.* lichen. In figure 2.3, two bacteria derived molecules are shown in the cartoon model. One is a lipid molecule and it was also found in one of the *Peltigera sp.* isolates. From the MS/MS spectrum in figure 2S8, it was similar to the MS/MS spectrum of a sterol lipid and showed a lot of 14 Dalton mass shift in the low mass range. The distribution of this molecule is represented by purple dots in figure 2.3, and they are localized beneath the chlorophyll distribution. By comparing the distribution of figure 2.3 (B), (C) and (D), it is obvious that the distribution intensity of this molecule has changed vertically, where top piece lichen has more intense concentration while bottom piece of lichen does not has much distribution. A possible hypothesis of such distribution pattern is that this bacteria-derived sterol lipid was a material for cyanobacteria usage. According to Wang et al 2001[22], cyanobacteria in lichen are capable to synthesis some UV resistance molecules like Vitamin D3 and Vitamin D2, and this distribution has suggested a possible mechanism of how sterol like lipid was made in

Peltigera sp. It is a pity that non vitamin D2/D3 has been detected in the lichen extract, but the distribution of 385 m/z, M+H of vitamin D3, ion on the three dimensional model has clear distribution on the cyanobacteria region as well as light-exposed surface shown in figure 2SX, where will be exposed to a lot UV light. The other bacterial derived molecule is colored in light blue shown in figure 2.3. This molecule has been produced by one of the lichen bacterial isolates, but its structure and function are still not known. According to its three dimensional map shown by figure 2S8, this molecule seems to have strong molecule distribution at the bottom piece of lichen, but the type of molecule and its structure still need to be further investigated.

For a polymicrobial community, such as *Peltigera sp.* lichen, it is reasonable to observe antibiotic in the community. In addition, it is reported that amount of species in lichen will keep a steady relationship from each other because alternation of the balance between photobiont and mycobiont will endanger the symbiotic relationship[3]. One of the known antibiotics, novobiocin, is observed in the *Peltigera sp.* lichen extract, and its MS/MS is shown matched with previous reported data shown in figure 2S8. For lichen species, it was previously reported to produce novobiocin analog, aminocoumarin, inside *Cladonian gracilis*[23], but no literature has been reported the production of novobiocin in lichen previously. In figure 2S8, the distribution of novobiocin is putatively based on m/z 615.23 [M+3] from the three dimensional molecular map of *Peltigera sp* lichen because of the

ambiguity between the isotope of one of the multiple acetylated sugar, 613.24 [M+2], and the parent mass of novobiocin, 613.23 [M+1]. According to the distribution pattern shown in figure 2S8, novobiocin's distribution is mainly localized at the top piece of lichen than the middle and bottom pieces. The top piece of lichen would be consider source rich layer because of large quantity of photosynthesis carried by cyanobacteria, and, as a result, fast microbial growth rate within this lichen piece would be expected, especially fast grow bacteria species. It will make sense that the existence of novobiocin is to suppress some species growth in order to maintain they own nutrition. Specifically, the putative distribution of novobiocin is closed to the cyanobacteria distribution, and the reason behind this is now not clear. Indeed, less is known about the antibiotic effect of novobiocin to cyanobacteria in general. However, it is reported that novobiocin could not do much effect on DNA replication of *Synechocystic* 6803, a cyanobacterium[24]. Indeed, *Synechocystics* 6803 could up-regulate expression of large amount of stress-inducible genes when it stressed by cold, heat and salt environment with present of novobiocin as response to acclimatization[25, 26]. Further study is needed for identifying the effect of presence of novobiocin around cyanobacteria distribution area. One possibility would be the presence of novobiocin could help cyanobacteria to adapt the sudden change of environment. Because novobiocin is a DNA gyrase antibiotic, the present of novobiocin around cyanobacteria might help cyanobacteria to defense themselves.

Also, for a lichen species, it would be extremely common if the whole polymicrobial community encounter poor nutrient environment or suddenly environmental change such as sudden iron deficiency or sudden dehydration. Lichen has been reported to obtain self-nutrition enrich system such as siderophore production or ion uptake activation[27, 28]. A potential siderophore like polypeptide molecule has been observed and its distribution layer, deep blue dots shown on figure 2.3 (B), has shown within the chlorophyll layer as well as penetrating both the fungal lipid layer at its top and another lipid layer at its bottom. In the figure, ion 1101 m/z has been shown only distributed on the lichen surface exposed to sun, but not to the surface attached to ground. However, the polypeptide layer did not distribute distinctly at the middle and the third piece, represented by figure 2.x! (C) and figure 2.x! (D). Because it's within the chlorophyll layer, which must produced by cyanobacteria in lichen, this polypeptide are it could be a bioactive molecule secreted by the cyanobacteria for defending themselves from surrounding environment. Also, as top piece in the lichen section slide is facing to sun and expose to air while bottom piece is growing with grass, this peptide could be a prominent peptide for lichen polymicrobial community because it was locating at the air lichen surface interface. Because this peptide is unique and distributed only at the top piece of *Peltigera sp.* lichen, high resolution MS/MS was collected by LTQ-FT MS and Bruker Amazon MS. Several distinguished amino acid was purposed according to the MS2

spectrum shown in figure 2S7. Due to limited of samples, purified 1101 m/z molecules from *Peltigera sp.* lichen sample are not sufficient for NMR analysis. Instead, a well performed MS/MS spectrum has been collected. In figure 2S7, the 156 Dalton mass shift is suspected to be formyl-hydroxyl ornithine residue, which is commonly used as siderophore residue. More study is needed to confirm its molecular identity.

In short summary, the three dimensional molecular distribution map of *Peltigera sp*. lichen directly visualized the chemistry dynamics inside *Peltigera sp*. lichen polymicrobial community such as photosensitive molecule, lipids from both fungal and bacterial species, antibiotic, polypeptide, antioxidant and photosensitive molecules. As lichen species is inherently a three dimensional object, it would be expected and, indeed, observed gradient change in lichen across various molecules. In addition, with the spatial distribution available, a lot of biology reference could make sense and the some scientific hypothesis could be drawn.

2.4 Conclusion:

The chemistry of lichen polymicrobial community has been studied through traditional chemistry method such as combining organic solvent extraction followed by structure elucidation analysis. The novel three dimensional molecular maps of *Peltigera sp.* lichen technique has given a new way to investigate complete lichen molecular. Even though the 3D lichen

polymicrobial metabolites maps mainly unveil the chemistry world of lichen polymicrobial community, it could still guide us to identify some surprising biological information inside the polymicrobial community by co-relating some finger print molecules to its producers. This capability of bridging the lichen extract and lichen isolates help us to further investigate the chemical structure and hypothesize the spatial location of these particular microbial. In conclusion, the three dimensional molecular map of *Peltigera sp*. lichen is a promising method for investigating both chemical and biological information of the *Peltigera sp.* lichen, and it might be applied on some other similar polymicrobial system as well.

2.5 Method:

Lichen spacemen preparation:

Lichen spacemen used were completely dried sample under double seals. A dimension of 3cm by 1.8cm lichen fragment was cut off from whole piece of lichen by sterile scissors. Vine was taken off by sterile clips from the back of lichen spacemen, and then, the spacemen was ready to go for 3D modeling.

3D Model of Peltigera sp. Lichen Preparation:

Prepared *Peltiegra sp.* lichen spacemen was placed on a pin at the center of the stage of NextEngine 3D scanner. Then the lichen spacemen was scanned by 360 degree rotation at -15, 0, 15 degree stage angle. 16 scans were collected in each rotation, and thus 48 scans in total for assembling the lichen model.

Lichen model was filled holes and polished by Geomagic 2013 Trial Version. Mesh Doctors function was used as a main tool for detection and elimination for small holes.

Outward Metabolites 3D Map of Peltigera sp. Lichen:

Lichen sample was placed in a p-tri dish and fixed by long metal clips. A BD PrecisionGlide 1.6mm x 40mm needle was used to scrape off a small piece of fragment from lichen surface. Then, this small piece of fragment is collected and transferred to an eppendorf tube that contained 50uL, 40: 60, and water to acetonitrile mixture solution for extraction. A total number of 110 samples were collected on the lichen surface. All collected fractions were put into 4C fridge for overnight extraction. 1uL of overnight extracted lichen fraction was well mixed with 1uL Dihydroxylbenzoic Acid (DHB) MALDI matrix. Then, 1uL of the mixture was spotted on 384 Ground MALDI metal plate, and MALDI data was obtained. The same process was applied to the rest 109 samples. In order to extrapolate spatial XYZ information of all scraped spots on the 3D model, all scraped spots position were physically recorded by marking on high resolution pictures, and the whole process of

scraping was videotaped. For helping to extrapolate XYZ coordinates information, a 3D model editing software, Geomagic 2012, was used[29]. All 110 extrapolated XYZ coordinates was collected and ran by algorithm with corresponding LC/MS data.

Internal Metabolites 3D Map of Peltigera sp. Lichen:

A 2.45mm x 1.85mm x 3.03mm lichen block was cut off by a sterile blade from the same scrapped lichen piece. This piece of lichen block was embedded into 25mg/ml gelatin water solution, and immediately let it float on isopropanol-dry ice liquid mixture bath for freezing to prevent diffusion of metabolites. Frozen block was placed into Leica cryostats at 21C for 2 hours to equilibrate the temperature between the block and its environment. Then, the block was sectioned into 12um thickness slices by cryostats, and each slice was transferred to an ITO conductive glass slide. A total 6 slices were collected; each collected slice was about 100um to 150um away from its consecutive collected slices. DHB matrix was applied on the sample containing ITO glass slide with matrix sublimation method in sublimator. Then, the matrix covered ITO glass slides were sent to Bruker Autoflex for MALDI imaging mass spectrometry with 15um raster steps resolution. All MALDI imaging mass spectrometry data was spatially aligned and visualized in 3D SCiLS lab software.

Metagenomic Sequencing of Peltigera sp. Lichen:

A small amount, 1cm x 1cm, of lichen piece sample were performed metagenomic sequencing by paired-end Illuminum sequencing method. The whole genome information of this *Peltigera sp*. was acquired. This piece of genomic data were performed quality control and data preprocessing by Prinseq-lite 0.20.3. Preproccessed data was uploaded to MG-RAST online metagenomic server for assembling and taxonomy identification.

2.6 Reference:

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2.7 Supplement Figures:

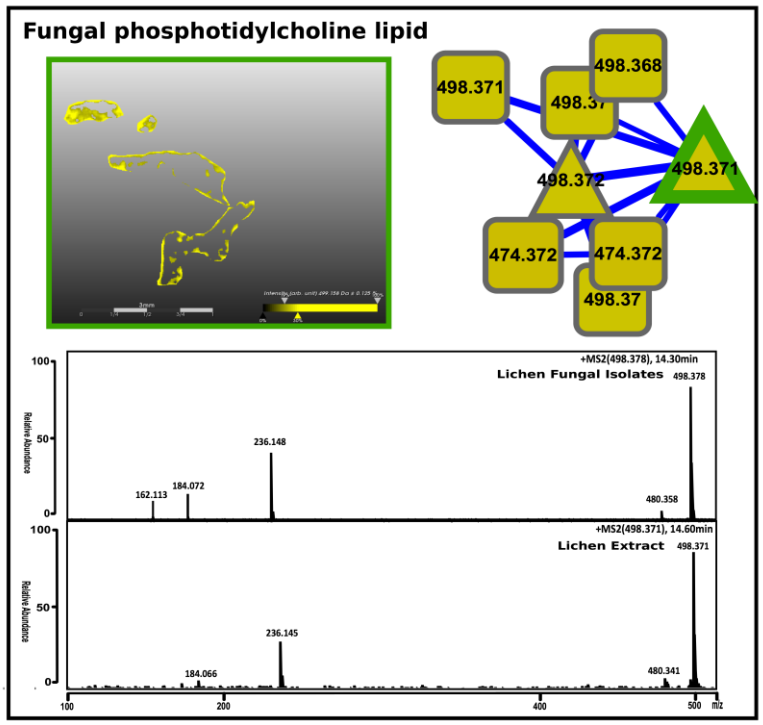


Figure 2S1: Fungal Phosphotidylcholine Lipid

The top left figure showed distribution of a fungal phosphotidylcholine lipid of *Peltigera sp*. lichen, and most intense area are edges of each piece of *Peltigera sp*. lichen. Top right showed a molecular networking cluster of m/z 498 and its derivatives. Two ms/ms spectrums of m/z 498 from the highlighted node have been shown at the bottom of the figure. Upper spectrum showed ms/ms pattern of m/z 498 from an lichen fungal isolate, and it matched with lower spectrum, which showed ms/ms pattern of 498 from lichen extract. The retention time are the same for these two spectrums, and this further confirm that two m/z 498 molecule are same molecules, and the lichen fungal isolate might be one of the producers to produce this molecule in lichen polymicrobial community.

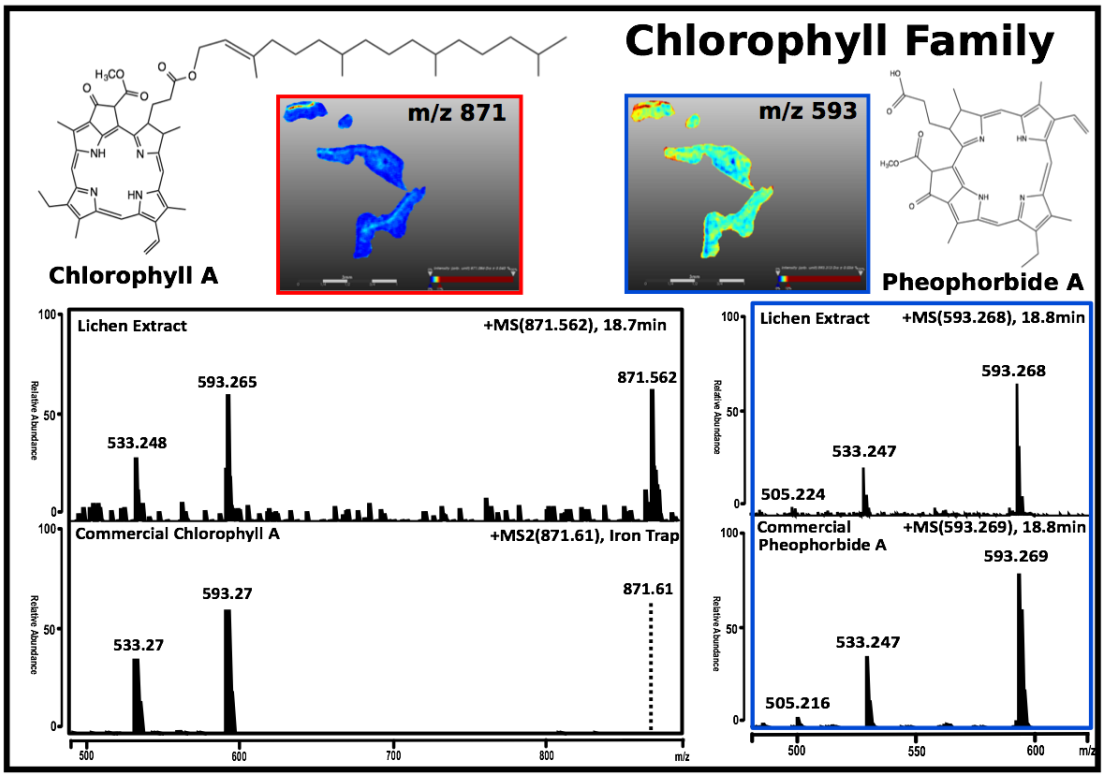
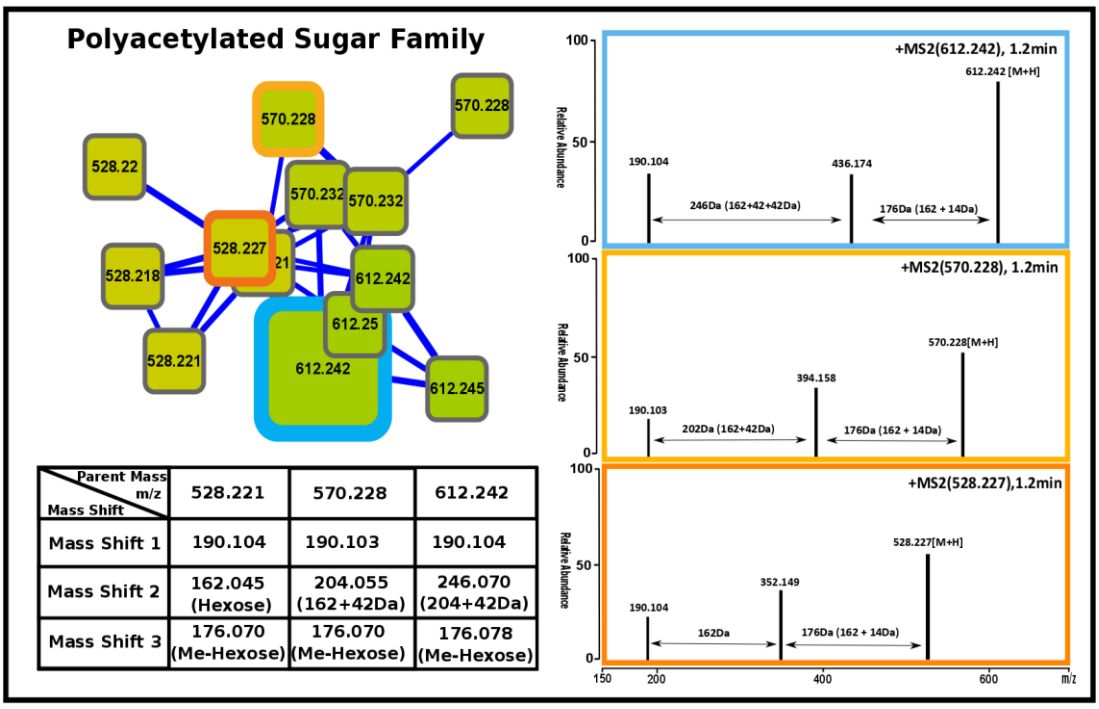


Figure 2S2: Chlorophyll Family

This figure has shown ms/ms spectrums of two chlorophyll molecules and their molecular distribution on one slice of *Peltigera sp*. lichen. Both chlorophyl molecules ms/ms spectrums from lichen extract are confirmed by purchased commercial products. The fragmentations of commercial chlorophylls are matched with two lichen extract molecules, and retention time matched as well. Both chlorophyll distributions are extremely similar to each other, and most of chlorophyll molecules are distributed at the top piece of lichen. Because it is expected that middle piece and bottom piece of lichen are less exposed to sunlight, so observed chlorophyll distribution reduced obviously as shown in both distribution figures.



Parent Mass m/z / Mass Shift	528.221	570.228	612.242
Mass Shift 1	190.104	190.103	190.104
Mass Shift 2	162.045 (Hexose)	204.055 (162+42Da)	246.070 (204+42Da)
Mass Shift 3	176.070 (Me-Hexose)	176.070 (Me-Hexose)	176.078 (Me-Hexose)

Figure 2S3: **Polyacetylated Sugar Family**

This figure showed a molecular networking cluster of polyacetyalted sugar mass of m/z 528.242, m/z 570.242 and m/z 612.248. Their MS2 spectrums were shown on the left hand side. From their MS2 pattern, this series of molecules contained three parts, a m/z 190 daughter mass, an acetylated mass shift, which are m/z 162, m/z 204 and m/z 246, as well as a m/z 176 mass shift, which is putatively methylated hexose. The acetylated mass shift have indicated a polyacetylation addition mechanism because all mass shift were different by 42 Da. In addition, from the second fragment of m/z 528 molecule, the acetylation addition was started from a hexose, 162 Da.

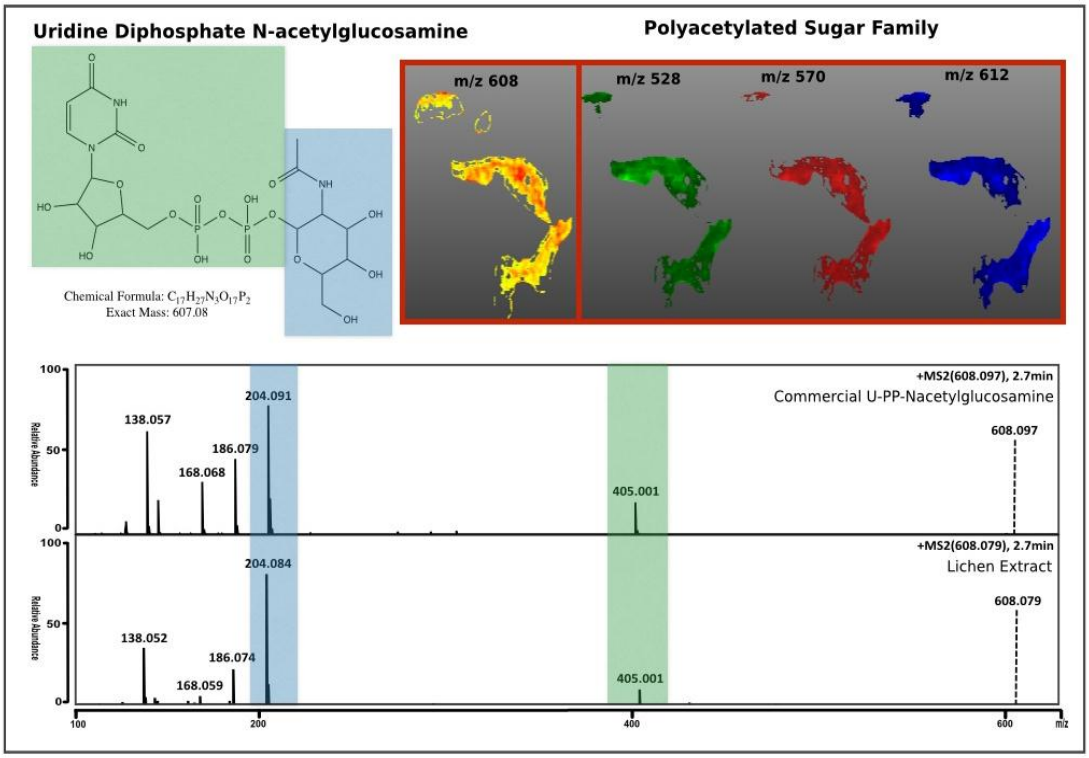


Figure 2S4: **Uridine Diphosphate N-glucosamine**

Figure has shown molecular distribution of four molecules in *Peltigera sp*. lichen slice. m/z 608 is Uridine Diphosphate N-acetylglucosamine, and m/z 528, m/z 570 and m/z 612 are polyacetylated sugars. The structure of Uridine Diphosphate N-acetylglucosamine is shown on the left-top side of the figure, and the ms/ms pattern of Uridine diphosphate N-actylglucosamine and its commercial product are shown at the bottom of the figure. Two major fragment peaks, m/z 405 & m/z 204, are highlighted, and the retention time of these molecules are matched. The distribution of Uridine diphosphate N-aceylglucosamine and all polyacetylated sugar molecules detected in *Peltigera sp*. lichen have similar distribution as shown in the top right figure.

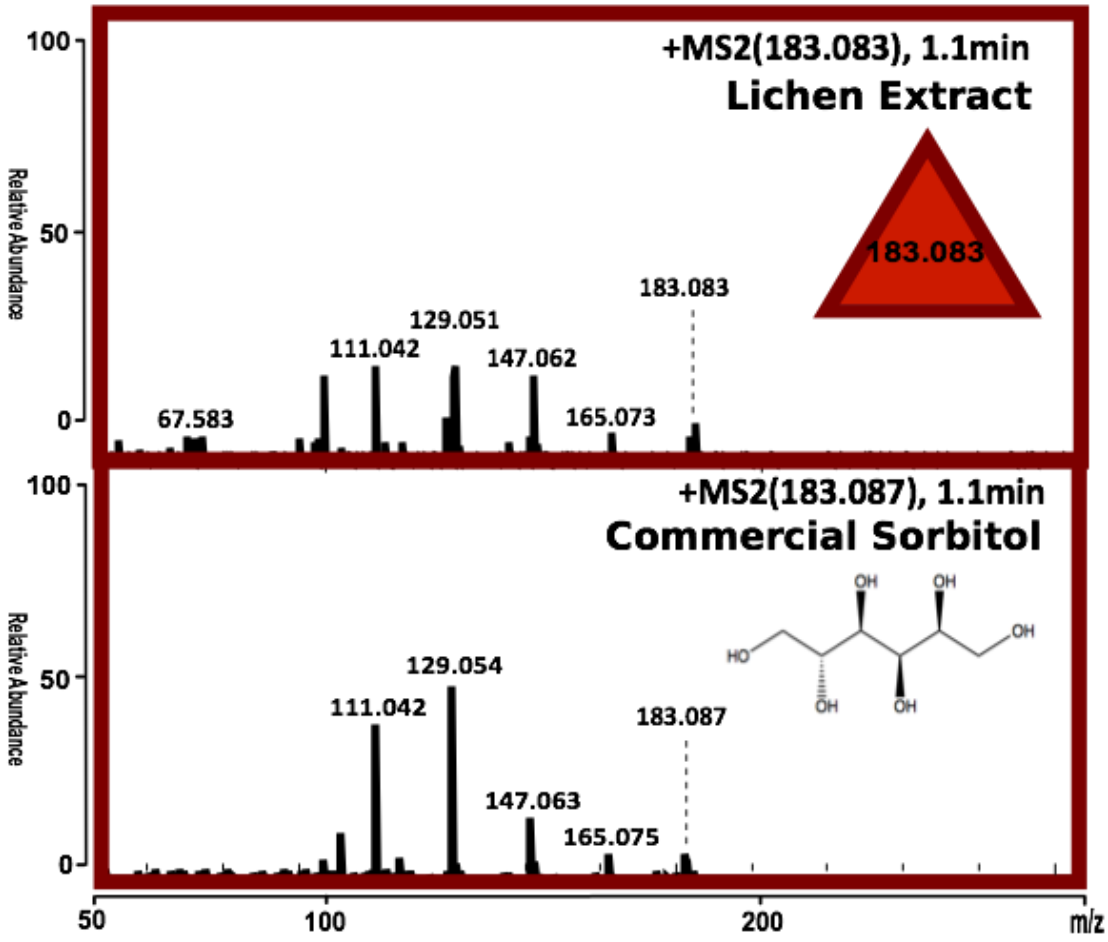


Figure 2S5: Hexose Alcohol

This figure showed a two ms/ms spectrum. One is *Peltigera sp*. lichen extract. The other is from commercial sorbitol. Two ms/ms fragmentation completely matched indicating the lichen extract molecule is sorbitol, or it is one of the hexosal alcohol family.

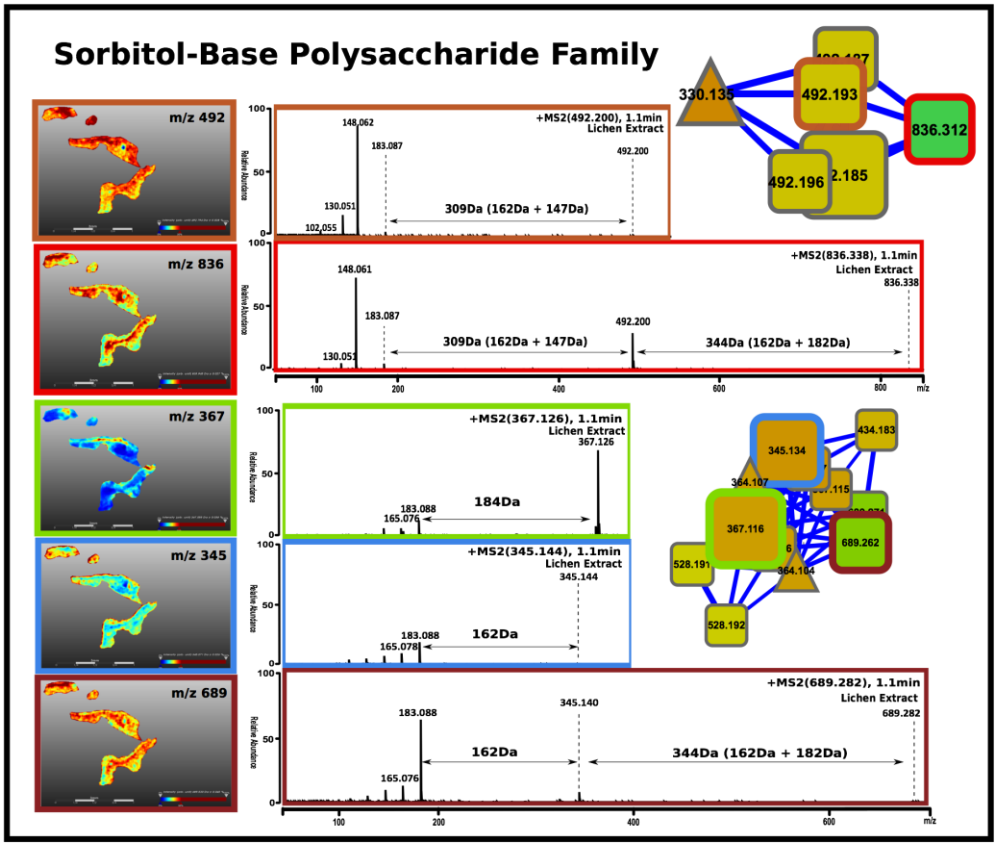


Figure 2S6: **Hexose-base Polysaccharide Family**

This figure has shown two clusters of sorbitol-base polysaccharide from the molecular networking in figure 2.3. Five ms/ms of sorbitol-base polysaccharide molecules were shown and highlighted with different colors. All polysaccharide has 183 m/z in their fragment pattern. This confirmed that these are sorbitol-based polysaccharide. The mass shift 162 Da between peaks in the ms/ms spectrum represented a dehydrated glucose. 182 Da mass shift is a sorbitol residue, and 147 Da was unknown residue, but it was suspended to be five carbon sugar alcohol because m/z 148 was shown in top two ms/ms fragmentation pattern. Left hand side showed all the distributions of corresponding sugar molecules located at the right hand side.

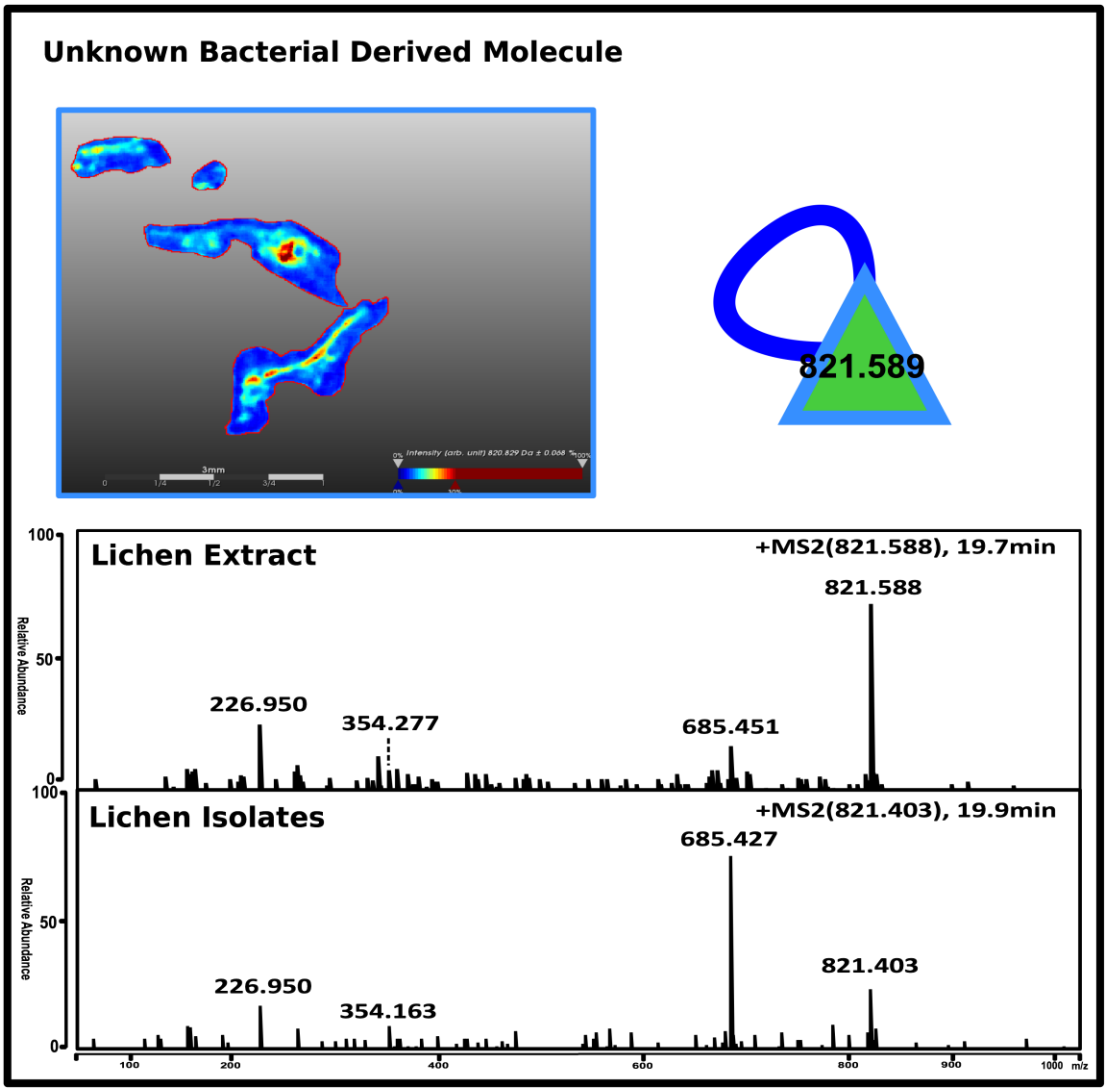


Figure 2S7: **Unknown Bacterial Derived Molecule**

This figure has shown ms/ms spectrums and its internal distribution of a bacterial derived unknown molecule, m/z 821. The ms/ms spectrums from both lichen extract and lichen isolate were shown at the bottom. The peaks matched perfectly with these two ms/ms spectrum. The left-top right side showed a distribution of m/z 821 molecule. It's majorly distributed at the middle and bottom pieces of the *Peltigera sp*. lichen.

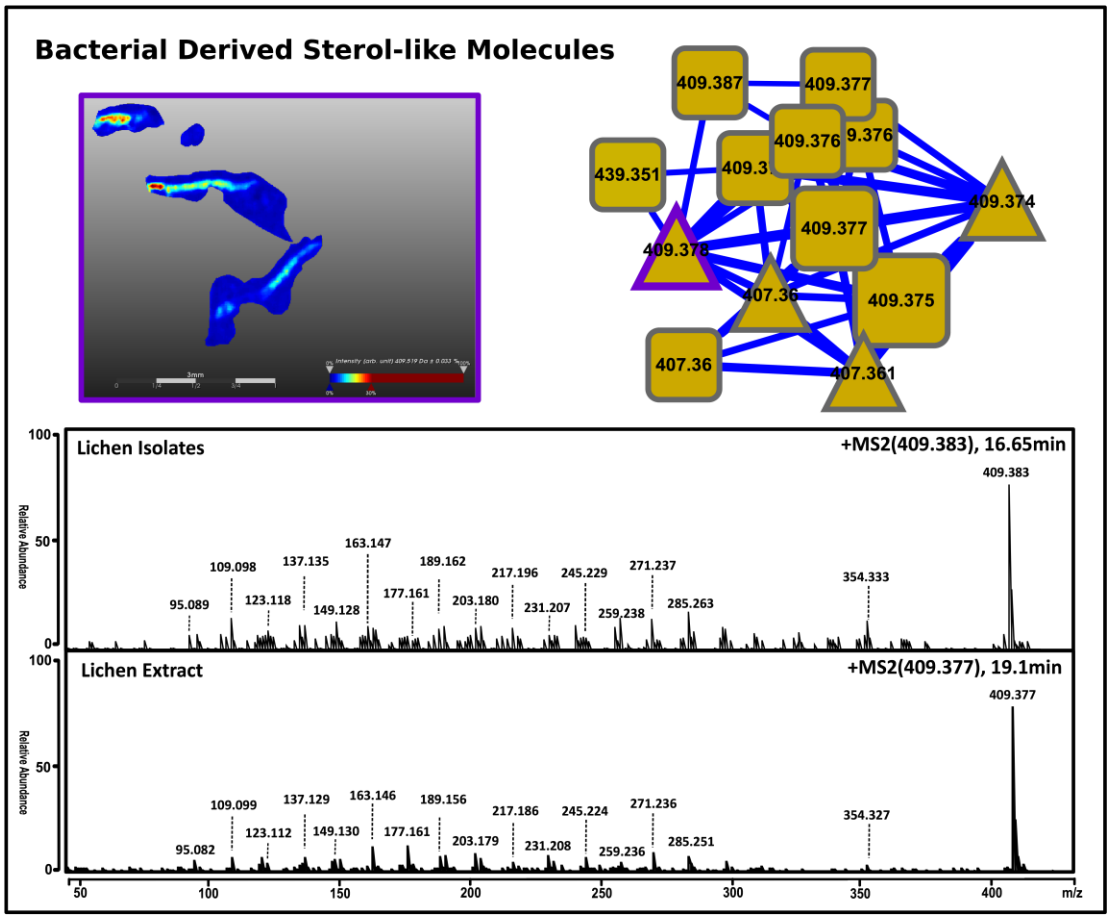
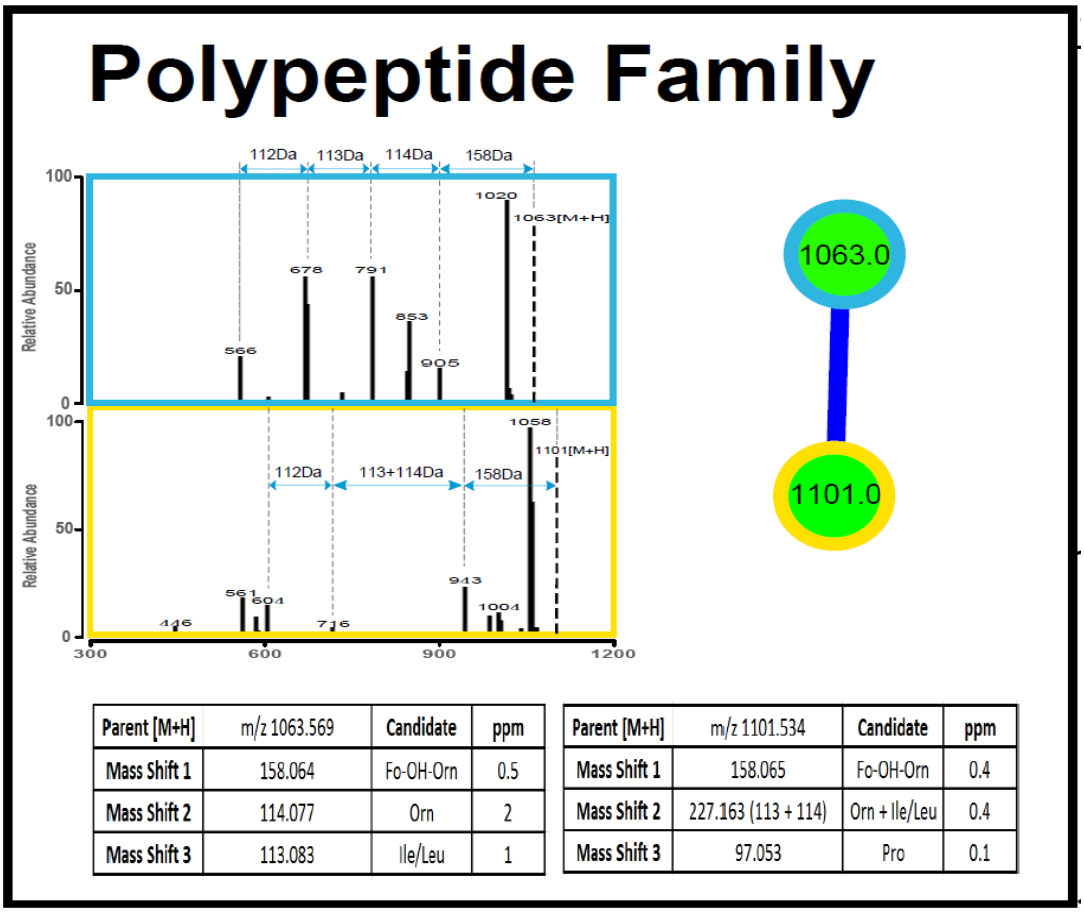


Figure 2S8: **Bacterial Derived Sterol-like Molecules**

This figure has shown ms/ms spectrums and its internal distribution of a bacterial derived sterol-like molecule, m/z 409. The ms/ms spectrums from both lichen extract and lichen isolate were shown at the bottom. The peaks matched perfectly with these two ms/ms spectrum. The left-top right side showed a distribution of m/z 409 molecule. It's majorly distributed right beneath the chlorophyll layer shown in 2S2 of the *Peltigera sp*. lichen.



Parent [M+H]	m/z 1063.569	Candidate	ppm
Mass Shift 1	158.064	Fo-OH-Orn	0.5
Mass Shift 2	114.077	Orn	2
Mass Shift 3	113.083	Ile/Leu	1

Parent [M+H]	m/z 1101.534	Candidate	ppm
Mass Shift 1	158.065	Fo-OH-Orn	0.4
Mass Shift 2	227.163 (113 + 114)	Orn + Ile/Leu	0.4
Mass Shift 3	97.053	Pro	0.1

Figure 2S9: **Polypeptide Family**

This figure has shown ms/ms spectrums of an observed polypeptide molecules. These ms/ms were collected in LTQ-FT instead of Bruker Maxis Impact because it received better fragmentation signal of this molecule. m/z 1101 is potassium add-up of m/z 1063. A table of some putative residues of this polypeptide has been shown at the bottom of the figure.

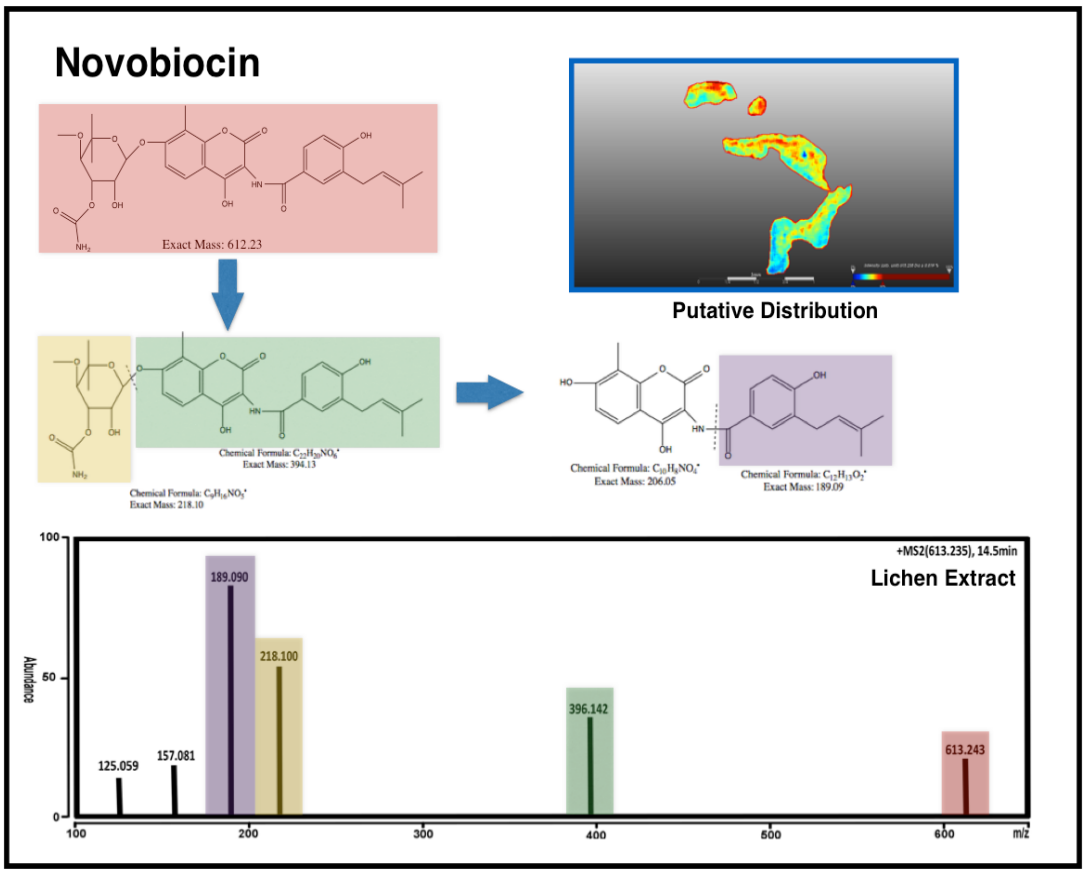


Figure 2S10: **Novobiocin**

This is an MS2 figure of novobiocin found in lichen extract. All colored shape rectangle indicated different fragments of novobiocin, and MS2 pattern has matched with previously published result. Top right hand side showed novobiocin distribution on one of *Peltigera sp.* sectioning slice. The distribution indicated most novobiocin tended to distributed on the top piece of *Peltigera sp.* lichen.

Chapter 3 :

Future Directions

CHAPTER 3: Future Directions

3.1 Abstract:

In this chapter, I will show how an extended application on the three dimensional molecular map to investigate possible objects of applying three dimensional molecular map. In previous chapters, we have introduced a method to combining 3D model, MALDI-TOF IMS and LC/MS to generate a three dimensional molecular map of a *Peltigera* lichen object. However, this piece of lichen is relatively flat and the idea of 3D modeling might not be conveyed if another object with different shape. Here, we have showed a modified technique to obtained a three dimensional map of a Brazil wasp. Different from previous chapter, the three-dimensional molecular map is constructed based on MALDI TOF IMS analysis. The breakthrough technique has been shown here would be method for extrapolating xyz coordinates from three dimensional wasp model and correlate them with the MALDI IMS data set.

3.2 Research Goals:

The ultimate research goal for this project is to study the chemical profile on wasp. From the advantage of constructing three dimensional molecular map, it would be excited to see the molecular distribution on a wasp and the gradient change. This might help us to understand some habitat of the wasp. This current research is for investigating the possibility of constructing three dimensional

molecular map on an object that has uneven surface, for example a wasp. Therefore, I will focusing on constructing a molecular 3D map of a wasp.