UNIVERSITY OF CALIFORNIA, SAN DIEGO

Study of Specialized Metabolites Produced by *Pseudomonas aeruginosa* Using Mass Spectrometry

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Chemistry

by

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The Thesis of Jinshu Fang is approved, and it is acceptable in quality and form for the publication of microfilm and electronically:

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Chair

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DEDICATION

To my parents, for all your love and support.

To my husband, for everything.

And to Lord Jesus, the One who loves us in forever.

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LIST OF ABBREVIATIONS

DHB	2,5-Dihydroxy benzoic acid
CID	Collision Induced Dissociation
Da	Dalton
ESI	Electrospray Ionization
ESI-MS	Electrospray Ionization Mass Spectrometry
IMS	Imaging Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MS	Mass Spectrometry
m/z	Mass-to-Charge
MALDI	Matrix Assisted Laser Desorption Ionization
MALDI-IMS	Matrix Assisted Laser Desorption Ionization Imaging Mass Spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
μL	microliter
MS/MS	Tandem Mass Spectrometry
MS^2	Tandem Mass Spectrometry
TOF	Time of Flight

CF	Cystic Fibrosis
PYO	Pyocyanin
PCA	Phenazine-1-carbonxylic acid
PQS	*Pseduomonas* quorum sensing
HHQ	2-Heptyl-4(1H)-quinolone
PCN	5-N-methyl-l-hydroxyphenazine
Pch	Pyochelin
AHL	*N*-acylated homoserine lactones
QS	Quorum sensing
HQNO	2-Heptyl-4-hydroxyquinolone-*N*-oxide
AHQ	2-Alkyl-4-quinolones
CFU	Colony forming unit
GnPS	Global Natural Products Social Molecular Networking
UPLC	Ultra-performance liquid chromatography

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ABSTRACT OF THE THESIS

Study of Specialized Metabolites Produced by *Pseudomonas aeruginosa* Using Mass Spectrometry

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Professor Pieter C. Dorrestein, Chair

Specialized metabolites produced by pathogens such as *Pseudomonas aeruginosa* play multiple roles such as signaling molecules in biosynthesis and chemical communications, virulence factors, or functional molecules in order to develop antibiotic resistance. The characterization and quantitative analysis of the specialized metabolites

production would help us study the virulence of pathogens, or the chemical responses of bacteria under antibiotic treatment. Also, metabolomics study of the specialized metabolites production in large scale of *P.aeruginosa* samples collected from multiple patients at different time frame would guide us to understand the dynamic change of metabolomics profiles of different *P.aeruginosa* strains.

Here we use two mass spectrometry techniques and an advanced mass spectrometry data analysis tool molecular networking to dereplicate and quantitatively compare different levels of specialized metabolites production from various cystic fibrosis related pathogen *P.aeruginosa* strains and the chemical responses of *P.aeruginosa* under different levels of antibiotic treatment.

Chapter 1

Specialized Metabolites Produced by *Pseudomonas areuginosa* and Mass Spectrometry

Pseudomonas aeruginosa is a well-studied Gram-negative bacterium that may infect human beings and causes various kinds of clinical diseases with its compromised natural defenses. It can utilize a broad spectrum of nutrients, and its ability of to form biofilms to further protect the bacteria from antibiotics and from the attack of host immune system. For example, *P.aeruginosa* usually founded in the respiratory system of cystic fibrosis (CF) patients because of the mucus environment caused by the gene defect disease. Various clinic diseases are caused by *P.aeruginosa* infection in different organs, such as in eyes, ears, urinary traction systems and the blood systems. Most severely is the infection in the respiratory system, such as in lung. The chronic infection caused by *P.aeruginosa* in cystic fibrosis patient always associates with accelerated decline lung function. Chronic lung colonization and infection also occur in bronchiectasis, a disease characterized by irreversible dilation of the bronchial tree, and in chronic obstructive pulmonary disease, which is characterized by narrowing of the airways and abnormalities in airflow. Patients with CF usually have airway mucus produced in the respiratory system, which is the major reservoir of bacteria growth. In both upper airway such as in the paranasal sinuses and the lower airways such the conductive and respiratory zones to the bronchi where the bacteria could growth, the upper airway has less airflow and exposure to the host immune system and to antibiotics (1, 2).

As the *P.aeruginosa* moves from the environment to the CF airway, it could only survive if the bacteria could adapt the stressful environment conditions. Fundamental conditions such as antibiotics and the host immune system both affect the colonization of bacteria. Also, competing with other co-exist microorganisms and changing environment of the CF airway as the infection developed are also factors that would affect the adaption

and evolution of *P.aeruginosa* (3). Among all these factors**,** antibiotics not only affect the environment *P.aeruginosa* existing in CF airway, but also highly associate with *P.aeruginosa*-related CF infection as the major treatment nowadays. Since *P.aeruginosa* is recognized as one of the CF-associated pathogens, the infection by *P.aeruginosa* is highly influenced by the usage of antibiotics as the major treatment. Patients with CF always treated with large amount of antibiotics, so antibiotics-resistance as well as the antibiotics may both highly affect the adaptation of *P.aeruginosa* in CF airway (4). As antibiotics and antibiotics resistance are playing roles in *P.aeruginosa* adaptation, works have been done due to the urge to understand the antibiotics resistance mechanism. As one of the common pathogens of infection diseases, *P.aeruginosa* shows highly therapeutic challenge in choosing appropriate antibiotic treatment because of its ability to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection (5). Study has shown that infections with drug-resistance *P.aeruginosa* may associate with significant increases in morbidity, mortality, need for surgical intervention, length of hospital stay and chronic care, and overall cost of treating the infection (6). Furthermore, the prevalence of multiple-resistance strains which show resistance to more than one antibacterial drug classes raise more concern in therapeutic challenge (7).

P.aeruginosa has a complex multiple-signaling system control its metabolism, coordinate its virulence and persistence. And as one of the most complex signaling systems among all microorganisms, it provided a good model to study the effects of the signaling specialized metabolites to the adaptation of strains in host system (8). Specialized metabolites in *P.aeruginosa* refers to these compounds that, different from

common metabolites involved in primary metabolism, are only found under specific conditions. And mostly, specialized metabolites are classes of compounds such as alkaloids, polyketides, nonribosomal peptides that shown antibiotic, or antifungal properties. Other specialized metabolites produced by *P.aeruginosa* such as quinolones, phenazines, rhamnolipids, which are essential for cell-cell communications, metal complexing or modulate bacteria communications (8, 9). In here, we mainly focus on four groups of specialized metabolites that correlated to the signaling system, as the production of the following specialized metabolites would reflect the growth or adaptation of *P.aeruginosa* to the environment such and the host immune system, the antibiotic treatment and so on.

Quinolones Among the complex signaling systems of *P.aeruginosa*, quorum sensing (QS) is considered as a significant regulating system controlling the production of specific genes in a density-dependent manner. In *P.aeruginosa*, the quorum sensing function through cell-to-cell signaling molecules (autoinducers) that consist of homoserine lactones with fatty acid side chain. Such as quorum sensing signaling system (*las* and *rhl*) that operate via the autoinducers, N-(3-oxododecanoyl)-l-homoserine lactone and N-butyryl-l-homoserine lactone. Studies show these two systems could control the activate proteins that induce *P.aeruginosa* virulence genes. Also, another group of signaling molecules, quinolones, was found that its unique cell-to-cell signal system controlling the expression of *lasB* gene, which encodes for the major virulence factor, LasB elastase. It is shown that one of the 4-quinolone molecules, 2-heptyl-3-hydroxy-4-quinolone can function as an intercellular signal sheds light on the role of secondary metabolites and shows that *P. aeruginosa* cell-to-cell signaling, which is why

the quinolone is also considered as *Pseudomona* quorum sensing molecule (PQS) (10). Unlike the *N*-acylated homoserine lactones (AHL) signaling system which is common among Gram-negative bacteria, the 2-alkyl-4-quinolones signaling system (AHQ) is more restricted that only been detected in certain bacteria such as *P.aeruginosa*. It may indicate that The AHQ signaling system is an important factor establishing the chronic infection (11, 12). PQS has shown the function to control the production of some virulence secondary metabolites such as pyocyanin (PYO), rhamnolipids and siderophore pyoverdine, which will be discussed more about their functions later. Besides, the biosynthetic precursor of PQS, 2-heptyl-4(1H)-quinolone (HHQ) has also been demonstrated as a signaling molecule in *P.aeruginosa* as well as the molecule modulate the swarming mobility and biofilm formation in *Pseudomonas aeruginosa*. Although PQS is the molecule that was considered as the signaling molecule of the virulence factor of *P.aeruginosa*, study has shown it is not the sole inducer of another essential transcriptional regulator protein that required for producing QS-controlled virulence factors since it regulates several of virulence factors generation such as pyocyanin (PYO), as well as 4-hydroxy-2-alkylquinolones (HAQs) biosynthesis (13). Both HHQ and PQS are shown to have the ability to potentiate the DNA-binding activity of the MvfR protein, as MvfR ligand *in vivo*. Also, even HHQ is the precursor of PQS, it is highly produced *in vivo* and never fully converted (14). Besides HHQ and PQS, other 4-hydroxy-quinolones are still under in unclear mechanisms that require more research.

Phenazines Besides quinolones, there are also some other secondary metabolites that play important roles in *P.aeruginosa* infection. For example, phenazines are one group of the pigmented signal molecules which shown antimicrobial activities.

Phenazines display a broad spectrum of toxicity toward prokaryotic and eukaryotic organisms. They could be produced from various bacteria, but their toxic activity is based on the substitution positions on the heterocyclic ring of phenazines. Phenazines act as electron shuttles to alternate terminal acceptors that could also modify cellular redox state, as cellular signal molecules that regulate gene expression, and contribute to biofilm formation to enhance the bacteria survival. In nature, there are more than 100 different phenazines has been identified (15). Because of the interesting physicochemical properties as pigment signal molecules and redox-activate molecules, as well as a group of metabolites that contributes the biofilm formation and its antibiotic properties, phenazine metabolites have been well studied for years (16). Blue redox-active secondary metabolite pyocyanin (PYO) is the first and well-studied member of the phenazines family, produced only by *P.aeruginosa* (17). Studies have shown that PYO has a crucial role in *P.aeruginosa* infection using alternative *in vivo* host model (18-20). The biosynthesis of PYO is regulated by QS signaling system as mentioned before. Individual study that involve making mutants of QS regulating genes has shown that the *lasR*, *rhlR* and *mvrR-haq* QS systems are all have regulation on pyocyanin production (21). In model study, pyocyanin also shows cruel effects on lung infection in animal model (22). It also has been proved that its presence is associated with high morbidity and mortality in CF patients (23). The biosynthetic precursor of PYO, phenazine-1-carboxylic acid (PCA) is also has been proved to have the ability to increase oxidant formation, cytokine IL-8 release, and adhesion molecules ICAM-1 expression by human airway which contribute to the disease pathogenesis (24). Another phenazine that produced by pseudomonas to defense the environment is 1-hydroxyphenazine (1-HP), which also have

been proved to have toxicity to the host (25). However, since phenazines are produced among various virulence factors during infection, the biological function during infection of all of the phenazines are not clear.

Rhamnolipids Glycolipidic biosurfactants rhamnolipids are exoproduced secondary metabolites that produced by various bacteria. There has been more than 60 different rhamnolipids cogeners or homologues discovered to be produced at different concentration by various pseudomona species. Even the exact physiological function of rhamnolipids is still not clear, most identified activities are derived from the surface activity, wetting ability, detergency and other amphipathic-related properties of these molecules, which provide antimicrobial activities and are involve in surface motility and in biofilm development (26-28). Rhamnolipids are always produced as a mixture of the homologues L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C10-C10), L-rhamnopyranosyl-β-hydroxydecanoate (Rha-C10), 2-O-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10), and 2-O-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C10) as classified, and in *P.aeruginoa* the monorhamnolipids are the predominant component, and other monorhamnolipid with different fatty acid chains are minor components in the mixture, but the percentages are not clear (29).

Siderophores Also, iron-chelating molecule siderophores are crucial for bacterial survival because it is necessary for them to be feed with enough iron ions as necessary cofactors in a lot of enzymatic reactions. It has also been proved that *P.aeruginosa* could produce various kinds to siderophore molecules. However, since the host did not freely provide iron ions during infection but predominantly found in iron-binding proteins such

as ferritin, in order to grow and survive, pathogens need to produce iron-chelating molecules as siderophores. In *P.aeruginosa*, pyoverdine (Pvd) and pyochelin (Pch) are the two siderophores produced to promote biofilm development and bacterial virulence (30). Especially, pyoverdine is the major siderophore that could evade innate defense of host, such as binding to apo-siderophore neutrophil-gelatinase-associated lipocalin (18) from host to protect bacterial from clean out by host. And the other siderophore pyochelin, besides showing moderate affinity in binding iron ions (16), it illustrates strong chelate ability in binding other ions such as zinc (II) and copper (II) (31).

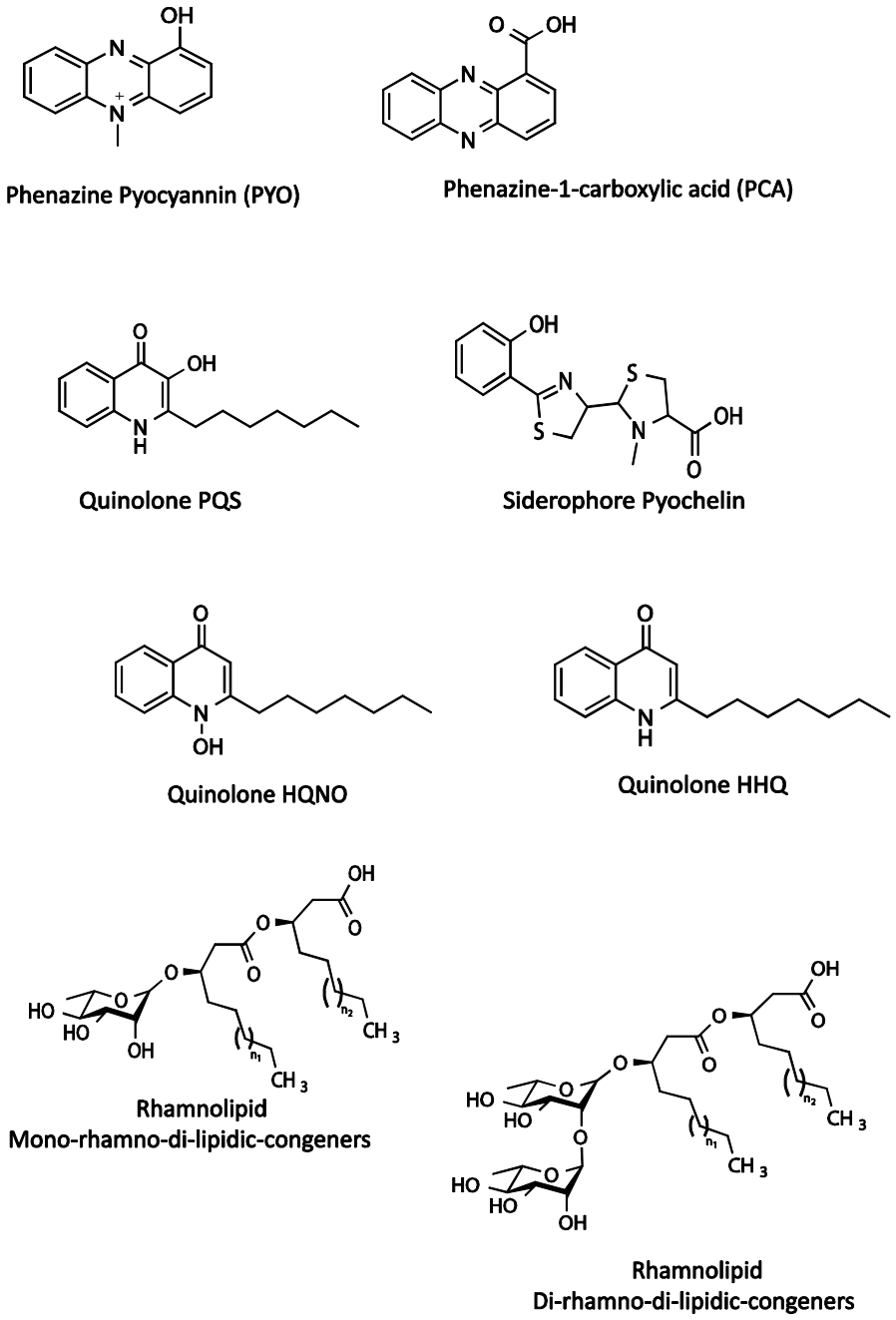

Figure 1-1. Examples of specialized metabolites produced by *Pseudomonas aeruginosa* Phenazines pyocyanin and PCA, quinolones PQS, HQNO and HHQ, mono- and di-rhamnolipids, and siderophore pyochelin.

Mass spectrometry has been proved to be a powerful tool to study the specialized metabolites produced by microorganism, such as using liquid chromatography with mass

spectrometry (LC-MS) to understanding the intricate complexities of microbial metabolism, protein-protein interactions and post-translational modifications (32-43). And with needs to directly analysis of microbial colonies to capture the partial distribution of specialized metabolites, advanced mass spectrometry tools such as imaging mass spectrometry (IMS) has been developed and widely applied in microbial study. For example, matrix assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) has been applied in characterizing specialized metabolites from microbial monocultures or tracking the metabolite transfer within polymicrobial colonies and biofilms (36, 44, 45).

Here in this thesis, we described application of two types of mass spectrometers to study the specialized metabolites produced by *P.aeruginosa*: electrospray ionization mass spectrometry (used in projects described in Chapter 2 and Chapter 3), and matrix-assisted laser desorption ionization imaging mass spectrometry (used in project described in Chapter 3).

Mass spectrometers are consisted of ion source, analyzer and detector as basic components. Mass spectrometry is a technique that generates ions from a sample of either organic or inorganic compounds and detects these ions based on their mass-to-charge ratios (m/z). Analytes from sample can be ionized by bombardment of energetic neutral atoms, or by dissolving sample in a solvent and spraying it out of a capillary. Upon entering the mass spectrometer, ions are separated and sorted based on the behavior of the ion's m/z within the presence of an electric or magnetic field. Eventually these ions would pass by or hit a surface that causes an induced charge to be detected whereupon this information is digitized and converted into a mass spectrum as the output. With this

information, molecular weight and chemical composition can be determined, as a hint to predict the structure of molecule.

In here, MALDI-IMS was used to exam the specialized metabolites production by different *Pseudomona aeruginosa* strains under different concentrations of antibiotic azithromycint. Under MALDI-IMS, it is easy to correlate the metabolite production level with the phenotype of bacteria. To run an analysis of sample in MALDI, the sample is first covered with a layer of matrix. The matrix is a UV absorbing organic acid such as 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid that is simply used to assist the ionization of analyst. In microbial imaging, the thickness of agar media is limited to 0.5-1.5 mm before applying matrix (45) **(Figure 1-2).** This is a physical limitation due to the insertion of the MALDI target plate into the instrument and there is not much space between the vacuum chamber and the MALDI plate during insertion. The mass analyzers for MALDIs are typically time-of-flight analyzers and come in two varieties, linear as well as reflectron analyzers. Detection in these instruments occurs when ions collide with a resistive material, which results in the release of secondary particles that intensify the original signal to be recorded and digitized as a mass spectrum. The principles of both linear and reflectron analyzers are the same: a set of ions with different *m/z* is accelerated towards a detector all with the same amount of energy. The ions of differing *m/z* are dispersed in time as they fly down the TOF tube along a path of known length. Because all the ions start with the same amount of kinetic energy, smaller ions that are less massive will travel along the TOF tube at a greater velocity, thus reaching the detector earlier. In a linear analyzer, the ions are accelerated straight towards the detector, however, a reflectron detector increases the time ions require to reach a

secondary detector by reflecting the ions back in the opposite direction they originally came from (46).

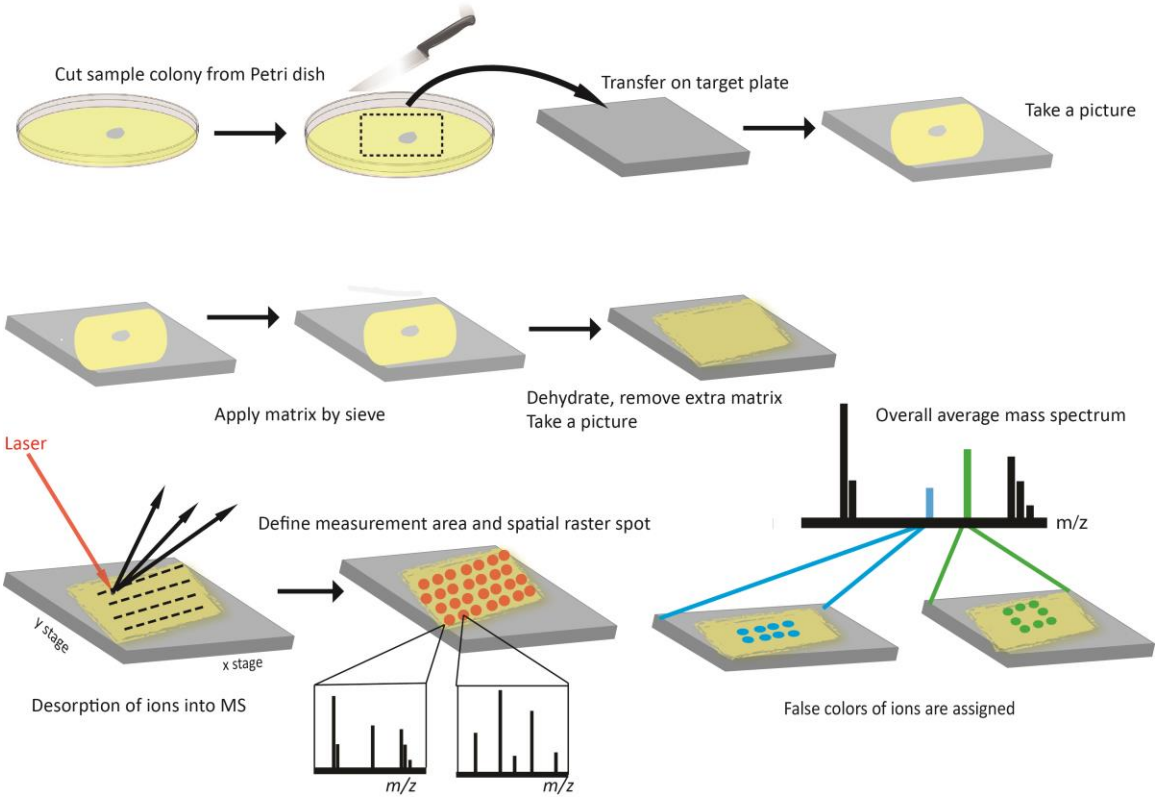

Figure 1-2. Workflow of MALDI-IMS. Sample colony grown on agar in Petri dish is cut out and then transferred onto a MALDI target plate, covered with MALDI matrix by sieve, and waited to be dehydrated before put into MALDI-IMS. In MALDI-IMS, the raster region will be measured as defined. Individual mass spectra at each raster point are collected and the overall results are the averaged out overall spectrum. Each m/z in mass spectrum can be labeled in false color overlapping of the optical image of the sample plate, representing the unique distribution of each ion.

In MALDI-IMS, we were able to detect the spatial distribution of specialized metabolites produced by *P.aeurginosa* under various concentrations of antibiotic azithromycin treatment. However, to identify these specialized metabolites we are interested, and to quantitatively compare the different level of production, a higher resolution mass spectrometry instrument and a liquid chromatogram would be necessary,

as an orthogonal method of specialized metabolites identification. Electrospray ionization mass spectrometry (ESI-MS) is another technique for generating intact molecular ions where a sample is dissolved in an electrospray solvent (a mixture of water with volatile organic solvent and a small percentage of acid) and sprayed out of a fine capillary or needle that is held at high voltage. The emerging liquid is charged and forced into a fine spray of droplets directed towards the inlet of the mass spectrometer. Generally, a heated inert gas is circulated near the inlet of the mass spectrometer to help vaporize the droplets and the vaporized droplets are pushed through ion transfer stages to refocus the electrospray of samples and get into the mass spectrometer analyzer, a hybrid quadruple with orthogonal accelerated time-of-flight mass spectrometer. The ion transfer stages consist of three stages of glass capillary that transmit analytes ions with dry gas with small among of solvent into the vacuum system. The RF voltage applied at the capillary effective confines the ion beam into the funnel. And DC voltage applied at the first and last plate controls the direction of the ion beam to going through the funnel to the next stage. As the ions going through the transfer stages, the ion beam will go through the analytical quadruple and collision cells to isolate and fragment parent ions before going into the TOF-mass spectrometer. The analytical quadruple is the first analyzer that filter the sample of the analysis by isolating a certain ion mass. As in the collision cells, parent masses are fragmented by Collision Induced Dissociation CID) as a collision with neutral gas such as nitrogen to give MS^2 data. After going through the multiple ions transfer stages, quadruples and collision cell, the ion beam with both parent ions and fragmented ions are refocused before into the TOF-mass spectrometer and get to the detector. Combined with pre-separation liquid chromatogram system, the high-resolution ESI-

qTOF (quadruple time-of-flight) tandem mass spectrometry is able to give high resolution MS^2 or even pseudo MS^3 data of sample.

To summarize, the goals of this thesis are to illustrate the applications of the mass spectrometry techniques, MALDI-IMS and ESI-MS to analysis the specialized metabolites produced by *P.aeruginosa*. Chapter 2 introduces the metabolomics study by using MS/MS analysis with powerful data analysis tool molecular networking to quantitatively study the metabolites production patterns of different *Pseudomonas.aeruginosa* clinic isolates. And Chapter 3 briefly describes the applications of these mass spectrometry methodologies to visualize the spatial distribution of those specialized metabolites produced by *P.aeruginosa* when antibiotic azithromycin is treated in MALDI-IMS, and then quantitatively compare the level of production using LC-MS/MS.

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Chapter 2

Metabolomics Assisted Characterization of *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients

2.1 Abstract

Cystic fibrosis (CF) is a common genetic disorder disease affecting more than 30,000 patients in United States alone. Among all the effects caused by CF, frequent lung infection caused by various pathogens such as *Pseudomonas aeruginosa* mostly affects patients since very young age and lead to lung transplants or death eventually, even with aggressive antibiotics treatments. As one of the well studied opportunistic pathogens that commonly found in the sputum or lung tissues of CF patients and cause severe conditions, we would like to study the dynamic evolution of *P.aeruginosa* isolated from CF patients, by tracking the specialized metabolites production patterns among various patients, at different time points after the patient has been diagnosed with cystic fibrosis, and also compared the transition between the specialized metabolites production and the genome mutation. The study of metabolites production pattern would make a good comparison with the genome study of mutations in patient isolated *P.aeruginosa* strains, and give a guideline of the metabolites profiles of CF patients, in assistant of CF diagnoses and treatment.

2.2 Introduction

Cystic fibrosis (CF) is a common genetic disorder caused by one of 1,500 possible mutations of cystic fibrosis trans-membrane conductance regulator (CFTR) gene, about 30,000 individuals are affecting by this disease in United States alone. The mutation causes defect of CFTR protein, resulting in defective chloride ion transport across epithelial cell surface. It decreases the volume of periciliary fluid in the lower respiratory

tract, which in turn interferes with the mucociliary clearance of inhaled microorganisms. Early recruitment of inflammatory defense mediators such as antibiotics is caused by the impairment of the non-inflammatory defenses of the respiratory tract (1). If not treated, most patients with CF die at young age due to airway infections. Although there is no cure for CF for now, aggressive treatment with antibiotics has successfully extended the lifetime of patients to a median age of 40 years in the 2000s (2).

The human airways system distinguished into highly compartmentalized environments with different habitats, from the upper airways paranasal sinuses to the lower airways conductive and respiratory zones. At different region of the respiratory system, airway mucus could be produced. This viscous mucus layer is the major reservoir of bacteria reproduction especially at the respiratory tract, where the presence of bacteria often linked to substantial lung infection disease (3). The microbiota of the respiratory tract in CF patients exhibits a complex diverse multispecies ecosystem. The study of CF lung infection associated pathogens focusing on three bacterial species, *Pseudomona aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenza*. Among them, *Pseudomona aeruginosa* is the major pathogen in adults, with its ability to develop resistance to antibiotics, form impenetrable biofilm and release a large arsenal of virulence factors. Especially, infections with *Pseudomona aeruginosa* always developed into chronic infection, eventually leading to respiratory failure, lung transplantation or even death (4). Chronic infection is shown as continuous reinfection of *P.aeruginosa* in respiratory airways and development of *P.aeruginosa*-specific antibiotics (5). The reinfection of *P.aeruginosa* in the chronically infected lung leads to immune complex-

mediated chronic inflammation, which is the major cause of lung tissue damage and decreased lung function in addition to the damage caused by the bacteria.

As mentioned in Chapter 1, *P.aeruginosa* could only survive if the bacteria could adapt the stressful environment conditions in host. Antibiotic treatment, host immune system, polymicrobial environment would all affect the colonization of bacteria (6). Among all these factors**,** antibiotics not only affect the environment *P.aeruginosa* existing in CF airway, but also highly associate with *P.aeruginosa*-related CF infection as the major treatment nowadays. Since *P.aeruginosa* is recognized as one of the CF-associated pathogens, the infection by *P.aeruginosa* is highly influenced by the usage of antibiotics as treatment. Patients with CF always treated with large amount of antibiotics, so antibiotics-resistance as well as the antibiotics may both highly affect the adaptation of *P.aeruginosa* in CF airway (7). *P.aeruginosa* shows highly therapeutic challenge in choosing appropriate antibiotic treatment because of its ability to develop resistance to multiple classes of antibacterial agents, as the function of adaptation and evolution based on the external environment.(8). Study has shown that infections with drug-resistance *P.aeruginosa* may associate with significant increases in morbidity, mortality, need for surgical intervention, length of hospital stay and chronic care, and overall cost of treating the infection (9). The prevalence of multiple-resistance strains which show resistance to more than one antibacterial drug classes raise more concern in therapeutic challenge (10). Besides antibiotic resistance, the polymicrobial environment *P.aeruginosa* exists is also affecting the adaptation and evolution of *P.aeruginosa*. In CF patients, besides *P.aeruginosa*, other microbial such as *Staphylococcus aureus* and *Haemophilus influenza* are also commonly present.

Clearly, these environmental factors play important roles in the evolutionary dynamics of the pathogen *P.aeruginosa*, which means under various ecology environments, *P.aeruginosa* strains would present different metabolites profiles associated with the physiological environment. In order to study the complexity metabolites profiles of isolated *P.aeruginosa* strains, metabolomics study is the strategy we are using to approach. Metabolomics study is rapidly growing in the past twenty years due to the advanced in analytical and informatics technologies. Metabolomics study has been used into investigate microbial, plants, mammalian and environment systems. The experimental strategies to apply metabolomics into complex systems are in three categories: targeted analysis, semi-targeted analysis and untargeted analysis (also known as metabolic profiling or metabolite profiling) (11). The basic workflow of metabolomics study is using liquid chromatography (LC) tandem mass spectrometry (MS/MS) technique to acquire quantitative metabolites information from samples and combined with statistically analysis software to do data visualization, chemical identification or pathway modeling of the sample system. Different experimental strategies may have various requirements of sample preparation, data accuracy and precision, primary hypothesis and study objective. One major difference between untargeted data mining and targeted or semi-targeted data mining is the need to chemical identification and structure elucidation. For targeted and semi-targeted analysis, the chemical identification is known before the samples being assayed. By applying metabolomics method, we would like to study the dynamic evolution of the secondary metabolites production profile of *P.aeruginosa* isolated from CF patients, based on our hypothesis that different *P.aeruginosa* strains would have different levels of specialized metabolites production.

To study the dynamic evolution of specialized metabolites production of *P.aeruginosa*, we received 101 *P.aeruginosa* clinical isolates from CF patients collected from 1973 to 2013 by Dr. Søren Molin's lab from Technical University of Denmark. The isolates were grown under lab condition and their crude extractions were run under ultra–performance liquid chromatogram (UPLC) tandem mass spectrometry (MS/MS) to acquire the metabolites production profiles.

As one of the leading cause of morbidity and mortality in CF patients, *P. aeruginosa* metabolites classes such as phenazines, quinolones and rhamnolipids are described as signaling molecules which regulating the virulence and persistence. The study and detection of those molecules could give advantage to map the global virulence factors signaling network of *P.aeruginosa* which pose a major threat to human beings (12). As described in Chapter 1, there are different categories of specialized metabolites produced by *P.aeruginosa* that affect the virulence of the pathogen, as well as a reflection of the function signaling quorum sensing (QS) mechanisms, such as the quinolones quorum sensing system and the quinolone quorum sensing molecule (PQS), and those specialized metabolites controlled by the alkyl quinolone signaling system (AHQ) such as phenazine pyocyanin, rhamnolipids and siderophores as described in Chapter 1. Based on previous study on the secondary metabolites produced by *P.aeruginosa*, it is a fair hypothesis that under various physical conditions which may induce the mutation of bacterial to development higher resistance to survive, different *P.aeruginosa* strains isolated from different patients would show various specialized metabolites production level as the reflection of different levels of dynamic evolution of pathogens. It will also be interesting to correlate the metabolites production profile of *P.aeruginosa* isolates with

their genome sequencing information, and the physiological condition of patients that induce the variation of metabolites production.

2.3 Results and Discussion

2.3.1 FindMolecularFeatures and bucket calculation

The primary challenge of this project is to process and analysis this massive MS data. In our project, DataAnalysis (Version 4.1, Bruker) and ProfileAnalysis (Version 2.1, Bruker) were used. The FindMolecularFeature function in DataAnalysis could apply an algorithm to extract background free molecular features based on mass-to-charge value (m/z), retention time in chromatogram, intensity, area under the curve and different analytes from raw data, transferring the complex raw data into a table format. The compound features were detected based on S/N threshold of 5, correlation coefficient threshold of 0.8 which checked for time correlation of neighbored isotopes, minimum compound length of 10 which defined the size of peak cluster, and smooth width of 5 for peak detection. By applying these features, a list of compound spectra can be generated. After that, the compound spectra representing molecular features were loaded into ProfileAnalysis to create a bucket table. In ProfileAnalysis, each bucket e represents a m/z-retention time pair with its area under the curve of that compound spectra. The bucket table generate from ProfileAnalysis was based on parameters of m/z range from 100 Da to 1500 Da, within retention time range from 60.0 s to 720.0 s. By applying these two functions, the LC-MS raw data has been transformed into a 2D statistic table that allowed further data processing.

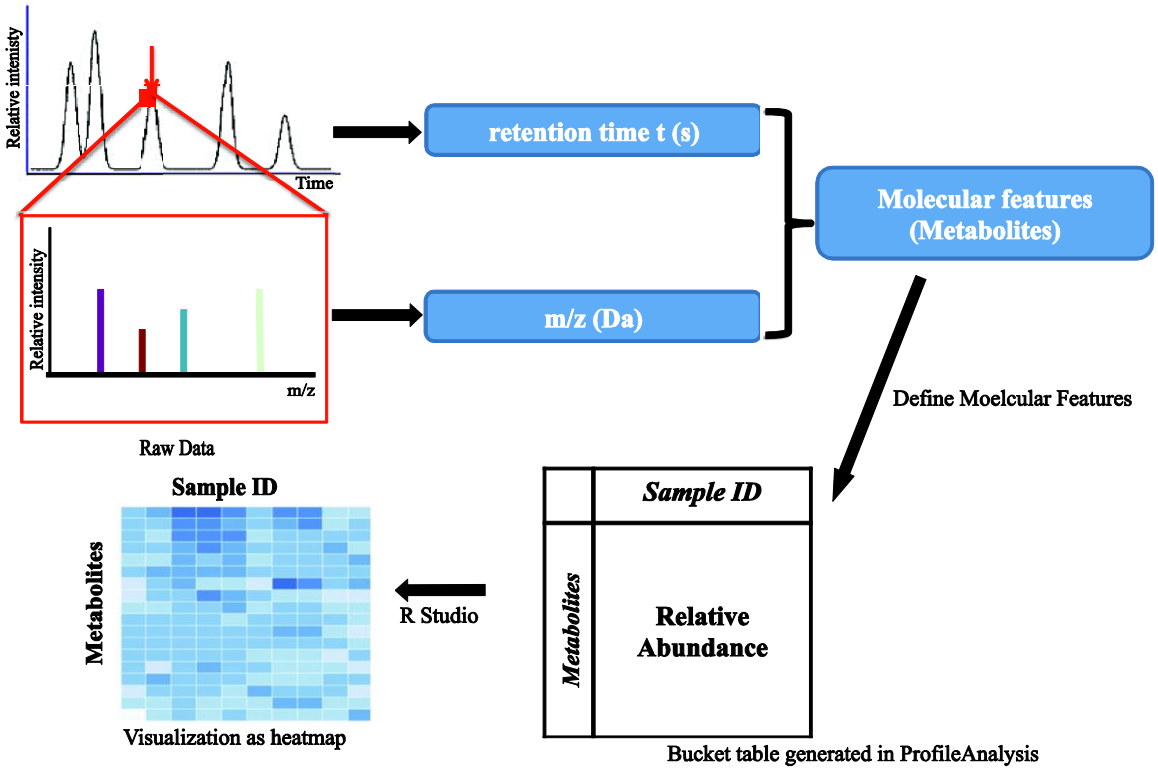

Figure 2-1. Workflow of heatmap generation. The molecular features are defined by the retention time and m/z of ions collected in raw data. All molecular features that above the defined detecting cutoff would be considered as a real metabolites. The abundance of each metabolite from all samples will be summarized as a table in ProfileAnalysis. The table will then be converted to a heatmap to visualize the relative abundance of each metabolite.

2.3.2 Heatmap

To visualize the difference of molecular features in each sample and find possible patterns of specialized metabolites production, we used heatmap to illustrate the molecular features from raw data. The excel file of bucket table from ProfileAnalysis was first exported and then loaded into R program**.** The area under the curve value of each bucket exported from ProfileAnalysis was converted into log2 value and color-coded in R program**.** Pattern based hierarchical cluster was also applied in heatmap.

The heatmap of molecular features illustrated the diversity of possible metabolites produced from each sample, and also the variety of amount of same metabolites produced from sample to sample. The heatmap was clustered based on similarity of metabolic profiling patterns **(Figure 2-2).** However, in this heatmap, since all possible molecular features were included, it is expected to see all of media compounds, primary metabolites and unknown metabolites shown as molecular features. In the overall heatmap include sample plate 1 and its duplicate sample plate 2, the hierarchical clusters based on patterns of molecular features, however, did not cluster the duplicates together. The results indicated the plate effect in sample preparation. Since samples and their duplicates were cultured and extracted on two different 96-well plates, the plate effect indicated possible inconsistency in growth condition, or inconsistent of extraction methods. It was possible that the inconsistent metabolites profile between samples and their duplications was due to different bacteria growth level, or inconsistence sample injection volume in LC-MS in instrument. Overall, the general heatmap of all samples with duplicates did not illustrate the possible profile based on biological difference, but possible variety caused by inconsistence sample preparation process.

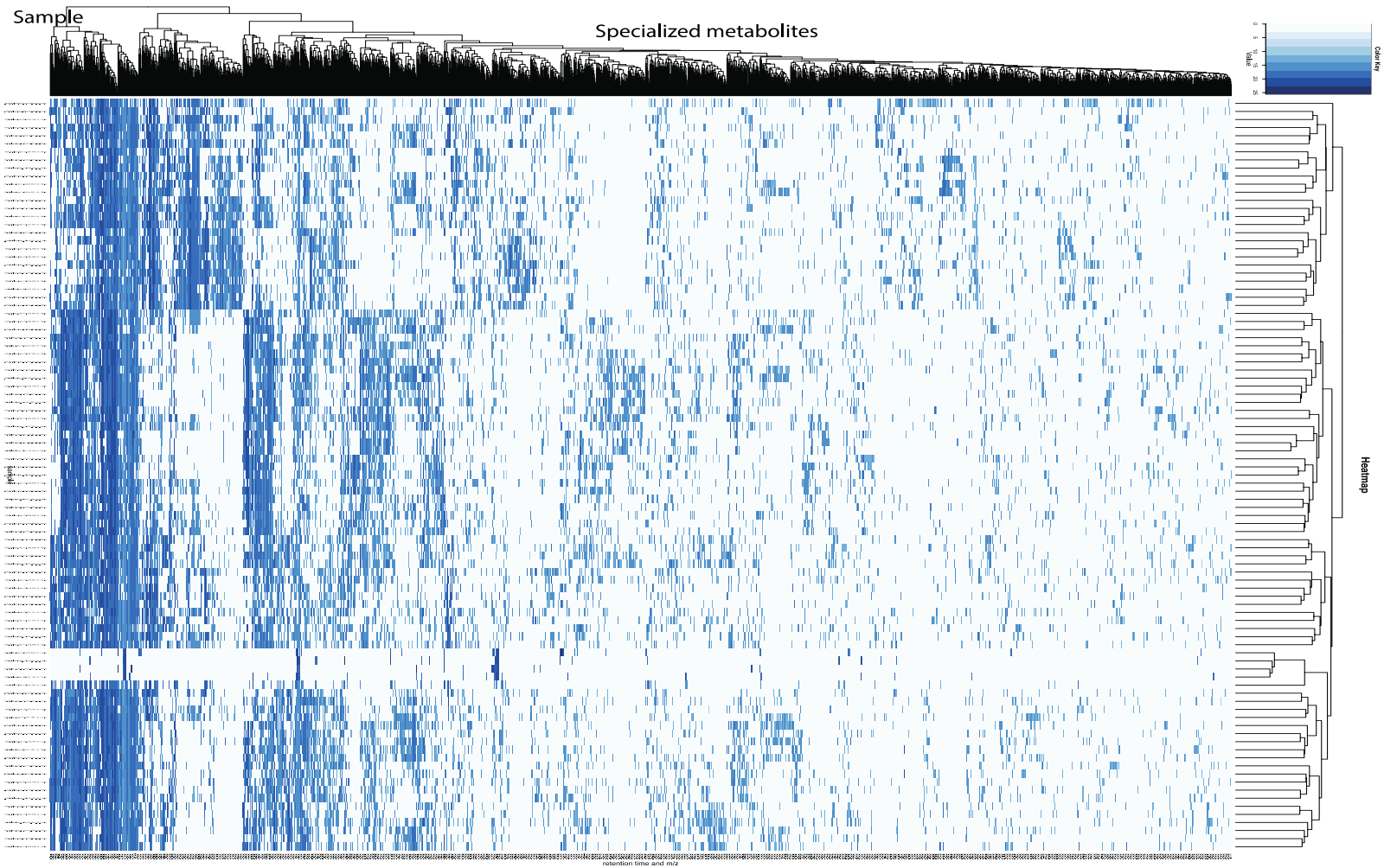

Figure 2-2. General heatmap with hierarchical clusters X axis refers to metabolites, Y axis represents samples

By assuming the inconsistency on the same 96-well plate would be minor, future data analysis would be focus on one plate instead of both. A general heatmap of **Plate 1** was generated using same parameters. Three different hierarchical cluster settings were used to show similarity of metabolites profile patterns among samples. By clustering based on samples, the heatmap shown the similarities of metabolites profiling among different samples; by clustering based on molecular features, the heatmap shown for specific metabolite, the variety in production among all samples. In **Figure 2-2** which shown two-way hierarchical cluster, it was able to visualize the most abundant metabolites produced among all samples. However, with more than 3000 possible molecular features generated from ProfileAnalysis, it was hard to decide a biological metabolites production pattern from heatmap. Although from the statistic data, we could locate the abundant metabolites generate among all samples, it was not possible to interpret the biological meanings behind the observation without known what were those samples. Here we used an advanced MS/MS based data analysis tool to identify specialized metabolites generated from *P. aeruginosa*.

2.3.3 Molecular networking

Molecular networking is a powerful MS based data analysis tool that enables the detection and visualization of related compounds via MS/MS fragmentation spectra relationships within a dataset, as well as analysis individual MS/MS signals by finding diversity of molecules before their chemical structure is fully elucidated (13). MS/MS based molecular networking could guide the molecule identification based on the MS/MS

fragmentation patterns of molecules that were detected as ions. In molecular networking, it is able to visualize related compounds based on similarities of MS/MS spectra, and applying to a global analysis of microbial metabolites. Also, it is possible to provide a view of the composition of many uncharacterized microbial factors (14, 15). More importantly, by cross comparing to a high quality dataset includes identified spectrum, it is possible to dereplicate known metabolites from a massive data set without manually go through all the spectra, which is very time intensive and not practical considering that even one MS sample data may include thousands of spectra. In here, we demonstrated using molecular networking from Global Natural Products Social Molecular Networking (GnPS, http://gnps.ucsd.edu) as a powerful tool to rapidly identified characterized specialized metabolites produced by bacteria.

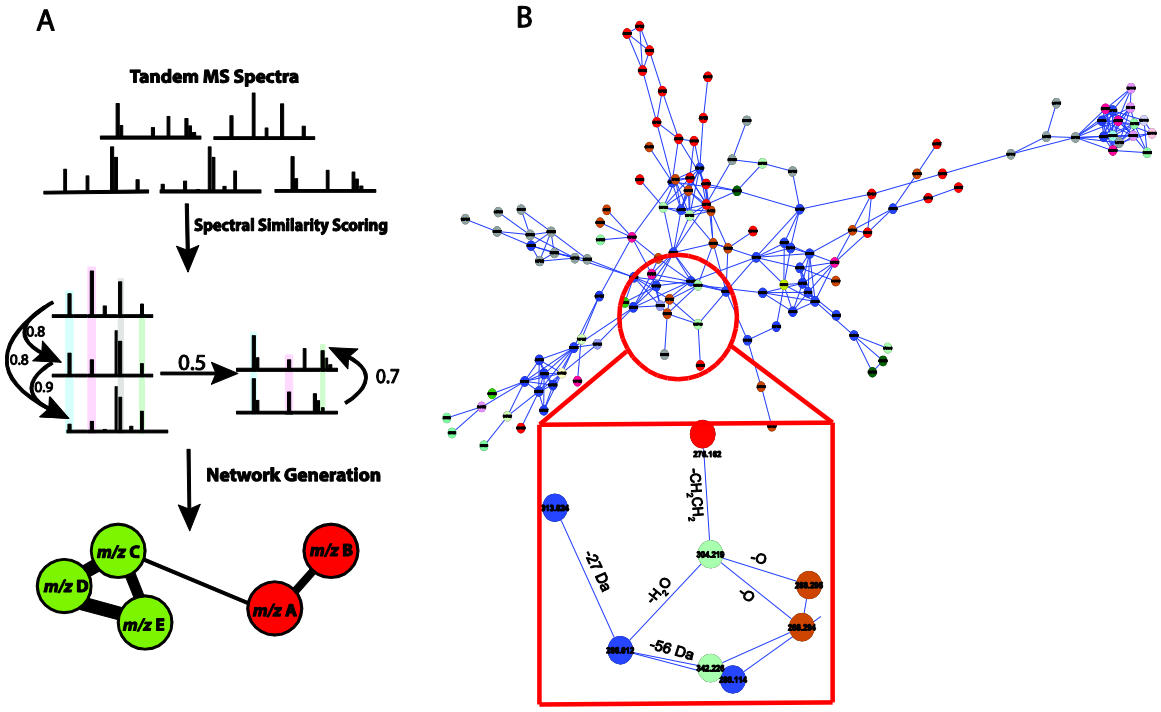

Figure 2-3. Generation and visualization of molecular networking. A) Workflow of generation of molecular networking. The cosine score of MS spectra alignment between 0 to 1 as well as the thickness of edges in network represent the similarity of certain spectra. Score in 1 or thick edge means high spectral similarity. Certain cut-off of cosine score (normally 0.5) usually applied. B) Cytoscape visualization of molecular networking. The number labeled on each node represent the parent mass. Different colors represent different samples that data generated from.

There are multiple steps to use molecular networking to analysis MS/MS data. Pairwise MS/MS spectra comparison is made to identify match fragmentation peaks, which also take into account the relative intensities and the m/z difference between two precursor ions. In default, the minimum matching fragmentation peaks should be six, but based on the fragmentation patterns of small molecules usually with m/z less than 600 Da, it is fair to set the number of matching peaks in to 4 in order to get fair results. The similarity of one pair of spectra was evaluated by cosine score. And each spectrum was combined with its identical spectrum from other sample, which then connected to its top ten scoring matches based on the cutoff of cosine score 0.65. In molecular networking,

maximum connected component size can be defined manually to avoid a 'hair-ball' connection in networking that was relatively difficult to analysis. Also, group mapping feature imported the parameters of sample sources, which helps visualization and comparison. To do a spectra match of our sample with database, similar MS/MS spectra matching called library search was applied with minimum match peaks of 4 and cosine cutoff of 0.5. Finally, in order to generate a background clear networking and achieve a better match only based on MS/MS spectra, the minimum peak intensity was set at 400.0 counts and the precursors were filtered out. The output of molecular networking can be visualized in free software Cytoscape (v2.8). In Cytoscape, each node represented same parent mass that was fragmented as well as its underlying MS/MS spectra, which labeled by the precursor mass. Nodes were connected to their related precursor ions with different parent mass but similar fragmentation patterns. The mass difference between a pair of nodes was labeled on the edges. The width of edge between a pair of nodes represented the cosine score (the thinnest represented the lowest score 0.65, and the thickest represented the highest score 1.0). Samples were grouped based on sample source information as patients ID provided by our collaborator Dr. Søren Molin's lab. The difference in grouping was illustrated as color code of nodes. And nodes included spectra with hits on library data were set with larger node size. Spectra belonging to controls such as media or solvent blanks were removed from Cytoscape. The final networking was shown in FM3 layout **(Figure 2-4).** Overall, in the molecular networking, there were 93 samples from six defined groups shown in 1416 nodes connected by 2098 edges.

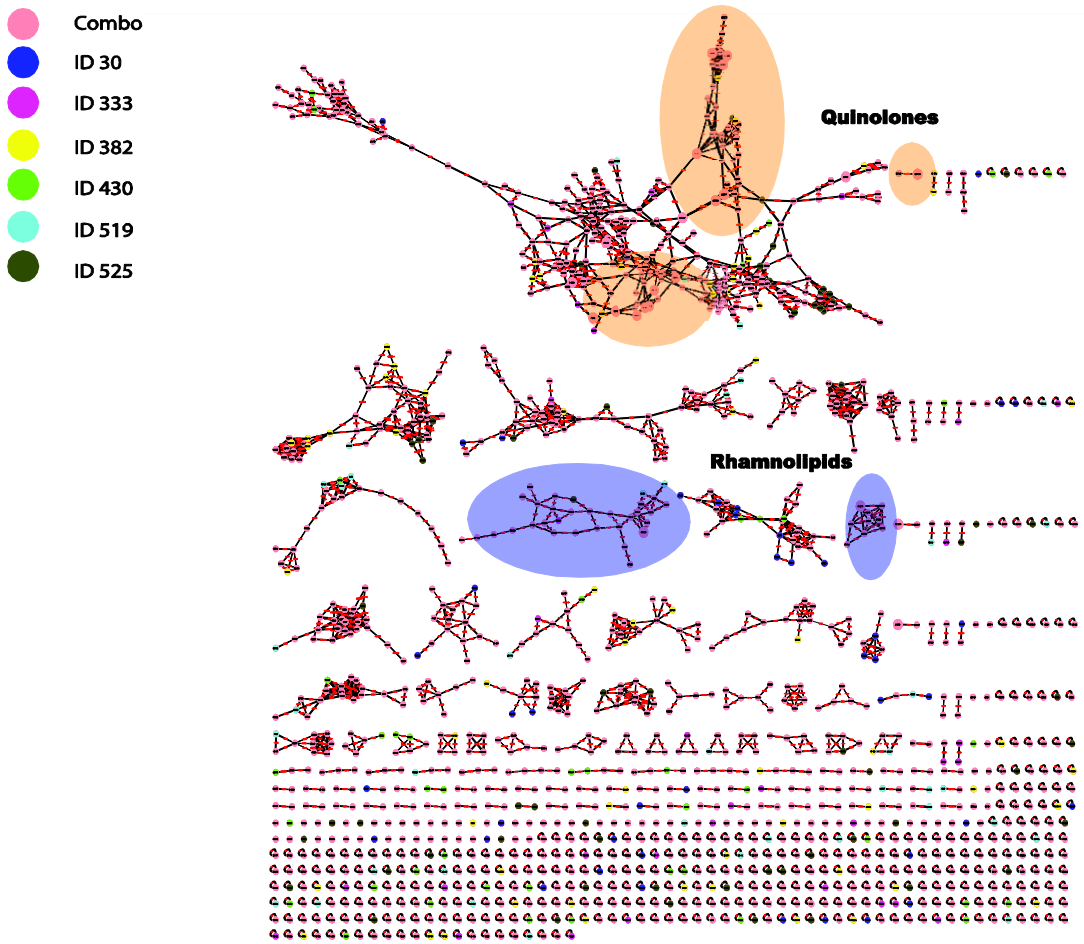

Figure 2-3. Molecular networking of all samples.

By matching to the database on GnPS, our data got 14 different matches with annotated spectra. Since the standard spectra are in high quality and well annotated, it gave promising results of the metabolites production from those specific samples, even the data was collected from different mass spectrometry instrument. There were seven pre-identified quinolone analogs, four rhamnolipid analogs, one unclear peptide, one phenazine and two cyclic amino acids matched with the database. From the known metabolites found in the networking and the linkage between them, it is possible to identify some unknown specialized metabolites. For example, metabolites with m/z of 258.1 Da may be a possible quinolone derivative with a double bond based on fragmentation pattern. Also, it is convenience to export out the file information consist of an individual node, such as the sample name and spectra number.

As a summary, molecular networking is a powerful tool for rapid mining a large dataset of unknowns, targeting the specialized metabolites from each sample without manually going through all files.

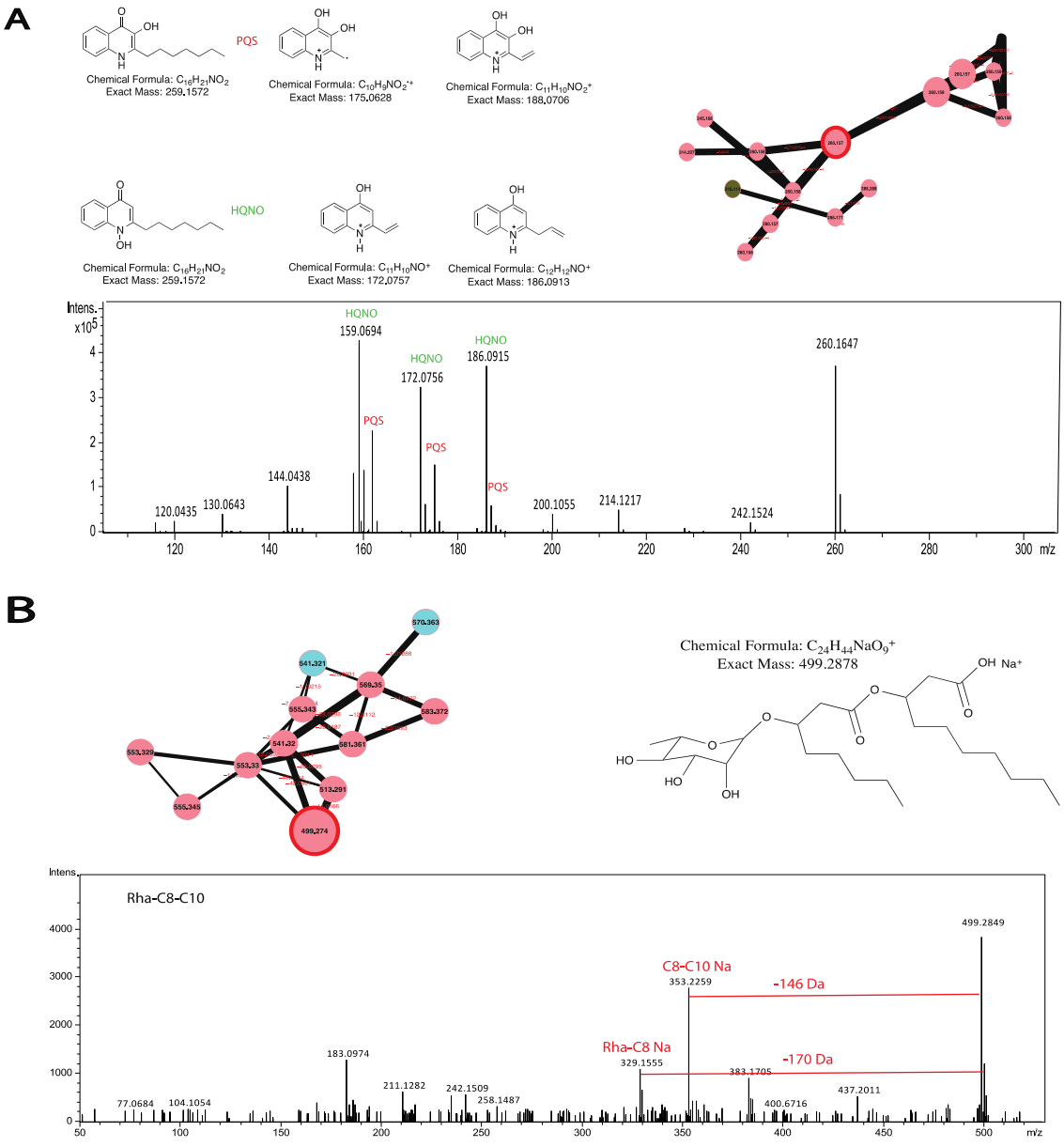

Figure 2-4. Example of specialized metabolites identification from molecular networking. A) Quinolone PQS/HQNO identified from molecular networking and confirmed the structure by MS^2. B) Rhamnolipid m/z 499 Da identified from molecular networking and confirmed the structure by MS^2.

Table 2-1. List of specialized metabolites identified from samples.

Metabolites	m/z (Da)	Retention time (min)	Form
Phenazines			
Pyocyanin (PYO)	211.086	2.70	H+
Phenazine-1-caboxylic acid (PCA)	225.060	4.61	H+
Quinolones			
HHQ	244.170	5.06	H+
PQS/HQNO	260.165	5.26	H+
C9:db NHQ	270.185	5.77	H+
NHQ	272.202	5.84	H+
C9:db-PQS, C9:db-NQNO	286.180	5.69	H+
C9-HQNO	288.196	5.90	H+
C9-PQS	288.196	6.16	H+
C11:db UHQ	298.217	6.16	H+
C11-PQS, UQNO	314.211	6.20	H+
Rhamnolipids			
Rha-C8-C10, Rha-C10-C8	499.287	6.34	Na+
Rha-C10-C10	527.319	6.89	Na+
Rha-C10-C12:db, Rha-C12:db-C10	553.335	7.32	Na+
Rha-C10-C12, Rha-C12-C10	555.350	7.57	Na+
Rha-Rha-C10-C8, Rha-Rha-C8-C10	645.345	5.99	Na+
Rha-Rha-C10-C10	673.377	6.46	Na+
Rha-Rha-C12:db-C10 Rha-Rha-C10-C12:db	699.392	6.82	Na+
Rha-Rha-C12-C10 Rha-Rha-C10-C12	701.408	7.06	Na+
Siderophore			
Pyochelin	324.060	4.70	H+

2.3.4 Specialized metabolites distribution

2.3.4.1 General specialized metabolites distribution

It is interesting to see the statistic results of these specialize metabolites production profiles among all samples. In here, specialized metabolites heatmap was used to visualize the distribution. Combined with statistic results, the heatmap showed that the two phenazines were basically equally distributed, but PCA was dominant. Both of them showed high abundance among all samples; Of all the quinolone derivatives, the PQS/HQNO was unexpectedly not shown in the highest abundance, even though it was predict to have major effects on the metabolic synthesis pathway and quorum sensing. Also, the results just showed the relative abundance of PQS and HQNO in the mixture, it is fair to predict that the PQS may even in a lower abundance that it has shown here. Rhamnolipids which most of them showed high abundance of all samples, the difference of abundance between these rhamnolipids was not clear among all samples.

2.3.4.2 Correlation of specialized metabolites with patients ID

From the general distribution of these specialized metabolites, it was not clear the how the metabolites profiles changed as results of evolution. So to take one step further, we would like to see the correlation between the specialized metabolites distribution of each patient at different time frame. We then generated heatmap for each patient to visualize the specialized metabolites distribution along the time frame. The results gave a patient specific correlation profile.

CF30 In this case, the time frame of sample collection lasted for more than twenty years from 1980 to 2012. In the heatmap of specialized metabolites, there were two clear boundaries that have very obvious metabolites profile change between 1980 and 1984, 2006 and 2009 respectively. From 1980 to 1984, most metabolites that not detectable at 1980 were appeared in 1984, but some metabolites such as quinolone m/z 260 was undetectable at 1984. To look into the MS2 spectra of compound m/z 260 and to compare the fragmentation patterns, we found that it was actually a mixture of two quinolone analogs, quinolone PQS and quinolone HQNO, which have same retention time and m/z. To compare the relative peak intensities of fragmentation from each of them, we found the relative abundance of PQS verses HQNO at the four different time points when compound m/z 260 was detected. In all four time points, quinolone HQNO shown higher relative abundance compared with quinolone PQS. However, the exact function of quinolone was not clear As one of the major virulence factors of *pseudomonas aeruginosa*, quinolone PQS did not show a great abundance as expected. On the other hand, the precursor of PQS, quinolone HHQ showed relatively high abundance especially from 1980 to 2006. Other specialized metabolites such phenazine pyocyanin (m/z 211) and PCA (m/z 225) were relatively widely produced in the beginning, but turned into undetectable in later 2009 **(Figure 2-6).**

CF333 samples collected from this patient showed very different metabolites production pattern compared with others. All samples were collected from 1991 to 2007, but the overall abundance of these specialized metabolites were much lower than other patients, which may indicate an aggressive inhibition of the *P.aeruginosa*'s growth in this patient. For example, one of the major phenazines pyocyanin was barely produced among

all samples. And unlike the data from other patients, two rhamnolipids (Rha-C8-C10 and Rha-C10-C12:db) analogs were also in low abundance. Although the metabolites production transition following the time frame was not clear, it is clear that the patient showed a different pattern compared with other five patients we worked on **(Figure 2-7).**

CF382 From this individual, similar patterns among all the samples showed within a slightly different time frame. Within five years from 2007 to 2012, it was clear from the heatmap that rhamnolipids and phenazines were commonly produced among these samples, but quinolones were shown various abundance among all samples. Unlike patient **CF525** and **CF519**, in this individual, those strains collected at the same year showed high similarity in the metabolites production patterns. For example, samples collected in 2008, those two strains showed very similar patterns of metabolites production. The six samples collected in 2011 were also shown similar patterns in most rhamnolipids production, but slightly different in metabolites production of quinolone analogs and phenazines **(Figure 2-8).**

CF430 Samples collected from this patient were follow similar transition patterns. Most rhamnolipids analogs and phenazines were commonly produced among all samples. But the quinolone analogs production showed various abundance among all samples. Especially, some quinolone analogs were widely produced from those samples collected in 2007 but not detectable from samples collected in 2009 and 2006 (C9:PQS and C9:HQNO). And in the sample collected in 2011 and one of the strains collected in 2012, only two or three specialized metabolites were detectable. **(Figure 2-9).**

CF519 In this patient, samples collected from 2007 to 2012, rhamnolipids showed great abundance among all samples besides one sample collected from 2012. Considering

other specialized metabolites such as quinolones and phenazines, the metabolites production patterns various for each of them. Even at the point the report is written, the gene mutation of each isolated strain is not clear, however, it is clear even the strains were collected at the same time point, the metabolites production pattern may varies based on the gene mutation they have. For example, compared the five strains isolated from year 2012, the production of quinolones and phenazines were different. Among all five samples, one of them showed low abundance of approximately all specialized metabolites. And for quinolone PQS/HQNO, only one sample among five had that compound detected, same as quinolone HHQ. Among all five different strains collected from 2012, none of them had identical metabolites production pattern. Compared to the results shown in 2011, which also had five strains isolated at the same year, the metabolites production patterns were more similar among those strains with only quinolone compounds showed major difference **(Figure 2-10).**

CF525 Strains isolated from this patient were all collected within five years from 2007 to 2012. In this individual, the rhamnolipids analogs all showed relative high abundance among all samples. For example, rhamnolipids m/z 673 was shown in all samples, with slightly various of intensity. Other groups of specialized metabolites such as phenazine pyocyanin and siderophore pyochelin showed various abundance at different time point. Compared quinolone PQS and its precursor HHQ, even at certain time points both of them showed high intensity, but overall, they showed unclear production relation and quinolone HHQ had relative higher abundance among all samples **(Figure 2-11).**

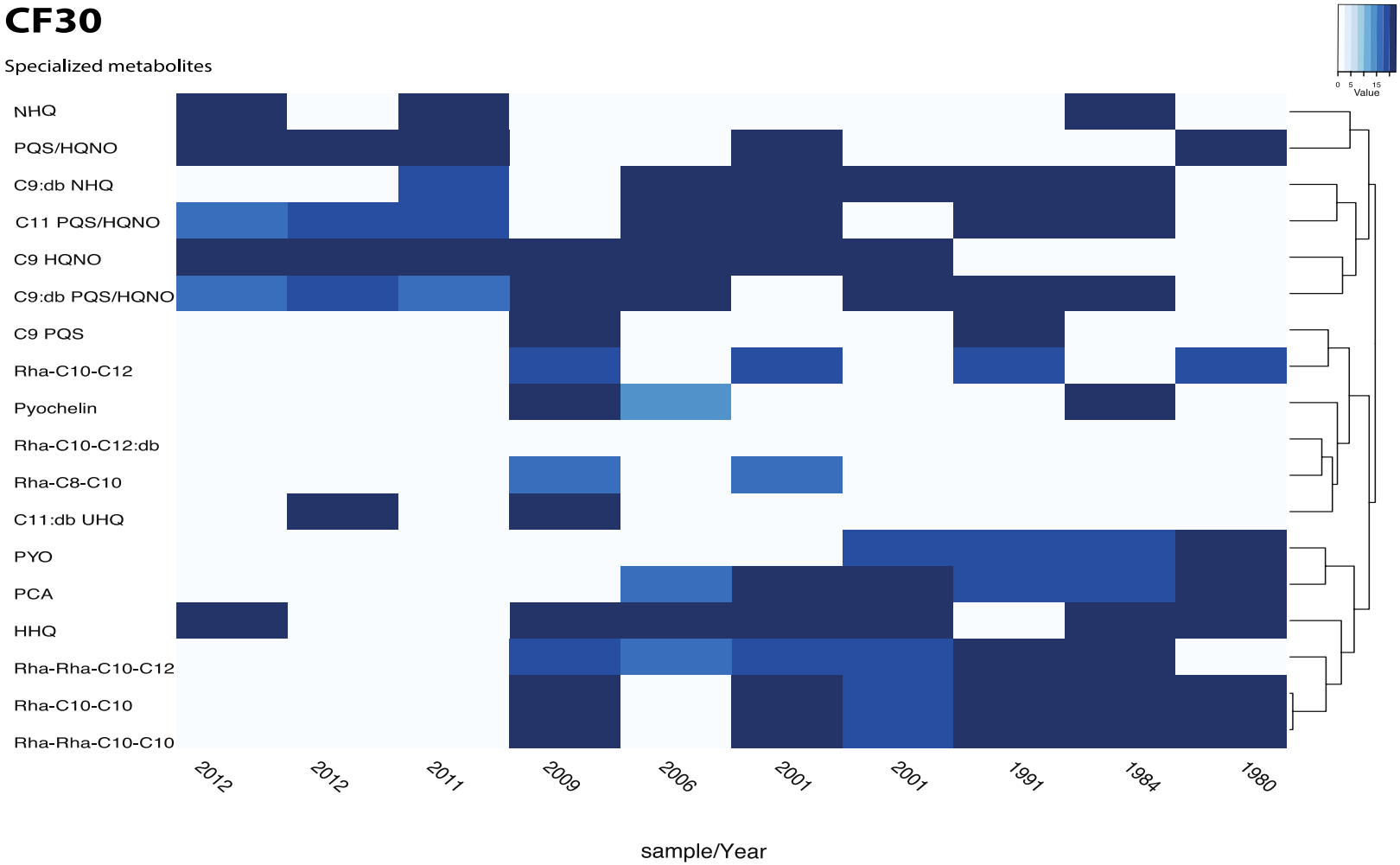

Figure 2-5. Specialized metabolites heatmap of patient CF30.

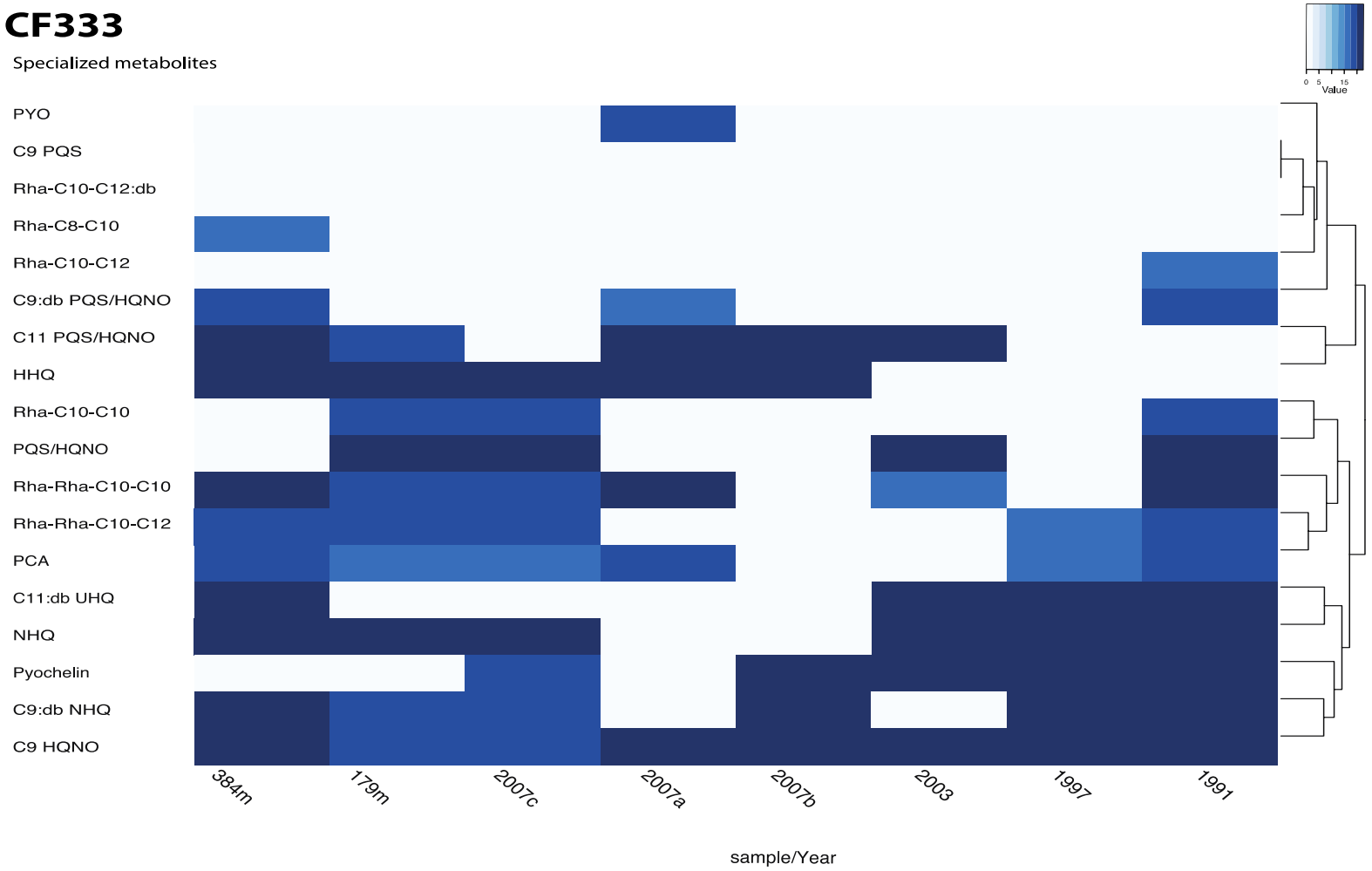

Figure 2-6. Specialized metabolites heatmap of patient CF333.

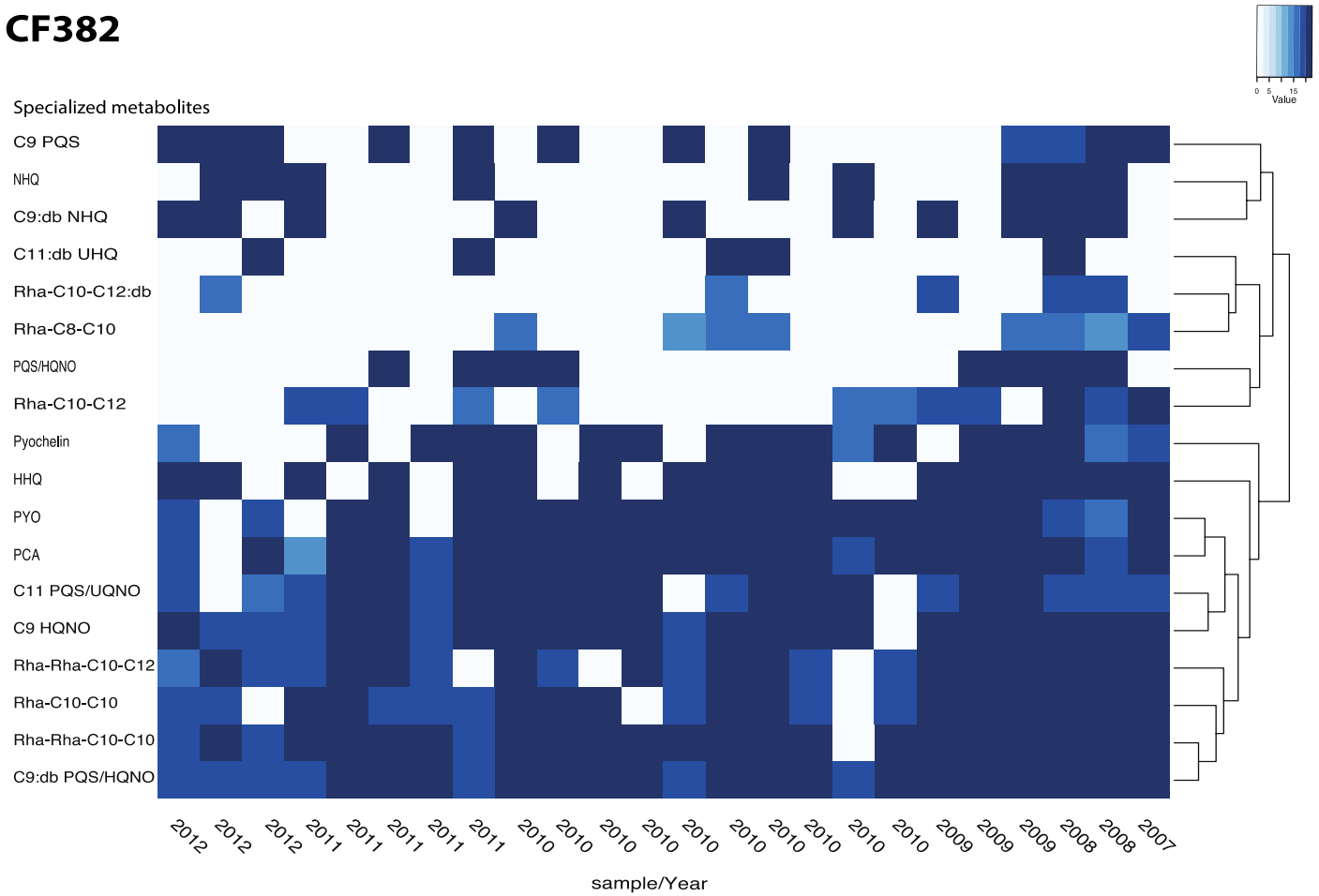

Figure 2-7. Specialized metabolites heatmap of patient CF382.

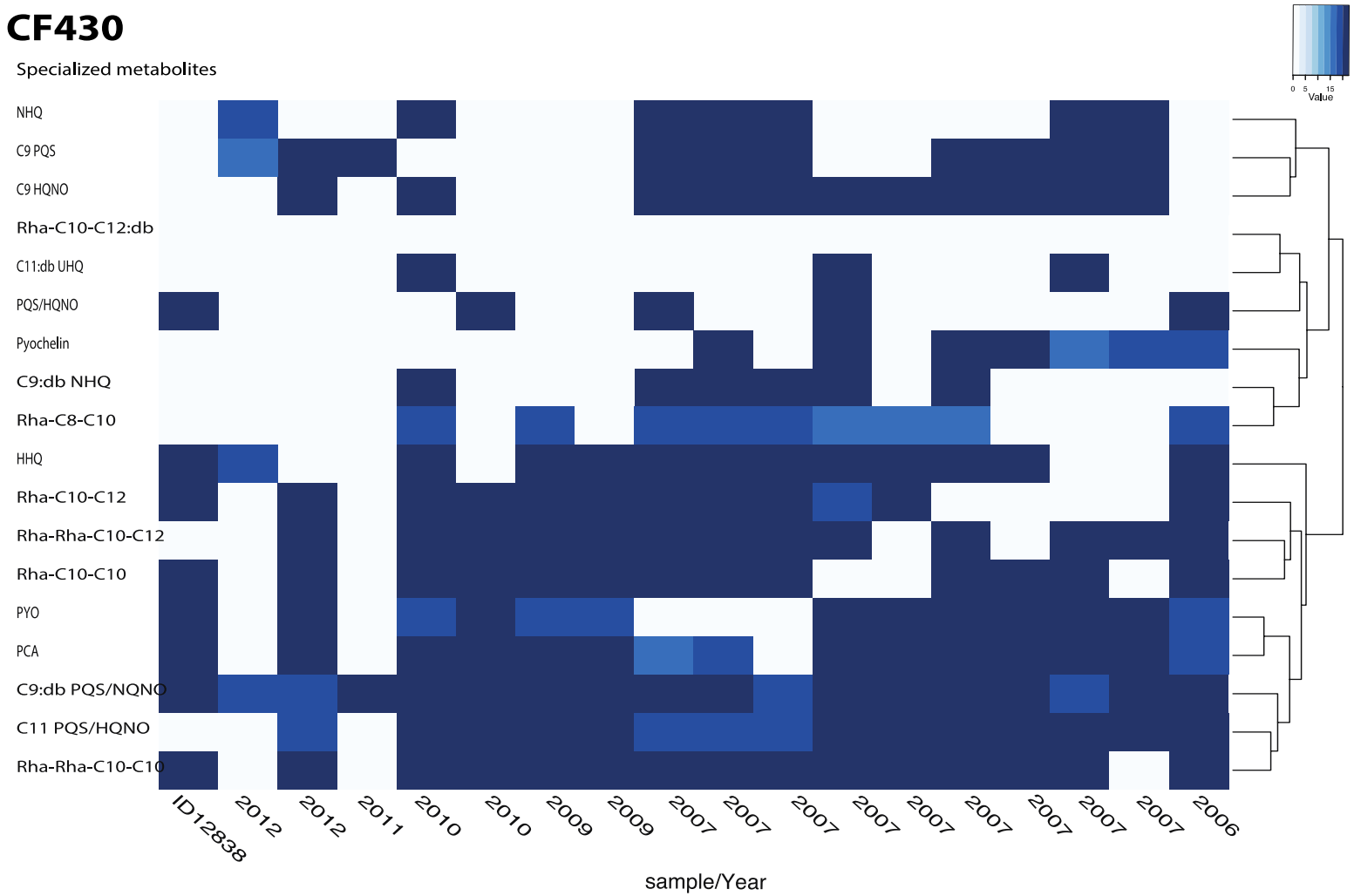

Figure 2-8. Specialized metabolites heatmap of patient CF430.

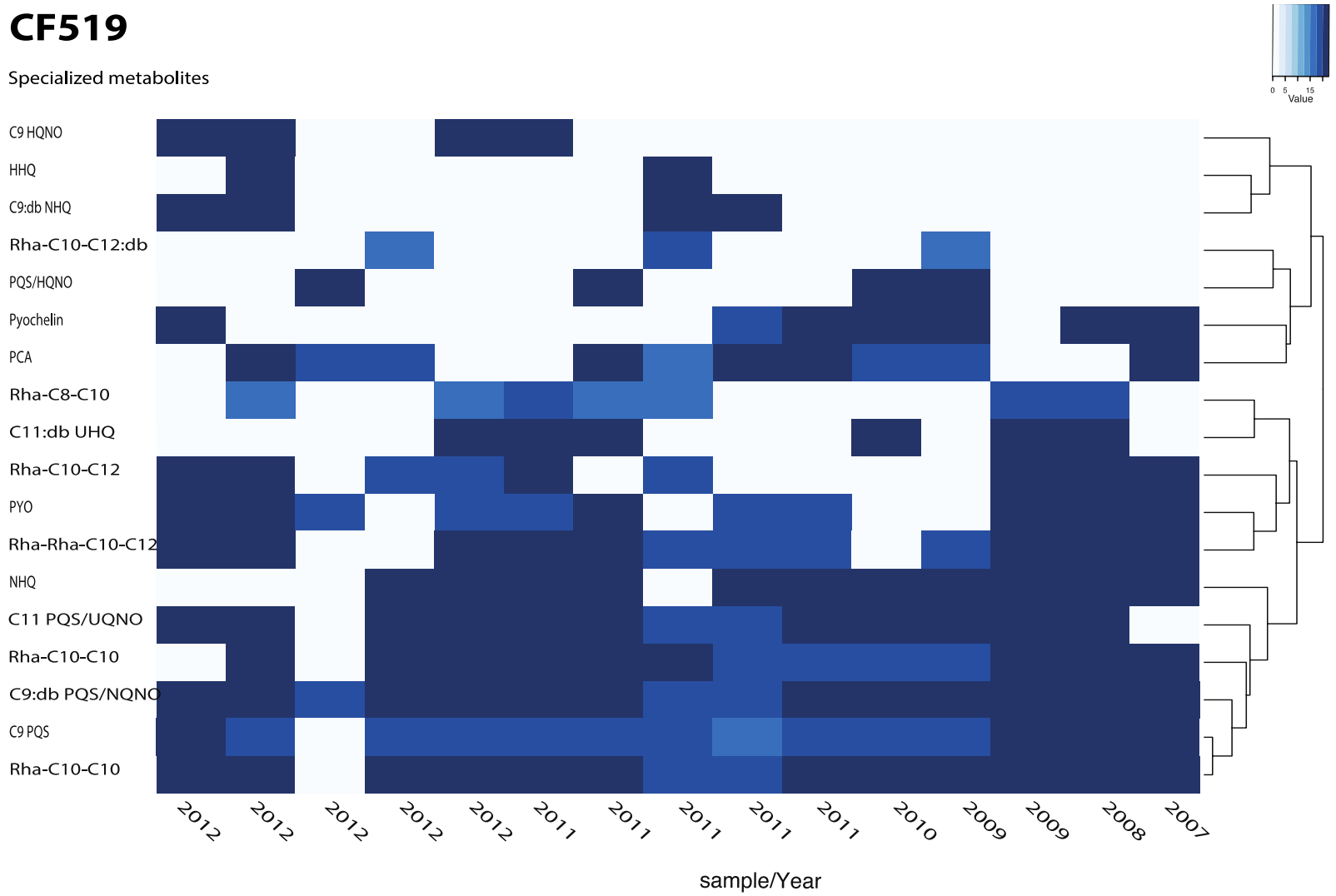

Figure 2-9. Specialized metabolites heatmap of patient CF519.

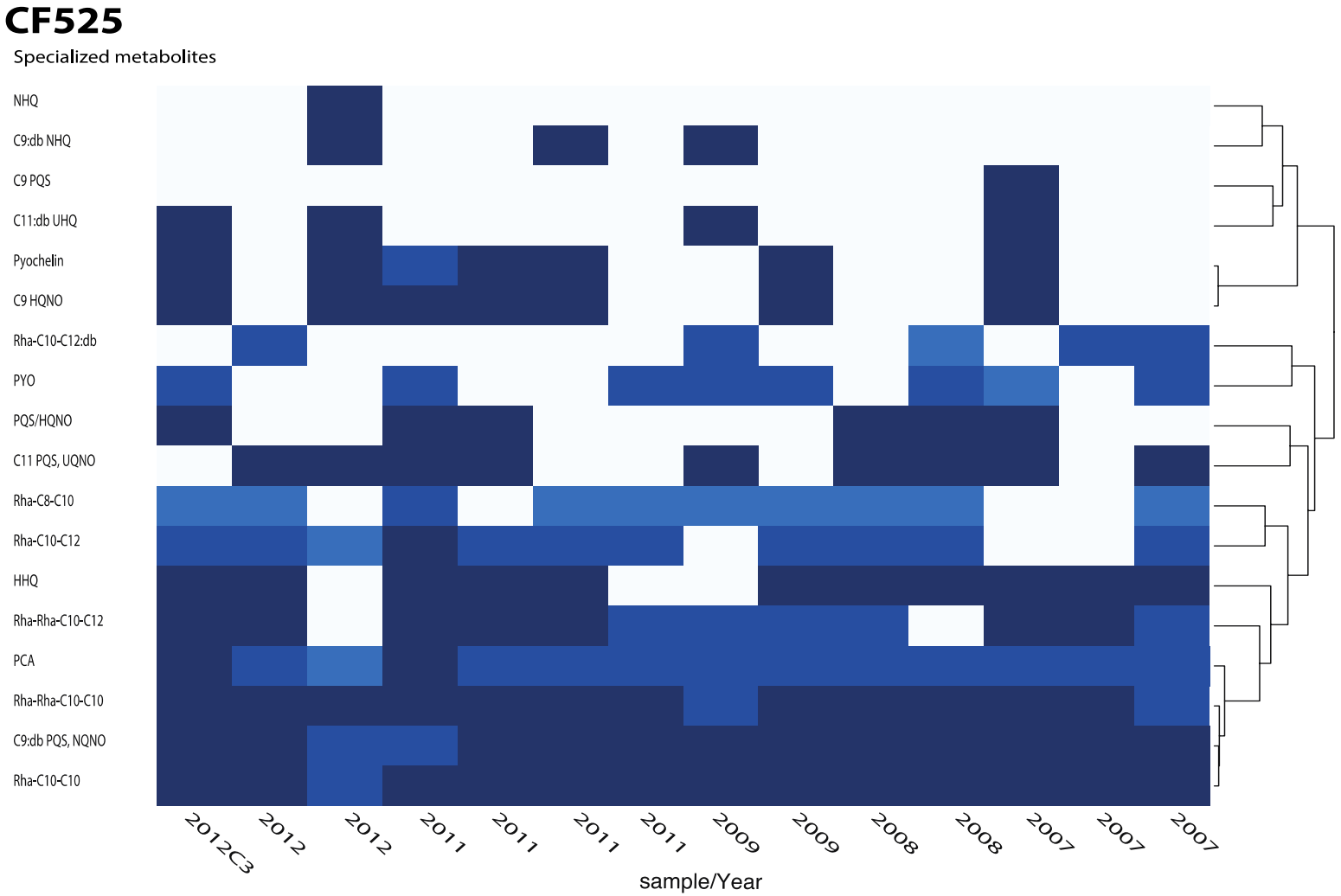

Figure 2-10. Specialized metabolites heatmap of patient CF525.

2.4 Conclusion and future work

In this project, we were able to analysis more than 100 LC-MS/MS samples using metabolomics method, visualizing the general and specialized metabolites production profiles of all samples, and identifying known specialized metabolites produced by *P.aeruginosa* such as phenazines, quinolones and rhamnolipids. To study the production of specialized metabolites of those strains, we were able to track the transition of strains from patient to patient, and at various time points of infection, which was very patient specific as we showed. However, without knowing more information about patients such as their ages, years of infection been diagnosed, it is difficult to have more conclusion correlating the specialized metabolites production profiles with the patients. One limitations of our study is based on the extraction solvent we used to extract specialized metabolites from samples, it is impossible to get all metabolites into mass spectrometry. So it is unable to compare all identified specialized metabolites produced by *P.aeruginosa*. Also, even molecular networking is a powerful and effective tool to dereplicate known compounds from hundreds of LC-MS samples, it is still a time consuming work to identify uncharacterized specialized metabolites in this samples scale, in which may contain more information of the specialized metabolites production patterns. For future work, following up data mining may reveal more clear metabolites production profiles of *P.aeruginosa* strains from CF patients. Also, genomic analysis of the mutations has been done to reveal the adaptive revolution of *P.aeruginosa* in CF patients by our collaborators (17), it would be interesting to compare the adaptations of pathogen *P.aeruginosa* in respect to antibiotic therapy or other physical condition change

of patients in genomic and metabolomics aspects to see if they have similar transition path.

2.5 Materials and Methods

Sample culture and extraction

All 202 samples were consists of 95 *Pseudomoas aeruginosa* clinic isolate strains isolated from six different CF patients, 3 controls of *P.aeruginosa* PAO1 strains and 3 blank controls with their duplicates. All samples were cultured and extracted by Anna Lewińska (Dr. Molin Lab, Technical University of Denmark). The *P.aeruginosa* strains were cultured in 300 µl liquid ISP2 overnight under 37 °C in 96-well plate, extracted by 300 µl ethyl acetate (EtOAc), then sealed and vortexed for 1 hour. After completely vortex, the 96-well plate was spinned down in centrifuge under 4 °C for 15 minutes. The supernatant was then transferred into another 96-well plate and air dried under fume hood. The remained material was extracted by 300 µl methanol (MeOH) and the plate was sealed and vortexed for another hour. Then the plate was spinned down in centrifuge under 4°C for 15 minutes. The supernatant then combined with ethyl acetate extracts and air dried under fume hood. The crude extraction samples were stock in 96-well plate in -20 °C before using.

LC-MS/MS

LC-MS/MS analysis was performed on a Dionex (Thermo scientific) high performance liquid chromatography (HPLC) with an UltiMate 3000 Series pump system

with a 96-well plate autosampler. The analytical column was a reverse phase ultra-performance liquid chromatography (UPLC) column (1.7 μm C18 100A 30 x 2.10 mm, Phenomenex). The system was operated isocratically with a flow rate of 5 mL/min of (A) 98% H_2O, 2% acetonitrile and 0.1% formic acid and (B) 98% acetonitrile, 2% H_2O and 0.1% formic acid. The mass spectrometry (MS) system used for detection was MaXis from Bruker control. Both LC and MS system were controlled by Hystar. The MS was operated in the positive ion mode for analytes and internal standard detection. All general extraction samples were run in one batch to avoid inconsistence of general conditions.

Data Analysis and heatmap generation

The raw data was firstly processed by compactable Bruker software DataAnalysis (version 4.1) and ProfileAnalysis (version 2.1) to generate molecular features and buckets table. The molecular features were calculated in DataAnalysis (v4.1), the compound detection features were set as S/N = 5, correlation coefficient threshold = 0.8, minimum compound length = 20 spectra and smoothing width = 20. And buckets table of molecular features was generated from ProfileAnalysis (v2.1). The buckets were defined by retention time range from 60 seconds to 720 seconds of the entire chromatography, and the mass range of interests was m/z from 100 Dalton to 1500 Dalton. The advanced bucketing filter was set based on molecular feature alignments. Total 1684 buckets were generated from ProfileAnalysis for total 202 samples. Bucket table in text format was exported from ProfileAnalysis. The text file was converted into output format in Perl, and used as input file to generate a heatmap in Rstudio. In heatmap, the relative abundance of each metabolite was represented as log2 of peak area under the curve in raw data.

Molecular networking and dereplication

The networking was created by using GnPS (http://gnps.ucsd.edu) and visualized in Cytoscape (v2.1). 101 samples were used in creating the network, grouping in six groups of patients, one group for control MPAO1 and on group of blank control. In the final network, MPAO1 and blank control group were removed from the network. Database was used match to dereplicate the specialized metabolites of *P.aeruginosa*. The network after removing MPAO1 controls and blank controls was created with minimum match peak number of 4, minimum component size of 300 connections, cosine score of 0.65 and the precursors were filtered. The library match was also with minimum library match peak of 4, threshold of 0.5. In total, the network gave 1416 nodes and 2098 edges.

2.6 Acknowledgements

We acknowledge Dr. Søren Molin and his lab from Technical University of Denmark to provide all the *P.aeruginosa* isolated strains and patients information, and Dr. Vanessa Phelan for designing the extraction protocol and all others helps for this project. Financial support was provided by the National Institute of Health K01GM103809 (VP).

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Chapter 3

Effects of Azithromycin on *Pseudomonas aeruginosa*

3.1 Introduction

Cystic fibrosis (CF) patients are easily infected with a number of pathogens because of the mucus condition caused by the mutations of cystic fibrosis trans-membrane conductance regulator (CFTR) genes. As the disease progresses, *Pseudomonas aeruginosa* becomes a common pathogen for CF patients, because of its ability to use a wide range of different compounds as energy source for growth (1). *Pseudomosas aeruginosa* is a Gram-negative bacteria and an important pathogen in chronic respiratory infections like CF, it is difficult to eradicate from the CF lung once established and typically forms life-long infection. Like other bacteria infections, infection with *Pseudomosas aeruginosa* is commonly treated with antibiotics that can kill it or stop it from reproducing. However, the treatment of *P.aeruginosa* is not easy, because it can form a biofilm that provides further defense against antibiotics (2, 3). In past decades, antibiotic treatment was commonly used in treating cystic fibrosis patients (4, 5).

The macrolide antibiotics such as azithromycin have significant benefits in patients with cystic fibrosis. Although early study shows that macrolide antibiotics have no direct killing effects against the Gram-negative bacteria *P.aeruginosa*, however, reports suggest that the macrolide antibiotic might have a role in CF through indirect anti-pseudomonal properties. Clinic studies about azithromycin on CF patients show that azithromycin demonstrated a significant improvement in lung function. Also, azithromycin might have the effect to reduce the airway mucus production and altering the biofilm phenotype of *P.aeruginosa* (6-8). Since the minimum inhibitory concentrations (MICs) of azithromycin for *P.aeruginosa* strains are almost all in excess 64 mg/L (9), which are unachievable in vivo, we are

interested in how azithromycin under sub-inhibitory concentrations (sub-MICs) could affect the growth of *P.aeruginosa*. Previous study suggest that azithromycin could affect production of some virulence traits of *P.aeruginosa* under sub-inhibitory concentrations (sub-MICs) by testing the motility of strains (10, 11). It will be interesting to see how azithromycin affects specific metabolites production as virulence factors such as phenazines, quinolones, and rhamnolipids (have already discussed in Chapter 1 and Chapter 2) produced by *P.aeruginosa*.

In our project, we tested the effects of antibiotic azithromycin to specialized metabolites production under different sub-inhibitory concentration (sub-MICs). Especially, we used two well-studied lab cultured *P.aeruginosa* strains PA14 and MPAO1 with one clinic isolate *P.aeruginosa* strains FLR01 to compare their different levels of antibiotic resistance reflected as the abundance of specialized metabolites productions.

3.2 Results and Discussion

3.2.1 Influence of azithromycin on phenotype of *P. aeruginosa*

By increasing the concentration of antibiotic azithromycin in the media, the change on phenotype of *P.aeruginosa* after growing in 48hrs was obvious. Compared to the wide type, PA14 strain did not form the whole yellowish colony when the concentration of azithromycin was higher than 4 μg/mL, the phenotype was shown as several sticky colorless droplets on the agar. In another lab strain MPAO1, the bacteria formed the colony circle as the wide type, but the color was much lighter with higher concentration of azithromycin, and the size of colony was smaller. With 8 μg/mL azithromycin, the colony of MPAO1 was small and transparent. However, the

phenotype was different in the clinic isolate strain FLR01. The form and color of colony on the agar did not change that much with the difference of azithromycin concentration in the media, the strain formed the same yellow circle in 8 μg/mL azithromycin agar as well as in the wide type, only with lighter color and smaller size. In summary, with the azithromycin up to 8 μg/mL, the antibiotic had a severe inhibition on the colony formation of PA14 with the increasing of azithromycin, as well as a relatively high inhibition on MPAO1. On the other hand, the colony did not show much difference in FLR01.

Based on the difference shown in phenotype, we believed there must be some difference in metabolites generation caused by the antibiotic. After growing under 30 ^{o}C for 48hrs, the whole agar piece containing the colony was cut out, treated with matrix and subjected to MALDI-TOF IMS to examine the distribution of secreted metabolites. At 48hrs, more than 50 ions were detected localized on and around the colony, indicating a complex metabolites production in the organism. Some metabolites generated by *P.aeruginosa* were well characterized. In addition, extract of the selected area in each sample was analyzed by LC-MS/MS to confirm the prediction of metabolites.

3.2.2 Specialized metabolites production under different concentration of azithromycin

P.aeruginosa is known to generate a wide variety of secondary metabolites. Here we chose three main categories of metabolites produced by *P.aeruginosa* to illustrate the differences of specialized metabolites production caused by antibiotic azithromycin. Phenazines, a major class of *P.aeruginosa* metabolites, showed a

severe suppress of production in PA14. Even with 2 μg/mL of azithromycin, almost no phenazine metabolites among pyocyanin (PYO), phenazine-1-carboxyamine (PCN), phenazine-1-carboxylic acid (PCA) and 5-N-methylated PCA (5-MPCA) was detectable. The phenomenon was similar in MPAO1. However, 1-Hydroxyphenazine (1-HP) was shown not be effected by the antibiotic. Those four phenazines were barely produced with 2 μg/mL of azithromycin, showing very low intensity in imaging, as the concentration increased, the phenazines were all undetectable. However, the phenomenon was different in FLR01. Obviously, the clinic isolates strain showed higher resistance of antibiotic than lab strains PA14 and MPAO1. Even with lower intensity, which indicated that less phenazines were produced with azithromycin from 2 μg/mL to 6 μg/mL, the metabolites were produced on the colony and diffused around, but the intensity was lower with the increasing amount of antibiotic. With 8 μg/mL of azithromycin added in the media, the production of phenazines was highly suppressed that only PCN with very low intensity could be detected.

Quinolones, another group of metabolites of *P. aeruginosa*, were also observed and analyzed. Several quinolones were identified as quorum-sensing (QS) signaling molecules (PQS and 2-heptyl-4-hydroxyquinoline, HHQ), iron chelators (PQS), and antimicrobials (2-heptyl-4-hydroxyquinoline-*N*-oxide, HQNO), but most quinolones remain uncharacterized. In PA14, the quinolone production was completely suppressed with 4 μg/mL or higher concentration of azithromycin. But at 2 μg/mL of azithromycin, quinolones were well produced on the colony. The results were corresponding to the phenotype of the colony. It is possible that quinolones were responsible for the colony formation of PA14. In MPAO1, similar results were shown. Interestingly, unlike the production of quinolone HHQ (m/z 224 Da) and

quinolone HQNO (m/z 260 Da) in wide type, which were localized both on the colony and diffused around, these two quinolones were almost only detected on the colony under the influence of 2 μg/mL azithromycin. Unlike in PA14 and MPAO1, the inhibition of quinolones production was not severe in FLR01. The quinolones were detectable in all samples with various amount of azithromycin. Only with 8 μg/mL of azithromycin, the difference was clear based on the intensity of signals. In **Figure. 3-3**, we could see quinolone HHQ and HQNO were highly diffused around the colony but quinolone NHQ and C9:db HQNO were only produced on the colony.

Another group of metabolites detected was rhamnolipids. More than 10 rhamnolipids were produced by *P aeruginosa* and secreted extensively in the media, as exemplified by m/z 673 Da. These bio-surfactants have been reported to promote uptake and biodegradation of insoluble compounds, affect swarming and biofilm formation, act as virulence factors, and inhibit a variety of microorganisms, including fungi as described in previous chapters. As shown in MALDI-IMS, rhamnolipid m/z 673 Da was well produced on and around colony in wide type PA14, but highly suppressed with azithromycin since no signal was detected in antibiotic media samples. In MPAO1, rhamnolipid with m/z equals to 673 Da was widely diffused into the agar rather than located on the colony. Even very low intensity signal was shown in 2 μg/mL azithromycin sample, the production of rhamnolipids was also suppressed by the antibiotic. Like MPAO1, the rhamnolipids were also diffused into agar in FLR01. However, even with the increasing of azithromycin in the agar, rhamnolipids produced less and less, the inhibition was not so severe as in MPAO1 and PA14.

In summary, the MALDI-IMS data confirmed our hypothesis that along with the inhibition on formation of colony, some major metabolites production must be suppressed by antibiotic azithromycin. Also, as the difference shown in phenotype,

the clinic isolates strain FLR01 has higher resistance over azithromycin than MPAO1 and PA14. With 4 μg/mL of azithromycin, almost no major metabolites were detectable in PA14 and MPAO1. However, on the other hand, most quinolones metabolites, phenazine PCN and rhamnolipids were detected even with 8 μg/mL azithromycin.

3.2.3 Quantitative analysis of metabolites production

Although MALDI-IMS was able to reveal the inhibition of azithromycin over *P. aeruginosa* by suppressing the production of major metabolites based on the distribution and intensity of signals on the agar, the technique was not enough to do a quantitative comparison of metabolites production and confirm the prediction of metabolites. So LC-MS/MS analysis was done as an orthogonal method to confirm the metabolites shown in IMS and also support the quantitative analysis. By applying LC-MS/MS, we were able to separate all metabolites in liquid chromatogram and identify each metabolite according to the m/z value and MS/MS fragmentation pattern. With unique ion chromatogram, the intensity of each metabolite could be represent as the integrate area of the ion peak.

All samples were inoculated and incubated under the same condition as what we did in IMS experiment, and samples were extracted by acetyl acetate (EtOAc) and methanol (MeOH) and then injected into LC-qTOF-MS/MS. The LC-MS/MS data was analyzed in DataAnalysis (v4.1, Bruker). The integrate results were an average of three biological replicate trials in order to minimize personal errors. Each sample was calibrated by internal standard (m/z 922.0 Da) in DataAnalysis. Most integrate results were generated from XCMS (https://xcmsonline.scripps.edu), some of them (FLR01

with 8 μg/mL azithromycin, PA14 with 2 μg/mL azithromycin and all PYO results) were integrated manually from DataAnalysis. With all integrate results, the ratio of specific metabolites production level in azithromycin sample over wide type sample were calculated, and log2 values of ratio were calculated to represent the inhibition level of different concentrations of azithromycin on specific metabolites production.

In *P. aeruginosa* PA14, as what was shown in MALDI-IMS results, azithromycin highly suppressed the production of metabolites as the concentration getting higher and higher. At 2μg/mL condition, the log2 values were larger -3.0. As the concentration increased to 8 μg/mL, the log2 values were mostly lower than -6.0, which represented a high inhibition level. Among these metabolites, pyocyanin (PYO) showed very high inhibition level, which was undetectable in all azithromycin samples. There was an unknown phenazine compound with same m/z value detected, which was not pyocyanin (PYO) based on MS/MS data. Another two phenazines PCN and PCA showed similar levels of inhibition. On the other hand, the quinolones showed various levels of inhibition. Quinolone HHQ was less affected than the others, with the log2 value larger than -5.0. Also, the log2 value was fluctuated rather than consistently getting larger while the concentration getting higher. The other quinolone C9:db HQNO had quite high inhibition level which represenedt as the log2 value lower than 8.0 with 8 μg/mL of azithromycin. Compared to PA14, MPAO1 was inhibited much seriously. In MPAO1, the pyocyanin was not detectable when the azithromycin higher than 4 μg/mL. Phenazine PCA was more inhibited than PCN under same condition, and quinolone NHQ was the metabolite that severely affected by azithromycin. As shown in IMS, the clinic isolates strain FLR01 had less inhibition level than PA14 and MPAO1. The results from LC-MS/MS were consistent with IMS. For example, phenazine pyocyanin was detectable under all condition,

which showed low log2 value at -3.2 with 8 μg/mL azithromycin, meaning high levels of inhibition. The other metabolites all showed log2 value larger than -3.0 even with highest concentration of azithromycin we tested.

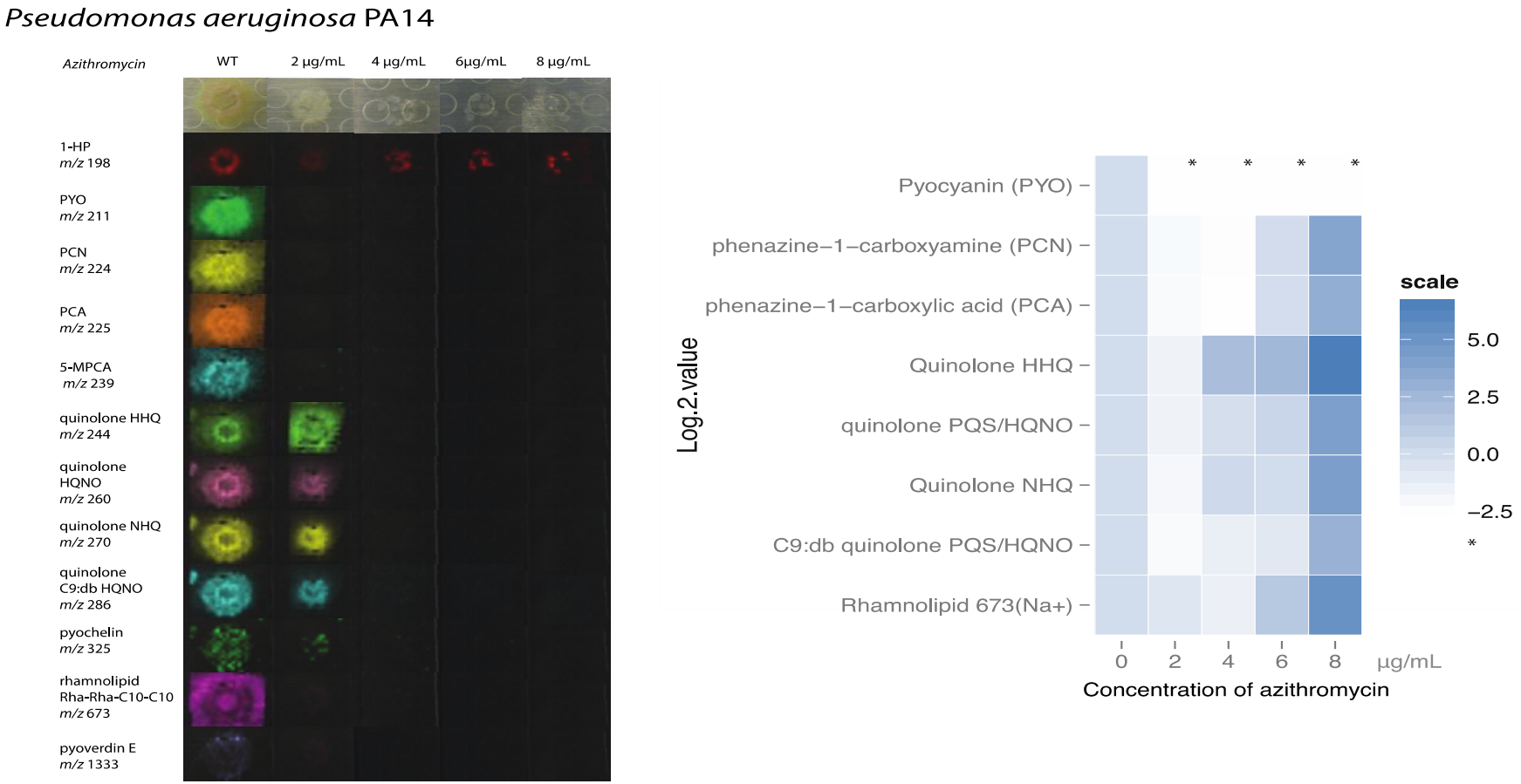

Figure 3-1. *P.aeruginosa* PA14: MALDI-IMS of selected specialized metabolites and quantitative comparison of production normalized by CFU

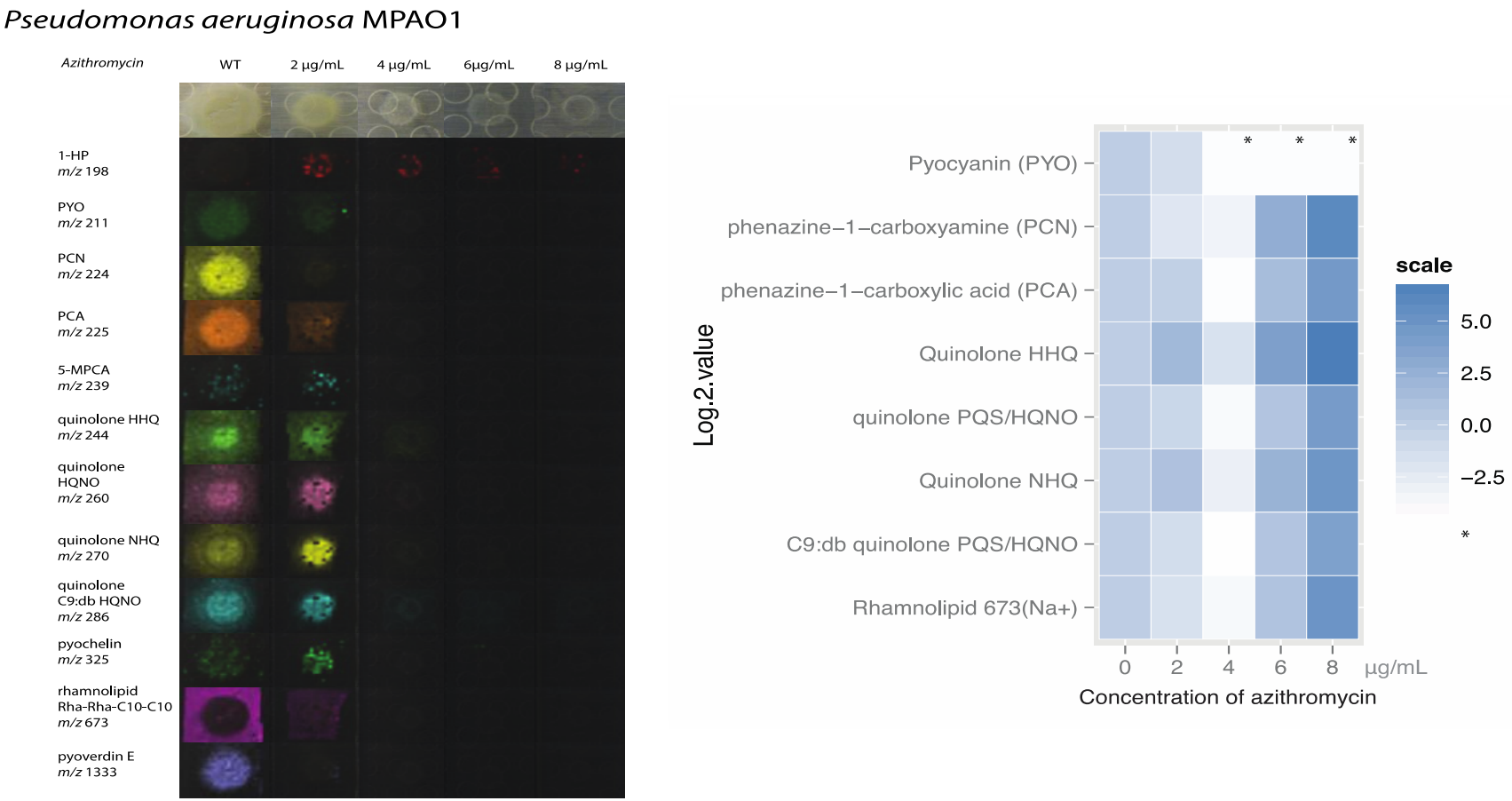

Figure 3-2. *P.aeruginosa* MPAO1: MALDI-IMS of selected specialized metabolites and quantitative comparison of production normalized by CFU

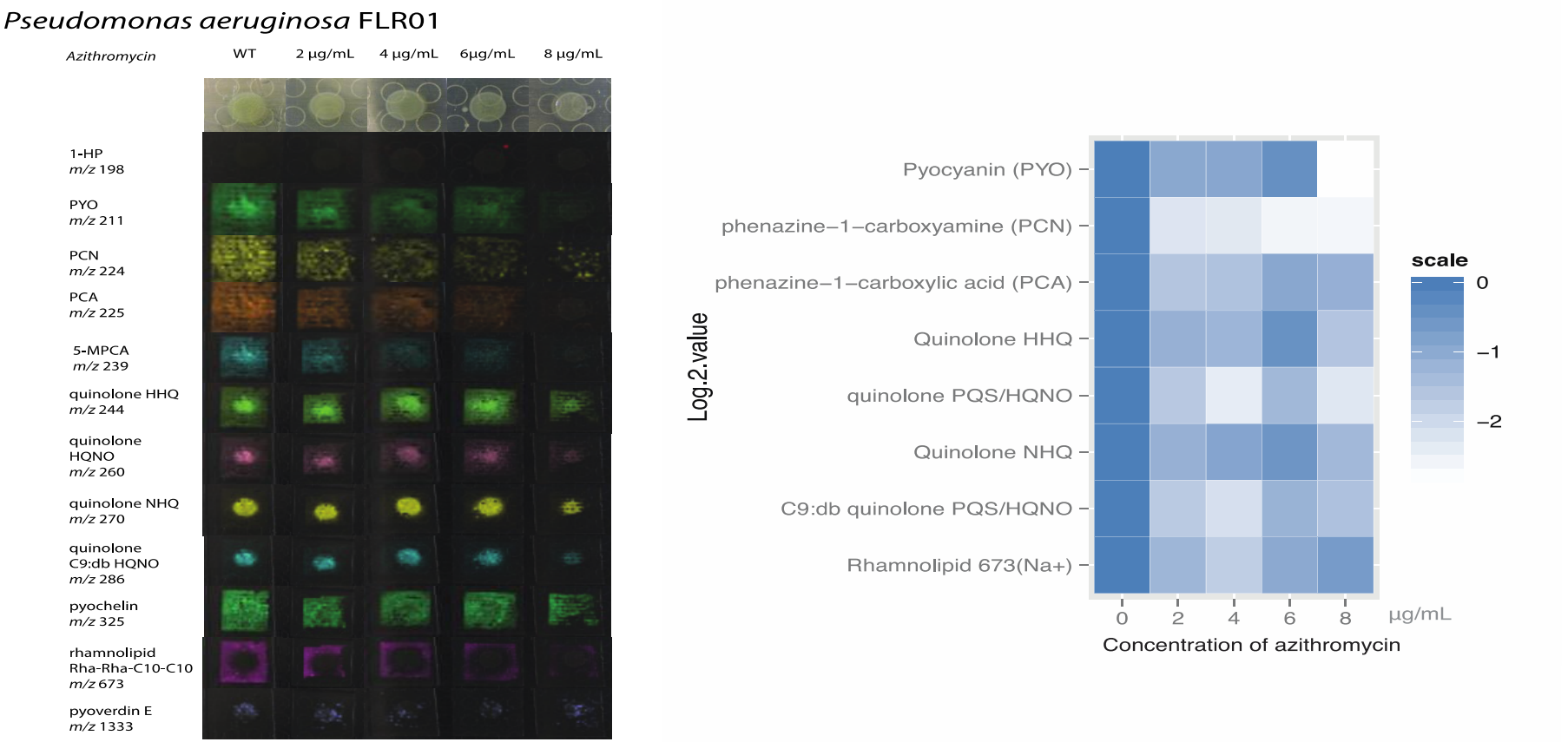

Figure 3-3. *P.aeruginosa* FLR01: MALDI-IMS of selected specialized metabolites and quantitative comparison of production normalized by CFU

Especially, phenazine PCA had the smallest fold change compared to others, which indicated lower inhibition level it had caused by azithromycin **(Figure 3-1, Figure 3-2 and Figure 3-3).**

Based on the results shown from MALDI-IMS and LC-MS/MS, antibiotic azithromycin had obvious inhibition on *P. aeruginosa* strains PA14, MPAO1 and clinic isolates FLR01 proportional to the concentration of azithromycin in the media.

3.3 Materials and methods

Strains and materials

Pseudomonas aeruginosa lab strains PA14 and MPAO1 were both provided by Dr. Vanessa Phelan, UCSD. Clinic isolated *Pseudomonas aeruginosa* FLR01 (non-mucloid) also provided by Dr. Vanessa Phelan, UCSD. Azithromyzin hydride (formula weight, 748.9 Da) was purchased from Sigma-Aldrich. All chemicals used for ISP2 media were purchased from Sigma-Aldrich.

Bacteria growth

Glycerol stocks of *Pseudomonas aeruginosa* MPAO1, PA14 and FLR01 were prepared by growing each strain in liquid LB culture overnight and diluted into 60% glycerol/water, and stored at -80^{o}C. For the MALDI-IMS the stocks were used directly after the sample went back to room temperature. The antibiotic ISP2 agar plates were prepared by adding azithromycin solution into the ISP2 media and then pouring the media in 100 O.D. x 25 mm Petri dishes (Fisher brand). The azithromycin solution was prepared by dissolving azithromycin hydride in methanol. *P aeruginosa* PA14, MPAO1 and FLR01 were inoculated on the ISP2 agar (10 mL) with

azithromycin in Petri dishes. The three strains were also inoculated on ISP2 agar (10 mL) without azithromycin in Petri dishes as controls. Samples were incubated for 48 hrs at 30 ^{o}C. For MALDI-IMS, a region of agar was cut off (range from 1.3 cm x 1.3 cm to 1.5 cm x 1.5 cm) covering the whole organism and laid on the top of MALDI MSP 96 anchor target plate.

MALDI imaging mass spectrometry analysis

A photograph was taken of the sample colony after cutting out and transferring onto a MALDI MSP 96 anchor plate. Another photograph was taken after applying a thin layer of Universal matrix (DHB) on top of the sample on the anchor plate using a 53 μm molecular sieve. Samples were dried at 37 ^{o}C overnight.

Samples of three different *P.aeruginosa* strains with azithromycin concentration gradient (2 μg/mL, 4 μg/mL, 6 μg/mL and 8 μg/mL) in ISP2 agar and wild type controls were subjected to Microflex (Bruker Daltonics) MALDI-TOF imaging mass spectrometry for data acquisition. All samples were run in positive reflectron mode, with 500 μm laser intervals in XY and a mass range of m/z between 100 Da to 2500 Da. The data was analyzed using FlexImaging (v3.0, Bruker). Intensity of metabolite ions in MALDI-IMS depended on various factors including concentration, and ionization efficiency.

Extractions protocol of LC-MS/MS analysis

In order to identify ions observed in MALDI-IMS, and quantitatively compare the metabolites productions in each sample, all samples were subjected to the an extraction protocol to be analyzed in LC-MS/MS. Fifteen agar plates containing PA14, MPAO1 or FLR01 with different concentrations of azithromycin (range from 2

μg/mL to 8 μg/mL) were inoculated. All plates were incubated for 48 hrs at 30 ^{o}C. Agar in each Petri dish with sample was cut into small slices and placed into 20 ml glass vial. The agar pieces were firstly extracted by 10 mL of ethyl acetate (EtOAc) for 1 hour, then the supernatant was taken out from the mixture, transferred into another 20 mL glass via and vaporized the solvent in vacuo. After that, the original agar pieces were extracted by 10 mL of methanol (MeOH) in the 20 mL glass vial for another hour. The supernatant was then transferred into the glass vial with extracts from EtOAc before vaporizing the solvent by vacuo again. Crude extracts were kept under -20 ^{o}C before using.

LC-MS/MS analysis of extracts

Chromatography was performed on a Dionex (Thermo scientific) high performance liquid chromatography (HPLC) with an UltiMate 3000 Series pump system and a vial autosampler. The analytical column was an UPLC column (1.7 μm C18 100A 30 x 2.10 mm, Phenomenex). The system was operated isocratically with a flow of 5 mL/min of solvent (A) 98% H_2O, 2% acetonitrile and 0.1% formic acid and (B) 98% acetonitrile, 2% H_2O and 0.1% formic acid. The mass spectrometry used for detection was MaXis (Bruker). Both the LC and MS systems were controlled by Hystar system,. The MS was operated in the positive ion mode and internal standard detection.

Data analysis and heatmap generation

The integrate analysis of LC-MS/MS data was done on XCMS online, Scripps Center for Metabolomics (https://xcmsonline.scripps.edu). Several of them that not able to be plotted out were manually integrated in DataAnalysis (v4.1, Bruker). The

output data was converted into text file. The text file was converted into output format in Perl, and used as input file to generate heatmap in R studio.

3.4 Conclusion and future work

In this preliminary study, we tested the effects of azithromycin under sub-MIC on three different *P.aeruginosa* strains, including both lab strains and clinic isolate. Under four different clinic achievable concentrations of azithromycin at 2 ug/ml, 4 ug/ml, 6 ug/ml and 8 ug/ml, there was a trend that the major metabolites phenazines, quinolones and rhamnolipids produced in lower abundance with the increasing concentrations of azithromycin after inoculated under 30 ^{o}C for 48 hours. Research showed that azithromycin has exposure-dependent bactericidal activity on *P.aeruginosa*. After 48 hours, the viability of bacteria was severely decreased. Interestingly, the metabolites production normalized by colony forming unit (CFU) in *P.aeruginosa* PA14 and MPAO1 showed that azithromycin may have bacteriostatic property under the concentration of 2 ug/ml and 4 ug/ml, but bactericidal property when the concentration of azithromycin increased to 6 ug/ml and 8 ug/ml. The preliminary results gave more solid prove about the inhibition effects of azithromycin on *P.aeruginosa*.

On the other hand, growing evidence suggests that the associated pulmonary infections should be considered from a microbial community perspective, rather a single pathogen infection. Bacterial pathogens such as *Pseudomonas aeruginosa*, and fungus like *Aspergillus fumigatus* are all considered relevance in CF airway infection (12). Fungal usually colonizes in the respiratory tract of CF patients, but their involvement in respiratory infections remains controversial and largely unsolved. In a polymicrobial community, microbial intend to form polymicrobial biofilms to

increase resistance to antibiotic therapy, immune responses and disinfectants (13), which makes the treatment becomes even harder. Many of the interactions within the polymicrobial biofilms are dependent upon secreted molecules. Yet, little study has been done addressing the convergence of multiples signals produced by multiple microorganisms as a community. With previous study on the polymircobial communication of *P.aeruginosa* and *A.fumigutus* (14), It would be interesting to further study polymicrobial interactions between common cystic fibrosis pathogens such as *P.aeruginosa*, *A.fumigutus* and *E.coli* under antibiotics treatment.

3.5 References

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