RAMAN SPECTROSCOPY BASED TOOLKIT FOR MAPPING BACTERIAL SOCIAL INTERACTIONS RELEVANT TO HUMAN AND PLANT HEALTH

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Sneha Polisetti Couvillion

Paul W. Bohn, Director

Graduate Program in Chemical and Biomolecular Engineering
Notre Dame, Indiana
June 2016
© Copyright 2016

Sneha Polisetti Couvillion
RAMAN SPECTROSCOPY BASED TOOLKIT FOR MAPPING BACTERIAL SOCIAL INTERACTIONS RELEVANT TO HUMAN AND PLANT HEALTH

Abstract

by

Sneha Polisetti Couvillion

Bacteria interact and co-exist with other microbes and with higher organisms like plants and humans, playing a major role in their health and well being. These ubiquitous single celled organisms are so successful, because they can form organized communities, called biofilms, that protect them from environmental stressors and enable communication and cooperation among members of the community. The work described in this thesis develops a toolkit of analytical techniques centered around Raman microspectroscopy and imaging representing a powerful approach to non-invasively investigate bacterial communities, yielding molecular information at the sub-micrometer length scale.

Bacterial cellular components of non-pigmented and pigmented rhizosphere strains are characterized, and regiospecific SERS is used for cases where resonantly enhanced background signals obscure the spectra. Silver nanoparticle colloids were synthesized in situ, in the presence of the cells to form a proximal coating and principal component analysis (PCA) revealed features attributed to flavins. SERS
enabled in situ acquisition of Raman spectra and chemical images in highly autofluorescent P. aeruginosa biofilms. In combination with PCA, this allowed for non-invasive spatial mapping of bacterial communities and revealed differences between strains and nutrients in the secretion of virulence factor pyocyanin. The rich potential of using Raman microspectroscopy to study plant-microbe interactions is demonstrated. Effect of exposure to oxidative stress, on both the wild type Pantoea sp. YR343 and carotenoid mutant ΔcrtB, was assessed by following the intensity of the 1520 cm⁻¹ and 1126 cm⁻¹ Raman bands, respectively, after treatment with various concentrations of H₂O₂. Significant changes were observed in these marker bands even at concentrations (1 mM) below the point at which the traditional plate-based viability assay shows an effect (5-10 mM), thus establishing the value of Raman microspectroscopy as a tool for high sensitivity studies of bacterial environmental stressors. The use of PCA in Raman imaging can also discriminate between spectral contributions from plant and bacterial cells. Finally, spectroscopy compatible microfluidic corral platforms are fabricated and a simple microfluidic technique is demonstrated for capturing bacterial cells. This opens up the possibility of studying bacterial communication in settings where it is possible to control population size and microenvironment.
Dedicated to the lotus feet

of my Gurudev
CONTENTS

Figures ............................................................................................................................................................ v

Acknowledgments ........................................................................................................................................... viii

Chapter 1: Introduction ..................................................................................................................................... 1
  1.1 Motivation to Study Bacteria .................................................................................................................. 1
    1.1.1 Bacterial Behavior— Communities and Interactions ................................................................. 1
    1.1.2 Economic Relevance of Bacteria ................................................................................................. 2
  1.2 The Need for Collaborative and Interdisciplinary Research ......................................................... 4
  1.3 Raman Spectroscopy ........................................................................................................................... 6
    1.3.1 Background ..................................................................................................................................... 6
  1.4 Raman Spectroscopy in Microbiology .............................................................................................. 9
  1.5 Objective and Organization of Text .................................................................................................. 10
  1.6 References ............................................................................................................................................ 12

Chapter 2: Raman Toolkit for Looking at the Microbial Cell ................................................................. 15
  2.1 Introduction .......................................................................................................................................... 15
    2.1.1 Theoretical Background .............................................................................................................. 16
    2.1.2 Enhancement of the Raman Signal ............................................................................................. 19
    2.1.3 Chemometrics for Data Analysis ............................................................................................... 20
    2.1.4 Research Overview and Practical Challenges ........................................................................... 21
  2.2 Experimental Section .......................................................................................................................... 26
    2.2.1 Materials and Methods .................................................................................................................. 26
  2.3 Results and Discussion ......................................................................................................................... 29
    2.3.1 The Importance of Control Experiments .................................................................................... 29
    2.3.2 Substrate Selection ....................................................................................................................... 30
    2.3.3 Raman Spectroscopy of Planktonic Cells ................................................................................. 30
  2.4 Concluding Remarks ............................................................................................................................ 40
  2.5 References ............................................................................................................................................ 42

Chapter 3: Looking Outside the Microbial Cell— Investigations of the Secretome for Small Molecule Metabolites ................................................................................................................................. 50
  3.1 Introduction .......................................................................................................................................... 50
    3.1.1 Theoretical Background .............................................................................................................. 52
      3.1.1.1 Pyocyanin in P. aeruginosa ................................................................................................. 52
    3.1.2 Research Overview ....................................................................................................................... 54
  3.2 Experimental Section ............................................................................................................................ 56
<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 Materials and Methods</td>
</tr>
<tr>
<td>3.3 Results and Discussion</td>
</tr>
<tr>
<td>3.3.1 Circumventing the Issue of Autofluorescence</td>
</tr>
<tr>
<td>3.3.2 Incorporating Electron Microscopy and PCA in SERS Imaging of <em>P. aeruginosa</em> Biofilms—The Superior Approach</td>
</tr>
<tr>
<td>3.3.3 Strain and Nutrient Based Differences in Pyocyanin Production</td>
</tr>
<tr>
<td>3.4 Concluding Remarks</td>
</tr>
<tr>
<td>3.5 References</td>
</tr>
<tr>
<td>3.6 Chapter 4: Raman Imaging of the Rhizosphere Bacterium <em>Pantoea</em> sp. YR343—Cell Response to Oxidative Stress and Co-Culture with <em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>4.1 Introduction</td>
</tr>
<tr>
<td>4.1.1 Theoretical Background and Research Overview</td>
</tr>
<tr>
<td>4.1.1.1 Friends and Foes in the Rhizosphere: Plant-Microbe Interactions</td>
</tr>
<tr>
<td>4.1.1.2 Relevance of Oxidative Stress in Rhizosphere</td>
</tr>
<tr>
<td>4.2 Experimental Section</td>
</tr>
<tr>
<td>4.2.1 Materials and Methods</td>
</tr>
<tr>
<td>4.3 Results and Discussion</td>
</tr>
<tr>
<td>4.3.1 Role of Carotenoids in YR343 in Withstanding Oxidative Stress</td>
</tr>
<tr>
<td>4.3.2 Pantoea-Arabidopsis Co-cultures</td>
</tr>
<tr>
<td>4.3.3 Indole Acetic-Acid (IAA) Production and Detection</td>
</tr>
<tr>
<td>4.3.3.1 Using Raman Spectroscopy for Detection of IAA</td>
</tr>
<tr>
<td>4.4 Conclusions</td>
</tr>
<tr>
<td>4.5 References</td>
</tr>
<tr>
<td>4.7 Chapter 5: Towards Microfluidic Platforms for Raman Spectroscopy</td>
</tr>
<tr>
<td>5.1 Introduction</td>
</tr>
<tr>
<td>5.1.1 Theoretical Background and Research Overview</td>
</tr>
<tr>
<td>5.2 Experimental Section and Results</td>
</tr>
<tr>
<td>5.2.1 Materials &amp; Methods</td>
</tr>
<tr>
<td>5.3 Results and Discussion</td>
</tr>
<tr>
<td>5.3.1 Micro-corral Fabrication</td>
</tr>
<tr>
<td>5.3.2 Bacterial Cell Capture in Corrals</td>
</tr>
<tr>
<td>5.4 Conclusions</td>
</tr>
<tr>
<td>5.5 References</td>
</tr>
<tr>
<td>5.8 Chapter 6: Conclusions and Outlook</td>
</tr>
<tr>
<td>6.1 Suggested Future Studies</td>
</tr>
<tr>
<td>6.2 References</td>
</tr>
</tbody>
</table>
FIGURES

Figure 2.1: Energy level diagram depicting absorption, scattering and photoluminescence processes ................................................................. 17

Figure 2.2: Raman spectrum generated from planktonic cells of wildtype *Pseudomonas* sp. GM41 ........................................................................................................... 31

Figure 2.3: Overlay of resonance Raman spectra generated from planktonic cells of wild type *Pantoea* sp. YR343 (top) and mutant strain ΔcrtB (bottom). Spectra have been offset for comparison ................................................................. 34

Figure 2.4: Principal component loading plots from a sample of *Pantoea* sp. YR343 planktonic cells mixed with pre-formed Ag colloid (A) and in situ Ag-coated *Pantoea* sp. YR343 cells (B) ........................................................................................................... 36

Figure 2.5: SEM images. (A) *Pantoea* sp. YR343 planktonic cell; (B) Silver-coated *Pantoea* sp. YR343 planktonic cell; and (C) Higher magnification image of silver clusters on surface of a *Pantoea* sp. YR343 planktonic cell. Ag cluster identity was confirmed by EDS ........................................................................................................................................................................... 38

Figure 2.6: TEM images of thin cross-sections of *in situ* Ag-coated *Pantoea* sp. YR343 cells with preferential accumulation of Ag on the inner membrane indicated with circles ........................................................................................................................................................................... 39

Figure 3.1: Spatially averaged SERS spectrum of pyocyanin standard with molecular structure (inlay) ........................................................................................................... 54

Figure 3.2: Comparison of spectra generated from a *P. aeruginosa* bacterial community under normal Raman conditions (top) and with the addition of ~12 nm Ag colloid to generate SERS (bottom). Raman and autofluorescence spectra have been scaled differently to appear on the same plot ........................................................................................................................................................................... 59

Figure 3.3: SEM image taken from a representative region of a 24 h FRD1 pellicle biofilm grown in glucose and decorated with 12 nm Ag nanoparticles showing the spatial distribution and state of aggregation of Ag nanoparticles ........................................................................................................................................................................... 61

Figure 3.4. (A) SERS image constructed as a heat map of the integrated intensity in the range 2800-3000 cm⁻¹, generated from a 48 h FRD1 pellicle biofilm
grown in glucose. (B), (C) and (D) SERS spectra of individual voxels obtained from different spatial locations in the image displayed in A. (E) SERS spectrum obtained by averaging all of the spectra obtained within the image in (A), *i.e.* the spatially-averaged spectrum.

Figure 3.5. SERS imaging and PC analysis of a representative area of a 48 h FRD1 pellicle biofilm. (A) and (D) are loading plots of PC1 and PC2 generated from the SERS image (C) which is integrated over the 2800-3000 cm⁻¹ filter; (B) and (E) are heat maps of PC1 and PC2, respectively.

Figure 3.6. Analysis of 24 h pellicle biofilms of *P. aeruginosa* FRD1 and PAO1C strains in response to the source of carbon. Raman spectra (i), principal component heat maps (ii), and SERS images (iii) integrated over 2800-3000 cm⁻¹ for representative regions of pellicle biofilms derived from *P. aeruginosa* FRD1 (A-B) and PAO1C (C) are shown. Glucose (A and C) and glutamate (B) were provided as carbon sources.

Figure 4.1: Wild type YR343 and ΔcrtB mutant microcolonies on agar plates and in microcentrifuge tubes, showing visual difference in pigmentation.

Figure 4.2: Raman spectrum of pure zeaxanthin standard.

Figure 4.3: Cell survival after H₂O₂ exposure as measured by Bac-Titer Glo plate-based viability assay. YR343 depicted in red and ΔcrtB in blue.

Figure 4.4: Normalized Raman spectral overlay of (A) YR343 and (B) ΔcrtB mutant after treatment with various concentrations of hydrogen peroxide.

Figure 4.5: (A) Overlay of Raman spectra generated from wild type Pantoea sp. YR343 and ΔcrtB mutant with circles indicating the peaks selected for comparing the effect of varying hydrogen peroxide concentration; (B) Normalized intensity of selected peaks in YR343 and ΔcrtB as a function of H₂O₂ concentration (M).

Figure 4.6. Raman spectra of senescent samples dried for (A) 5 days and (B) 15 days. Each panel shows an overlay of *Arabidopsis thaliana* plant (bottom) and *Arabidopsis thaliana* inoculated with *Pantoea* sp. YR343 (top). Spectra were background subtracted after fitting to a 3rd order polynomial.

Figure 4.7. SEM images of *Pantoea* sp. YR343 colonizing *A. thaliana* root showing spatial distribution and growth of bacteria on the root at different magnifications: (A) 1,200x, (B) 15,000x and (C) 50,000x.

Figure 4.8. Chemical imaging of *Arabidopsis thaliana* root inoculated with wild-type *Pantoea* sp. YR343. (A) Raman image integrated over 2800-3050 cm⁻¹; (B) principal component heat map and (C) corresponding loading plot.
representing the principal component obtained from the Arabidopsis regions; (D) Raman spectrum showing the 2800-3050 cm-1 wavenumber region; (E) Raman image integrated over 1507-1546 cm-1, (F) principal component heat map and (G) corresponding loading plot obtained from regions dominated by Pantoea. (H) Combined Raman image produced using a combination of the spectral filters used in Figures 4.8(A) and 4.8(E), showing the spatial distribution of bacteria (yellow) on a plant root (green).........................99

Figure 4.9: Salkowski colorimetric test showing red color in presence of IAA and indoles (left) and indole production in YR343 and ΔcrtB mutant as measured by colorimetric assay...........................................................................................................101

Figure 4.10: Overlay of Raman spectra of IAA and L-tryptophan. Offset for comparison...........................................................................................................................................................................103

Figure 5.1: Schematic describing the sequence of steps in micro-corrall fabrication121

Figure 5.2: (A) 10 μm and (B) 50 μm corral arrays with a depth of 10 μm, fabricated using photolithography .................................................................................................................................122

Figure 5.3: Overlay of Raman spectra generated from the plain glass slide (blue), base of micro-corrall (green) and regions on the substrate between the corrals containing SU-8 (pink). ........................................................................................................................................123

Figure 5.4: (A) Schematic for microfluidic capture of bacterial cells in micro-corrrals. (B) Bright-field image of Pseudomonas sp. GM41 cells captured in 50 μm corrals..................................................................................................................................................125

Figure 5.5: Pseudomonas sp. GM41 cells imaged in 25 μm corrals. (A) Two cells captured using a low-density culture; Cells adhering to the corral’s base (B) and walls (C)..............................................................................................................................................126

Figure 5.6: Overlay of Raman spectra generated from the SU-8 region between corrals (red) and the region inside a corral containing cells using a 40x (green) and 60x (blue) objective .............................................................................................................................127

Figure 5.7: Overlay of Raman spectra of GM41 cells in a micro-corrall. The three spectra were collected sequentially, top-down..................................................................................................................129
ACKNOWLEDGMENTS

As I look back upon the last five years spent at Notre Dame, I am grateful to many people who have made this journey fruitful and positive. My research advisor Dr. Paul Bohn has been an incredible source of inspiration and support. He has been a wonderful mentor, giving the right balance of guidance and independence during my doctoral work. All the members of the Bohn research group have contributed to my betterment as a scientist and as a person. Dr. Sean Branagan, Dr. Chaoxiong Ma, Dr. Bei Nie, Dr. Barrett Duan, Dr. Jing Zhao, Dr. Dane Grismer, Dr. Rachel Masyuko, Dr. Larry Gibson, Dr. Nick Contento, Dr. Tai-Wei Hwang, Dr. Dorothy Alhf, Dr. Larry Zaino, Dr. Donghoon Han, Kayla Shaw, Yang Yang, Min Yu, William Wichert, Lauren Gibson, Jenny Hu, Yang Song, Garrison Crouch, Kaiyu Fu, Wei Xu, Erick Foster, Chelsea Ayres and Abby Cao have all been good colleagues.

My collaborators were not only excellent scientists but also very enjoyable people to get to know and work with. I would like to thank Nameera Baig, Dr. Amber Bible, Dr. Jennifer Morrell-Falvey, Dr. Scott Retterer, Dr. Robert Standeart and Dr. Mitch Doktycz. Dr. Joshua Shrout and Dr. Nydia Morales-Soto were generous with providing lab space and guidance with microbiological methods apart from being delightful collaborators.
I wish to thank the members of my committee, Dr. Norman Dovichi, Dr. Hsueh-Chia Chang and Dr. Jason Hicks for their helpful suggestions, guidance and time. I am thankful for the assistance provided by William Archer and Tatyana Orlova from NDIIF for the electron microscopy experiments. I feel lucky to have taken courses and been a TA for many wonderful faculty members in Chemical Engineering. The administrative staff in the CBE department has always been very helpful- special thanks to Lauren Gibson, Chelsea Ayres, Nadia Casas, and Anne Veselik. Thanks to the patient feedback and help from Shari Hill Sweet and her team, the dissertation submission process was straightforward.

I am indebted to my family and friends for being unconditionally loving and supportive. If not for them, it would be hard to imagine moving to the other side of the world and getting a PhD. My Guru's and my parents’ blessings have been my pillars of strength. My maternal grandfather, the first engineer in his village, has been my inspiration. Pierre, my wonderful husband, has been by my side through the ups and downs during the last two years and made everything seem better. I am blessed to have the most loving parents-in-law, both PhDs, who have always been enthusiastic and encouraging about my research. I look back fondly at the time spent with dear friends- Nadia Suzuki, Golnaz Karbazian, Pradeepa Vennam, Kaavya Polisetti and Vandhana Raju- I couldn’t have survived South Bend’s long winters without their cheerful presence.
CHAPTER 1:
INTRODUCTION

1.1 Motivation to Study Bacteria

Bacteria have a simple cell structure, lacking a nucleus or membrane bound organelles, and their entire genetic information is contained in a single loop of DNA. Despite this seemingly simple organizational plan, these single celled organisms are one of the most adaptable, widespread and successful life forms on the planet, playing a crucial role in plant and animal health,\(^1\) cycling of nutrients\(^2\) and smooth functioning of global ecosystems.\(^3\)\(^4\) In short, the well being of all life forms seems to depend directly or indirectly on these microorganisms. Initially bacteria were studied in the context of disease and pathogenesis and since the 19\(^{th}\) century microbes have been known to cause infectious diseases.\(^5\) Recent evidence points to their possible role in cardiovascular disease,\(^6\) cancer,\(^7\) dementia,\(^8\) diabetes\(^9\) and other seemingly unconnected illnesses. Research is increasingly establishing the relationship between the human gut microbiome and the overall health of humans including susceptibility to chronic disease.\(^10\)

1.1.1 Bacterial Behavior— Communities and Interactions

Just as multicellular organisms like humans and animals can survive more successfully in groups or societies, bacteria also tend to form communities, the most
common form being biofilms, and are seldom found as single entities or free-floating ‘planktonic’ cells. Bacterial biofilms are comprised of a group of cells that find a surface to attach to and surround themselves with a protective armor of extracellular substances. Biofilms are complex and highly successful, with the cells actively communicating among themselves using a chemical vocabulary. Bacterial cells in biofilms are phenotypically very differently than their planktonic counterparts and are known to show 1000 times higher resistance to antibiotic treatments. Although both have the same genes, the gene functions change dramatically between the planktonic and biofilm states, and studies suggest that as much as 40% of the genes may be up-regulated (turned on) or down-regulated (switched-off) when the planktonic-to-biofilm transition occurs. This strongly suggests that laboratory studies carried out on planktonic bacterial cells do not represent the behavior of their biofilm counterparts. The same holds true with the development of antibiotics, because most persistent infections are caused by biofilms. There is a huge motivation to study the phenotypic differences between individual cells and bacterial communities and understand the complex interactions and cooperation in biofilms to get a molecular picture of bacterial.

1.1.2 Economic Relevance of Bacteria

Bacteria have a significant presence in the food industry, be it in the production of probiotic and fermented foods, the markets for which continue to increase rapidly, or as a matter of concern in food spoilage and safety. The pharmaceutical industry uses bacteria to produce medically valuable vaccines,
antibiotics and enzymes, which have drastically increased average life expectancy and quality of life. The worldwide sale of medical and pharmaceutical drugs of microbial origin exceeds US $13 billion annually.²⁰ The advent of genetic engineering and biotechnology has opened up endless possibilities in the production of useful biological substances for human health, environmental bioremediation, biofuels, and agricultural pest-control. In the 1940s Mary Shorb’s work with *Lactobacillus lactis* resulted in the discovery of vitamin B12 and led to its isolation by Merck and Co. from *Streptomyces griseus*.²¹ In the mid fifties, Kinoshita et al. reported the production of monosodium glutamate (MSG) using bacteria and the fermentation method continues to be used in industrial production.²² With the discovery of recombinant DNA methodology in the 1970s, genetically engineering bacteria were then also used to make insulin and human growth hormone, resulting in the boom of the biotechnology industry.²³-²⁴ According to the consulting firm Ernst & Young, in the US alone, as of 2014 there were 2519 biotech companies with revenues of publically traded Biotechs reaching US$ 93.1 billion - a 29.1% increase from the prior year.²⁵ According to the National Institute of Health (NIH), more than 65% of all microbial infections are caused by biofilms.²⁶ Centre for Disease Control and Prevention (CDC) estimates that antibiotic-resistance adds $20 billion per year to healthcare costs and as much as $35 billion per year to society for lost productivity in the United States.²⁷

As Louis Pasteur put it, “Never underestimate the power of the microbe”. The ubiquitous role of harmful and useful bacteria is a compelling motivation to identify,
study and understand the structure and function of these fascinating life forms, both individually and collectively in their biofilm communities.

1.2 The Need for Collaborative and Interdisciplinary Research

Although it makes more sense to study bacteria in the context of biofilms, just as they naturally tend to exist, research on biofilms has been growing slowly owing to many intrinsic challenges. Analytical methods, both conventional and emerging, are required to understand the physical and biochemical composition and structure of biofilms and the bacterial cells that they are comprised of. This may require the collective expertise of analytical chemists, biomedical engineers, clinicians and microbiologists for a multi-pronged approach to studying these complex systems. In addition to intricate chemical networks of communication, biofilms have well developed physical architectures. Hall-Stoodley et al., in a Nature Reviews article, describe the difficulty of culturing biofilms in the laboratory since it involves careful calculations of diffusion, flow rates and fluid forces acting on them. Although this is something that may not be appealing to a traditional microbiologist, a chemical engineer may be up for the task. This is just one example of why expertise from various fields of science and engineering is needed to move this area of research forward. There is also a pressing need for non-invasive techniques and tools to investigate molecular basis of bacterial behavior in situ or in samples that simulate the microenvironment. Many of the methods and assays that are commonly used in microbiological laboratories have not changed much for decades and a many of them are still based on visual assessment. Confocal
microscopy offers the advantages of sensitivity, high resolution and easy integration with flow cells but requires use of labels and optically transparent flat samples and cannot probe the interior of biofilms, which can be several hundred micrometers thick. Scanning electron microscopy (SEM) can visualize the biofilm without the need of stains or labels at a very high resolution (~few nm) but requires extensive sample preparation and cannot be used in real time. Atomic force microscopy (AFM) can be used to study surface morphology and test adhesion forces. These techniques are low-throughput and cannot provide any information about the chemical composition of the sample. Genetic assays are rapid compared to culture methods and capable of giving species specific information but provide little information about the biofilm matrix or secreted metabolites. Different forms of mass spectrometry and mass spectrometry imaging offer the sensitivity and specificity to characterize the microbe metabolome. Depending on the modality used, spatial resolution, matrix interference, sample preparation and the destructive nature of the MS technique may be potential problems. Raman spectroscopy and imaging are also capable of providing chemical information about multiple analytes in the same spectrum and offer good spatial resolution (< 1 μm) but suffer from low sensitivity and may require enhancing strategies. Microfluidic technologies can be used to apply physical and chemical stimuli to recreate environmental conditions and measure the response. This has the potential to lessen the gap between in vivo and in vitro measurements. The use of microfluidic platforms also enables the study of single cells and small-molecule metabolites produced in low concentrations.
It is evident that no single technique has all the required capabilities to study microbial populations but a combination of the abovementioned methods can shine light on different aspects thus giving a comprehensive picture of these complex and fascinating communities.

1.3 Raman Spectroscopy

Vibrational spectroscopic methods like Raman spectroscopy and Fourier transform infrared (FTIR) are increasingly being used to identify and characterize bacteria based on the vibrational modes of characteristic molecules. The attractiveness of these techniques is that they are non-invasive and non-destructive while being capable of providing a whole organism fingerprint.\textsuperscript{30} Raman spectroscopy is well suited for different types of samples including solids, liquids and even gases, which can be analyzed through transparent containers like glass vials. This is very useful for samples stored under inert gases due to their tendency to oxidize or in testing potentially hazardous substances while contained. The emergence of small, portable spectrometers, which allow recording of spectra from a distance, has made it a technique of choice in quality control, forensic analysis and anti-counterfeiting.

1.3.1 Background

In 1930 Sir C. V. Raman was awarded the Nobel Prize for his work on the inelastic scattering of light by a fluid. He used filtered sunlight as the light source and recorded the spectrum on photographic plates using exposures ranging up to 24
hours. Instrumental innovations, enhancing methodologies and developments in data analysis have brought Raman spectroscopy out of specialized research laboratories, and the technique is being adopted in industry, forensics, medical settings as well as fundamental research.

It is useful to remember that the Raman effect results from a vibrationally modulated change in molecular polarizability, which distinguishes it from the other principal vibrational method, infrared spectroscopy, that relies on a change in the permanent dipole moment. These physical mechanisms give rise to selection rules that can be used to determine which vibrations in a molecule are Raman active and which are infrared active, with quantum and group theoretical approaches being used to determine activity of complex molecules. In common practice it is known that vibrations that result in very strongly intense infrared bands tend to be weak Raman scatterers. Another difference between the two vibrational techniques is that water weakly scatters but absorbs strongly, making Raman more amenable to hydrated samples. This is especially useful while choosing between the methods for study of biological samples in their native state.

The primary challenge to using Raman spectroscopy is the fact that it is inherently weak, with only one in $10^6$-$10^8$ photons being inelastically scattered for a typical molecule. The intensity of a Raman band is dependent on two groups of parameters, one of which is sample related and the other, instrument related. The instrument parameters include laser frequency and laser power, numerical aperture of collection optics, efficiency of optical components especially the transfer efficiency and the quantum efficiency of the detector. Because the intensity of the
Raman signal is proportional to the intensity of the laser irradiating the sample, it is useful to use the highest laser intensity possible without degrading the sample due to thermal effects or photodecomposition. This becomes especially important in analytes that are light or heat sensitive and are present in low concentrations in the sample. Fluorescence, a stronger effect, is a significant source of interference when using Raman spectroscopy. This issue is amplified in biological samples that commonly are auto-fluorescent, which can strongly interfere, and even overwhelm the Raman spectrum. Autofluorescence is a common issue faced in Raman analysis of biological samples especially in in situ studies where sample pre-treatment is not possible. Confocal systems and judicious choice of the laser wavelength can help along with post-processing data analysis techniques. Changing the excitation wavelength can circumvent this issue and commonly longer excitation wavelength lasers like 785 and 1064 nm, are used for this reason. However, the intensity of the Raman signal, which is proportional to the fourth power of the laser frequency, also drops at longer wavelength excitations. Sometimes photobleaching can be used to destroy the fluorophore, if its absorption spectrum is sufficiently different from the analyte molecule by irradiating the sample with the laser prior to spectral data collection. Mathematical tools are also used in the data processing stage to eliminate fluorescence background from the spectrum. In this work, one or more of these strategies are used. The two most commonly used mechanisms to enhance the Raman signal are resonance enhanced Raman spectroscopy (RR) and surface enhanced Raman spectroscopy (SERS), both of which have been used in this work.
1.4 Raman Spectroscopy in Microbiology

Integrating confocal microscopy within Raman instruments has resulted in the emergence of confocal Raman microscopy (CRM), which allows for obtaining molecular information with micrometer-scale resolution in all three Cartesian directions. This is useful for obtaining a spectral footprint of a single bacterium. A majority of the studies using Raman spectroscopy in microbiology have targeted microbial identification. This is because the Raman spectrum is rich with vibrational information on various cellular components and contains bands that can be assigned to nucleic acids, proteins, carbohydrates and lipids. The technique is sensitive enough to show slight differences in the positions of these peaks between different species and even different strains. This offers the microbiologist a quick (order of seconds to minutes) method of identification as opposed to traditional techniques (order of days) that involve plating and culture, which may not even be possible for most strains that cannot be cultivated in a laboratory setting. Additionally, Raman spectroscopy requires little sample preparation and is non-invasive. The spectrum can be collected from live cells (in culture, on plates or on a environmental specimen) without disturbing the sample, which can continue to grow. This allows the same sample to be studied over time, which can be beneficial in a variety of experiments.

Although it offers so many advantages, Raman spectroscopy is not a commonly used laboratory technique for studying bacterial cells or communities. Partly, this is because the use of the technique requires trained personnel especially for experimental design and data interpretation. In fact, much of the published work
on microbial Raman spectroscopy has been done by analytical scientists who chose a biological system to study. This has mostly limited the use of spectroscopy to identification, detection and characterization of the bacterial cell, when in fact Raman can be used to map a variety of molecular scale phenomena as demonstrated in the following chapters. This work aims to illustrate the potential of Raman spectroscopy and its use in studying various aspects of bacterial communities and their interactions. There is much to be gained by collaborative research between microbiologists and spectroscopists in this field of study. It opens up a whole new world of fascinating questions to answer, for the analytical chemist/engineer and provides powerful new tools, which are capable of giving spatio-temporal chemical information to the microbiologist.

1.5 Objective and Organization of Text

This work intends to demonstrate the competencies of using Raman spectroscopy, RR, SERS and imaging along with complementary techniques like multivariate analysis, electron and optical microscopy and microfluidic platforms for investigating various facets of bacterial communities and interactions, many of which, until now, have only been studied with conventional microbiological techniques. Thus, in addition to demonstrating unique applications of Raman spectroscopies in microbiology, this thesis will also serve as a practical guide for designing novel bio-imaging studies, overcoming experimental challenges and avoiding common problems arising in data interpretation. This work mainly focuses on bacterial strains relevant to human and plant health.
Chapter 2 introduces the Raman toolkit consisting of various techniques in addition to Raman spectroscopy, that are used in this work. The focus in this chapter is on the bacterial cell and its components. Chapter 3 demonstrates the use of the toolkit to look outside of the cell and investigate the bacterial secretome for small molecule metabolites and chemical signals used for bacterial cell-to-cell interactions. In Chapter 4, novel spectroscopic approaches are presented for studying rhizobacteria and their intra-species interactions with plants. Chapter 5 discusses the preliminary work done towards designing spectroscopy-compatible microfluidic platforms for studying bacterial communities and their behavior. Investigations worth pursuing in the future and experimental improvements to better investigate these systems are suggested in Chapter 6.
1.6 References


15. Cunningham, A. B., Ross, R. J., Biofilms: the hypertextbook. *Montana State University, Bozeman, MT 2006*.


CHAPTER 2:
RAMAN TOOLKIT FOR LOOKING AT THE MICROBIAL CELL

This chapter is based on previously published material from:

2.1 Introduction

The study of the bacterial cell is applicable to general microbiology, identification and discrimination of strains in both routine and medically relevant settings, and in studying biochemical changes inside the cell in response to external stress. Single cell microbiology has become important for studying uncultivable organisms and also for understanding heterogeneity in microbial populations, which is the basis of their superior adaptability.\textsuperscript{1} Raman spectroscopy is a powerful bioanalytical tool for studying bacterial cells and cellular components and monitoring changes in them over time. It is capable of providing a wealth of information in a single spectrum. Advances in microscopy have made it possible to obtain vibrational information from single bacterial cells producing spatially-resolved Raman microspectra, which contain bands assigned to nucleic acids,
proteins, carbohydrates and lipids. Indeed, the intensity and placement of these bands can be used in microbial identification at the strain level.$^{2-5}$

This chapter focuses on the studies and approaches used to overcome common challenges in elucidating bacterial cellular components. The reader is introduced to the toolkit of techniques used in conjunction with Raman spectroscopy that will be employed in chapters that follow. Raman and Resonance Raman spectroscopy are used to characterize non-pigmented and pigmented bacterial cells isolated from the rhizosphere of Polulus deltoides and prove successful mutagenesis in an engineered mutant strain. A SERS based approach is demonstrated for targeted detection of bacterial cellular components in the presence of resonantly enhanced carotenoid pigments, with principal component analysis (PCA) and electron microscopy contributing significantly to data analysis and interpretation.

2.1.1 Theoretical Background

Interactions of light (electromagnetic radiation) with matter can result in scattering, absorption and transmission. Figure 2.1 illustrates the energy level diagram for these processes in an example molecule, where E=0 and E=1 are the ground and first excited electronic energy levels, respectively, and the vibrational energy levels in increasing order of energy are denoted by ν=0,1,2 and 3. In Raman spectroscopy, an intense monochromatic beam of light, typically a laser, is focused on the sample, and the intensity of the scattered radiation is measured as a function of wavelength. Most of this light is scattered elastically in Rayleigh scattering where
the frequency and wavelength of the scattered radiation remains unchanged from the incoming radiation. A small fraction of the scattered light is scattered inelastically, in which there is a shift in the frequency of the scattered photons due to their interaction with molecular vibrations, resulting in the Raman effect. This shift can be Stokes or anti-Stokes depending on whether the inelastically scattered photon is shifted to a lower or higher frequency upon interaction with the molecular rotational or vibrational state.6
Plotting the measured intensity of the scattered radiation as a function of the Raman shift or wavenumber $\Delta \bar{\nu} \text{ (cm}^{-1}\text{)}$ generates a Raman spectrum.\textsuperscript{7}

$$\Delta \bar{\nu} = \frac{\nu_m}{c} - \frac{\nu_0}{c} \quad \text{Eq. 2.1}$$

where, $\nu_m$ and $\nu_0$ represent the frequency of measured and incident radiation and $c$ is the speed of light. Being a difference value, the Raman shift is independent of the frequency of the incident radiation. This enables comparison of spectra collected across common excitation sources such as UV (244 nm, 325 nm), visible (488 nm, 514 nm, 532 nm, 633 nm) and near infrared (785 nm, 830 nm, 1064 nm). The intensity of the Raman signal $I_R$, however, is wavelength dependent and can be expressed as,\textsuperscript{6}

$$I_R \propto I_0 (\bar{\nu}_0 \pm \bar{\nu}_{vib})^4 N \quad \text{Eq. 2.2}$$

where, $\bar{\nu}_{vib}$ is the vibrational frequency, $I_0$ is the intensity of the incident radiation and $N$ is the number density of molecules. This indicates that high frequency or shorter wavelength excitation will achieve higher Raman scattering efficiency. UV sources and detection systems, however, can be expensive, and UV sources are more likely to cause photodamage when compared to longer wavelength sources. Fluorescence that can overwhelm the Raman spectrum is another major concern at wavelengths in the UV and visible region. Ultimately, each excitation wavelength offers some advantages over others and the choice depends on the sample under study.
2.1.2 Enhancement of the Raman Signal

Despite the several advantages that Raman offers, even for very strongly scattering molecules only 1 in about $10^7$ photons will undergo inelastic (Raman) scattering while the rest participate in competing processes like fluorescence, (Rayleigh) elastic scattering, etc., making the Raman signal inherently weak.\(^8\) Enhancing techniques like Resonance Raman (RR), surface enhanced Raman scattering (SERS) and coherent anti-stokes Raman scattering (CARS) have been developed to overcome this drawback. These enhancing techniques make it possible to probe analytes at biologically relevant concentrations and in their native environment. They have opened up the possibility of using Raman for a much wider range of applications and are the key reason that Raman is becoming a mainstream technique in areas such as microbiology, forensics and quality control.

By selecting a laser wavelength that coincides with an allowed transition to an electronic excited state of the analyte molecule, it is possible to significantly enhance the Raman intensity. This phenomenon, known as Resonance Raman, provides the user with the ability to selectively enhance a specific molecule in the sample, if one has access to the appropriate excitation wavelength. An enhancement of the signal can be observed even when the wavelength does not match exactly but is within the pre-resonance region of a few hundred wavenumbers below the electronic transition.\(^9\)

The Raman signals can also be greatly enhanced by surface enhanced Raman scattering (SERS), which intensifies the signal by factors ranging from $10^6$-$10^8$ (averaged over many molecules) to $10^{14}$ (special cases of single molecules in...
metallic nanogaps) when the molecule of interest is in close proximity to an enhancing surface feature.\textsuperscript{10} It is known that both electromagnetic and chemical effects contribute to SERS enhancement, although the relative contributions are still a matter of debate in the literature. The choice of an effective SERS substrate is dependent on many factors. The size and/or spacing and material determine the localized surface plasmon resonance (LSPR) wavelength, which must match the laser wavelength in use.\textsuperscript{11} Generally silver is preferred for blue and green wavelengths and gold for red and near-IR wavelengths. Other metals like copper, platinum and palladium have also exhibited enhancing characteristics. The charge of the particles or surface can affect the adsorption of the analyte and therefore the enhancement.\textsuperscript{12} Intimate contact of the analyte with the substrate is crucial, as the enhanced fields decay very rapidly from the nanostructure surface, with a characteristic decay length of a few nanometers.\textsuperscript{13} For an in-depth description of the theory behind RR and SERS, which is beyond the scope of this thesis, the reader is directed to excellent reviews in the field.\textsuperscript{13-14}

2.1.3 Chemometrics for Data Analysis

Multivariate statistical methods of data analysis have become increasingly widely used in the field, as they are a powerful approach to examine a large number of spectra simultaneously and extract trends and relationships in the data that may not be apparent on casual inspection. This decreases the inherent error as a selected wavenumber range or whole spectrum is utilized rather than a single band as in the case of univariate analysis. Chemometric methods can be qualitative or quantitative.
Qualitative methods like cluster analysis, classification and principal component analysis (PCA) are used to distinguish different constituents in the sample. Quantitative methods like multiple linear regression, principal component regression and partial least squares can give quantitative information, about the sample composition like the concentrations of individual constituents. In this work PCA has been used for analysis of Raman spectra and images. Its inherent advantage for biological samples is that it can filter out the data variance that is not sample related, reducing the background noise and autofluorescence and thus increasing the signal-to-noise ratio. Another attractive feature is the similarity between loading plots and actual Raman spectra, which facilitates visual comparison and assignment, even in cases where user has limited knowledge of sample constituents prior to analysis. A detailed explanation and excellent review of PCA in spectroscopy is found elsewhere.

2.1.4 Research Overview and Practical Challenges

A majority of the published work on Raman spectroscopy of bacteria has been directly or indirectly motivated by microbial identification. Approximately 30% of the papers, which are outcome of the search terms ‘bacteria’ and ‘Raman spectroscopy’ on Web of Knowledge contain ‘identification’ among their topic terms. This is not surprising, as Raman spectroscopy can yield a large amount of information on the biochemical components of the microbial cell in a single spectrum, with clearly observable differences that can be used to discriminate between strains. Conventional microbiological methods are often time consuming
and cultivation dependent, as described in Chapter 1. They typically involve visual inspection of colony morphology and tests for examination of nutritional, biochemical and physiological properties. Vibrational spectroscopy is among the next generation methods like PCR, gene sequencing, mass spectrometry, fluorescence spectroscopy, in situ hybridization, matrix assisted laser desorption ionization, electrospray ionization and flow cytometry that are been increasingly used for reliable strain-level identification in the last two decades.

In 1990 Puppels et al. combined Raman spectroscopy and optical microscopy and introduced the technique of Raman microspectroscopy to non-invasively obtain chemical information from a small volume of less than 1 μm³. This made it possible to study single cells and generated a lot of interest around using Raman in microbiology. Bacterial identification and classification have been carried out among colonies of oral streptococci, filtered waterborne strains and foodborne microorganisms on food surface. Harz et al. demonstrated the use of Raman as a biomedical assay to analyze bacteria in cerebrospinal fluid in patients with bacterial meningitis. They used chemometrics via hierarchical cluster analysis (HCA) to characterize the bacteria without interference from the cerebrospinal fluid. Heterogeneity in microbial populations has been studied at the single cell, colony and biofilm levels using Raman microspectroscopy. Raman spectroscopy has also been used in combination with other analytical techniques to yield complementary information. Huang et al. combined fluorescence in situ hybridization with Raman microscopy for cultivation-independent microbial identification. Raman analysis of fluorescently stained single cells has also been
reported, which has opened the possibility of performing Raman on samples that have been labeled with fluorescent tags, a common practice in microbiology.\textsuperscript{34-35} Previous studies in our group have correlated imaging by secondary ion mass spectrometry (C60-SIMS) and matrix assisted laser desorption ionization mass spectrometry (MALDI) with confocal Raman microscopy for spatiotemporal characterization of bacterial biofilms.\textsuperscript{36-37}

UV Resonance Raman (UVRR) has been used to obtain resonantly enhanced spectra dominated by nucleic acids and aromatic amino acids which strongly absorb in the UV range.\textsuperscript{38-39} Neugebauer \textit{et al.} used UVRR and chemometrics to track bacterial growth and monitor the interaction of an antibiotic which targeted DNA.\textsuperscript{40} The use of RR has also been reported for studying bacterial photosynthetic reaction centers,\textsuperscript{41-42} cytochrome c oxidase\textsuperscript{43} and bacterial pigments.\textsuperscript{44-45} A large number of SERS studies have targeted the identification, detection and characterization of bacteria.\textsuperscript{46-50} Zeiri \textit{et al.} demonstrated the selectivity offered by SERS by depositing silver nanoparticles in a targeted manner on bacteria.\textsuperscript{51-52} SERS has been used to differentiate between live and dead cells,\textsuperscript{53} discriminate bacteria on the species and strain level\textsuperscript{5} and distinguish between gram-positive and gram negative types.\textsuperscript{54} SERS spectra have been used to study the biochemical changes in lactic acid bacteria in response to antibiotics.\textsuperscript{55} There is also a trend towards development of SERS based biosensors.\textsuperscript{56-57}

For studying cells in an aqueous environment (liquid cultures, drinking water samples, body fluids etc.) using Raman microspectroscopy, it is necessary for the cell to remain within the confocal probe volume while the signal is being
collected. This has been achieved by immobilizing the cell physically or chemically on a substrate\textsuperscript{2} or solid media\textsuperscript{58} or by the use of optical trapping\textsuperscript{59} and microfluidic capture methods.\textsuperscript{60} Interpretation of the Raman spectrum of complex biological samples is a major challenge, as molecular vibrations generate the Raman signals, and many molecules exhibit overlapping bands. Biomacromolecules, even in a single cell spectrum, have many overlapping peaks. This results in broad bands, thus making band assignment challenging, even if the user knows the sample constituents and has access to Raman spectra of pure chemical standards. Oftentimes, the spectrum contains much more information than the user can interpret and translate into physically significant conclusions. There have been efforts to document Raman band assignments from pure standards and create databases for biological molecules, but much remains to be done to standardize how biological Raman spectra are interpreted.\textsuperscript{3-4, 61} Raman measurements are sensitive to the bacterial phenotype, which is determined by both the genotype (genetic makeup) and environmental influences such as growth conditions (temperature, pH, nutrients), stress, antimicrobial agents and host immune response. This can induce changes in the physiology, behavior and genetics of the bacterial cell, which are reflected in the Raman spectrum. This is both an advantage and a reason to exercise care while using Raman as a bioanalytical tool. It is important to ensure that bacteria strains are grown under the same conditions and measurements are replicated to eliminate variations that could arise from environmental influences. The same caveats apply to the comparison of results with other studies in literature.
There is a vast amount of literature describing the use of various substrates and metal nanoparticle colloids for SERS of bacteria and cellular components. It is, in practice, quite a challenge to bring a live bacterial cell in intimate contact with a SERS substrate for enhancing the cellular bands. Addition of nanoparticle colloids to the sample and drying cells on substrates are commonly chosen routes. The SERS spectra obtained from colloidal nanoparticles must be interpreted with caution as strong bands can arise from the colloid or from impurities in the sample. Since native biological samples can be heterogeneous and can contain unknown components, it is critical to carefully interpret the source of the peaks in relation to the nanoparticle binding site. The lack of a standard approach in the use of substrates or in the collection and interpretation of SERS spectra have made the field of SERS as much of an art as it is a science. There are so many possible ways of engineering SERS substrates, and a host of factors can influence the performance. The objective of this work is not to improve on the current understanding of SERS substrates or test their performance, but rather to evaluate the use of well-established enhancing methods to gain deeper understanding of the biological system. Nanoparticle colloids were chosen for their ease of use with biological samples and reasonable performance. SERS enhancement factors are not calculated in most cases presented here, because it was not possible to obtain the normal Raman spectrum necessary for the calculation. Another issue that arises with the use of SERS is the reproducibility of measurements even from different points on the same sample. In this chapter and others that follow, the sources of SERS bands have been thoroughly examined through judicious control experiments and sample
preparation, and PCA has been used in conjunction with large scale Raman imaging of the samples in order to circumvent the issue of reproducibility and ensure that the spectra are representative.

2.2 Experimental Section

2.2.1 Materials and Methods

**Bacterial strains.** Wild type *Pseudomonas* sp. GM41, *Pantoea* sp. YR343 and mutant strain Δ*crtB* were grown on Lysogeny broth (LB) plates under aerobic conditions at 28°C. *Pseudomonas* sp. GM41 and *Pantoea* sp. YR343 were previously isolated from the rhizosphere of *Populus deltoides*.62 A mutant defective in the production of carotenoids was constructed from *Pantoea* sp. YR343 by deleting the *crtB* gene, which encodes phytoene synthase, an enzyme required for carotenoid biosynthesis.63

**Materials.** Sodium borohydride (NaBH₄), and silver nitrate (AgNO₃) were purchased from Sigma Aldrich (St. Louis, MO) and used as received. Silicon substrates were purchased from WRS Materials (San Jose, CA) as 3-inch diameter wafers of Si (100) orientation, then scored and broken into 2 cm x 2 cm tiles before use.

**Nanoparticle synthesis.** Colloidal silver solutions were prepared according to the protocol of Lee and Meisel.64 A solution of 8.5 mg of AgNO₃ in 50 mL water was added dropwise to 150 mL of 1 mM NaBH₄ while stirring in an ice-bath.65 The resultant pale yellow solution was stirred and maintained at 4°C for 1 h. UV-visible
spectra were used to estimate the size of the nanoparticles. The above method was expected to produce 10-14 nm nanoparticles, with the observed absorbance maximum at 390 nm, indicating particles smaller than 12 nm. Samples were then decorated by exposure to a solution containing ~ 3 x 10^{12} Ag nanoparticles mL^{-1}. The colloid solution was stable for a few months when stored in a clear vial at 4°C.

**In situ silver coating of cells.** Planktonic cell samples were washed three times to remove remnants of the culture growth media and extracellular metabolites, which could possibly interfere with the Raman spectra. Colloids were added to *Pantoea* sp. YR343 using both ex situ and in situ protocols. In the ex situ protocol, Ag nanoparticles were synthesized separately and added exogenously to the bacterial samples. In the in situ approach, the Ag nanoparticles were synthesized in the presence of the cells to form an intimate nanostructured coating on the cell. In the in situ procedure, *Pantoea* sp. YR343 cells collected from an agar plate were washed in deionized (DI) water, pelleted by centrifugation at 3000 g for 10 min., and resuspended in DI water. Following the external coating protocol described by Efrima *et al.*, the cells were suspended first in 0.1 M aqueous NaBH₄ followed by suspension in 0.05 M aqueous AgNO₃ solutions with intermediate wash steps to remove excess precursors.⁵² The cells were dispersed onto silicon substrates and dried before confocal Raman imaging.

**Scanning Electron Microscopy and Transmission Electron Microscopy.** Bacterial cultures or plant-bacteria co-cultures were fixed and dehydrated using the protocol described by Schrand *et al.*⁶⁶ The samples were dispersed on filters (MF-Millipore membrane filter and Swinnex filter holders, EMD Millipore Corp.) with a
pore size of 0.22 μm and sputter coated with 4 nm of Ir. Scanning electron microscope (SEM) (Magellan 400 FESEM, FEI, Netherlands) images were acquired with an electron beam accelerating voltage of 2 kV and a current of 3.1 pA for the undecorated cells and 5 kV and 6.3 pA for the bacteria-on-plant samples. Morphological-chemical characterization of Ag-coated cells was carried out using the SEM instrument’s energy dispersive spectroscopy (EDS) capability, and SEM images were acquired using an accelerating voltage of 15 kV and current of 25 pA. For transmission electron microscopy (TEM, Jeol 1220), the fixed and dehydrated cells were embedded in Spurr’s low viscosity medium (Low Viscosity Kit 18300–4221, Pelco International, Redding, CA) in polyethylene embedding capsules (BEEM, Better Equipment for Electron Microscopy Inc., Bronx, NY). Ultra-thin (70-100 nm) sections were obtained using a RMC MT-X ultra-microtome and a Diatome Diamond knife. They were collected onto 200 mesh copper grids and stained with uranyl acetate and lead citrate. TEM images were acquired at 25,000x magnification.

**Instrumentation and Data Analysis.** RR and SERS imaging were performed using a laser scanning confocal Raman microscope (Alpha 300R, WITec, GMBH, Germany), equipped with a 532 nm focused Nd:YAG laser. The laser radiation was delivered to the microscope using a polarization preserving single-mode optical fiber, deflected through a dichroic beam-splitter and focused onto the sample through the microscope objective. The Raman scattered radiation was collected using the same objectives and delivered through a 50 μm diameter multi-mode fiber to a UHTS 300 spectrometer equipped with a 600 groove mm⁻¹ grating and a back-illuminated CCD camera (Newton DU970 N-BV, Andor Inc., cooled to −60°C). SERS
images were acquired using a Nikon 40x objective (NA 0.6). Images were obtained by acquiring a full Raman spectrum from each image pixel (80 x 80 pixels or 1600 spectra) over a 25 µm x 25 µm region on the sample with an integration time of 100 ms/spectrum. MATLAB was used to perform Principal Component Analysis (PCA) using previously described custom scripts. The SERS spectra were rendered in IGOR Pro 6.3.

2.3 Results and Discussion

2.3.1 The Importance of Control Experiments

Biological samples are often heterogeneous and complex in composition, resulting a very information rich Raman spectrum. It is important to keep in mind, the possible interference that can arise from components other than the sample of interest and possibly mislead the user during data interpretation. These unwanted sources include the substrates like glass, growth media in biological samples, petri dishes etc. Marotta et al. has reported how liquid growth media used for growth of cultures can influence the spectrum. In this work, it was a standard practice to carry out rigorous controls and collect spectra of various possible sources of background Raman scattering arising from components that were used during sample preparation and spectrum collection. This was found to be very useful during spectral band assignment to determine which bands were analyte related. In addition to this, caution was exercised during sample preparation to use minimal growth media, which did not contain biological extracts in cases where it was not
possible to wash samples, especially in the study of secreted metabolites described in later chapters.

2.3.2 Substrate Selection

Various substrates have been tested in the literature for use as sample substrates.\textsuperscript{69-70} Silicon substrates were chosen for most of the experiments carried out in this work for their optical properties and biological compatibility. The wavenumber window between 500-1800 cm\textsuperscript{-1} in the Raman spectrum is the primary region of interest in biological samples. The silicon wafers with a 2-3 nm native silicon oxide layer have characteristic second order peaks between 900-1100 cm\textsuperscript{-1}. Although this falls in the spectral region of interest, it was not found to be a significant impediment. Microscopic glass on the other hand was avoided because it results in a broad background in the 800-1100 cm\textsuperscript{-1} region and obscures less intense biological peaks because of its transparency. Oftentimes glass substrates also contribute to autofluorescence. Polystyrene petri dishes used for growing biological samples were avoided during spectral collection as they resulted in an intense Raman spectrum. Although it wasn’t necessitated for the current work, around 100 nm of thermally evaporated gold coating on the silicon substrate considerably reduced the silicon background.

2.3.3 Raman Spectroscopy of Planktonic Cells

\textit{Wild-type Pseudomonas sp. GM41}. Planktonic cells of \textit{Pseudomonas} sp. GM41 wildtype were investigated by Raman microspectroscopy without silver decoration. The confocal setup offers a lateral spatial resolution better than 1 \( \mu \text{m} \),
which indicates that, spectra like that shown in Figure 2.2 are collected from a single bacterial cell or at most two overlapping cells. The key bands in the spectrum and their tentative assignments based on a thorough review of literature are listed in Table 2.1. There are contributions from the major building blocks of the cell including nucleic acids, proteins, and lipids.

Figure 2.2: Raman spectrum generated from planktonic cells of wildtype *Pseudomonas* sp. GM41
A shoulder peak at 1002 cm\(^{-1}\) is present on the broad SiO\(_2\) band between 900-1100 cm\(^{-1}\). Although this is typically assigned to phenylalanine in literature,\(^{72-73}\) no assignment is made here due to the peak being obscured by the substrate Si-O stretching band.
Wild-type Pantoea sp. YR343 and Phytoene Synthase Mutant. Raman spectra of both wild-type cells and the ΔcrtB mutant excited at 532 nm are shown in Figure 2.3. Strong bands at 1520 cm⁻¹ ($\nu_1$), 1155 cm⁻¹ ($\nu_2$), and 1002 cm⁻¹ ($\nu_3$) arising from conjugated C=C stretching vibrations ($\nu_1$), C-C vibrations coupled to C-CH₃ stretches or C-H in-plane bending ($\nu_2$), and in-plane CH₃ rocking vibrations ($\nu_3$) associated with carotenoids are the dominant features in the microspectrum of wildtype Pantoea sp. YR343. These pre-resonantly enhanced scattering peaks are observed in the carotenoid family of bacterial wall pigments, because the wavelength of excitation of 532 nm is just below the energy of their electronic transitions, which are in the range 400-500 nm for various members of the family.⁷⁴-⁷⁵ In contrast, the microspectrum of the ΔcrtB carotenoid mutant is completely devoid of these three bands, indicating successful disruption of phytoene synthase, one of the enzymes required for carotenoid biosynthesis, and elimination of the carotenoids from these bacteria. In addition, both wild-type and ΔcrtB mutant spectra contain bands related to bacterial cellular components, especially DNA/RNA (745, 1126, 1310, 1447, and 1585 cm⁻¹) and proteins (~1640 cm⁻¹) that have been assigned in Table 2.1 for wildtype GM41.
Figure 2.3: Overlay of resonance Raman spectra generated from planktonic cells of wild type Pantoea sp. YR343 (top) and mutant strain ΔcrtB (bottom). Spectra have been offset for comparison.

Although Raman spectroscopy, in this case, is an effective tool to distinguish the ΔcrtB mutant from wild-type Pantoea sp. YR343, the remarkably intense RR spectra makes it difficult to obtain in situ Raman microspectra of components other than the carotenoids. Typically these other fragile components cannot be studied in their native state in the protective biological matrix, but instead must be processed by common techniques, such as chromatography and mass spectrometry, which involve harsh extraction steps.76-77 Thus, samples containing resonant pigments, like carotenoids, pose special challenges to the study of non-resonant components, because the carotenoid features overwhelm the weaker features in the Raman spectrum.
**Regiospecific SERS.** In a recent study, Kumar *et al.* utilized laser-induced photo-bleaching to eliminate the carotenoid signatures from pigmented cocci bacteria.\(^{78}\) Although this approach may be suitable for taxonomic studies, the use of intense laser radiation raises the risk of photodegradation of non-carotenoid cellular components and small molecule metabolites that are of interest in plant-bacteria interactions. In an effort to circumvent this problem, we investigated the use of SERS to study *Pantoea* sp. YR343 cells, under conditions that otherwise would give rise to pre-resonant enhancements of the carotenoid pigments.\(^{49,79}\) In previous studies, we found that the combination of SERS with multivariate statistics makes it possible to obtain informative Raman spectra from complex auto-fluorescent microbial samples,\(^{80}\) (see chapter 3). Here, PCA was employed to analyze SERS spectral data acquired under two different sets of SERS-enhancing conditions – an *ex situ* approach in which SERS spectra were obtained by mixing *Pantoea* sp.YR343 planktonic cells with pre-formed Ag colloid, **Figure 2.4(A),** and *in situ* Ag-coated *Pantoea* sp.YR343 cells, **Figure 2.4(B).** A typical SERS experiment obtained from wild type cells decorated with preformed ~ 12 nm silver colloids produces the loading plot show in **Figure 2.4(A),** which exhibits features very similar to the RR spectra of the carotenoids and, thus, does not add new information about the sample. This observation might result if the Ag nanoparticles are not in intimate contact with the cells, or if the nanoparticles are positioned such that they further enhance the carotenoid resonance bands through the surface enhanced resonance Raman (SERRS) effect. This latter explanation is especially appealing given the
strong wavelength overlap of the excitation laser, the localized plasmon resonance of 12 nm Ag nanoparticles, and the proximity of the carotenoid absorption bands.\textsuperscript{80}

Figure 2.4: Principal component loading plots from a sample of \textit{Pantoea} sp. YR343 planktonic cells mixed with pre-formed Ag colloid (A) and in situ Ag-coated \textit{Pantoea} sp. YR343 cells (B).

Subsequently, the coating strategy was altered to form a proximal coating of enhancing Ag colloid by synthesizing the Ag nanoparticles in the presence of the bacterial cells, using the \textit{in situ} procedure described by Efrima \textit{et al.}\textsuperscript{52} SERS imaging
and PCA of these in situ Ag-coated cells produce the loading plot shown in Figure 2.4(B). This plot reveals principal component loadings that match well with the spectral features attributed to flavins, specifically flavin adenine dinucleotide (FAD). In particular, bands at 730 cm\(^{-1}\) and 1330 cm\(^{-1}\) match bands of similar spectra by Picorel et al., Zeiri et al and Kahraman et al.\(^{48, 51, 81-82}\) FAD is known to be present at the surface of the cytoplasmic (inner) membrane in gram negative bacteria and plays a major role in many important oxidoreductase enzymes.\(^{83}\) A significant observation in the SERS spectra obtained with both Ag-coating strategies is the lack of interference from autofluorescence. In addition, one might anticipate some pre-resonance enhancement of the isoalloxazine moiety of FAD (\(\lambda_{\text{max}} \sim 460\) nm) in the in situ coated samples when excited at 532 nm, consistent with the observation of FAD SERRS.\(^{84}\)

A key question raised by the obvious differences in the spectra in Figures 2.4(A) and 2.4(B) concerns the physical location of the enhancing Ag nanoparticles relative to the components observed. As indicated in the SEM images in Figure 2.5, the ex situ Ag-coating produces a rough and non-uniform external decoration of the cells with Ag. The cells remain intact with their original structure preserved; despite treating them with silver and no lysed or damaged cells were observed. Instead, SEM images show Ag clusters formed on the exterior of the Pantoea cells in contact with the outer membrane. Correlating the position of the enhancing Ag nanoparticles in the ex situ decorated cells shown in Figures 2.5(B) and 2.5(C) with the typical PCA loading plot shown Figure 2.4(A), and considering the distance dependence of SERS enhancement, which falls off with distance, \(r\), approximately as
.. \[ r^{12,85} \] strongly suggests that carotenoids are located in the outer membrane. A further question concerns the ability of the \emph{ex situ}-prepared Ag nanoparticles to infiltrate the bacterial membrane and, thus, enhance features on the inner membrane as well. In addition to the fact that we have not observed evidence of \emph{ex situ} nanoparticles on the inner surface, we note that it is quite difficult to get a silver nanoparticle inside an intact cell, as evidenced by the lack of reports of successful infiltration of \emph{ex situ} Ag nanoparticles into bacterial cells without damaging or lysing the cell membrane.\textsuperscript{86} Exceptions include special cases of metal accumulating bacteria like \emph{P. stutzeri} with an innately high silver resistance.\textsuperscript{87-88}

![Figure 2.5: SEM images. (A) \textit{Pantoea} sp. YR343 planktonic cell; (B) Silver-coated \textit{Pantoea} sp. YR343 planktonic cell; and (C) Higher magnification image of silver clusters on surface of a \textit{Pantoea} sp. YR343 planktonic cell. Ag cluster identity was confirmed by EDS.](image)

TEM images of thin cross-sectional slices were collected to understand the distribution and possible permeation of the silver inside the cell membrane.
produced by the *in situ* Ag-coating procedure. The typical TEM image in **Figure 2.6** shows Ag nanoparticles located just inside the cell, with preferential accumulation near the (inner) cytoplasmic membrane. The observation of Ag clusters in close proximity to the inner membrane in the *in situ* coated samples contrasts with the Ag cluster location in the *ex situ* samples and is consistent with the large differences between the SERS loading plots in **Figures 2.3(A) and 2.3(B).**

![TEM images of thin cross-sections of *in situ* Ag-coated *Pantoea* sp. YR343 cells with preferential accumulation of Ag on the inner membrane indicated with circles.](image)
Furthermore, it suggests that the internal Ag complexes are proximal to the flavins, which is consistent with previous suggestions that flavins may act as nucleation sites for metal deposition. From the point of view of chemical imaging, it is apparent that altering the method of nanoparticle application provides a route to control the regiospecificity of the SERS effect, thus enabling the observation of other cell-membrane components even in the presence of a very strong, pre-resonance Raman effect in chromophores, like the carotenoids. One might question whether Ag ions affect the cells used for the in situ preparation. Ag is a well known bactericidal agent, where it results in disintegration of cell membrane, lysis and cell death. To address this point, we confirmed, by SEM, that the cell morphology is conserved and, by TEM, that the nanoparticles form on the inner membrane where FAD is located. While these electron imaging experiments cannot address the question of viability, they do indicate that the Pantoea sp. YR343 cells maintain their structural integrity through the imaging process.

2.4 Concluding Remarks

Raman spectroscopy is an effective technique to characterize the cellular components of non-pigmented and pigmented rhizosphere bacterial isolates. Resonance Raman spectra are competent to distinguish the wild-type pigmented rhizosphere bacterium Pantoea sp. YR343 from the ΔcrtB mutant which lacks the phytoene synthase gene needed for biosynthesis of the carotenoid membrane pigments. Furthermore, judicious use of Ag nanoparticle-based SERS and SERRS, together with SEM and TEM images, make possible the regiospecific identification of
carotenoids in the outer membrane and flavins, such as FAD, in the inner membrane of *Pantoea*. Raman spectroscopies, in combination with PCA and electron microscopy is a promising toolkit for further studies of microbial populations and co-culture systems which will be detailed in Chapters 3 and 5.
2.5 References


42


36. Lanni, E. J., Masyuko, R. N., Driscoll, C. M., Dunham, S. J., Shrout, J. D., Bohn, P. W., Sweedler, J. V., Correlated imaging with C60-SIMS and confocal Raman


CHAPTER 3:
LOOKING OUTSIDE THE MICROBIAL CELL— INVESTIGATIONS OF THE
SECRETOME FOR SMALL MOLECULE METABOLITES

This chapter is based on work previously submitted for publication:
Polisetti, S., Baig, N. F., Morales-Soto, N., Shrout, J. D., Bohn, P. W., Spatial Mapping of
Pyocyanin in Pseudomonas aeruginosa Bacterial Communities by Surface Enhanced Raman

3.1 Introduction
Bacterial metabolites, which are typically small molecule intermediates and
products of reactions involved in intricate metabolic pathways, can provide a
comprehensive picture of cellular functions including, microbial behavior, defense
mechanisms, communication and cooperation.¹ Secondary metabolites in bacteria
are not required for the basic physiological processes of the cell for survival and
growth. However, they are involved in a variety of interesting ecological functions
and can thus provide a chemical fingerprint of these processes in bacterial biofilms
and co-cultures. These small diffusible bioactive molecules are produced and
released by the microbe in response to external stimuli and play a role in quorum
sensing in biofilm communities, cooperation, competition and defense in co-cultures
with other bacterial species and host organisms (e.g. humans, plants). The use of Raman spectroscopies in this field is relatively unexplored and it is motivating to evaluate the possible role of Raman in advancing of understanding of bacterial behavior though the secretome. A major part of this thesis targets this goal with selected systems relevant to human and plant health.

Previously, Chapter 2 described the use of Raman spectroscopy and related techniques to study the microbial cellular components. In the current chapter, small molecule metabolites, secreted by bacterial cells, are investigated. An approach for in situ SERS spatial and temporal mapping of P. aeruginosa bacterial communities is presented that shows interesting strain- and nutrient-based differences in the production of key chemical signatures, as exemplified by pyocyanin. SERS imaging of a large area of the sample (25 μm x 25 μm) is employed in contrast to the more common practice of collecting microspectra from a single spot. This large area vibrational spectroscopic mapping provides a more comprehensive picture of the cell-cell relationships in the community, and this is then followed by principal component analysis (PCA) in order to classify the major factors influencing spectral heterogeneity within the community. The combination of SERS and PCA applied to large area data collections produces additional information when compared to averaging single spectra, avoids the random contaminant spectra often seen in SERS, and also provides critical insight into the spatial distribution of metabolites and their role in organizing microbial communities. The following chapter (Chapter 4) addresses the potential of using the Raman toolkit for investigation of auxins in plant-bacteria co-culture systems and the challenges encountered in doing so.
3.1.1 Theoretical Background

3.1.1.1 Pyocyanin in *P. aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative bacterium and opportunistic human pathogen that can cause severe chronic infections, leading to a high rate of mortality, in patients afflicted with burns, wounds, acute leukemia and cystic fibrosis (CF).³ The success of *P. aeruginosa* as a pathogen depends partly on the production and secretion of an arsenal of virulence factors which include the phenazines – a class of redox-active heterocyclic small-molecule metabolites.⁴ Phenazines have also been implicated in several functions critical to the survival of the bacteria, such as biofilm formation and growth. Pyocyanin (5-N-methyl-1-hydroxy-phenazine) is the most widely studied phenazine primarily due to its role in pathogenesis.⁵ The molecular structure and SERS spectrum of pyocyanin standard is shown in **Figure 3.1**. The presence of pyocyanin in CF lung isolates has been associated with lung damage, which is one of the hallmarks of the disease.⁶ High amounts of pyocyanin are typically found in sputum of patients with *P. aeruginosa* infections. It is thought to play an important role in chronic infection - causing progressive loss of lung function, which is the principal cause of death in > 80% of CF patients.⁷

The pathogenicity of *P. aeruginosa* is strain dependent, with some strains being more virulent than others. For example, between two common laboratory strains, *P. aeruginosa* PA14 is often observed to be more virulent than *P. aeruginosa* PAO1.⁸ The nutrients available to the bacteria during growth may also influence
phenotypes associated with cell-cell signaling, motility, biofilm formation and the production of virulence factors.\textsuperscript{9} \textit{P. aeruginosa} in CF sputum preferentially catabolizes amino acids like L-alanine, L-arginine and L-glutamate over other carbon sources like lactic acid and glucose.\textsuperscript{10} Furthermore, aromatic amino acids in the sputum of CF patients may promote synthesis and secretion of the signaling molecule 2-heptyl-3-hydroxy-4-quinolone commonly known as the \textit{Pseudomonas} quinolone signal (PQS). Caldwell \textit{et al.} demonstrated that many CF clinical isolates of \textit{P. aeruginosa} produce more pyocyanin than laboratory strains, like PAO1,\textsuperscript{11} and Huang \textit{et al.} showed the effect various carbon sources had on catabolite repression of pyocyanin biosynthesis.\textsuperscript{12} Thus, together the phenazines, typified by pyocyanin, and the quinolones/quinolines play important, but still not well understood, roles in mediating the interactions of \textit{P. aeruginosa} with other organisms and its spatial and temporal organization at the community level.\textsuperscript{13}
3.1.2 Research Overview

There are far fewer studies on Raman spectroscopic investigations of the bacterial secretome, compared with those of bacterial cells and cellular components. This is partly due to the fact that very few strains have well understood metabolic pathways, and it is challenging to identify or assign a molecule using Raman spectroscopy when little is known about the biology of the bacterial strain. In addition, there is the challenge of in situ detection in cultures, which are often a complex mixture of growth media and other autofluorescent biomolecules that can obscure the spectrum. Bacteria use extracellular small molecule signals in communication and quorum sensing as they synchronize gene expression in communities. Two main classes of signaling molecules include acylhomoserine
lactones (AHLs) and the Pseudomonas quinolone signals (PQSs).\textsuperscript{14} Claussen \textit{et al.} demonstrated SERS detection of biologically relevant concentrations of AHLs in ultrapure water and simulated physiological conditions although no \textit{in situ} measurements are reported.\textsuperscript{15} The Bohn research group at the University of Notre Dame has been spearheading the use of Raman spectroscopy in conjunction with other techniques to further understanding of biology in bacterial communities through chemical imaging of the secretome. Previous studies in our group have effectively used multimodal imaging combining secondary ion mass spectrometry (SIMS) and confocal Raman microscopy (CRM) to map quinolone distributions in \textit{P. aeruginosa} biofilms and cross-validate the observations from both techniques.\textsuperscript{16} Masyuko \textit{et al.} used a combination of matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) and CRM to characterize the presence of rhamnolipids in biofilms and linked their production to the presence of quorum sensing mechanisms.\textsuperscript{17} Baig \textit{et al.} demonstrated how CRM could be used to guide MS analysis for \textit{in situ} analysis of co-localized, isomeric quinolones thus demonstrating the advantages Raman spectroscopy offers to this field of study.\textsuperscript{18} Wu \textit{et al.} reported a culture-free method of diagnosing \textit{P. aeruginosa} infections by SERS detection of pyocyanin as a biomarker. Raman microspectroscopy has been used to detect the antifungal antibiotic amphotericin B, produced by \textit{Streptomyces nodosus}.\textsuperscript{19}
3.2 Experimental Section

3.2.1 Materials and Methods

**Materials.** Silicon substrates were purchased from WRS Materials (San Jose, CA) as 3-in-diameter wafers of Si (100), then scored and broken into 2 cm x 2 cm tiles before use. Pyocyanin standard, sodium borohydride (NaBH₄), and silver nitrate (AgNO₃) were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification.

**Colloid synthesis.** Colloidal silver solutions were prepared according to the protocol of Lee and Meisel.²⁰ A solution of 8.5 mg of AgNO₃ in 50 mL water was added dropwise to 150 mL of 1 mM NaBH₄ while stirring in an ice-bath.²¹ The resultant pale yellow solution was stirred and maintained at 4°C for 1 h. UV-visible spectra were used to estimate the size of the nanoparticles. The above method was expected to produce 10-14 nm particles, with the observed absorbance maximum at 390 nm, indicating particles smaller than 12 nm. Samples were then decorated by exposure to a solution containing ~ 3 x 10¹² Ag nanoparticles mL⁻¹. The colloid solution was stable for a few months when stored in a clear vial at 4°C.

**Pellicle biofilm growth and sample preparation.** *P. aeruginosa* strains PAO1C (laboratory strain)⁹ and FRD1 (cystic fibrosis lung isolate)²² were used in this study. Cell cultures were grown overnight at 37°C with shaking at 240 rpm in modified Fastidious Anaerobe Broth (FAB) culture medium⁹ supplemented with either 30 mM filter sterilized glucose or glutamate as the source of carbon. Pellicle biofilms (biofilms that form at the air-liquid interface) of FRD1 and PAO1C were
grown in a test tube by inoculating 6 mL fresh FAB medium containing 150 μL of 1.2 M glucose or glutamate, with 200 μL of the overnight cell culture (OD=1 at 600 nm) followed by incubation (without shaking) at 37°C until the desired growth time had elapsed. For SERS analysis, 50 μL of the growth at the air-liquid interface was carefully pipetted onto sterile 2 cm x 2 cm silicon wafers, 100 μL of the colloid was added to the sample and allowed to dry in the dark.

**Scanning Electron Microscopy.** Dried samples were sputter coated with 2 nm of Au/Ir and scanning electron microscope (SEM) (Magellan 400 FESEM, FEI, Netherlands) images were acquired with a 2.1 mm working distance. The electron beam was operated at an accelerating voltage of 3 kV and a current of 6.3 pA.

**Instrumentation and Data Analysis.** SERS imaging was performed using a laser scanning confocal Raman microscope (Alpha 300R, WITec, GMBH, Germany), equipped with a 532 nm focused Nd:YAG laser. Laser radiation was delivered to the microscope using a polarization preserving single-mode optical fiber, deflected through a dichroic beam-splitter and focused onto the sample through the microscope objective. The Raman scattered radiation was collected using the same objective and delivered through a 50 μm diameter multi-mode fiber to a UHTS 300 spectrometer equipped with a 600 groove mm⁻¹ grating and a back-illuminated CCD camera (Newton DU970 N-BV, Andor Inc.), cooled to −60°C. SERS images were acquired using a coverslip corrected Nikon water immersion 60x objective (NA=1). Images were obtained by acquiring a full Raman spectrum from each image pixel (80 x 80 pixels or 1600 spectra) over a 25 μm x 25 μm region on the sample with an integration time of 100 ms/spectrum. MATLAB was used to perform Principal
Component Analysis (PCA) using previously described custom scripts. The SERS spectra were rendered in IGOR Pro 6.3.

3.3 Results and Discussion

3.3.1 Circumventing the Issue of Autofluorescence

Autofluorescence is a common problem posed by biological samples due to the natural emission of fluorescence by certain molecules. It can strongly interfere with Raman signals, often completely obscuring the spectra with broad bands when visible excitation wavelengths are used. Although the use of near-infrared (NIR) excitation wavelengths dramatically reduces the autofluorescence emission, in some studies the Resonance Raman (RR) effect or the increased band intensities at the lower excitation wavelength warrant and necessitate the use of visible range laser excitation. In these cases, SERS truly shines as a double-pronged approach to both enhance the Raman signal and reduce autofluorescence.

The biofilm matrix encases bacterial cells in extracellular polymeric substances (EPS), a complex agglomeration of secreted material that include small molecule metabolites, fluorescent pigments and complex carbohydrates. Pseudomonas biofilms produce, among other components, rhamnolipids, quinolines/quinolones, and phenazines, with multiple roles being assigned to each class of compounds, depending on the time and local environment. The first problem to be overcome in the Raman analysis of bacterial communities arises from interference from fluorescent pigments. Fortuitously, as shown in Figure 3.2, SERS
enables Raman spectral acquisition from highly fluorescent biofilm matrices, presumably because the Ag nanoparticles\textsuperscript{30-31} quench sample autofluorescence, as well as providing the signal enhancements that increase sensitivity compared to unenhanced Raman spectroscopy.\textsuperscript{32} Because the Raman signal enhancement in SERS falls off with distance between the molecule being probed and the SERS enhancing site, $r$, approximately as $r^{-12,33}$ it requires that the nanoparticles be in close proximity to the analyte molecule.

Figure 3.2: Comparison of spectra generated from a \textit{P. aeruginosa} bacterial community under normal Raman conditions (\textit{top}) and with the addition of $\sim 12$ nm Ag colloid to generate SERS (\textit{bottom}). Raman and autofluorescence spectra have been scaled differently to appear on the same plot.
3.3.2 Incorporating Electron Microscopy and PCA in SERS Imaging of *P. aeruginosa* Biofilms—The Superior Approach

Scanning electron microscopy (SEM) images of *P. aeruginosa* biofilm samples coated with 10-12 nm diameter Ag colloid were obtained to understand how the nanoparticles were distributed in the sample relative to the cells and the EPS. A representative SEM image in **Figure 3.3** shows that the nanoparticles are distributed heterogeneously on the sample surface with regions of aggregated clusters interspersed among areas of sparse coverage, which is typical when colloidal particles are used as the enhancing medium. High SERS enhancement is expected from the regions where the clusters are colocalized with the analytes of interest. The spatial length scale is set in bacterial communities and biofilms by the size of *P. aeruginosa* bacterial cells, *i.e.* 1-2 μm. As **Figure 3.3** illustrates that there are relatively few areas this large that are not in contact with Ag colloid.
Figure 3.3: SEM image taken from a representative region of a 24 h FRD1 pellicle biofilm grown in glucose and decorated with 12 nm Ag nanoparticles showing the spatial distribution and state of aggregation of Ag nanoparticles

SERS imaging can also be coupled with multivariate statistical approaches, such as PCA, to provide more detailed insight into the chemical makeup of the EPS. The problem in Raman imaging of extended objects, such as bacterial biofilms, at high spatial resolution with laser scanning confocal Raman microscopy is that a typical volume element (voxel) represents a volume of the order 1 μm$^3$ while the entire biofilm may occupy a volume 10$^8$ times larger (1 mm x 1 mm x 100 μm). Thus, it is important to know how to optimally integrate and interpret the spectral data acquired from individual 1 μm$^3$ voxels.
In this context, it is possible to obtain chemical information from principal components that is not available from a single spectrum, or even by averaging spectra collected over different locations, owing to the inherent sample heterogeneity and the need to tradeoff signal-to-noise (S/N) ratio (exacerbated by interference from background and fluorescence) vs. acquisition time. This issue is illustrated in Figure 3.4 which shows a 25 μm x 25 μm image of the sample generated from the integrated intensities over the 2800 – 3000 cm⁻¹ window of the ν(C-H) spectral region. Three individual spectra from different locations of the image in Fig. 3.4A are shown in Figs. 3.4B-D. Clearly, they are all distinctly different from each other. Figs. 3.4B and C illustrate spectra dominated by unknown sample components, while Fig. 3.4D contains bands corresponding to pyocyanin, as determined by comparison to the spectrum of pure standard compound.

This heterogeneity in spectral response is a common issue in SERS microscopy, and there are a number of different ways that it has been addressed, for example searching for suitable spots that yield “typical” spectra or averaging spectra from multiple locations. Obviously, trying to represent typical spectra is subject to operator bias, while averaging, in addition to being cumbersome and inefficient, can produce misleading results. For example, Fig. 3.4E shows a spectral response obtained by averaging all 6400 spectra in the image in the area covered by Fig. 3.4A. The averaging results in improved S/N ratio when compared to single spectra, but it does not resemble any of the individual spectra in Figs. 3.4B-D, although it does contain the pyocyanin marker band at 1354 cm⁻¹, suggesting that pyocyanin is
present at comparatively high levels throughout the imaged region. Nevertheless, the fidelity of information transfer from the averaging process is clearly less than ideal.
Figure 3.4. (A) SERS image constructed as a heat map of the integrated intensity in the range 2800-3000 cm⁻¹, generated from a 48 h FRD1 pellicle biofilm grown in glucose. (B), (C) and (D) SERS spectra of individual voxels obtained from different spatial locations in the image displayed in A. (E) SERS spectrum obtained by averaging all of the spectra obtained within the image in (A), i.e. the spatially-averaged spectrum.
As an alternative to deterministic data processing approaches, classification approaches based on multivariate statistics show great promise. These approaches have been used extensively in diagnostics, for example bacterial identification from spectral data.\textsuperscript{36-37} Principal component analysis is especially attractive in addressing large multispectral datasets such as those acquired from confocal Raman imaging of heterogeneous biological samples, like bacterial biofilms, where it is important to sample large regions of the sample,\textsuperscript{38} and there may be many individual components secreted by the bacteria in the biofilm. This is shown in Fig. 3.5A, which is the loading plot of the first PC generated from the SERS image in Fig. 3.5C. The heat map (Fig. 3.5B) corresponding to PC1 shows that the contribution from PC1 is localized to a small spot on the sample. This, along with the features in the loading plot, indicates that it likely corresponds to a contaminant – a valuable conclusion in that it may disregarded so that other principal components can be examined in greater detail.

The second principal component (PC2) has features in the loading plot (Fig. 3.5D), that resemble Raman bands characteristic of pyocyanin in the sample, and the corresponding heat map (Fig. 3.5E) shows a relatively homogeneous distribution across the imaged area. Thus, PC2 is chemically relevant to the sample under study. Briefly, this is the strategy we apply to obtain useful information from the sample. The principal component loading plots offer spectral features of much superior quality than averaging of spectra and enable observation of subtle changes in bacterial communities of closely related strains as shown in greater detail below. The PC heat map in Fig. 3.5E, in particular, indicates that pyocyanin is
present in high abundance throughout the imaged region of the biofilm with a small number of isolated regions of extra high intensity.
Figure 3.5. SERS imaging and PC analysis of a representative area of a 48 h FRD1 pellicle biofilm. (A) and (D) are loading plots of PC1 and PC2 generated from the SERS image (C) which is integrated over the 2800-3000 cm\(^{-1}\) filter; (B) and (E) are heat maps of PC1 and PC2, respectively.
3.3.3 Strain and Nutrient Based Differences in Pyocyanin Production

Having established the utility of mapping the principal components to elucidate behavior in complex multicomponent samples, SERS/PCA mapping was used next to investigate the behavior of two *P. aeruginosa* strains, PA01C and FRD1, grown using either glucose or glutamate as carbon source. PA01C pellicle biofilms grown in glucose did not exhibit detectable levels of pyocyanin (data not shown). However, PA01C grown in glutamate and FRD1 grown in either glucose or glutamate produced detectable levels of pyocyanin (*Fig. 3.6*). The first principal components generated from PC analysis of SERS images collected from pellicle biofilms of FRD1 and PA01C reveal high z-score features at \( \sim 1352, 1511, 1572 \) and 1600 cm\(^{-1}\) (*Figs. 3.6A(i), 3.6B(i), 3.6C(i)) that are in concordance with vibrational bands representing a combined C-C/C-N stretch and C-H in-plane bend at 1352 cm\(^{-1}\), CH\(_3\) wag and C-H in-plane bend at 1511 cm\(^{-1}\) and ring deformation stretch at 1572 and 1600 cm\(^{-1}\)\(^{19,39}\) present in the SERS spectrum of pyocyanin standard as shown previously in *Figure 3.1*. This suggests that a substantial amount of pyocyanin is secreted by the bacteria within the imaged regions of the biofilm. This point is emphasized by comparing the heat maps of the principal components identified as being dominated by pyocyanin (*Figs. 3.6A(ii), 3.6B(ii), and 3.6C(ii)) with the corresponding Raman intensity maps (*Figs. 3.6A(iii), 3.6B(iii), and 3.6C(iii)). There is an obvious concordance between the positions of high intensity in the heat maps and in the Raman images, even though the two were selected to examine different features of the biofilm. The Raman images, which are integrations over the
2800-3000 cm$^{-1}$ region are meant as a proxy for the total density of organic material at a given location.
Figure 3.6. Analysis of 24 h pellicle biofilms of *P. aeruginosa* FRD1 and PAO1C strains in response to the source of carbon. Raman spectra (i), principal component heat maps (ii), and SERS images (iii) integrated over 2800-3000 cm$^{-1}$ for representative regions of pellicle biofilms derived from *P. aeruginosa* FRD1 (A-B) and PAO1C (C) are shown. Glucose (A and C) and glutamate (B) were provided as carbon sources.
Furthermore, in addition to features corresponding to the vibrational bands of pyocyanin, the most significant principal components from SERS images of FRD1 pellicle biofilms (Figs. 3.6A(i) and 3.6B(i)) exhibit a distinct feature at \( \sim 935 \text{ cm}^{-1} \), that does not appear in the PAO1C biofilm grown on either carbon source. This feature in the loading plot corresponds to vibrational bands associated with C-C-H and C-O-H modes of carbohydrates.\(^{40-41}\) The presence of carbohydrates in greater abundance in FRD1 biofilms is associated with the mucoid phenotype characteristic of the FRD1 strain, which is considered to provide protection to the bacteria against the host immune system, phagocytosis and antibiotics,\(^{42}\) allowing the bacteria to cause persistent infections in the host. Beyond its chemical interpretation, the presence of the 935 cm\(^{-1}\) feature and its association with a non-pyocyanin source serves to highlight the fact that principal components are not necessarily derived from a single molecular source and that they must, therefore, be interpreted with care. Finally, the obvious differences in pyocyanin production from FRD1 and PAO1C strains grown on the two carbon sources suggest corresponding differences in the biosynthetic pathways utilized at the sub-species level. Whereas PAO1C is competent to synthesize phenazines (of which pyocyanin is the most abundant member) from glutamate, but not glucose, FRD1, along with other morphological differences in EPS production, is clearly competent to biosynthesize pyocyanin from either carbon source.
3.4 Concluding Remarks

SERS imaging is an effective tool to produce high quality data from spatially distinct regions of single biofilm samples and across multiple biofilm samples, making it a suitable technique for chemometric analysis. The use of SERS in conjunction with PCA allows rapid sample analysis, eliminating the need to find an optimal location thus bypassing inherent difficulties associated with the analysis of biological samples, such as autofluorescence from cellular metabolites. Studies of bacterial communities, like biofilms, can especially benefit from Ag nanoparticle induced SERS, as it offers similar enhancement factors as engineered SERS substrates fabricated using e-beam or chemical assembly, but uses cost-effective metal nanoparticle colloids, which can be easily synthesized in most laboratory settings. Furthermore, Ag nanoparticle mediated SERS samples, provide effective reduction in background fluorescence, a particularly important attribute in light of the fluorescent nature of the phenazine class of secreted virulence factors. Finally, the special nature of SERS, the media and geometries that are efficient at enhancing Raman scattering, and the potential for biased sampling is noted. Given the highly non-linear nature of SERS hot spots, it is at least possible that molecules with a strong propensity to bind to Ag nanoparticles will experience a disproportionate enhancement. This would be an aid in the study of phenazines, but it presents a factor which must be weighed in interpreting the data.
3.5 References


44. Kneipp, K., Kneipp, H., Dresselhaus, M. S., Lefrant, S., Surface-enhanced Raman scattering on single-wall carbon nanotubes. *Philosophical Transactions of the*

CHAPTER 4:

RAMAN IMAGING OF THE RHIZOSPHERE BACTERIUM PANTOE SP. YR343 — CELL RESPONSE TO OXIDATIVE STRESS AND CO-CULTURE WITH ARABIDOPSIS THALIANA

This chapter is based on previously published work from:


And

4.1 Introduction

The rhizosphere is the zone of the soil immediately adjacent to the plant root and is rich with microbial activity.\(^1\) The interactions between plant and microbes can be quite complex with different strains having different effects on plant health and growth. These plant-associated bacteria can be neutral, beneficial, or pathogenic to the plant host.\(^2-3\) Some beneficial bacteria, or plant growth promoting rhizobacteria (PGPR), can enhance plant development with their ability to increase nutrient availability and synthesize plant phytohormones, like indole-3-acetic acid.\(^4\) Other PGPR may produce a variety of secondary metabolites with antibacterial and antifungal activities. PGPR have recently drawn considerable scientific attention for the possibility to replace or supplement the use of chemicals in agriculture.\(^5-7\) Thus, understanding the behavior and function of rhizobacteria is important for their role in influencing plant growth and controlling plant susceptibility to disease, offering potential developments in green biotechnology.\(^8-9\)

There are several factors that suggest that Raman microspectroscopy could be useful as a tool to study plant-microbe rhizobacterial ecosystems. Raman microspectroscopy and confocal Raman microscopy (CRM) have the capacity to non-invasively probe these complex, dynamic ecological niches in models that closely mimic the \textit{in situ} environment, and they could be applied to the study of numerous microbe/plant systems. This is advantageous, because most environmental strains have not been cultured in the laboratory.\(^10\)

This chapter describes the use of Raman spectroscopy based studies of a newly characterized strain of \textit{Pantoea}, named \textit{Pantoea} sp. YR343, a non-pathogenic
bacterial strain which was found to be a robust colonizer of plant roots. For the first time, Raman spectroscopy is used to measure the cells’ response to oxidative stress alongside conventionally used techniques and emerges as a valuable method of analysis. A comparison between the wild type YR343 and a constructed mutant in which the phytoene synthase gene, \textit{crtB}, was deleted, reveals increased sensitivity of the mutant to reactive oxygen species. Raman imaging is also used to characterize senescent green Arabidopsis thaliana plant roots inoculated with \textit{Pantoea} sp. YR343, and PCA is employed to distinguish spectral contributions from plant and bacterial cells, thereby establishing the potential of Raman imaging to visualize the distribution of rhizobacteria on plant roots. Finally, the potential of using Raman for detecting auxins produced by rhizobacteria is investigated and the challenges involved are presented.

4.1.1 Theoretical Background and Research Overview

4.1.1.1 Friends and Foes in the Rhizosphere: Plant-Microbe Interactions

The rhizosphere is the site of a complex network of plant-microbe and microbe-microbe interactions, which ultimately influence plant health and productivity. Microbes can be considered pathogenic, neutral, or beneficial depending on the plant host with which they associate \textsuperscript{11}. Among these bacteria is a group of plant growth-promoting bacteria (PGPB), which can colonize within the plant, on leaf or root surfaces, or in the surrounding rhizosphere \textsuperscript{12-14}. These bacteria can promote plant growth via phytohormone production, nitrogen fixation,
and/or enhancement of water and mineral uptake \(15-18\). For example, the ability of microbes to influence root development via biosynthesis of the auxin indole-3-acetic acid (IAA) is well documented and multiple pathways for microbial IAA production have been described \(19\). Although well-studied genera like *Azospirillum* and *Rhizobium* are included in the PGPB group, the mechanisms by which these bacteria interact with and influence plant growth are not yet fully understood.

Recently the bacterial community associated with mature *Populus deltoides* roots was analyzed and shown to be dominated by *Proteobacteria*, *Acidobacteria*, and *Verrucomicrobia* \(20\). Among the organisms isolated from the *Populus* rhizosphere was the \(\gamma\)-proteobacterium *Pantoea* sp. YR343. Different species within the *Pantoea* genera have been shown to be either beneficial or harmful in association with plants \(21\). For example, *P. stewartii* and *P. ananatis* are responsible for Stewart’s bacterial wilt disease on corn, leaf blotch disease in Sudangrass, and center rot in onion \(22-23\). A number of *Pantoea* strains, including *P. rwandensis*, *P. rodasii*, *P. vagans*, and *P. eucalypti*, have been implicated as the causal agents of bacterial blight, leaf lesions, and dieback in eucalyptus \(24-26\). In contrast, *P. vagans* C9-1 is used as a biocontrol agent to protect against fire blight \(27\) and *P. agglomerans* can protect against plant pathogens like *Pseudomonas syringae* pv. *Syringae* \(28\). Strains of *P. agglomerans* have also been shown to promote plant growth in wheat, rice, and cotton \(29-33\). It is thought that *P. agglomerans* promotes plant growth by enhancing root architecture, as well as total root mass, which increases the amount of minerals and water that can be taken up by the plant \(34\). Evidence supports the hypothesis that the ability of *P. agglomerans* to produce IAA is responsible for its plant growth-promoting
abilities. Additional studies have shown that production of exopolysaccharides by P. agglomerans contributes to soil aggregation and moisture control, which also enhances plant growth.

4.1.1.2 Relevance of Oxidative Stress in Rhizosphere

Plants produce reactive oxygen species as a by-product of their metabolism, and these molecules have been implicated in plant defense mechanisms, stress responses, and the establishment of symbiotic relationships. Many soil-dwelling and plant-associated bacteria, including Pantoea sp., produce carotenoids, which are pigment molecules found in the cellular membrane. Carotenoids are well known for their antioxidant activity, which is important in protection from reactive oxygen species. In addition, carotenoids can modulate membrane fluidity and may play a role in the formation of membrane domains. Zeaxanthin (both mono- and diglucoside forms) was found to be the predominant carotenoid present in Erwinia herbicola and Pantoea stewartii. The carotenoid biosynthesis pathway has been well characterized in Pantoea and consists of six enzymes: geranylgeranyl diphosphate (GGPP) synthase CrtE, phytoene synthase CrtB, phytoene desaturase CrtI, lycopene cyclase CrtY, β-carotene hydroxylase CrtZ, and the zeaxanthin glucosyltransferase CrtX. In P. ananatis, it was found that deletion of the phytoene synthase gene, crtB, resulted in the loss of yellow pigment and increased sensitivity to environmental stress factors.
4.2 Experimental Section

4.2.1 Materials and Methods

**Materials.** Silicon substrates were purchased from WRS Materials (San Jose, CA) as 3-in-diameter wafers of Si (100), then scored and broken into 2 cm x 2 cm tiles before use. Indole-3-acetic acid standard and L-tryptophan were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification.

**Bacterial isolation and growth conditions.** Fine roots and associated rhizosphere samples were harvested from native *Populus deltoides* trees at the Yadkin River in North Carolina in May 2011 and bacteria were isolated from both the endosphere and rhizosphere as described \(^{20}\). A rhizosphere isolate designated YR343 was restreaked three times to R2A plates to obtain a purified strain and a draft genome sequence was obtained \(^{52}\). This bacterial strain is referred to as *Pantoea* sp. YR343 in this paper. *Pantoea* sp. YR343 was cultured at 28°C in R2A medium (R2A Broth Premix, TEKnova, Inc.) or on R2A agar plates (R2A Agar, VWR). Cultures were also grown in standard LB or M9 media, TY media (per 1 liter, 10 g tryptone, 5 g yeast extract), or SOBG medium (per 1 liter, 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.4 g MgSO\(_4\), 0.186 g KCl, 50 ml of 40% v/v glycerol).

**Indole-3-acetic acid (IAA) production assay.** IAA production was measured as previously described \(^{53}\). Briefly, 500 μl of overnight cultures was diluted into 50 ml of M9 minimal media plus L-Tryptophan (200 μg ml\(^{-1}\) final concentration), and incubated overnight at 28°C. IAA was detected in the supernatant using Salkowski’s reagent (500 ml dH\(_2\)O, 300 ml concentrated H\(_2\)SO\(_4\), 2.03g FeCl\(_3\)·6H\(_2\)O) and
absorbance was measured at 535 nm using a UV-Vis spectrophotometer (Cary, Agilent technologies). All measurements were performed in triplicate and compared to a standard curve generated from IAA (Sigma-Aldrich).

**Root colonization assays.** *Arabidopsis thaliana* ecotype Col-0 seeds were germinated and grown, as previously described. Sterilized seeds were soaked in double distilled H₂O containing 0.1% w/v agar at 4°C for 4 days and then germinated on Murashige and Skoog (MS) agar containing 0.25% w/v sucrose. Seedlings were incubated in a growth chamber at 24°C with a 12 hour light and 12 hour dark photoperiod. After 7 days, 4 - 6 *A. thaliana* seedlings of equivalent root lengths were transferred to new MS agar plates containing 0.25% w/v sucrose and grown for an additional 7 days. An overnight culture of *Pantoea* sp. YR343 was then pipetted in a line across the bottom of the plate.

**Treatment of Cells with Hydrogen Peroxide.** Wild type Pantoea sp. YR343 and mutant strain ΔcrtB were grown on Lysogeny broth (LB) plates under aerobic conditions at 28 °C. Cell cultures were grown overnight at 28°C with shaking at 240 rpm in modified Fastidious Anaerobe Broth (FAB) culture medium supplemented with 30 mM filter sterilized glucose as the source of carbon. Overnight cultures of YR343 and ΔcrtB were mixed in equal volume amount with various concentrations of H₂O₂ (1 M, 100 mM, 10 mM, 1 mM, 0.1 mM) and pure water for the control experiment. After 1 hr the samples were centrifuged and the cells were spotted on a silicon wafer.

**Scanning Electron Microscopy.** Plant-bacteria co-cultures were fixed and dehydrated using the protocol described by Schrand et al. The samples were

---

84
dispersed on filters (MF-Millipore membrane filter and Swinnex filter holders, EMD Millipore Corp.) with a pore size of 0.22 μm and sputter coated with 4 nm of Ir. Scanning electron microscope (SEM) (Magellan 400 FESEM, FEI, Netherlands) images were acquired with an electron beam accelerating voltage of 5 kV and a current of 6.3 pA for the bacteria-on plant samples.

**Instrumentation and Data Analysis.** Raman microscopy was carried out on an Alpha 300R confocal Raman microscope (WITec GmbH, Ulm, Germany) with a 40×, NA = 0.6 air-immersion objective using a frequency-doubled Nd:YAG laser (λ = 532 nm) delivered through a single-mode optical fiber, dichroic beam splitter, and focused onto the surface of the sample using a microscope objective operating in epi-illumination geometry. The backscattered radiation was transmitted through a 50-μm diameter multimode fiber to a UHTS 300 spectrometer with a 600 groove mm⁻¹ diffraction grating and back-illuminated CCD camera cooled to −65 °C (Newton DU970 N-BV, Andor Technology Ltd., Belfast, UK). The incident laser power was adjusted to 10 mW. Images were acquired by collecting a full Raman spectrum at each pixel in an 80 × 80 array, with a 10 ms integration time per pixel, over a 25x25 μm² area on sample. An average of the 6400 spectra in the image was obtained using WITec Project 2.1 software (WITec GmbH, Ulm, Germany) and processed using Igor Pro 6.32A (Lake Oswego, OR).
4.3 Results and Discussion

4.3.1 Role of Carotenoids in YR343 in Withstanding Oxidative Stress

*Pantoea* sp. YR343 produces a yellow pigment under all growth conditions tested; however, it is the most apparent when cells are grown to stationary phase in LB medium. Carotenoids play a vital role in the survival of cells under harsh conditions, such as extremes in pH and resistance to toxins \(^{58-59}\). Moreover, the transient production of reactive oxygen species (oxidative bursting) is a common feature during the early stages of plant-microbe interactions (reviewed in \(^{60}\)). Thus, it was hypothesized that carotenoids may play a role in rhizosphere survival and/or plant association. To test the function of carotenoids in *Pantoea* sp. YR343, we deleted the *crtB* gene which encodes phytoene synthase, the enzyme responsible for the conversion of geranylgeranyl pyrophosphate into phytoene and serves as a precursor for the synthesis of other carotenoids \(^{61}\). As predicted, the Δ*crtB* mutant no longer produced a yellow pigment and the colonies appeared white as shown in Figure 4.1. Colonies lacking yellow pigmentation were screened by PCR to verify that the *crtB* gene was deleted.
Figure 4.1: Wild type YR343 and ΔcrtB mutant microcolonies on agar plates and in microcentrifuge tubes, showing visual difference in pigmentation

This was further confirmed by analyzing the UV-Vis profiles of pigments extracted from wild type and ΔcrtB mutant cells (data not shown). The pigments extracted from wild type cells showed spectra consistent with zeaxanthin as has been described for other Pantoea strains. Conversely, little to no pigment was extracted from the ΔcrtB mutant. To further characterize this carotenoid, Raman spectroscopy was used as a non-destructive in situ method of analysis. Representative Raman spectra of wild type Pantoea sp. YR343 and the ΔcrtB mutant are shown (and explained in detail) in Figure 2.3 (Chapter 2). To recapitulate, the
spectrum for the wild type exhibits three prominent bands at 1520 cm$^{-1}$, 1155 cm$^{-1}$, and 1002 cm$^{-1}$ characteristic of carotenoid compounds arising from in-phase C=C and C-C stretching and in-plane CH$_3$ rocking vibrations, designated $\nu_1 = 1520$ cm$^{-1}$, $\nu_2 = 1155$ cm$^{-1}$, and $\nu_3 = 1002$ cm$^{-1}$ 62-66. In contrast, these bands are absent in the $\Delta$crtB mutant. **Figure 4.2** shows a Raman spectrum of pure zeaxanthin which contains the abovementioned characteristic carotenoid bands.

![Figure 4.2: Raman spectrum of pure zeaxanthin standard](image)

Because carotenoids are known to function as antioxidants in many organisms 40-42,59, it was interesting to study the role of this pigment in the survival
of *Pantoea* sp. YR343 under various conditions. Indeed, it was found that this carotenoid was important to the survival of *Pantoea* sp. YR343 in the presence of oxidative stress created by hydrogen peroxide. Compared to the wild type, the Δ*crtB* mutant was more sensitive to the effects of increasing concentrations of hydrogen peroxide as determined by a viability assay as shown in Figure 4.3. The cell viability assay is a conventional microbiological technique used to measure the viable microbial cells after applying the oxidative stress. The assay relies on quantitation of ATP to indicate metabolically active cells. Briefly, in a 96 well plate, 50 µl of log phase or stationary phase cells were combined with various concentrations of hydrogen peroxide and incubated for 1 hour with shaking at 28°C. Afterwards, each well was treated with 100 µl of Bac-Titer Glo reagent (Promega) according to the manufacturer’s instructions and luminescence was measured on a BioTek Synergy 2 microplate reader. Though this technique is quick (~5 min) and easy to use, it does not provide any information beyond cell viability. The user can only conclude if the cell was alive or dead but could make no inference about the extent or nature of damage in a live cell. In other words, the gradual cell damage that occurs between the completely healthy and dead stages was totally inaccessible.
To demonstrate that hydrogen peroxide has a specific effect on carotenoids, we examined the effect of hydrogen peroxide on wild type and mutant cells using Raman spectroscopy. For purpose of comparison, the average spectrum from each sample was normalized using the area under the C-H stretching peak (2800-3000 cm\(^{-1}\)). The Multi-peak Fit 2 package in Igor Pro was used to approximate baselines, calculate the area under peaks and measure peak amplitudes. In the overlay of spectra shown in Figure 4.4, each spectrum is offset from the next for ease of visualization.
Figure 4.4: Normalized Raman spectral overlay of (A) YR343 and (B) ΔcrtB mutant after treatment with various concentrations of hydrogen peroxide.
By this experiment, a steady decrease in the peak intensity for carotenoids was observed (Figure 4.4 (A)), which correlates to the loss of cell viability in YR343. In the ΔcrtB mutant, a steady decrease in peak intensity was observed in multiple cell-related (DNA, protein) peaks as seen in Figure 4.4 (B). Furthermore, in contrast to the viability assays which show a precipitous decrease from 5-10 mM H$_2$O$_2$, the Raman spectra in Figure 4.4 clearly show progressive loss of cell-related peaks beginning at H$_2$O$_2$ concentrations as low as 1 mM. These data are consistent with the protective role of carotenoids in the presence of reactive oxygen species $^{40-42,59}$.

As indicated in Figure 4.5 (A), for YR343, the intensity of carotenoid peak at 1520 cm$^{-1}$ was used for comparison and for ΔcrtB the DNA/protein related band at 1126 cm$^{-1}$ was used. The 1126 band has been assigned to ν(C–O–C) stretching vibration from symmetric glycosidic linkages$^{67}$ or to ν(C–N) stretching vibrations.$^{68}$ The intensity of the tracked peaks decreases as the H$_2$O$_2$ concentration is increased. In Figure 4.5 (B), the intensities of the selected peaks (carotenoid in YR343 and a cellular/protein peak in ΔcrtB) are plotted as a function of log of H$_2$O$_2$ concentration in order to see the effect of peroxide concentrations on the cells. We can see that lower concentrations of H$_2$O$_2$ don’t have much of an observable effect on ΔcrtB peak while they do affect the carotenoid peak in YR343. This experiment is a great illustration of the advantages accessible by using the Raman toolkit over conventional biological plate-based assay formats. The population of cells in a culture is heterogeneous and all the cells respond in different ways and time courses.
to various stresses or toxins. The data from a plate-based assay reflects the average of the signal from the cell population, whereas Raman spectroscopy can be used to both gain a comprehensive population level picture as well as study smaller numbers of cells or even individual cells. Additionally, Raman affords the ability to understand which components of the cell are affected in time under oxidative stress.
Figure 4.5: (A) Overlay of Raman spectra generated from wild type Pantoea sp. YR343 and ΔcrtB mutant with circles indicating the peaks selected for comparing the effect of varying hydrogen peroxide concentration; (B) Normalized intensity of selected peaks in YR343 and ΔcrtB as a function of H$_2$O$_2$ concentration (M)
4.3.2 Pantoea-Arabidopsis Co-cultures

In order to evaluate Raman spectroscopy as a method of studying plant-bacteria interactions, *Arabidopsis thaliana* was chosen as the model plant for *Pantoea* co-cultures. Plant roots were inoculated with the *Pantoea* sp. YR343 rhizobacterium and then imaged by confocal Raman and scanning electron microscopics in order to gain chemical and spatiotemporal information from the co-culture samples with minimum sample processing. Initially *Arabidopsis* plants were tested, but roots of fresh green plants were found to exhibit unacceptably high levels of autofluorescence. This is consistent with the findings of Schmidt *et al.* who overcame the autofluorescence in Raman imaging of *Arabidopsis* with visible wavelength excitation by using senescent samples.69 Thus, inoculated *Arabidopsis* plant roots were aged for periods ranging from 5 to 15 days, which proved sufficient to obtain informative data using a combination of CRM and PCA.

As shown in Figure 4.6, sample aging effectively eliminates sample autofluorescence so that bands corresponding to cellulose (1100 cm\(^{-1}\), 1130 cm\(^{-1}\), and 1326 cm\(^{-1}\)) and lignin (1600 cm\(^{-1}\)) can be observed in the *Arabidopsis* control spectra (bottom spectra in Figures 4.6(A) and 4.6(B)). The spectra obtained from the *Pantoea* YR343-inoculated *Arabidopsis* samples exhibit three additional strong bands that correspond to the \(\nu_1-\nu_3\) pre-resonance Raman bands previously assigned to carotenoids in the bacterial outer membrane. Comparison of the spectra shown in Figures 4.6(A) and 4.6(B) show that the relative intensities of *Arabidopsis*-derived bands to *Pantoea* YR343-derived bands increases with sample aging, with the 15 day samples showing much clearer plant related cellulose and lignin bands.
Figure 4.6. Raman spectra of senescent samples dried for (A) 5 days and (B) 15 days. Each panel shows an overlay of *Arabidopsis thaliana* plant (bottom) and *Arabidopsis thaliana* inoculated with *Pantoea* sp. YR343 (top). Spectra were background subtracted after fitting to a 3rd order polynomial.
Figure 4.7 shows SEM images of an *Arabidopsis* root colonized with *Pantoea* sp. YR343, showing good coverage and affinity of the rhizobacteria towards the root. Careful comparison of Figures 4.7(A) and 4.7(B) shows that the *Pantoea* cells predominantly colonize the crevices on the surface of the root and Figure 4.7(C) shows the distribution of individual cells on the root.

![Figure 4.7. SEM images of *Pantoea* sp. YR343 colonizing *A. thaliana* root showing spatial distribution and growth of bacteria on the root at different magnifications: (A) 1,200x, (B) 15,000x and (C) 50,000x.](image)

Figure 4.8 illustrates typical Raman microspectra and chemical images obtained from the *Arabisopsis-Pantoea* co-cultures. Figures 4.8(A) and 4.8(E) show Raman images with filters integrated over the 2800-3050 cm\(^{-1}\) and 1507-1546 cm\(^{-1}\) spectral regions, corresponding to the C-H stretching and carotenoid-related \(\nu_1\) assigned to conjugated C=C stretching vibrations, respectively. The Raman spectrum in Figure 4.8(D) shows the 2800-3050 cm\(^{-1}\) wavenumber region. The principal component heat maps in Figures 4.8(B) and 4.8(F) corresponding to loading plots.
4.8(C) and 4.8(G) contain features that closely resemble plant cell (bands corresponding to lignin\textsuperscript{70}, pectin\textsuperscript{71}, and chlorophyll\textsuperscript{72}) and bacterial carotenoids, respectively. In particular, the Raman images in Figures 4.8(A) and 4.8(E) are well-matched to the PCA heat maps in Figures 4.8(B) and 4.8(F), respectively, thereby confirming the assignments of the principal components in Figure 4.8(C) to (predominantly) Arabidopsis and Figure 4.8(G) to Pantoea. A combined Raman image produced using a combination of the spectral filters used in Figures 4.8(A) and 4.8(E), shown in Figure 4.8(H), clearly illustrates the non-uniform spatial distribution of Pantoea on the Arabisopsis root. In particular, note that the high intensity region of the (Arabidopsis) heat map in Figure 4.8(B) (bottom center) matches a low intensity region of the (Pantoea) heat map in Figure 4.8(F), and that the same region of Figure 4.8(H) is devoid of bacteria. Given that the images are acquired by confocal scanning Raman microscopy with a limited confocal depth (\(\sim 1\) \(\mu\text{m}\)) and that the bacteria are clearly on the root surface, as shown in Figure 4.7, the composite image in Figure 4.8(H) shows bacteria where they are present, and the underlying plant root surface only in locations where there are no bacteria.
Figure 4.8. Chemical imaging of Arabidopsis thaliana root inoculated with wild-type Pantoea sp. YR343. (A) Raman image integrated over 2800-3050 cm⁻¹; (B) principal component heat map and (C) corresponding loading plot representing the principal component obtained from the Arabidopsis regions; (D) Raman spectrum showing the 2800-3050 cm⁻¹ wavenumber region; (E) Raman image integrated over 1507-1546 cm⁻¹, (F) principal component heat map and (G) corresponding loading plot obtained from regions dominated by Pantoea. (H) Combined Raman image produced using a combination of the spectral filters used in Figures 4.8(A) and 4.8(E), showing the spatial distribution of bacteria (yellow) on a plant root (green).
4.3.3 Indole Acetic-Acid (IAA) Production and Detection

Many plant associated bacteria can produce phytohormones, such as indole-3-acetic acid (IAA), which can stimulate root growth and therefore enhance water and nutrient uptake. Indeed, *P. dispersa* and *P. agglomerans* have previously been shown to produce IAA. For this reason, the question of whether *Pantoea* sp. YR343 was able to produce IAA, which is synthesized from the amino acid tryptophan, and is commonly found in plant root exudates was examined. A colorimetric assay was used to measure the production of indolic compounds, including IAA, in *Pantoea* sp. YR343 when grown in the presence of tryptophan. The cultures treated with the Salkowski reagent showed a red color due to formation of an iron complex. The amount of indoles was quantified by constructing a calibration curve by preparing standard solutions of IAA of varying concentrations and treating with the Salkowski reagent which resulted in a color change, as shown in Figure 4.9, followed by measuring the absorbance. The first five samples from the left contain varying concentrations of IAA while the sample to the far right is a tryptophan control which was colorless after addition of the reagent, thus confirming that only the presence of indoles resulted in the change of color. The tryptophan-dependent production of IAA was confirmed by GC-MS analyses and measured at approximately 0.5 µg/ml. Somewhat unexpectedly, it was found that the ΔcrtB mutant was defective in IAA production as determined by this colorimetric assay. Indeed, a 3-fold decrease in indole production was observed from 4.35 ± 0.2 µg/ml indoles in wild type cells compared to 1.52 ± 0.02 µg/ml indoles in ΔcrtB as shown in Figure 4.9. This was unexpected since there is no
obvious connection (e.g. common enzymes or substrates) between the pathways involved in carotenoid production and the pathways involved in IAA production. In addition to their protective role, however, carotenoids have been implicated in modulation of membrane fluidity and may play a role in the formation of membrane domains^{43-48}. From this perspective, the decrease in IAA production by the ΔcrtB mutant may be a consequence of changes in membrane fluidity or organization. For example, uptake of tryptophan which is a precursor to IAA production may be defective in the ΔcrtB mutant.

![Figure 4.9: Salkowski colorimetric test showing red color in presence of IAA and indoles (left) and indole production in YR343 and ΔcrtB mutant as measured by colorimetric assay](image)

4.3.3.1 Using Raman Spectroscopy for Detection of IAA

In principle, Raman spectroscopy would be an ideal method of non-invasively studying plant-bacteria interactions, *in situ*, through the spatio-temporal
mapping of auxins. Evaluating this possibility has been especially attractive and motivating given our groups’ success with chemical imaging of small molecule chemical metabolites like quinolones and pyocyanin in bacterial communities.\textsuperscript{77-79}

An initial attempt towards the use of Raman spectroscopy to study auxin production in plant-bacteria interactions presented some challenges. As shown in Figure 4.10, the spectrum of IAA is visually very similar to the spectrum of tryptophan, which is an essential precursor for the production of IAA. This brings up an important question: can we tell them apart? The IAA assays contained 200 mg/L of extraneously added tryptophan and resulted in about 4.0 µg/ml of indoles being produced by YR343. Even the use of techniques such a PCA to distinguish between IAA and tryptophan presents a significant challenge when looking molar ratios around 1:40.
Ag colloids were used in an attempt to enhance the Raman spectra of a 1 mM pure standard solution of IAA but did not yield a clear SERS spectrum or PC from spectral analysis. IAA plate assays experiments were designed where instead of using liquid cultures, agar plates with tryptophan were used and the YR343 cell cultures were pipetted over the plate in an attempt to contain the tryptophan in the agar while measuring the IAA from the cells on top. Although this resulted in production of IAA as confirmed by the colorimetric test, the strong tryptophan bands were still being picked up by the Raman spectra. Despite the challenges encountered in the preliminary attempts described above, it would still be a worthwhile effort to explore alternative enhancing structures, excitation
wavelengths, multivariate statistical techniques, electrochemical-SERS and assay recipes further down the road.

4.4 Conclusions

This chapter detailed experiments that made use of Raman spectroscopies in innovative, non-traditional ways such as measuring the effect of oxidative stress in the cell, chemical imaging of root colonization in plant bacteria co-cultures, for the first time. This demonstrates the untapped potential of Raman spectroscopy as a powerful bioanalytical tool.

To better understand the mechanisms that promote plant association and to demonstrate the genetic tractability of *Pantoea* sp. YR343, a mutant defective in carotenoid production was constructed by deleting the *crtB* gene that encodes for phytoene synthase. The production of carotenoids by *Pantoea* sp. YR343 proved to be a distinguishing feature for Raman spectroscopic analysis, which could be used to follow the effect of H$_2$O$_2$ on carotenoids. In these analyses, a steady decrease in the Raman spectra associated with carotenoids was observed as the cells were exposed to increasing concentrations of H$_2$O$_2$. The decrease in Raman signal intensity was apparent even at H$_2$O$_2$ concentrations that did not result in decreased cell viability, indicating that even low concentrations of H$_2$O$_2$ have an effect on the cells. Thus Raman spectroscopy is an effective method to measure the effect of oxidative stress on both pigmented and non-pigmented bacterial cells and is capable of providing more perceptive insight into cell damage than conventionally used plate-based assays. High-resolution chemical images of inter-kingdom co-cultures between a
plant (*A. thaliana*) and a bacterium (*Pantoea*) are also presented. Clearly Raman microspectroscopy and chemical imaging constitutes a promising approach for further studies of co-culture systems. Further work is required to evaluate the possibility of detecting and identifying Raman active metabolites, like the auxins, which are implicated in cross-kingdom communication in the rhizosphere.
4.5 References


CHAPTER 5:
TOWARDS MICROFLUIDIC PLATFORMS FOR RAMAN SPECTROSCOPY

5.1 Introduction

Understanding how observable biological processes, transpiring over wide-ranging spatio-temporal scales, result from molecular scale events represents a grand challenge facing biological research. Developments in microfluidic technology have made it possible to manipulate single bacterial cells and control the microenvironment.\textsuperscript{1-2} Structures in the micrometer to millimeter range are well suited for microbiological investigations as the size matches that of a microbial cell, and many important physical and chemical interactions occur at those length scales.\textsuperscript{3-4} Microfluidic platforms provide the ability to monitor and control the flow of materials and their presentation to the biological system of interest and to measure responses to environmental perturbations in the samples, which opens up a relatively unexplored facet for bioimaging studies.\textsuperscript{5} Modern lab-on-a-chip devices are now capable of miniaturizing many common bench-scale operations like separations, reactions, concentration, detection and sensing.\textsuperscript{6-7} As microfluidics has made inroads into modern microbiology, the field is benefitting from the ability to study small numbers of cells, which are either uncultivable or which can provide unique information that cannot be obtained from ensemble average measurements.
In the study of bacterial populations, the use of microfluidic platforms offer the indispensable ability to control the microenvironment in a field of study where the samples are heterogeneous and involve a number of both known and unknown factors that can influence the measurement. In the context of Raman spectroscopy, microfluidic platforms offer the potential to integrate not just fluidic but also electronic, plasmonic and photonic structures for enhanced and correlated imaging.

In the previous chapters, successful application of Raman spectroscopies and related techniques for studying and imaging the bacterial cell, its secretome and inter and intra-species interactions was demonstrated. This chapter presents preliminary work done towards developing Raman spectroscopy compatible with microfluidic platforms and discusses practical considerations and the rich potential of integrating such platforms in studies of bacterial communities. Micro-corral (well-defined geometries constructed of materials that are biocompatible and spectroscopy-friendly) fabrication and use is demonstrated as an easy method to organize, manipulate and investigate small well-defined subsets of bacterial communities.

5.1.1 Theoretical Background and Research Overview

The microenvironment is the immediate surroundings that influence the bacterial cell and can be sensed by it. This includes the physical and the chemical factors that are perceived by the cell. For example, motility is known to play an important role in surface attachment and microcolony formation in biofilms of *Pseudomonas aeruginosa*. The substrate properties like surface charge and
roughness can play an important role in motility and biofilm formation.\textsuperscript{10-11} Many factors of the microenvironment, like nutrient availability, temperature, pH, oxygen, iron etc. are also known to influence biofilm formation.\textsuperscript{9} The ability of \textit{P. aeruginosa} to form biofilms, is partly responsible for its persistence in cystic fibrosis patient infections.\textsuperscript{12} Bacteria can secrete and detect signaling molecules known as autoinducers (AIs), which enables them to perceive the population density and respond to environmental factors.\textsuperscript{13} AI concentrations reach a critical level at high population densities resulting in the onset of quorum sensing (QS) and altered gene expression in unison. Due to receptor specificity, bacteria can recognize the population sizes of their own kind (intra-species) and also of other species of bacteria (inter-species) and alter their behavior accordingly.\textsuperscript{14} Pathogenic bacteria use quorum sensing to control virulence gene expression.\textsuperscript{15} QS plays a crucial role in \textit{P. aeruginosa} pathogenicity by regulating the production of multiple virulence factors, aiding in biofilm maturation and antibiotic resistance.\textsuperscript{16} Studies have shown that bacteria can also communicate across species and therefore assess the population densities of other species of bacteria.\textsuperscript{17} Quorum sensing controls gene expression\textsuperscript{18} and is known to influence processes such as biofilm formation, motility, virulence, symbiosis and antibiotic production. Thus, there is substantial motivation to study bacterial cell-to-cell interaction and the role of molecular diffusion in quorum sensing and biofilm formation in individual cells or small bacterial communities that are confined.

Microfabrication and microfluidics have widespread use in multiple fields including microelectronics, lab-on-a-chip (LOC) applications micrototal analytical
systems and microscale bioanalytical assays. Microfluidics has become a powerful tool in biological research, because it offers precise control of the chemical, mechanical and electrical microenvironment.\textsuperscript{8} A variety of lithographic and etching techniques have been used to fabricate microscale and nanoscale structures with photolithography being the dominant technology.\textsuperscript{19} Microfluidic devices have been used to study the effect of spatial constraints and chemical gradients on microbial communities.\textsuperscript{8} More recently, researchers have recognized the need to study small communities of spatially organized microorganisms. This approach can help gain insights into the dynamics of growth and survival of a small number of cells which is characteristic of early stages of biofilm formation and infections. It can also help in unraveling the processes that occur during on the onset of QS and biofilm behavior. This is key to the design of therapeutic drugs based on quorum sensing inhibition of drug-resistant bacteria.\textsuperscript{12}

A comprehensive review article by Wessel \textit{et al.} describes the methods various groups have used to spatially organize and confine small groups of bacteria and the analytical technique used to study them.\textsuperscript{20} Boedicker \textit{et al.} used microstructures to confine single cells and demonstrated high density quorum sensing and heterogeneity in genetically identical populations.\textsuperscript{21} Microfluidic mazes were constructed to demonstrate that bacteria could form a quorum by using chemotaxis to accumulate in confined geometries.\textsuperscript{22} Flickinger \textit{et al.} combined microfluidics with hydrogels to show that \textit{P. aeruginosa} cells could sense acyl-homoserine lactone quorum sensing molecules from 8mm distance.\textsuperscript{23} Small numbers (~150 cells) of \textit{P. aeruginosa} confined in lobster traps (fabricated using
multiphoton lithography) displayed a high resistance to an antibiotic that successfully exterminated a bulk culture grown in a test tube (~10^7 cells).\textsuperscript{24} Individual \textit{Staphylococcus aureus} cells, confined in lipid-silica structures were shown to initiate quorum sensing due to spatial confinement.\textsuperscript{25} It is interesting to note that a majority of the abovementioned studies utilized measurements based on green-fluorescent protein (GFP) reporters. Though fluorescence and luminescence based measurements continue to be used extensively in microbiological research, it is known that fluorescence reporter systems can interfere with normal gene expression and behavior in bacterial cells.\textsuperscript{26} In autofluorescent samples, fluorescence based GFP signals are hard to detect without high expression, which may be toxic to the cells and interfere with virulence (I. Haufert, J. M. Sidebotham, and J. C. D. Hinton, unpublished data).\textsuperscript{27} Thus it is crucial to push towards the development of truly non-invasive, sensitive and selective methods to visualize cellular events.

5.2 Experimental Section and Results

5.2.1 Materials & Methods

\textbf{Materials.} All chemicals were obtained from Sigma Aldrich (St. Louis, MO) and used as received. Silicon substrates were purchased from WRS Materials (San Jose, CA) as 3-in-diameter wafers of Si (100). Soda lime glass cover slips (Corning) measuring 24 mm x 60 mm were purchased from VWR. Photoresist SU-8 2010
(Microchem) and PDMS (Dow Corning) were used following manufacturers’ procedures.

**Bacterial strains.** Wild type *Pseudomonas* sp. GM41 were grown on Lysogeny broth (LB) plates under aerobic conditions at 28°C. *Pseudomonas* sp. GM41 was previously isolated from the rhizosphere of *Populus deltoides.* Over night cultures were grown at 28°C with shaking at 240 rpm in modified Fastidious Anaerobe Broth (FAB) culture medium supplemented with 30 mM filter sterilized glucose as the source of carbon and adjusted to OD$_{600}$ = 1.0 using a Cary UV-Vis spectrophotometer.

**Instrumentation and Data Analysis.** Raman microscopy was carried out on an Alpha 300R confocal Raman microscope (WITec GmbH, Ulm, Germany) with a 40×, Nikon air objective (NA=0.6), and a coverslip-corrected Nikon water immersion 60× objective (NA = 1), using a frequency-doubled Nd:YAG laser ($\lambda$ = 532 nm) delivered through a single-mode optical fiber, dichroic beam splitter, and focused onto the surface of the sample using a microscope objective operating in epi-illumination geometry. The backscattered radiation was transmitted through a 50-μm diameter multimode fiber to a UHTS 300 spectrometer with a 600 groove mm$^{-1}$ diffraction grating and back-illuminated CCD camera cooled to −65 °C (Newton DU970 N–BV, Andor Technology Ltd., Belfast, UK). The incident laser power was adjusted to 10 mW. Each Raman spectrum recorded was an accumulation of 100 spectra acquired with integration time of 0.5 s each at 5 mW incident laser power using WITec Project 2.1 software (WITec GmbH, Ulm, Germany) and processed using Igor Pro 6.32A (Lake Oswego, OR).
5.3 Results and Discussion

5.3.1 Micro-corrals Fabrication

Square micro-corrals arrays of different dimensions (5, 10, 25, 50, 250 μm side) were fabricated using photolithography to capture and isolate small groups of cells. The design was adapted from a study by Boedicker et al. where quorum sensing was shown in single cells under confinement using fluorescent reporters. SU-8 2010, an epoxy based negative photoresist was chosen, because it is known to be biocompatible, and because it can be used to pattern high aspect ratio structures using standard contact lithography. All the fabrication steps were carried out in the cleanroom setting of the Notre Dame Nano Fabrication facility. A photomask was generated with opaque chromium patterns designed using L-Edit software, in a Mann 3600F Photomask Pattern Generator. A thin film of SU-8 2010 photoresist was spincoated onto piranha (3:1 mixture H₂SO₄:H₂O₂) cleaned glass substrates, followed by pre-baking, UV light exposure and postbaking steps, thus transferring the mask pattern onto the photoresist layer. A Karl Suss MJB-3 contact mask aligner was used for the exposure. The pattern was developed long enough to remove any residual SU-8 from the base of the micro-corrals. The sequence of photolithographic steps is shown in Figure 5.1. A Tencor P-6 profilometer was used to measure the micro-corrals' dimensions and check for any defects and images of 10 μm and 50 μm side micro-corrals, both of which have a depth of 10 μm, are shown in Figure 5.2.
Fabrication of SU-8 Micro-corrals

Figure 5.1: Schematic describing the sequence of steps in micro-corrall fabrication
Figure 5.2: (A) 10 µm and (B) 50 µm corral arrays with a depth of 10 µm, fabricated using photolithography

Raman spectroscopy was used to characterize the micro-coral substrates and perform control experiments to avoid ambiguity in future peak assignments. **Figure 5.3** shows an overlay of the spectra of the glass slide used for fabrication (blue), SU-8 photoresist on glass (pink) and the center of micro-coral base (green). The spectra of the micro-coral base is identical to that of the glass slide and this helped confirmed that there was no undeveloped SU-8 remaining on the micro-coral base after the exposure and development steps. It was important to ensure that there was no residual photoresist in the regions of the micro-coral where the sample spectra would be collected, because SU-8 can potentially obscure the spectral features from biological samples.
5.3.2 Bacterial Cell Capture in Corrals

Bacterial cell capture in the micro-corrals was accomplished modifying a microfluidic technique previously described by Park et al.\textsuperscript{31} A 2 mm wide, 150 μm deep PDMS channel, fabricated using soft-lithography (SU-8 2050 master) was used to flow an overnight culture of bacteria (adjusted to OD\textsubscript{600} = 1.0) into the micro-corrals. After allowing the bacteria and the nutrient media to settle into the wells for about five minutes, the excess solution above the wells is gently pushed out of the channel with an air bubble using a disposable pipette, leaving just the wells filled
with cells in culture media. This graphic is shown in Figure 5.4 (A). Although the success of this method of filling cells relies on the ability to determine the optimal pressure that is to be applied manually to push the air bubble and excess liquid, it offers a quick and easy route which does not require and specialized pumps, tubing or complex microfluidic architecture. Figure 5.4(B) shows a bright-field microscopic image (Olympus IX70) of the GM41 cells in 50 μm corrals after the cell capture. A large number of cells were successfully captured and were found to be actively motile in the wells. The capture technique effectively pushed the excess liquid out, resulting in negligible amount of cells or media remaining outside of the corrals.
Figure 5.4: (A) Schematic for microfluidic capture of bacterial cells in micro-corrals. (B) Bright-field image of *Pseudomonas sp.* GM41 cells captured in 50 μm corrals.

The GM-41 cells were stained with SYTO9 (ThermoFischer Scientific) nucleic acid fluorescent stain, filled into 25 μm corrals and imaged using a Nikon A1-R confocal microscope. By varying the size of the micro-corral and the density of the bacterial cell culture, numbers ranging from a single cell to hundreds of cells can be
captured and retained in each well by this approach. **Figure 5.5 (A)** shows two cells captured in a 25 µm corral using a dilute cell culture with OD$_{600}$ = 0.1. After about an hour, it was found that the cells attached to the glass base (**Figure 5.5 (B)**) and the SU-8 walls (**Figure 5.5 (C)**) of the corrals. This behavior is consistent with initial stages of biofilm formation, when free-swimming planktonic cells adhere to surfaces.  

![Figure 5.5: Pseudomonas sp. GM41 cells imaged in 25 µm corrals. (A) Two cells captured using a low-density culture; Cells adhering to the corral's base (B) and walls (C).](image)

An array of 250 µm corrals were filled with GM41 cell culture and investigated using Raman spectroscopy. The overlay of spectra collected from the SU-8 region (red spectrum) and the corrals with cells is shown in **Figure 5.6**. The green spectrum and the blue spectrum were collected using a 40x and 60x objective respectively. Both contain bands (748 cm$^{-1}$, 1126 cm$^{-1}$, 1310 cm$^{-1}$, 1586 cm$^{-1}$) that are assigned to bacterial cellular components as explained in detail in Chapter 2,
although predictably the spectrum collected in blue has a better signal to noise ratio and peaks of higher intensity. The SU-8 walls clearly do not influence the spectra collected from the center of the micro-corrals as seen by the absence of the distinguishing aromatic C-C stretch mode at 1608 cm\(^{-1}\) in SU-8.\textsuperscript{33}

Figure 5.6: Overlay of Raman spectra generated from the SU-8 region between corrals (red) and the region inside a corral containing cells using a 40x (green) and 60x (blue) objective

Consecutive spectra collected on the same spot, with a gap of not more than 1 min between each spectrum and overlaid and shown for comparison in Figure 5.7.
It was found that the cell-related Raman peaks gradually decreased in intensity with increasing integrated laser flux on the cells. Puppels et al. have observed similar photodamage with the use of 514.5 nm and 532 nm laser irradiation for single cells or small numbers of cells and attribute it to unknown photochemical reactions in the cell, which are initiated at the abovementioned wavelengths.34 A simple solution to this issue is the use of higher excitation wavelengths such as 785 nm. It is interesting to note that no sample or substrate heating or burning, as evidenced by a rapid increase in the background or the typical carbon D and G bands, was observed. It is worthwhile to keep this photodamage phenomenon in consideration while designing future micro-corral experiments that involve small numbers of cells or small quantities of the analyte.
Figure 5.7: Overlay of Raman spectra of GM41 cells in a micro-corral. The three spectra were collected sequentially, top-down.

5.4 Conclusions

Arrays of micro-corral structures, fabricated using photolithography are suitable for spectroscopic and optical imaging. A simple microfluidic technique is effective to capture bacterial cells in culture media, into the corrals. Raman spectroscopy and fluorescence imaging of the cells in the corrals has been successfully demonstrated. Photodamage of cells occurs due to laser irradiation at 532 nm wavelength and results in gradual decrease in cellular peak intensities.
Research on bacterial behavior and quorum or diffusion sensing in small groups of cells in confined geometries, although still at its infancy, has the potential to answer many fundamental questions on signaling and the biology of biofilms. A thorough understanding of early stage biofilm formation is crucial for the development of therapeutic agents that target the biofilm phenotype in chronic infections. With a combination of powerful techniques like Raman spectroscopy, SERS, electron microscopy, electrochemistry and mass spectrometry, there is abundant potential to obtain a comprehensive picture of bacterial behavior and communication in small groups of cells. The integration of microfluidic platforms in creates the potential to extract information that conventional studies in bulk cultures have not been able to access due to interference from intercellular signaling and inherent spatial and temporal heterogeneity and complexity.
5.5 References


CHAPTER 6:
CONCLUSIONS AND OUTLOOK

The novel use of Raman spectroscopy, enhancing methodologies, and imaging for investigating bacteria and their social behavior in inter- and intra-species interactions, was demonstrated by the work described in this thesis. Complementary methods of sample examination and data analysis, such as electron microscopies and principal component analysis, were incorporated to develop more thorough and efficient methods of processing and interpreting data than those currently used. To summarize, this work examines the use of previously established analytical techniques in mapping bacterial communities in order to better understand these complex systems and gain important perspectives on the social elements of microbial communities. The studies described here establish the strong potential of the Raman toolkit to probe aspects of bacterial interactions relevant to plant and human health. This work also serves as a practical guide for improved experimental design and data treatment, which are crucial in studies of heterogeneous and complex systems. There is much to be studied and understood in the field, and the work in this dissertation motivates the use of confocal Raman imaging and related analytical techniques to explore the little understood, but hugely significant, world of microbial social behavior.
In Chapter 2, the toolkit was used to characterize the bacterial cellular components in the presence of pigments that interfere with spectral analysis. Electron microscopy was utilized to aid in SERS peak assignments, which can often be ambiguous. Once the cell itself was characterized in Chapter 2, Chapter 3 detailed the use of SERS imaging to obtain chemical information about small molecule metabolites in *P. aeruginosa* biofilms. This technique of mapping distinct regions of the biofilm, presents a marked improvement over existing studies, which tend to collect a spectrum from a chosen hotspot of the heterogeneous biofilm. It is cumbersome to ‘search’ for a hotspot and the spectrum does not necessarily provide an accurate representation of the sample. The results also provided chemical information in contrast to the commonly applied microscopy methods used to study biofilms, which provide morphological information. The biofilm samples were studied *in situ* without any pre-preparation steps except for addition of the Ag colloid. In Chapter 4, Raman and SERS imaging are used for the first time to image plant-microbe interactions on senescent green plant roots. Spectroscopic measurement of oxidative stress was used to produce more information than conventional plate-based viability assays, displaying yet another way Raman spectroscopy can be applied to study bacterial systems. Microfluidic corral platforms that are compatible with spectroscopy were described and tested in Chapter 5. Bacterial cells were successfully captured using a simple microfluidic technique, and their Raman spectra were collected from the micro-corrals.
6.1 Suggested Future Studies

While conducting the experiments that make up the body of this work, there were many opportunities to either further explore an observed phenomenon or change the approach used. Although beyond the scope of the current dissertation, these possibilities are worth considering for future work.

**Nanoparticles for SERS.** Silver nanoparticle colloids were used as the key enhancing modality for SERS studies of bacteria and bacterial biofilms as described in Chapter 2 and Chapter 3. This choice was primarily made based on the available laser excitation source at 532 nm, which matches well with the plasmon resonance of silver nanoparticles. After the recent addition of a 785 nm near-IR laser in the laboratory, it is worth exploring the use of gold nanoparticles, which exhibit plasmon resonances in the near-IR. The benefit of using colloidal nanoparticles in place of nano-structured substrates fabricated by e-beam lithography is the ability to disperse the particles throughout the three dimensional biofilms and also to achieve intimate contact with the bacterial cell by the coating method detailed in Chapter 2. Gold is recommended, as it is known to be highly inert, non-toxic and biocompatible.\(^\text{1}\) It is also useful to carry out cell viability studies to ensure compatibility with the samples.

Another important factor that can be varied is the surface charge of the nanoparticle. The charge on the nanoparticle is important in enhancement, as it affects the adsorption of the analyte molecule and also determines nanoparticle aggregation.\(^\text{2}\) A variety of analytes can be studied with nanoparticles of different charges. The Ag colloids used in this work were synthesized using sodium
borohydride as the reducing agent, using a protocol that results in a net negative charge on the nanoparticle. The bacterial cells have a net negative charge because of the presence of carboxyl, phosphate groups. Studies have shown that positively charged silver nanoparticles show higher interaction and bactericidal action, while neutral and negatively charged nanoparticles have a negligible effect. Thus, care must be exercised to control the sign and magnitude of the surface charge on silver nanoparticles for SERS in samples containing bacteria.

**Microfluidic Platforms.** Microfluidic platforms integrated with enhancing strategies can be used to attempt to collect and concentrate signaling molecules, which are produced in very low concentrations, for offline detection. There is also a great advantage in designing methods of environmental control (temperature, humidity) for bacterial cultures to be maintained over time in micro-corrrals. This will facilitate culturing different sized populations under varying physical and chemical environments accompanied by *in situ* Raman measurements over time.

**Biofilms of YR343.** A difference was observed in the ability of wild type YR343 and mutant ΔcrtB mutant to form biofilms. The ΔcrtB mutant was impaired in forming pellicle biofilms. In addition, as described in Chapter 4, the mutant was also found to be impaired in IAA production. These observations warrant further investigation to elucidate possible differences in the chemical makeup of biofilms of both mutant and wild type using Raman spectroscopy.
6.2 References


