Systematic Study of Bacterial Phenotypes Caused by
Pseudomonas aeruginosa Prophage Adaptive Genes

by

Yu-Fan Tsao

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

© Copyright by Yu-Fan Tsao 2015
Systematic Study of Bacterial Phenotypes Caused by 
_Pseudomonas aeruginosa_ Prophage Adaptive Genes

Yu-Fan Tsao

Master of Science

Department of Molecular Genetics

University of Toronto

2015

Abstract

In addition to being the major predator of bacteria, some bacteriophages adapted a symbiotic relationship with their bacterial hosts through the formation of prophages. Unique genes in prophages known as Prophage Adaptive Genes (PrAGs) function to alter host biological processes, and they contribute greatly to bacterial pathogenicity. I aimed to investigate the phenotypic effects of PrAGs on _Pseudomonas aeruginosa_, an opportunistic human pathogen. Individual PrAGs in _P. aeruginosa_ PA14 or PAO1 strain background were screened for bacterial phenotypes related to virulence and survivability, including phage resistance, motility, production of biofilm and pyocyanin, and virulence in _Drosophila melanogaster_. I found that 11 out of 17 PrAGs screened modified the host phenotype in some manner. In addition, some of them were observed to affect multiple phenotypes or cause bacterial strain-specific changes, which suggests that PrAGs interact with host components to cause the phenotypes observed.
Acknowledgments

My journey through Master’s study has been a wonderful one, and I want to thank all the people who made this possible. Without any of you, I would not be standing where I am today. First of all, I would like to express my gratitude to my supervisors Dr. Karen Maxwell and Dr. Alan Davidson for allowing me to work in their labs and providing me with all the resources and guidance for my graduate study. Their continual support, both academically and mentally, has helped me pass numerous obstacles in the past few years and is the key to the successful completion of this project.

I would also like to thank my committee members Dr. Trevor Moraes, Dr. Alex Ensminger and Dr. John Parkinson for their helpful advice throughout my study. They guided me forward in my project while keeping me on track. Every committee meeting was valuable to me as they always clear away confusion and inspire me with new ideas. Special thanks to Dr. Trevor Moraes and his lab members for their help with structural studies. I have learned a lot about protein structures and X-ray crystallography through working with the Moraes lab. In addition, I would like to thank Dr. Paul Sadowski for sharing his experiences in experimental techniques and other helpful comments.

Thanks to all the past and present members of the Maxwell and Davidson labs for their input and inspirations at the lab meetings and elsewhere. They made the lab environment and my experience there really positive and enjoyable, and they have also filled my life outside of the lab with laughter and happiness. Especially, I need to thank Diane Bona for guiding me through my early days in the lab as well as assisting me with my experiments, and Joe Bondy-Denomy for his work on prophages that led to my project and his valuable guidance. I also wish to thank Nichole Cumby, Senjuti Saha, Smriti Kala and Kris Hon for answering thousands of my questions, helping and mentoring me through my study, and being such great friends. My graduate school life will be very different without them.

Finally, I would like to thank my parents Charles Tsao and Jennifer Fu, my brother Oliver Tsao, my boyfriend Dan Cheng and all my friends for their love and encouragement through stressful times which made my journey so much easier and wonderful.
Table of Contents

Acknowledgments .................................................................................................................................................. iii

Table of Contents ................................................................................................................................................ iv

List of Tables .................................................................................................................................................. vi

List of Figures ................................................................................................................................................ vii

List of Abbreviations ....................................................................................................................................... viii

Chapter 1 Introduction ..................................................................................................................................... 1

1.1 Bacteriophages and their Life Cycle ........................................................................................................ 1

1.2 Bacteriophages and Human Health ........................................................................................................ 2

1.3 The Importance of Bacteriophages in Bacterial Physiology ................................................................. 3

1.4 Prophage Adaptive Genes (PrAGs) ........................................................................................................ 5

1.5 Pseudomonas aeruginosa – An Opportunistic Human Pathogen .......................................................... 7

1.5.1 P. aeruginosa and Human Health ...................................................................................................... 7

1.5.2 Virulence Factors of P. aeruginosa ................................................................................................... 8

1.6 P. aeruginosa Prophages and PrAGs .................................................................................................... 10

1.7 Thesis Objectives .................................................................................................................................... 10

Chapter 2 Materials and Methods .................................................................................................................. 12

2.1 Construction of Expression Plasmids and Protein Test Expression in E. coli ................................. 12

2.2 Transformation of plasmids into P. aeruginosa .................................................................................... 12

2.3 PCR Reactions and Colony Screening ................................................................................................ 13

2.4 Bacterial Growth .................................................................................................................................... 13

2.5 Twitching Motility Assay ....................................................................................................................... 14

2.6 Swimming Motility Assay ...................................................................................................................... 14

2.7 Induction of P. aeruginosa Lysogens and Plaques .............................................................................. 15
List of Tables

Table 1. Summary of PrAGs included in this study. ................................................................. 19

Table 2. Increase in phage resistance caused by PrAG expression in PA14 compared to no PrAG expression. ................................................................. 25

Table 3. Fold increase in phage resistance caused by PrAG expression in PAO1 compared to no PrAG expression. ................................................................. 26

Table 4. Difference in phage resistance caused by PrAG expression in PA14 and PAO1 ........ 28

Table 5. List of drugs tested in drug resistance assay ................................................................. 29

Table 6. Summary of host phenotypic changes caused by PrAGs in P. aeruginosa PA14 and PAO1 strains. ................................................................. 43
List of Figures

Figure 1. The life cycle of a bacteriophage................................................................. 2
Figure 2. Example of PrAGs identified in phage genomes. ........................................ 6
Figure 3. Genome comparison between related phage strains and positions of PrAGs. .......... 18
Figure 4. Protein expression of PrAGs in E. coli............................................................ 20
Figure 5. PrAG expression affect bacterial growth rate. ................................................ 23
Figure 6. Some PrAGs affect twitching motility of P. aeruginosa. ................................. 30
Figure 7. PrAGs could alter swimming motility of the bacteria....................................... 32
Figure 8. PrAGs cause changes in bacterial biofilm production. ..................................... 34
Figure 9. JBD5-4 alters the levels of pyocyanin produced by P. aeruginosa. .................... 35
Figure 10. PrAGs have effects on bacterial virulence towards Drosophila malenogaster....... 37
Figure 11. PrAG expression affect various bacterial elements.......................................... 44
Figure 12. Detail genome alignment for phages used in this study.................................... 53
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>C. botulinum</td>
<td>Clostridium botulinum</td>
</tr>
<tr>
<td>C. diphtheria</td>
<td>Corynebacterium diphtheriae</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>D. malenogaster</td>
<td>Drosophila malenogaster</td>
</tr>
<tr>
<td>EV</td>
<td>empty vector</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
</tr>
<tr>
<td>gp</td>
<td>gene product</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
</tbody>
</table>

viii
\( h \)  
\text{hour}

\text{IPTG}  
isopropyl \beta-D-1 thiogalactopyranoside

\text{kDa}  
kilodalton

\( \lambda \)  
Enterobacterophage lambda

\text{LB}  
lysogeny broth

\text{NaCl}  
sodium chloride

\( \mu g \)  
microgram

\( \mu L \)  
microliter

\( \mu M \)  
micromolar

\text{mL}  
milliliter

\text{mm}  
millimeter

\text{mM}  
millimolar

\text{NCBI}  
National Center for Biotechnology Information

\text{OD}  
optical density

\text{OD}_{595}  
optical density measured at 595 nm

\text{OD}_{600}  
optical density measured at 600 nm

\text{ORF}  
open reading frame

\text{PCR}  
polymerase chain reaction

\text{PrAG}  
Prophage Adaptive Gene

\text{P. aeruginosa}  
Pseudomonas aeruginosa
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssRNA</td>
<td>single-stranded ribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>RNAseq</td>
<td>ribonucleic acid sequencing</td>
</tr>
<tr>
<td>S. enterica</td>
<td><em>Salmonella enterica</em></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. mitis</td>
<td><em>Streptococcus mitis</em></td>
</tr>
<tr>
<td>T4P</td>
<td>Type IV pili</td>
</tr>
<tr>
<td>V cholerae</td>
<td><em>Vibrio cholera</em></td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 Bacteriophages and their Life Cycle

In the early 1900s, Twort and d’Herelle first discovered bacteriophages (phages), the viruses that specifically infect bacteria. They are the most abundant biological entity on earth with an estimated population of $10^{31}$, outnumbering their bacterial host by approximately 10 fold. Their population is widely spread as they can be identified in all places where their bacterial hosts are found, including soil, water, plants, animals and all non-sterile sites in the human body. According to the International Committee on Taxonomy of Viruses, phages were classified into different families based on their virion morphology and form of nucleic acid. Phages virions can be binary (tailed), cubic, helical or pleomorphic. Most of the phages contain double-stranded (ds) DNA as their genetic material, while some phages have single-stranded (ss) DNA, dsRNA or ssRNA. Phages, having narrow host range and high population in a given environment, are actively involved in shaping the composition of the bio-community, genetic transfer, nutrient and energy recycling and they are an important factor in bacterial evolution.

Phages are suspected to have originated from bacterial gene clusters that escaped from cellular control resulting in a lifeform that do not have the necessary machinery to replicate themselves. In order for a phage to replicate its genome, it must infect a bacterial host. Upon infection, phages inject their genetic material into the bacterial cell, where they can go into two different lifecycles: the lytic cycle or the lysogenic cycle. According to how phages behave during infections, they can be virulent or temperate. Virulent phages only go through the lytic cycle, while temperate phages are able to survive with either life cycle. During the lytic cycle, the phage proteins are actively produced and assembled into phage virions inside the bacterial cell. Eventually, the phage particles exit the host, usually through lysis which kills the host cells. In the lysogenic life cycle, the phage genome is either maintained as a plasmid or gets integrated into the bacterial genome, forming a prophage. In this state, most of the phage genes are silenced by a repressor protein. The prophage is replicated passively when the bacterial cells divide. Although prophages can remain in the bacterial genome for numerous generations, temperate phages are able to switch from the lysogenic cycle to the lytic cycle when the bacterial cell is
under certain stress conditions, including DNA damage, changes in temperature or pH, and exposure to heavy metal or oxidative radicals.

Figure 1. The life cycle of a bacteriophage.

When a phage infects a bacterial cell it injects its genetic material into the host cell and it can replicate itself through two different life cycles: the lysogenic cycle or the lytic cycle. In the lysogenic cycle, the phage DNA gets incorporated into the bacterial genome, and it is replicated passively with the host genome through host cell divisions. In the lytic cycle, the phage genome is replicated and phage proteins are produced and assembled into phage virions. Eventually, the mature phage particles exit the host cell via lysis. Phages can switch from lysogenic to lytic cycle upon induction by cell stress, such as DNA damage.

1.2 Bacteriophages and Human Health

Although bacteriophages do not infect any other organisms except bacteria, they can exert profound effects on mammalian health indirectly through their bacterial hosts. Bacteriophages are found in any environments where bacteria reside, including inside human bodies. Up to $10^{15}$ bacteriophage particles are found in the human gut, and the phage populations in the body are sometimes referred to as the phageome. The relationship between bacteriophages, bacteria and humans is very complex, and our knowledge on the impact of phages on human health is limited. Phages are known to encode genes that modify various biological processes in the bacterial life cycle. Based on how phages infect and alter the physiology of their host, they are likely
involved significantly in the shaping of the number and diversity of bacteria in the human microbiome. In one example, studies have shown that both the phage and bacteria population in the gastrointestinal tract of healthy individual and Crohn’s disease patients were noticeably different\textsuperscript{13-16}. It is possible that differences in the phageome contribute to the difference in microbiota and the onset of the disease, but further studies are required to confirm this hypothesis.

In addition to the natural residence in human bodies, phages have been used to treat bacterial infections as an alternative to antibiotics as early as 1921\textsuperscript{17}. Recent successful use of phage therapy was reported in \textit{Pseudomonas aeruginosa} and \textit{Staphylococcus aureus} infection models\textsuperscript{18-21}, which suggests the potential of phage therapy becoming more common in the future, especially against antibiotic-resistant bacteria. Phages have also been investigated as direct treatments or vectors to deliver vaccines and anticancer agents in order to prevent or treat human diseases\textsuperscript{22-24}. There are also cases where phages were used in food safety, where they were added during food production to reduce pathogens in the food chain\textsuperscript{25,26}. Phages play important roles in bacterial and human biology, and as potential disease treatments and clinical biomarkers, their applications in human health will likely increase as our knowledge of them expands.

1.3 The Importance of Bacteriophages in Bacterial Physiology

As previously mentioned, some phages replicate themselves by infecting and lysing bacterial cells, and the number of phage exceeds the bacteria by approximately 10 fold. As a result, phage killing has been the major way of how phage affect bacterial physiology, and it is also one of the most common factors limiting bacterial population levels.

While phages are seen as parasitic to bacteria in the traditional view, the prophage exists in a symbiotic relationship with the bacterial host. There are numerous known cases where prophage genes make the bacteria more fit by enhancing its survivability or virulence. The most common way of phages contributing to bacterial virulence is the encoding of extracellular toxins within prophage genomes. Prophages were found to encode various toxins produced by both Gram-positive and Gram-negative bacteria, including \textit{C. botulinum}\textsuperscript{27}, \textit{C. diphtheria}\textsuperscript{28,29}, \textit{V. cholerae}\textsuperscript{30} and \textit{E. coli}\textsuperscript{31-33}. The phage-encoded toxins aid the bacteria in invading their targets via various mechanisms, such as inhibiting protein synthesis (e.g. shiga toxin\textsuperscript{34}) or interfering with mammalian immune-response (e.g. enterotoxins\textsuperscript{35}), and they are often the main cause of
diseases. Prophages can also encode proteins which help their host during initial bacterial infection, for example, *S. mitis* surface proteins *PblA* and *PblB* that are important for adhesion onto target cells\textsuperscript{36,37} as well as the Group A Streptococcal hyaluronidase that is essential for invasion\textsuperscript{38} are both encoded by prophages. Once the bacteria is inside the target cell, the prophage can protect the bacterial host from the human immune system via a variety of methods. Some prophages encode enzymes that alter the bacterial antigens to help evade immune-responses, such as the O-antigen acetylase (*oac*) encoded by *Shigella flexneri* Sf6 prophage, which is able to convert the serotype of its bacterial host\textsuperscript{39,40}. Other prophages encode proteins that interfere with components of the mammalian immune system. For example, the chemotaxis inhibitory protein encoded by *Staphylococci* prophage binds to neutrophil receptors and attenuates its activity\textsuperscript{41}. The *S. aureus* prophage encodes another such protein known as Panton-Valentine leucocidin, a cytotoxin that directly targets and attacks human phagocytes\textsuperscript{42}. There are also examples where a prophage protects bacterial hosts from oxidative stress generated by mammalian immune system, such as the superoxide dismutase (*SodC*) encoded by *S. enterica* Gifsy-2 prophage which neutralizes superoxide radicals in phagocytes\textsuperscript{43}. Thus, prophages contribute greatly to bacterial pathogenicity by a variety of mechanisms.

In addition to protecting their hosts during bacterial infections, prophages were also found to increase the survivability of the host in different environments. Through increasing the fitness of their hosts, the phages themselves gain benefit as more copies of the prophage are replicated when the bacterial cells divide. Therefore, the most successful phages likely express genes that enhance the fitness of their host. Prophages encode proteins that protect the bacteria from further phage infections. For example, the superinfection exclusion protein gp15 in *E. coli* is encoded by the HK97 prophage\textsuperscript{44}. Since phage killing is one of the main factors limiting bacterial population levels, blocking phage infections results in higher survivability for the bacterial host. It is believed that phages may be involved in the transduction of antibiotic-resistance genes among bacteria. Studies have shown that the rate of phage transduction of non-phage genetic elements is much higher than previously known\textsuperscript{45}, and gene sequences related to antibiotic-resistance were found in viral population in human gut and lungs\textsuperscript{46}. In addition, antibiotic treatment has been observed to increase the number of antibiotic-resistance genes detected in the phage genome\textsuperscript{47}. These findings suggest that phages may contribute indirectly to the distribution of host resistance genes against various antibiotics in both environmental and clinical bacterial strains.
Multiple prophage elements can be found integrated in a bacterial genome, averaging around 3-10% of the host genome\textsuperscript{48}. An extreme case involves the enterohemorrhagic \textit{E. coli} 0157:H7, which harbours a total of 18 prophages, making up 16% of its entire genome content\textsuperscript{49}. While single prophages can cause a significant effect on bacterial phenotypes, the interactions between multiple prophages can lead to very diverse phenotypic outcomes. Therefore, in order to understand the physiology of bacteria, it is essential to note the importance of prophages, as they contribute greatly to bacterial diversity and evolution.

### 1.4 Prophage Adaptive Genes (PrAGs)

As previously mentioned, prophages cause a wide variety of phenotypic effects in their bacterial hosts, and they are able to do so through a class of genes we call Prophage Adaptive Genes (PrAGs). Some of these genes have also been referred to as “accessory genes” or “morons”\textsuperscript{12,50}. PrAGs are phage genes that are not essential for the lytic or lysogenic phage life cycle. Since they are non-essential genes, the presence of PrAGs is not necessarily conserved among related phage strains, and their positions in the genome can be variable. These genetic elements are thought to be inserted into the genome from a foreign source via horizontal gene transfer, as many PrAGs have homologs in the bacterial genome outside of the prophage-encoded regions. For example, the \textit{Pseudomonas} phage D3-encoded O-antigen acetylase (Oac) has several bacterial homologs, including the \textit{Pseudomonas} acetyltransferases (WbpC proteins)\textsuperscript{12,51}. Since related phage genomes tend to have conserved gene orders, PrAG elements can be easily spotted through genome alignments as they are usually only present in one or a few genomes (Figure 2). PrAGs have been identified in many phage genomes, and they can be diverse in sequence and function.
Figure 2. Example of PrAGs identified in phage genomes.

Since the presence of PrAGs are not necessarily conserved, they can be easily spotted through genome alignment of closely related phages that have conserved gene orders, such as *P. aeruginosa* phages JBD30, JBD23, JBD5 and DMS3. Gene JBD30-30 (red arrow a) and DMS3-25 (red arrow b) are PrAGs identical in sequence and are found between the phage small and large terminase genes in JBD30 and DMS3, but they are not present in JBD23 or JBD5. Note that the small terminase genes share more than 90% identity and the large terminase gene share 35-99% identity in the phage genomes examined.

When expressed from a prophage, PrAG elements may modify the physiological properties of the host cell. In fact, many of the effects on bacterial virulence and survivability mentioned previously that are caused by prophages arose from the expression of PrAGs. In some examples, prophages protect their hosts from further phage infections by expressing PrAGs to inactive receptors on bacterial cell surface required for phage entry (e.g. Cor proteins expressed by *E. coli* phages HK022, N15 and ϕ80 inactivate FhuA receptors), or through other mechanisms (e.g. PrAG gp15 from *E. coli* phage HK97 was hypothesized to block the formation of the phage entry complex in the bacterial cell membrane as it has been shown to be responsible for the superinfection exclusion phenotype observed in the lysogen). In other examples, PrAGs help their bacterial host in invading mammalian cells (e.g. PrAG-expressed λ Lom protein in *E. coli* and SopE protein expressed by *Salmonella* phages) or surviving the mammalian immune system (e.g. Bor protein expressed from λ PrAG protect the host from serum killing). The previously described bacterial toxins encoded by prophages are also PrAGs, including cholera, diphtheria, botulism and shiga toxins, which directly enhance the virulence of the bacterial hosts. Although a number of PrAGs have been shown to be of great importance in bacterial biology, the biological roles of most PrAGs are still unknown.
1.5 Pseudomonas aeruginosa – An Opportunistic Human Pathogen

*Pseudomonas aeruginosa* is a ubiquitous, gram-negative bacterium that can be found in diverse environments, including soil, water and inside animal hosts. *P. aeruginosa* and its phages are great model organisms for the studies of prophages, PrAGs, and their effects in bacterial pathogenesis, because this bacterium is well-studied with many fully-sequenced bacterial and phage strains. *P. aeruginosa* is also known to produce a large number of virulence factors, most of which are well-characterized with techniques and assays available for related studies. In addition, *P. aeruginosa* infects a variety of hosts, including several common model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, which can be used to measure the virulence level of *P. aeruginosa* with infection models.

1.5.1 *P. aeruginosa* and Human Health

*P. aeruginosa* is one of the most common opportunistic human pathogens. It is able to infect humans who are immuno-compromised or elderly, as well as patients with burns, lung diseases, open fractures or other injuries. *P. aeruginosa* infections often lead to complication of disease states or secondary fungal infections, and in some cases can result in the death of the patient.

This bacterium especially causes serious issues among cystic fibrosis patients. Cystic fibrosis (CF) is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, resulting in non-functional chloride ion channel important for the production of sweat, digestive fluids and mucus. The disease mainly affects the lungs, but it can also affect intestines, liver, kidneys and pancreas. *P. aeruginosa* can worsen CF conditions, since it is commonly found in the lungs of the patients, where it can cause both acute and chronic infections in the respiratory systems. *P. aeruginosa* infections among CF patients can be severe and persistent as the bacterial strains often possess high resistance to multiple antibiotics. In addition, the ability of the bacteria to grow in various conditions significantly increases the occurrence of new infections and outbreaks in hospitals. Because of its infectious and highly drug-resistant nature, *P. aeruginosa* is a huge concern for public health, and many studies have been done in the hope of developing effective treatments against these infections.
1.5.2 Virulence Factors of *P. aeruginosa*

*P. aeruginosa* expresses various virulence factors, some of which help the bacteria invade a wide spectrum of hosts, including mammals, plants and insects, while others enable the bacteria to survive environmental stresses, such as antibiotic treatment and mammalian immune response. The expression of virulence factors in *P. aeruginosa* is controlled by the quorum sensing system\(^6^6\). The quorum sensing system is an intercellular communication network that is responsible for the regulation of bacterial virulence based on the cell density in the surrounding environment\(^6^6\). This study focused on investigating the phenotypic effects of PrAGs on three *P. aeruginosa* virulence factors: bacterial motility, production of biofilm and pyocyanin.

1.5.2.1 Bacterial Motility

In *P. aeruginosa*, motility is mediated by two cell surface structures known as the Type IV pilus (T4P) and the flagellum. The T4P is responsible for twitching motility, which describes the ability of the bacteria to move across solid surfaces. The main T4P structure is a polymer of Type IV pilins (PilA), and it is connected to an ATPase motor (PilT, PilB and PilU) associated with the inner cell membrane\(^6^7,6^8\). The ATPase motor enables pilus retraction motion that moves the bacteria on surfaces\(^6^9\). The flagellum is involved in bacterial movement in liquid environment, which is termed swimming motility. The flagellum is a long helical filament made of thousands of flagellins (FliC), and the swimming motility is achieved by reversibly rotating the flagellum driven by membrane rotor proteins (MotA and MotB)\(^7^0\). The expressions of both the T4P and the flagellum are regulated by the quorum sensing system in *P. aeruginosa*.

Twitching and swimming motility is important in *P. aeruginosa* virulence, because it allows the bacterial cells to be motile, meaning that the bacteria can more easily gain access to nutrients and host cells during infection. Bacterial pili and flagella are also involved in adhesion\(^7^1\), biofilm and microcolony formation\(^7^2\), which protect the bacteria from external threats, such as anti-microbial agents. In addition, since these surface structures are often targets for the host immune system and phage attachment, through down-regulation of gene expression or alteration of their structures, the bacteria can use pilus and flagellum as a method for immune escape and phage resistance\(^7^3\). Indeed, studies have shown that there are significant differences in pilin alleles between *P. aeruginosa* isolates from CF and non-CF patients\(^7^4\), suggesting that bacteria possesses different pilin types that may be advantageous in different situations.
1.5.2.2 Biofilm

Biofilm is an extracellular polymeric substance (EPS) matrix produced by *P. aeruginosa* and many other bacteria to facilitate growth in a protected environment. The EPS matrix is composed of polysaccharides, extracellular DNA and proteins\(^ {75,76}\). They function to hold the bacterial cells together, forming a microcolony. The production of biofilm in *P. aeruginosa* is mainly regulated by the quorum sensing system\(^ {77}\), but there are also reports of the involvement of T4P and flagellum in biofilm development and maturation\(^ {78}\). As an important virulence factor, biofilm protects the bacteria from a wide variety of environmental stressors, including antibiotics and the host immune responses\(^ {79,80}\). The biofilm also promotes genetic exchanges between bacterial cells\(^ {81}\) and aids in the acquisition of nutrients by concentrating cell densities. Due to the nature and function of the biofilm, it plays a key role in the establishment of chronic infections in CF patient lungs\(^ {82}\).

1.5.2.3 Pyocyanin

Pyocyanin is a redox-active secondary metabolite produced by *P. aeruginosa*, and it is also a blue color pigment that gives the bacterium its unique bluish-green color. Pyocyanin is important for the bacteria as it is known to be involved in various biological processes, for example, it participates in the reduction and release of iron from transferrin in bacterial iron metabolism, and it acts as an electron shuttle during bacterial respiration\(^ {83}\). The biosynthesis of pyocyanin is mainly mediated by the *phzABCDEFG* operons and extra genes, and the expression of these genes are regulated by the quorum sensing network involving multiple regulatory pathways\(^ {84,85}\). As a virulence factor and a bacterial toxin, pyocyanin contributes greatly during *P. aeruginosa* infections; it has been recovered from CF patient sputum in large quantities and was shown to be crucial for lung infection in mice\(^ {84,86}\). Pyocyanin generates reactive oxygen intermediates, creating oxidative stress inside target cells, which results in target cell death\(^ {84}\). In the mammalian system, pyocyanin is known to cause multiple cellular damages, including inhibition of cell respiration, interference with ciliary function\(^ {87}\) and disruption of calcium homeostasis\(^ {88}\). In addition to its role in bacterial infections, pyocyanin has anti-bacterial and anti-fungal activities\(^ {83,89,90}\), which provides advantages for *P. aeruginosa* when competing with other microorganisms.
1.6 *P. aeruginosa* Prophages and PrAGs

Although there are numerous examples of prophages involved in the modification of bacterial phenotypes, there have been no systematic studies performed. Joe Bondy-Denomy, a former student in the Davidson lab, conducted a systematic study of prophages in *P. aeruginosa*, where he created lysogens, each with a different phage in the same bacterial strain background. He observed differences in various bacterial phenotypes related to the virulence and survivability of *P. aeruginosa*, in the presence compared to the absence of prophages. For example, when Joe inserted phage JBD26 into *P. aeruginosa* strain PA14, the bacteria gained increased resistance against several phages, including JBD24, JBD23 and JBD5. JBD26 and JBD16C lysogens were also observed to cause defects in twitching and swimming motility, respectively. Even the insertions of closely related phages in the host genome could result in different host phenotypes. For example, phages JBD26 and JBD30 have very similar genomic composition, however, their lysogens display significantly different phage resistance patterns. Since related phages have conserved gene orders, Joe was interested in pinpointing the cause of phenotypic modifications through comparisons of the phage genomes. Because of the high sequence identity conserved between these phages, Joe was able to identify a number of PrAGs that were not conserved between strains, and thus were likely responsible for the host phenotypic changes. Through studies of PrAGs and their effects on bacterial physiology, we hope to gain insight into *P. aeruginosa* infections and the development of successful therapies.

1.7 Thesis Objectives

My thesis project aims to determine the specific genes responsible for the phenotypic changes arising from prophage integration. I conducted a systematic study of PrAGs cloned from the collection of JBD phages and characterized the effects of their expression on a variety of host cell phenotypes, including: growth, resistance to phage infection and antibiotics, twitching and swimming motility, biofilm production, pyocyanin production, and virulence in a *Drosophila melanogaster* model of disease.

As the expression of phage genes and the movement of phages between bacterial strains are common, they can cause variability in bacterial infections and make them difficult to treat. Depending on the phages present in a given clinical isolate, different types of treatments may be most effective. A catalog of PrAG phenotypes and other knowledge gained from my work will
help in understanding the contribution of phages to bacterial pathogenesis, and form the basis of future research for personalized treatment against *P. aeruginosa* infections.
Chapter 2
Materials and Methods

2.1 Construction of Expression Plasmids and Protein Test Expression in E. coli

The 17 PrAGs were cloned into p15TV-L E. coli expression plasmids, expressed under the control of the T7 promoter and tested for protein expression by Diane Bona. In brief, the gene coding sequences were amplified from PA14 lysogens using pfX DNA polymerase (Invitrogen) in polymerase chain reactions (PCR) and inserted into p15TV-L plasmids with In-Fusion HD EcoDry™ Mix (Clontech) following the product protocol. The ligation mixtures were then transformation into E. coli calcium chloride competent DH5α cells. The colonies obtained were PCR screened for the correct size gene insert. Positive plasmids were purified using Qiagen technologies and the correct gene insert was confirmed by DNA sequencing.

Protein test expression was performed to confirm the ability of the PrAGs to produce proteins in E. coli strain BL21. Overnight cultures grown in LB media were sub-cultured 3 in 100 into fresh LB media and grown at 37°C shaking until OD$_{600}$ = 1. 0.8 mM IPTG was then added to the culture to induce protein expression at 37°C for 3-4 hours. To assess solubility, 1 mL of induced culture was spun down and resuspended in 200μL BugBuster Protein Extraction Reagent (Novagen). The samples were incubated at room temperature for 30 minutes, and the cell debris was collected by centrifugation. 50 μL of supernatant was mixed with 50 μL of 2x SDS-PAGE loading buffer to prepare SDS-PAGE samples. For the whole cell fractions, 500μL of induced culture was spun down and resuspended in 200μL 2x SDS-PAGE loading buffer. All SDS-PAGE samples were boiled for 5 minutes at 100°C before being loaded on to the SDS-PAGE gel. The expressions of the PrAG proteins were confirmed if protein bands of the expected size were observed on the coomassie brilliant blue-stained SDS-PAGE gels.

2.2 Transformation of plasmids into P. aeruginosa

The 17 PrAG constructs were obtained from Joe Bondy-Denomy. The constructs have each of the 17 PrAGs cloned into the pHerd30T expression plasmids individually. The expressions of the PrAGs were under the control of a pBad promoter, which can be turned on by the addition of arabinose. The plasmids were transformed into P. aeruginosa PA14 or PAO1 strains through
electroporation. To create electro-competent *P. aeruginosa* cells, overnight culture grown in LB was spin down and washed twice with 300 mM sucrose, and then resuspended in 1/10th the initial volume of 300 mM sucrose solution. Fresh made electro-competent cells were placed on ice for 5 minutes before the addition of 1 μL plasmids into 100 μL of cells. The mixture was incubated on ice for 20 minutes, followed by electroporation at 2500 V. The electroporated cells were immediately transferred into 900 μL LB and incubated at 37°C, with shaking for 1 hour. The cells were spin down and plated on LB + gentamycin (50 μM/mL) plates. After overnight 37°C incubation, any colonies grown on the plate could contain the desired plasmids, and were further confirmed using PCR colony screens and sequencing.

### 2.3 PCR Reactions and Colony Screening

Bacterial colonies potentially containing the desired plasmids were screened using PCR before sequencing. PCR reactions were set-up with 1x Taq buffer with ammonium sulphate (from 10x Thermo Scientific buffer stock), 3.5 mM magnesium chloride, 2.5 U Taq DNA polymerase, 0.2 mM dNTPs, 1x PCR Enhancer solution and 2 μM of appropriate primers, and inoculated with bacteria colonies. pBad forward and M13 reverse primers were used in all colony PCR reactions with pHerd30T constructs, and the T7 promoter forward and reverse primers were used in colony PCR reactions with p15TV-L constructs. The PCR program used consisted of 94°C for 2 minutes, 30 cycles of: 30 seconds at 94°C, 30 seconds at 50°C, 1 minute at 68°C, followed by 72°C for 10 minutes. The sizes of amplified PCR fragments were determined by running on standard DNA gels.

### 2.4 Bacterial Growth

The growth rates of different strains of bacteria were determined by monitoring cell densities at multiple time points through growth. Overnight cultures were diluted 1:100 into LB + Gentamycin (50 μM/mL) + 0.1% (w/v) arabinose. 200 μL of diluted cultures were grown in 96-well plates in Infinite F200 microplate reader (Tecan) with shaking at 37°C for 8 hours. The growth curves were generated by measuring cell density at OD$_{595}$ every 15 minutes during growth.
2.5 Twitching Motility Assay

Twitching motility assay was used to assess the ability of the bacteria to move on solid media. Thin plates containing LB broth + 1% (w/v) agar were prepared on the same day of the assay. Gentamycin (50 μM/mL) and 0.1% (w/v) arabinose were added to the media prior to plate pouring. Freshly poured plates were solidified in the biosafety cabinet with lids off for 20 minutes. Plates were inoculated by stabbing isolated colonies grown on overnight LB plates to the bottom of the twitch plate, and the plates were incubated for 24 hours at 37°C. The agar was carefully removed from the plates with a spatula, and the cells adhered to the plates were stained with 1% crystal violet for 1 minute followed by 3 washes with water. The plates were dried inverted overnight. The distance traveled by the bacteria was then determined by measuring the diameter of the stain.

The twitching motility assay was performed with 3 technical replicates in each experiment, and the experiments were repeated 3 separate times (biological replicates). The measurements from all experiments were analyzed using a T-test, and the values were considered significantly different from the empty vector control if P-value < 0.005.

2.6 Swimming Motility Assay

To study the movement of the bacteria in liquid media, swimming motility assays were performed. The media used in this assay contained: 0.3% (w/v) agar, 1% (w/v) tryptone and 0.5% (w/v) NaCl. Thin plates were prepared on the same day of the assay. Gentamycin (50 μM/mL) and 0.1% (w/v) arabinose were added to the media prior to plate pouring. Freshly poured plates were solidified in the biosafety cabinet with lids off for 20 minutes. Plates were inoculated by stabbing isolated colonies grown on overnight LB plates to the bottom of the swimming plate, and the plates were incubated for 24 hours at 37°C. The diameter of the area traveled by the bacteria was then measured.

The swimming motility assay was performed with 3 technical replicates in each experiment, and the experiments were repeated 3 separate times (biological replicates). The measurements from all experiments were analyzed using a T-test, and the values were considered significantly different from the empty vector control if P-value < 0.005.
2.7 Induction of *P. aeruginosa* Lysogens and Plaquing Assays

*P. aeruginosa* lysogens were grown overnight in LB media at 37°C. A few drops of chloroform were added to the overnight culture, and cells were shaken for 30 minutes at 37°C. Cell debris was collected by centrifugation and the supernatant containing phages was collected and stored at 4°C.

The sensitivities of bacteria to various phages were assessed using the spotting assay. 150 μL of overnight bacteria culture was mixed with 3 mL top agar (LB broth + 0.7% (w/v) agar) and poured on top of thick 1.5% LB agar plates. 2 μL serial dilutions of freshly prepared *P. aeruginosa* phage lysates were spotted on the surface of the plates. The plates were incubated inverted at 37°C overnight. The following day, the phage titres were calculated by noting the highest dilution where plaques were observed. The phage titres were compared between different bacterial strains, and the fold changes in phage sensitivities were recorded.

2.8 Drug Resistance Assay

150 μL of overnight culture grown in LB media was mixed with top agar (LB broth + 0.7% (w/v) agar) and poured on top of thick LB plates (LB broth + 1.5% (w/v) agar + 50 μM/mL gentamycin + 0.1% (w/v) arabinose). Circular drug-containing discs (Sensi-Disc, BD) were placed on top of the dried plates, and the plates were incubated at 37°C overnight. The drug sensitivity of the bacteria was assessed by measuring the diameters of clear zones around the drug discs.

2.9 Biofilm Production Assay

The assay protocol was adapted from O’Toole (2011). Overnight bacterial culture grown in LB medium was diluted 1:100 into M63 minimum medium (3g monobasic potassium phosphate + 7g dibasic potassium phosphate + 2g ammonium sulphate dissolved in water into 1L solution) supplemented with 1mM magnesium sulphate, 0.2% (w/v) glucose and 0.5% (w/v) casamino acids. 100 μL of diluted culture was distributed into 96-well vinyl microtitre plates (Corning) and incubated at 37°C for 24 hours without shaking. After incubation, the bacterial culture was removed, the plates were washed 3 times with water and air dried. The wells were stained with 100 μL 0.1% crystal violet solution for 15 minutes at room temperature, followed by 3 washes with water to get rid of excess stain. The plates were again air dried and the dye was dissolved in
125 μL 30% acetic acid for 15 minutes at room temperature. The dissolved dye was transferred to Infinite F200 microplate reader (Tecan)-compatible 96-well plates, and the levels of biofilm produced were determined through quantifying the amount of dye present in the wells by measuring the OD at 595nm.

The biofilm production assay was performed with 3 technical replicates in each experiment, and the experiments were repeated 3 times at different occasions (biological replicates). The measurements from all experiments were analyzed using T-test, and the values were considered significantly different from the empty vector control if P-value < 0.05.

2.10 Virulence Assay

The fly feeding virulence assay was used to determine the virulence of different P. aeruginosa strains. This assay models the chronic P. aeruginosa infection in cystic fibrosis patient lungs. The assay protocol was modified from Shen et al. (2012)\textsuperscript{57}. In preparation for the fly feeding experiment, bacteria were inoculated in LB and grown overnight at 37°C. 5 mL of molten sucrose agar solution (5% (w/v) sucrose + 2.5% (w/v) agar) was poured into polystyrene Drosophila vials (VWR) and let solidify overnight. The following day, bacterial cultures were spun down, resuspended in 100μL 5% sucrose, and spotted onto sterile filter paper (Whatman GF/A, 21 mm) on top of solidified sucrose agar in the fly vials. The volume of bacterial culture used varied according to the cell density of different strains so that similar numbers of cells were distributed in each vial. The vials were incubated at 37°C for 30 minutes, followed by 25°C for another 30 minutes. Twenty pre-starved 3-5 day old male W1118 Drosophila melanogaster flies were transferred into each vial using carbon dioxide pads. The vials were placed at 25°C humidity-controlled dark environment, and the number of dead flies were recorded every 24 hours. Each experiment was performed with 100 flies (5 vials with 20 flies each), and the assay includes at least 3 individual experiments.
Chapter 3
Results

3.1 Acknowledgement

A number of experiments in the study was performed with the help of Diane Bona and Smriti Kala as outlined below:

(1) The cloning and protein test expression of PrAGs in *E. coli* was performed by Diane Bona.

(2) The drug resistance assay was conducted by Diane Bona and Smriti Kala.

(3) The virulence assay (fly feeding assay) was performed with the help of Diane Bona and Smriti Kala.

3.2 *P. aeruginosa* PrAGs possess diverse genomic and bioinformatics properties

The PrAGs identified in *P. aeruginosa* phages were distributed throughout the entire phage genomes (Figure 3). They were commonly found in the transposase, gam, terminase and tail regions of the Mu-like phage genome (Figure 3A and B), and between the DNA helicase and protein NinG in phage JBD44 (Figure 3C). Some of the PrAGs had homologs in several phages, while some were unique and only present in one phage genome. Each of the 17 PrAGs I studied are small in terms of gene sizes, and they produce proteins of less than 300 amino acids (Table 1). PrAGs are diverse and highly mobile genetic elements, they can frequently be transferred between bacterial and phage genomes, even among different bacterial species. Some of the PrAGs I studied have over 100 homologs found in a wide variety of Gram-negative bacterial strains (Table 1). Within the *P. aeruginosa* phages I examined, the percent identity among PrAG proteins range anywhere from 47% to 100% (Table 1). The genomic positions, sequence identities and numbers of homologs suggests that PrAGs plays important roles in the life cycle of *P. aeruginosa* and their presence is greatly influenced by evolution.
Figure 3. Genome comparison between related phage strains and positions of PrAGs.

The genome alignment of (A and B) Mu-like phages: JBD26, JBD30, JBD23, JBD5 and JBD24 and (C) phages JBD44 and JBD25. Genomes of close related *P. aeruginosa* phages were aligned based on sequence similarities and gene functions. The light grey arrows represent genes of known functions, and the dark grey arrows represent hypothetical genes. The putative PrAGs were colored differently according to their homologies. The PrAGs studied in this project were numbered.
Table 1. Summary of PrAGs included in this study.

<table>
<thead>
<tr>
<th>PrAG Number</th>
<th>PrAG</th>
<th>GenBank #</th>
<th>Protein Length (amino acids)</th>
<th>Protein Size (kDa)</th>
<th>% Identity range for homologues examined</th>
<th>Number of homologs by BLAST*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JBD30-4</td>
<td>AFQ21918.1</td>
<td>108</td>
<td>12.5</td>
<td>97-98%</td>
<td>4</td>
</tr>
<tr>
<td>2A</td>
<td>JBD26-5</td>
<td>AGC24045.1</td>
<td>136</td>
<td>14.4</td>
<td>47-94%</td>
<td>16</td>
</tr>
<tr>
<td>2B</td>
<td>JBD5-4</td>
<td>AFQ21801.1</td>
<td>69</td>
<td>7.6</td>
<td>47-94%</td>
<td>16</td>
</tr>
<tr>
<td>2C</td>
<td>JBD24-4</td>
<td>AFQ21860.1</td>
<td>143</td>
<td>15.1</td>
<td>47-94%</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>JBD30-9</td>
<td>AFQ21923.1</td>
<td>211</td>
<td>23.6</td>
<td>95-100%</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>JBD23-13</td>
<td>N/A</td>
<td>125</td>
<td>13.6</td>
<td>97%</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>JBD26-14</td>
<td>AEY99459.1</td>
<td>93</td>
<td>10.0</td>
<td>77-100%</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>JBD30-14</td>
<td>AFQ21928.1</td>
<td>67</td>
<td>7.5</td>
<td>N/A</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>JBD26-15</td>
<td>AEY99428.1</td>
<td>102</td>
<td>11.2</td>
<td>100%</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>JBD5-15</td>
<td>AFQ21812.1</td>
<td>73</td>
<td>8.1</td>
<td>N/A</td>
<td>33</td>
</tr>
<tr>
<td>9</td>
<td>JBD24-17</td>
<td>AFQ21873.1</td>
<td>223</td>
<td>23.9</td>
<td>N/A</td>
<td>&gt;100</td>
</tr>
<tr>
<td>10</td>
<td>JBD26-30</td>
<td>AEY99449.1</td>
<td>83</td>
<td>9.0</td>
<td>100%</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>JBD26-31</td>
<td>AEY99485.1</td>
<td>83</td>
<td>9.3</td>
<td>100%</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>JBD30-30</td>
<td>AFQ21944.1</td>
<td>164</td>
<td>18.3</td>
<td>100%</td>
<td>&gt;100</td>
</tr>
<tr>
<td>13</td>
<td>JBD26-61</td>
<td>AEY99477.1</td>
<td>74</td>
<td>8.5</td>
<td>85-99%</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>JBD44-8</td>
<td>N/A</td>
<td>52</td>
<td>5.7</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>JBD44-9</td>
<td>N/A</td>
<td>77</td>
<td>8.6</td>
<td>N/A</td>
<td>1</td>
</tr>
</tbody>
</table>

*Proteins with BLAST E-value lower than 10^{-4} are considered homologs.

3.3 The PrAGs all express and make proteins in *E. coli*

Since the PrAGs that I am screening were mostly identified through genome comparison and bioinformatics, it is important to determine if these open reading frames (ORFs) actually encode proteins. All 17 PrAGs were cloned into *Escherichia coli* expression vectors and most of them were shown to express and produce proteins (Figure 4). JBD30-9 may be expressed at a low level, which could not be captured by the Coomassie blue dye.
The PrAGs were cloned into p15TV-L expression vectors and induced in *E. coli* BL21 strain. Protein expression of individual PrAGs was examined in whole cell lysate (w) and soluble fraction (s) on Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. All PrAG protein bands observed are close to the expected sizes (Refer to Table 1 for PrAG protein sizes). Note that JBD26-61 is known to proteolyze at a fast rate, resulting in two protein bands observed in the soluble fraction.
3.4 Some PrAGs introduce growth defects in the bacterial host

To determine if the expression of any individual PrAG affects the growth of *P. aeruginosa*, I examined the growth rates of each of the strains expressing a PrAG from a plasmid. *P. aeruginosa* PA14 and PAO1 strains overexpressing individual PrAGs of interest were grown in LB medium at 37°C for 8 hours, and cell density was monitored at OD$_{595}$ in the TECAN microplate reader during growth. While most PrAGs have little effects on host growth, four PrAGs, JBD26-15 and JBD30-14 in PA14 and JBD44-8 and JBD5-4 in PAO1, slowed bacterial cell growth (Figure 5). The four PrAGs have varying levels of effects on bacterial growth. At the eighth hour, the cell densities of bacterial strains expressing JBD30-14 and JBD5-4 were 19% and 17% lower than strains expressing no PrAGs (empty vector control), respectively, which is mild compared to the effects of JBD44-8 and JBD26-15. When JBD44-8 was expressed in PAO1, the bacterial cell density was more than 30% lower than PAO1 empty vector control at the 8-hour mark (Figure 5D). The expression of JBD26-15 in PA14 strain causes the most serious growth defect among all the PrAGs examined. After 8 hours of growth, the cell density of PA14 strain expressing JBD26-15 was less than 45% of the empty vector control strain, and it reached the bacterial growth log phase after 6 hours of growth, which was approximately 4 hours later than the control strain (Figure 5B). These bacterial growth experiments demonstrated that PrAGs are able to influence the growth rate of bacterial hosts. It is unclear if the growth defects affected other phenotypic screens performed, but it is important to take them into account during analysis and interpretation.
A.

B.
Figure 5. PrAG expression affect bacterial growth rate.

Different PrAGs were expressed in (A and B) PA14 or (C and D) PAO1 \textit{P. aeruginosa} strains, and the cell density of the growing liquid culture was monitored every 15 minutes for 8 hours. Most of the PrAGs had little effects on bacterial growth rate (A and C), while some slowed the growth of the host (B and D).
3.5 PrAGs increase bacterial survivability by protecting their hosts from phage infection

As prophages and their bacterial hosts exist in a symbiotic relationship, the survivability of the bacteria is important to both. The longer the lysogen lives and replicates, the more copies of the phage genome there will be. Since phage infection and killing is one of the most common factors in limiting the sizes of bacteria populations, resistance against further phage infection can therefore provide a great advantage in bacterial survivability. Thus, I examined the effects of PrAG expression on bacterial resistance against a panel of *P. aeruginosa* phages. I spotted serial dilutions of 31 different phages atop lawns of PA14 and 18 phages on lawns of PAO1 expressing individual PrAGs. I found five PrAGs in PA14 (Table 2) and six PrAGs in PAO1 (Table 3) that increased the bacterial resistance against several phages. JBD30-30, JBD26-5, JBD26-31, JBD26-61 and JBD24-4 all caused increased phage resistance in both PA14 and PAO1, while JBD44-8 only had an effect in PAO1. The levels and patterns of phage resistance caused by PrAGs differ from PrAG to PrAG even within the same bacterial strain. For example, the expression of JBD26-61 and JBD24-4 led to increased resistance against more phages than the expression of JBD30-30, JBD26-5 and JBD26-31 in PA14 (Table 2). In addition, JBD44-8 only caused a slight increase in phage resistance in PAO1, while JBD24-4 was able to protect its host completely from infections by most phages in our collection (Table 3). Interestingly, the expression of JBD26-61 and JBD24-4 resulted in identical phage resistance patterns, suggesting that they may be modifying the host phenotypes via similar mechanisms. Since phage infection largely depend on bacterial cell surface structures – T4P and O-antigen, as they serve as common phage binding sites during initial adsorption, the data of phage spotting on PA14 knockouts of T4P (*pilA*) and O-antigen (*wbpL*) adapted from study done by Bondy-Denomy\textsuperscript{81} is also included for clarity (Table 2).
Table 2. Increase in phage resistance caused by PrAG expression in PA14 compared to no PrAG expression.

<table>
<thead>
<tr>
<th>PA14 Knockout*</th>
<th>Phage</th>
<th>PrAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>wbpL</td>
<td>pilA</td>
<td>JBD30-30</td>
</tr>
<tr>
<td>JBD16C</td>
<td>JBD63</td>
<td>JBD5</td>
</tr>
<tr>
<td>JBD33</td>
<td>JBD35C</td>
<td>JBD59a</td>
</tr>
<tr>
<td>JBD95b</td>
<td>JBD86</td>
<td>JBD63c</td>
</tr>
<tr>
<td>JBD30</td>
<td>JBD88a</td>
<td>JBD88b</td>
</tr>
<tr>
<td>JBD44a</td>
<td>JBD62b</td>
<td>JBD69a</td>
</tr>
</tbody>
</table>

*Data adapted from study done by Bondy-Denomy. Only complete resistance is shown.
**JBD10 infects PA14 poorly.

Legend

<table>
<thead>
<tr>
<th>Resistance Level</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>Light grey</td>
</tr>
<tr>
<td>Slightly resistant (&gt;100-fold)</td>
<td>Orange</td>
</tr>
<tr>
<td>Intermediately resistant (&gt;10^4-fold)</td>
<td>Dark orange</td>
</tr>
<tr>
<td>Highly resistant (&gt;10^6-fold)</td>
<td>Red</td>
</tr>
<tr>
<td>Complete resistant</td>
<td>Black</td>
</tr>
</tbody>
</table>
Table 3. Fold increase in phage resistance caused by PrAG expression in PAO1 compared to no PrAG expression.

<table>
<thead>
<tr>
<th>Phage</th>
<th>JBD30-30</th>
<th>JBD44-8</th>
<th>JBD26-5</th>
<th>JBD26-31</th>
<th>JBD26-61</th>
<th>JBD24-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBD16C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD35C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD59a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD60a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD95b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD63c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD93a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD88a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD88b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD44a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD62b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD69a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD70a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBD80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend**

- **Sensitive**
- **Slightly resistant** (>100-fold)
- **Intermediately resistant** (>10^4-fold)
- **Highly resistant** (>10^6-fold)
- **Complete resistant**
- **Phages that do not infect wild-type PAO1**
Some PrAGs were found to have different effects on phage resistance in different *P. aeruginosa* strains. I compared phage resistance caused by PrAGs in PA14 and PAO1 and found that in most cases where PrAGs caused increased phage resistance, it was observed in both strain backgrounds tested. However, there were some examples of resistance only seen in PAO1 (Table 4). Among the phages that are able to infect both PAO1 and PA14 strains, the only case of a PrAG affecting phage resistance only in PA14 is the case of JBD26-31, which only causes resistance in PA14 against phage JBD60a (Table 4). I have also noted that in PAO1 three PrAGs that caused complete resistance against all the phages I tested: JBD26-5, JBD26-61 and JBD24-4. However, these PrAGs did not cause complete resistance in PA14 (Table 2 and 3). The results from the phage resistance screens suggests that multiple PrAGs function to increase the resistance of the bacteria against various phages. As the patterns of resistance are unique for each PrAG, they are likely acting through different mechanisms, or interacting with different host components in the bacterial cell.
Another major factor determining the survivability of the bacteria is how well the bacteria can survive and replicate in the presence of antibiotics. The ability to withstand antibiotics increases the chance of successful infection and propagation of the bacteria inside mammalian systems.

We examined 9 antibiotics commonly used to treat bacterial infections (Table 5) using the disk diffusion method and determined that none of the 17 PrAGs had effects on drug resistance.
Table 5. List of drugs tested in drug resistance assay.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Amount (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>15</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>75</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>30</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>75</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>100</td>
</tr>
<tr>
<td>Colistin</td>
<td>10</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>30</td>
</tr>
</tbody>
</table>

3.6 Bacterial motility is often modified by PrAGs

Bacterial motility is considered a virulence factor for *P. aeruginosa* as the motility of the bacterial facilitates invasive infection of their mammalian hosts.\(^{71,73}\) I performed two motility screens to study the ability of the bacteria to travel in different environments when expressing PrAGs of interest. Twitching motility describes the motility of the bacteria on solid media. Through the twitching motility screen, I found that more than half of the PrAGs I screened decrease bacterial twitching. JBD26-15, JBD23-13, JBD44-8, JBD26-5, JBD26-61, JBD5-4 and JBD24-4 cause twitching defects in both PA14 and PAO1 strains, and JBD30-4 and JBD24-17 only affects PAO1 (Figure 6). Within the same *P. aeruginosa* strain, different PrAGs modified twitching motility to different degrees. JBD26-5, JBD26-61, JBD5-4 and JBD24-4 completely abolished twitching motility, comparable to the control where the pili was knocked out, while the other PrAGs only cause mild to moderate defects (Figure 6). Given the differences in degree of modification within and between bacterial strains, it is possible that the PrAGs are able to work through several different mechanisms to alter bacterial motility.
Figure 6. Some PrAGs affect twitching motility of *P. aeruginosa*.

Twitching motility of *P. aeruginosa* (A) PA14 and (B) PAO1 strains over-expressing different PrAGs were measured as diameter traveled by the bacteria over 24 hr on solid medium. *P. aeruginosa* strains with no PrAG expressed (empty vector) and Type IV pilus knockout control (*pilA* knockout) were also included. T-test was used to determine statistically significant results compared to empty vector control and they are marked with asterisks (P<0.005), error bars represent the standard deviation of sample groups.
In addition to twitching motility, I examined the effects of PrAG expression on bacterial swimming motility, which describes the ability of bacteria to move in liquid media. I performed the swimming motility assay using semi-solid medium to mimic liquid environments, which allow the measurement of distance traveled by the bacteria. Three PrAGs caused swimming defects in both PA14 and PAO1, including JBD30-4, JBD26-15 and JBD44-8, while JBD23-13 only has an effect in PAO1 (Figure 7). Similar to the twitching motility screen, the PrAGs altered bacterial swimming motility to different degrees. However, the effects of PrAGs on swimming motility were mostly mild to moderate, and no PrAGs were able to cause swimming defects as serious as the flagellum knockout negative control (Figure 7).

Using the bacterial motility screens, I found a number of PrAGs can modify the ability of the bacteria to travel on solid or in semi-solid medium, and some can affect both.
Figure 7. PrAGs could alter swimming motility of the bacteria.

Swimming motility of *P. aeruginosa* (A) PA14 and (B) PAO1 strains over-expressing different PrAGs were measured as diameter traveled from central point by the bacteria over 24 hr in low percentage medium. *P. aeruginosa* strains with no PrAG expressed (empty vector) and flagellum knockout control (*fliC* knockout) were also included. T-test was used to determine statistically significant results compared to empty vector control and they are marked with asterisks (P<0.005), error bars represent the standard deviation of sample groups.
3.7 PrAGs are capable of altering the production of bacterial virulence factors

*P. aeruginosa* produces a number of virulence factors to aid in the invasion of mammalian systems, which include biofilm and pyocyanin. Biofilm mediates bacterial attachment to target mammalian cells and it can also help the bacteria in evading mammalian immune systems and antibiotics\(^79,80\). To investigate if PrAG expression alters the level of biofilm produced by the bacterial host, I measured the biofilm production of PA14 and PAO1 strains expressing different PrAGs. I found seven PrAGs affected the amount of biofilm produced by *P. aeruginosa*. JBD23-13, JBD26-61, JBD5-4 and JBD24-4 in PA14 and JBD30-4, JBD26-15, JBD26-5 and JBD26-61 in PAO1 decreased bacterial biofilm production (Figure 8). The only case where PrAG increased level of biofilm production by the host is when expressing JBD23-13 in PAO1 (Figure 8B). Interestingly, JBD23-13 has an opposite effect when expressed in PA14 where it decreased biofilm production (Figure 8A). JBD26-61 is the only PrAG that behaved similarly in both PA14 and PAO1 strains, where it was able to lower the production of biofilm to the level comparable with the pili and flagella knockout controls (*pilA* and *fliC* knockout, Figure 8). One thing to note here is that PAO1 strains seem to grow better in the minimal media I used for this particular assay, therefore they have overall higher biofilm production than PA14 strains. The results of the experiments show that PrAGs are able to either increase or decrease the production of biofilm by their hosts, and the effects can vary depending on the bacterial strain.
Figure 8. PrAGs cause changes in bacterial biofilm production.

The level of biofilm production for *P. aeruginosa* (A) PA14 and (B) PAO1 strains over-expressing different PrAGs were measured. *P. aeruginosa* strains with no PrAG expressed (empty vector) serves as wild-type control. As controls, Type IV pilus and flagellum knockouts (*pilA* and *fliC* knockout) were included. T-test was used to determine statistically significant results compared to empty vector control and they are marked with asterisks (P<0.05), error bars represent the standard deviation of sample groups.

Pyocyanin produced by *P. aeruginosa* is a toxin as well as a colour pigment that is responsible for the greenish blue color of the bacteria. The amount of pyocyanin produced by the bacteria is
dependent on many factors, including the growth conditions and quorum sensing systems. However, because of the color of pyocyanin, a significant change in the production level can be observed by eye. One really interesting PrAG – JBD5-4 changed the level of pyocyanin production in both PA14 and PAO1 depending on the environment the bacteria is growing in. When the bacterial host was grown on solid medium, the expression of JBD5-4 greatly increased pyocyanin production (Figure 9A), while JBD5-4 decreased pyocyanin production when the bacteria were grown in liquid medium (Figure 9B). This observation suggested that JBD5-4 may be able to interact with host components that the bacteria uses to sense growing environment, and alter the amount of pyocyanin produced accordingly.

Figure 9. JBD5-4 alters the levels of pyocyanin produced by *P. aeruginosa*.

*P. aeruginosa* PA14 strains expressing PrAG JBD5-4 or no PrAG were grown on (A) solid medium and (B) liquid medium, and the amount of pigment produced was compared.
3.8 Two PrAGs modify bacterial virulence in *Drosophila melanogaster*

Phages are known to alter the degree of bacterial virulence through a variety of different mechanisms, ranging from encoding genes for the production of toxins in their genomes to helping their host evade mammalian immune system. To investigate if the expression of PrAGs has an effect on the virulence level of *P. aeruginosa*, I infected *Drosophila melanogaster* (fruit flies) with *P. aeruginosa* PA14 strain expressing different PrAGs to mimic the bacterial chronic infection seen in the lungs of cystic fibrosis patients. Because of the striking similarities between mammalian and *Drosophila* innate immunity responses, the flies provide an excellent model organism to study pathogenesis of chronic *P. aeruginosa* infections\(^{58}\). Through the virulence assay, we found two PrAGs that were able to modify the bacterial host virulence. I observed that fruit flies infected with PA14 expressing JBD5-4 live longer than flies fed with PA14 empty vector control. Approximately half of the flies in the control group died within the first eight days post infection, when 91% of the flies were still alive in the JBD5-4 group (Figure 10A). By contrast, JBD44-8 had the opposite effect on bacterial virulence. JBD44-8 increased the rate of fly killing by the bacteria. By day 9, almost all the flies infected by JBD44-8-expressing PA14 strain were all dead, while 67% of the flies infected with the empty vector strain survived (Figure 10B). There exists some discrepancies between the fly survival profiles in the control group from experiments done on different days, which were most likely due to the conditions of the bacteria and flies on particular days. Since the experiments were repeated on at least three different occasions, and we observed the same trend in all of them, the differences in results between controls and samples we seen were likely due to PrAG expression. The virulence assay demonstrated that the effects of PrAGs on their host are significant enough to modify bacterial virulence in the chronic infection of fruit flies, which suggests that PrAGs could also play crucial roles in bacterial infection of mammalian systems.
Figure 10. PrAGs have effects on bacterial virulence towards *Drosophila melanogaster*.

*P. aeruginosa* PA14 strains expressing different PrAGs or no PrAG (empty vector, EV) were fed to *D. melanogaster*. The number of dead flies were recorded every 24 hours. (A) The survival rate of flies fed with PA14 strain expressing JBD5-4 (○) was compared with PA14 strain harbouring an empty expression vector (■). (B) The survival rate of flies fed with PA14 strain expressing JBD44-8 (Δ) was compared to PA14 strain harbouring an empty expression vector (■).
Chapter 4  
Discussion and Future Directions

Phages have been shown to play very important roles in bacterial ecosystems. While prophages are known to express PrAGs that function to alter the physiology of their hosts, no systematic studies had investigated the bacterial phenotypes caused by expressions of a variety of PrAGs. The experiments described in this thesis aimed to assess the diversity of PrAGs in a closely related group of *P. aeruginosa* phages and determine their effects on bacterial phenotypes related to survivability and virulence in a systematic manner. Through a number of phenotypic screens, I found that *P. aeruginosa* PrAGs are able to modify various host phenotypes, including swimming and twitching motility, phage resistance, biofilm production, pyocyanin production, and degree of virulence against *Drosophila* fruit flies. More than half (11/17) of the PrAGs I studied were observed to affect host phenotypes in at least one screen (Table 6), and they possibly alter other bacterial phenotypes that were not tested in this study, which suggests that the role of PrAGs in bacterial biology may be more prevalent than previously appreciated. The phenotypic effects of PrAGs vary based on the individual PrAGs and the bacterial strain that the PrAG is expressed in. Some PrAGs, such as JBD30-30 and JBD26-31, were only able to modify a single host phenotype while others, like JBD26-61 and JBD24-4, can alter multiple phenotypes (Table 6), which likely depends on the mechanism by which each PrAG functions. It is also noted that a number of phenotypic modification are bacterial strain-specific. For example, the expression of JBD23-13 in strain PA14 resulted in decreased biofilm production but it increased the production of biofilm in strain PAO1 (Table 6), suggesting that PrAGs interact with host elements to cause changes in phenotypes.

Although various functional studies are still required to characterize the biological roles of the PrAGs, it is possible to make predictions on how the PrAGs function to alter host phenotypes based on the results of the phenotypic screens. One example includes PrAGs JBD24-4, JBD26-5 and JBD26-61, all of which caused decreased twitching motility and biofilm production and increased bacterial resistance against phages (Table 6). The bacterial T4P mediates twitching motility\(^{68}\) and it is involved in the biofilm synthesis\(^{72}\). In addition, as a cell surface structure which serves as a binding site during phage infections\(^{93}\), the pilus is essential for certain phages to infect. It is very likely that these three PrAGs function to down-regulate or affect the proper functioning of the host T4P, which leads to the reduction in twitching motility and biofilm
production, and protects the host from phages that use pili to infect bacteria. A recent study has shown that protein gp05 of *P. aeruginosa* phage D3112, which is identical to JBD26-5 in sequence, interacts and interferes with the activity of the host T4P assembly/extension ATPase, PilB\(^{94}\). This result is consistent with the phenotypes I observed through the screens. However, the functional amino acids or regions in gp05 responsible for the interaction are still unclear, and the PrAG may have other functions in bacterial cells that are not yet identified.

Another example is JBD5-4, which was observed to decrease bacterial twitching motility, biofilm production and cause increased or decreased level of host pyocyanin production when grown in liquid or on solid medium, respectively (Table 6). All three biological processes of the bacteria are controlled by the quorum-sensing system\(^{66}\). Therefore JBD5-4 may be interacting with host components regulating the quorum-sensing system. In their 2002 publication, Scott A. Beatson and his colleagues found that a regulator of quorum-sensing in *P. aeruginosa*, Vfr, was able to alter level of pyocyanin production based on different growth media\(^{95}\). It is possible that there exist a quorum-sensing regulator that can sense the difference between liquid and solid medium which JBD5-4 is interacting with. Indeed, a more recent study has shown that mutations in the *mexT* or *mexF* gene in *P. aeruginosa* strain PAO1 could lead to defective MexEF-OprN pump, and results in enhanced pyocyanin production in liquid medium and attenuated production on solid medium\(^{96,97}\). Since the MexEG-OprN pump has been suggested to be involved in the efflux of quorum sensing signalling molecules\(^{98}\), it is a likely candidate to interact with JBD5-4.

According to protein sequence alignments, JBD5-4, JBD26-5 and JBD24-4 are protein homologs. They share a central domain with 40-60% identity, while JBD26-5 has an extra N-terminal domain, and JBD24-4 has a C-terminal domain. Although the three PrAGs all caused twitching defects, their effects on phage resistance, biofilm and pyocyanin production differ, despite being protein homologs. The results of the screens suggested that the differences in phenotypic modifications may be caused by the non-homologous sequences in the PrAGs, and this also suggests the possibility of one PrAG interacting with multiple host components to affect bacterial phenotypes.

Among all the PrAGs I studied, two sets of PrAGs have very similar phenotypic effects on *P. aeruginosa*. First, JBD24-4 and JBD26-61 both abolished twitching motility, increased host resistance against the same phages and decreased biofilm production in the PA14 strain (Table
6). The only difference was JBD26-61 also affected biofilm production in PAO1, but JBD24-4 did not. This is interesting because JBD26-61 and JBD24-4 have no sequence similarity, and their genome positions are very different. The other set of PrAGs: JBD26-31 and JBD30-30 both increased phage resistance of the host without causing defects in bacterial motility or biofilm production (Table 6), which suggests that they might function at subsequent steps downstream of cell binding during phage infections. JBD26-31 and JBD30-30 have no sequence similarity, however they occupy the same genome position right between the small and large terminase (Figure 3). It is fascinating in the evolutionary point of view how different PrAGs may evolve to function through similar mechanisms to affect host phenotypes in similar ways, which indicates that the biological roles of PrAGs may be more relevant than previously known.

One PrAG, JBD23-13, has opposite effects on level of biofilm production under PA14 and PAO1 background. It decreased host biofilm production in PA14, however when expressed in PAO1, it increased the production of biofilm. In addition, JBD23-13 causes a twitching defect in strain PA14 and a swimming defect in strain PAO1 (Table 6). Since all the phenotypic effects caused by JBD23-13 were strain-specific, it is possible that the PrAG interacts with strain-specific host components or its activity is induced by strain-specific signals. More studies are required to explain how JBD23-13 functions in the host cell. Besides JBD23-13, differences between phenotypes observed in strains PA14 and PAO1 were also seen for other PrAGs. For examples, JBD30-4 caused twitching defects in PAO1 only, and similarly, JBD5-4, decreased biofilm production in PA14 but not in PAO1 (Table 6). Although the two commonly used lab strains are very similar in genome sequences, approximately 4-8% of the genome is unique to only one strain and may be responsible for the differences observed in this study99. It is known that the PA14-specific genomic regions contain a few pathogenicity islands, which account for the difference in pathogenicity between PA14 and PAO1100. PrAGs that were observed to cause strain-specific effects may interact with components found in unique regions, including Type IV pilin, Type IV fimbrial assembly protein, ABC transporter-like protein, DNA helicase-like protein, O-antigen biosynthesis proteins, putative transcriptional regulators and other genes of unknown functions100,101. For example, in the phage resistance screen, the expression of JBD24-4 in PAO1 resulted in complete host resistance against all phages tested. However, JBD24-4 only increased the resistance of PA14 against some of the phages. Since the bacterial T4P is involved
in initial phage infections, JBD24-4 possibly interferes with strain-specific components of the pilus system, such as the pilin (PilA) or fimbrial assembly protein (pilC), or related regulators.

Two other PrAGs, JBD26-15 and JBD44-8, have effects on twitching and swimming motility in both PA14 and PAO1 strains (Table 6), suggesting that they may play a role in quorum-sensing, which is essential for T4P and flagellum functions. Although JBD26-15 and JBD44-8 interfered with bacterial pili and flagella, they did not have significant effect on phage resistance except JBD44-8 slightly increased the phage resistance of PAO1 strain. The PrAGs possibly affect the proper functioning of the pili and flagella rather than the synthesis, so phages can still bind to the host cell during infections. While pilus and flagellum are considered to be important virulence factors during bacterial infections, it is interesting how JBD44-8 causes motility defects but increased virulence in fruit flies. As previously mentioned, JBD44-8 likely interacts with components in the quorum-sensing system. Therefore one possible explanation is that while it interferes with bacterial motility, JBD44-8 enhances the functions of other virulence factors controlled by quorum-sensing (e.g. exotoxin, protease), which were not captured by the screens performed in this study.

Interestingly, the motility and biofilm production screens generated results which were opposite to what is expected. Since bacterial motility and the production of biofilm often translates into bacterial virulence, I was expecting that the PrAGs increase bacterial motility and the level of biofilm production to aid bacterial infection, so the phages can replicate with their hosts. It is possible that the phages are sacrificing these two virulence factors for something that provides more advantages to themselves. For example, as previously mentioned in the case of JBD24-4, JBD26-5 and JBD26-61, the phages might be down-regulating bacterial T4P to protect the host from further phage infections, but it also resulted in motility and biofilm production defects. One thing to note is that most phages and their P. aeruginosa host live in natural environments, and Pseudomonas does not need to infect to survive. Therefore, the ability to resist phage infections and increase survivability is likely more important for both the phage and its host than virulence against other organisms.

Through the experiments done in this study, several PrAGs with interesting phenotypic effects in P. aeruginosa were identified. However, only a small number of PrAGs were screened for specific phenotypes, there are a lot more PrAGs identified in P. aeruginosa that were not
included in this study, and PrAGs likely affect the hosts in other ways that were not captured by the screens performed. Another limitation of the study is that the phenotypes observed in the screens were due to the overexpression of PrAGs, and it is not clear if the same phenotype modifications occur outside of lab environments, since we do not know the endogenous expression levels of PrAGs in *P. aeruginosa* at this point. However, the study does show that the PrAGs have the potential to cause bacterial phenotypic changes, and whether they happen or not depends on multiple factors, including environmental conditions, external cues and interactions between PrAGs and other prophage or host components. In the study, I have only investigated the phenotypic effects of the expression of individual PrAGs. In natural environments, it is common for more than one PrAG to be expressed inside the host cell. Based on the findings of the study, I cannot draw any conclusion on how the expression of multiple PrAGs interact with each other and what the resulting bacterial phenotypes will be.

In order to expand our knowledge of the biological roles of PrAGs in *P. aeruginosa*, follow-up studies are required to understand the mechanisms employed by the PrAGs to modify bacterial physiology. Based on the phenotypic screen results, it is likely that PrAGs function through interacting with host components. Therefore a potential next step in the study will be to identify the physical interacting partners of PrAGs through protein pull-down assay, or use mutagenesis to pinpoint genetic interactions between PrAGs and host genes. One can also take advantage of RNAseq technology to study the effects of PrAGs on host genetic expression profile, which may provide clues for the functions of the PrAGs. In addition, structural studies may prove useful in determining signature motifs and PrAG function predictions. Combining phenotypic screens with various biochemical and genetic approaches, one can more efficiently characterize the functions of the PrAGs. As previously mentioned, in addition to the PrAGs studied in my thesis, there are a lot of putative PrAGs with unknown functions, and new PrAGs are continuously being identified. In the future, the systematic study can be expanded to include more PrAGs and screen for more host phenotypes, such as swarming motility and production of other virulence factors like pyoverdine and elastase. It will also be interesting to investigate the interaction between different PrAGs when expressed simultaneously and the phenotypic consequences.

PrAGs are known to play important roles in bacterial physiology by altering different biological processes of the host life cycle, however no systematic studies have been done to address the effects of PrAGs on their bacterial hosts. Multiple screens were employed in this study to
systematically examine the phenotypic effects of PrAG expression in bacterial cells. The results have shown that many *P. aeruginosa* PrAGs have the ability to modify bacterial phenotypes related to survivability and virulence (Figure 11), and these events may contribute significantly to bacterial infection and disease progression. Therefore, when studying human diseases caused by bacteria, it is important to take into account the bacteriophages and what they can do through PrAG expression. I hope the facts learned from this study will offer inspiration for more research on bacteriophages and their contribution in human diseases, which may become the foundation for possible treatment in the future.

### Table 6. Summary of host phenotypic changes caused by PrAGs in *P. aeruginosa* PA14 and PAO1 strains.

<table>
<thead>
<tr>
<th>PrAG</th>
<th>Phage Resistance</th>
<th>Twitching Motility</th>
<th>Swimming Motility</th>
<th>Biofilm Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA14</td>
<td>PAO1</td>
<td>PA14</td>
<td>PAO1</td>
</tr>
<tr>
<td>JBD30-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD30-9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD30-14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD30-30</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD26-14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD26-15</td>
<td>-</td>
<td>-</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD23-13</td>
<td>-</td>
<td>-</td>
<td>(\downarrow)</td>
<td>-</td>
</tr>
<tr>
<td>JBD26-30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD5-15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD24-17</td>
<td>-</td>
<td>-</td>
<td>(\downarrow)</td>
<td>-</td>
</tr>
<tr>
<td>JBD44-8</td>
<td>-</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD44-9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD26-5</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD26-31</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JBD26-61</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD5-4</td>
<td>N/A</td>
<td>N/A</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>JBD24-4</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
</tbody>
</table>
Figure 11. PrAG expression affect various bacterial elements.

The phenotypic screens conducted in this study have shown that the expression of PrAGs modifies multiple host elements related to survivability and virulence. The PrAGs found to affect specific elements are listed.
References


52


Figure 12. Detail genome alignment for phages used in this study.

Genomes of closely related JBD phages were aligned and known gene functions were labeled. Genes without function labeled represent hypothetical genes (ORFs) or genes with unknown function. The numbers in black bordered boxes represent gene number, and blank boxes indicate that the gene homolog does not exist in a specific phage. Phage JBD26 was used as the reference genome, and the percentages shown are the differences in DNA sequence of phage genes compared to homologs in phage JBD26. However, for genes with no homologs in JBD26, I compared them to their homologs in JBD5. The genes labeled in yellow are the PrAGs examined in this study.