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Experimental Evolution of Specialism in a Wild Virus

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EXPERIMENTAL EVOLUTION OF SPECIALISM IN A WILD VIRUS

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ABSTRACT

A pathogen's ability to successfully replicate and persist within a new host population is fraught with obstacles. While an expanded host-range allows for a greater chance at successful replication, such generalists are typically outcompeted by species which have specialized and adapted host-specific features. Although the most ubiquitous species on earth, very few bacteria-infecting viral species (bacteriophages) with truly broad host-ranges have been identified. As such, the processes of expanding as well as narrowing host-range are not well understood despite their profound importance for furthering our understanding of viral pathogens.

Following the successful isolation of novel bacteriophage named ΦHabibi, we identified this generalist species' broad host-range, including several *Enterobacteriaceae*. To examine the cost of generalism, we propagated ΦHabibi either in isolation with a single host species, *Escherichia coli* C or *Pseudomonas aeruginosa*, or alternating between the two hosts (serving as a control). While no reduction in fitness was observed on either host population for the control lines, this was not the case for either of the experimental conditions. Lines propagated through *E. coli* C cultures were more fit in *E. coli* C. Likewise, lines propagated through *P. aeruginosa* cultures were more fit in *P. aeruginosa*. Moreover, specialization as well as changes in phage development, from lytic to lysogenic and pseudolysogenic, was observed as a result of restricting host diversity thus providing insight into the cost and consequences of host-range.

CHAPTER ONE

INTRODUCTION

Bacteriophages

Bacteriophages are the most abundant species on Earth, infecting bacteria found in the polar icecaps to hot springs, from the natural environment to the human microbiome. Phages are extremely diverse. While some phage species have single stranded genomes, some are double stranded; genomes can be DNA or RNA, linear or circular, singular or segmented. Morphologically, phages vary just as greatly. Furthermore, phages can differ in their reproductive strategy as well as their host(s) predilections, neither of which is static.

Phages demonstrate two key life cycles (in addition to several other intermediate lifestyles): lytic and lysogenic. Lytic phages utilize the host's translational machinery to produce progeny phage and then kill (burst) the host cell to releasing virons (Bobay *et al*. 2014). In contrast, lysogens incorporate their own (viral) genetic material into the bacterial genome without causing harm to the bacterial host (Refardt *et al*. 2011). Both of these life cycles can have profound impact on bacterial hosts, and therefore the ecosystem. The lytic process dictates that the bacterial cell is destroyed. On a large enough scale, this mediation of mortality can have consequences for nutrient cycling, primary productivity and biological processes such as photosynthesis. Lysogenic phages

are often seen as having a positive interaction for both the phage and host. Research has shown that some of these positive interactions can include making the host antibiotic tolerant (Wang *et al*. 2010), as well as aiding the host when nutrients are scarce (Edlin *et al*. 1977). Furthermore, the lysogenic phage can actually protect the bacterial host from other potential viral infections (Bossi *et al*. 2003).

Lysogenic pathogens are also known as temperate phages. These temperate phages possess the ability to switch between a lytic and lysogenic life cycle (Ali *et al*. 2014). Induction of the lysogen (i.e. conversion to lysis) may take place at a later point. Various factors, such as environmental signals or bacterial density, can determine which life cycle is more beneficial at any given moment (James *et al*. 2014). When discussing lytic and lysogenic reproduction, lytic is often described as the productive life style and lysogenic is referred to as reductive (Koskella *et al*. 2013, Dang *et al*. 2014). The lytic stage is responsible for creating viable progeny to infect other hosts (Koskella *et al*. 2013). The lysogenic stage preserves the phage chromosome within the bacterial host (Morelli *et al*. 2009; Dang *et al*. 2014). This temperate life cycle provides the opportunity for the phage to thrive under a variety of environmental pressures.

Viral Host-Range

The number of hosts a viral pathogen can successfully infect and propagate itself in varies greatly. It may be broad or narrow: a generalist or a specialist, respectively. Given the high mutation rates and frequent recombination (Domingo & Holland 1997; Roosinck 1997), one would hypothesize that most viruses would be generalists, able to quickly adapt to new hosts. The comprehensively studied Cucumber mosaic virus (CMV) has a broad host-range capable of infecting more than 1000 species among different taxa

of plants (Elena *et al*. 2009). Besides being able to infect different species, true generalist viruses have been shown to "jump" to higher taxonomical groups (Elena *et al*. 2009). An even more well-known virus exhibiting a broad host-range is the Influenza A virus. Like CMV, Influenza A has been known to jump between taxonomically distant hosts, particularly between classes of birds and mammals (Bernard *et al*. 2006). While infection necessitates appropriate host cell receptors, successful propagation and proliferation is frequently the result of rapid acquisition of beneficial mutations.

At the other end of the spectrum, some viruses are highly specialized, infecting only one, or very few, closely related host species (Brown *et al* 2012). The expectation is that a specialized virus has a restricted niche range. One such example is that of the human papillomavirus (HPV) which is limited in its host-range as it can only infect humans. There are, however, numerous papillomaviruses with a wide variety of host species. As prior research has shown (Ong *et al*. 1993, Bernard *et al*. 2006), once a papillomavirus has become host specific, the virus rarely or never crosses host species barriers. There has never been a documented case of HPV infecting another animal species, nor an animal PV successfully infecting a human (Bernard *et al*. 2006). Within the HPV types/strains/species, the genes that are involved in transcription regulation vary greatly; so extensively, in fact, that these genes share essentially no homologous sequence (Ong *et al*. 1993).

Variation in host-range is not exclusive to eukaryote-infecting viruses as the previous examples given here. When bacteriophages were first discovered, it was believed that the phage could only infect one bacterial species and possibly a subtype of the natural host (Bielke *et al*. 2007). Empirical evidence, however, has found

bacteriophages capable of infecting multiple bacterial species (Hall *et al*. 2011; Remold *et al*. 2012). Nevertheless, there is no single phage can infect all bacteria. Thus, bacteriophage species predate on both niche host populations as well as diverse complex communities.

Specialist Viruses

A specialist is able to successfully utilize the translational mechanisms of the ancestral host without having to be opportunistic among alternative hosts. This specialist lifestyle is preferred when the environmental conditions favor a constricted host density (Guyader *et al*. 2008). Even when there are a wide variety of bacteria hosts, some pathogens still opt for a specialized lifestyle, but why? The prominent theory for this phenomenon is the optimal foraging model. This model predicts that a predator should be specialized on a narrow prey range, which is highly profitable (Guyader *et al*. 2008). It is thus not surprising that model phage species propagated within laboratory conditions for decades exhibit a very narrow host-range. By being constrained to a few bacterial hosts, a specialized pathogen will be able to utilize a bacterium far more efficiently than a generalist pathogen. Specialism would thus provide the pathogen with an advantage as it would be able to out-compete a generalist by infecting and propagating within a bacterial host more efficiently.

The specialist lifestyle is only plausible if a pathogen has the means to readily adapt to a host. For example, as infection begins to favor a particular bacterial host, or even a particular sub-strain, the phage will lose the ability to infect ancestral hosts (Weitz *et al*. 2013). By having an advantageous adaptation in a selective environment, the pathogen would have an increase the likelihood of a successful infection (Presloid *et al*.

2008). Antagonistic pleiotropy can explain this acquisition of genetic material that would account for the innovative performance of the pathogen (Presloid *et al*. 2008). In a given environment there can be one beneficial adaptation, and conversely cause an unfavorable outcome in another environment (Kubinak *et al*. 2012). The lifestyle of a specialized organism relies heavily on the diversification between organisms, so that the competition can remain low. Limor-Waisberg *et al*. demonstrated that within cyanophages there are different strategies that are utilized for effective translation, including a specialization strategy within Podovirus cyanophages.

Yet the specialized lifestyle is far from optimal, particularly when bacterial host abundance is low. When observing a specialist pathogen in a wider density of bacterial hosts, the replication time of specialists dwindles (Guyader *et al*. 2008). At low host density, it takes more time for the specialized pathogen to locate a suitable bacterial host for replication to occur. Specialists are also at a disadvantage when host populations shift, as the cost of specialization reduces the species' ability to adapt to these changing conditions.

Generalist Viruses

A broad host-range allows the pathogen to sustain productivity within a diverse environment; the generalist is not dependent upon the presence of a single host species and thus a broad host-range can aid in its survival. Living in a vast, complex environment, a generalist phage would be more likely to persist by a long-term coevolution with the numerous host species available (Hall *et al*. 2011). The generalist virus is often seen as opportunistic. Generalism can thus reduce competition, allowing access to a greater diversity of host cells (Refardt *et al*. 2011). In a given population that

is experiencing intense competition, whether it be intraspecific or interspecific, frequency-dependent selection will favor the pathogen that can exploit an underutilized niche (Duffy *et al*. 2006, Wilson *et al*. 1986). Most of the time the new source is of a poorer quality than the ancestral host, however, it will still be a more beneficial source due to the decreased competition (Bono *et al*. 2012). An emerging pathogen will thus have an initial fitness that will be low relative to the ancestral host (Duffy *et al*. 2006), but the pathogen will experience a decreased competition that will allow for successful replication over time (Dennehy *et al*. 2006).

Generalism, nevertheless, comes at a cost. Generalists adapt more slowly to bacterial host defenses as they have other options for replication; this is of course not true for viral species which can only infect a single host species (Elena *et al*. 2009). Acquisition of host-specific genetic content may increase the complexity (size as well as regulatory mechanisms) of the virus, thus potentially decreasing the speed and/or efficiency of transcription and translation. Moreover, adaptations to one host may be detrimental in another environment - antagonistic plietropy. If the bacterial community composition within an environment is not diverse, a virus with a limited host-range will out-compete a generalist. As such, over time the generalist virus would more than likely be driven off by the specialist.

Although domesticated laboratory phage strains typically infect just a single bacterial species or strain, some show a broader host-range. For instance, the domesticated *Pseudomonas*-infecting bacteriophage Φ6 was exposed to a highly competitive environment; as a result of competition a novel generalist mutant capable of infecting multiple strains of the same host species arose (Bono *et al*. 2012). Phage

isolated directly from the environment exhibit broader host-ranges (Koskella *et al*. 2013). Most notable are those bacteriophage isolated from wastewater/sewage. In the study of Bielke *et al*. (2007), phage were found capable of infecting strains of the family *Enterobacteriaceae*. An even broader host-range has been observed including phage capable of infecting multiple phylogenetically distant genera (Jensen *et al*. 1998). Here are just a few examples of phage with broad host-ranges; nevertheless, the number of true generalist phage known is very few despite their very likely prevalence in nature. The breadth of generalists remains unknown, as does the principles governing the set of host that a given virus can infect.

Changes in Host-Range over Time

Host-range is not necessarily a fixed trait as selection often pushes viruses across the fitness landscape. It is possible that a broad host-range virus may have an infective advantage for one host over another. Such a virus would then co-evolve with its "favorite" host, eventually becoming specialized to the preferred host (Nikolin *et al*. 2012), and no longer being able to infect others. With the rise of emerging infectious diseases, there has been considerable effort to understand how pathogens have developed the ability to infect multiple hosts, for example, severe acute respiratory syndrome (SARS), and Ebola. It is rare for a novel virus to cause successful host to host transmission in a new host population: rather, most emerging viruses are the result of a "spillover" event (Parrish *et al*. 2008). The recent spillover for H5N1, for example, occurred due to a reassortment event between the avian influenza virus (HPAI), found in domestic poultry, and the seasonal flu (CDC, 2015).

Experimental Evolution of Host-Range

As part of this project, five generalist bacteriophages were isolated from Lake Michigan. By exposing the phage to multiple different bacterial species, the viruses were determined to be true generalists, capable of infecting species from several genera of bacteria. The genomic resources constricting and/or expanding a virus' host-range are largely unknown as are the costs associated with specialism and generalism. Using one of the generalist phages isolated from the environment, designated ΦHabibi, I have experimentally explored the phenotypic and genotypic signals of host-range via a resource limiting experiment. By exposing a true generalist bacteriophage to an artificially restrictive environment, I have begun to elucidate the genotypic and phenotypic responses leading to specialization.

CHAPTER TWO

METHODS

Viral Isolation from Lake Michigan

Water was collected from nearshore waters throughout the Chicago area during the summer 2013. No specific permits or permissions were required for the water samples collected from the Chicago Lake Michigan nearshore waters. 4 L collections were then filter via successively smaller membranes, first a 0.45 μm bottle-top cellulose acetate membrane filter (Corning Inc, Corning, NY) followed by a 0.22 μm polyethersulfone membrane filter (MO BIO Laboratories, Carlsbad, CA). To isolate viral particles a third filtration was conducted through a 0.10 μm polypropylene filter (EMD Millipore Corp, Billerica, MA) using the Labscale™ tangential flow filtration (TFF) System (EMD Millipore Corp, Billerica, MA). 100 μl of the 5 ml of viral concentrate retrieved from the TFF was then aliquoted into 2 ml of *Escherichia coli* C (ATCC 8739) in exponential growth. The *E. coli* C culture was grown in LB broth at 37°C, as determined by the optical density (OD_{600}). The culture was grown within a shaking incubator at 37°C for 48 hours prior to treatment with 20 μl of chloroform. Lysate was then plated as follows: 3 ml LB-soft agar, 1 ml *E. coli* C and 100 μl were mixed poured over a 1.7% LB agar plate. The plates were then incubated overnight at 37°C. Plates producing visible plaques were next harvested and suspended in 0.8% NaCl solution. Viral DNA was extracted using the

MO BIO Laboratories UltraClean® Microbial DNA Isolation Kit (Carlsbad, CA). The protocol recommended by the manufacturer was followed with the exception of an additional heat treatment in a waterbath at 70°C overnight prior to initial vortexing. In doing so, only plaques produced by DNA-viruses would remain. 400 µl of *E. coli* C spheroplast solution was incubated with 5 µl of the isolated viral DNA (suspended in nuclease free water) for 20 min at 37°C; SOC medium (3 ml, pre-warmed to 37°C) was added and the preparation was incubated for 90 min. The phage was released using a 1:10 dilution into water then titered and spotted on LB plates with *E. coli* C lawns (following the same protocol as previously listed) and incubated overnight at 37°C. Individual plaques were picked from these plates, suspended in saline solution, chloroformed (10 µl), vortexed, and again grown in 2 ml *E. coli* C cultures. This process was repeated several times to guarantee only a single viral species was in solution. In total, five individual isolates were obtained. Herein, one of these isolates was used for the experiment, ΦHabibi, which originated from the nearshore waters of Loyola Beach collected on May 21, 2013.

Assessing Phage Host-Range

Chloroform was added to samples of phage-induced bacterial lysate (200 µl) and spotted onto bacterial lawns, which were incubated as above and examined for the presence of plaques after 24 hours (Hyman & Abedon 2009). All bacterial strains used in this study were propagated in LB medium and incubated at 37°C in static or shaking incubators (unless otherwise stated): *Escherichia coli* C (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 15692), *Pseudomonas syringae* (NCPPB 2849), *Escherichia coli* K12, *Shigella boydii* (ATCC 9207), *Shigella flexneri* (ATCC 12022), *Achromobacter* sp.

(obtained from D. Castignetti, LUC). Pseudomonad strains LM C1, C3, L2 and L1 were isolated from unfiltered Lake Michigan water onto Cetrimide Agar (Oxoid, Basingstoke, UK) for strains C1 and C3 and LB for strains L1 and L2. For those strains which did not have corresponding 16S rRNA sequences already present in the NCBI Genbank database (*Achromobacter* sp., LM C1-L1), DNA was extracted using the MO BIO Laboratories UltraClean® Microbial DNA Isolation Kit (Carlsbad, CA) according to the instructions of the manufacturer, and the 16S rRNA gene was amplified via PCR, using the 63F, 1387R primer set (Marchesi *et al*. 1998). Amplicons were sequenced by Genewiz (South Plainfield, NJ), sequence data were checked for homology to records in Genbank via BLAST[33] and subsequently analyzed in MEGA6 (Tamura *et al*. 2013).

One-step growth curves were generated using *P. aeruginosa*, which was grown to the end of exponential growth and mixed with each phage at a multiplicity of infection (m.o.i.) of <1 according to the protocol detailed in Abedon & Hyman 2009. Burst size was estimated from the resulting one-step growth curves.

Phage Characterization

In an effort to determine the taxonomic classification of the bacteriophage isolates, PCR primers were used for targeted amplification of conserved proteins within various families of phages. PCR primers previously described (Sullivan *et al* 2008) were used to assess for the presence of the coliphage portal vertex protein (gp20). PCR primers were also designed for three different tail proteins, PB1_gp39 (putative tail protein containing transglycosylase), PB1_gp47 (tail fiber component), and the Lambda phage tail fiber. These three tail proteins were selected as they are common amongst freshwater phage. Primers were designed using Primer3 (Rozen 1998); coding sequences for these

three tail proteins were retrieved from NCBI (GenBank records NC_011810 and CP008739). The PB1_gp39 primers are GGACTCGCGCATATCCTG and CTGGATGGCAGTGGTCAG with an expected amplicon of 776bp; the PB1_gp47 primers are CATCCACGCCATGGTGAC and CGAATCTGCTGCCTCCAG with an expected amplicon of 306 bp. The Lambda phage tail fiber primers include GTGCCGACCACCTACGAC and GCGATCGAGCGTTACCAC (expected amplicon length 746bp). All PCR primers were synthesized by Eurofins (Huntsville, AL). Two different thermal cycling conditions were used for all three primer pairs: (1) 94°C 5 min, 35 cycles: 94°C 30 sec, 52°C 30, 72°C 1 min 15 sec, and 72°C 5 min and (2) 94°C 5 min, 35 cycles: 94°C 30 sec, 50°C 30, 72°C 1 min 15 sec, and 72°C 5 min. In addition to amplification of the five isolated phage DNA, two negative controls – nuclease-free water and whole *P. aeruginosa* cells – were included for each primer pair and their respective thermal cycling conditions.

Imaging Phages via TEM

Purified viral lysate was applied to pioloform coated copper grids and left to dry at room temperature. Samples were positively stained with 2% (w/v) uranyl acetate (Ackermann, 2009) and observed at 80 kV using a Hitachi H-600 Transmission Electron Microscope (Loyola University Chicago).

Selection Experiment

Phage populations were either cultured in the presence of the *P. aeruginosa* host or the *E. coli* C host. As a control, populations were passaged through alternating hosts; phage populations were cultured in one of the hosts for two days (approximately 16 generations) followed by two days in the other host, and so on. These three regimes are

referred to henceforth as Pa, Ec, and AH (alternating host). Each regime was conducted in ten replicates thus resulting in 30 independent lines. Figure 1 illustrates the propagation strategies employed.

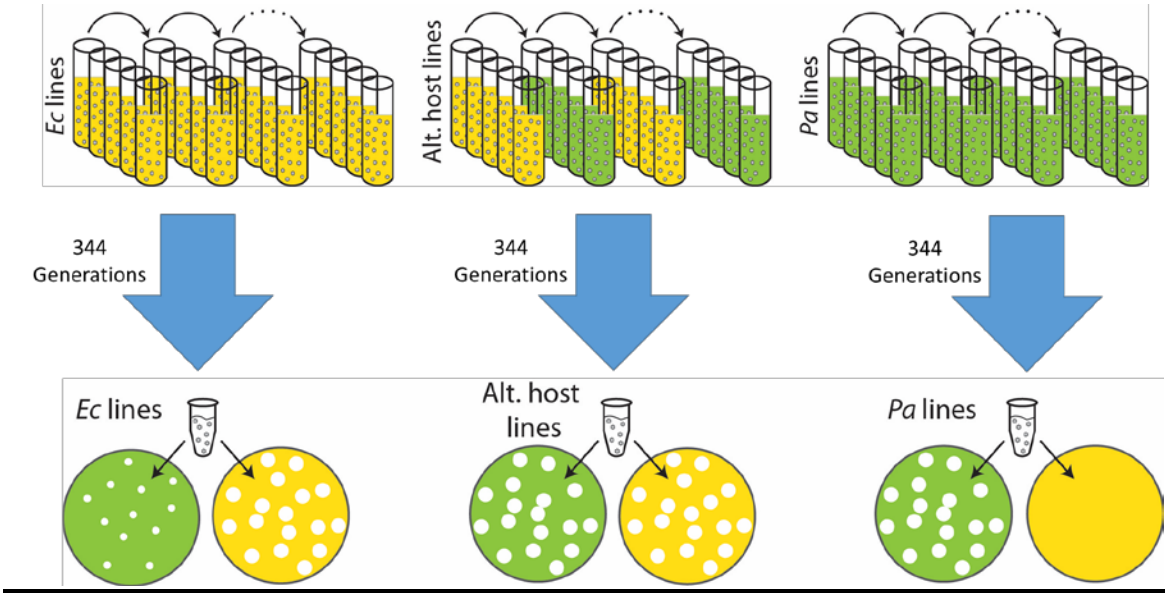

Figure 1. Diagram of Propagation and Plating of ΦHabibi. The top represents the three bacterial host serial passage lines for ΦHabibi. The experimental regime including serial passage through *E. coli* C (yellow), *P. aeruginosa* (green) or alternating hosts. The bottom represents the plating results after T43 (344 generations).

Host Populations: The beginning of the experiment began with 3mL of bacterial host (either *P. aeruginosa* or *Escherichia coli* C). *P. aeruginosa* was grown for 24 hours at 37°C prior to inoculation. An initial amount of 10 mL of *E. coli* C was grown 22 hours and 2 hours prior to the phage inoculation, the reminder of *E. coli* C was grown at 37°C. While the *E. coli* C cultures were in the exponential phase of growth, the *P.* aeruginosa culture was in stationary phase. Prior work within the lab confirmed these two stages of the bacterial host were ideal for infection. Both bacterial host cultures were grown in a

shaking incubator at 250 RPMs. Glass beads were added to the *P. aeruginosa* culture to prevent biofilm formation. Host populations were prepared, selecting a colony from a plate, daily.

Passage Protocols: The experiment started with 3 ml of bacterial host and 300 µl of ancestral ΦHabibi in a 5ml cryogenic tube. The bacterial host and bacteriophage ΦHabibi was allowed to grow for 24 hours in a shaking incubator (37°C at 250 RPMs). After the 24 hour growth, 50 µl of chloroform was added followed by 5 seconds of vortex. The tube was then allowed to settle for five minutes. Next, 300 µl of lysate was collected and added to the next tube containing 3 mL of a turbid, naïve bacterial host culture. This was then placed in the shaking incubator for 24 hours.

Qualifying Changes in Host-Range: In order to verify that the bacteriophage had successfully grown, 10 µl of lysate was aliquoted and plated on lawns of both naïve *E. coli* C and *P. aeruginosa* (similar to that illustrated in Figure 1). In doing so, we could assess the virulence of the phage both within the host lineage it was being propagated through as well as the host it was not being grown in. Plating protocols and incubation was identical to previous protocols listed.

Genome Sequencing

ΦHabibi was grown in 5ml of a 24 hour culture of *P. aeruginosa* overnight at 250rpm at 37°C. The culture was chloroformed (100 µl) and vortexed for 1 minute. The entire lysate was then transferred to 300 ml of *P. aeruginosa* culture (grown for 24 hours prior) and incubated at 37°C overnight in an Erlenmeyer flask containing glass beads. The entire culture was then filtered using a 0.22 µm bottle-top cellulose acetate membrane filter (Corning Inc, Corning, NY) to remove bacterial cells from solution. The

filtrate was next filtered using the TFF system and a 0.10 μm polypropylene filter producing 5 ml of viral concentrate for each of the five isolates. 300 μl of concentrate was then treated with 5 μl of OPTIZYME^{TM} DNase I (Fisher BioReagents) for 30 minutes at 37°C. This was performed to degrade DNA from lysed *P. aeruginosa* cells. DNase was then inactivated via the addition of 50 mM OPTIZYME EDTA at 65°C for 10 minutes. DNA was extracted using the MO BIO Laboratories UltraClean® Microbial DNA Isolation Kit following the same modified protocol as listed prior. Extracted DNA was quantified using a Nanodrop and tested for *P. aeruginosa* contamination via 16S rRNA targeted PCR. As no amplification of 16S rRNA was observed, the DNA was next prepared for sequencing. Library construction and sequencing was conducted at the University of Texas Medical Branch (Galveston, TX). DNA was fragmented using NEBNext Fragmentase into the size of 300 to 400 bp. Libraries were prepared using the NEBNext® UltraTM DNA Library Prep kit for Illumina®. Each of the five isolates was multiplexed and sequenced using the Illumina MiSeq platform via the MiSeq Reagent Kit v2 (500 cycle), producing paired-end reads each 250 nucleotides in length.

Genome Assembly and Annotation

Initially, sequence assembly was performed using the Velvet software (Zerbino *et al*. 2008) and numerous large (>50kbp) contigs were produced. Each of these larger contigs were BLASTed manually via the web interface only to find identical or near identical hits to the *P. aeruginosa* PAO1 genomic sequences. Thus, despite DNase treatment, *P. aeruginosa* DNA remained within the DNA isolated and sequenced. Therefore, the raw reads were first filtered – removing *Pseudomonas* sequences – using the PhagePhisher pipeline developed in our laboratory (Hatzopoulos *et al*. in review). The

genome of the *P. aeruginosa* PAO1 RefSeq [GenBank: NC_002516] was used as the filter. Those reads which passed through the PhagePhisher pipeline were then assembled using the *de novo* assembly functionality available through the Geneious software (Biomatters, Auckland, New Zealand). Assembly was performed at the medium-low sensitivity for speed. Reads contained within contigs greater than 1000 nucleotides in length were then BLASTed against the RefSeq viral protein database locally using the BLAST+ software. Those contigs containing reads identified as viral in nature were selected to scaffold the genome. The genome was then finished using the software SSPACE (Boetzer *et al*. 2014). This tool takes the scaffolding contigs along with the complete set of sequenced reads in order to close gaps in the genome. PCR primers were designed for a number of the original contigs in the scaffold. Amplicons were sequenced (Genewiz, South Plainfield, NJ) confirming the completion of the genome. The genome sequence of ΦHabibi was annotated using the RAST web-service (Overbeek *et al*. 2014). Genome comparisons were conducted via WebAct (Carver *et al*. 2008).

CHAPTER THREE

RESULTS AND DISCUSSION

Microbial Host Information

The generalist phage ΦHabibi, isolated from the nearshore waters of Lake Michigan's Loyola Beach, was found to be capable of infecting two bacterial laboratory strains commonly used in our lab, *Pseudomonas aeruginosa* and *Escherichia coli* C. As detailed in Table 1, these two species are similar, in that they are both aerobic gram-negative species. They are, however, taxonomically distinct, members of two different genera, and vary significantly in their genomic composition and biases. The biggest difference between *E. coli* C and *P. aeruginosa* is the outer membrane; *P. aeruginosa* contains pili, which is used for adhering to surfaces. Nevertheless, ΦHabibi was found to be capable of both infecting and replicating within these two different hosts.

Organism	**Bacteriological Characteristics**	**Isolated from**	**Outer Membrane Characteristics**	**Genome Characteristics**
Escherichia coli C ATCC 8739	Aerobic Rod-shaped	Feces	Rough lipopolysaccharide (LPS) Gram-Negative	4.6 Mbp 50% GC Content
Pseudomonas aeruginosa ATCC 15692	Aerobic Rod-shaped	Infected wound	Protein F (OprF) Pilus Gram-Negative	6.2* Mbp 65% GC Content

Table 1. General description of the two microbial hosts. (*Finished genome sequence for *P. aeruginosa* strain is not complete; genome size estimate based upon other sequenced strains.) Host characteristic information about *E. coli* C and *P. aeruginosa* was obtained from Bergey *et al*. 1984.

Phage populations were either cultured in the presence of the *P. aeruginosa* host or the *E. coli* C host. By removing alternative hosts, the propagated viral population is artificially under selection to maintain and/or improve fitness within a single host species. Thus, through this direct manipulation, selection for generalism is removed. As a control, populations were also propagated through alternating hosts in which phage populations were cultured in one of the hosts for two days followed by two days in the other host, and so on. Each of the three regimes, henceforth referred to as the Pa, Ec, and AH lines, was conducted in ten replicates thus resulting in 30 independent lines. After each transfer (post-24 hours of growth), the phage lysate was plated on both hosts thus providing a means for accessing the fitness of the selected lineages as well as the control lines.

Phenotypic Response to Alternating Host Line

No phenotypic response to selection was observed in the control lines; ΦHabibi was able to successfully infect and lyse both of the bacterial hosts (Figure 2). In these alternating host (AH) lines, ΦHabibi was exposed to only one bacterial host at a time, either *P. aeruginosa* or *E. coli* C, for two transfers (roughly 8 generations each) and then exposed for two transfers to the alternate host. This would prohibit ΦHabibi to develop an affinity for a particular host. While one of the lines is shown in Figure 2, identical results were observed for all replicates.

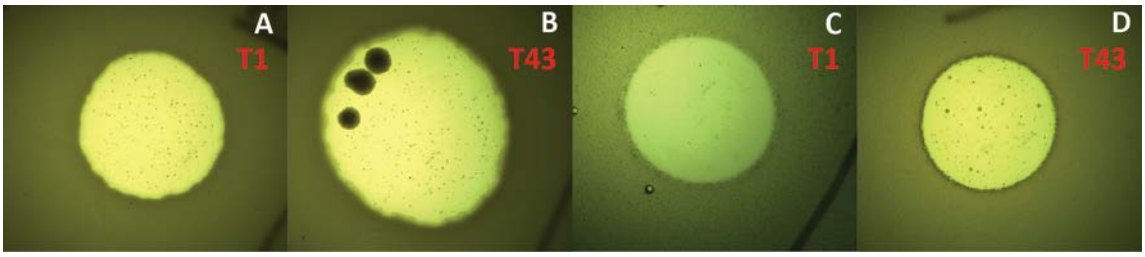

Figure 2. Microscope Images of AH Line. Preservation of plaque forming units size and lysis efficiency as a result of selection under propagation through alternating host regime. A and B: Representative lines plated on an *E. coli* C lawn after 1 transfer and 43 transfers respectively. C and D: Representative lines plated on a *P. aeruginosa* lawn after 1 transfer and 43 transfers respectively.

Phenotypic Response to Propagation Limited to the *E. coli* C Host

Figure 3 presents a summarization of the phenotypic responses observed for the Ec lines. Figure 3 A-D represent ΦHabibi that was grown in *E. coli C* that were plated on *E. coli C*. The ΦHabibi sample from Transfer 1 (T1) shown in panel A had a very successful infection rate, capable of lysing the bacterial host. After T14 (Figure 3B), ΦHabibi still had the capacity to successfully infect the *E. coli C* host. There was a change in lifestyle though, as ΦHabibi appeared to alter to a lysogenic lifestyle. As the exposure to a restrictive *E. coli C* environment continued, ΦHabibi developed a pseudolysogenic lifestyle. The pseudo-lysogenic lifestyle can be seen in the T42 and T43 samples, Figure 3C and 3D respectively. ΦHabibi was able to efficiently infect *E. coli C,* but ΦHabibi was not always able to successfully lyse the host. There is a buildup of infected bacterial cells surrounded by bacterial hosts that were lysed by ΦHabibi.

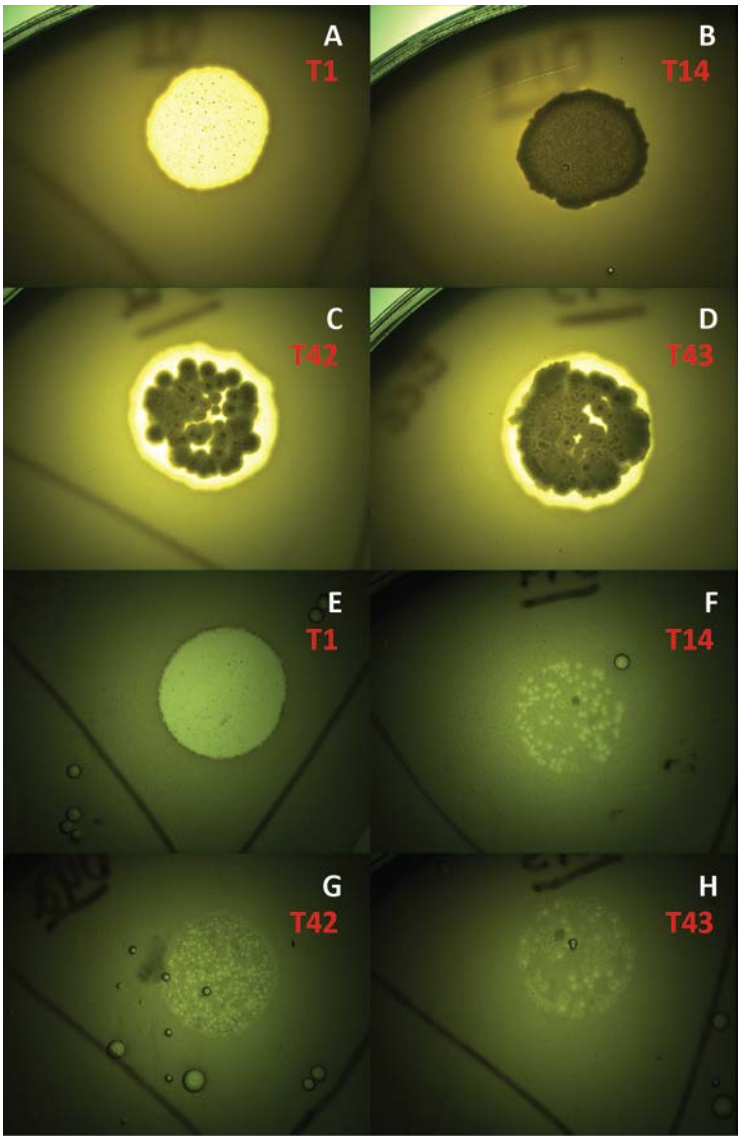

Figure 3. Microscope Images of Ec Line. Reduction in size of plaque forming units (PFU) as a result of selection under propagation through only the *E. coli* C host. A-D: Representative lines plated on an *E. coli* C lawn after 1 transfer, 14 transfers, 42 transfers and 43 transfers respectively. E-H: Representative lines plated on a *P. aeruginosa* lawn after 1 transfer, 14 transfers, 42 transfers and 43 transfers respectively.

Figure 3 panels E-H show ΦHabibi that was grown in *E. coli C,* but was plated on a *P. aeruginosa* bacterial lawn. The first sample, T1 (Figure 3E) shows a lytic lifestyle for ΦHabibi, as well as a high successful infection rate. After the 14th transfer (T14,

Figure 3F) a reduced infectivity rate for ΦHabibi is evident; although some of the viral population is able to lyse the host lawn. ΦHabibi from T42 (Figure 3G) displays pathogens that are able to produce PFUs. Also though, ΦHabibi from T42 displays pathogens that are not able to successfully produce a burst. Thus, the pathogens are capable of infecting the *P. aeruginosa* host but are not able to lyse the cell. ΦHabibi from T43 (Figure 3H) has a reduction in infectivity when compared to T42, as well as a reduced capacity to lyse the bacterial host. While the infectivity for the T43 ΦHabibi was reduced, some of the pathogens were able to infect *P. aeruginosa.*

Thus, when comparing the evolved Ec lines' ability to infect the two hosts, one immediately observes a preference for the *E. coli* C host. However, it appears that within the *E. coli* host, ΦHabibi has a predilection towards pseudolysogeny, particularly visible between T21 (not shown) to T43 (Figure 3D). When plated on *P. aeruginosa*, evidence of pseudolysogeny is also evident (Figure 3H). However, the virus' ability to infect is significantly reduced within this host. Seen as early as T14 (Figure 3F), the PFU morphology is smaller and hazier, suggestive of more inefficient lysis. The diminishing plaque size continues throughout the selection experiment. Thus, by constricting the host-range of the virus to only *E. coli* C, ΦHabibi adapts a pseduolysogenic lifestyle which has greater fitness in the *E. coli* host than in *Pseduomonas*.

Phenotypic Response to Propagation Limited to the *P. aeruginosa* Host

Figure 4 presents a snapshot of the phenotypic responses observed for the Pa lines. Figure 4, panels A-D, show a representative Pa line plated on an *E. coli* C lawn after 1, 14, 42 and 43 transfers, respectively. While the number of PFU decreases over the course of the selection experiment, lifestyle changes to lysogeny and/or

pseudolysogeny are not observed. Rather, phage able to infect the other host – in this case *E. coli* C – for which it has not been in contact with for as little as 14 transfers dwindle in number. While phage are still able to infect and lyse *E. coli* after 42nd transfers, after the 43rd transfer there is no evidence that ΦHabibi is capable of infecting the *E. coli* host cells. Of important note is the fact that this was observed in all 10 lines; by the 43rd transfer no PFU were observed in any of the Pa lines.

The second group of images (Figure 4, panels E-H) consists of ΦHabibi that were plated on a *P. aeruginosa* bacterial lawn. There was no fitness loss (with respect to plaquing efficiency) when infecting the *P. aeruginosa* host throughout the course of the experiment. ΦHabibi was able to maintain a highly successful infectivity rate and also maintain a lytic lifestyle.

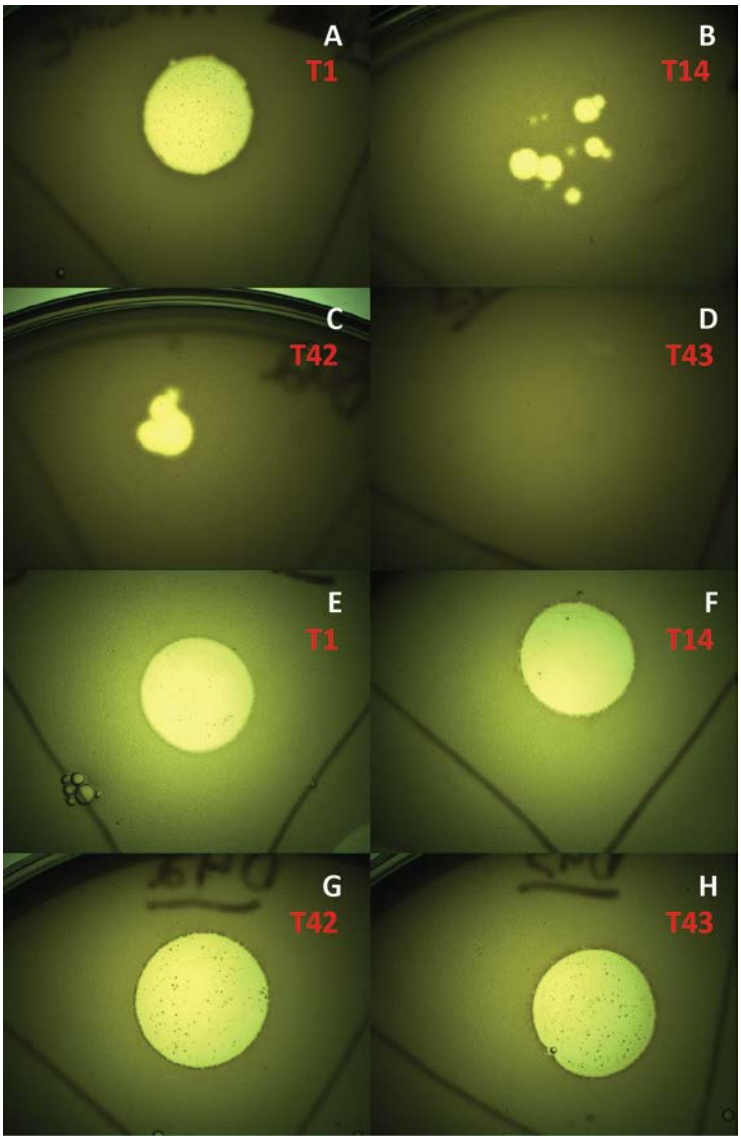

Figure 4. Microscope Images of Pa Line. Reduction in size of plaque forming units (PFU) as a result of selection under propagation through only the *P. aeruginosa* host. A-D: Representative lines plated on an *E. coli* C lawn after 1 transfer, 14 transfers, 42 transfers and 43 transfers respectively. E-H: Representative lines plated on a *P. aeruginosa* lawn after 1 transfer, 14 transfers, 42 transfers and 43 transfers respectively.

Characterizing the Generalist Phage

To further investigate the generalist phage and potential modes of infection of the two genera, a variety of methods were employed. Using Loyola's Jeol JEM 2100

Transmission Electron Microscope (TEM), we were able to clearly identify the structure of the phage. As shown in Figure 5, ΦHabibi possess a tail; more importantly ΦHabibi demonstrates a contractile tail. The TEM images also provide information on the length of the various components of ΦHabibi. The capsid appears to be in the form of an icosahedral capsid. The length of the capsid is approximately 60 nm in length. The diameter of the capsid appears to be greater than the length of the capsid, and is approximately 65 nm. The length of the tail sheath of ΦHabibi is roughly the same length as the 50 nm scale bar.

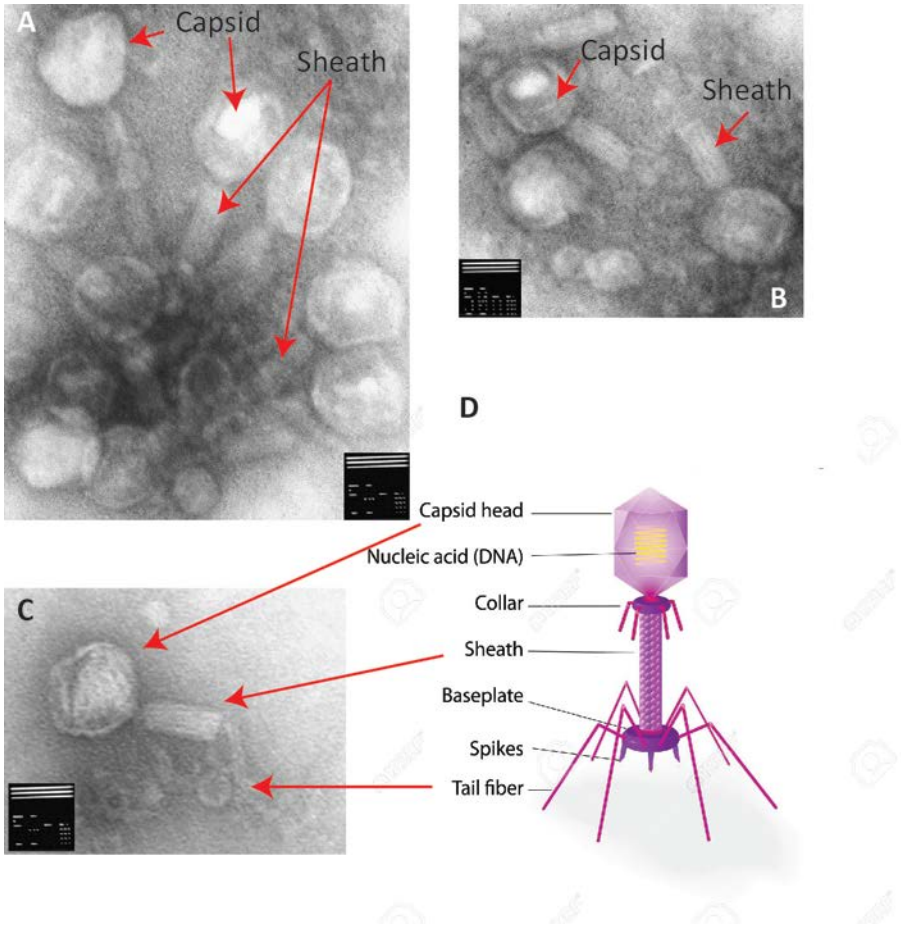

Figure 5. TEM Images of ΦHabibi. ΦHabibi is a myovirus. A-C: Transmission electron micrographs of ΦHabibi. D: Cartoon representation of a myovirus.

The tail observed in the TEM images then prompted us to investigate the type of tail present. Three PCR primers were designed for *Pseudomonas*-infecting phages as well as *Escherichia*-infecting phages typically found in freshwater environments given that ΦHabibi came from a freshwater environment. Table 2 lists the primers as well as the tail-associated gene that they were designed to amplify. After initially testing all three of the tail primers on ΦHabibi, the amplicon for the PB1-gp47 annotated tail protein successfully occurred at the initial annealing temperature of 52°C. In order to further examine if the other tail primers would anneal with ΦHabibi, the annealing temperature was lowered to 50°C. After this thermal cycling condition was applied, the PB1-gp39 primer pair also produced an amplicon of the correct size. The PCR primers for the lambda tail fiber gene failed to amplify at this lowered condition. Thus, ΦHabibi contains tail fiber genes that is similar to the for *Pseudomonas* phage PB1.

Gene	**Expected Amplicon (bp)**	**Primer Set (5' - > 3')**	**Annealing Temperature (^{o}C)**
PB1-gp39	776	GGACTCGCGCATATCCTG CTGGATGGCAGTGGTCAG	50
PB1-gp47	306	CATCCACGCCATGGTGAC CGAATCTGCTGCCTCCAG	52
Lambda tail fiber gene	746	GTGCCGACCACCTACGAC GCGATCGAGCGTTACCAC	50

Table 2. Targeted PCR of viral tail genes to infer putative taxonomy of ΦHabibi.

Growth of ΦHabibi in *Pseudomonas*

Because ΦHabibi maintained its fitness throughout the course of the experiment when isolated to the *Pseudomonas* host, we next wanted to quantify the virulence of this phage. ΦHabibi is very efficient at infecting and lysing *P. aeruginosa*. Compared to the

other *Pseudomonas* infecting phages, ΦHabibi is more efficient (results not shown). As Figure 6 demonstrates, between the 1st and 2nd burst (lyse of the host cell) ΦHabibi's 2nd burst is roughly two and a half times larger than the 1st burst indicative of either increased lytic potential or a large burst size (the number of progeny produced per infected host cell). If the burst analysis were to continue, I hypothesize that the burst would even get larger.

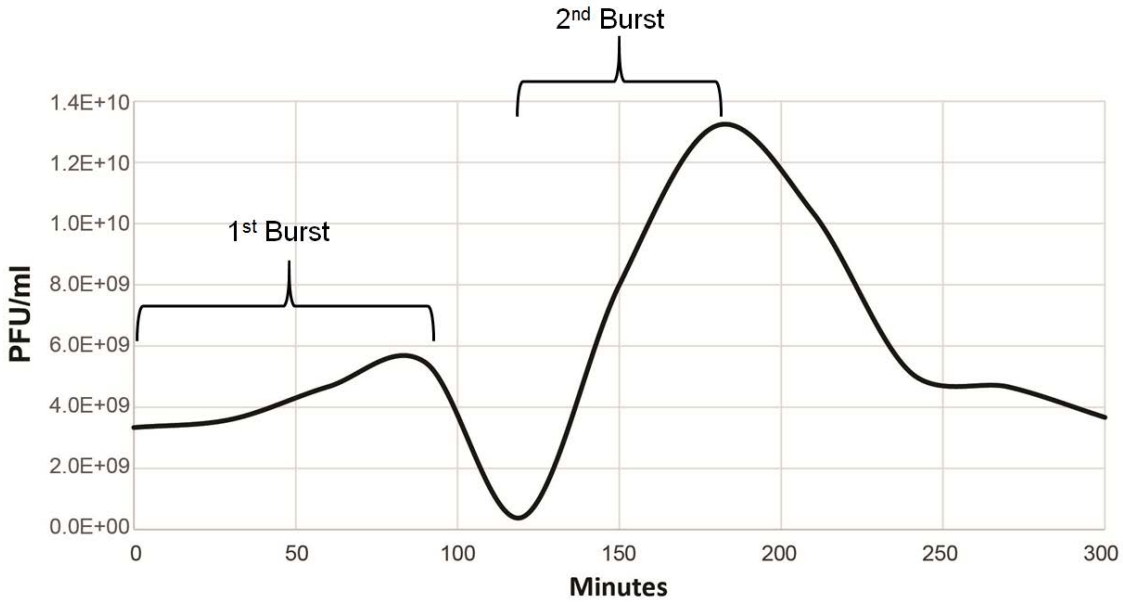

Figure 6. Burst Chart of ΦHabibi Growth in *Pseudomonas aeruginosa*.

Genome Sequencing, Assembly and Annotation

ΦHabibi was sequenced using the Illumina MiSeq platform. The genome was then assembled and annotated (see Methods). Because the PCR primers designed to recognize the PB1-like virus tail proteins were successful, we hypothesized that ΦHabibi was a closely related to the Pbunalikeviruses which includes Pseudomonas phage PB1. Thus, the complete genome of ΦHabibi was compared to PB1's genome (Figure 7). In

this figure, the top line represents the GC content for ΦHabibi. The top bar represents ΦHabibi, which has a genome size of 56 Kbp. The bottom bar represents Pseudomonas phage PB1, which has a genome size of 65 Kbp. The lines connecting the two genomes show the homologous relationship between the bacteriophages. (Two colors are used to differentiate between overlapping lines thus making it easier to follow the location when rearrangements occur.) As evident from this graph, ΦHabibi is likely a close relative of the PB1 virus which was isolated from sewage in Edinburgh, Scotland (Ceyssens *et al*. 2009), although there is significant genome rearrangement between the two.

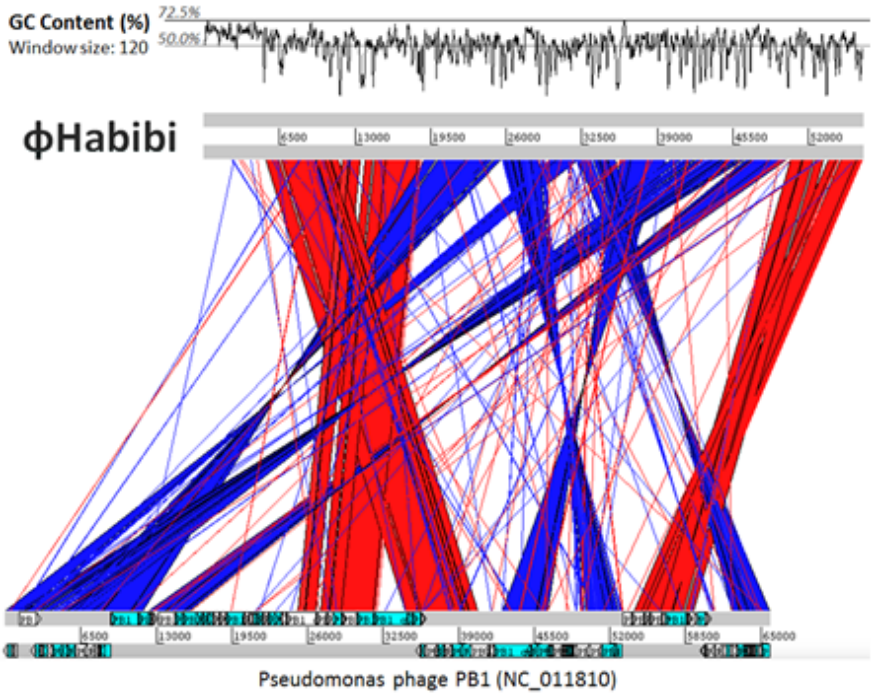

Figure 7. Genome Comparison of Assembled ΦHabibi and Pseudomonas phage PB1. Comparative genomics visualization produced by WebAct (Carver *et al*. 2008).

CHAPTER FOUR

CONCLUSIONS

Bacteriophages, or phages, are a vital entity in aquatic ecosystems, shown to drive the diversification of bacterial organisms as well as many nutrient cycling processes for which all life depends. By exploiting the generalist lifestyle of the ΦHabibi virus isolated in our lab, we were able to explore the cost and consequence of host-range, empirically rather than theoretically. Herein I have demonstrated that a true generalist can give rise to a specialist. Moreover, alternative replication lifestyles can be explored as phage evolve within the fitness landscape. Initially, there was no reduction in fitness for either of the Ec and Pa ΦHabibi lines. However, as the experiment progressed, the Ec ΦHabibi lines switched from an entirely lytic population to a pseudolysogenic lifestyle on *E. coli* C; the evolved strains also exhibited pseudolysogeny, albeit at a reduced fitness, within the *P. aeruginosa* host. The Pa ΦHabibi lines, in contrast, did not become either lysogenic or pseudolysogenic over the course of the selection experiment. Maintaining a virulent phenotype within the *P. aeruginosa* host population, the Pa lines became specialized, losing the ability to efficiently lyse *E. coli* C cells. While many studies have attempted to broaden the host-range of a laboratory viral pathogen, this is the first study to our knowledge which attempted the converse. In doing so, the experiment exemplifies that generalist bacteriophages do not have a uniform method for effective infection. Furthermore, in order to survive, the phage must readily adapt to the host it is infecting.

Both of these lines provide evidence that host-range is a readily adaptable trait; specialization and adaptations were observed in less than 400 generations. As the potential for emerging diseases becomes a worldwide concern, understanding the mechanisms in which a pathogen can specialize to host has far reaching impacts.

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