ASSESSING LIFE HISTORY TRADEOFFS IN HOST RANGE MUTANTS OF RNA BACTERIOPHAGE PHI6

By

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ABSTRACT OF THESIS

ASSESSING LIFE HISTORY TRADEOFFS IN HOST RANGE MUTANTS OF RNA BACTERIOPHAGE PHI6

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Studies on host range mutation and its effects on phage fitness are common for bacteriophage phi6. Expanding to a novel host can occur through a large number of mutations which provide a new resource for use typically at a decreased level of reproduction. Secondary mutations developed during evolution on the novel host can raise phage fitness but may also lead to specialization on that host. Again, the mutations that are beneficial on the novel host are usually detrimental on the initial host. Studying phage life history traits such as fecundity (reproduction rate and size) as well as competitive ability on the hosts may help determine where fitness is lost. Here I used host range mutants of phi6 that were previously evolved on *P. pseudoalcaligenes* East River Isolate A (ERA). I performed paired growth assays on original host *P. syringae* pathovar *phaseolicola* (PP) and burst assays on both PP and ERA to determine the effects of these mutations on the phage's reproductive ability. My selection of phages includes a

specialist on PP, a specialist on ERA, and generalists capable of infecting more than one host. Paired growth assays showed that two of the five mutants suffer a fitness cost relative to the wild type ancestor on PP. However, burst assays showed no significant difference between the wild type and evolved mutants on PP. Burst assays on ERA showed that several mutants improved their burst time and all mutants improved their burst sizes compared to their ancestor on that host. The evolved mutants were not able to separate themselves from one another. Mutants separated by one mutation showed significant effects from these differences. My results suggest that burst and fitness assays can be a component for assessing differences in phage life history but need to be supplemented with further experimentation.

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Table of Contents

List of Tables

List of Figures

Introduction

Viruses are a ubiquitous and versatile parasitic component of the microbial world capable of surviving and infecting organisms in most environments. Their small genomes, high replication rate, and high mutation rates make viruses an ideal system of study for changes in life history traits. These traits include: fecundity (number of progeny produced), lysis timing, life cycle style, and survival outside of the host cell (Dennehy and Turner 2004, Goldhill and Turner 2014). Mutations that affect these traits can have a profound effect on a parasites' fitness: ability to infect hosts, speed of reproduction, clutch size, and transmission of progeny to new hosts. It is important to study the evolution of these traits in the lab because new viruses can emerge in hosts we care about, such as humans, often without warning. Evolution of these traits can be studied in labs using human infecting viruses but there is a chance for creating an outbreak. It is safer to use bacteriophages, viruses that infect bacteria, because bacteriophages cannot infect humans. Much of the emergence of RNA viruses in novel hosts is due to their high mutation rate.

Previous studies have shown that both competition (Bono 2013) and host variation (Kassen 2002, Turner et al., 2012) are drivers of viral emergence on new hosts. These strategies work by promoting adaptation to a new host, however the successful emergence of a novel host is often accompanied by trade-offs the virus experiences on other hosts or environments (Dennehy et al., 2006, Duffy et al., 2006). During emergence events, most viruses maintain their ability to infect the original host. Organisms that can infect multiple hosts or species are called generalists (Legget et al., 2013). Although their

initial fitness on the novel host is usually low, in laboratory experiments, there is no competition for resources from parasites that are specialized on that host (Dennehy et al., 2006). During prolonged exposure to the novel host, secondary mutations occur and organisms that carry beneficial mutations which improve fitness are selected for within populations. Selection occurs because most point mutations can be deleterious in nature (Domingo-Calap et al., 2009). Over time, prolonged exposure to one host environment can lead to a fitness decline on the original host or specialization on the novel host (Duffy et al. 2007, Turner et al., 2012).

There is much debate concerning whether specialism or generalism is a better strategy for a pathogen (see Elena et al., 2009, Remold 2012 and Bedhomme et al., 2015 for reviews). On the one hand, specialists are perceived to have an advantage over generalists in any given single environment because they can optimize their fitness in that environment (Abedon and Culler 2007b**)** and outcompete all competition. However, it is more difficult for specialists to adapt to a new environment because transitioning to generalism might require mutations that might decrease fitness in their one niche.

On the other hand, generalists can infect and transmit in multiple environments. It has also been shown that generalists are more welcoming to adding additional niches (Turner et al., 2010). Generalists may carry antagonistically pleiotropic mutations that are beneficial in one niche but detrimental in another (Duffy et al., 2006, Bloom et al., 2010, Dessau et al., 2012, Alto et al., 2013, Goldhill et al., 2014). While this has been seen, studies have shown generalists that appear to suffer few or no costs compared to specialists do exist (Ketola et al., 2013) but these instances are rare. Maintaining a high

fitness in multiple environments is best generated through environmental fluctuation (Turner and Elena 2000, Crill et al., 2000, Turner et al., 2012). In one instance, a specialist was generated during variable exposure to a less productive resource (Jasmin and Kassen 2007).

Mutation in viruses has been shown to influence attachment (Bono et al., 2013, Ford et al., 2014), host range (Duffy et al., 2006, Duffy et al., 2007), thermal tolerance (McBride et al., 2008, Dessau et al., 2012, Alto et al., 2013, Goldhill et al., 2014), antiviral resistance (Bloom et al., 2010), and reproduction size (Dennehy and Turner 2004, Heineman and Bull 2007, Bruns et al., 2014). Any of these changes can influence fitness positively or negatively, and all will aproximately affect viral fitness by altering life history traits. Research using bacteriophage phi6 has delved into elucidating the effects of point mutations throughout the genome.

Phi6

Bacteriophage (phage) Φ6 (phi6) is a lipid coated double stranded RNA lytic bacterial virus and the type phage of the family *Cystoviridae* (Vidaver et al., 1973). Its lipid coating is derived from the host of phi6: the many pathovars of the gram negative plant pathogen *Pseudomonas syringae* (Laurinivicus et al., 2004). The phi6 genome is composed of three segments labeled Small (2948bp), Medium (4061bp) and Large (6374bp) (Mindich 2006). The genome contains 12 essential genes with different functions (Table 1). Packaging and replication of the phi6 genome was covered in a 2004 paper (Mindich 2004). Phi6 infects the bacterium by attaching to the host's type IV pilus using protein P3 (Mindich 2006), fusing to the host membrane when the pilus retracts

using P6 (Cvirkaite-Krupovic et al., 2010), then enters the host cell with help from P5 (Mindich 2006). This entry into the host cell is fostered by nucleocapsid protein P8 (Romantschuk et al., 1988, Poranen et al., 1999). The genome is replicated using the RNA dependent RNA polymerase which lacks proofreading ability. This inability to proofread contributes to the high mutation rate seen in phi6. This rate varies between 10^{-4} and 10^{-7} depending on the host on which the rate is viewed (Duffy et al., 2006, Ferris et al., 2007, Ford et al., 2014).

Segment	Protein	Function
Large	P1	Major structural protein
	P2	RNA Dependent RNA Polymerase
	P4	NTPase, genomic RNA packaging
	P7	Procapsid stabilization
	P14	Nonessential protein
Medium	P3	Attachment to Type IV pilus
	P6	Fusion to host membrane
	P10	Membrane protein needed for lysis
	P13	Minor membrane protein
Small	P5	Peptidoglycan lysin
	P8	Nucleocapsid shell protein
	P9	Major membrane protein
	P12	Morphogenic membrane protein

Table 1: Phi6 proteins
Proteins in the phi6 genome including the segment, name, and their function.
Source: Mindich 2004, 2006

Previous Research with phi6

Previous studies have examined the phage's ability to adapt to different environmental conditions. Experiments have examined adaptation to a novel host (Duffy et al., 2006, Duffy et al., 2007, Ferris et al., 2007, Bono et al., 2013), growth in a structured (Dennehy et al., 2007, Abedon and Culler 2007a) environment, high temperature (Dessau et al., 2012), and at variable multiplicities of infection (MOI)

(Turner and Chao 1998, Turner et al., 1999, Dennehy et al., 2004, Montville et al., 2005, Goldhill et al., 2014). Many of these experiments have shown that spontaneous point mutations are the most important factor in the phage's adaptation. These mutations allow the phage to expand its host range and infect other pathovars of *P. syringae* such as pathovar *glycinea* (Ferris et al., 2007, Bono et al., 2013) and in one case a distant relative in the genus *P. pseudoalcaligenes* (Turner and Chao 1998). Infection in *P. pseudoalcaligenes* is interesting because it does not have a type IV pilus to which the phage can attach (Bamford et al., 1987).

Several experiments have explored the phage's ability to mutate to infect novel hosts to determine the mutational landscape of the phage (Ferris et al., 2007, Ford et al., 2014). In one, they isolated 40 mutants and found 16 distinct mutations in the attachment protein. Phage fitness was assessed using several methods and it was determined that there was a trade-off in fitness on the ancestral host after mutating (Ferris et al., 2007). In the other experiment, 69 mutants were isolated with 17 different mutations at three main sites (Ford et al., 2014). Attachment assays were carried out here as well and showed it to be the main cause in differences in phage fitness.

Numerous experiments have shown that varying the virus to host ratio can influence fitness. An evolution experiment using high and low MOI, 5 and .002 respectively, illustrated that cheater phages develop at a high MOI. These cheater phages carry incomplete genes and become reliant on having helper phages when coinfection occurs (Turner and Chao 1998). This helps deleterious mutations persist in the population. These populations can undergo selection when evolved at a low MOI

(Froissart et al., 2004). Viruses evolved at a high MOI have also been shown to be less adaptive to new environments (Montville et al., 2005, McBride et al., 2008, Goldhill et al., 2014) and have a decreased fitness when out of their element (Dennehy and Turner 2004, Turner and Chao 1998). This loss of fitness comes from incomplete or defective proteins.

These experiments with varying MOI are also important because they help to determine the robustness of a population (Dennehy et al., 2013). Robustness is the ability of a population to adapt to its environment. A robust population is genetically diverse and can survive adverse conditions better than a less diverse population. Evolution can occur at a faster rate in robust populations (McBride et al., 2008, Goldhill et al., 2014).

Several papers have studied the effects of heat shock on brittle and robust phi6 populations (Goldhill et al., 2014). Phi6 was evolved at both high and low MOI for a set number of generations. For two of the papers, end point phages were subjected to mutation accumulation then exposed to either heat shock or grown at a high temperature to analyze adaptation and survivability (Dessau et al., 2012, Goldhill et al., 2014). In the most recent paper, phages were given five minute heat shocks daily and grown at standard temperature (Goldhill et al., 2014). Phages were analyzed genetically to determine how survival occurred. Sequencing showed that a single mutation in the peptidoglycan lysin changed one of the amino acids leading to stabilization of the protein at the higher temperature (Dessau et al., 2012). This mutation was antagonistically pleiotropic and decreased overall fitness at 25ºC. Antagonistic pleiotropy is common in thermally adapted mutants (Kashiwagi et al., 2014, Alto et al., 2013, McBride et al.,

2008, Ketola et al., 2013, Arribas et al., 2014). Overall, the robust lines adopted the lysin mutation quicker than brittle lines (Goldhill et al., 2014).

In my study, I used spontaneous and selected mutants of the RNA bacteriophage phi6 to explore the effects of host range mutations (both generalism and specialism) on the life histories of these RNA viruses. My strains differ from one another by multiple mutations resulting from up to 30 days of selection on a novel host. My strains contain a specialist for the original host, a specialist for the novel host and five strains that can infect both, which I term generalists. Of those generalists, there are two pairs that differ by only one point mutation. These singular differences allow for the direct connection between genotype and life history phenotype. Additionally, I knew that 30 days of exposure to a novel host improved phage fitness on that host, but it is rarely known how viruses improve their fitness in novel environments. I ascertained that replicate lineages gained fitness in comparable ways by increasing their burst size.

Methods

Bacterial Culturing and Storage

Pseudomonas syringae pathovar *phaseolicola* HB10Y (PP) [American Type Culture Collection 21781] (Duffy et al., 2006, 2007) was used as the standard laboratory host of bacteriophage phi6 and its host range mutants. *Pseudomonas pseudoalcaligenes* East River Isolate A (ERA) (Mindich et al., 1976) and *Pseudomonas syringae* pathovar *tomato* (Tomato) (Duffy et al., 2006) were used as alternate hosts for some of the mutants. Bacteria were cultured in 10 ml of LC broth (10 g/L Tryptone, 10 g/L NaCl, 5 g/L Yeast Extract, pH 7.5) (Mindich et al., 1976) in 50 ml flasks covered with a 25 ml

beaker. Fresh cultures were made by selecting a single colony from quadrant streak plates (LC + 1.5% agar). Bacterial stocks were archived in glycerol stored at -80ºC 4:6 glycerol/LC (v/v) in triplicate.

Phage Strains, Culturing and Storage

Phages used are descended from the original phi6 isolate (Vidaver et al., 1973). The wild type phage used in the lab is ATCC 21781-B1 (Duffy et al., 2006). Mutants were derived in previous experiments (Duffy et al., 2006, 2007). Briefly, wild type phi6 was grown on nonpermissive bacterial host Tomato and spontaneous point mutations in P3 allowed for infection. Ten single plaques, indicative of phage with host-range mutations, were isolated from this plate and stored in glycerol, named with a preceding "T" for Tomato (T1-T10). Paired growth assays were used to assess the fitness of the mutants on the original host, PP. Mutant T1, which was able to infect PP and the novel hosts Tomato and ERA, was selected for experimental evolution on novel hosts.

A high titer lysate of T1 was used to found four experimental lines that were grown for 30 consecutive days on ERA alone (Duffy et al., 2007). Lines were monitored for genetic and phenotypic changes, and over the 30 days, the lines developed new mutations which altered some of their host ranges. These new mutants were named for their experimental line (1-4), with an "e" for ERA, and for effects on their host range (Narrow [N], Wide [W]). Mutants with an unchanged host range do not receive a letter. Mutants from that experiment (Table 2) are utilized here for burst and fitness assays.

Phage	P3 Mutation	Host(s)	Ancestor
Phi6	*N/A*	PP	
T1	E8G	PP, ERA, Tomato	Phi6
1eN	T47S **(G247A)**	ERA	T1
1eW		PP, ERA, Tomato	T1
2e	G8K	PP, ERA, Tomato	T1
3eN	**A31T**	PP, ERA	T1
3eW		PP, ERA, Tomato	T1

Table 2: Genotypes used in the experiment
Wild type phi6 and its host range mutants. Mutations for phages with T1 as the ancestor have those mutations in addition to the E8G from T1. Mutations in bold confer a narrowed host range. The only mutation found on the Medium genomic segment of 3eW is in the untranslated region (t3338c), and 1eW had no mutations on the Medium segment relative to its immediate ancestor, T1. Host range of each mutant shown as: *P. syringae* pv. *phaseolicola* (PP), *P. pseudoalcaligenes* East River Isolate A (ERA), *P. syringae* pv. *tomato* (Tomato)
Source: Duffy et al., 2007

Bacteria Preparation

Bacterial stocks were stored at -80C. To prepare cultures, the glycerol stock was placed on ice then an inoculating loop was sterilized and used to scrape off enough of the stock to fill the loop. Quadrant streak plates were made and incubated at 25ºC for 48 hours. A single colony was selected from the quadrant streak and added to 10 ml LC in a 50 ml flask. Cultures were incubated overnight at 25ºC with shaking at 120 rpm. After 18 hours, overnights were considered ready for use.

Phage Preparation

Mutant phages were plated on their host of isolation from glycerol stocks stored at -20ºC. The required amount of bacteria to form an opaque lawn after overnight incubation (200ul for PP, 5ul for ERA, 100ul for tomato) was added to a tube of top agar. A platinum loop was sterilized and used to add ~1ul of the phage glycerol stock to the top agar. The mixture was poured on top of an LC plate then incubated overnight at 25ºC.

The following day, the top agar was scraped from the plate using a bent metal rod sterilized using 99% ethanol and added to a 15 mL centrifuge tube. 3 ml of LC broth was added to the plate, swirled on the plate surface and then poured into the same centrifuge tube. After vortexing, tubes containing top agar and broth were centrifuged for ten minutes at 3000 rpm. The resulting supernatant was syringe filtered through a 0.22 um filter creating a high titer lysate (~10^{10} plaque forming units [pfu]/ml). The lysate was then serially diluted to 10^{-8}. These dilutions were used to determine lysate titer on the host of interest. After overnight incubation (18-24 hours), plaques on the titer plates were counted and the results recorded. The lysate, its dilution series and the titers thereof were used to prepare dilutions for any experiments that followed for up to one week after initial preparation.

Bacterial Growth Curves

Overnight bacterial cultures were prepared and incubated at 25ºC for 24 hours, which ensured the bacteria were in stationary phase. 300 ul of the overnight culture was then added to 29.7 ml LC and incubated at 25ºC with shaking. 1:10 dilutions of the overnight cultures were also made and the absorbance observed at at 600 nm. Hourly for the next 12 hours, 30 ul of the growing cultures were used to prepare serial dilutions in three ml of LC and the absorbance of the growing cultures were measured. From these dilutions, spread plates of 10^{-5} - 10^{-8} were prepared. All plates were incubated at 25ºC for 48 hours. Single bacterial colonies were counted and results were graphed against time. Bacterial growth curves were performed in triplicate. This was done to delineate the

range of absorbances at which the bacterial cultures were exponentially growing for future experiments.

Phage Fitness Assessment on Original Host

Paired growth assays (Duffy et al., 2006, 2007) were used as a method of assessing phage fitness on PP. Phage competitions were produced by mixing approximately 10^3 pfu each of phi6 and a test phage, excluding 1eN, in a 2 ml microfuge tube. The volume was then increased to one ml by adding the appropriate amount of LC. Phages were tested by plating 100 ul of the mixture on a 200ul PP + 1ul ERA (200:1 PE) lawn and 150 ul on 200 ul of PP. Wild type phi6 is unable to grow on ERA, so it produces turbid plaques while mutants produce clear plaques on PE plates. Plaque counts were recorded the following day and phages were harvested from the PP plates after 24 hours of incubation. Phage lysates were prepared from the harvested plate and serially diluted to 10^{-6}. 5 ul was plated from the 10^{-6} dilution onto 200:1 PE. Plates were incubated overnight at 25ºC and values recorded. Fitness (W) was calculated as the ratio of clear to turbid plaques at the start (R_0) and end (R_{24}) of the experiment. This method allows evaluation of fitness on PP through phenotypic enumeration on PE plates. Fitness assays were carried out in triplicate using three independently prepared competitions for each test phage.

Phage Burst Assays

Each burst assay is conducted from a single mixture that is serially diluted into different flasks as the experiment progresses for ease of sampling and plating as phage concentration rises. For assays on PP, there were five 50ml flasks containing different

volumes of LC need to be prepared: two with 9.9 ml (for sampling at the 20 and 30 min timepoints), two with 10 ml (flasks A and C), and one with 9 ml (flask B). ERA assays used four flasks, as the lower productivity of phi6 on this novel host obviated the need for a flask C.

Overnight cultures of PP and ERA were diluted in duplicate 1:100 (100 ul in 9.9 ml) then used immediately or placed in the refrigerator at 4ºC for storage. PP dilutions were incubated at 25ºC for ~8 hours and ERA dilutions for ~6 hours, times determined from growth rate assays to ensure the bacteria would be in exponential growth. Diluted cultures were grown to an approximate concentration of $4x10^8$. The phage of interest was diluted to $4x10^5$ PFU/ml in a 5 ml snap cap tube. Sterile LC was then added to the tube to increase the volume to 1 ml. The tube was vortexed and 500 ul was added to a fresh snap cap tube.

At t = 0, 500 ml of exponential phase bacteria was added to the aforementioned 500 ml of $4x10^5$ phage making the concentrations in the tube $2x10^8$ and $2x10^5$ for bacteria and phage, respectively, for a MOI of 0.001. The tube was incubated with shaking in an Erlenmeyer flask. After 20 and 30 minutes, the falcon tube was removed from the incubator, vortexed, and 100 ul was added into the appropriate flask to dilute the phage mixture by 100-fold. The falcon tube was then returned to the incubator. 200 ul of PP or 5ul ERA from overnight cultures was added to top agar and 100 ul from the flask (a 10^{-3} dilution of the burst assay mixture) was overlaid on an LC plate. After 40 minutes, the falcon tube was removed from the incubator and diluted into the prepared flasks: 100 ul from the tube was added to flask A (10^{-2}), 1 ml from A to B (10^{-3}), and 100 ul from B

to C (10^{-5}). The three flasks were then incubated at 25ºC with shaking for the remainder of the experiment. Different dilutions were plated from this point forward with the experiment terminating after 200 minutes (Table 3A and 3B). All plates were incubated overnight at 25ºC and plaques counted the following day.

Table 3A: Bursts on PP

Time (min)	Dilution Rate			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
20	X			
30	X			
60	X			
80	X	X		
90	X	X		
100	X	X		
110	X	X	X	
120		X	X	
130		X	X	
140		X	X	X
150			X	X
160			X	X
170			X	X
180			X	X
190			X	X
200			X	X

Table 3B: Bursts on ERA

Time (min)	Dilution Rate		
	10^{-3}	10^{-4}	10^{-5}
20	X		
30	X		
60	X		
80	X	X	
90	X	X	
100	X	X	
110	X	X	X
120		X	X
130		X	X
140		X	X
150		X	X
160		X	X
170		X	X
180			X
190			X
200			X

Table 3: Plating protocol for burst assays on PP and ERA
The plating protocol used for burst assays conducted on PP (A) and ERA (B). The length of the experiment was 200 minutes. 10^{-3} corresponds to plating of 100ul from the 20 and 30 flasks as well as from the A flask. 10^{-4} is 100 ul from flask B, 10^{-5} is 10 ul from flask B and 10^{-6} is 100 ul from flask C. Plating was carried out until t = 200 minutes.

Statistical Analysis

T-tests and analyses of variance (ANOVA) were carried out using Microsoft Excel for all assays.

Results

Bacterial Growth Rate

Growth curves of three bacterial hosts were performed to establish bacterial concentrations at an approximate time or absorbance. Analysis provided equations which could be used to estimate the concentration at a given absorbance: PP CFU/ml = 2.6768x

+ 6.5591 R^2 = 0.95347, ERA CFU/ml = 3.9061x + 6.8329 R^2 = 0.76559, Tomato CFU/ml = 1.426x + 6.976 R^2 = 0.790 where "x" is the absorbance at 600 nm. The optical density was assessed intermittently at 600 nm to confirm population density. Growth curves showed that an optical density of between 0.60-0.65 is $4x10^8$ CFU/ml for PP and 0.45-0.50 is $4x10^8$ CFU/ml for ERA.

Paired Growth Assays

Paired growth assays were used to compare growth of mutant phages to the wild type as a means of assessing fitness on the original host *P. syringae* pathovar *phaseolicola*. The mean relative fitness of the mutants (Figure 1) all fall below the fitness of their ancestor, but only the fitness of T1 and 2e are statistically distinguishable from wildtype phi6 (one-tailed, one sample t-test, P = 0.025, 0.027 respectively). The mutation in P3 found in 2e has been observed in several previously characterized phi6 mutants, and the loss of mean fitness relative to the ancestor observed here is similar to previously published values (Duffy et al., 2006). Mutants 3eN and 3eW show fitness results very close to that of the wild type ancestor and one another (two-tailed t-test, P = 0.726), indicating that their difference in host range on Tomato does not impact their fitness on PP. Excluding 2e, evolution on ERA appears to qualitatively increase fitness on the unselected host compared to their ancestor T1.

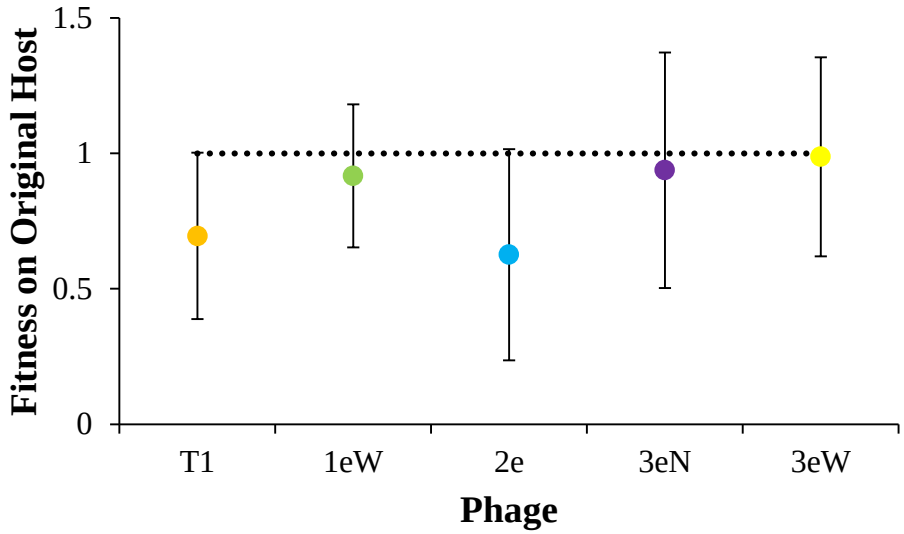

Figure 1: Fitness of Host Range Mutants on the Original Host
Mean fitness of the five host range mutants which can use both novel hosts Tomato and ERA. Wild type phi6 fitness is illustrated by the dotted line. Error bars show 95% confidence intervals.

Burst Assays on PP

Burst assays on two hosts were used to assess whether there was a difference in the average burst size or burst period length at a given bacterial concentration. Burst size was measured as the difference between the initial concentration of phage (log 10 PFU) and the concentration of phage after growth leveled off after one burst, and the burst length was measured as the time between the last time point when the phage was at the initial concentration and the first time point when it was at the final, stable concentration.

Burst lengths for mutants were qualitatively longer than the wild type on PP (Figure 2) but an ANOVA shows little difference among all genotypes tested ($P = 0.137$, d.f. 5, $F = 2.094$). A comparison of the two ancestral phages, phi6 and T1, suggests thatT1 has a somewhat longer burst time (one-tailed t-test, $P = 0.061$).

There is also no significant difference between the burst size values based on an ANOVA (P = 0.135, d.f. = 5, F = 2.107). Mean values for these data (Figure 3) show that, overall, the lines are similar. 3eN has a larger burst size than the isogenic 3eW, indicating that the one nonsynonymous mutation between them (A31T in P3) confers a measurable phenotypic effect (one-tailed t test, P = 0.071).

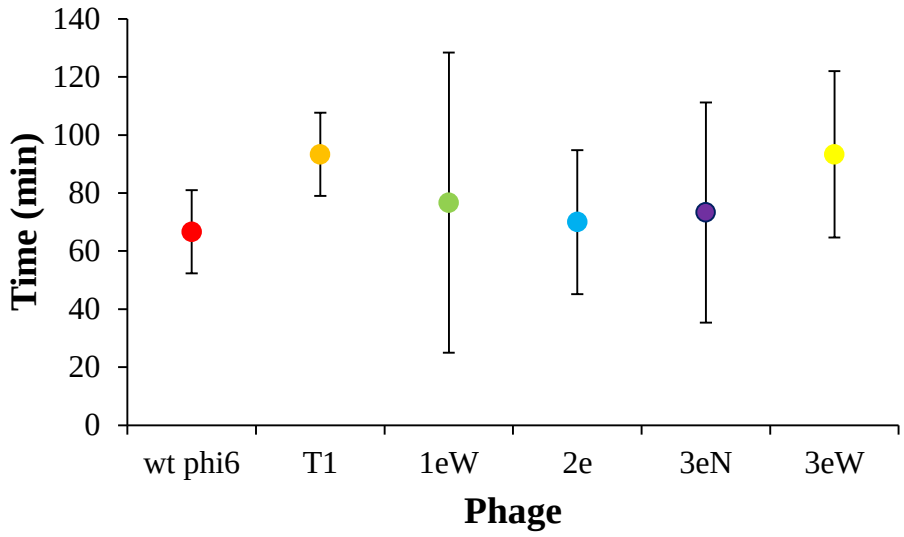

Figure 2: Burst Length on PP
Average burst length of phages capable of growth on PP. Error bars represent 95% confidence intervals calculated from triplicate measurements.

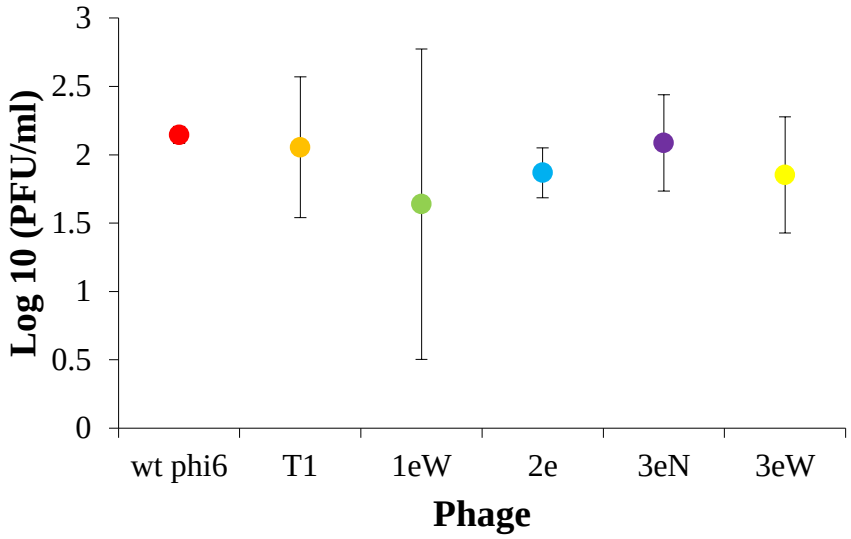

Figure 3: Burst Size on PP
Average log burst size of six phages capable of utilizing PP. Error bars represent 95% confidence intervals calculated from triplicate measurements.

Burst Assays on ERA

Assays were performed on ERA similarly to those on PP with a major difference being including the ERA specialist 1eN and excluding wildtype phi6, which cannot infect ERA. 1eN had the shortest burst time of all phages on ERA (Figure 4), especially in comparison to the wider host range isolate from the same lineage, 1eW (one-tailed t test, P = 0.0151). Similarly, 3eN has a somewhat shorter burst length than 3eW (one-tailed t test, P = 0.0569). An ANOVA run on the group as a whole shows that there are differences overall among the isolates in burst length (P = 0.0227, d.f. = 5, F = 4.008).

The ancestor T1, which had not previously been exposed to ERA, has the lowest burst size (Figure 5) with all its evolved descendants showing improvement (P = 0.0024, d.f. = 5, F = 7.236). However, a comparison of the mutants without T1 present (P = 0.1579, d.f. = 4, F = 2.084) suggests that there are no significant differences between the

evolved lineages. This is further supported by t-tests performed between each of the narrow and wide mutant pairs. Both 1eN vs 1eW (two-tailed t-test, P = 0.605) and 3eN vs 3eW (two-tailed t-test, P = 0.362) show no significant differences between their burst sizes. Therefore, while evolution on ERA led to increased burst size in all three lineages, the success of the narrowed host range genotypes 1eN and 3eN over their wide host range counterparts appears to be in shortening burst length and not in further increasing burst size.

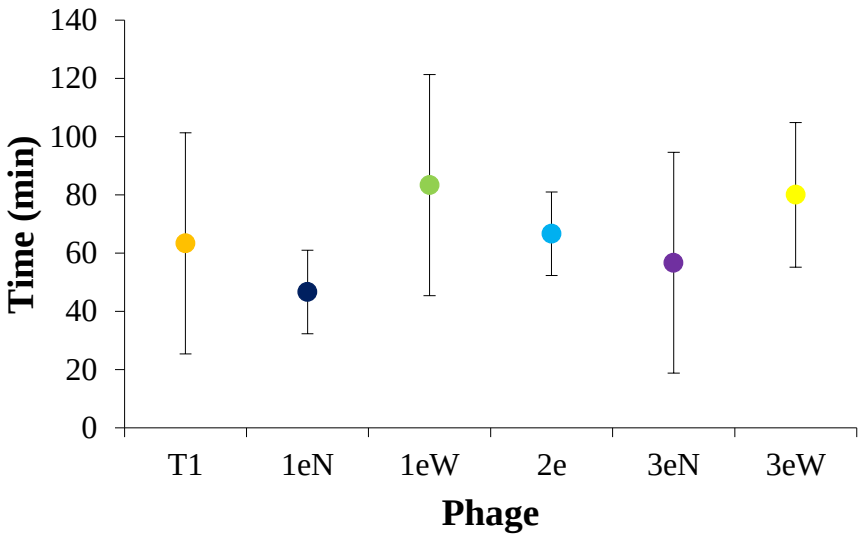

Figure 4: Burst Length on ERA
Average burst length data for assays performed on ERA. Error bars represent 95% confidence intervals calculated from triplicate measurements.

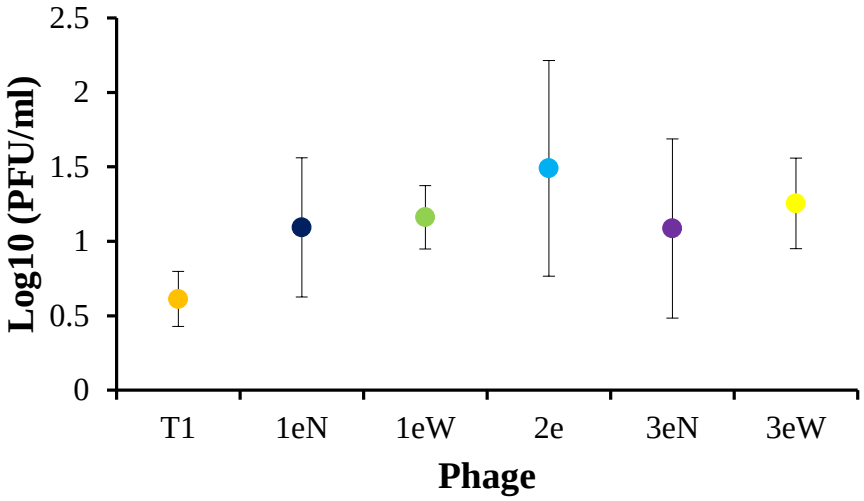

Figure 5: Burst Size on ERA
Average burst size of three lines for mutants that can grow on ERA. Error bars represent 95% confidence intervals calculated from triplicate measurements.

Discussion

My experiment assessed the reproductive time and output of host range mutants of phi6. I also assessed mutant phage ability during direct competition with the wild type. While I confirmed that carrying expanded host range mutations usually lowers the fitness of phi6 on its usual host, PP, I further dissected the costs. Results show that both fecundity decreases and replicative time extends when phi6 carries mutations that expand their host ranges. Assays on the novel host suggest that the secondary mutations that occurred during prolonged exposure to the novel host boosted fecundity on the novel host, with all mutants outperforming their T1 ancestor. The phage specialized on the novel host showed the shortest burst time of all mutants on the novel host suggesting a potential advantage during direct competition on that host.

Fitness on Original Host

Paired growth assays (Chao 1990, Turner and Chao 1998, Duffy et al., 2006, 2007) have frequently been used in phi6 as a method of examining a phage's ability when exposed to competition. These fitness assays integrate a large number of phage phenotypes over the several rounds of phage bursting that occur during overnight growth. My burst assay experiments can tease apart different parts of the phage phenotype underlying fitness losses and gains. Previous experiments (Ferris et al., 2007, Shao and Wang 2008, Bono et al., 2013) have shown that attachment rate is an important factor during competition as the phage that attaches faster is more likely to have a shorter lysis time (Shao and Wang 2008). However, this factor is difficult to measure independently in phi6, as it is statistically indistinguishable from zero (Ferris et al., 2007). My results show that mutations in the host attachment protein P3 do affect fitness, but to varying degrees. This antagonistically pleiotropic effect was previously shown for T1 and other mutants (Duffy et al., 2006), including for those with a lysine in the 8^{th} residue in P3 seen in 2e (Duffy et al., 2006, Duffy et al., 2007, Ferris et al., 2007, Ford et al., 2014) carries a substantial fitness cost during competition. On the other hand, the three genotypes that had statistically indistinguishable fitness on PP relative to the wild type phi6 fitness support the concept that generalism does not always come at a fitness cost (Duffy et al., 2006). Though these lineages were descended from T1, which had a lower fitness on PP, the secondary mutations they fixed during prolonged exposure to ERA increased their fitness on the unselected host PP. Increased fitness in the original environment is usually attributed to compensatory mutations (Bloom et al., 2010, Turner et al., 2012) or

reversions that occur after growth in that original environment (Burch and Chao 1999). The mutations here appear to be compensatory, perhaps stabilizing the mutant P3 proteins, even though they were not selected during replication on PP.

Effects of Mutations on Life History Traits

Burst assays were used to assess the replication time and progeny produced lysed. Evolutionary biologists are very familiar with measuring analogous values in macroscopic organisms: time to sexual maturity and clutch size. If we consider lytic phage to be semelparous organisms, burst length and burst size are the life history traits of phi6.

Previous studies have shown that burst (Dennehy and Turner 2004, Wang 2006) or growth assays (Heineman and Brown 2012) are reliable indications of phage fitness at a specific host concentration, and these values are mostly used as proxies for overall fitness. However, measuring fecundity and time to reproduction separately allows us to dissect phage phenotype on a finer level than just relative fitness, and can help determine the functions of mutations fixed during adaptation to a novel host.

I used a low MOI of 0.001 to limit the possibility of coinfection which may select for mutants with a shorter latent period (Abedon et al., 2003, Dennehy et al., 2007). Phages with a shorter latent period may replicate faster and infect more of the available bacteria within a plaque (Dennehy et al., 2007, Abedon and Culler 2007b, Garcia-Villada and Drake 2013) thus limiting the resources of others during competition. My burst assay results suggest the PP specialist phi6 and ERA specialist 1eN gain their fitness advantages by shortening their burst times; both phages tend to reproduce faster than the

generalist mutants on their respective hosts. While these specialists outperformed the generalists on their respective hosts in the burst assays, all ERA-evolved genotypes performed better than T1 on ERA. Prolonged exposure to one host is well known to lead to increased viral fitness on that host, due to natural selection (Turner and Elena 2000, Duffy et al., 2007, Agudelo-Romero et al., 2008). The genotypes show no significant difference in burst size when compared to one another suggesting that they are near an optimal clutch size on ERA, though fitness gains still appear possible through shortened burst lengths (e.g., 1eN vs 1eW, 3eN vs. 3eW).

It was expected that 1eN would perform better than the mutants on ERA because it can only utilize the one resource, and its host range narrowed during evolution on ERA, under selection for better performance on ERA but no direct selection on host range. This means selection could fix mutations that raise its fitness on the one host even at the expense of fitness in other environments. Generalist phages are considered to be at a disadvantage because it is more difficult to adapt to a new host while maintaining fitness in other environments. Beneficial mutations on ERA may lead to a big fitness loss on another host in the range of these generalists. Specialist 3eN and generalist 3eW diverged on the same experimental line and differ by one nonsynonymous mutation. While their burst sizes are similar, the burst time for 3eN is shorter than that of 3eW, showing a clear link between a single mutation and an effect on a life history trait. The improved ability most likely stems from improved attachment to the host allowing more efficient infection (Ford et al., 2014). The cost of this mutation is that 3eN can no longer infect the host Tomato, but that cost is not realized during replication on ERA or PP. 1eN suffers a

larger trade-off, as it cannot infect any host other than ERA. While it is significantly faster at bursting than its 1eW counterpart, there are several mutations that differ between these genotypes and no single mutation can be unambiguously identified as being responsible for the reduced burst length.

Further experimentation is needed to assess the effects of more individual mutations on life history traits. Future experiments could also utilize a variety of phage to bacteria MOIs in an effort to examine life history traits under different environmental conditions.

Conclusions

I have demonstrated that phages can gain fitness on an unselected host (their original host PP) without exposure to that host. I have also showed that burst size and time assays can assess changes in virus life history traits, and these phenotypic changes can be associated with their genetic basis. Burst assays show that there is little difference between the wild type phage and ERA-evolved host range mutants on their original host, though the ancestral host range mutant T1 had a lengthened burst time. Additionally, the evolved genotypes improved their performance on the novel host compared to the unevolved ancestor by increasing their average burst size (fecundity). Further fitness increases among the ERA-evolved lineages on ERA occurred subsequently through shortening of the burst time.

Appendix

Burst Data on PP

Phage	Burst Length (t)	Burst Size
Phi6	70	2.13
	70	2.13
	60	2.17
T1	100	2.17
	90	2.18
	90	1.81
1eW	100	1.48
	60	1.29
	70	2.15
2e	80	1.88
	70	1.94
	60	1.79
3eN	90	2.04
	70	1.97
	60	2.25
3eW	100	1.69
	80	1.83
	100	2.03

Table 1: Size and Time values for all burst assay lines on *Pseudomonas syringae* pathovar *phaseolicola* (PP)

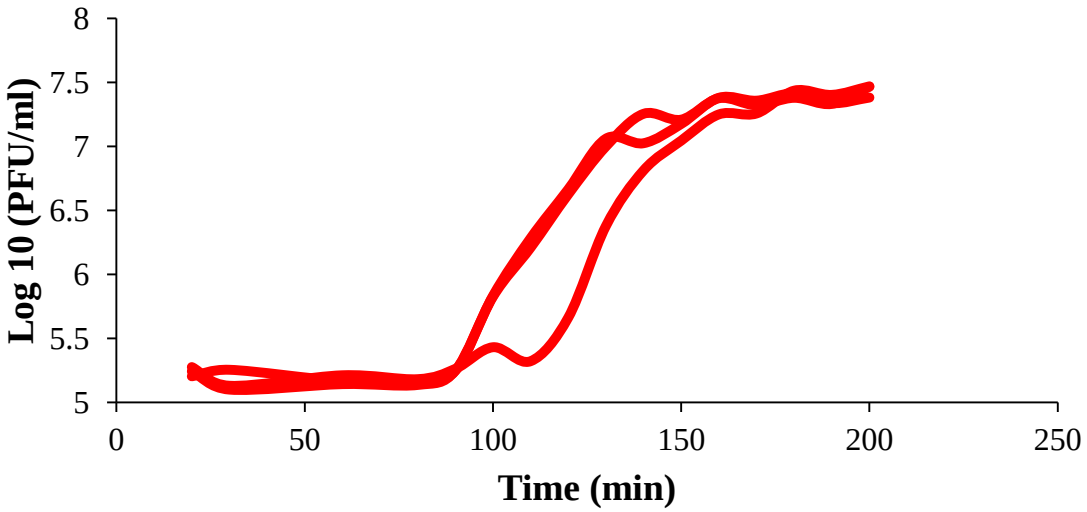

Figure 1A: Burst results for wild type phi6 on PP

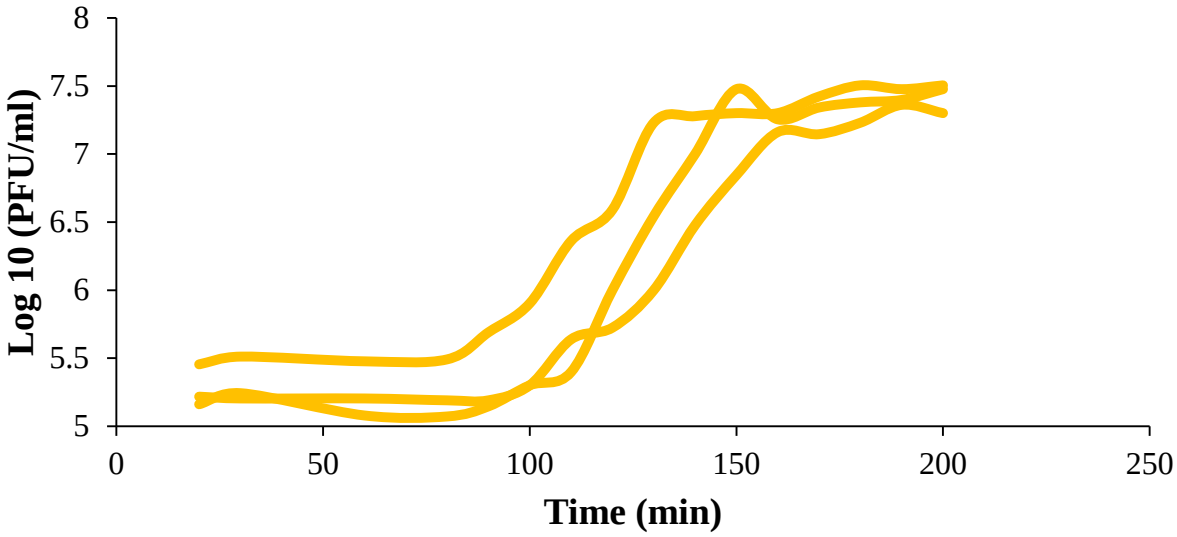

Figure 1B: Burst results for T1 on PP

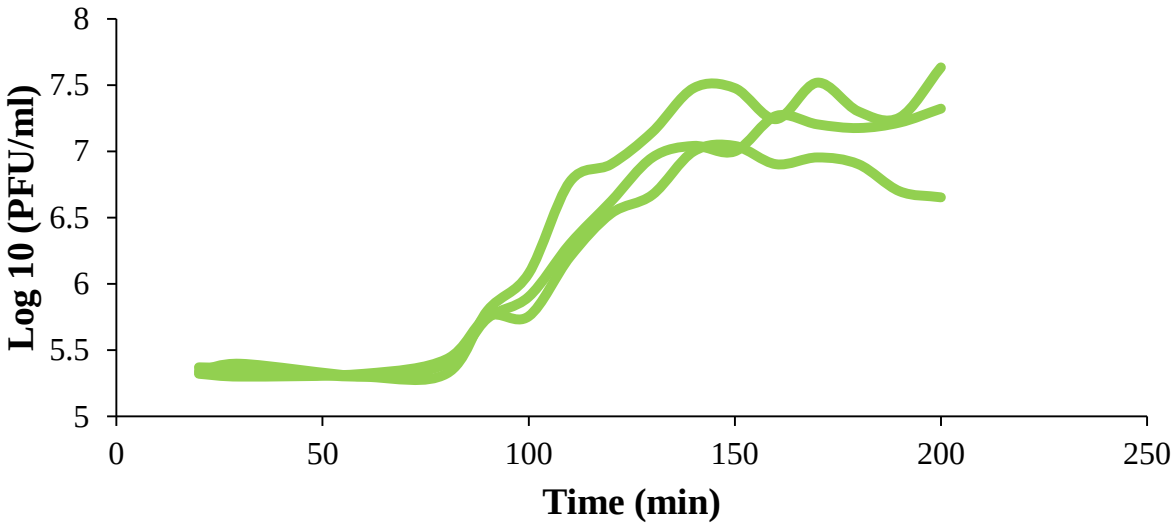

Figure 1C: Burst results for 1eW on PP

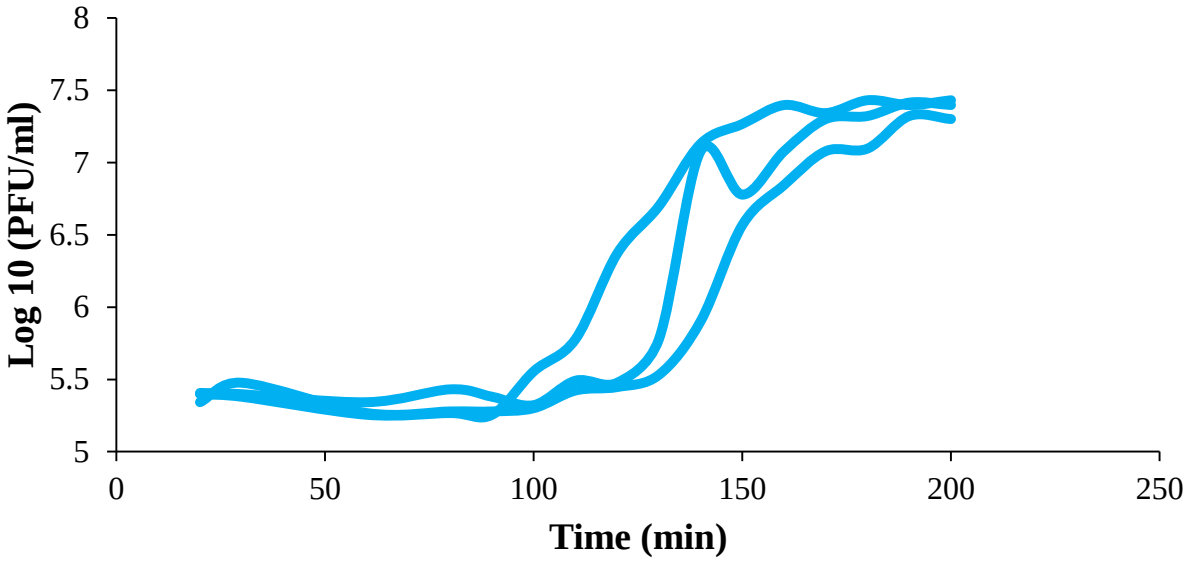

Figure 1D: Burst results for 2e on PP

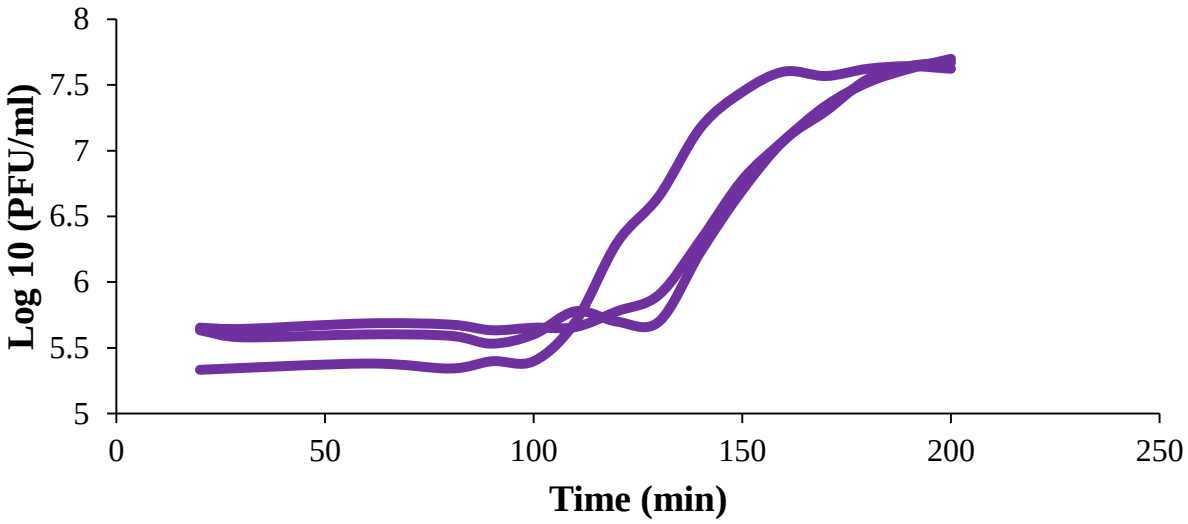

Figure 1E: Burst results for 3eN on PP

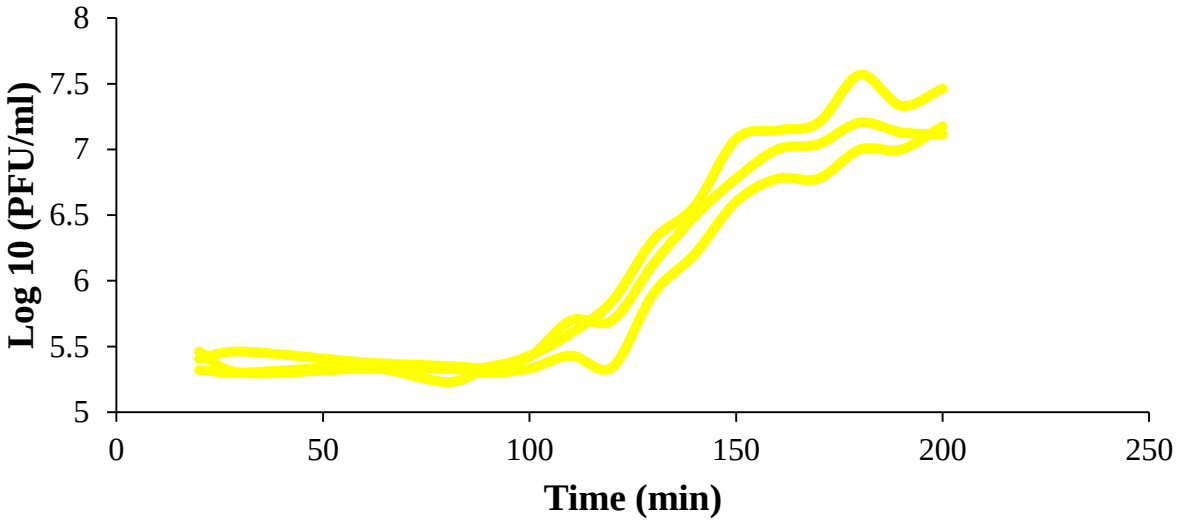

Figure 1F: Burst results for 3eW on PP

Burst Data on ERA

Phage	Burst Length (t)	Burst Size
T1	60	0.56
	80	0.58
	50	0.70
1eN30	50	1.04
	40	0.93
	50	1.30
1eW30	100	1.07
	70	1.18
	80	1.24
2e	60	1.16
	70	1.71
	70	1.60
3eN	60	1.08
	70	1.33
	40	0.85
3eW	90	1.12
	80	1.30
	70	1.35

Table 2: Burst length and log 10 burst size for phages on *P. pseudoalcaligenes* East River Isolate A. Burst length is the difference between the start and end of the burst period. Burst size is the difference between the log 10 of the titer at the start and end points.

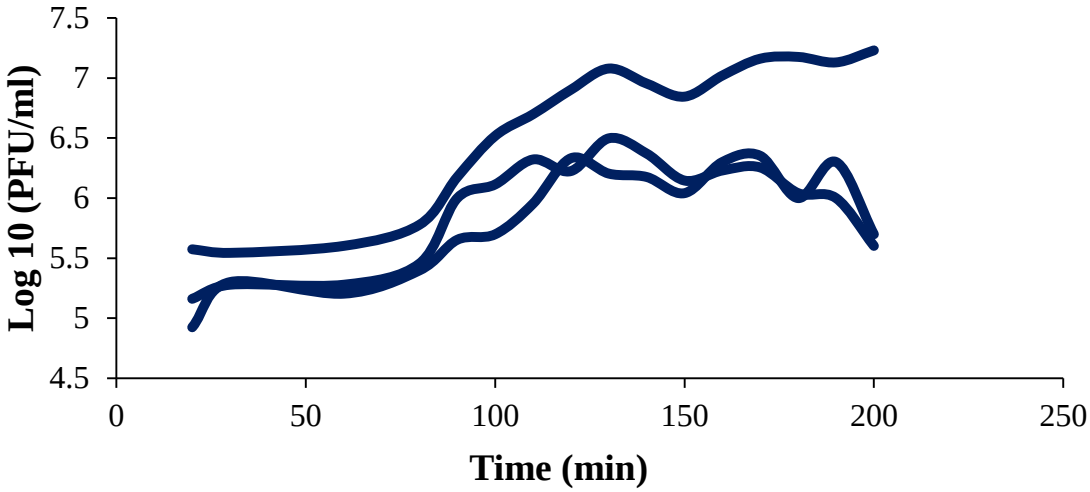

Figure 2A: Burst results for 1eN on ERA

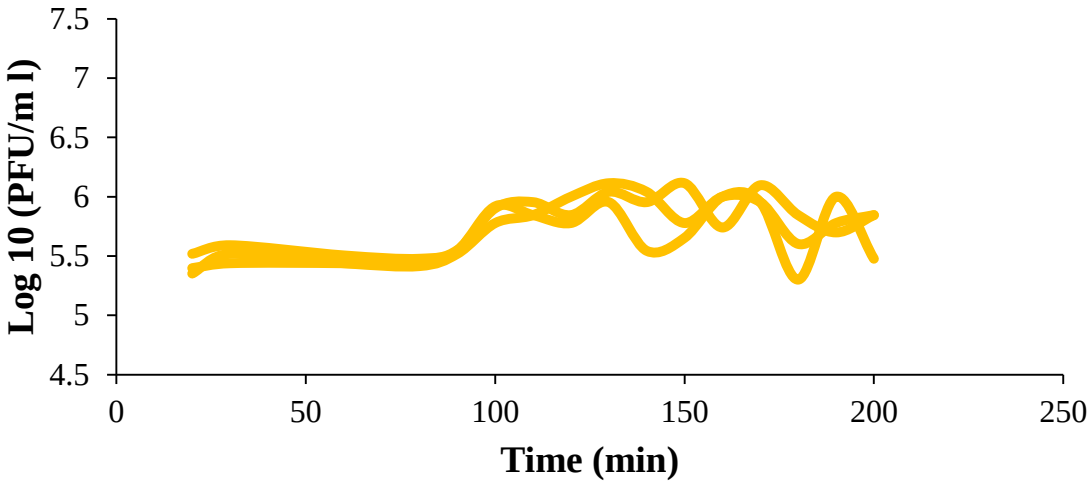

Figure 2B: Burst results for T1 on ERA

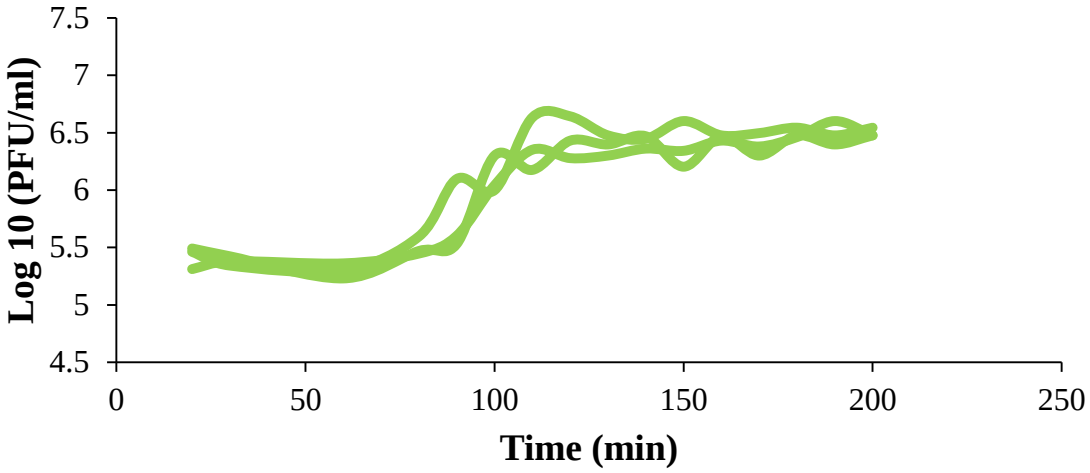

Figure 2C: Burst results for 1eW on ERA

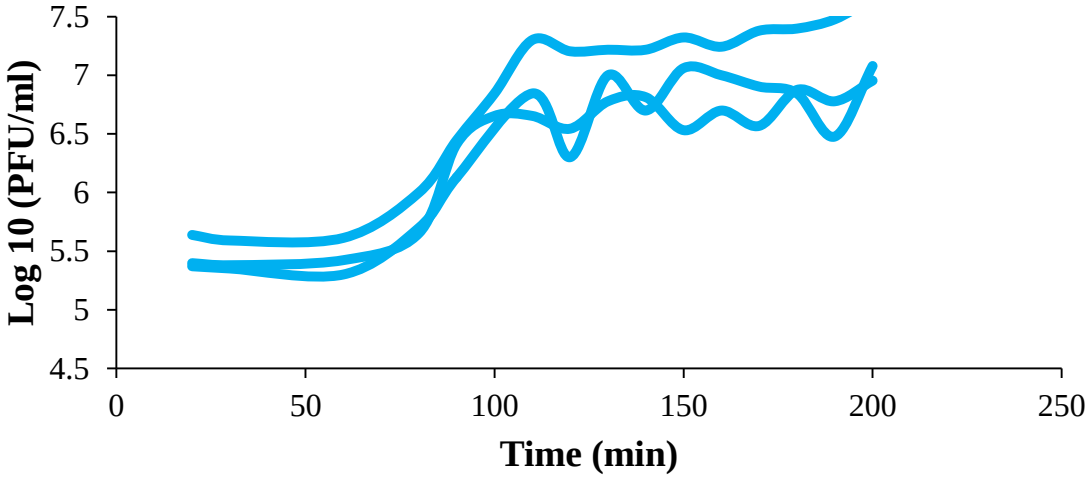

Figure 2D: Burst results for 2e on ERA

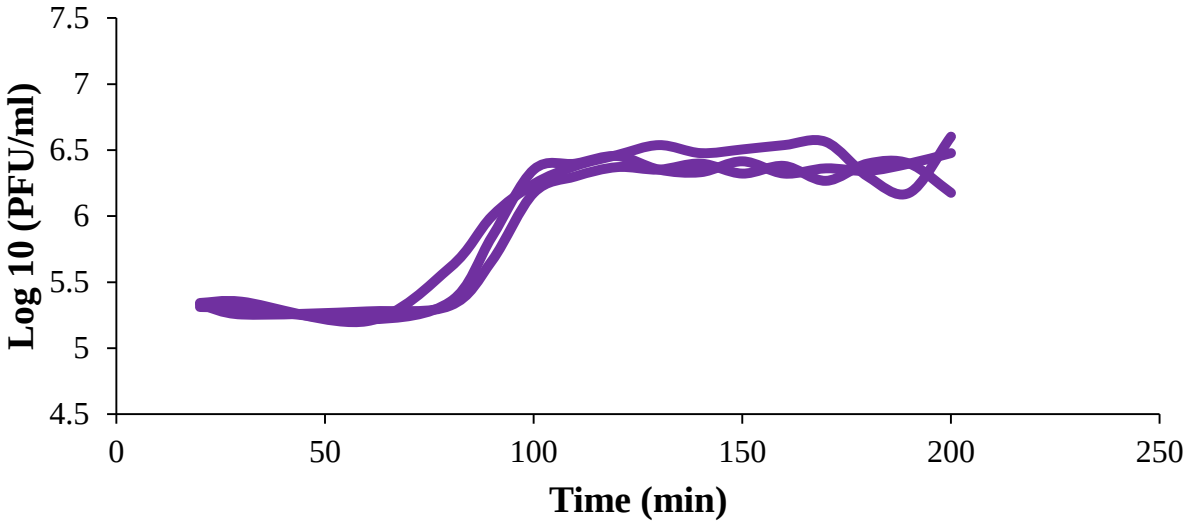

Figure 2E: Burst results for 3eN on ERA

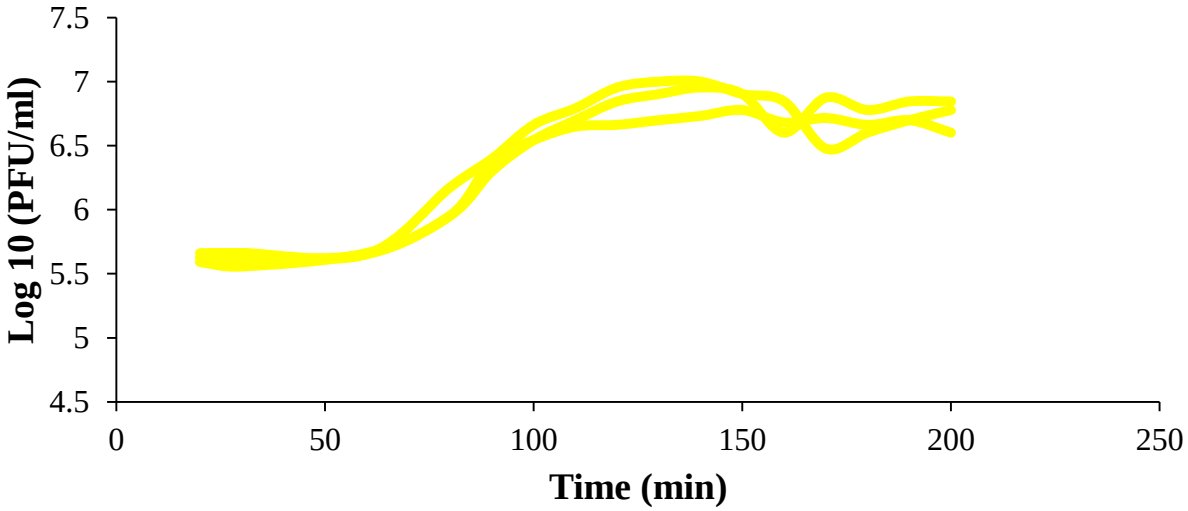

Figure 2F: Burst results for 3eW on ERA

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