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## Effects of Differing Environmental Conditions on Bacteriophage Jenika

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## Abstract

The dynamic role of bacteriophage in different environmental areas poses important questions about viral interactions with and control of bacteria. Bacteriophage play a vital role in the evolutionary track of bacteria. The evolutionary role of bacteriophage is affected by lysogenic conversion, transduction, mediated gene transfer, and the exertion of selective pressures. The objective of this research was to determine if there were changes in growth of the bacterial host *Mycobacterium smegmatis* and its bacteriophage *Jenika* given isolated changes in environmental conditions. Growth of the host, *M. smegmatis*, was documented using changes in optical density and growth of one of its bacteriophages, *Jenika*, was documented using viral titer. Additionally, changes in bacteriophage morphology were documented using electron microscopy. There were differences in the rate of growth of *Mycobacterium smegmatis* among different temperatures. Specifically, the *M. smegmatis* culture grown at 25° C showed significantly less growth than all other temperatures. However, despite these differences, all cultures began the exponential phase at approximately hour 15 after inoculation. The titer of *Jenika* was also affected by temperature as a result of the host changes; it showed a shift in the time necessary to start its exponential growth. Temperatures below the standard growth temperature showed a one-hour shift in when the exponential phase started. The electron microscopy showed similar tail lengths and head diameters for isolated phage. However, given the low statistical power of the sample size, an ANOVA was not performed on the results. The electron microscopy work should be repeated in the future with the goal of obtaining measurements on a larger sample size to increase statistical

power. The results presented here suggest that changes in temperature may have an effect on the growth of the bacterial host and its bacteriophage's ability to attach to and/or infect its host.

MONTCLAIR STATE UNIVERSITY

Effects of Differing Environmental Conditions on Bacteriophage *Jenika*

by

Gerard Nasser

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

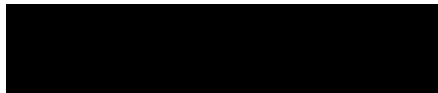
Master of Science

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College of Science and Mathematics

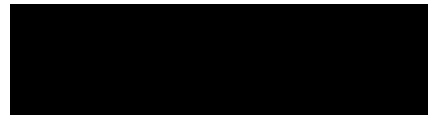
Thesis Committee:

Department of Biology



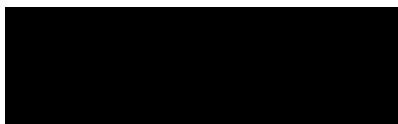
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Effects of Differing Environmental Conditions on Bacteriophage *Jenika*

A Thesis

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Montclair, NJ

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## CHAPTER ONE

### INTRODUCTION

The dynamic role of bacteriophage in different environmental conditions poses important questions about the virus' interactions with and control of their bacterial hosts. Bacteriophage play a vital role in the evolutionary track of bacteria (León & Bastías 2015). The evolutionary role of bacteriophage is affected by lysogenic conversion, transduction, mediated gene transfer, and the exertion of selective pressures (León & Bastías 2015 and Ogunseitán et al. 1990). Bacteriophages are also central in the control of bacterial populations (Yu et al. 2017). Studies have shown how they infect and replicate in ways that drive changes in bacterial populations environmentally, such as recent studies showing populational culling of *V. cholerae* as a result of bacteriophage increases (Silva-Valenzuela & Camilli 2018). These studies support the idea that on a microbial level, bacteriophage drive many aspects of bacterial communities in the environment.

Bacteriophages are central not only for bacterial evolution but also in the shaping of ecosystems (Silva-Valenzuela & Camilli 2018). For instance, humans interact with various microbes, either in ways that are beneficial, neutral, or harmful. Studies have investigated the medical and industrial aspects of these relationships in detail, as well as the relationships between bacteriophage and environmental conditions within organisms and natural and man-made environments. One such study, conducted by Fisher et al. (2016), investigated the environmental effects on P100 bacteriophage as a control mechanism for *Listeria monocytogenes*. However, there seems to be a gap in the ecological and environmental knowledge of naturally occurring bacteriophage in

changing conditions. After investigating the literature revolving around bacteriophage, it appears that there are no published studies on how environmental variations in natural soil ecosystems can affect bacteriophage and their bacterial hosts. Given consistent increases in global temperatures (Banerjee & Sharma 2021), it is important to understand how these changes will affect bacteriophage.

Changing environmental factors around the globe have begun to affect many aspects of life on Earth. These changing environmental factors include temperature changes and the availability of nutrient resources. The global temperature has risen 1.8°F from 1901 – 2016 and is predicted to continue rising (USGCRP 2018). Nutrient availability is also changing as a response to climate change, and a recent example of this has been documented in a forest ecosystem. Forests, which are important carbon sinks, can start to become carbon sources as a result of increased temperature, droughts, and other climate changes (Jansson & Hofmockel 2020). Given that soil microbes have important roles in nutrient cycles, they have been seen to respond to these climate and nutrient changes, including decreases in abundance and diversity as a result of the shifts in nutrient availability (Freedman et al. 2015, Frey et al. 2014, Llado et al. 2017, Wu et al. 2015). There are still questions that lie in how the environment plays a role in the bacteriophage itself (such as in biochemistry, gene expression, growth/replication rate and morphology). These questions pose importance for environmental studies because any changes in the bacteriophage due to environmental conditions may affect how the bacteriophage interacts with its host or the bacteriophage's ability to infect its host.

This study investigated the effects of temperature change on the growth of a soil-dwelling bacteria, *Mycobacterium smegmatis* (*M. smegmatis*) as well as one of its

bacteriophages, *Jenika*. This study was done to gain an initial understanding as to how environmental stressors of the host translate to the bacteriophage. *M. smegmatis* is a non-pathogenic bacterium that is used as a model to identify possible bacteriophage that could be used to treat the congeneric pathogen *Mycobacterium tuberculosis* (Fujiwara et al., 2012). *Jenika* was isolated by Jessica Eucker and Nikita Patel from a soil sample as part of the Howard Hughes Medical Institute's SEA-PHAGES program at MSU. *Jenika* is a clone of the bacteriophage ShiVal, isolated by Shivani Patel and Valerie Paschalis as part of the Howard Hughes Medical Institute's SEA-PHAGES program at MSU. The specific goals of this study were to investigate the effects of different temperatures on the growth rates of *M. smegmatis* and *Jenika*, and to determine the effects of these temperatures on the morphology of *Jenika*. Understanding how these conditions affect bacteriophage can help lead to a more comprehensive understanding on how long-term changes in environmental conditions can take its toll on soil microbiomes and affect the role bacteriophage play in them.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 *Jenika* Stock Culture

In order to create a stock culture of *Jenika*, a 2.5 $\mu$ l initial high lysate titer was collected using previously acquired lysate stored at 4°C. In order to propagate the bacteriophage, a web plate was made on a Luria agar plate using 3ml top agar, 250  $\mu$ l of a saturated *M. smegmatis* culture, and 2.5 $\mu$ l of *Jenika* lysate. The top agar was made with 20 mL of 2X TA (Top Agar), 20 mL of 7H9 Neat and 1ml of 0.1M CaCl<sub>2</sub>. To prepare the top agar, both the 2X TA and 7H9 Neat were brought to the same temperature of 50°C. Once the 2X TA and 7H9 Neat were at equal temperature, 20 $\mu$ l of each were added to a 50 mL conical tube. After incubation for 24 hours at 37°C, the plate was flooded with 3mL of 1X phage buffer. The 1X Phage buffer consists of 10 mL 1M Tris at pH 7.5, 10 mL 1M MgSO<sub>4</sub>, 4g NaCl, and 980ml of ddH<sub>2</sub>O, with the addition of 1ml 0.1M CaCl<sub>2</sub> before use. The plate was incubated for 4 hours at room temperature to maximize bacteriophage concentration, then filtered through a 0.22  $\mu$ m syringe filter to isolate purified bacteriophage. The titer was then determined by calculating the plaque forming units per milliliter of culture (pfu/mL). The pfu/mL is calculated by taking the number of plaques counted at the highest dilution, divided by the amount plated in milliliters times the dilution. The titer of the final stock using these calculations was 2x10<sup>10</sup> pfu/mL.

#### 2.2 *Mycobacterium smegmatis* Growth Curves

The growth curves of *M. smegmatis* were assessed at 22, 25, 37, and 40°C by measuring the optical density of cultures hourly using a GENESYS 20 spectrophotometer by Thermo Scientific over the course of 24 hours. A 50ml flask of 7H9 complete media

was made using 44.5mL of 7H9 Neat, 5ml of AD supplement, 0.5ml of 100 $\mu$ M CaCl<sub>2</sub>, and 5 $\mu$ l of carbenicillin (CB) and cycloheximide (CHX). CB and CHX are an antibiotic and antifungal, respectively. The 7H9 complete media was then inoculated with 250 $\mu$ l of *M. smegmatis* from a previously made saturated working culture grown at 37°C. The cultures were incubated at their appropriate temperatures in a shaker at 225 RPM. The first curve generated was the standard at 37°C, the optimal temperature for *M. smegmatis*. 7H9 complete media was used as a blank for all spectrophotometer measurements for this portion of the study. At each time point, one mL of the bacterial culture was removed and added to a 1ml plastic cuvette and measured at a wavelength ( $\lambda$ ) of 600 nm. Before each measurement, the blank was checked to make sure the spectrophotometer was calibrated. There were 20 total time points, one for inoculation and every hour after. Following the creation of a standard growth curve under standard conditions, the approximate start to exponential growth was identified. Following the construction of the growth curve at 37°C, curves for 40, 25, and 22°C were generated from hours 0 to 19 under the same conditions as the initial standard curve. The curves and 95% confidence intervals were created using RStudio and the ggplot2 package (v3.3.3; Wickham, 2016).

### 2.3 *Jenika* Titer Curves

To create bacteriophage titer curves, bacterial cultures were made for each temperature following the methods used to generate the growth curves for *M. smegmatis*. In addition, each culture was inoculated with 2.5  $\mu$ l of the stock lysate with titer of  $2 \times 10^{10}$  pfu/mL. At each time point, 1ml of the culture was removed and filtered through a 0.22  $\mu$ m syringe filter into a 3ml Eppendorf tube. After the culture was syringe filtered, the lysate was then serial diluted seven times to achieve a -7 dilution. This was done by

putting 90  $\mu$ l of phage buffer in seven 1.5ml microcentrifuge tubes, then taking 10  $\mu$ l of the original lysate and performing serial dilutions in each tube. The tubes were vortexed briefly between each dilution, after which, a spot titer plate was prepared by mixing 250  $\mu$ l of *M. smegmatis* with 3ml of 1X Top Agar. Once the agar was solidified, the bottom of the plate was marked in a grid pattern to show 9 sections of the plate. Each section was labeled to identify the plated dilutions. In each area of the grid, 5 $\mu$ l of lysate was dropped onto the plate. The plates were then incubated for 24 hours at 37<sup>o</sup> C. At 19 hours for each temperature, 1ml of the culture was collected and placed at -80<sup>o</sup> C to use for Electron Microscopy. The curves and 95% confidence intervals were created using the RStudio packages ggplot2 and ggpubr (v0.4.0; Kassambara, 2020 & v3.3.3; Wickham, 2016).

#### 2.4 *Jenika* Electron Microscopy

The electron microscopy of *Jenika*, cultured at each temperature, was conducted by Dr. Laying Wu using a NANOSPRT12 electron microscope. All usable images of intact bacteriophage were analyzed for tail length and head diameter. The measurements were taken by hand using a ruler. The head, tail, and image scale bar were all measured in millimeters and then converted to nanometers based on the scale bar for that image.

## CHAPTER THREE

### RESULTS

#### 3.1 *Mycobacterium smegmatis* Growth Curves

The growth of the host was observed at the standard and experimental temperatures using optical density readings at hourly intervals. The optical density data for the standard growth curve were collected first and showed the growth of *M. smegmatis* at 37°C in 7H9 complete medium. This set a standard that was used to compare the effects of experimental temperatures on host growth. As seen in Figure 1, the approximate start of exponential growth began at approximately hour 14 – 15 for all temperatures. This provided a starting point for observation of *Jenika* during the host exponential phase. The results also show that there is notable similarity between the OD readings for 37°C and 40°C.

Growth curves for 37° C and 22° C were very similar for hours 1 to 15. However, growth curves for these temperatures began to diverge around hour 15 with 37° C growth decreasing and 22° C growth continuing to increase. The growth curve for 40° C began with less growth compared to 22° and 37° C until approximately hour 15. At hour 15, the 40° C culture had growth similar to 22° C and higher growth than 37° C. Throughout all time points, 25° C had the lowest growth compared to all other temperatures. A one-way ANOVA was performed using RStudio, resulting in a P-value of 0.0284. A paired one tailed T-Test between all the temperatures results in a P-value of  $6.9 \times 10^{-11}$ ,



$3.9 \times 10^{-05}$ ,  $6.1 \times 10^{-08}$ , between the temperatures of 37 and 25, 40 and 25, and 25° C and 22° C, respectively. Given that 25° C was the common temperature between the statistically significant T-tests, 25° C had the greatest effect on optical density readings.

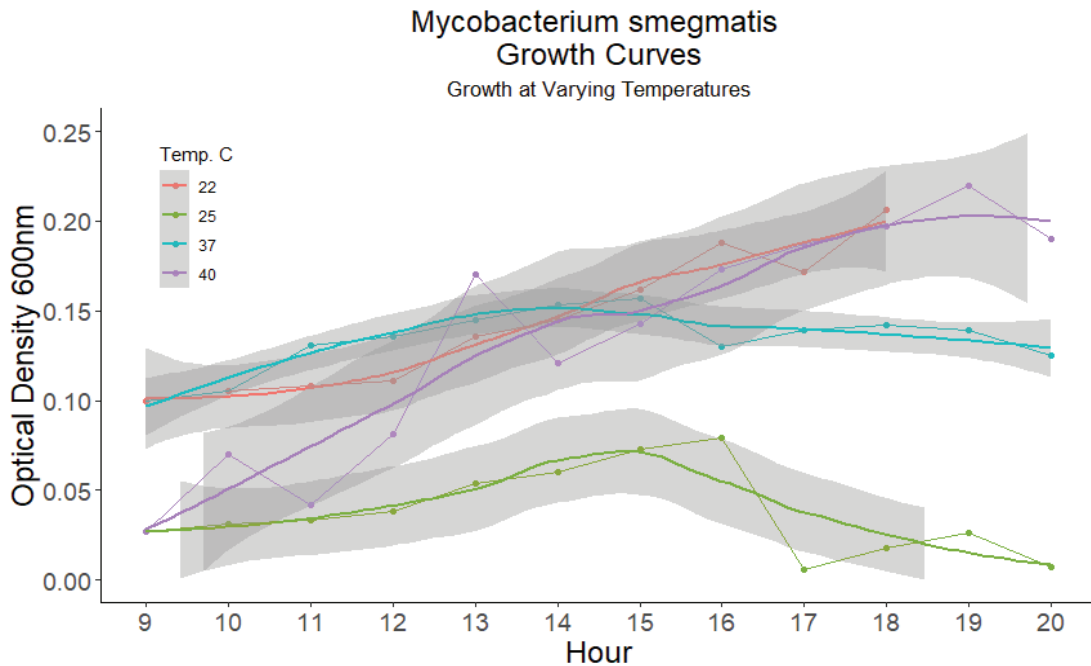
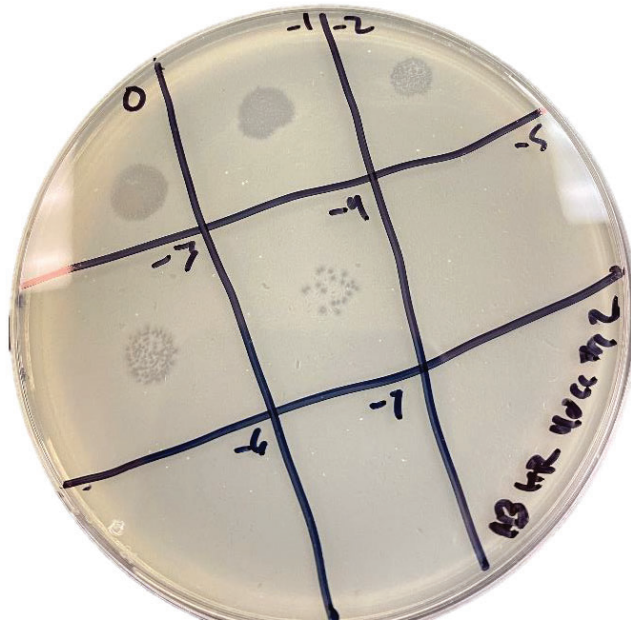


Figure 1 fix axis m smeg and spelling

The above graph displays the OD measurements of *Mycobacterium smegmatis* at 600nm wavelength with 95% confidence intervals shown in gray. The measurements were taken hourly starting 9 hours after inoculation.

### 3.2 Jenika Titer Curves

Following the incubation period, the plaques were counted at each dilution and the titer was calculated. The titer is measured with the unit pfu/mL, also known as plaque forming units/mL (Fig. 2). In order to calculate the titer in this unit, the number of plaques counted at the highest dilution is divided by the amount plated in milliliters times the dilution. An example of this is the calculations used to find the stock titer,  $1 \div [0.05 \times (1 \times 10^{-8})]$ . This results in a titer of  $2 \times 10^{10}$  pfu/mL. The results at each temperature were then graphed with a 95% confidence interval as seen below in Figure 3.



*Figure 2*

An example of the spot titer performed at each time point in order to generate the titer curve. The resulting titer is  $4 \times 10^7$  pfu/mL.

As seen in Figure 3, the titer changed relative to the growth of the host at varying temperatures. The starting point in which the exponential phase of bacteriophage propagation is seen shifts as temperature decreases. The overall titer after 19 hours also decreases as the temperature deviates further from the optimal  $37^\circ\text{C}$ . The graph was split for Figure 3 due to the large variation in the Y-axis. A one-way ANOVA was performed using RStudio, resulting in a P-value of 0.0405. A paired one tail T-test between all the temperatures showed no statistical significance for any particular temperature.

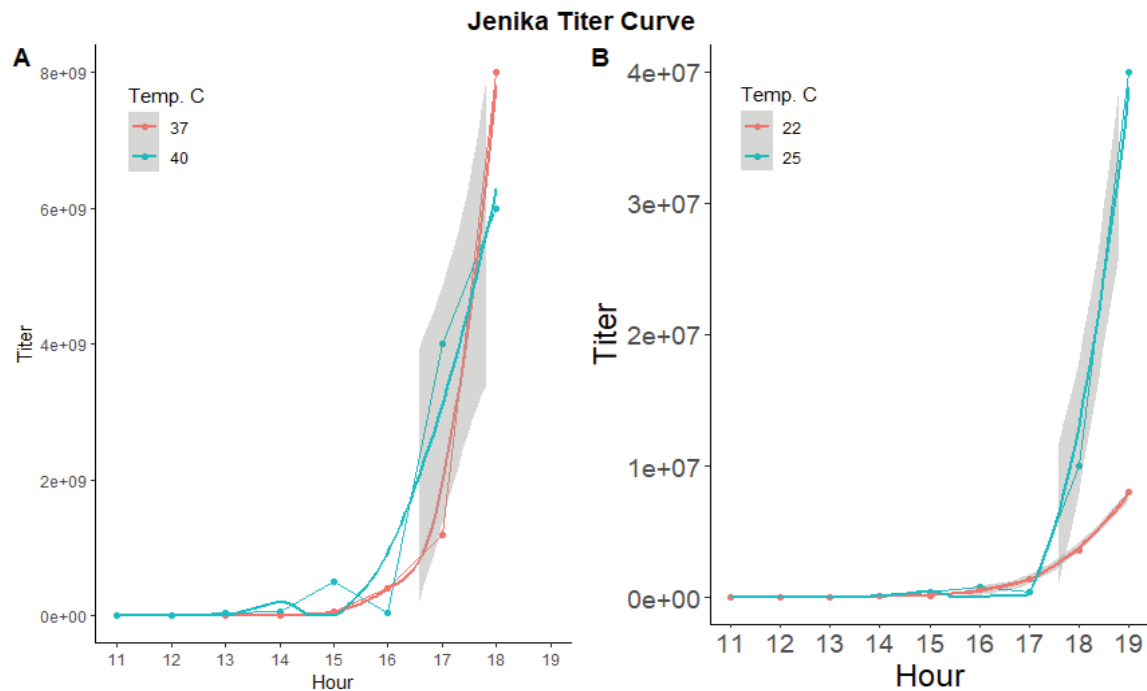
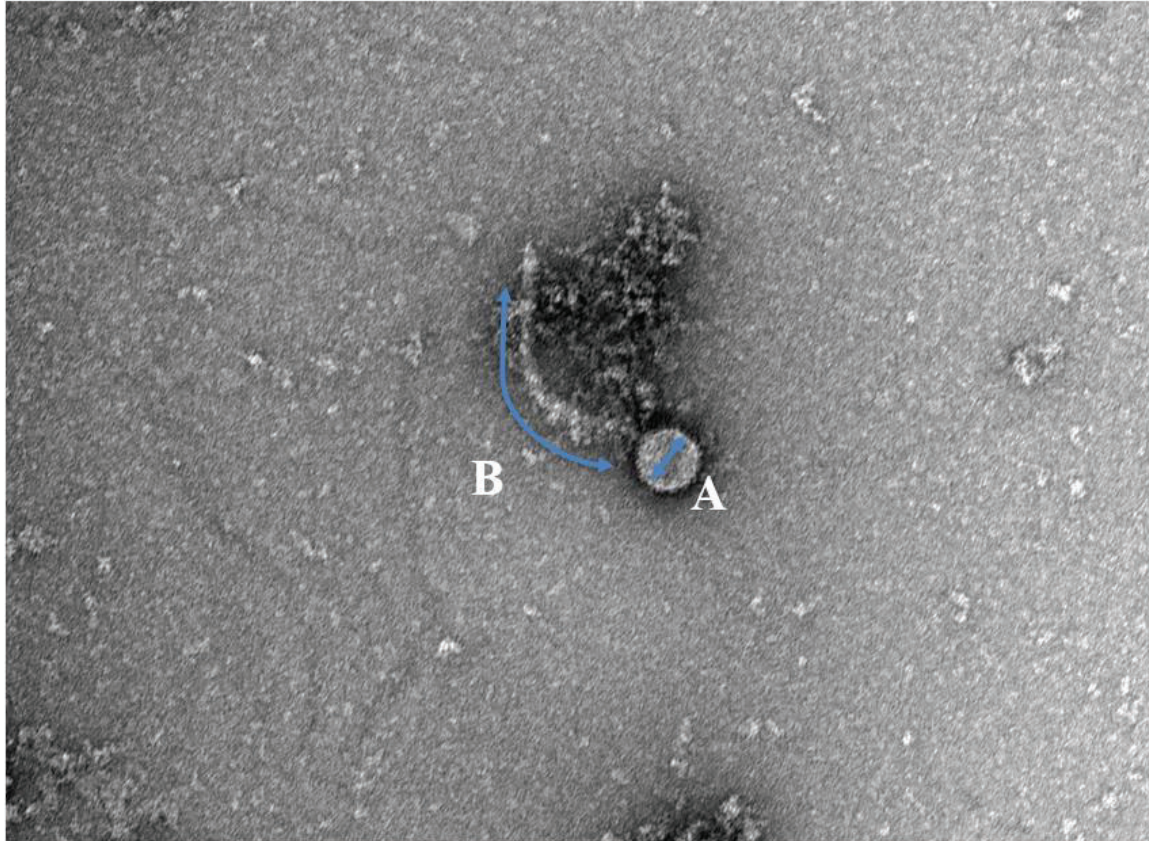


Figure 2

The above graph displays the titer of *Jenika* at each timepoint under the varying temperature conditions after 24 hours of incubation at 37°C with 95% confidence intervals shown in gray.

### 3.3 *Jenika* Electron Microscopy

The Electron Microscopy showed the morphology of bacteriophage *Jenika* after exposure to each temperature condition for 18 hours. The head of *Jenika* at 37, 40, and 25° C measured 71.4, 76.5, and 77.8 nm, respectively. The tail of *Jenika* at 37, and 40° C measured at 278.6, and 241.2 nm, respectively. Statistical tests were not run on these measurements due to the low statistical power of such small sample sizes. Due to image quality, the tail was not measurable at 25° C. No usable images were obtained for *Jenika* cultured at 22° C.



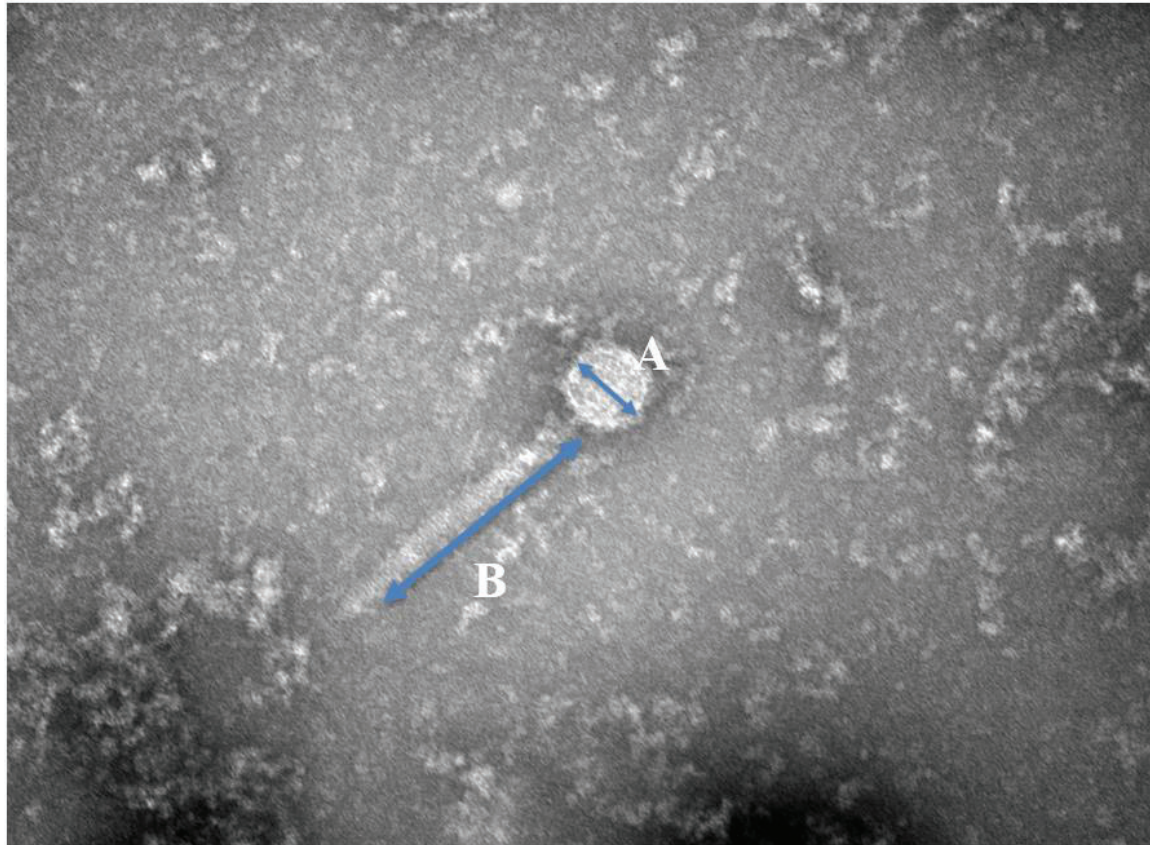
Jenika 37-1.tif  
Cal: 0.326056 nm/pix  
11:44 4/30/2021

Camera: NANOSPRT12, Exposure: 1600 (ms) x 4 std. frames, Gain: 1, Bin: 1  
Gamma: 1.00, No Sharpening, Normal Contrast

100 nm  
HV=100kV  
Direct Mag: 20000 x

*Figure 4*

Electron Microscopy image of *Jenika* after 18 hours of treatment at 37°C. Line A represents the diameter of the head, which measures at 71.4 nm. Line B represents the length of the tail, which measures approximately at 278.6 nm.



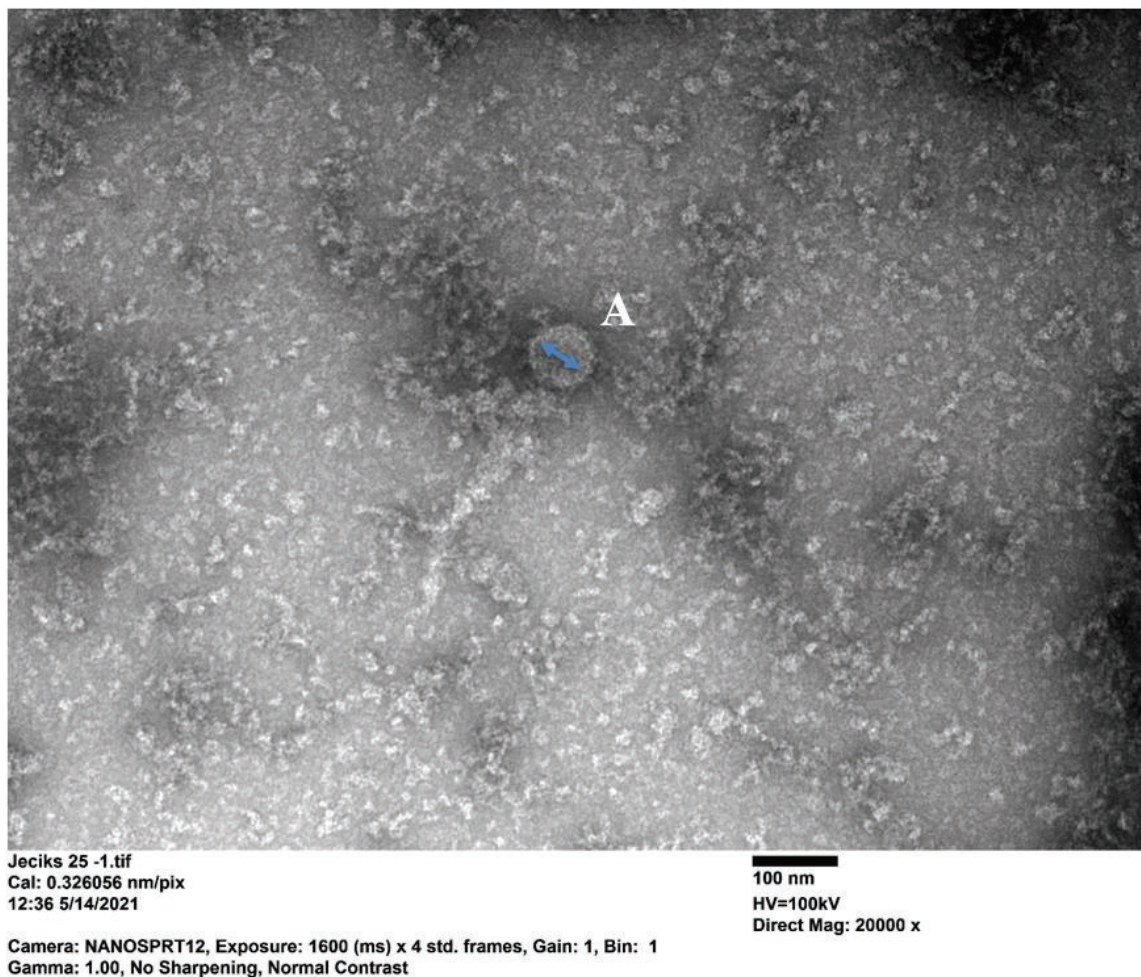
Jenika 40-2.tif  
Cal: 0.260845 nm/pix  
11:07 5/3/2021

Camera: NANOSPRT12, Exposure: 1600 (ms) x 4 std. frames, Gain: 1, Bin: 1  
Gamma: 1.00, No Sharpening, Normal Contrast

100 nm  
HV=100kV  
Direct Mag: 25000 x

*Figure 5*

Electron Microscopy image of *Jenika* after 18 hours of treatment at 40°C. Line A represents the diameter of the head, which measures at 76.5 nm. Line B represents the length of the tail, which measures approximately at 241.2 nm.



*Figure 6*

Electron Microscopy image of *Jenika* after 18 hours of treatment at 25°C. Line A represents the diameter of the head, which measures at 76.5 nm. The tail was not clearly visible in any image, and thus was not measured.

## CHAPTER FOUR

### DISCUSSION

#### 4.1 *Mycobacterium smegmatis* Growth Curves

The overall growth for *Mycobacterium smegmatis* increased over the course of the 19 hours as expected. The observable point at which the exponential phase began based on the optical density data was shown to be in the same approximate time frame

despite the temperature conditions. This is important as it narrows down an observational point for *Jenika* replication. The variability and decreases in OD towards the end of the curves can be attributed to clumping of bacterial cells. *Mycobacterium smegmatis* clumps together as the culture becomes more saturated despite the presence of a detergent in the solution. This can also account for the optical density readings showing the growth curves for these temperatures diverging around hour 15 with the optical density at 37 ° C growth decreasing while the 22 ° C growth continuing to increase. Clumping of *M. smegmatis* is a possible explanation for the apparent decrease in population size over time. Despite any possible clumping by *Mycobacterium smegmatis*, the confidence of the curves tightened showing an increase in accuracy as the culture propagated. Overall, the growth curves at varying temperatures have the expected results of a general increase in *M. smegmatis* cells until a point of saturation is reached when clumping may begin.

A one-way ANOVA was performed with temperature being the dependent variable, resulting in a P-value of 0.0284. Given that this value is below 0.05, the null hypothesis is rejected, and we see there is a statistically significant difference in optical density measures as a result of temperature. The results of the pairwise T-tests showed P-values of  $6.9 \times 10^{-11}$ ,  $3.9 \times 10^{-05}$ ,  $6.1 \times 10^{-08}$ , between the temperatures of 37 and 25, 40 and 25, and 22° C and 25° C, respectively. These results suggest 25° C had the most significant effect on the growth of *M. smegmatis*.

#### 4.2 *Jenika* Titer Curves

The titer of *Jenika* after host exposure at each temperature was reflective of the growth of the host population. When compared to the growth curves of *Mycobacterium*

*smegmatis*, the temperatures that showed lower overall host growth showed lower overall titers. Lower overall titers as a result of the overall host growth suggests that stress on the host can translate to the bacteriophage. Reduced host growth is also a plausible explanation for a notably different y-axis for the 37 and 40 ° C cultures compared to those of the 22 and 25 ° C conditions. This could also be due to a direct effect on the bacteriophage. It is possible that *Jenika* is directly experiencing the effects of the temperature change, possibly resulting in a decreased ability to attach and/or infect the host.

A one-way ANOVA was performed with temperature being the dependent variable, resulting in a P-value of 0.0405. Given that this value is below 0.05, the null hypothesis is rejected, and we see there is a statistically significant difference in bacteriophage titer as a result of temperature. Given the ANOVA showing a statistical significance, paired one tail T-tests were performed. The T-tests showed no individual temperature having a significant statistical value. This shows an observable trend, however due to small sample size of only one measurement at each time point for each temperature the statistical tests may be unreliable.

The titer curves also showed at what time point the bacteriophage population began to exponentially grow. The beginning of the exponential curve for each temperature varies despite a similar starting point in the host curves. This could indicate that there could be more affects despite changes in population increases relative to those of the host or independently as a result of temperature. It is possible that the changes in temperature conditions could cause any of the bacteriophage mechanisms, such as attachment or infection, to move at an altered pace.



A change in *Jenika*'s ability to attach to or infect *M. smegmatis* could lead to decreases in population control that bacteriophage exert on bacterial populations in environments where temperature may be decreased. Global warming and increased temperatures can lead to sudden drops and increased winter severity as seen in a recent study by Cohen et al. (2018). Their work linked the warmer arctic episodes as a result of increased temperatures to an increase in the frequency of extreme winter weather in the United States (Cohen et al. 2018). Given the importance of bacterial populations in nutrient cycling, changes in overall population sizes of different species in soil microbiome can cause adverse effects on other species in that given environment. It can also lead to disproportional abundance in a soil microbiome, leading to altered soil conditions which may further affect other species in the area. Sudden changes in environmental temperatures may have a negative impact on both the bacterial host and its bacteriophage's ability to attach and/or infect the host.

#### 4.3 *Jenika* Electron Microscopy

The electron microscopy of bacteriophage *Jenika* after each temperature treatment showed no noticeable change in the morphological shape. The length of the tails and the diameter of the head were measured for each of the images. There appear to be minimal differences in the length and diameter, however due to the small sample size and missing images for the 22 °C treatment, no statistical tests were able to be performed. These conditions should be repeated in the future with the aim of attaining a higher sample size. If a repeat of these test shows a significant difference in a one-way ANOVA, pairwise comparisons will be done between the measurements to determine statistical differences between each temperature.

## CHAPTER FIVE

### CONCLUSIONS AND FUTURE WORK

Based on the results presented here, there appears to be a negative effect of temperature on growth of the bacterial host *M. smegmatis*, and a corresponding negative effect on the replication of its bacteriophage, *Jenika*. Changing environmental conditions, especially temperature, may have a negative impact on other bacterial hosts and their respective bacteriophage in natural environments. Given the essential role bacteria play in nutrient cycling and given the essential role bacteriophage play in the control of bacterial populations, future work should focus on investigating the effects of additional temperatures on host/bacteriophage growth (particularly additional higher temperatures). Additionally, future work should also focus on investigating how changing temperature may be affecting host and bacteriophage growth by specifically looking at differences in gene expression of host and bacteriophage genes. Retesting of the titer curves in order to increase the statistical power of the sample size is another focus that should be done. Given the inability to find statistical significance using T-tests despite ANOVA results, larger sampling to create curves that use average readings would help better understand the effect temperature has in the bacteriophage.

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