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Environmental Effects on Mycobacteriophage Cepens

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Environmental Effects on Mycobacteriophage Cepens - Host Interactions

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Abstract

Bacteriophage (phage) are viruses that infect and use bacterial hosts for viral replication. Study of these bacterial viruses has led to such discoveries as knowing DNA is the molecule of inheritance[12]. Study of phage has also led to discovery and implementation of novel therapeutic treatment of infections. Known as "phage therapy", phage have been utilized in curing a patient of the highly virulent infection known as MRSA[11]. Tuberculosis, caused by *Mycobacterium tuberculosis*, is a growing concern as antibiotic-resistant strains become more prevalent in the population. Cepens is a lytic phage that infects members of the Mycobacterium genus. This study analyzes Cepens and its interactions with its host bacteria, *Mycobacterium smegmatis*, in an effort to understand how environmental conditions impact infection and replication efficiency. First, infection benchmarks were established by conducting a serial dilution and plaque assay under standard conditions. Baseline titer calculations were used for comparison after alteration of incubation temperature and pH of phage, both prior to and during infection. Extreme temperatures and pH were expected to lead to a decrease in infectivity as well as potential degradation of the phage itself. Analysis of this data indicates that extremes in either pH or temperature decrease phage viability as well as its ability to establish successful infection.

Introduction

Before the rise of antibiotics, a group of viruses known as bacteriophages (phage) were being studied for their effects on bacteria. Bacteriophage are viruses which infect bacterial cells, commonly causing the cell to lyse and release virions into the area surrounding the cell. These virions move on to infect more bacterial cells in the surrounding area. The continued release of virions leads to amplification of the virus and a decrease in viable bacterial cells. Ernest Hankin first published about phage in 1896. Hankin wrote about an unknown substance contributing to antibacterial activity in various rivers in India [1]. Fredrick Twort later hypothesized that the antibacterial activity could be related to the processes of a virus. Twenty-one years later another scientist, Felix d'Herelle, was the first to test the virus phenomenon on humans and animals. After testing his mixtures on agar plates, d'Herelle was also the first to discover the clear areas that form when the virus is introduced to bacterial samples. These clear areas are now referred to as plaques[1]. After the parasitic nature of bacteriophages was realized, the phenomenon was expanded. d'Herelle started utilizing bacteriophages experimentally to treat human infections such as dysentery[1]. However, concerns with bacteriophage therapy rose after discovering their transduction capabilities, and the possibility of causing mutations in host cells[6]. After controversial opinions on bacteriophage use in humans and animals, along with the rise of antibiotics, the drive for research on bacteriophage therapy declined in most areas. However, in parts of the world such as Eastern Europe and the Soviet Union bacteriophage use continued therapeutically in combination with or in replacement of antibiotic treatment[1].

The recent push for the reintroduction of bacteriophage research is driven by the rise in antibiotic resistant bacterial pathogens. The initial incline of antibiotic use was due to their effectiveness, ability to treat a broad spectrum of bacterial infections, and stability[6]. However, researchers and medical professionals are now realizing that prolonged antibiotic use can lead to further increases in antibiotic resistance and damage to the gut biome, resulting in chronic enteric inflammatory diseases[8]. As a solution to this rising issue, bacteriophage can be used therapeutically in replacement of antibiotics to treat bacterial infections[6]. Bacteriophage therapy gains its effectiveness through the viral lifecycles, which can be classified two ways. If the bacteriophage inserts itself into the host genome forming a prophage then it is a lysogenic bacteriophage. The prophage is later excised and replicated to produce viral particles. This process can result in transduction which is harnessed to incorporate gene segments into pathogenic bacterial genomes[2]. On the other hand, if the bacteriophage enters the host cell and immediately uses the host machinery to start producing viral particles then it is considered a lytic bacteriophage. Lytic bacteriophage can be applied directly to infection sites to cause bacterial cell lysis[3]. Today, bacteriophages are also being used as model organisms for genetics research as well as studying temperate bacteriophages to be used in cancer treatments or mutation corrections[3].

This study focuses on mycobacteriophage, which are a classification of phage that specialize in infection of mycobacterial hosts. These bacteriophages are split into about 30 clusters and infect microorganisms such as, *Mycobacterium smegmatis*, *Mycobacterium Tuberculosis* and *Mycobacterium leprae*[1]. Prior to this study, mycobacteriophage Cepens was isolated by Ethan Strickland on the University of North

Georgia campus in 2016. Cepens is a lytic cluster W mycobacteriophage from the Siphoviridae family. Members of this family have characteristic long non-contractile tails, and isometric heads[13]. Cepens forms 2mm clear round plaques, and transmission electron microscopy imaging shows a tail length of 235nm and head size of 52 nm. Cepens was isolated and studied using host organism *M. smegmatis* mc^2155 (ATCC 700084). Typically, *M. smegmatis* is used as a model organism for other pathogenic microorganisms from the mycobacteria family such as *M. tuberculosis* and *M. leprae*. *M. smegmatis* has a characteristic thick and waxy hydrophobic cell wall, similar to *M. tuberculosis*. It is also a generally nonpathogenic environmental species found in soils, and contributes to degradation of organic materials[5]. However, since 1986 there have been about 25 reported cases of *M. smegmatis* related infections in the soft tissue of the skin[5]. Use of *M. smegmatis* as a host organism allows researchers to learn more about the interactions of cluster W phages and mycobacterial pathogens without the dangers of working directly with those pathogens in the lab.

Before bacteriophages can be used therapeutically, an effective method of administration must be determined. Most antibiotics are administered orally, however once the drug is ingested it becomes exposed to the increased acidity of the digestive system. If a bacteriophage is expected to be administered the same way, it would need to be capable of maintaining infectivity after exposure to the hostile conditions of the gut[9]. Also, the average internal body temperature of humans is 37 ˚C, so the bacteriophage must be able to successfully infect host cells at this temperature. Another primary concern is the temperature conditions under which the phage is capable of being stored until therapeutic use. Understanding the conditions in which bacteriophage Cepens is able

to maintain infectivity against *M. smegmatis* could shed light on potential administration methods and storage conditions during bacteriophage therapy.

Methods

M. smegmatis mc^2155 (ATCC 700084) cells are cultured using Middlebrook 7H9 broth and shaken at an incubation temperature of 37˚C. Infections by Cepens are completed using lysate stored at 4 ˚C on sterile Difco Luria Agar plates at a pH of 6.5 using 1X soft top agar with the addition of $CaCl_2$. For normal infection conditions, samples are incubated at 37 ˚C for 48 hours.

Titer Calculations

First, bacteria were serially diluted in a 1:10 fashion up to 10^{-7}. After dilution, aliquots of 10^{-5},10^{-6}, and 10^{-7} were mixed with *M. smegmatis*, $CaCl_2$ and 1X soft top agar then spread via swirling onto LB agar and plated in duplicate. Samples were incubated at 37˚C for 48 hours. After incubation, plaques were counted and averaged.

M. smegmatis Growth Curve

A standard curve of *M. smegmatis* was constructed to indicate the number of cells (colony forming units) present per ml of solution. This curve was generated by serial dilution plating and OD_{600} measurements. Samples were analyzed for colony number and absorbance at dilutions of 1:400-1:1600. First, *M. smegmatis* was inoculated and shaken in a 37 ˚C incubator for 48 hours. Next, the sample was aliquoted out and diluted in 7H9 growth media. A spectrophotometer was used to determine the absorbance in each

dilution factor. Finally, each sample was plated in duplicate onto LB plates. After incubation at 37 ˚C, colonies were counted using the quadrant method.

One step bacteriophage growth curve

Cepens lysate was added at a multiplicity of infection of 1.0 to an aliquot of *M. smegmatis* at an OD of 0.4, and allowed to adsorb for 10 minutes. Next, the sample was centrifuged at 6000G for 10 minutes. Supernatant was removed and the pellet was resuspended in 7H9 growth media. The resuspended sample was centrifuged again at 6000G for 10 minutes, and the supernatant was removed. Finally, sample was shaken at 37 ˚C for six hours. Samples were plated in duplicate each hour.

Temperature Stability of Lysate

Cepens lysate was diluted to 10^{-5} and incubated at 4˚C, 37˚C, 45˚C, 55˚C, and 65 ˚C for two hours[7]. First, lysate was aliquoted into five sterile microcentrifuge tubes. After incubation, each aliquot was cooled to 4 ˚ C, and then added to *M. smegmatis* samples and allowed to adsorb for 10 minutes at room temperature and then plated in triplicate. Plates were incubated at 37 ˚C for 48 hours and then analyzed.

pH Stability of Lysate

To determine the pH stability of bacteriophage Cepens, pH of 7H9 growth medium was adjusted using filter sterilized HCl and NaOH to pH levels of 2,3,5,7,9, and 10. Target pH values were confirmed using pH strips. Cepens lysate was incubated at each pH, undisturbed at room temperature for 24 hours[7]. Following treatment, *M. smegmatis* was infected with lysate and incubated at 37˚ C for 48 hours. Plaques were counted and averaged for each pH condition.

Incubation Temperatures and Infection

Cepens lysate was first added to *M. smegmatis* and allowed to adsorb for 10 minutes. Samples were plated in triplicate and incubated at 25˚C, 30˚C, 37˚C, 45˚C and 50 ˚C for 48 hours. After incubation, average plaque numbers were used to calculate titer.

pH and Infection

Cepens was plated on plates with pH values between 5.0-8.1 and then incubated for 48h at 37 ˚C. pH of LB agar base was adjusted using filter sterilized 5M HCl and NaOH. pH strips were used to confirm ideal pH levels. Samples were plated in triplicate and incubated at 37 ˚C for 48 hours. After incubation, plaques were averaged to be used in titer calculations.

Results

Lysate was calculated to be at a titer of $2.2x10^8$ pfu/ml (*Figure 1*). This titer was used in all future experiments as a control. The completed standard curve of *M. smegmatis* (*Figure 2*) provides the number of colony forming units (CFU) in relation to the OD value of the sample. This value can be used to calculate PFU to determine multiplicity of infection (MOI) of a sample. This value indicates the number of active plaque forming units per cell in a sample. These data indicate that when M. smegmatis reaches an OD_{600} of 0.25 there are approximately 370,000 colony forming units (CFU) in solution (*Figure 2).*

In order to observe typical replication kinetics of phage Cepens, a one-step growth curve was attempted. This procedure did not provide a clear plaque pattern despite many attempts (*Figure 3*). Therefore, the results from this test are not reliable in indicating phage infection kinetics. Since the results from the one step bacteriophage growth curve were discounted, the baseline titer calculation under standard conditions will be used to indicate variability in infection.

Statistical analysis using a single factor ANOVA was performed to determine if environmental conditions lead to a significant change in infectivity. Samples that indicated a significant difference were then tested using a Tukey's HSD post hoc analysis. Any pairwise comparison which indicated significant difference from baseline conditions are indicated by an asterisk on the corresponding figure. Error bars on each figure represent the calculated standard error of data collected.

The titer of plaques observed indicates that exposure of Cepens lysate to temperatures above 4˚C leads to a trend wise decrease in infectivity (*Figure 4*). Statistical analysis shows that samples exposed to 45˚, 55˚, and 65˚ C have a p-value < 0.05 indicating significant difference when compared to the control. Cepens also demonstrated an observed decrease in infectivity after exposure to pH below 5.5 or above 9.5 before infection (*Figure 5*). Results from statistical analysis indicate that exposure to pH of 2.0-3.0 before infection results in a statistically significant p-value < 0.05. The effect of incubation temperatures during infection indicate that Cepens requires a temperature of 30˚C for maximum infectivity (*Figure 6*). This effect could be due to inhibition of growth of host cells or inhibition of infection kinetics of the virus. This data showed a p-value < 0.05 after exposure to 25˚, 30˚, 45˚, and 50˚C when compared to the baseline infection temperature. When testing the effect of pH on infection, a minimal trend wise decrease in titer was observed on plates with pH above 6.0 (*Figure 7*). However, after analysis of the data it did not indicate a significant difference in infectivity.

Figures

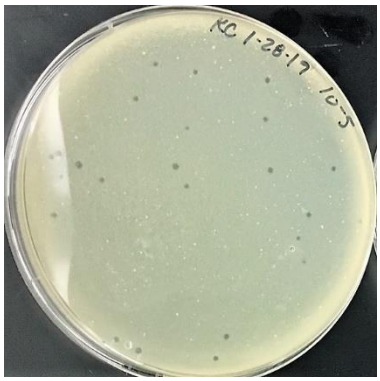

Figure 1. 1:10 serial dilution of phage lysate was completed. Plaques were counted from plate showing abundant individual plaques and used to calculate titer.

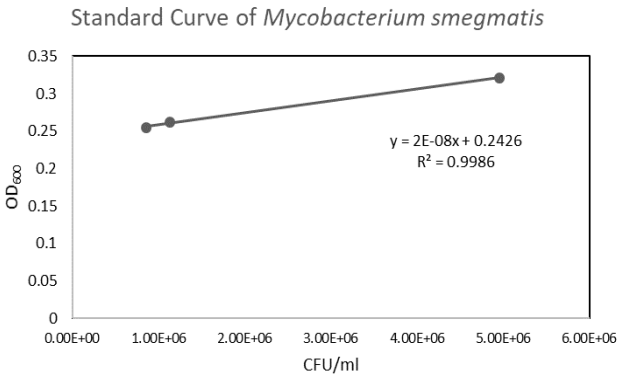

Figure 2. Cells were incubated and shaken at 37°C. Samples were analyzed at dilutions of 1:400-1:1600 for OD600 and colonies were counted using the quadrant method.

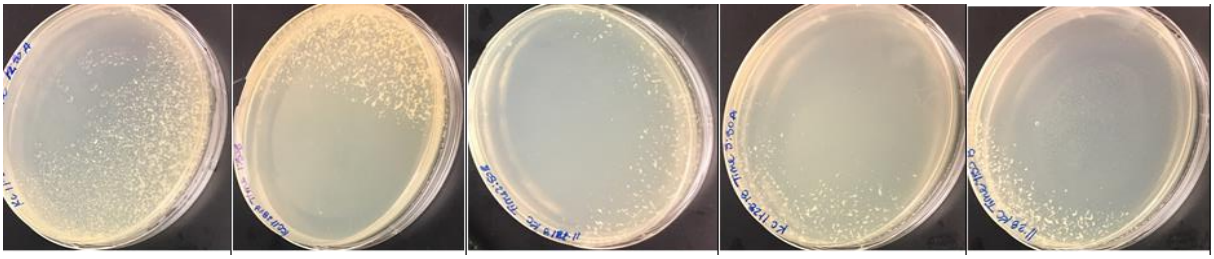

Figure 3. Initial plaques (on the left) are frequent and small in size but not overlapping. Over subsequent plates begin to overlap and plaque number increases in size and number. Plaque presence is not clear and indicative of the expected viral lifecycle pattern.

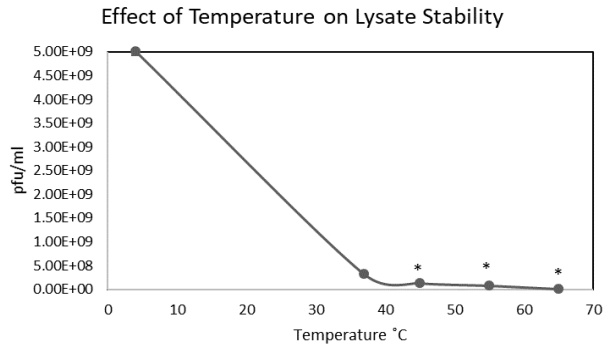

Figure 4. To determine the temperature stability of Cepens, lysate at 10-5 pfu/ml was exposed to 4˚C, 37˚C, 45˚C, 55˚C, and 65˚C for two hours. After exposure, each aliquot was cooled to 4˚C and then plated in triplicate. Plaques were counted for titer calculation and comparison. Increasing temperature decreases titer value. * indicates statistical significance after post hoc analysis.

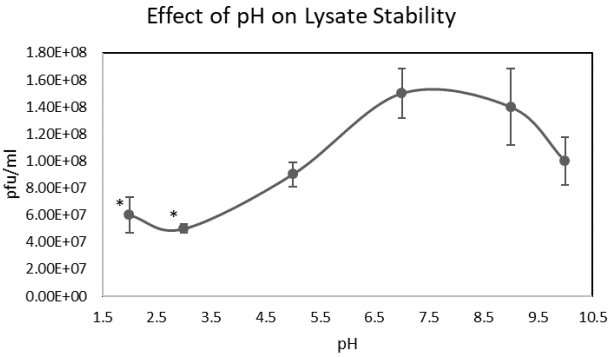

Figure 5. To determine the pH stability of Cepens, lysate was exposed to pH 1,2,3,5,7,9,10 and then plated in triplicate. Plaques were then counted for titer calculation and comparison. Extreme pH shows decrease in titer values. * indicates statistical significance after post hoc analysis.

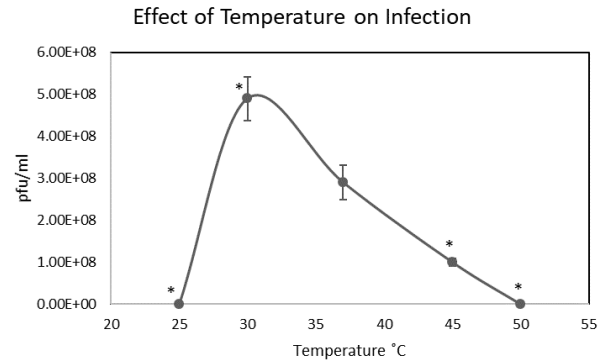

Figure 6. To determine the effects of variations in temperature on infection, lysate was plated in triplicate and then incubated in 25, 30˚, 37˚, 45˚, and 50˚ C. After incubation plaques were counted and variations in titer were calculated. * indicates statistical significance after post hoc analysis.

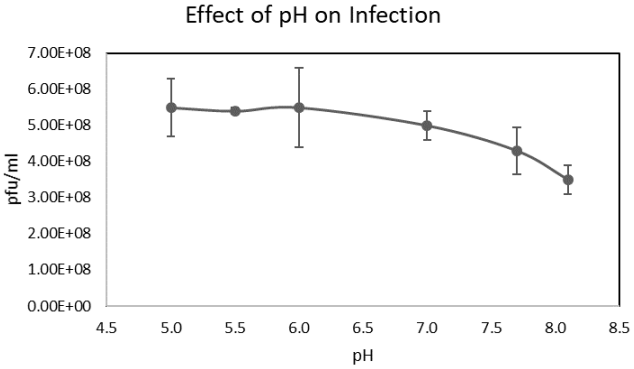

Figure 7. Infection of M. smegmatis by Cepens was performed on plates with pH 5.0-8.1 in triplicate. After plating, samples were incubated at 37°C for 48 hours and then plaques were counted for titer calculation.

Conclusion

The data in this study provide insights into the proper storage of Cepens lysate as well as possible limitations on delivery of phage to a patient. Cepens was sensitive to temperatures outside of the host cell. This indicated potential difficulty of lysate storage for therapeutic use without the presence of climate control mechanisms. Cepens was also highly sensitive to changes in pH outside of the host cell, which would suggest this organism could not be delivered orally for therapeutics. However, pH did not seem to inhibit replication of the virus within the host. Finally, Cepens replicates optimally outside the natural human body temperature but it is still able to produce a successful infection at that temperature. While there is limited data from the single round of experiments completed here, the data collected does suggest mycobacteriophage similar to Cepens could have the capability of therapeutic use under the right conditions. Repeating this study with Cepens and other mycobacteriophage and their pathogenic hosts could lead to major breakthroughs in the conditions required to maintain proper host interactions during bacteriophage therapy.

The potentials for utilization of bacteriophage for medical and environmental use are vast. Bacteriophages are one of the most abundant organisms on earth, but there are still vast amounts that have yet to be isolated[3]. Therefore, continuing studies on new bacteriophages could lead to genetic and medical breakthroughs that will change the way we treat diseases all around the world. Knowing how to use the host specificity and efficiency of bacteriophages could result in quicker and safer patient treatment as well as additional treatment of antibiotic resistant microorganisms. There are still concerns of potentially negative transduction events during therapeutic use; but furthering the

understanding of mycobacteriophage will lead to a better understanding of the relationship between phage and pathogenic mycobacterial hosts *M. tuberculosis* and *M. Leprae*.

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