Isolation of Mycobacteriophage from Local Soil

Christina Kling
Faculty Advisor: Donna Rhoads-Frost
Department of Biology and Environmental Science
College of Arts and Sciences

Abstract:

The goal of this experiment was to isolate bacteriophage from local soil that were specific for *Mycobacterium smegmatis* (*M. smegmatis*), a common model organism for *Mycobacterium tuberculosis*, the bacterial pathogen that causes tuberculosis. Soil was collected near large bodies of water, such as the Mill River in Hamden and East Rock Park and the Branford Supply Ponds. Soil extracts were prepared using phage buffer and co-plated with *M. smegmatis* using brain-heart infusion top agar. Phages were identified on these plates based on the presence of replicable clear zones (plaques) on the bacterial lawn. Two probable phage were isolated from soil samples obtained near the Mill River in Hamden. The DNA of these phage can be sequenced to confirm that they are indeed mycobacteriophage, and then pure lysates of these phage can be used to study the toxins that are produced when *M. smegmatis* is lysed by these phage to give a better understanding of the sepsis observed in the phage treatment of tuberculosis in animals.

Introduction:

Bacteriophage (phage) can be used both for the identification and the treatment of a variety of infectious diseases. Currently, there are effective treatments for *Staphylococcus* (including methicillin resistant *Staphylococcus aureus* aka MRSA), *E. coli*, *Salmonella*, *Shigella*, *Brucella* and many other infections in the Republic of Georgia (of the former USSR), where phage therapy has been pursued for many years.[1] The idea of using phages for the treatment of infectious disease originated in the 1920s[1], but was not thoroughly investigated because of the discovery of antibiotics, particularly in Western countries (Western Europe and the United States of America). Due to the recent surge of antibiotic resistance among many potentially lethal pathogens, phage therapy is something that should be investigated again.

Phage therapy has multiple advantages over traditional antibiotic therapy. Unlike antibiotics, each type of phage targets only specific bacteria, which reduces the impact of the treatment of bacterial infections on the microbes with which we naturally live in symbiosis. Phage therapy is also much easier to modify than antibiotic therapy. When an organism becomes resistant to a chemotherapeutic antimicrobial agent, a new agent must be invented, tested for harmful physiological effects, go through extensive human trials and be approved before it can become useful to those who need it. Phages are significantly easier to modify to overcome resistance than traditional antibiotics. Physiological effects are minimal due to the specificity of phages. In most situations, culturing the phage and the newly resistant bacteria together for several generations will be enough to encourage a mutation in the phage that enables it to once more target the pathogen and eliminate it.[1]

There are still an enormous number of phages in nature that we have not discovered and an enormous number of bacteria diseases for which we do not have phage treatments.[1] One of the most lethal and widespread ailments for which there is no phage treatment is tuberculosis. Tuberculosis is caused by a bacterium from the genus *Mycobacterium* and it is suspected that as many as two billion people may have latent tuberculosis infections.[2] Of this group, approximately ten percent will develop an active infection.[2] A small amount of these people will have

multidrug resistant or extremely drug resistant tuberculosis and for these people, there is little that can be done.[2] Research into finding bacteriophage that can be used not just to diagnose tuberculosis, as is done now[3], but to ultimately cure resistant drug-resistant tuberculosis infections is necessary.

The Mycobacteriophage Database is an organization that collects and shares data associated with the discovery, sequencing and characterization of mycobacteriophage. The goal of this project was to isolate and sequence the DNA of any phage isolated to determine whether or not they were novel phage, and if they were novel phage, to upload any information gathered from these phage to the database to examine their evolutionary relationship with other phage that have been discovered. In addition to this, pure phage extracts could then be used for further study in regards to potential phage treatment of tuberculosis.

Materials and Methods:

The methods used in this project were predominately modified from those provided by the Mycobacteriophage Database for their colligate phage hunting program.[4] *M. smegmatis* was plated on variations of both tryptic soy agar and brain heart infusion agar until an effective protocol for growing them was established. Soil samples were collected from 12 locations around West Haven, North Haven, New Haven, and Branford as shown in Figure 1. Most of the samples were taken from moist soil near large bodies of fresh or mildly brackish water. The temperature of each area was recorded approximately 4 cm from the surface of the soil. Soil was taken from 1 – 4 centimeters deep, placed in a sterile petri plate which was then wrapped in parafilm and stored at approximately 4°C until it was used.

Soil pH was measured at the same time as when the soil extracts were prepared. Soil pH was measured using a soil analysis kit. Soil extracts were prepared by mixing approximately 5 mL of soil with 15 – 20 mL of phage buffer solution (10 mM Tris HCl, 10 mM Magnesium sulfate, 68 mM Sodium chloride, and 1 mM Calcium chloride) and shaking vigorously. The soil was allowed to settle and then the supernatant was passed through a 0.22 micrometer syringe filter into two 1.5 mL microcentrifuge tubes for storage at 4°C.

The extracts were plated by mixing 1.5 mL of a brain heart infusion broth culture of *M. smegmatis* with 50 µL of the soil extract, adding that mixture to 4.5 mL melted brain heart infusion top agar (50:50 brain heart infusion agar: brain heart infusion broth), cooled to about 55°C. This mixture was vortexed and poured onto a room temperature brain heart infusion agar plate. Once the top agar solidified, these plates were incubated at 35°C for 48-72 hours.

After incubation, the plates were inspected for the presence of clear zone that indicated a possible phage. Samples were taken from each clear zone using a sterile pipette tip, mixed with 100 µL fresh phage buffer and 10 µL of this mixture was added to a fresh mixture of *M. smegmatis* and brain heart infusion broth. After multiple rounds of purification that followed the protocols set forth by the Mycobacteriophage Database, plates were obtained that contained only one type of plaque.

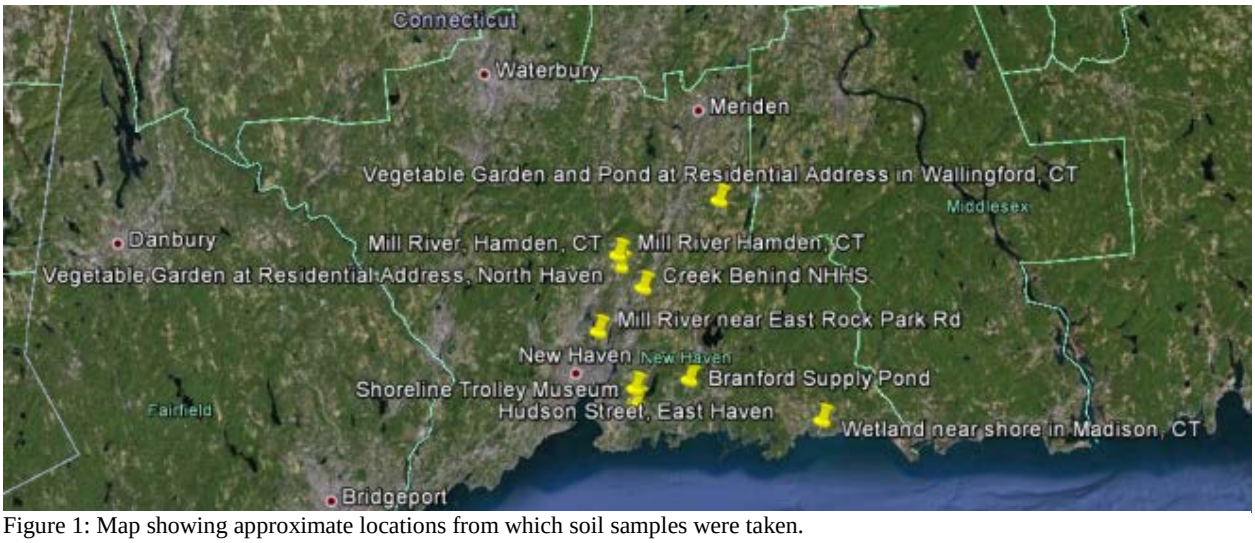

Figure 1: Map showing approximate locations from which soil samples were taken.

Results:

Two phages appear to have been isolated from soil collected near the Mill River in Hamden, CT. The two phages were isolated and purified based on plaque morphology. One phage produced pin-prick shaped plaques that were less than a millimeter in diameter (see Figure 2 and Figure 3). The second phage produced plaques that were closer to a centimeter in diameter. These plaques consisted of an inner zone of normal bacterial growth surrounded by a halo of clear zone (see Figure 4 and Figure 5). No other soil sample produced phage with consistent colony morphology that could be used for purification purposes. Sequencing was not performed due to time limitations associated with this project so it is unconfirmed if these are novel phage.

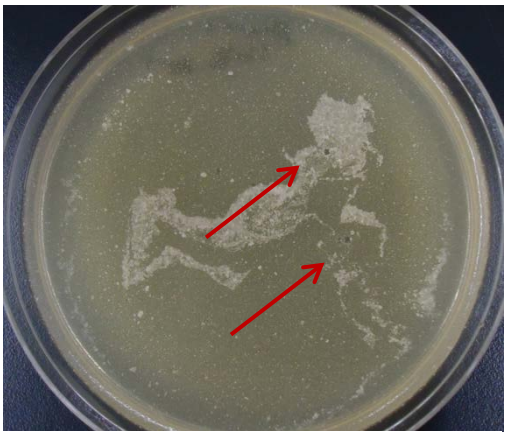

Figure 2: Initial purification using concentrated sample of pin-prick type phage from Mill River soil sample, arrow denotes plaque.

Figure 3: Purification of pin-prick type phage from Mill River soil sample, arrow denotes plaque.

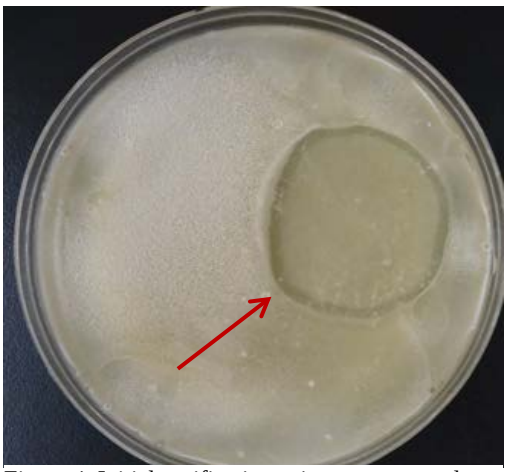

Figure 4: Initial purification using concentrated sample of bulls-eye type phage from Mill River soil sample, arrow denotes plaque.

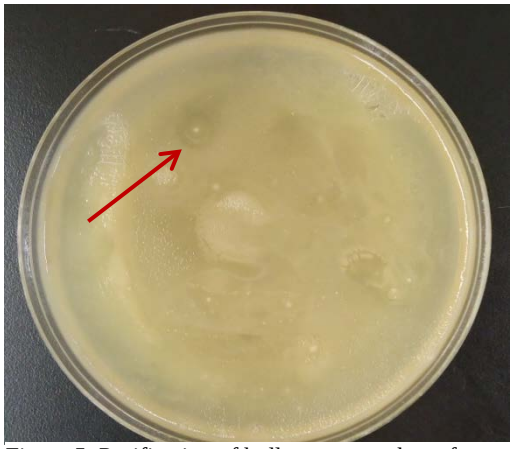

Figure 5: Purification of bulls-eye type phage from Mill River soil sample, arrow denotes plaque.

Conclusions and Future Work:

Two types of phage specific for *M. smegmatis* were successfully isolated over the course of this project. The plates containing one phage type each and the phage buffer samples used to produce these plates were preserved and will be used to produce a concentrated lysate that can be used for DNA isolation and sequencing. Once sequencing is completed, analysis of the phage genome will indicate whether or not it is a novel phage and determine whether it is eligible to be added to the Mycobacteriophage Database.

Once a pure lysate is obtained, freezer stocks of these phage can be established and more work can be done to analyze the lysing process and

the various toxins produced during the lysing process that lead to sepsis in animal phage treatment trials. If the identities of these toxins can be determined, it might be possible to find a way to mitigate their physiological effects to reopen the possibility of using phage to treat tuberculosis. In addition to looking at directly mitigating the effects of any toxins released upon lysing, various methods of delivering phage can be examined to determine if there is a way to administer phage at a rate in which the body can deal with the amount of bacteria being lysed.

Acknowledgements:

This project was funded by the Summer Undergraduate Research Fellowship at the University of New Haven. Thank you to the faculty in the Department of Biology and Environmental Science and the Department of Chemistry and Chemical Engineering for providing additional materials and guidance.

References:

1. Phage Therapy Center. Available from http://www.phagetherapycenter.com/pii/PatientServlet?command=static_home
2. Pommerville, J.C. Alcamo's Fundamentals of Microbiology 9th edition, March 8, 2010. Joans and Bartlett Publishers
3. Mole RJ, Maskell T. *Phage as a diagnostic—the use of phage in TB diagnosis*. J Chem Technol Biotechnol 2001;76:683–8.
4. Mycobacteriophage Database. Available from http://phagesdb.org/
5. Hanauer, D; Jacobs-Sera, D; Pedulla, M; Cresawn, S; Hendrix R; Hatfull, G. 2006. *Science.* [online] Available from: http://www.sciencemag.org/content/suppl/2006/12/19/314.5807.1880.DC1/Hanauer.SOM.pdf
6. Pope WH, Jacobs-Sera D, Russell DA, Peebles CL, Al-Atrache Z, et al. (2011) *Expanding the Diversity of Mycobacteriophages: Insights into Genome Architecture and Evolution*. PLoS ONE 6(1): e16329. doi:10.1371/journal.pone.0016329
7. Revie, D. 2012. *Determination of phages in soil* [online]. Available from: http://public.callutheran.edu/~revie/virology/Soil-phage-isolation.pdf
8. Froman S, Will DW, Bogen E. *Bacteriophage active against virulent Mycobacterium tuberculosis. I. Isolation and activity*. Am J Public Health Nations Health. 1954;44(10):1326-33.
9. World Health Organization: Tuberculosis Fact Sheet. http://www.who.int/mediacentre/factsheets/fs104/en/index.html
10. Kamala T, Paramasivan CN, Herbert D, Venkatesan P, Prabhakar R (1994a). *Evaluation of procedures for isolation mycobacteria from soil and water*. Appl. Environ. Microbial. 60: 1021-1024
11. Rahbar, M. Lamei2, A. Babazadeh2, H. Shoherh A. Y. *Isolation of rapid growing mycobacteria from soil and water in Iran.* African Journal of Biotechnology Vol. 9(24), pp. 3618-3621, 14 June, 2010 Available online at http://www.academicjournals.org/AJB DOI: 10.5897/AJB10.064 ISSN 1684–5315 © 2010 Academic Journals
12. University of Pittsburgh Phagehunting Program Protocols

Biography:

Christina Kling is currently at junior at the University of New Haven majoring in Biotechnology and Biochemistry. She hopes to pursue a Ph.D. in Infectious Disease, Epidemiology or a similar field. She is also considering pursuing an MD simultaneously. This was Christina's first experience with scientific research and, despite the dilemmas she experienced, she still aspires to pursue a career in research.