Identification and characterization of three bacteriophages that infect *Mycobacterium smegmatis*

Robert H. Frazier ’26 and Michael J. Wolyniak

Department of Biology, Hampden-Sydney College, Hampden-Sydney, VA 23943

Introduction

Bacteriophages, also known as phages, are viruses that infect bacteria hosts to replicate. Consisting primarily of a head and a tail, phages are the most abundant life-form on planet Earth. The head of a phage contains the DNA while the tail acts as both the binding mechanism and pathway of DNA between the head to the cytoplasm of the attacked bacteria cell. Phages reproduce through two distinct cycles. First, and the most prominent, is the lytic cycle, where the phage cell membrane inserts its DNA, and uses the cell’s ribosomal host machinery to create many new phages. Second is the lysogenic cycle, when the phage’s nucleic acid inserts itself into the host’s genome, followed by the lytic cycle. The phages populate until the cell lysates, or bursts open, leaving the cell killed and populating the environment with many new phages (Hatfull, *et al*.)

Phage Therapy

The most novel feature of phages is that the virus attacks and kills only bacteria specific to the individual phage. This feature has given rise to what is known as “phage therapy,” where a specific phage is introduced to an organism to stop illness caused by the bacteria specific to the given phage. A notable example was when phages found by undergraduate students of the SEA-PHAGES program were used to treat the symptoms of illness caused by a *Mycobacterium* infection. The patient, a 15-year-old with cystic fibrosis, along with a multitude of other health problems, was infected with *Mycobacterium abscessus*. To combat the infection, intravenous antibiotics were used but these only resulted in worsening symptoms of infection. The health team turned to phage therapy and began a search for phages found by the SEA-PHAGES program that infect *Mycobacterium*. Using three phages named Muddy, ZoeJ, and BPs, a phage “cocktail” was created as an alternative treatment, given intravenously every 12 hours for 32 weeks at 10^9 pfu concentration. As this treatment continued the symptoms of illness began to decrease and the patient was discharged (Dedrick, *et al*.).

This example serves as the first known use of phage therapy to treat a human mycobacterial infection. As antibiotic resistance grows within humanity, phage therapy continues to be the most evident candidate to replace antibiotics to stop human bacterial infections. For phage therapy to continue, and their effectiveness in stopping human infections and side effects on the human body, further phages are needed to be identified and characterized (Sparks). As this process of identification and characterization is simple yet informative, the SEA-PHAGES program exists to allow undergraduate students to perform this process. This project, using the procedures and guidance of the SEA-PHAGES program, identified and characterized three bacteriophages that infect *Mycobacterium smegmatis*. *M. smegmatis* was specifically used as the species’ DNA genome is over 90% identical to *M. tuberculosis*, a pathogen that is labeled a public health crisis according to the World Health Organization (Tuberculosis). Identifying and characterizing just three new phages will help the viruses become the answer to antibiotic resistance, such as the case described before.

Methods

The project began by following Protocol 5.1: Collecting Environmental Samples. In short, a quart-size plastic bag was used to collect 9 samples of fresh mulch from the east entrance garden of the Pauley Science Center in Hampden-Sydney, VA. The phages were isolated from the environmental samples using Protocol 5.5: Enriched Isolation. The protocol involved centrifuging 15 ml of sample and 20 ml of liquid *M. smegmatis* together to allow the phages to propagate. Enriched isolation was used over direct isolation as the protocol creates an abundance of the same phage for future protocols, rather than using a single phage sample. After centrifuging, the lysate was filter sterilized, resulting in a solution of only phages and *M. smegmatis*. The solution was incubated within flasks for 5 days at 220 rpm and 37 ℃. After incubation, 1.4 ml of

solution was transferred to two microcentrifuge tubes, microcentrifuged at 1,500 rpm for 1 minute, and transferred to a new tube without the resulting pellet of debris (Phage).

All nine samples were then subject to Protocol 5.6: Spot Test, where 250 µl of *M. smegmatis* and 3 ml of Middlebrook top agar were mixed and transferred onto Luria agar plates. The top agar was allowed to solidify for about 30 minutes. 10 µl of each solution from Protocol 5.5 was transferred onto the top agar and incubated for 24 hours at 37 ℃. To confirm the results of the spot test for purity and concentration of phages within samples 4, 6, and 8, each sample was subject to Protocol 6.2: Serial Dilutions. Each solution from Protocol 5.5 was diluted 10-fold through a series of microcentrifuge tubes from 10^{-1} to 10^{-8} concentration. After, 10 µl of the individual dilution, 250 µl of *M. smegmatis* and 3 ml of top agar were mixed and transferred onto their own Luria agar plate and allowed to solidify before incubating for 24 hours at 37 ℃. Also, samples 4, 6, and 8 were subject to Protocol 6.4: Spot Titer, to determine the correct concentration of dilution to make the necessary webbed plates. The protocol involved transferring 250 µl of *M. smegmatis* and 3 ml of Middlebrook top agar to a Luria agar plate. After the plate was solidified, 10 µl of each solution from Protocol 5.5 was placed within the corresponding location on the plate (Phage).

To extract DNA from the phage samples, Protocol 7.1: Making Webbed Plates from a Lysate of Known Titer was done. To start, five plates for each sample were made, containing 10 µl of 10^{-1} dilution, 250 µl of *M. Smegmatis*, and 3 ml of Middlebrook top agar mixed and transferred on top of a Luria agar plate. After 24 hours of incubation at 37 ℃, Protocol 6.3: Collecting Plate Lysates was done. Each plate was “flooded” with 8 ml of phage buffer and incubated at 4 ℃ for 24 hours. After, the plates were harvested with 5 ml syringes while being filter sterilized using attached 0.22 µm filters. The DNA from each sample was then extracted following Protocol 9.1: Phage DNA Extraction. First, the DNA was degraded by mixing 5 ml of nuclease mix with 1 ml of phage lysate from Protocol 6.3 before incubating at room temperature for 1 hour. Second, the protein capsid to remove the DNA was then degraded by adding 2 ml of the DNA clean-up resin with the lysate/nuclease mix before inverting the solution for 2 minutes. Lastly, the genomic DNA of the individual phages was isolated. To start, 1.5 ml of lysate/nuclease/resin solution was filtered through two columns to have the columns contain the DNA. Then the DNA within the columns was washed three times with 2 ml of 80% isopropanol to “clean” the DNA. The residual isopropanol in the columns was removed by centrifuging at 10,000 rpm for 10 minutes before being placed into a heat black at 90 ℃ for 1 minute. The DNA was then diluted from the column by adding 50 µl of water at 90 ℃, incubated at room temperature for 1 minute, and spun at 10,000 rpm for 1 minute. The solution of the two columns was added together to have a resulting solution of the isolated phage DNA in 100 µl of water (Phage).

To determine the amount of phage DNA isolated, a Biotek Epoch Spectrophotometer was used. The machine quantified the DNA using ultraviolet spectrometry and the results were analyzed with Biotek Epoch 1.09.8 software in µg/ml. To determine whether the isolated DNA was intact, a gel electrophoresis was run, where the DNA of all three phages was compared to a 1kb ladder.

To prepare to visually examine the phages, Protocol: 8.1: Mounting Phage Samples for TEM and Staining with Uranyl Acetate was done. To do so, 100 µl of phage lysate from Protocol 7.1 was centrifuged at 10,000 rpm for 22 minutes within an environment of 4 ℃ to remove any phage debris. Then, 5 ml of the centrifuged lysate was pipetted on top of a copper grid, specifically a Formvar/Carbon 200 Mesh grid. After letting the phages adhere to the copper grid for 5 minutes, the phage lysate was rinsed off the grid by filter paper. Then, 5 µl of Uranyless was pipetted on top of the mesh grid and left to stain the grid for 5 minutes, before rinsing off the grid by filter paper (Phage). The resulting grids with adhered phages were then taken to Mary Washington University in Fredericksburg, VA, where the samples were observed under an electron microscope at 36,000x magnification.

Results

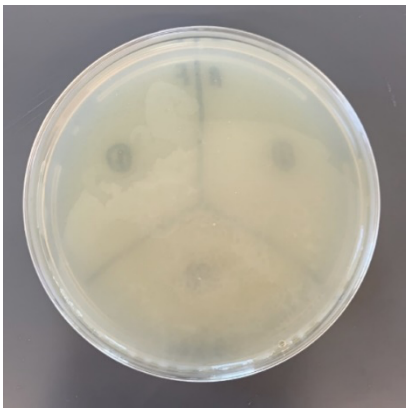


Figure 1: Spot test for samples 1,2, and 3

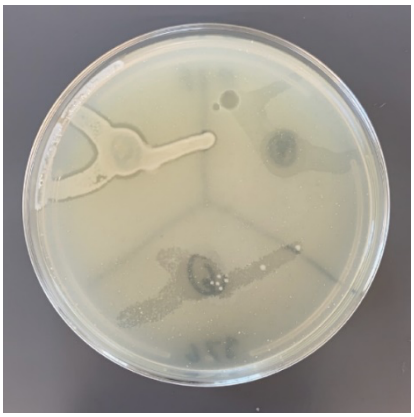


Figure 2: Spot test for samples 4,5, and 6

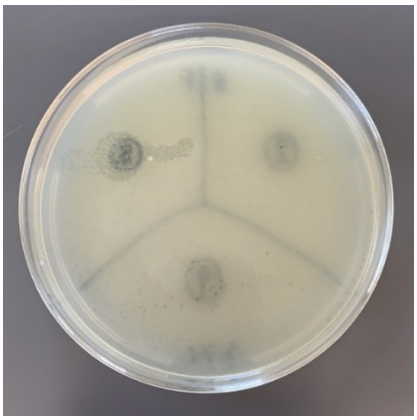


Figure 3: Spot test for samples 7,8, and 9

Figure 1 shows the results of the spot test for samples 1, 2, and 3. There are no clearings on the "lawn" of *M. smegmatis*, only a "haze" that covers the plate. Figure 2 shows the results of the spot test for samples 4, 5, and 6. There are clearings on the "lawn" of *M. smegmatis* for all three samples. Figure 3 shows the results of the spot test for samples 7, 8, and 9. There is only a clearing on the "lawn" of *M. smegmatis* in sample 8.

Figure 4

Figure 4 shows the representative results of the serial dilutions done for samples 4, 6, and 8. Decreasing in dilution from top to bottom, the results show 10^{1} dilution plate having the most clearings on the "lawn" of *M. smegmatis*, to the 10^{-8} dilution plate with the least clearings on the "lawn" of *M. smegmatis*.

Figure 5 shows the representative results of the spot titer done for samples 4, 6, and 8. There is less of a clearing on the "lawn" of *M. smegmatis* as the dilution increases from 10^{-1} to 10^{-8}.

Figure 6 shows the representative webbed plates made for extracting phage DNA. The webbed plates, at 10^{-1} dilution, were produced to provide enough phages to extract DNA from.

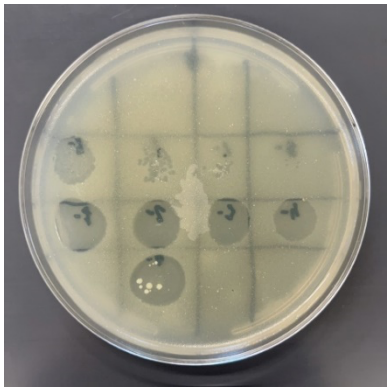


Figure 5: Spot titer of samples 4, 6, and 8

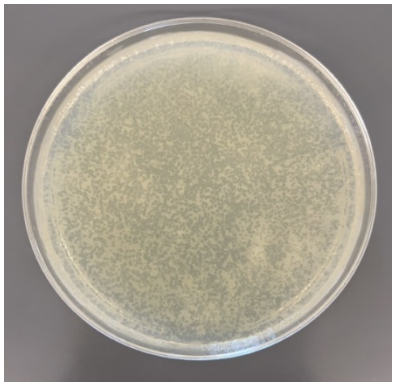
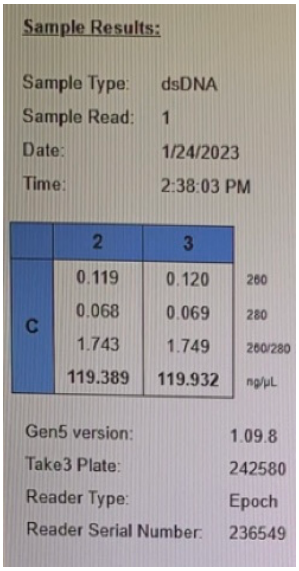


Figure 6: Webbed plate of samples of samples 4, 6, and 8



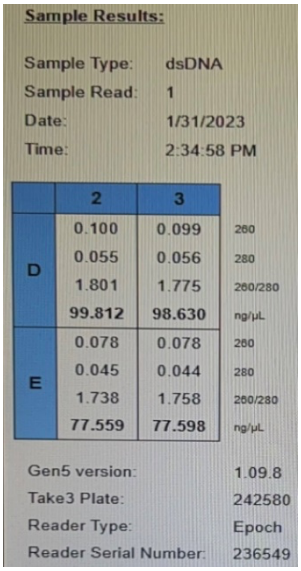
Sample Results:

Sample Type: dsDNA
Sample Read: 1
Date: 1/24/2023
Time: 2:38:03 PM

	2	3	
C	0.119	0.120	260
	0.068	0.069	280
	1.743	1.749	260/280
	119.389	119.932	ng/µL

Gen5 version: 1.09.8
Take3 Plate: 242580
Reader Type: Epoch
Reader Serial Number: 236549

Figure 7



Sample Results:

Sample Type: dsDNA
Sample Read: 1
Date: 1/31/2023
Time: 2:34:58 PM

	2	3	
D	0.100	0.099	260
	0.055	0.056	280
	1.801	1.775	260/280
	99.812	98.630	ng/µL
E	0.078	0.078	260
	0.045	0.044	280
	1.738	1.758	260/280
	77.559	77.598	ng/µL

Gen5 version: 1.09.8
Take3 Plate: 242580
Reader Type: Epoch
Reader Serial Number: 236549

Figure 8

Figure 7 shows the results of the amount of phage DNA extracted from sample 4. The table shows an average ng/µl measurement of 119.661. Figure 8 shows the results of the amount of phage DNA extracted from samples 6 and 8. The table shows an average ng/µl measurement of 99.221 for sample 6 in the “D” row and an average ng/ul measurement of 77.578 for sample 8 in the “E” row.

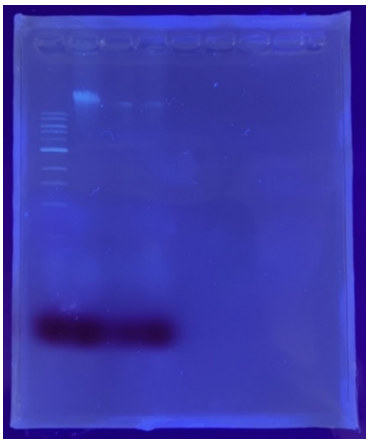


Figure 9: Gel electrophoresis of samples 4,6 and 8

Figure 9 shows the results of the gel electrophoresis done for samples 4, 6, and 8. From left to right, the first column is the 1 kb ladder, the second is sample 4, the third is sample 6, and the fourth is sample 8. In the second, third, and fourth rows, there is only a single line within the respective row, indicating that the DNA is intact for samples 4, 6, and 8.

Figures 10, 11, and 12 show the phages under an electron microscope at 36,000x magnification. Clearly distinct are the heads and tails of the phages, including differences in head diameter and tail length. By visual confirmation, the head shape of all three phages is siphoviridae. The phage from sample 4 was named Bosample4, the phage from sample 6 was named Bosection6, and the phage from sample 8 was named Boselection8.

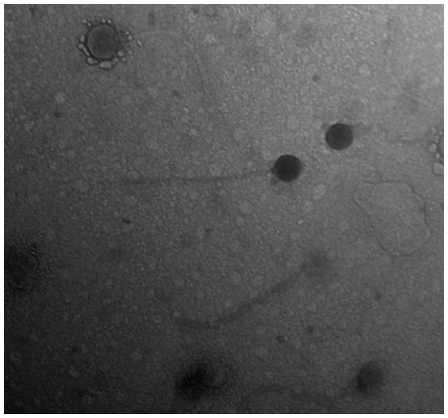


Figure 10: Bosample4

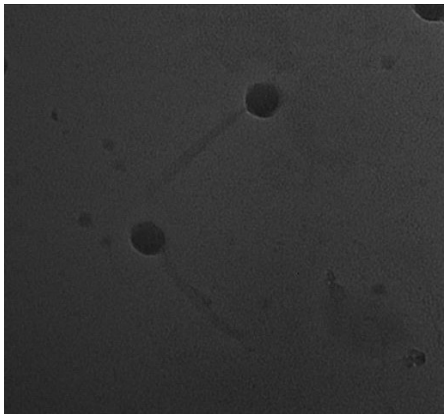


Figure 11: Bosample6

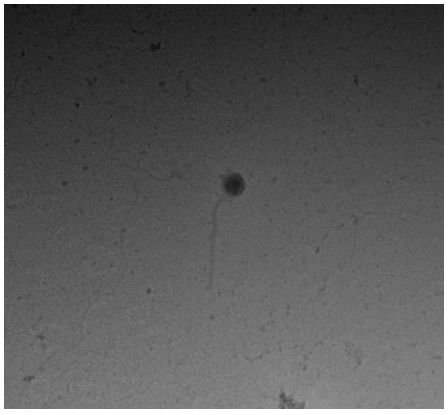


Figure 12: Boample8

Discussion

In conclusion, the project identified three new bacteriophages from nine samples. Starting with a spot test of the nine samples, we could identify the phages from the clearing on the "lawn" of *M. smegmatis*. As phages are specific to bacteria, phages that only attack and kill bacteria they are "programmed" to attack and kill. Therefore, clearings on the "lawn" of *M. smegmatis* were caused by phages specific to *M. smegmatis*. Of all nine samples, samples 4, 5, 6, and 8 had clearings on the "lawns" of *M. smegmatis*. Although the phage from sample 5 had a great amount of clearing on the "lawn", we could not continue with it due to a "yellowish" containment that covered the inside of the clearing it caused. Evident in the remaining three phages was the different amount of *M. smegmatis* left within the surface area the phages were exposed to. Bosample4 had the most "clearing" within the surface area infected, followed by Bosection6, and then Boselection8.

To confirm the purity and concentration of all three phages, serial dilutions and spot titer protocols were used. As per Figure 4, the amount of clearing on the "lawn" of *M. smegmatis* decreased as the dilution factor increased. Parallel to the results of the serial dilutions were the results of the spot titers. As per Figure 5, the amount of clearing in the grid spaces decreased as the dilution factor increased. After these two tests for all three phages, we were able to confirm that we did have three isolated phages that infect M. smegmatis. Also, from the spot titer test we were able to confirm that we had a concentration of each phage that would be at least 1.0×10^{10} pfu/ml. With Bosample4 and Bosection6 having a concentration of 4.0×10^{10} pfu/ml, and Boselection8 having a concentration of 1.2 x 10^10 pfu/ml, we were able to confirm that we had a good enough concentration to continue with future protocols.

To extract the phage DNA for sequencing, webbed plates were made. Webbed plates, plates where the "lawn" of bacteria, or in this case *M. smegmatis*, covers less than 25% of the surface of the plate because of phages, were made from a dilution factor of 10^{-1}. As per Figure 6, we did have webbed plates for all three samples containing phages. After the samples were turned into lysate and the DNA was extracted, we used ultraviolet spectrophotometry to read the amount of phage DNA extracted in ng/ul. We extracted the most DNA from Bosample4, at an average concentration of 119.661 ng/ul. For Bosection6, we had an average concentration of 99.221 ng/ul, and Boselection8 had an average concentration of 77.578 ng/ul. Even though there was an unequal amount of phage DNA extracted between all three samples, all samples were at least 50 ng/ul, a good enough concentration for the phage DNA to be sequenced. Afterward, gel electrophoresis was used to determine whether the extracted phage DNA was intact. As per columns 2-4 in Figure 9, there is only one band within the boundaries of the columns, defining that the DNA of all three phages was intact. What is also worthy of noting is the decreasing amount of color of each sample matches the decreasing amount of phage DNA concentration, as Bosample4 had the most phage DNA extracted and the most visible band, while Boselection8 has the least phage DNA extracted and the least visible band.

Under the electron microscope, the phages were individually visible to the naked eye. All three phages had a siphoviridae head shape, the most common shape. Where the three phages differed was in head diameters and tail lengths. For the head diameter, Boselection8 had the largest, followed by Bosample4, and Bosection6, and for the tail lengths, Bosample4 had the longest, followed by Boselection8, and Bosection6. These differences not only visually confirm the presence of three individual phages but suit the different needs of attacking different strains of *M. smegmatis*.

Future Directions

The scope of this project was limited by the time frame given to students to do research between the 2023 spring and fall semesters of Hampden-Sydney College. Therefore, the complete characterization of all three phages will be completed once the fall semester starts. First will be to send the isolated DNA of all three phages from Protocol 6.3 to the University of Pittsburgh to be sequenced. After, Dr. Michael Wolyniak will use the DNA genomes of all three phages to be the basis of his Biology 313: Genomics and Bioinformatics class of spring 2024 at Hampden-Sydney College. In his class, the DNA genomes will be identified and compared to the genomes of other phages found by the SEA-PHAGES program.

References

Dedrick, Rebekah, *et al*. “Engineered bacteriophages for treatment of patient with a disseminated drug resistant *Mycobacterium abscessus*”. *Nature Medicine*, vol. 25, 2019, pp. 730-733. *National Library of Medicine*, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6557439/.

Hatfull, Graham, *et al*. “Phage Therapy for Antibiotic-Resistance Bacterial Infections”. *Annual Review of Medicine*, vol. 73, 2022, pp. 197-221. *Annual Review*, https://www.annualreviews.org/doi/full/10.1146/annurev-med-080219-122208#_i2.

“Phage Discovery Guide”. The SEA-PHAGES Program, 2023. https://seaphagesphagediscoveryguide.helpdocsonline.com/home.

Sparks, Ian. “Mycobacterium smegmatis: The Vanguard of Mycobacterial Research”. *Journal of Bacteriology*, vol. 25, 2023. *National Library of Medicine*, https://pubmed.ncbi.nlm.nih.gov/36598232/.

“Tubercolosis”. *World Health Organization*, 2023. https://www.who.int/news-room/fact-sheets/detail/tuberculosis.