Isolation and Analysis of a Mycobacteriophage Specific to Mycobacterium Smegmatis

ANGELA J. COLLINS, MOHAMMED DANISH GHAZALI, YI LI, SOO HA,
DR. KARI L. CLASE, and DR. HATFULL

IT 22600, Industrial Technology Department, Purdue University
West Lafayette, IN, 47907

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Over the course of the Fall 2015 semester of IT 26600, my lab partner, Mohammed Danish Ghazali, and I conducted research regarding mycobacteriophages. We successfully isolated a novel bacteriophage from a soil sample collected on the campus of Purdue University, and then continued on to isolate, purify, restrict, and analyze the genomic DNA of the phage. This bacteriophage is now documented on the Bacteriophage Database as FelixElFago at http://phagesdb.org/phages/FelixElFago/. Throughout this report, I will discuss our experimental procedures and results, as well as the implications of our findings and future possibilities regarding this research experiment.

INTRODUCTION

Bacteriophages are viruses that kill bacteria; the Greek root of the word literally means "eater of bacteria" (Griffiths, 1999). Bacteriophages "eat" bacteria by attaching to the surface of a bacterium, injecting its DNA into the bacterium, replicating, and then causing the microbe to lyse, which sends out countless new bacteriophages to infect other hosts (Howard Hughes Medical Institute). They are perhaps the most abundant species on the planet, outnumbering "all the bacteria, all the humans, whales, trees, et cetera, put together" (Travis, 2003). It is estimated that there are roughly 10^{31} types of bacteriophages in the world, and that every bacterium has an average of 10 bacteriophages that can infect it (Travis, 2003).

Specifically, a mycobacteriophage is a type of bacteriophage which has a mycobacterial species as its host (Hatfull). Examples of mycobacterial species include *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, which is the bacterial culture that we used in our research. Already, through programs such as Phage Hunters Integrating Research and Education (PHIRE) and the Howard Hughes Medical Institute Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (HHMI SEA-PHAGES), several thousand distinct mycobacteriophages have been isolated from the environment using Mycobacterium smegmatis as the host (Hatfull), and we are excited to add yet another novel bacteriophage to the count. Currently, due to the extensive research being conducted by these programs, there are 30 known clusters, or types, or mycobacteriophages which all contain very distinct genetic structures (Hatfull). Furthermore, even though more than 50,000 different bacteriophage genes have been analyzed and sorted, researchers "have yet to saturate the diversity of this particular population" (Hatfull)." There are still countless mycobacteriophage populations that need to be isolated, which is what makes our research project so significant.

Bacteriophages were discovered about 100 years ago in 1915 by two European scientists named Twort and D'Herelle (Veiga-Crespo, 2007), and early on they were thought to serve as "treatments for infectious diseases" (Travis, 2003). Then, when antibiotics were discovered, many scientists from the West began researching antibiotics more in depth and stopped researching

bacteriophages (Veiga-Crespo, 2007). However, decades later, antibiotics are not as effective as they used to be because first, the FDA has been approving fewer and fewer antibiotics, and second, the microorganisms are becoming resistant to these antibiotics. For example, "today, penicillin fails to completely eradicate streptococci in up to 35% of patients" (Veiga-Crespo, 2007). Therefore, the study of bacteriophages is once again on the rise, but this time a lot more research has been directed towards it. It is important to study mycobacteriophages more in-depth because they have an incredibly untapped potential to "offer a new hope for medicine in the struggle against illness" (Veiga-Crespo, 2007). One potential use for bacteriophages in medicine includes multiple forms of therapy, such as that used to cure MRSA, or methicillin-resistant staphylococcus aureus, which is caused by "a type of staph bacteria that's become resistant to many of the antibiotics used to treat ordinary staph infections" (Benson, 2013). Other potential uses for bacteriophages include eradication of bacterial contaminants in food products, ulcer treatments, the healing of open wounds, control of bacterial growth during fermentation, and much more (Veiga-Crespo, 2007).

Therefore, it is apparent that our phage research is important because phages have so many significant applications in medicine, yet the vast variety of bacteriophages is relatively untapped and undiscovered. As we discover new bacteriophages as a class, scientists will then be able to further research our phages and the functionality of their genomic DNA, and apply these discoveries to find medical solutions.

MATERIALS AND METHODS

For the entirety of this semester, our class has captured, isolated, and purified a bacteriophage species from the local environment of West Lafayette, Indiana. Upon isolating a single bacteriophage species, we then isolated, purified, and analyzed the genomic DNA of the phage. Finally, we logged our findings into the bacteriophage database and sent our DNA to the Sequencing Center.

Throughout this experiment, we followed very closely the procedures which were outlined in the Howard Hughes Medical Institute (HHMI) Sea-Phages Laboratory Manual. The specific steps which we executed are as follows:

Sample Collection: To begin the process of discovering a novel bacteriophage, we first had to collect soil and water samples from local areas around Purdue University. To do this, we used a spatula to fill a 15-mL conical tube about two-thirds full with soil or water. We also took a picture to document the sample site, and noted several characteristics about the sample. Important aspects we noted were the data and time of the sample collection, GPS coordinates of the site location, depth, approximate moisture content of the soil, whether the sample site was urban or rural, proximity to foot traffic, cement, major roads, and trees, and whether the site was sloped (Howard Hughes Medical Institute).

Direct Plating: Once we had obtained our samples, we plated the samples directly. The HHMI lab manual offers another method of plating the samples in which the environmental sample in enriched by seeding it with host bacteria. However, we only used the method of direct plating. For each sample, we first labeled a 15mL conical tube, and then filled the tube approximately half-full of the sample using a spatula. Next, we added 10mL of phage buffer (PB) to the tube to flood the sample, mixed it well for 5 seconds using a vortexer, and let the sample set undisturbed for 20 minutes to allow the liquid later to separate from the solid layer. After 20 minutes, we used a syringe to remove 2mL of the liquid only from the sample tube, and then filtered the solution through a filter attached to the end of the syringe and into a sterile microcentrifuge tube. To prepare the negative control, 1 mL of PB was filtered into a labeled microcentrifuge tube (Howard Hughes Medical Institute).

Next, we infected a bacteria culture with these prepared samples. By using a micropipette, 50 µL of each sample and 50 µL of the control was added to labeled culture tubes containing 0.5 mL of an *M. smegmatis* culture. Then, each tube was well mixed by using a vortexer, allowed to sit for 30 minutes at room temperature to allow ample time for the possible bacteriophages in the samples to infect the bacteria culture. Meanwhile, agar plates were labeled for each sample and the control. After 30 minutes had passed, we obtained a 50 mL bottle of top agar (TA),

prepared by our graduate teaching assistants Yi Li and Soo Ha, and used a 5 mL pipette to transfer 4.5 mL of TA into each culture tube. Immediately after transferring the agar into each tube, the tube was swirled gently, and the mixture was poured onto the corresponding agar plate. Finally, the plate was swirled gently to allow the TA to spread evenly and cover the entire surface of the plate. After following this procedure for every sample, the plates were left alone for at least 20 minutes so that the agar could solidify before being inverted and incubated at 37°C. It is important to note that in this step, our execution of the procedure differed slightly from the protocol in the HHMI lab manual. In the manual, it states to suck the top agar/bacteria/sample mixture back into the pipette and then transfer the mixture to the plate using the pipette, but we simply poured the contents of the tube directly onto the plate. We elected to make this slight adjustment because it plated the agar faster, which prevented the agar from solidifying too early, and it prevented the creation of air bubbles that often results from the pipette transfer method (Howard Hughes Medical Institute).

Finally, after waiting at least 24 hours, during the next lab period we checked our plates for plaque growth. We were looking for plaques on the samples plates, and no growth on the control plate. Once we found growth that we suspected to be a plaque, the HHMI lab manual states to perform the spot test protocol to determine whether or not the unit is actually a plaque. However, due to the larger size and unique morphology of the units we found, we were positive that the grow was plaque growth, and therefore we elected to skip this step and proceed to the plaque streak protocol (Howard Hughes Medical Institute).

Streak Plates: The objective of this step was to isolate a single bacteriophage population. This entire procedure required the aseptic technique. First, a few isolated plaques were selected to streak from, and their size and morphology was noted in our lab notebooks. To practice streaking, a micropipettor and tip was first used to prepare a negative control plate. To perform this streaking, the tip of the micropipettor was used to draw a chain of three linked "Z" formations around the plate. Then, to prepare the sample plating, a similar yet more concise procedure was used. First, a new sterile tip was attached to the micropipettor, and using this tip, we touched the center of a plaque we had selected for streaking. Then, using this same tip, a "Z" formation was drawn lightly in the agar on one-third of the plate without lifting the tip from the agar. Then, this tip was discarded and a new sterile tip was used to draw a second "Z" formation to the right of the first, with only the initial stroke overlapping the original strokes. Finally, a third new tip was used to draw a third "Z" formation, with only the initial stroke overlapping the previous formation. After the streaking has been completed, the end streak was marked to indicate where the most dilute section of the plate was. The finish the plate, a 5 mL pipette was used to add 4.5 mL of TA to a culture tube containing 0.5 mL of the *M. smegmatis* bacteria, and then the mixture was poured onto the agar plate at the most dilute site, as previously marked on the plate. Unlike in the direct plating protocol, the plate was not swirled to distribute the agar because we did not want to affect the concentration of bacteriophage sample in the different areas of the plate. This procedure was repeated for every plaque that we wanted to streak. After allowing the TA in each plate to solidify for 20 minutes, the plates were inverted and incubated at 37°C (Howard Hughes Medical Institute).

After at least 24 hours, or in our case during the next lab period, we checked the plates for plaques. The most isolated plaques were then marked and used to perform the next set of streaking. This entire procedure was repeated 9 times. Due to rampant contamination, during the 7^{th} streaking we also performed a serial dilution and filtration on our sample plates from the 5^{th} streaking to attempt to decrease the level of contaminants. We then had to return to the 5^{th} streak plates and restreak from them to limit the contamination. After performing two more streaks, which was the 7^{th} streak but the 9^{th} streak total, we determined that the plaque morphology was consistent, and that was could confidently conclude that we had isolated a single phage population and could advance to the next step (Howard Hughes Medical Institute).

Serial Dilutions/Titer: Once we had isolated a single bacteriophage population, used a phage-titer assay and serial dilutions to determine

the titer, or concentration of plaque forming units (pfu) in the original sample. To begin, 100 μL of phage buffer was put into a microcentrifuge tube. Using a sterile tip on a micropipettor, a sample of phage was taking by poking a plaque and then inserting the tip into the phage buffer. The tube was then vortexed and used to perform a serial 10-fold dilution (Howard Hughes Medical Institute).

To perform this dilution, 90 μL of phage buffer was added to four microcentrifuge tubes, previously labeled with -1, -2, -3, and -4. Then, 10 μL of the phage sample is transferred into the -1 microcentrifuge tube and vortexed well. 10 μL of this solution is then transferred into the -2 microcentrifuge tube and vortexed well. This process is repeated until 10 μL is added to the -4 microcentrifuge tube (Howard Hughes Medical Institute).

Next, add 10 μL of each dilution solution to culture tubes containing 0.5 mL of the *M. smegmatis* bacteria culture. Also add 10 μL to a to culture tube containing 0.5 mL of the *M. smegmatis* to prepare the control. After allowing the phage to infect the bacteria for 20 minutes, add 4.5 mL of top agar to each tube, swirl, and quickly pour the contents onto the correspondingly labeled agar plates. Allow the plates to solidify for 20 minutes, and then invert the plate and incubate them at 37°C (Howard Hughes Medical Institute).

After 24 hours, or in our case during the next lab period, we checked for plaque growth, counted the number of plaques on each plate, and calculated the titer. These calculations are shown in the results section of this report (Howard Hughes Medical Institute).

MTL Harvest and Titer: After the titer of the original sample was calculated, the plate which was used to calculate the titer was then used to make the Medium Titer Lysate (MTL). The MTL was harvested by flooding the bacterial lawn with 8 mL of phage buffer, incubating the plate overnight at 37°C, and then transferring a filtered solution of the phage buffer from the plate into a conical tube via a 5-mLsyringe and filter. Next, an agar plate is prepared by drawing gridlines to create 20 boxes which are labeled from 10^{-1} to 10^{-10}, as well as a control block. Then, 4.5 mL of top agar was added to 0.5 mL of *M. smegmatis* bacteria in a conical tube, swirled, and transferred onto the agar plate. Then, we allowed the plate to solidify while we prepared the tenfold dilution with 10 μL of the harvested MTL. The procedure for this dilution is the same as that of the tenfold dilution in the previous Serial Dilutions step, except we diluted up to a factor of 10^{-10} instead of 10^{-4}. Then, once the dilutions were created, we transferred 5 μL of each dilution onto the corresponding grid of the agar plate by using a micropipettor. For the control grid, 5 μL of phage buffer was added instead. Once the droplets soaked into the solidified agar, the plates were inverted and incubated at 37°C overnight. During the nest lab period, we counted the number of plaques in each grid, and calculated the titer of the MTL. These calculations are shown in the results section of this report (Howard Hughes Medical Institute).

Empirical Test: Once the MTL titer was calculated, we calculated the volume and dilution of MTL that was needed to form a web plate. We also calculated the volumes of MTL needed to perform two 2-fold dilutions above and below the calculate web plate. These calculations are shown in the results section of this report (Howard Hughes Medical Institute).

Then, to perform the empirical assay, we performed another serial tenfold dilution based on our calculations. The specified amounts of each dilution were then transferred into culture tubes containing 0.5 mL of *M. smegmatis* bacteria. After allowing the phage to infect the bacteria for 20 minutes, 4.5 mL of phage buffer was added to each tube, swirled, and the contents were poured onto the correspondingly labeled agar plates. The plates then set for 30 minutes to let the agar solidify, and were incubated at 37°C. The next lab period, we analyzed the plates, chose the plate with the best web plate pattern, and used that volume and dilution combination to perform the 10 plate infection (Howard Hughes Medical Institute).

10 Plate Infection: To perform the 10 plate infection, we infected 5.0 mL of *M. smegmatis* with the appropriate amount of MTL, as calculated based on the dilution needed for a web plate. After waiting for the phage to infect the bacteria for 20 minutes, 45 mL of top agar was added to the mixture and swirled gently to mix well. 5 mL of the mixture was then added to each of ten appropriately labeled agar plates. After

allowing the top agar to solidify for 15 minutes, we inverted the plates and incubated them at 37°C overnight (Howard Hughes Medical Institute).

HTL Harvest and Titer: Within 24 hours, or in our case the following morning, we harvested the HTL. We did this by extracting the phage buffer from the agar plates with a 25 mL pipette, and then draining the extracted HTL into 2 50 mL conical tubes. We then centrifuged the tubes to 20 minutes to allow the debris to collect together, and drained the HTL into a 100 mL sterilization unit, leaving the debris pellet behind in the conical tube. Finally, we filter sterilized the HTL using suction, capped the tube containing the sterilized HTL, and stored the HTL at 4°C (Howard Hughes Medical Institute).

To calculate the titer of the HTL, we performed a serial tenfold dilution of the HTL. We then added 10 μL of each dilution to 0.5 mL of *M. smegmatis*, allowed the phage to infect the bacteria for 20 minutes, added 4.5 mL of top agar to each tube, and then poured the solution onto correspondingly labeled agar plates. After allowing the top agar to solidify for 10 minutes, we incubated the plates at 37°C. During the next lab period, we counted the number of plaques that had formed on the plates and calculated the titer of the HTL (Howard Hughes Medical Institute). These calculations are shown in the results section of this report.

Isolation of DNA: To isolate and purify the phage genomic DNA, we first transferred 10 mL of HTL into an Oak Ridge tube, added 40 μL of Nuclease Mix, and mixed by gently inverting several times. Next, we let the mixture incubate at 37°C for 30 minutes, and then we let it sit for one hour at room temperature. Finally, we added 4.0 mL of phage precipitant solution to the lysate, mixed by gently inverting it several times, and then incubating it overnight at 4°C (Howard Hughes Medical Institute).

During the next lab period, we centrifuged the lysate at 10,000xg for 20 minutes, and gently drained the solution, making sure to leave the pellet that had formed. Drain any remaining fluid from the pellet by inverting the tube and allowing it to drain for 3 minutes. Once the pellet has drained, add 0.5 mL of sterile ddH_2O to the pellet and break the pellet apart by pipetting it and the water up and down several times. After the mixture set untouched for 5 minutes, we added 2 mL of DNA Clean Up Resin and gently pipetted up and down once more. All of this was done to uncoat the genomic DNA of the phage (Howard Hughes Medical Institute).

Finally, to isolate the DNA and prepare a sample to analyze with the spectrophotometer, we added 1.25 mL of the solution to two columns attached to a syringe. A plunger was then used to push the solution through the column, which collected the DNA. Next, after disconnecting the syringe from the column and removing the plunger, the syringe was reattached and 2 mL of 80% isopropanol was added to each column. Using the plunger, we then pushed the isopropanol through the column to strip the DNA of any salts and proteins. To dry the isopropanol off of the DNA, we then centrifuged the columns in a microcentrifuge tube (with the cap open) at maximum speed for 5 minutes. To dry off the alcohol, the columns were then placed in clean microcentrifuge tubes and centrifuged for another minutes at maximum speed. Next, after transferring the columns to two more sterile microcentrifuge tubes, we added 50 μL of 80°C TE to the columns, allowed the TE to dissolve the DNA for 60 seconds, and then centrifuged the columns for 60 seconds to finish purifying the DNA sample. This purified sample was then analyzed and quantified by using a Nanodrop spectrophotometer (Howard Hughes Medical Institute). The results of this test are shown in the results section of this report.

Restriction Enzyme Digest: To prepare the DNA sample for electrophoresis, we first had to perform several reactions to digest the phage genomic DNA. The specific details regarding these reactions are published in the results section of this paper. However, before the reaction were carried out, we had to prepare the DNA sample by vortexing it on low, incubating it for 10 minutes at 65°C, and then placing the tube in ice before performing a quick spin. Then, the reactions were carried out by adding the specific volumes of each solution, as indicated in the table in Figure 8.1 in the results section. Finally, we quick spun the tubes again, and incubated them in a 37°C water bath for 2 hours before quick spinning them again and placing them in the freezer (Howard Hughes Medical Institute).

DNA Agarose Gel Electrophoresis: In this step, one of our graduate teaching assistants,

Yi LI, prepared the gel apparatus for us. Once he finished setting up the gel, we used a micropipettor to load 10 µL of each sample into pockets in the gel. The electrophoresis test was then run at 100 V until the bromophenol blue (BPB) was within 1 cm of the end of the gel, which took approximately 45 minutes (Howard Hughes Medical Institute). A photograph of the results of this test is shown in the results section of this report.

Electron Microscopy: To analyze our bacteriophage under the electron microscope, we prepared our phage sample by first transferring 1.0 mL of the HTL into a microcentrifuge tube. We then centrifuged the tubes for one hour at 4°C and 10,000xg. After centrifuging, all but 20 µL of solution was removed without disturbing the pellet that had formed. 100 µL of phage buffer was then added, and mixed by gently pipetting up and down. This sample was stored overnight at 4°C. The following day, Mrs. Laurie Miller helped us analyze the bacteriophage under an electron microscope. She placed a 5 x 5 cm piece of parafilm on a Petri dish and a PELCO Tab on top of the parafilm. She then placed a grid on the edge of the tab and placed 10 µL of the phage sample on the grid. After allowing the solution to attach to the grid for 30 seconds, she absorbed the excess solution with a piece of filter paper. Finally, she added 10 µL of 1.0% uranyl acetate to the grid, let it stain for 2 minutes, and absorbed the excess stain. The prepared sample was then loaded into the electron microscope (Howard Hughes Medical Institute). The photographs taken of the physical structure of the bacteriophage are shown in the results section of this report.

RESULTS

Sample Collection:

During this step, we gathered eight samples total – two water samples and six soil samples. The water samples were collected from the fountain next to the Bell Tower on Purdue's campus, and a low-lying stream located near the celery bog near Purdue's campus. However, neither of these samples resulted in plaque growth through direct plating. The six soil samples were taken from various areas on Purdue's campus, ranging from a flower bed near the Hello Walk to one of the Cattail hiking trails, but only one sample produced plaque growth after direct plating. This sample, Sample 3, was acquired on September 10, 2015 at 2:01pm from a flower bed next to the Third Street Suites, which is an urban area of campus. The soil was taken from a depth of about 4 cm and was near a small mushroom located roughly 1.5 feet from a cement sidewalk and foot traffic. The soil bed was level, but contained a mixture of clay, soil, and mulch. The coordinates of the sample location are 40.427758 N, -86.920437 E. An overview of this information is listed below, as well as picture of the sample cite which are shown in Figure 1.

Sample 3 Data:

- Date: 9/10/2015
- Time: 2:01pm
- Location: 40.427758 N, -86.920437 E
- Depth: 4cm
- Approximate Moisture Content of Soil: pretty damp
- Urban/Rural: Urban
- Proximity to Foot Traffic: 1.5ft
- Sloped/Level: Level
- Other: mulch, clay, mushrooms

Figure 1: Flower bed where Sample 3 was collected. The right photo shows a close-up of where Sample 3 was collected, including the nearby mushroom growth.

Direct Plating:

During this step, we plated a total 29 plates for 8 different samples. Sample 3 was the only sample that resulted in plaque growth, as shown by the pictures in Figures 2.1, 2.2, and 2.3. When we proceeded to the plate streaking protocol, we streaked from the Sample 3C plate.

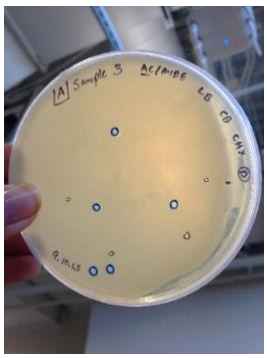

Figure 2.1: Sample 3A

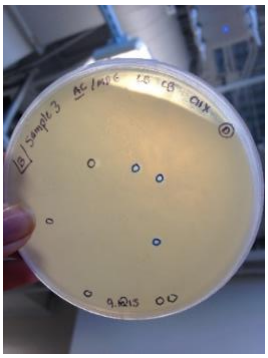

Figure 2.2: Sample 3B

Figure 2.3: Sample 3C

Streak Plates:

At the beginning of this procedure, we streaked from the Sample 3C plate shown in Figure 2.3. To keep track of the origins of the streak plates, we named the initial three plaques that we streaked from; Violet 1, Violet 2, and Felix. After streaking from each of these plaques and analyzing their resulting plaque growth, we decided to continue the streaking procedure with Felix because that plate had the most isolated plaques with the most consistent plaque morphology. Therefore, that is how the official name of our phage, FelixElFago, originated.

We performed a total of 9 streak tests. On the same day that we performed the 7th streak, we also performed a dilution series and filtration on the 5th streak plate (Figure 3.1) to eliminate the contamination. Although the dilution series and filtration did not completely eliminate the contamination, it did successfully reduce the amount of contamination. Therefore, we scrapped the existing 6th (Figure 3.2) and 7th (Figure 3.3) streak plates and returned to streak from the purified 5th streak plate. After remaking the 6th and 7th streak plates, we concluded that the 5th, 6th, and 7th streak plates all had a consistent plaque size and morphology. Every plaque had a small clear center surrounded by thick opaque/cloudy edges. The plaque diameters ranged in size from 1mm to 4 mm, with an average diameter size of 3mm.

Figure 3.1: 5th Streak Plate; The left image shows the plate before the serial dilution and filtration was performed on it. The right image shows the dilution plate, 10^{-4}, that we then used to proceed to the 6th streak plate

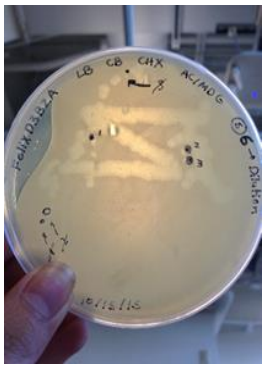

Figure 3.2: 6th Streak Plate

Figure 3.3: 7th Streak Plate

Titer Calculation of the Original Sample:

After performing the serial dilution, the HHMI lab manual instructs to count the number of plaques on each plate and use the plate with between 20 and 200 plaques for the calculation of the original sample titer. The dilution series plate which was used for the titer calculation had 38 plaque forming units (pfu) and a dilution of 10^{-1}. Therefore, the dilution factor of the titer was 10^{1}. Another important aspect to note is that 10 μL of sample volume was used in the dilution series. The calculations for the original sample titer are shown below:

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{pfu}{\#\mu L}\right) * \left(\frac{1000\mu L}{mL}\right) * (dilution\ factor)$$

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{38\ pfu}{10\ \mu L}\right) * \left(\frac{1000\ \mu L}{mL}\right) * (10^1)$$

$$Titer = 3.8 * 10^4\ pfu/mL$$

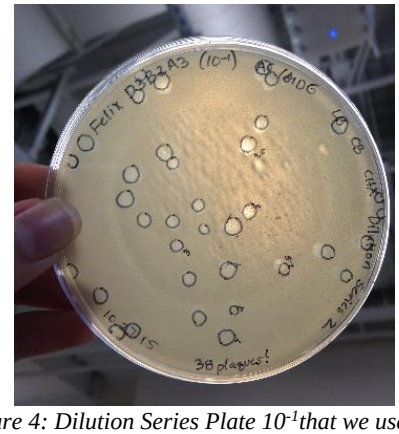

Figure 4: Dilution Series Plate 10^{-1}that we used for the titer calculation of the original sample

Medium Titer Lysate (MTL) Harvest and Titer:

After performing the high-dilution spot test, in which we added 5 µL of each dilution sample to the corresponding grid (Figure 5), the HHMI lab notebook instructs to find the dilution grid that has between 5 and 50 plaques on it. We counted 8 pfu on the 10^{-5} dilution grid. Therefore, we were able to calculate a new titer. The calculations for the MTL titer are shown below:

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{pfu}{\#\mu L}\right) * \left(\frac{1000\mu L}{mL}\right) * (dilution\ factor)$$

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{8\ pfu}{5\ \mu L}\right) * \left(\frac{1000\mu L}{mL}\right) * (10^5)$$

$$Titer = 1.6 * 10^8\ pfu/mL$$

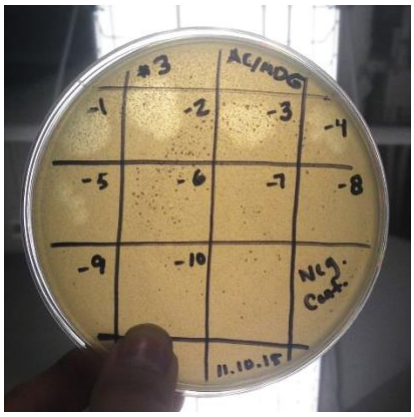

Figure 5: The high-dilutions spot test plate that was used to calculate the MTL titer

High Titer Lysate (HTL) Harvest and Titer:

In order to create the High Titer Lysate (HTL), we had to perform an Empirical Test. The measured diameter of the bottom of the agar plates was 86 mm, and the average diameter of the plaques was 3 mm. We used this information to calculate the areas of each, and therefore the approximate number of pfu that was needed in order to cover the entire agar plate.

$$Area = \pi\left(\frac{Diameter}{2}\right)^2 =$$

$$Area_{Agar\ Plate} = \pi\left(\frac{86\ mm}{2}\right)^2 = 5808.8\ mm^2$$

$$Area_{Plaque} = \pi(\frac{3\ mm}{2})^2 = 7.0686\ mm^2$$

$$pfu_{desired} = \frac{Area_{Agar\ Plate}}{Area_{Plaque}} = \frac{5808.8\ mm^2}{7.0686\ mm^2}$$

$$pfu_{desired} = 821.77\ pfu/plate$$

Once we determined how many pfu was needed to cover the entire agar plate, we used the MTL titer to calculate the volume of MTL necessary to from a web plate.

$$Volume_{MTL} = pfu_{desired} * \frac{1}{Titer_{MTL}}$$

$$Volume_{MTL} = 821.777\ pfu * \frac{1\ mL}{1.6 * 10^8\ pfu}$$

$$Volume_{MTL} = 5.136 * 10^{-6}\ mL$$

$$Volume_{MTL} = 5.136 * 10^{-3}\ \mu L$$

Using this volume, we calculated how to make two 2-fold dilutions above and below the calculated pfu. These calculations are shown in the table in Figure 6.1.

Empirical Assay Calculations			
Dilution of Empirical Assay	Pfu Content	Volume of Phage Stock	Concentration of Phage Stock
0.25x	205.5 pfu	12.84 µL	10^{-4}
0.5x	411.0 pfu	25.68 µL	10^{-4}
1x	822.0 pfu	51.36 µL	10^{-4}
2x	1644 pfu	10.27 µL	10^{-3}
4x	3288 pfu	20.54 µL	10^{-3}

Figure 6.1: Table of empirical test calculations

After incubating the empirical test, we concluded that the 2x and 4x plates yielded the best web plate patterns (Figure 6.2). However, the 2x plate was not quite covered enough and the 4x plate was slightly too covered. Therefore, we used a hypothetical 3x plate, with a dilution of the $15.41*10^{-3}$ µL to perform the 10-plate phage infection, and thus make the higher-concentrated phage stock.

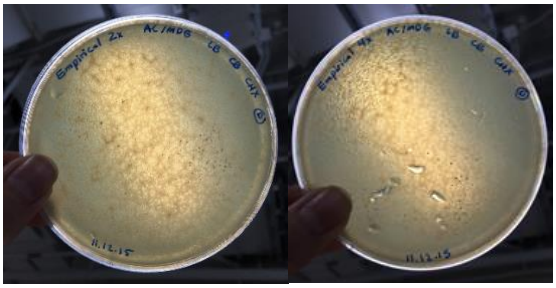

Figure 6.2: The 2x and 4x concentrated web plates from the Empirical Test

After harvesting the HTL, we performed another dilution series to calculate the titer of the HTL. The plate with a dilution of 10^{-5} had 4 pfu on it (Figure 6.3), and the plate with 10^{-6} had no plaques on it, so we used the 10^{-5} plate to calculate the titer of the HTL.

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{pfu}{\#\mu L}\right) * \left(\frac{1000\mu L}{mL}\right) * (dilution\ factor)$$

$$Titer\left(\frac{pfu}{mL}\right) = \left(\frac{4\ pfu}{10\ \mu L}\right) * \left(\frac{1000\ \mu L}{mL}\right) * (10^5)$$

$$Titer = 4.0 * 10^8\ pfu/mL$$

Figure 6.3: The 10^{-5} dilution plate used to calculate the HTL titer

Isolation of DNA:

After isolating and purifying the phage genomic DNA, we analyzed the sample and quantified the DNA using a Nanodrop spectrophotometer. The results of this test concluded that the concentration of DNA in our HTL was 107.9ng/µL, as shown in Figure 7. This concentration was high enough to proceed to restriction analysis and sequencing protocols.

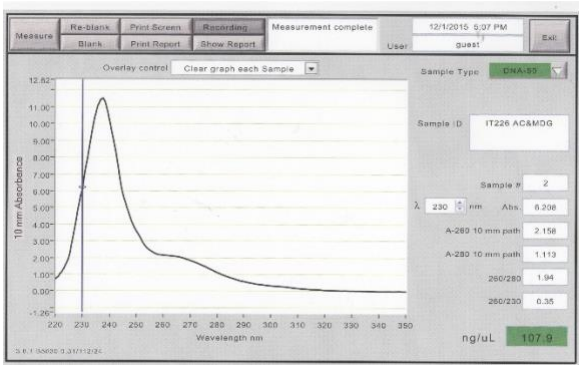

Figure 7: The results of the Nanodrop spectrophotometer, what quantified the DNA concentration of the HTL as 107.9ng/µL

Restriction Enzyme Digest:

In order to digest the phage genomic DNA, several reactions were set up, as shown in the table in Figure 8.1. Once the solutions were ready, we used agarose gel electrophoresis to separate and analyze the DNA fragments. The results are shown in Figure 8.2.

Solution:	Tube, amount					
	1	2	3	4	5	6
10X Reaction Buffer	2µL	2µL	2µL	2µL	2µL	2µL
Phage Genomic DNA	4.6µL	4.6µL	4.6µL	4.6µL	4.6µL	4.6µL
10X BSA	2µL	2µL	2µL	2µL	2µL	2µL
BamHI	---	0.5µL	---	---	---	---
ClaI	---	---	2.0µL	---	---	---
EcoRI	---	---	---	0.5µL	---	---
HaeIII	---	---	---	---	1.0µL	---
HindIII	---	---	---	---	---	0.5µL
ddH_2O	11.4µL	10.9µL	9.4µL	10.9µL	10.4µL	10.9µL
Total	20µL	20µL	20µL	20µL	20µL	20µL

Figure 8.1: Table of volumes to add to each tube in order to set up the reactions for the phage DNA digest

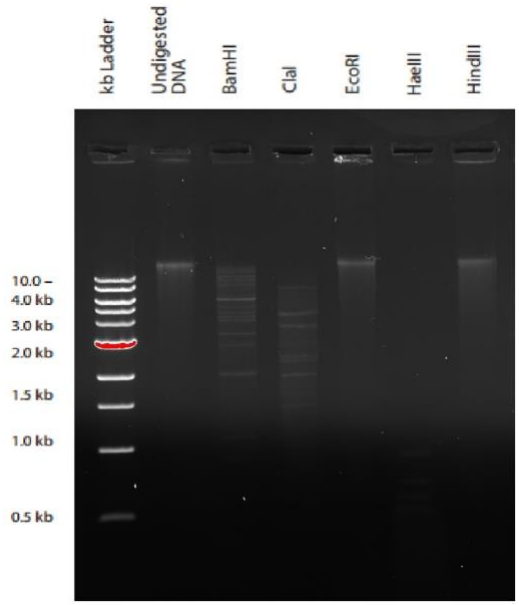

Figure 8.2: The results of the agarose gel electrophoresis

Electron Microscopy:

After months of hard work, this step finally allowed us to see the physical structure of FelixElFago, the bacteriophage we had isolated. When we were first viewing his structure, we were worried that there were actually two different bacteriophages present. However, upon further analysis, we were able to discern black, clumped areas of contamination, which is what had originally thrown us off. Therefore, we were able to confidently conclude that we had, in fact, successfully isolated a single population.

Two pictures of the FelixElFago are shown below in Figure 9. Each bacteriophage has a perfectly spherical head with a diameter of roughly 50 nanometers, as well as a long, thin tail, which is approximately 109 nanometers.

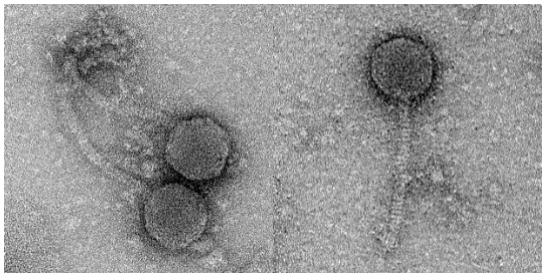

Figure 9: Images of FelixElFago under an electron microscope using a uranyl acetate solution stain

DISCUSSION

In conclusion, the direct plating of a soil sample found on the campus of Purdue University near a mushroom lead to the discovery of a potentially novel bacteriophage. This bacteriophage, named FelixElFago, produces turbid, or cloudy, plaques which are an average of 3mm in diameter. The plaques also have a small, clear center, which is approximately one-third the diameter of the entire plaque. The cloudiness of these plaques suggests that FelixElFago is a temperate phage (Howard Hughes Medical Institute, pg. 41). Temperate phages, which describe the majority of bacteriophages, switch between two types of behavior in regards to replication and survival (Howard Hughes Medical Institute, pg. 41). One "strategy" is exactly the same as that of lytic phages; upon injection of its DNA into the host bacteria, the DNA can replicate and form new bacteriophages, which causes the bacterial cell to lyse, or burst (Howard Hughes Medical Institute, pg. 41). These new bacteriophages then continue on to infect and lyse other bacteria. However, another "strategy" is for the phage DNA to integrate itself with the genetic material of the bacterial cell through a process called lysogeny (Howard Hughes Medical Institute, pg. 44). The phage then lies in this quiescent, or dormant, phase until it enters the lytic cycle, causing phage replication and eventually lysis of the bacterial cell. Therefore, temperate phages produce turbid plaques because not all of the infected bacteria lyses (Howard Hughes Medical Institute, pg. 41).

Further analysis of the physical structure of FelixElFago reveals more information about

its genomic DNA. According to an article published in the Quarterly Reviews of Biophysics, there is a strong correlation between the capsid size of a bacteriophage, and the length of the genomic DNA contained within (Nurmemmedov, 2008). Do to the fact that the capsid walls for all bacteriophages are of relatively similar thickness, it is concluded that smaller capsids can withstand more internal pressure than larger capsids (Nurmemmedov, 2008). Therefore, a large capsid cannot withstand a large amount of internal elastic energy "within the DNA condensate" (Nurmemmedov, 2008). According to the article, this lower tolerance for energy is "compensated by the larger genetic information encoded into" its genomic DNA (Nurmemmedov, 2008). Furthermore, a similar statement is made in the HHMI lab manual. In the section discussion electron microscopy, it states, "the area of the capsid (in nanometers) correlates with the size of the phage genome it contains" (Howard Hughes Medical Institute). Since the average phage has a capsid of about 6.5 nanometers in diameter ("Bacteriophage," 2013), and FelixElFago is huge is comparison. Our newly discovered phage has a round head with a diameter of roughly 50 nanometers. Therefore, we hypothesize that the genomic DNA length of our phage is very large. Also according to the Howard Hughes Laboratory Manual, "the length of the tail will correlate with the size (in base pairs) of the tape-measure gene found in all mycobacteriophages" (Howard Hughes Medical Institute). The tail of our bacteriophage was measured to be 109 nm long, and therefore we expect the size of the tape-measure gene to be rather large as well.

Furthermore, the microphotographs generated through electron microscopy reveal a lot about the complexity of the physical structure of the phage ("Electron Microscopes"). According to Ms. Laurie Miller, who helped us run the electron microscopy procedure, 10 µL of 1.0% uranyl acetate were used to stain the phage so that it could be viewed under the electron microscope. The way an electron microscope works is that it emits electrons toward the sample which are then collected on a screen on the other side of the sample. When the sample is stained with an element that is dense, such as uranium, the stain coats the surface and little crevices of the bacteriophage. In the areas where the stain is present, the electrons do not pass through the sample to the other side, but are trapped by the stain. Therefore, the electron microscope creates an image showing light areas – where all the electrons passed through – and dark areas – where the stain stopped the electrons from passing through. As a result, an outline of the details of the physical structure of the phage can be seen. However, some phages seem to be stained darker than others. This is because some phages have capsids with a higher level of surface detail. The more surface detail a capsid has, the more stain sticks to the capsid, and consequently more electrons are blocked, or absorbed, thus creating a darker image (Miller).

As shown in Figure 9, the tail of FelixElFago is not very dark, which reveals that the surface complexity of the tail is most likely not very high. However, there are visible lines running perpendicular to the length of the tail. This shows that there are most likely ridges running down the tail. In comparison, the head is much darker than the tail, which means the surface complexity of the capsid is much higher. We hypothesize that there is a lot of detail on the surface of the capsid which trapped a lot of uranyl acetate stain and therefore caused a darker image.

Unfortunately, due to the time constraint of the project, we were not able to effectively analyze the results of the agarose gel electrophoresis. If time had allowed, we would have compared our results with those logged on the Bacteriophage Database to determine whether FelixElFago is potentially a novel phage. However, the results of the genomic DNA sequence will hopefully prove that our bacteriophage is, in fact, a distinct population.

Although we have learned a lot about FelixElFago, there is still a lot more work to be done. Future directions for this research project are to evaluate the genomic DNA quality, as well as analyze the results from the sequencing center. Analyzing the genomic DNA sequence by using bioinformatics would reveal the functionality of FelixElFago's genomic DNA, and thus allow us to better hypothesize the potential impacts this discovery could have on the fields of biotechnology and medicine. Without the genomic sequencing of our phage, it is difficult to know what impact FelixElFago will make.

Furthermore, with the limited information we have collected, we cannot yet make a provisional cluster assignment for FelixElFago until we have a "completed genome sequence," (Jacobs-Sera). Therefore, in future research we would also use the genomic DNA sequence to determine which cluster of mycobacteriophages our phage belongs to. Another possible future direction for this research project includes further investigating whether or not FelixElFago truly is a temperate bacteriophage, and if he is, what exactly causes the small clear circle in the middle surrounded by a cloudy edge. Finally, one could further research why contamination was such a huge issue with our bacteriophage.

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