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The Isolation and Morphology of Imp48

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Bacteriophages, or phages, are viruses that infect bacteria (Fields et al, 1996). Like other viruses, phages infect their hosts by using the host's metabolism and biological processes to replicate the viral genome, thereby reproducing the virus (Lane, 2012). Viruses have two possible life cycles: lytic or lysogenic; in a lytic cycle, viruses replicate within the host cell until the host cell bursts; in a lysogenic cycle, the phage genetic code is integrated into the host until it is induced to enter the lytic cycle (Fields et al, 1996). As in all viruses, bacteriophages have specific species they can infect, called the host range (Lane, 2012).

Phages have the same basic elements as all viruses: genetic code, a head, and a protein coat (Lane, 2012). While its structure may be basic, each part of a virus performs a specific function. The protein coat, or capsid, is made of proteins that are virus specific (Mathews, 1971). These proteins cover the head of the virus and protect the viral genome (Davidson, 2013). As an additional level of protection, some viruses have an envelope made of lipids and proteins; the lipids are taken from the host cell (Davidson, 2013). The head of the virus is the container for the genetic information of the virus, which can be stored in several different forms (Mathews, 1971). It can be either single or double stranded and is made of either DNA or RNA (Lane, 2012). This information is integrated into the host genome; and when these genes are expressed, they reproduce the virus. Some viruses have tails that inject the genetic information into the host cell (Davidson, 2013).

Bacteriophages are the ideal variety of virus to study for several reasons. When lytic bacteriophages infect bacteria on an agar plate, they create plaques, which are distinct areas on a plate that have been cleared of bacteria (Mathews, 1971). The area that is cleared in an isolated plaque contains pure phage which can be used in further experiments (Mathews, 1971). Additionally, the growth medium, the bacteria, and the virus have very short incubation periods; a virus can infect a bacteria cell in as little as 20 minutes (Mathews, 1971). Due to the rapid replication of both the virus and the bacteria, it is ideal for researchers because there is a short turnaround time on experiments; they can be set up and results can be observed within 24 hours. Additionally, both bacteria and viruses are ideal vectors for genetic modification (Mathews, 1971). This allows researchers to test the effects of changing a single gene, or to insert different genes into the virus or bacteria and see how they respond.

Mycobacterium smegmatis is a non-pathogenic bacterium that is often used as a model for *Mycobacterium tuberculosis*¸ the bacteria responsible for the tuberculosis disease (Cardone et al, 2011). Viruses that infect *M. smegmatis* are likely to infect *M. tuberculosis*, and as such could be used to investigate how to treat and prevent tuberculosis. The goals of this project were to isolate and identify a unique bacteriophage that infects the bacteria *Mycobacterium smegmatis*. In order to accomplish this goal, a soil sample was obtained and procedures from the SEA-Phages Laboratory Manual were followed.

Materials and Methods

Phage Purification and Isolation

The first step in the project was isolating a single phage. A soil sample containing phage particles was collected. Details of collection site, including GPS coordinates, soil type, and general description of site, were recorded.

The soil sample was enriched in a flask using five mL of mc^2122 strain *M. smegmatis*, along with 0.5 mL of 0.1 M calcium chloride and about one half of a tablespoon of the soil sample (HHMI, 2013). Also added to the flask was 50 mL of 7H9, instead of water, glycerol broth, and AD supplement. The enrichment sample was incubated for 48 hours.

The sample was centrifuged for ten minutes and the supernatant was filtered using syringe filters. Tenfold dilutions of the filtered sample were made by placing 90 µL of phage buffer into microcentrifuge tubes labeled 10^{-1} through 10^{-5}. The dilutions were created by transferring ten µL of filtered sample into the 10^{-1} sample. From the 10^{-1} dilution, 10 µL was moved to the 10^{-2} tube (HHMI, 2013). This pattern of tenfold dilution was followed until the desired dilution was reached. During each transfer, a fresh pipet tip was used. In this manner, serial dilutions through 10^{-5} were created.

Seven test tubes containing five mL *M. smegmatis* were labeled negative control and 10^{0} through 10^{-5}. Each test tube of *M. smegmatis* was infected with 50 µL of the corresponding solution for 15 minutes (HHMI, 2013). Phage D9 obtained from instructors was the positive control and pure phage buffer was a negative control. Each sample was plated by pipetting 4.5 mL of top agar into the test tubes with *M. smegmatis* and immediately pipetting all five mL of the solution onto an agar plate. Plates were swirled gently to distribute the solution evenly on the plate. After the solution solidified, all plates were placed in a 37 °C incubator for 48 hours (HHMI, 2013). The goal of this step was to produce plates with distinct plaques.

The agar plates that yielded the best plaques were chosen for the next step. Streak plates were made by applying phage from the plaques of the chosen plates to new agar plates using sterile wooden sticks. The first wooden stick was touched to a plaque from a chosen plate, then rubbed gently across one third of the new plate. A new stick was used to gently rub another third of the plate, overlapping with the first section by approximately three strokes. A final stick was used to fill the last third of the plate in the same manner (HHMI, 2013). The final section, which had the least overlap and lowest phage concentration, was labeled with an X. The plate had 4.5 mL of top agar applied to it; however it was not swirled to maintain the concentration of phage obtained via streaking. A negative control plate was created using one bare sterile wooden stick using the same rubbing pattern as the experimental plates. Plates were observed after 24 hours. Two further rounds of streak plating were performed.

Phage Stock Amplification

To perform further steps in the project, the concentration of the phage needed to be increased. A spot titer test was performed using an eluted plaque from a chosen streak plate. A series of tenfold dilutions, through 10^{-10}, were created. The initial source of phage was obtained by touching the center of a plaque with a sterile pipet tip, then tapping that tip against the wall of a microcentrifuge tube with 90 µL phage buffer (HHMI, 2013). This was the 10^{0} sample of phage used for creating dilutions, as described above. A 12-square grid was drawn on the plate, with squares labeled 10^{0} through 10^{-10}, and control. The plate had 4.5 mL of top agar pipetted onto it, and was swirled gently to distribute the top agar evenly. Onto each labeled square, five µL of the appropriate dilution was pipetted, using phage buffer as a control (HHMI, 2013). The

plate was left undisturbed for 30 minutes to allow the phage solutions to soak into the top agar and placed in a 37 °C incubator for 24 hours. The number of plaques in each grid square was counted, and the titer (phage concentration per mL) of the 10^0, or neat sample, was calculated.

Titer Sample Calculation:

$$\frac{number\ of\ plaques}{\mu L\ solution} * dilution\ factor * \frac{1000\ \mu L}{1\ mL} = titer$$

$$\frac{8\ plaques}{10\ \mu L} * 10^4 * \frac{1000\ \mu L}{1\ mL} = titer$$

$$8.0 * 10^6\ \frac{pfu}{mL} = titer$$

The goal of the next step was to obtain web plates, that is, plates that were almost completely cleared of bacteria growth. The calculations from the spot titer plate allowed an estimation of which of the eleven dilutions would yield a web plate. Dilution plates were created by plating 50 µL of 10^0 through 10^{-4} dilutions previously created. M. smegmatis was infected and pipetted onto the plates as above. Dilution plates were performed in order to determine what specific concentration of phage solution would be needed to create web plates to create a medium titer lysate (HHMI, 2013).

Web plates were achieved by plating 50 µL of 10^{-1} dilution. Eight plates were then created as above, using this dilution, and incubated for 48 hours (HHMI, 2013). These plates were also used to determine plaque morphology and the average plaque size; morphology at 24 an 48 hours was recorded. Plates were then flooded with eight mL phage buffer and soaked at room temperature for two hours. The phage buffer was then carefully pipetted off of the plates. A spot titer test was performed with the medium titer lysate and the titer calculated from the results of that spot titer.

An empirical test was conducted creating a series of plates from bracketed dilutions, with the goal of the median dilution yielding a web plate (HHMI, 2013). In order to determine the amount and magnitude of dilution needed, the number of plaques needed for a web plate was calculated based on average plaque size, area of the plate, and the titer. After the concentration and amount were calculated, dilutions that were 0.25X, 0.5X, 2X, and 4X were created and M. smegmatis was infected with these calculated volumes.

Sample Empirical Test Calculation

$$pfu_{maxweb} = \frac{area\ (mm^2)_{plate}}{area\ (mm^2)_{plaque}} \qquad Volume = \frac{pfu_{maxweb}}{titer}$$

$$pfu_{maxweb} = \frac{42.5\ mm^2 * \pi}{0.75\ mm^2 * \pi} \qquad Volume = \frac{3211.11\ pfu}{8.0 * 10^9\ pfu/ml}$$

$$pfu_{maxweb} = 3211.11 \qquad Volume = 4.01389 * 10^{-4}\ \mu L$$

$$V_{pipet} = concentration * V_{maxweb} * 10^{-1}$$

$$V_{pipet} = \frac{1}{2} * 4.01389 * 10^{-4}\ \mu L * \ 10^{-1} = 20 * 10^{-5}\ \ \mu L$$

Table 1. Concentration and volume of phage solution used for each plate in first empirical test.

Sample Concentration	Pfu desired	Pfu to volume (µL of 10^0)	Pipette volume and dilution factor
1/4X	451	$2.0526 * 10^{-6}$	21 µL of 10^{-7}
1/2X	903	$4.1051 * 10^{-6}$	41 µL of 10^{-7}
Max web	1806	$8.2102 * 10^{-6}$	82 µL of 10^{-7}
2X	3612	$1.6421 * 10^{-5}$	16 µL of 10^{-6}
4X	7224	$3.2284 * 10^{-5}$	32 µL of 10^{-6}

Table 2. Concentration and volume of phage solution used for each plate in second empirical test.

Sample Concentration	Pfu desired	Pfu to volume (µL of 10^0)	Pipette volume and dilution factor
1/4X	802	$1.0035 * 10^{-4}$	10 µL of 10^{-5}
1/2X	1605	$2.0069* 10^{-4}$	20 µL of 10^{-5}
Max web	3211	$4.0139 * 10^{-4}$	40 µL of 10^{-5}
2X	6422	$8.0278 * 10^{-4}$	80 µL of 10^{-5}
4X	12844	$1.6056 * 10^{-3}$	16 µL of 10^{-4}

A high titer lysate is a solution with a phage concentration greater than 1 x 10^8 plaque forming units per mL. This is necessary to perform later DNA analysis and isolation. A high titer lysate was created from the medium titer lysate by plating seven plates with the concentration and amount of medium titer lysate that yielded a web plate. Infection, plating and incubation were performed as above. After 24 hours of incubation, the plates were flooded with eight mL of phage buffer and placed in a 4 °C fridge (HHMI, 2013). The liquid was pipetted off the plates and centrifuged for 20 minutes. The supernatant was filtered to remove any non-phage particles (HHMI, 2013). A spot titer test was performed with the resulting high titer lysate and titer was calculated from the results of that test.

DNA Isolation

Phage DNA was isolated from the high titer lysate. This was done using the Promega DNA Clean-Up Kit. To isolate DNA, 10 mL of the high titer lysate was placed into an Oak Ridge tube with 40 µL nuclease mix, incubated at 37 °C for 30 minutes, and let sit undisturbed at room temperature for an hour (HHMI, 2013). To this solution, four mL of phage precipitate solution was added, and the entire solution was stored for 48 hours at 4 °C. The solution was centrifuged for 20 minutes and the pellet was allowed to dry before being re-suspended with 0.5 mL sterile water (HHMI, 2013). The DNA was cleaned using two mL of 37 °C Clean Up Resin, and the entire solution was run through columns. To clean the DNA, two mL of 80% isopropanol was run through the columns. To remove the isopropanol, the column was centrifuged twice at max speed, first for five minutes, then for one minute (HHMI, 2013). The DNA was eluted from the dry column by adding 50 µL of 80 °C water, instead of TE.

The DNA was further cleaned by adding 10% of the DNA volume of 3M sodium acetate solution, 250% of volume of DNA and 95% ethanol. The solution was placed in the freezer for at least one hour. The microcentrifuge tube was spun on high for ten minutes in a centrifuge. The

supernatant was decanted and 100 µL of 70% ethanol was added, the solution was left at room temperature for ten minutes. The DNA was dried in a heated centrifuge and re-suspended in the original volume of water overnight. The concentration of DNA was determined by analyzing one µL of the DNA solution using the NanoDrop.

Phage Characterization

DNA digests for gel electrophoresis were performed with five different enzymes, *Bam* HI, *Cla* I, *Eco* RI, *Hae* III, and *Hind* III. The DNA solution was incubated at 65 °C for 10 minutes, then placed on ice. The digest reactions were set up with two µL of 10X reaction buffer, 0.5 ug DNA, two µL 10X BSA, two µL of enzyme, and enough water to equal 20 µL (HHMI, 2013). All reactions were incubated in a 37 °C water bath for two hours and stored in the freezer or placed on ice until ready to be loaded into the gel. Each reaction had four µL of tracking dye added. Reactions were incubated at 65 °C for five minutes and immediately placed on ice (HHMI, 2013). Using a fresh pipet tip for each digest, 12 µL of each reaction was loaded into its own lane in the gel. Gel was run and photographed by Robert Fleming. The length of DNA fragments were calculated by determining the slope of the logarithm of the length of the size ladder fragments and comparing that to distance traveled. To calculate the fragment size from the restriction digest, the distance traveled was put into the equation of the graphed size marker, then raising ten to the *y* power.

The phage was examined and photographed using electron microscopy. A PELCO Tab was used to hold a copper grid, onto which five µL of high titer lysate was placed. Each liquid placed on the grid was allowed to adsorb for two minutes, then wicked away using filter paper. After the high titer lysate, the grid was washed twice with five µL of sterile water and stained with five µL of 1.0% uranyl acetate. The grid was then examined with the electron microscope at Trinity College.

A 10^{-7} dilution was created, using the same procedure as above. Test tubes containing 0.5 mL *M. smegmatis* were infected with ten µL of the dilution for 20 minutes. Agar plates were plated out as above. This procedure was concurrently run for ten plates. After 48 hours, the two different plaque types were counted. The percentage of haloed plaques and percentage of small plaques were calculated.

Results

Phage Purification and Isolation

Soil sample was collected outside Raether Library on Trinity College campus on September 9th, 2013. The GPS coordinates of this location were 41°41’74” N 72°41’29”W. The sample was collected from surface containing woodchips, located near a sidewalk with heavy foot traffic, approximately five feet away from a tree and patch of lilies. The soil was very damp due to rain the previous day.

The initial plating of the enriched sample yielded plates with several different plaque types. These plaque morphologies included haloed, oblong plaques under 1.0 mm and plaques as large as 65.0 mm. The positive control plate, on which bacteria was infected with a known D9 phage sample, yielded no plaques. The 10^{-3} and 10^{-4} plates yielded the most isolated and distinct plaques. On the 10^{-3} plate, plaques were numerous and round with distinct edges. On the 10^{-4} plate, some plaques were haloed with clear centers.

The phage was purified through three rounds of streak plating. Two different plaques, phage A from the 10^{-3} plate and phage B from the 10^{-4} plate, were chosen. Phage B had round plaques under 0.10 cm in size, which were used in the second round of streak plating. Phage B

had consistent morphology through two rounds of streak plating, but in the third round five plaques appeared to have haloing.

After the first round, Phage A had two types of plaques after 48 hours – 0.30 cm haloed plaques and 0.10 cm round plaques – and a haloed plaque was used for the second round of streak plates. Between the 2nd and 3rd rounds, Phage A underwent a morphology change; haloed plaques were no longer obtained. Due to differences in morphology throughout the rounds of streak plating, a plaque from the second round of Phage A streak plating was used for further isolation. Phage A also had a more interesting morphology and was therefore chosen for isolation.

Phage Stock Amplification

For the spot titer test, a plaque from the second round of streak plating of phage A was used. The titer of the 10^0 sample was calculated to be $8.0 * 10^6$ plaque forming units per mL by using the formula in *Materials, Phage Stock Amplification*. The spot titer test had several cleared grid squares that could not be used for calculating the titer (Table 3). The 10^{-4} dilution was used in calculations.

The dilution plates yielded two different types of plaques; this was consistent across all three dilution plates. The 10^{-1} plate was almost a web plate, while the 10^{-2} plate was less cleared than the 10^{-1} plate but there were still too many plaques to count. All plates had a mix of haloed plaques that were between 4.1 mm and 1.0 mm and round plaques under 1.0 mm without halos.

Table 3. Results of second spot titer of phage A. Dilutions were created using an eluted plaque. Number of plaques created after 24 hours are given. Phage buffer was used as a negative control.

Dilution Concentration	Number of Plaques
Control	0
10^0	Cleared
10^{-2}	Cleared
10^{-3}	45
10^{-4}	8
10^{-5}	1
10^{-6}	0
10^{-7}	0
10^{-8}	0
10^{-9}	0
10^{-10}	0

A second round of streak plates were performed using haloed and non-haloed plaques from the dilution plates. The same procedures were followed as in the first round of streak plating. For the first plates grouping of streak plates, plates were incubated for 24 hours. The second plates were incubated for 48 hours. The haloed plaques were purified using a third round of streak plating that yielded a very high density of haloed plaques 2.0 to 3.0 mm in size.

Dilutions plates were made using an eluted plaque from the purified phage sample, through 10^{-4}. After 24 hours, both the 10^0 and 10^{-1} plates were cleared and the 10^{-2} plate was a web plate. The 10^{-3} plate was just shy of a web plate and the 10^{-4} plate had 107 plaques. The

plaques on these plates were round with distinct edges, 1.0 – 3.0 mm in size. There was no sign of haloing. Based on calculations using the number of plaques on the 10^{-4} plate, the titer of the 10^{0} stock was $2.14 * 10^{7} \frac{pfu}{mL}$. All plates except for the 10^{-2} plate were replaced in the incubator for another 24 fours. After 48 hours, there was no change on the 10^{0} or 10^{-1}plates. On both the 10^{-3} and 10^{-4} plates, both haloed and non-haloed plaques were observed, and plaques ranged from 1.0 mm to 5.0 mm in size.

A 10^{-2} dilution plate was remade, with the goal of achieving a web plate. However, a web plate was not achieved. Dilutions of 10^{-1} and 10^{-2} were remade from the 10^{0} stock, and then 10^{-1} and 10^{-2} plates were made. The 10^{-1} plate yielded a web plate after 24 hours, and was flooded to create the medium titer lysate solution.

The medium titer lysate solution was titered using a spot titer test. The titer of the medium titer lysate solution was calculated to be $2.2 * 10^{10} \frac{pfu}{mL}$. An empirical test was set up using the calculated titer. The goal was to create a web plate that would be replicated for the high titer lysate. On the first empirical test, the 1/4X, 1/2X, and max web plates had no plaques. The 2X plate had only two plaques, and the 4X plate had 3 plaques. Due to these results, a second empirical test was set up using volumes listed in Table 2. The 2X concentration plate yielded the best web plate, and that concentration and volume of phage solution was used to set up plates for the high titer lysate creation.

Table 4. Spot titer test of medium titer lysate results. Phage buffer was used as a negative control. Plaques were counted after 24 hours.

Dilution Concentration	Number of Plaques
Control	0
10^{0}	Cleared
10^{-2}	Cleared
10^{-3}	Cleared
10^{-4}	Cleared
10^{-5}	Cleared
10^{-6}	Web
10^{-7}	11
10^{-8}	1
10^{-9}	1
10^{-10}	0

Plates were set up for the high titer lysate solution creation and all seven of them were web plates. After flooding the plates and allowing them to soak in the fridge for 24 hours, approximately 50 mL of high titer lysate was harvested. This high titer lysate was spot titer tested (Table 4), and had a titer of $1.0 * 10^{11} \frac{\text{pfu}}{\text{mL}}$.

DNA Isolation

The first time phage DNA was isolated, the concentration of the DNA was 100.6 $\frac{ng}{\mu L}$. The volume of the DNA was 100 µL, meaning that a total of 10060 ng of DNA was isolated. This DNA was not washed using ethanol and sodium acetate until after the first digest was run.

The second time phage DNA was isolated, the concentration of DNA was 153.4 $\frac{ng}{\mu L}$, a total of 153400 ng total was isolated.

Phage Characterization

The plaque morphology of the phage after 24 hours was clear, round plaques with distinct edges. There was no haloing. The average plaque diameter was 2.1 mm, the smallest diameter was 1.0 mm and the largest was 3.0 mm.

After 48 hours, the plaque morphology changed considerably. There were two distinct types of plaques. The first type of plaque, which was significantly less numerous, was identical to those at 24 hours. The largest of these plaques was 1.5 mm, the smallest was 1.0 mm and the average 1.24 mm. The second, more numerous plaque type, was a lager plaque that was haloed with clear centers. The plaques were much larger, the average being 4.1 mm, largest was 5.0 mm, and the smallest was 3.0 mm.

The equation of the line produced by graphing the logarithm of the size marker compared to the distance traveled was $y = -0.0264x + 4.3281$. The distance traveled are *x*-values and the logarithm of the number of base pairs are *y*-values. *Cla I* digest yielded the most clear banding pattern, while the *Hae III* digest yielded a smear like pattern. Faint banding occurred in *Eco RI, Hind III,* and *Bam HI.* The uncut DNA was one solid band, a control to show that the DNA was both pure and uncontaminated by digest enzymes.

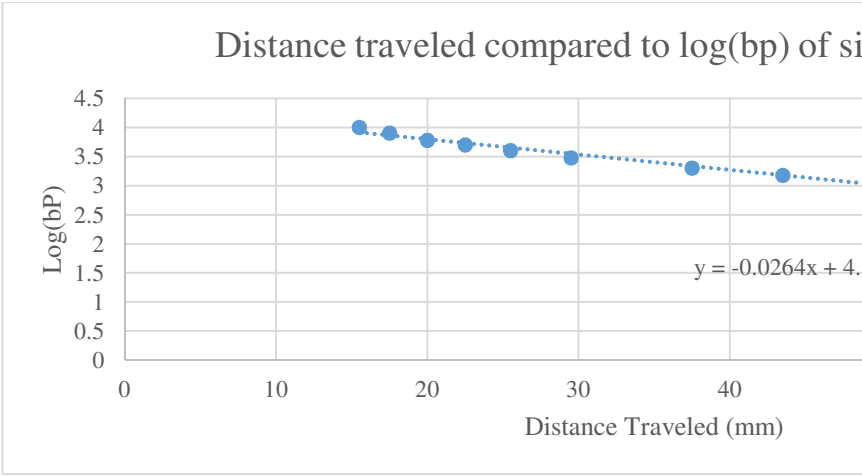

Figure 1. The logarithm of the number of base pairs was plotted against the distance those fragments traveled. The resulting equation was used to calculate the number of base pairs in restriction digests.

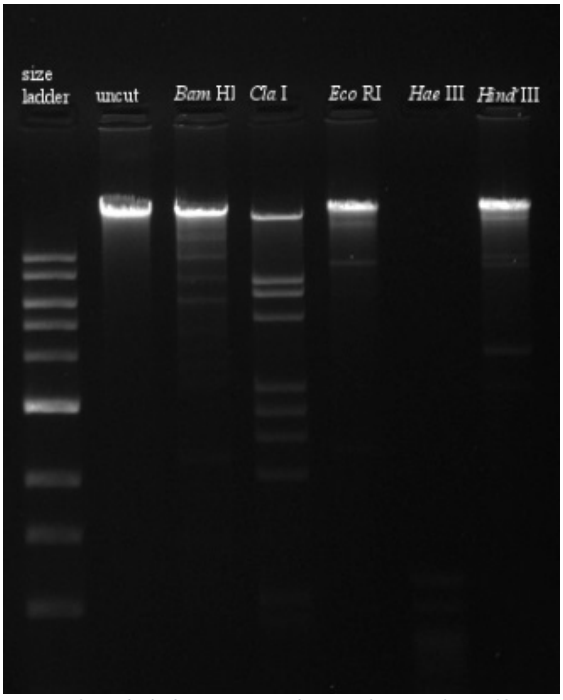

Figure 2. Labeled restriction digest of 5 uL of Imp48 DNA. Distance traveled by digests are in table to right.

Table 5. Distances traveled by DNA cut by different enzymes. Length of fragment calculated using equation in Figure 1. 1a refers to beginning of smear pattern of DNA, 1b is the end of that smeared pattern (Figure 2).

Enzyme used in Digest	Fragment number	Distance Traveled (mm)	Length of fragment (base pairs)
Uncut	1	11	10907

DNA			
Bam HI	1	12	10264
	2	16.5	7807
	3	19	6707
	4	31	3234
Cla I	1	12	10264
	2	18.5	6194
	3	19.5	6506
	4	21.5	5761
	5	28	3881
	6	31.5	3137
	7	33	2864
	8	37.5	2178
Eco RI	1	11.5	10580
	2	13	9658
Hae III	1a	61.5	506
	1b	77	197
Hind III	1	11.5	10580
	2	16	8048
	3	16.5	7807
	4	21.5	5761
	5	29.5	3542

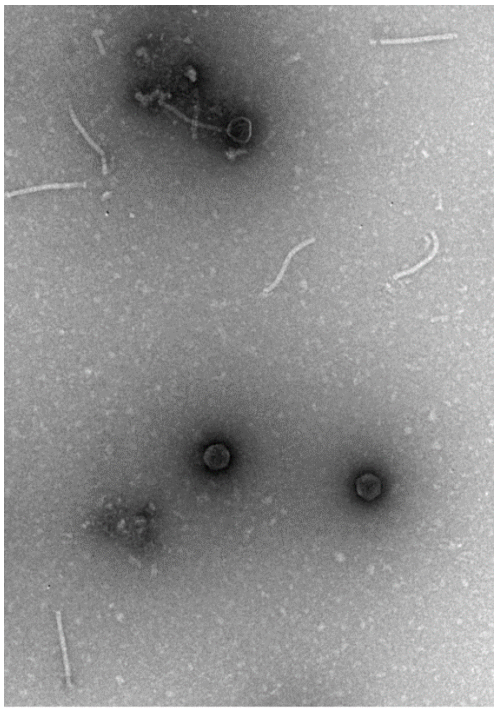

Figure 3. Imp48 viewed at 40000X magnification

The morphology of the phage under the electron microscope showed a phage with a head of approximately 66 nm diameter and a tail that is approximately 195 nm (Figure 3).

The first set of dilution plates used to determine the ratio of haloed plaques to non-haloed plaques produced plates that averaged 22 plaques per plate. There were not enough plaques to get an accurate ratio, so the experiment was repeated using a 10^{-6} dilution of the same volume. Between the two trials, a total of 4,366 haloed plaques and 323 non-haloed plaques were counted. The overall ratio of haloed to non-haloed plaques was 13.5 to 1. In the first trial, the ratio was 2.1 to 1, and the ratio of the second trial was 16.7 to 1.

Discussion

Naming of the Phage

The phage was named Imp48. This name references two aspects of the phage's morphology. Throughout the project, due to different incubation times, the phage only sometimes showed the two-plaque morphology. This was an impish quality of the phage and lent itself to the first half of the name. The "48" refers to the two-plaque morphology of the phage only appearing after 48 hours. Additionally, Imp was my grandfather's nickname, and he always encouraged my love of science.

Phage Purification and Isolation

The positive control plate with known phage, D9, never produced plaque growth. This was true for all participants. The D9 stock was from last year, and due to the long time period, including refrigeration, the phage degraded over time; therefore, the titer of the D9 stock decreased. Though the positive control did not work, it was not necessary because the enrichment sample yielded plaques and the negative control of only phage buffer did not; this made it clear that any virus particles were coming from the enrichment sample, rather than contamination.

A plaque from the second round of streak plates was used because both phages yielded a different morphology in the third round of streak plating. This could have been caused by eluting an impure phage sample from the previous streak plate.

The spot titer test was unsuccessful the first time. This was caused by not letting the dilutions soak into the top agar for a long enough time. Additionally, the original, rather than modified, procedure was followed. This meant that rather than only five µL of dilutions, 10 µL of each dilution was placed on the plate. Both the increased volume and not allowing the dilutions to soak in were contributing factors in the dilutions running together during transport to the incubator. In the second spot titer test, the volumes of dilutions were halved and the time for the dilutions to soak into the plate was doubled.

Looking back, it is clear that the two-plaque morphology on the dilution plates was not caused by error. The streak plates were observed after 24 hours only. The dilution plate was observed after 48 hours. The plaque morphology of this phage was different between 24 and 48 hours. Haloed plaques were observed after 48 hours, but never at 24 hours. This was merely caused by the morphology of Imp48, rather than an error. However, during the project it was unclear if this was caused by the phage or by error, so the streak plate procedure was repeated to ensure that the phage was pure.

Phage Stock Amplification

The first 10^{-2} plate did not yield a web plate. This was caused by not making fresh dilution stocks. When dilution plates were made fresh from the 10^{0} stock, web plates were achieved. This shows that the titer of a dilution stock decreases much more rapidly than a neat sample.

In the first empirical test, there was an error in calculation that caused web plates not to form. The diameter, not radius of the plaques, was used to calculate the number of plaque-forming units that would cause a max web plate. This caused the rest of the calculations, including the concentration and volume calculations, to be incorrect. Therefore an adequate amount of phage solution was not applied to the plates, so a web plate did not form. The second

empirical test used re-calculated values for volumes and concentrations of the infections. Titer was recalculated using the number of plaques formed in the first empirical test.

DNA Isolation

The second round of DNA isolation had a higher concentration of DNA than the first. This could have been caused by the addition of the sodium acetate and ethanol wash that was not used for the first isolation. Additionally, an increased familiarity with the DNA isolation procedure could also have yielded higher concentration the second time it was performed. The DNA solution with clean up resin did not go through the columns as easily during the second time DNA was isolated, which also suggests that there was a high concentration of DNA. This is consistent with the results from the NanoDrop.

Phage Characterization

One possible explanation of the Imp48's two-plaque morphology is the presence of mutations. In 1950, Seymour Benzer showed that the T4 phage also creates two different plaque types. This morphology is caused by a mutation of the phage genome. The mutant T4 phage yields a larger, ragged plaque (Griffiths et al, 2000). Conversely, the wild type phages created small round plaques. Additionally, the wild type and mutant viruses had slightly different host vectors (Griffiths et al, 2000). This morphology is somewhat similar to that of Imp48. However, this explanation is somewhat unlikely, as the two-plaque morphology was consistent throughout the experiment, rather than suddenly appearing. Also, the T4 phage studied by Benzer had been crossed with other phages, which was not done with Imp48 (Griffiths et al, 2000). When crossing wild type phages with mutants and plating them where only wild-type recombinant phages could grow, the recombination frequency was only 0.01% (Griffiths et all). Only 1 out of every 1000 viruses mutated to the extent that it changed their morphology; however the ratio of plaque types of Imp48 is 13 to 1, which is considerably higher. This suggests that rather than being a product of mutation, it is a product of Imp48's genes.

The phages Atlas and HufflyPuff also have a two-plaque morphology (PBI, 2013). Like Imp48, these phages have a haloed plaque and a smaller round plaque. However, these phages are in clusters C and E, respectively (PBI, 2013). Due to having a morphology that can be found in multiple clusters, the plaque morphology of Imp48 cannot be used to determine its cluster.

The total length of the uncut DNA is 10,264 base pairs long; this is considerably smaller than any sequenced phage, the shortest of which is 41,441 base pairs long (PBI, 2013). The phages with shorter genomes are in the G most frequently (PBI, 2013). However, digest patterns from the G cluster are very different from the digest of Imp48; cluster G phages have many narrow bands that travel the last half of the gel from the *Bam HI* digest, and no bands from the *Cla I* digest (PBI, 2013). Based on the digest patterns, it is more likely that Imp48 belongs in cluster A, which have similar *Bam HI* and *Cla I* patterns (PBI, 2013).

The structure of the phage from the micrograph is for the most part unremarkable. The head shape, head size, and tail shape are not characteristic of any one cluster. The micrograph shows many fragmented virus tails, which suggests that the high titer lysate was starting to degrade in quality, as the viruses were starting to break down.

The experiment to determine the ratio of plaque sizes was of my own design. The rationale behind this experiment was to gather as much data as possible about the morphology of the phage. There are very few other phages that have a two-plaque morphology, and as such gathering information about this morphology is important. The ratios obtained from the two

different trials were significantly different. The first trial, which had an average number of plaques, also had a lower ratio of haloed plaques to non-haloed plaques. This could mean that the small plaque morphology is density dependent; they were more numerous when there was a lower concentration of phage.

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