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Isolation and Characterization of a Bacteriophage The Flathead Lake Monster

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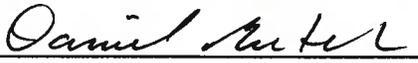
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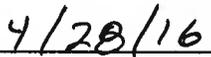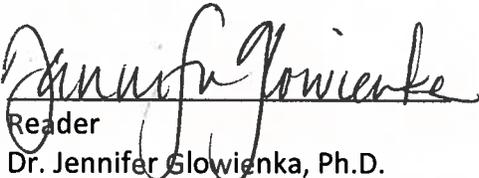
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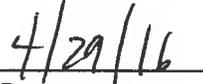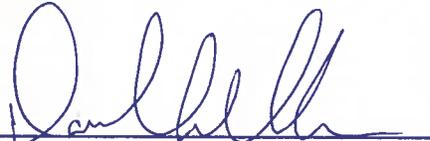
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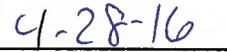
**Isolation and Characterization of a Bacteriophage
The Flathead Lake Monster**

Ian Lorang

Honors Thesis

Department of Life and Environmental Sciences

Carroll College, Helena, MT

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Abstract

It has been suggested that bacteriophages are the most abundant entities on the planet. The goal of this study was to isolate and characterize a novel phage. Few phages have been isolated, which provides this study a good chance to isolate a novel phage from Northwest Montana where a phage has never been isolated. Using plaque techniques, restriction digest, and phage enzyme tool a phage was isolated and named the Flathead Lake Monster (FLM) and is a novel phage based on results from the study. FLM had abnormally small plaque diameters and an unusually long tail. Compared to literature on other isolated phage's tail lengths, the FLM has the longest tail ever isolated using *M. smegmatis* as a host. This led to the investigation of a correlation between plaque diameter and phage tail length. Comparisons within our lab confirmed that there is a correlation. An additional question of this study was to see if tape measure gene length, which is highly conserved in all isolated phages, correlates with phage tail length. Genome analysis of the phage will help to answer that question for the FLM and possibly reveal genes that are unique.

Introduction

Earth is home to a diverse group of genetic entities, but the most abundant of them is a group of viruses called bacteriophages (Clokie et al., 2011). Estimates put phage numbers in the range of 10^{31} (Brussow and Hendrix, 2002). Phages consist of three different regions: a head that contains DNA, a tail to penetrate host membranes and deliver DNA, and tail fibers that act as recognition points to bacterial host cells (Yap and Rossman, 2014). In order to replicate, bacteriophages are dependent on a host and are specific to the host they infect (Hatfull et al., 2010). There are ten times more phages than bacteria, so a high specificity helps them infect their less abundant hosts (Stern and Sorek, 2011). Both Pedulla et al. (2003) and Hatfull et al. (2010) research groups illustrated this in their research as they isolated bacteriophages specific to a soil bacterium called *Mycobacterium smegmatis*.

M. smegmatis has been used as a host to isolate the majority of known mycobacteriophages (Hatfull et al. 2010). According to the phage data bank, there have been 5865 isolated phages to date. Of those, 879 have been sequenced, with 874 being specific to *M. smegmatis*. These data suggest that *M. smegmatis* is a good host for isolating diverse bacteriophages, even though they are only a small percentage of the total phages in our biosphere.

Every phage ever isolated has been unique (Pedulla et al., 2003). Unique phages have been referred to as having a mosaic genome that is assembled of their genes and those of their hosts (McNerney and Traore, 2005). This was

demonstrated by Hatfull et al. (2006) as they isolated and sequenced 30 phages, finding none that shared a single gene. This was credited to horizontal gene transfer between the phages and their hosts (Hatfull et al., 2006). In addition Pedulla et al. (2010) compared ten isolated phages to four known phage sequences and found that not only did most genes vary between the phages, but function for the majority of the genes was not known.

Mosaic relationships can be explored through comparing phage genomes and sorting them into clusters based on similarly shared gene sequences (Hatfull et al. 2010). Based on the phage database there are a total of 55 clusters, 14 of which have sub-clusters, which further categorize phages. This mosaic nature is further illustrated as the majority of the clusters in the database only have one phage assigned to them (PhagesDB.org).

While phage diversity is pronounced, they also display structural diversity. Phages can have three different types of tail structure (Brussow and Hendrix, 2002). Myoviridae have an extended tail with a coating allowing it to extend and contract (Brussow and Hendrix, 2002). Siphoviridae have long tails that are fixed and cannot extend. Finally, Podoviridae have stubby tails that are also fixed (Brussow and Hendrix, 2002).

Given the phage diversity on the planet, and the ability to use *M. smegmatis* as a suitable host, I hypothesized that I would be able to isolate and characterize a novel phage from the environment. In doing so I may further the small, but growing, phage data bank with unique genes that can further help us understand these diverse viruses and some of their functions.

While testing the original hypothesis, an atypical, small plaque size was observed in the *M. smegmatis* stimulating a second hypothesis investigating the small plaque size. Plaque size is influenced by three variables: absorption rate, lysis timing, and phage size (Romain et al., 2011). Therefore, phage size is a parameter I could readily evaluate. It has been suggested that small plaque size could be influenced by large phage size because the more mass a phage has the harder it would be to penetrate the agar layers to infect adjacent cells (Romain et al., 2011), although Romain et al. (2011) did not find a firm relationship between phage size and plaque diameter. I decided to evaluate the size of my phage to see if there was an inverse relationship between its size and the plaque diameters. This led me to my second hypothesis that there is a correlation between tail length and plaque size due to in lab observations regarding varied tail length.

Upon size analysis, I did observe an abnormally long tail on my phage. This led to a third hypothesis that a specific gene was responsible for this long tail length. A tape measure protein (TMP) has been found to assemble the phage tail and it is encoded by tape measure gene (Vessler and Cambillan, 2011). Since phages are extremely mosaic, it is interesting that the tape measure gene is highly conserved across all isolated phages, showing that this gene is critical to tail assembly in all phages (Vessler and Cambillan, 2011). When a portion of the tail-encoding gene is deleted, a shorter tail length is observed (Katsura and Hendrix, 1984). This suggests a direct relationship between tape measure gene and phage tail length. Based on this information, I hypothesize, that when sequenced, the tape measure gene in my phage will be unusually long.

<center>**Materials and Methods**</center>

Collecting a Soil Sample

Soil samples were obtained from the environment and the coordinates were recorded using a GPS. Pedulla et al. (2003) and Hatfull et al. (2010) have used the soil-bacterium *Mycobacterium smegmatis* as a host to isolate phages. The majority of sequenced phages were isolated using this host (phage data bank). Organic compound rich soil, such as compost, provide the highest likelihood of harboring a phage due to bacterial abundance (Otawa et al., 2012). Therefore, a compost pile existing in the backyard of my home was used as a sampling site. The sample was collected from the compost pile using a hand shovel to extract soil compost a few inches under the exposed crust. It was placed and stored in a plastic bag labeled with the GPS coordinates and the location name of the property.

Preparation of *M. smegmatis* Culture

To isolate a bacteriophage, *M. smegmatis* was used as a host. In a baffled flask, 100 mL of complete neat (Middlebrook 7H9 Liquid Medium, 10% albumin supplement, 50 μg/mL carbenicillin (CB), and 10 μg/mL cycloheximide (CHX)) were added to act as a medium. CHX inhibited the growth of any fungus. CB acted as an antibiotic, with no effect on *M. smegmatis* bacteria. One milliliter of $CaCl_2$ was added to the flask with the 100 mL of neat. Then 200 μL of sterile *M. smegmatis* cells were transferred. The culture was set on a shaker for approximately 24 hours, while incubating at 200 rpm and 37°C.

Isolation of Phage: Enrichment

To enrich a desired number of phages, the environmental sample was combined with culture bacteria to allow for phages to infect the cells and replicate. Half a gram of soil sample was added to a 15 mL centrifuge tube with 5 mL of cultured *M. smegmatis* cells. This was placed on the shaker for approximately 24 hours at 200 rpm and 437°C.

Viral Infection of *M. smegmatis*

Modifications in this section returned positive results, after negative prior trials. To determine if a phage was present, the enriched sample was mixed with cells and diluted out to 10^{-4} concentration. Five micro-centrifuge tubes were labeled 3^0 through 3^{-4} for diluting the enriched sample 3 (3 stands for the third environmental sample). Positive and negative control micro-centrifuge tubes were labeled. Seven glass test tubes were labeled to correspond to each micro-centrifuge tube. In a 15 mL centrifuge tube 2 mL of phage buffer and 20 μL of $CaCl_2$ were mixed. The phage buffer consisted of 10 mM Tris stock, 10mM $MgSO_4$, 68 mM NaCl, ddH_2O, and 1 mM $CaCl_2$. Every micro-centrifuge tube received 90μL of solution except the 3^0. In a 15 mL centrifuge tube, 7 mL of pre-cultured *M. smegmatis* were combined with 70 μL of $CaCl_2$. Each glass test tube received 0.5 mL. The 15 mL centrifuge tube (3) was spun in the micro-centrifuge at 3800 rpm for 10 minutes to isolate all the debris in the pellet and bacteriophages in the supernatant. Using a sterile syringe, 1mL of supernatant was removed and filtered into a correspondingly labeled 15 ml screw cap tube, separating the bacteriophages from other organic molecules and debris. From the filtered sample (3) 100 μL was transferred into the empty 3^0 micro-centrifuge tube. Then, 10 μL from the 3^0 tube was transferred to the

3^{-1} tube. They were vortexed between 8-9 speed for one second and then the same process occurred carrying the dilution out to 10^{-4}. The negative control did not receive any virus. A concentrated solution of, an already isolated virus, was transferred by touching a pipet tip to the isolated virus and then to the positive control. From the micro-centrifuge tube, 50 μl of the virus solution was transferred to the corresponding glass test tubes (previous trials with different samples used 20 μL of virus, but did not give positive results). After each transfer the glass test tube was vortexed for one second at 4-5 speed. The seven samples were placed on the workbench at 37°C for 30 minutes while being flicked periodically to mix. During the 30-minute incubation period, seven agar plates were labeled to match each glass test tube. The agar plate consisted of L-Agar base, ddH$_2$O, CB, and CHX. Each glass test tube received 4.5 mL of top agar (55°C) and mixed by pipetting up and down twice. The top agar was made up of 1 mM CaCl$_2$, neat, and 1X TA. It was transferred to the correspondingly labeled agar plate and gently spread completely covering it. If air bubbles formed, they were removed by the serological pipet. The plates sat at room temperature for 30-minutes to harden and were moved to the incubator for 24 hours. This allowed the bacteriophages to burrow holes in the *M. smegmatis layers* forming visible plaques.

Extraction of infected cells from plaques

Two isolated plaques from dilution of 10^{-3} of sample 3 were used. One 15 mL centrifuge tube was labeled A and another was labeled B and 100 μL of phage buffer were transferred into both. The cells were extracted from the isolated plaque by

poking it with a pipet tip, being careful to only pierce the top agar. The tip was transferred to tube A and the same was done for sample B.

Dilution of Isolated virus (round 1)

The purpose of this step is to dilute the virus out and plaque it in order to determine which dilution yields the best concentration of isolated plaques. A high titer was made based on the best concentration.

Seven micro-centrifuge tubes and glass test tubes were labeled B 10^0 through 10^{-4}, as well as a positive and negative controls. In a 15 mL test tube 2 mL of phage buffer with 20 μL of $CaCl_2$ were combined. All the micro-centrifuge tubes except for 10^0 (this was replaced by the infected cells obtained in the extraction process) received 90 μL of the solution. A 15 mL test tube received 7 mL of *M. smegmatis* cells and 70 μL of $CaCl_2$. Then 0.5 mL of the cell solution was added to each glass test tube. Cells from plaque B were chosen and became the B^0 tube. From B^0, 10 μL were transferred into the 10^{-1} tube and vortexed at 8-9 for a second. This was continued through the 10^{-4}. The negative control did not receive any virus. A concentrated solution, of an already isolated virus, was transferred by touching a pipet tip to the isolated virus and then to the positive control. It was vortexed at 8-9 for a second. Then 20 μL from each micro-centrifuge tube was transferred to its corresponding glass test tube and vortexed after each transfer at 4-5 for a second. The samples shook for 30 minutes at room temperature. Then 4.5 mL of top agar was transferred to the 10^0 glass tube and mixed by pipetting up and down. It was transferred to the corresponding agar plate, completely covering it. The same was done for 10^{-1}

through 10^{-4}, as well as the positive and negative controls. Plates were allowed to harden for 30 minutes before being moved to the incubator for 24 hours.

Dilution of Isolated virus (round 2)

The second round of dilutions was done to purify the virus further. Seven micro-centrifuge tubes, glass test tubes, and agar plates were labeled B 10^0 through B 10^{-4}, including a positive and negative control. In a 15 mL test tube 2 mL of phage buffer was combined with 20 μL of $CaCl_2$. Micro-centrifuge tube 10^0 received 100 μL and then 90μL into all the rest. A 15 mL test tube received 7 mL of *M. smegmatis* cells and 70 μL of $CaCl_2$. Each glass test tube received 0.5 mL of the cell mixture. An isolated plaque was pierced on the B 10^{-4} plate (from the first round of dilutions) and touched to the 10^0 micro-centrifuge tube using a pipet tip. Then it was vortexed at 8-9 for a second. Then, 10 μL were transferred from the 10^0 to the 10^{-1} and it was vortexed at 8-9 for a second. This was carried out through 10^{-4}. Nothing was added to the negative control. A concentrated solution, of an already isolated virus, was transferred by touching a pipet tip to the isolated virus and then to the positive control. A vortex at 8-9 for a second followed. Now 20 μL from each micro-centrifuge tube was transferred to its corresponding glass test tube and vortexed at 4-5 for a second. The samples shook for 30 minutes at room temperature. Then 4.5 mL of top agar was transferred into the 10^0 glass tube and mixed by pipetting up and down. It was then transferred to the corresponding agar plate, completely covering it. The same was done for 10^{-1} through 10^{-4}, as well as the positive and negative controls. Plates were allowed to harden for 30 minutes before being moved to the incubator for 24 hours.

Dilution Round 3: Determine concentration for a High Titer

  This round of dilutions was carried out to determine which dilution produced the best web pattern (countable individual plaques). Plaque B 10^{-2}, from the second dilution, was chosen and used to carry out this dilution. In a 15 mL screw cap tube 8 mL of phage buffer was mixed with 80 μL of $CaCl_2$. It was transferred to the B 10^{-2} plate, covering it and sat for three hours pulling the virus off the plate. Eleven micro-centrifuge tubes, glass test tubes, and agar plates were labeled B^0 through B^{-10}. A 15 mL tube received 2 mL of phage buffer and 20 μL of $CaCl_2$. Using a micropipet, 90 μL of the solution was added to all the micro-centrifuge tubes except for the B^0. A 15 mL test tube received 7 mL of *M. smegmatis* cells with 70 μL of $CaCl_2$. Each glass test tube received 0.5 mL of the cell mixture. The extracted phage solution was dispensed into a 15 mL screw cap tube. Half of the sample was extracted using a needle and syringe. The solution was filtered into a new 15 mL screw cap tube. This was done a second time to obtain approximately 6.5 mL of viral solution. The B^0 micro-centrifuge tube received 100 μL of the filtered solution. It was vortexed at 8-9 for a second and then 10 μL were transferred into B^{-1}. It was vortexed at 8-9 for a second. This was carried out through B^{-10}. Each corresponding glass test tube received 20 μL of the solution from the corresponding micro-centrifuge tubes and each was vortexed at 4-5 for a second. The samples shook for 30 minutes at room temperature. Each glass test tube received 4.5 mL of top agar and was mixed by pipetting up and down twice, before being transferred to the corresponding agar plate. The plates were allowed to harden for 30 minutes and then transferred to the incubator for 24 hours.

Determining Which Dilution Yielded the best Concentration of Virus

       Plaques were judged based on the ratio of visible countable plaques compared to open plaque space. It was hard to distinguish so two close dilutions were chosen (B^{-3} and B^{-4}). They were then diluted out again to determine which gave the best virus ratio, and ultimately which was used to create high titer of the virus.

Dilution For High Titer Determinate

       In order to make high titer, a dilution needed to be selected that provided the highest plaque to non-plaque ratio on the agar plates. Diluting out both samples helped determine which sample yields the most virus and which high titer should be used to purify virus. Five micro-centrifuge tubes were labeled B^0 through B^{-4}. This allowed the dilutions of 10^{-3} and 10^{-4} to be reached. Correspondingly 10 glass test tubes and agar plates were labeled: five B^{-3} and five B^{-4}. For later use, 50 mL of neat was mixed with 1 mL of $CaCl_2$ in a 50 mL screw cap tube and placed in a 55°C water bath. A 15 mL tube received 2 mL of phage buffer and 20 μL of $CaCl_2$ Micro-centrifuge tubes B^{-1} and B^{-2} received 90 μL, B^{-3} and B^{-4} received 180 μL, while B^0 received none (this will eventually get virus from sample B obtained in dilution three). A 15 mL screw cap tube received 7mL of *M. smegmatis* cells and 70 μL of $CaCl_2$. Each glass test tube received 0.5 mL of cell solution. A new solution of cells was made for upcoming procedures by mixing 100 mL of complete medium with 1 mL of $CaCl_2$ and 200 μL of *M. smegmatis* cells from the previous culture. They were dated and set on the shaker at room temperature for 24 hours. From virus B 100 μL were transferred into micro-centrifuge tube B^0. From there, 10 μL was transferred

into B^{-1} and vortexed at 8-9 for one second. The same was done for B^{-2}. From B^{-2} to B^{-3}, 20 µL was pipetted and then vortexed at 8-9 for one second. The same was done for B^{-4}. From $B^{\wedge-3}$ and B^{-4}, 20 µL were transferred to each of the corresponding five glass test tubes and then vortexed at 4-5 for one second. The samples shook for 30 minutes at room temperature. Neat was mixed with 2x top agar to create a 100 mL of 1x top agar for later plating. It was placed in the 55°C water bath. To plate the infected cells, 4.5 mL of top agar was mixed into each B^{-3} and B^{-4} glass test tube, pipetting up and down to mix after each transfer. Each solution was plated directly after mixing to the corresponding agar plate. The plates were allowed to harden for 30 minutes before being put in the incubator for 24 hours.

Dilution for high titer determinant (round 2)

A second and further dilution of B^{-3} and B^{-4} determined which high titer produced the best plaque to non-plaque ratio for countable plaques. The counted plaques were then calculated to determine how many plaques per mL the virus produced revealing which high titer was the most efficient. The plaques from the first high titer dilution were covered with 8mL of phage buffer each and allowed to extract virus for four hours. Ten micro-centrifuge tubes were labeled A^0 through A^{-9} (A represented the 10^{-3}) and 10 were labeled B^0 through B^{-9} (B represent the 10^{-4}). Twenty glass test tubes and 20 agar plates were labeled to correspond to the micro-centrifuge tubes. For later use, 50 mL of neat was mixed with 1 mL of $CaCl_2$ in a 50 mL screw cap tube and placed in a 55°C water bath. All the phage buffer from the B^{-3} was extracted with a serological pipet and placed into a 50 mL screw cap tubes labeled A. The same was done for the B^{-4} except it was labeled B. A 50 mL sterile

filter top was attached to the vacuum. The solution was filtered into a new 50 mL screw cap tube labeled A and the same process was done for sample B except the tube was labeled B. A 15 mL tube received 2mL of phage buffer and 20 μL of $CaCl_2$. All micro-centrifuge tubes received 90 μL of the solution except for both 10^0 tubes. Then, 11 mL of *M. smegmatis* cells were mixed with 110 μL of $CaCl_2$ in a new 15 mL screw cap tube. Each of the glass test tubes received 0.5 mL of cell solution. Micro-centrifuge A^0 and B^0 received 100 μL from the corresponding filtered A and B samples. Next, 10 μL was transferred from A^0 to A^{-1} and vortexed at 8-9 for one second. This was carried out through A^{-9}. The same was done for the B dilutions. Twenty micro-liters were transferred from micro-centrifuge A^0 to the corresponding glass test tube and vortexed at 4-5 for one second. This process was done the same way for the remaining 19 micro-centrifuge tubes and their glass test tubes. They shook for 30 minutes at room temperature. Then 4.5 mL of top agar was added to A^0 and pipetted up and down twice to mix before being transferred to the corresponding agar plate. This was done the same way for the remaining 19 samples. The plates were allowed to harden for 30 minutes and then put in the incubator for 24 hours.

Determining concentration of pfu (plaque forming units)

Calculations were done for four different plaques, but the one described in this section is for B^{-6} plaque dilution. The calculation determined how many plaque-forming units (Pfu) the virus produced per one milliliter. The number of isolated plaques were counted. This value was per 20 μL of virus. The (plaque amount)/20 μL had to be multiplied by 1000 μL to convert the denominator to milliliters. The

number then had to be multiplied by 10^6 because the plaques were counted on the 10^{-6} dilution and by doing this conversion the amount of Pfu produced by virus in the undiluted sample was obtained.

Phage Purification and Isolation

The purpose of this experiment was to isolate the phage DNA from the other bacteria particles such as DNA and RNA that were present in the solution. First, 10 mL of lysate was transferred into an Oak Ridge Tube. Then 40 µL of nuclease mix (NaCl, ddH$_2$O, DNase l, RNase A, Glycerol) was added to the Oak Ridge Tube. The DNase and RNase destroyed any bacterial DNA and RNA remnants from the lysis event. The solution was incubated in a 37°C water bath for 30 minutes and then at room temperature for an hour. Then 4 mL of phage precipitate (ddH$_2$O, Polyethylene Glycol 8000, NaCl) was transferred to the Oak Ridge tube. It was mixed by inversion and stored in the refrigerator for 24 hours. Prior to microcentrifuging, the sample was weighed. The sample spun in the microcentrifuge for 20 minutes at 8,500 rpm to create a phage DNA pellet. The supernatant was poured into a beaker, while making sure not to disturb the pellet. Inverting the test tube onto a piece of paper for three minutes allowed for a complete run off. The pellet received 0.5 mL of sterile DI water and was re-suspended by gently pipetting up and down. It sat at room temperature for 7.5 minutes. To denature the phage proteins 2 mL of pre warmed (37°C) DNA clean up resin was added. Two syringes each received 1.25 mL of phage-genomic-DNA water resin. A syringe plunger was used on each syringe to resin through a column. To rinse out salt and excess protein, 2 mL of 80% isopropanol was pushed through the column using a syringe plunger. The columns

should have contained the isolated DNA. Centrifuging for five minutes dried them.
They were placed on ice for one minute. Columns were removed and 50 μL of pre
warmed 80°C TE (ddh$_2$O, 10mM Tris-Cl stock, 1 mM EDTA stock) was added, and it
sat on the resin column for one minute to dissolve the DNA. The columns were
placed in two new topless micro-centrifuge tubes and centrifuged for one minute to
collect any purified genomic DNA. Both samples were combined into a labeled test
tube (my initials) and stored at 4°

Restriction Digest

The purpose of running the restriction digest is to cut the DNA into pieces
using specific enzymes and then run a gel to separate the fragments. The fragment
pattern can then be compared to known phage genomic patters to determine if the
isolated phage is novel or not. The steps reported in this procedure were the ones
that provided a sufficient digest.

Six micro-centrifuge tubes were labeled U (undigested), B (BamHI), C (ClaI),
E (EcoR1), Ha (HaeIII), and Hi (HindIII) to represent the enzymes being used to cut
the DNA. U is the only one without enzyme to act as a control. The purified DNA
from the last procedure was put into a 65°C water bath for 10 minutes and then a
quick spin was done to mix the DNA. Each tube received specific amounts of H$_2$O,
BSA, buffer, and DNA. The table below lists the amounts each tube received and they
received them in descending order of the table.

	U	B	C	E	Ha	Hi
H$_2$O	16	15	15	13	15	13

BSA	0	0	0	2	0	2
Buffer(2μL)	C.S.	3.1	C.S.	H	C.S.	2
DNA	2	2	2	2	2	2
Enzyme	0	1	1	1	1	1

*All units are in μL

The tubes were set in a 37°C water bath for 2.5 hours for digestion to be completed. Agarose gel was prepared by mixing 100mL of 1X TAE with 0.8g of agarose and place in a 50°C water bath. Once it reached 50°C, it was poured onto a gel plate (the eight welled plate was used) and hardened for 30 minutes. Each tube received 4 μL of loading dye, bringing the total in each tube to 24 μL. The 1KB ladder and the 6 test tubes were placed in a 65°C water bath for five minutes and then quickly spun and put on ice. Gel electrophoresis apparatus was assembled and filled with TAE filling both wells and covering the gel. The first and last lane received 6 μL of 1Kb ladder. All 24 μL of each tube were placed in order (U,B,C,E, Ha, Hi) in the remaining six wells. The gel ran for 1.5 hrs. and was placed in an ethidium bromide bath for 30 minutes for staining and then rinsed in a DI water bath for 20 minutes. Last, the gel was observed under a UV light and pictures were taken.

Electron Microscopy

At the University of Montana, pictures were taken from the isolated phage sample to see the physical morphology of the phage itself. This was done in the electron microscopy lab. The lab used an electron microscope, which shot electrons

through a very small sample, and the energy given off provided a micrograph of the page.

Categorizing the Phage

Phage Enzyme Tool, an online computer program, was used to categorize my phage by assigning it to a cluster, sub-cluster, or independent. First, the category of mycobacterium phage were selected to test against and fragments made by enzymes BamH1, Cla1, EcoR1, and HindIII in the restriction digest were entered in order to compare with other phage digest fragments. This returned a radar diagram and a list of the phages in the cluster and sub cluster that my phage was most similar to.

To create a phylogeny tree, the two closest phages and the digest fragments were entered to assign my phage within the tree compared to the phages in it's cluster.

Results

Analysis of the bacterial plates observed phage plaques. Therefore I had successfully extracted a bacteriophage from the environment (Fig 1a.) Three additional rounds of dilutions and re-plaquing produced a uniform phage size indicative of a pure phage sample. When compared to other viruses isolated in our lab (Fig. 1b) and with available plaque sizes on line, it was observed that my plaques were small. This lead to the investigation of the virus's size to, determine if there was an inverse relationship between its small plaque size and physical size.

Following the production of an amplified phage high titer stock, electron microscopy was performed to evaluate the phage's structure. The results (Fig. 2)

suggest the phage to belong to the group Siphoviridae since it appears to have a

long, non-contractile tail. The head appears to be icosahedral in shape. The phage in

Fig. 2 has a tail length of 375nm, which is of particular interest. Pedulla et al. (2003)

and Hatfull et al. (2010), whose combined research isolated 70 phages, recorded tail

lengths ranging from 120nm to 275nm. In comparison, the phage in this study has

an abnormally long tail. It was therefore named the Flathead Lake Monster based on

the location of isolation near Flathead Lake and in reference to the legendary

monster that is speculated to inhabit that body of water.

 The restriction digest results are shown in Figure 3. These enzymes are used

for comparative restriction pattern analysis between isolated phages and showed

unique cuts in comparison.

Further analysis of the restriction digest allowed for phylogenetic

characterization, using the Phage Enzyme Tool program. I found that my phage's top

26 matches were from cluster F and sub-cluster F1 (Fig. 4). The ray diagram in

Figure 4 overlapped other digest patterns with my digest to assign my phage into a

cluster. This also, showed that it was of the Siphoviridae class. It was also observed

that the Flathead Lake Monster is one step removed from the F1 sub-cluster (Fig 5)
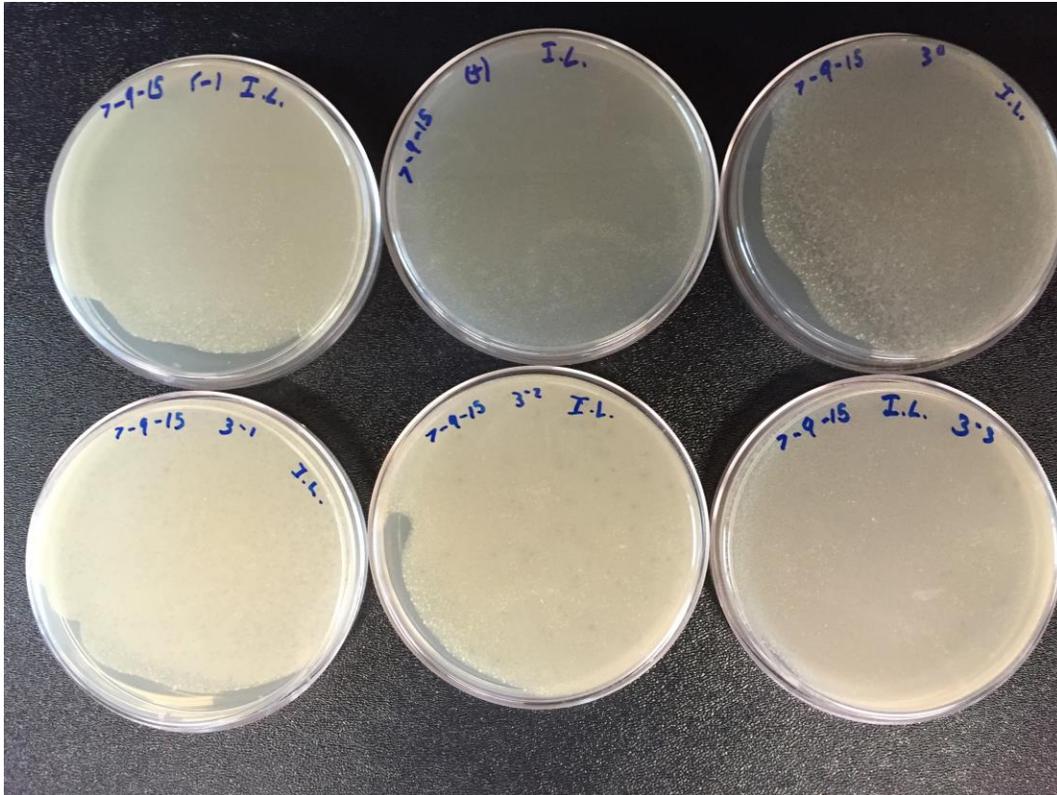
Figure 1a: A series of agar plates following a sample enrichment and *M. smegmatis* culture infection. Plaques in the bacterial lawn indicated a virus was present in the environmental sample. The upper left-most plate is the negative control, followed by the positive control and the serially diluted enriched samples. The 10^{-2} (bottom, middle) dilution clearly shows distinct individual plaques.
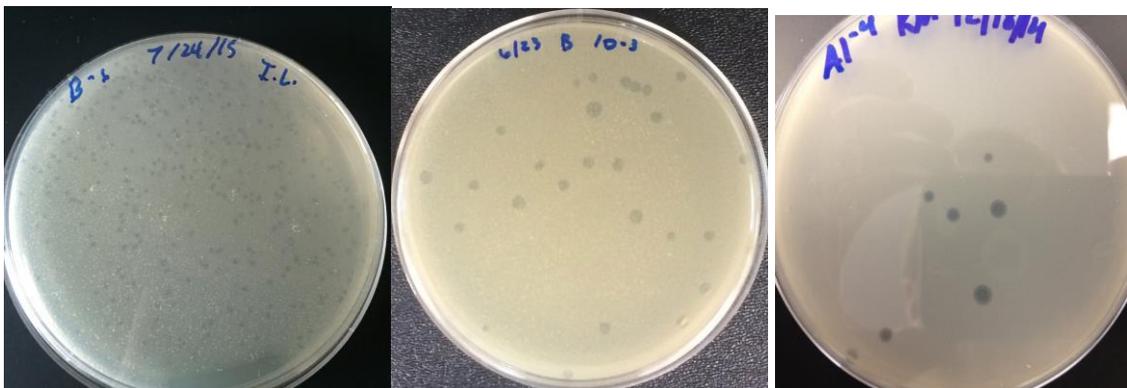

Figure 1b: A comparison in plaque size for the phage isolated in this study to others isolated in the same research group. My phage is in the far left, followed by Brad Gretch's phage and then Kevin McNamee's phage. (Data supplied by Kevin McNamee and Brad Gretch).
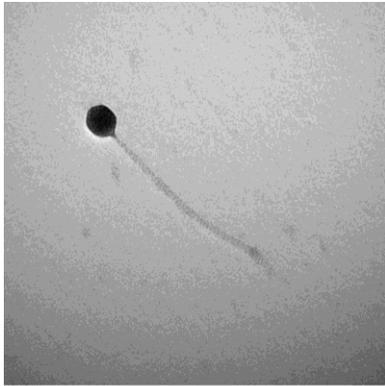
Figure 2: An electron micrograph image of the isolated phage from a purified sample. The tail length measured 375nm.
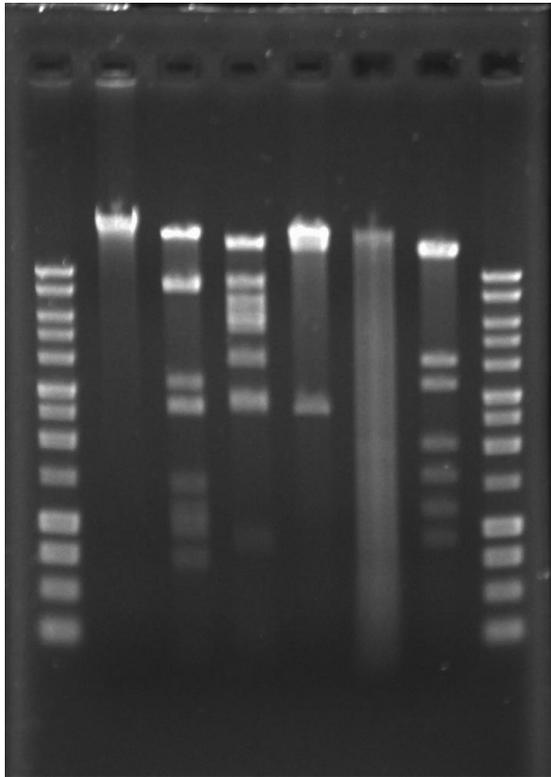
Figure 3: A restriction digest of the purified phage's genome. Specific digests were made in the genome by enzymes (from left to right) BamH1, Cla1, EcoR1, HaeIII, and HindIII. The two outer most bands, on the left and right, are base-pair marker ladders (1Kb). The pattern was compared to other phage digest patterns to help categorize the phage.
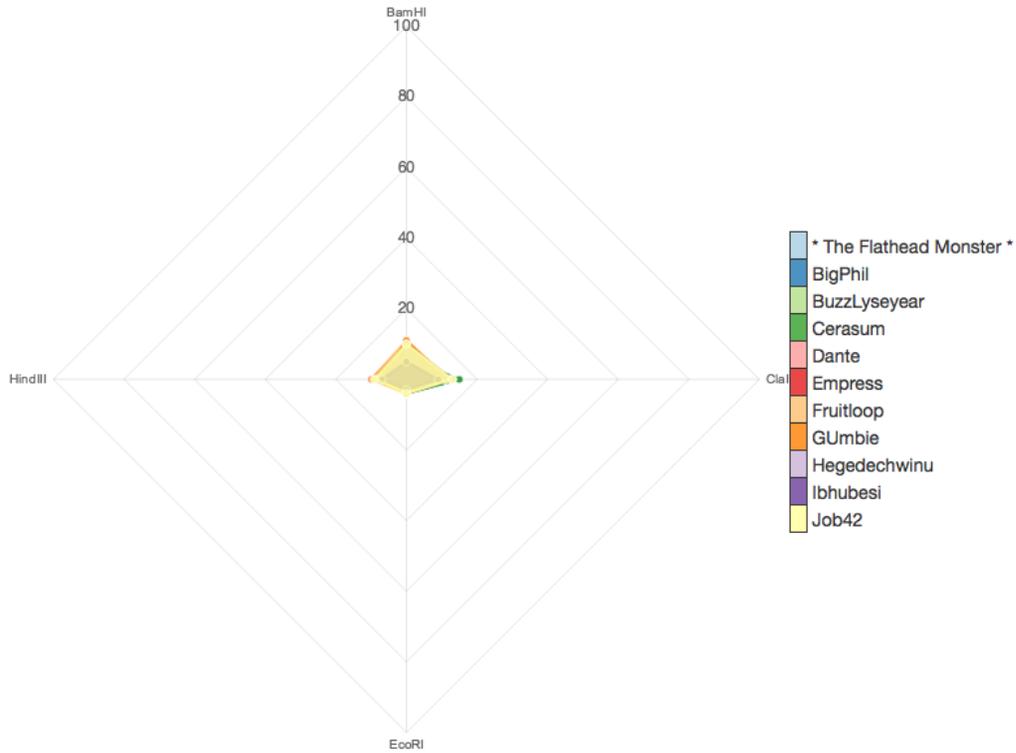
Figure 4: Ray Diagram produced by Phage Enzyme Tool based on digests with BamH1, Cla1, EcoR1, and HindIII. The diagram represents phages with similar restriction patterns and therefore genetic relatedness.
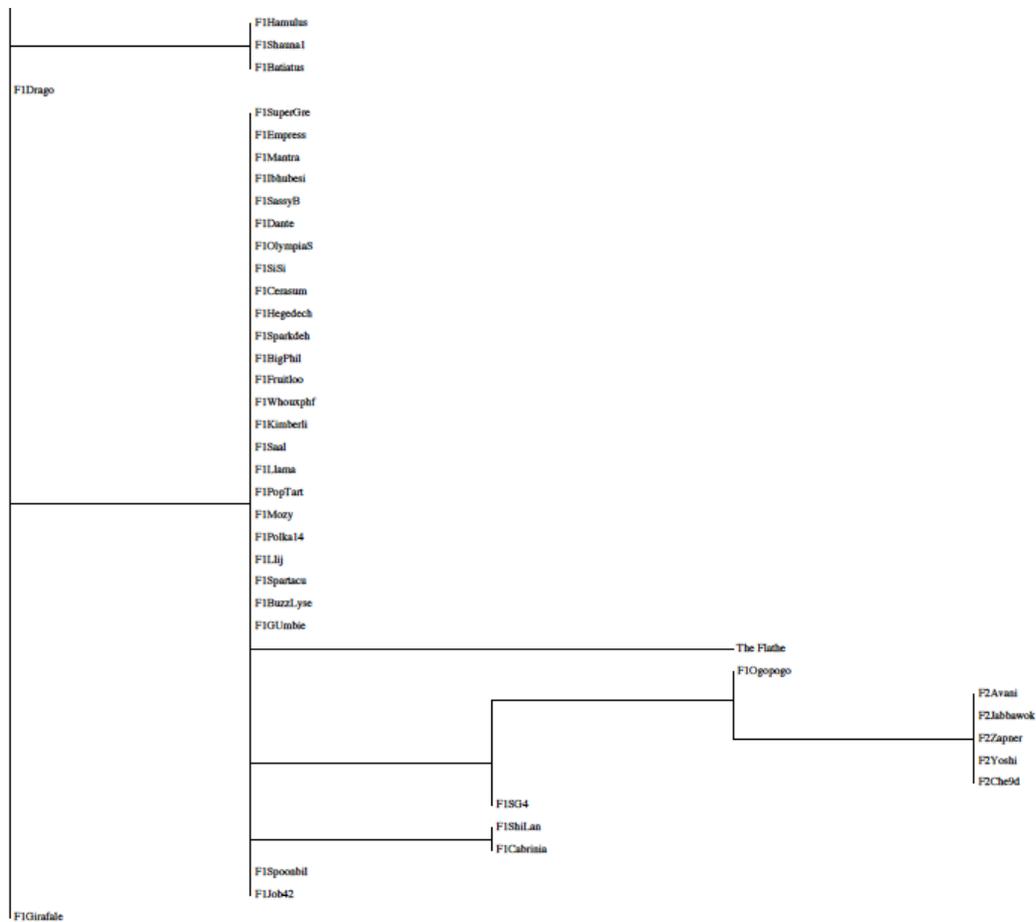
Figure 5: Tree phylogeny of sub-cluster F1, from cluster F. The Flathead Lake Monster's top 26 matches were from F cluster and F1 sub-cluster suggesting that it belongs to that phylogenetic group. The Phage Enzyme Tool's results also demonstrated the phage genome to be novel.

Discussion

The Flathead Lake Monster (FLM) was successfully isolated from an environmental soil sample using *M. smegmatis* as a host. Once purified, restriction digests of this phage genome using specific enzymes were compared to other isolated phages. All digests were unique suggesting that the FLM is unique phage that has never been isolated. A restriction digest pattern analysis suggested that FLM is most similar to the 26 F cluster/ F1 sub-cluster phages. This information

supports my first hypothesis that I would be able to isolate and categorize a novel phage from the environment. Genomic sequencing still needs to be completed, which would provide more concrete evidence that the Flathead Lake Monster is in fact a novel phage.

The average plaque diameter produced upon FLM infection was 0.785mm. This is a relatively small plaque diameter. The phage data bank had incomplete data regarding plaque diameter for isolated phages, so a comprehensive comparison was not possible. However, a comparison within our lab was completed. Brad Gretch's phage average plaque diameter was 2.12mm, 2.7 times larger than that of FLM (Plaque picture provided by Brad Gretch). Kevin McNamee's average plaque diameter was 2.9mm, 3.7 times larger than FLM plaques (plaque picture provided by Kevin McNamee) Refer to Figure 1b.

Since the physical structure of a phage can influence plaque diameter, electron microscopy was used to compare the three viruses. Indeed there was an inverse relationship between plaque diameter and phage tail length. FLM had an unusually long tail length of 375nm. The tail length for Brad Gretch's phage was estimated to be 100nm, 3.75 times smaller than the FLM. Kevin McNamee's phage tail length was estimated to be 105nm, 3.5 times smaller than the FLM.

With multiple variables potentially affecting plaque size (Romain et al., 2011) and little reported information from isolated phages it is difficult to reject or fail to reject my second hypothesis that plaque size is correlated with tail length. Although, within lab comparisons suggest that there is a correlation between plaque size and phage tail size. FLM is large in tail length and small in plaque size, while both other

phage's had smaller tails and larger plaques. The comparison made in this study supports the research on phage mass correlation with plaque size (Romain et al., 2011) suggesting that the bigger the phage, the harder it is to penetrate the agar plate, creating a smaller plaque.

My final hypothesis sought to examine the tape measure gene in FLM. Genome sequencing still is in the process of being completed. Analysis of the results will provide more evidence for novelty of the FLM and information to answer the third hypothesis. I will be able to observe if there is a correlation between base pair length of the tape measure gene and phage tail length.

My successful isolation using *M. smegmatis* supports Pedulla (2003) and Hatfull (2010) suggesting that *M. smegmatis* is a viable host for soil bacteria, such as the Flathead Lake Monster. Based on the research of Brussow and Hendrix (2002) on phage morphology and my analysis of the electron microscopy image, I suggest that the FLM phage is of the Siphoviridae type, having a long fixed tail. Compared to literature on other isolated phage's tail lengths (Hatfull et al. 2010), the FLM has the longest tail ever isolated using *M. smegmatis* as a host.

Overall, my research and data can be added to the growing phage data bank to provide more information of isolated phages. My research provides more possibilities in our lab to further examine the relationship between phage tail length and plaque size as well as tape measure gene length. A genome analysis needs to be completed in order to determine if the tape measure gene is conserved in FLM and if it is related to tail length. Further genome analysis of FLM could reveal more information on genome mosaics within the F cluster and provide information about

novel genes within this virus. Ultimately, blasting the genome against other genomes would reveal novel genes and possibilities to analyze those gene functions.

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