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Sucette: The Discovery and Classification of a Bacteriophage

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Introduction

Bacteriophages are, by an enormous margin, the most numerous organisms on earth. They are able to live and thrive in nearly any environment that our planet is capable of throwing their way due to millions of years of chemical and morphological evolution, and due to the tremendous diversity within their ranks. This variety is one of the foremost reasons that bacteriophage are becoming increasingly of interest to scientists across the world. Due to their prevalence we have investigated and classified only a minute fraction of these phage, and there remains much to be learned about one of the most abundant organisms ever discovered.

First, what are bacteriophages? As implied by their name, they are "eaters" of bacteria: *phagein* is Greek for "devour" (HHMI, 2012). They are viruses that act by injecting their genetic material within a bacterial cell and performing replication inside. Once the replication has occurred and the virus no longer has any use for the host, the bacterial cell will most often burst and release the newly produced viral phages. They have many practical uses to humans, including possible employment in medicine in addition to innovative electricity producing technologies. In this experiment, mycobacteriophages were the specific phages of interest. These phages use mycobacteria as their hosts, including *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis. Mycobacterium smegmatis* was chosen for the experiment due to its relatively rapid growth rate when compared to that of *Mycobacterium tuberculosis* in addition to the fact that it is not pathogenic to humans. It is possible that experimentation with *M. smegmatis* could yield us insight into the *M. tuberculosis* species as well, and could potentially help us move towards medications or cures for the disease this species causes in humans. The more we discover about these organisms in general, the more readily able we will become to research and develop possible uses for them.

Due to the relative novelty of the technology allowing us to study these organisms more closely, and subsequently the fact that so little is known about the many bacteriophage that populate the world, any and all information about a new phage is welcome in the community. As a result, experiments are being undertaken across America and the world, and the collective pool of knowledge is constantly growing. One of the foremost reasons the field has grown so rapidly is the founding of the Howard Hughes Medical Center, SEA PHAGES Program, which has fostered the growth of programs for bacteriophage experimentation and classification at colleges and universities across the country. The goals of the experiments undertaken are to isolate a phage, to conduct tests in order to determine several of its key characteristics, its classification, and to add this new-found information to the growing phage database, thereby adding to the scientific community's current pool of knowledge concerning these organisms.

Materials and Methods

Capture:

To begin, two soil samples were collected from the area outside the Life Sciences Center at Trinity College in Hartford, Connecticut, on 09/04/12 (HHMI, 2012). The area from which they were gathered was warm, dry, and the immediate area was predominantly dirt sparsely populated with grasses, weeds, and pebbles. The samples consisted of dirt collected from the first inch of topsoil at the site, of which the GPS coordinates were 41˚44'43.50"N 72˚41'29.41"W.

An enrichment culture was prepared in order to create conditions that would favor the replication of bacteriophages (HHMI, 2012). The enrichment culture was transferred to a conical tube to be centrifuged in order to separate the supernatant from the solids in the tube. The resulting supernatant was then used to make a ten-fold serial dilution (HHMI, 2012).

The solutions were then plated with *M. smegmatis* onto agar plates. After 48 hours of incubation, the plates were observed and the size, number, and morphology of any plaques present were noted.

Tame:

Following observation, two putative plaques were selected from the enrichment plates to be streaked. They were chosen due to observed characteristics including relative distance from other plaques, size, and morphology, amongst others. Using sterile wooden sticks, and aseptic technique, each of the two plaques were streaked onto agar dishes (HHMI, 2012). Each of the three were plated with *M. smegmatis* solution, and incubated for 48 hours in the hopes that a single purified phage population could be isolated from the sample (HHMI, 2012).

One plaque was chosen from one of the plates to be the subject of another streaking procedure, in the hopes of further isolating and purifying a single phage population. The plaque chosen was once again selected for its relative isolation from other plaques, its size, and morphology. The streaking procedure was then repeated, with the only difference being that the same plaque was streaked twice (on two separate plates) rather than only once (HHMI, 2012). Observations were taken for the plates after this time period had elapsed concerning the size, number, and morphology of the plaques on each plate. Then the streaking procedure was repeated for a third and final time (HHMI, 2012).

An isolated plaque was selected from one of the most recent steak plates, and this plaque was used to repeat a ten-fold serial dilution. The dilutions were each plated and were incubated at 37˚C for 48 hours (HHMI, 2012). Once the time period had passed, the plates were observed and the number, size, and morphology characteristics of the plaques were noted for each.

One plaque was chosen from the 10^{-4} dilution for its isolated nature and its size. This plaque was used to move forward and complete a ten-dilution spot-titer test in order to determine the titer of the phage sample (HHMI, 2012). Once the plate had been allowed to incubate, observations were made concerning the size, number, and morphology of the plaques on each section of the plate. The 10^{-5} dilution was found to have a countable number of plaques, and thus it was this section that was used to determine the titer of the phage solution (Archer, 2012).

Once the titer had been found, the next goal was to produce a web-plate, a plate with so many plaques that it demonstrates a web-like appearance, almost entirely consumed by the plaques. In order to accomplish this goal, a 5-plate dilution procedure was performed (HHMI, 2012). Observations were taken concerning the 4 dilutions and the negative control. It was found that one of the plates, the 10^{-1} dilution, demonstrated the web plate appearance that was the objective of this procedure.

In order to harvest the phage from this web plate and produce the medium-titer lysate (MTL), the web plate was soaked in phosphate buffer and the lysate was collected two hours later (HHMI, 2012).

Another spot titer test was performed in order to ascertain the titer of the MTL (Archer, 2012). The 10^{-8} dilution was found to have a countable number of plaques, and thus the titer could be calculated for the MTL (Archer, 2012).

Calculations were undertaken in order to attempt to predict the amount of MTL that would be necessary to use to yield a web plate (Archer, 2012). These are formally known as Pfu Maxweb calculations, or plaque-forming units maximum-web. A ten-fold serial dilution was then performed to produce the dilutions called for by the calculations. The five dilutions calculated were then plated and were incubated at 37˚C for 18 hours.

Once the time had elapsed, it was clear that none of the plates had yielded the web plate that was sought from this procedure. In addition, the MTL was accidentally left on the lab counter overnight rather than being replaced in the fridge and as a result it was determined that the lysate should be filtered to remove any impurities that may have grown as a result of this error and a 5 plate dilution should be undertaken in order to ascertain the titer and yield a web plate.

The newly filtered MTL was used in a new dilution series that could be used to attempt to produce 6 web plates in order to harvest the lysate for a High-Titer Lysate (HTL).

The 10^{-4} dilution was plated on 6 plates, and all 6 yielded the sought-after web plate appearance. All six plates were then soaked and the lysate was collected after two hours. In order to calculate the titer of the HTL, a ten fold serial dilution was performed. The 10^{-6} dilution had produced a countable number of plaques, and thus the titer of the HTL could be calculated.

Dissect:

In order to assess the morphology of the phage in great detail, electron microscopy was used. To prepare the phage for electron microscopic imaging, a procedure using a grid was employed (HHMI, 2012).

In order to isolate and purify phage genomic DNA the high-titer lysate was used (HHMI, 2012). A DNA collection procedure was employed to yield the isolated DNA (HHMI, 2012). The nanodrop device was used to ascertain the concentration and absorbance of the DNA that was produced.

It was found that the prodecure did not yield enough DNA, and this procedure was run through a second time. Once enough DNA had been produced, it was possible to move on to DNA digestion with gel electrophoresis.

The DNA was gently mixed with different enzymes: BamH1, Cla I, Eco RI, Hae III, and HindIII. Tracking dye was added to the enzyme mixtures, and they were loaded into the gel electrophoresis apparatus. The gels were run twice in an attempt to yield conclusive results.

Further Experimentation:

Due to the dry environment in which the phage was discovered, it seemed appropriate to perform a hydration test upon the phage. Six 10uL spots of HTL were spotted onto a clean dry petri dish. 2 spots were each assigned to 3 different treatments: 1 hour, 2 hours, and 3 hours of drying time. After each had reached its assigned drying time, the phage was re-suspended in

10uL of PB. They were then transferred to labeled microcentrifuge tubes, and a dilution series was performed for each of the treatments; 1uL of the 10^0 from each treatment was transferred to the 10^{-1} dilution (which already contained 9uL of PB), and this was repeated until the 10^{-3} dilution had been reached. This was repeated for all three time treatments. All twelve dilutions were then used to infect *M. smegmatis,* and were each plated. After they had incubated for 48 hours, they were observed and the resulting plaques were observed and recorded. Following this procedure, the same experiment was carried out using the same procedure but with 3 different drying time treatments: 24 hours, 48 hours, and 72 hours.

Results

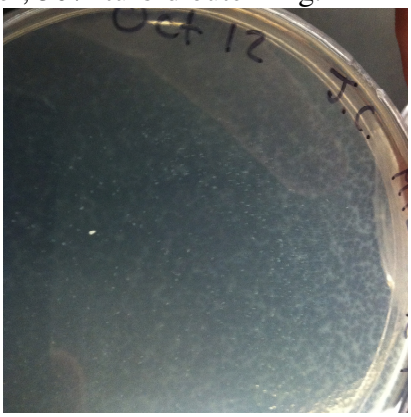
Sample Collection Data:

Table 1: Collection Details

Date of Collection	Location of Collection	GPS coordinates of Location	Characteristics of the Location	Depth from which the samples were taken
09/04/12	Directly outside Life Sciences Center, Trinity College, Hartford, CT	41˚44'43.50"N 72˚41'29.41"W	Warm, Dry, Sparsely populated with grasses, weeds, and pebbles. Grassy region and large Elm tree nearby	Samples were collected from the first inch of topsoil

Plaque Morphology:

Figure 1: Example image of experimentally produced plaque morphology. Plaque morphology was found to be quite consistent over time: consistently 3-6mm in diameter and composed of 50% clear center, 50% turbid outer ring.



Digestion / Gel Electrophoresis:

Figure 2: Gel electrophoresis of DNA Digestion; Trial 1

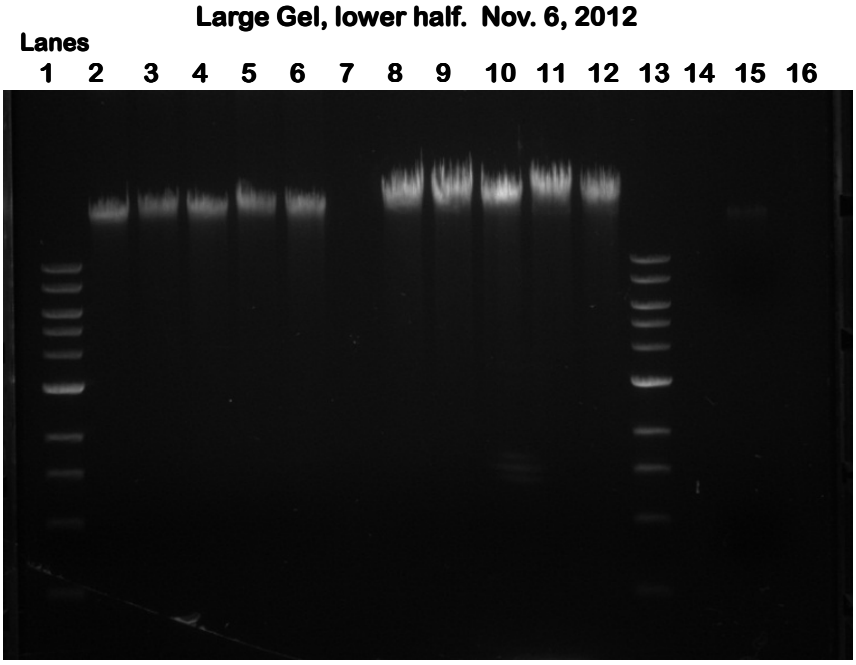
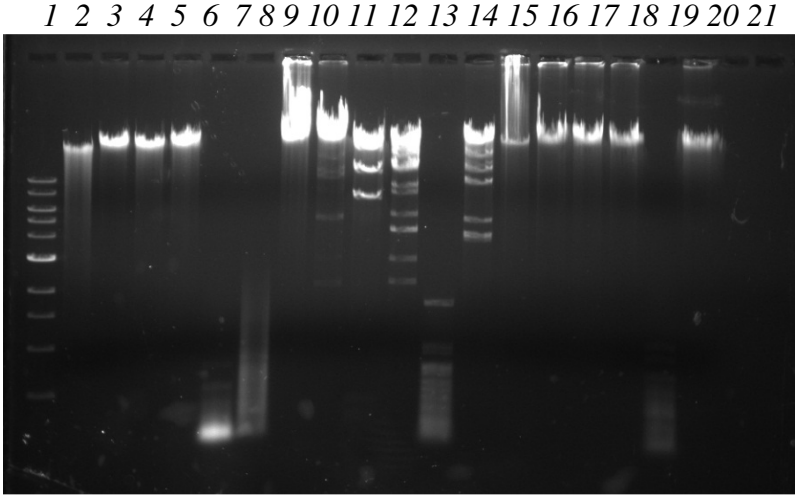


Figure 3: Gel electrophoresis of DNA Digestion; Trial 2



-The lanes occupied by this experiment's phage are lanes 2-6 in Figure 2 (trial 1), and lanes 14-19 in Figure 3 (trial 2). They each correspond to BamH1, Cla I, Eco RI, Hae III, and HindIII, in that order. As can clearly be seen, DNA is indeed present in both images, however, it did not travel as expected. Therefore, no conclusive inferences about the phage were able to be drawn from these procedures.

Phage Morphology:

Figure 4: Sample image of phage viewed using Transmission Electron Microscopy (EM)

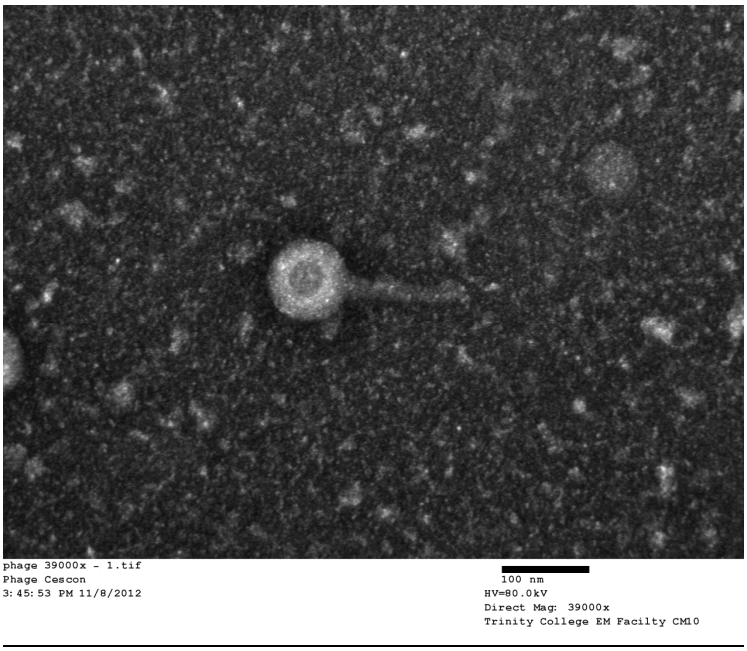


Table 2: Phage Morphology Characteristics

Phage Dimensions	Capsid Morphology	Phage Name
-Capsid: approximately 90nm in diameter total; dark center region 30nm in diameter, light outer ring accounts for the remainder. -Tail: 130nm in length, 15nm across	-Phage Capsid shows slight hexagonal shape, however it is more circular than most phage you usually see	-This morphology is the direct inspiration for the phage's name: Sucette. Sucette is the French word for lollipop.

Hydration Experiment Results:

Table 3: Hydration Experiment Results

First Round of Experiments (drying times < 3hrs)	Second Round of Experiments (drying times 24, 48, & 72hrs)
- Determined that phage HTL can be dried for up to three hours, and diluted to a 10^{-3} level and still result in a nearly webbed	-No plaques were found to have grown, and thus it could only be concluded that the point at which the phage can no longer be

plate. - All twelve plates in the first experimental round yielded a web plate.	re-suspended and grown is somewhere between 3 and 24 hours.

Discussion

The collection and experimental stages were conducted in an effort to successfully isolate a novel bacteriophage. Only once the phage had been isolated through many experimental procedures could data and information concerning the phage finally be assessed. These characteristics of interest included plaque morphology, phage morphology, DNA fragment sizes, and the phage's ability to survive in a hydration free environment.

Plaque morphology was assessed through observation of the plaques produced during the plating experiments that were carried out. Size, morphology, appearance, numbers, and dispersion were among the characteristics that were noted concerning the plaques (Fig.1).

The phage's individual morphology was investigated through the use of transmission electron microscopy. Figure 4 displays the results of this investigation, and characteristics of interest were the apparent density of the phage capsid, the size and length of the phage capsid and tail, whether the tail appeared to be contractile or not, the shape of the phage capsid, and the distribution and concentration of the phage. Most of these characteristics, visible in Figures 1 and 4, and Table 2, are un-remarkable and are quite average within the bacteriophage community. What was distinct, however, was the relatively circular capsid shape; most capsids are more obviously hexagonal than Sucette's appeared to be.

An inquiry was also made into the phage's DNA fragment sizes through an enzyme digestion and gel electrophoresis procedure. Unfortunately, these experiments did not yield results that could be used to form any conclusions for this phage. The reasons for this are not entirely clear, although a source of this error (the DNA not being digested) was likely the presence of remnants of some of the solutions that were used in the DNA preparation portion of the experiment. It is possible that these solutions inhibited the enzymes from performing their intended roles, and therefore the DNA did not move in the gel electrophoresis apparatus.

Conclusions about which cluster the phage might belong to were taken based solely upon the phage and plaque morphologies (Fig.1)(Fig. 4)(Table 2). Based on the phage morphology it appeared that the phage most likely belonged to either the A or F clusters, but once these findings were paired with plaque morphology it became clear that F cluster was the most similar to this phage. The phage capsid and tail sizes, in addition to its appearance, were consistent with F cluster, in addition to the plaque morphology produced and witnessed throughout the experimental process.

Endeavoring to learn more about the phage, a hydration experiment was conducted. As mentioned, the soil and location from which the sample and phage were collected was quite dry, and therefore it seemed appropriate to test the ability of the phage to survive in a hydration free environment, and subsequently be re-suspended in phage buffer and plated. The experiments yielded interesting results, showing that the phage could indeed survive and dry for extended periods of time and later grow at an equal capacity to those that had not been dried. This demonstrates the ability of several bacteriophages, and Sucette in particular, to survive under adverse conditions and in extreme environments. However, the precise point at which they are no longer able to do so could not be found; rather, only a range was able to be identified (3hrs-24hrs of drying time). If it were possible to perform continued experiments upon this phage, it would

be of interest to identify at what specific point in time this drop in phage growth ability actually occurs.

In conclusion, the phage was successfully isolated and much was learned concerning its morphology and other characteristics. These valuable findings have been added to the ever-growing database of bacteriophage online, adding to what little information is currently known about these fascinating and incredibly numerous organisms. These findings, in conjunction with many others, will likely lead to potential uses for bacteriophage in fields including electronics, curative and investigative medicinal employments (including studies involving *M. tuberculosis*), and many other potential benefits that have yet to be discovered.

Bibliography

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