Optimizing Phagehunting Methods to Isolate and Amplify Bacteriophages

Shriya Singh, Enrique Assad-Garcia, Nacyra Assad-Garcia, Lauren Oldfield, Sanjay Vashee, and Derrick E. Fouts
J. Craig Venter Institute, Rockville, MD

J. Craig Venter® INSTITUTE

Abstract

There are an estimated 10^{31} bacteriophages (phage), viruses that infect bacteria, on earth, thus comprising a significant portion of the biosphere. Of these, a mere 10,733 phages have been isolated and 2,061 phages have sequenced, complete genomes, with even fewer, only 1,073, presently publicly available in the NCBI GenBank database. "Phagehunting" is the process of isolation, characterization, and genomic analyses of phages that infect bacterial hosts. There is considerable interest in using phages as diagnostics and therapeutics of multi-drug resistant (MDR) bacteria. One such MDR bacterium is *Mycobacterium tuberculosis*, the causative agent of TB, whose phages can be isolated on the easy-to-grow non-pathogenic *Mycobacterium smegmatis*. Since current phagehunting procedures of *Mycobacterium* phages all use identical plating conditions, we would like to explore alternative phagehunting procedures to determine if that affects the type of phages isolated. To date, no phages within the *Podoviridae* family have been isolated that infect Mycobacteria. With these new optimized procedures, we would like to pilot a program, contingent on the success of this study, to expose high school students to the field of molecular and synthetic genomics. We examined the precise conditions required in each step of the phagehunting process to successfully obtain phage. With the use of careful phage isolation procedures and environmental samples from various locations, such as alternative media usage and modified plaque-picking methods, we isolated potentially novel phages. One of these phages, Kalon1, has been isolated and preliminarily characterized. The success of this study may provide the foundation for an improved program for high school students to foster their interest in research and gain invaluable experiences outside of the classroom while providing bacteriophages that can serve as the base chassis of synthetically engineered phages for diagnosis and treatment of MDR pathogens. Lastly, by creating a generalizable standardized process to obtain phages, we may broaden the horizon for new exploration of novel phages and advances in phage therapy of any MDR pathogen.

Background

Antimicrobial resistance in bacterial pathogens is steadily increasing and is recognized as one of the greatest threats to global public health. This is particularly true for multi-drug resistant tuberculosis (MDR-TB), caused by *Mycobacterium tuberculosis* (Mtb). The World Health Organization (WHO) estimates the prevalence of MDR-TB to be at approximately 50 million people worldwide (1) and expanding by nearly 500,000 new cases each year (2). The pervasiveness of tuberculosis worldwide paired with its limited diagnostic and therapeutic technologies necessitate the study of phages in the detection and treatment of tuberculosis. Mycobacterium phage, which has been used for tuberculosis diagnosis, can also be grown on *Mycobacterium smegmatis.* This provides for a non-pathogenic and easy-to-grow alternative that high school students can test on. However, the robustness of such methods need to be improved.

The goals for this project are:

1. To isolate phages as a tool for detection and treatment of MDR-TB.
2. To formulate improved and efficient methods that can be used by high school students on a non-pathogenic bacterium.

Methods

1. Preparation

Two different types of media were used to grow the phages: *Luria-Bertani* (LB), and Middlebrook 7H9 Broth. The samples plated with LB used LB plates and 0.7% T-Top Agar. The indicator host culture was made in the same manner, with liquid LB and *M. smegmatis* inoculated using a sterile inoculating loop. This culture should take two nights to fully grow. The 7H9 plates and liquid culture were made with 7H9 broth, Middlebrook ADC Growth Supplement, and 1mM $CaCl_2$. The culture has taken one night to grow, as compared to the two nights using LB. The top agar was made with an equal ratio of Middlebrook Top Agar (MBTA) and "Dilution" 7H9 (twice the normal concentration of $CaCl_2$).

2. Isolation

Environmental samples were collected from a variety of locations with variable weather conditions. Two of the samples, including Kalon1, were collected in spring, whereas the remaining samples were collected during the summer. 5~10 mL of the soil samples were collected in conical tubes, at least 1-2 inches below the surface. Based on the amount of soil samples, 5~10 mL of phage buffer was added to the tube and mixed. Once the particulate matter completely settled, liquid from the top of the tube was removed and filtered. In a separate tube, 50 µL of the filtered environmental sample was added to 500 µL of the 2-day old *M. smegmatis* culture. This was allowed to sit for 20 minutes to allow any phages to adsorb to the bacteria. Once the top agar was prepared, the contents of the tube containing *M. smegmatis* was pipetted into each 4.5 mL aliquot of top agar. The warm sample was poured onto a labeled agar plate and gently swirled in a circular pattern to spread the agar evenly across the surface of the plate. The plates were given 20 minutes to solidify before they were placed in the 37°C incubator and inverted.

After 18 hours, the plates were removed from the incubator and assessed for any putative plaques. If no plaques were visible, the plates were placed back into the incubator. However, if there were still no plaques found after an additional 24 hours, it was concluded that the sample did not contain any phage targeting *M. smegmatis*.

If plaques were found, they were picked using a glass Pasteur pipette or a micropipette tip and stored in 100 µL of phage buffer. The glass Pasteur pipette provides a high concentration of phage as it goes through the bottom agar, whereas the micropipette tip method just collects the plaque from the top agar.

Methods

3. Purification

Three rounds of purification using the streak plating purification method was necessary to obtain a pure lysate for each individual phage. To do so, 10 µL of the phage was dropped on one side of the plate and streaked out to get individual plaques. 4.5 mL of the top agar with 500 µL of the *M. smegmatis* culture was poured on the plate such that the agar moved from the point of highest to lowest dilution.

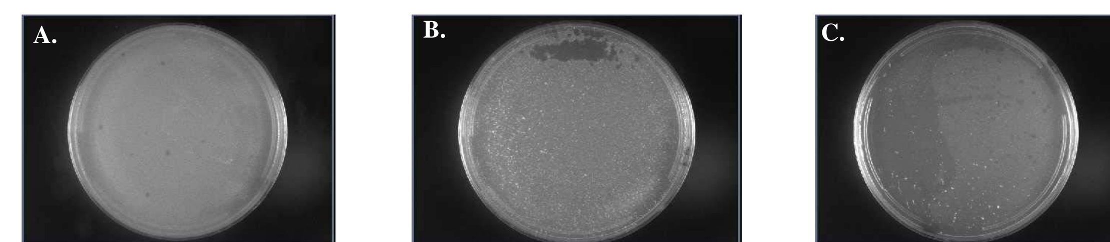


Figure 1. Screened Soil Sample and purifications of Kalon1. A. The plate of Kalon1's environmental sample. **B.** The first purification of Kalon1 by the streak method of one plaque from the plate in figure 1A. **C.** The second purification of Kalon1, the plaque was picked from the first purification plate and was streak plated.

4. Amplification

The phage was now amplified to obtain a high titer lysate. A phage lysate is a concentrated liquid sample obtained by infecting a plate of *M. smegmatis* with the phage, letting the phage lyse the cells, and adding phage buffer directly to the plate surface of 30 plates to collect the phages. High titer phage lysates yield sufficient quantities of DNA for sequencing. However, prior to this, the number of phages in a sample were to be quantified and expressed as PFU (Plaque Forming Unit)/mL. With an estimated titer, the concentration was manipulated to ensure there would be enough phages to form a "web" pattern. The highest-titer lysates come from plates that appear to have the aforementioned "web" pattern, where individual plaques are nearly still visible, but are so densely packed that they cover the whole plate. This indicates that several rounds of phage infection and lysis has taken place, and that the phages and bacteria have the most time and space to produce the highest maximum yield. To obtain this ideal, webbed plate, empirical titer values were tested prior to the 30-plate amplification.

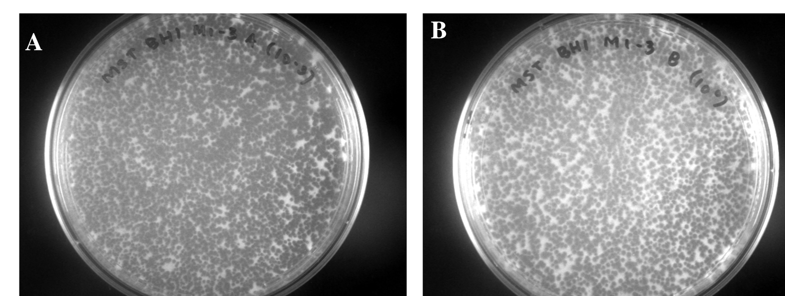
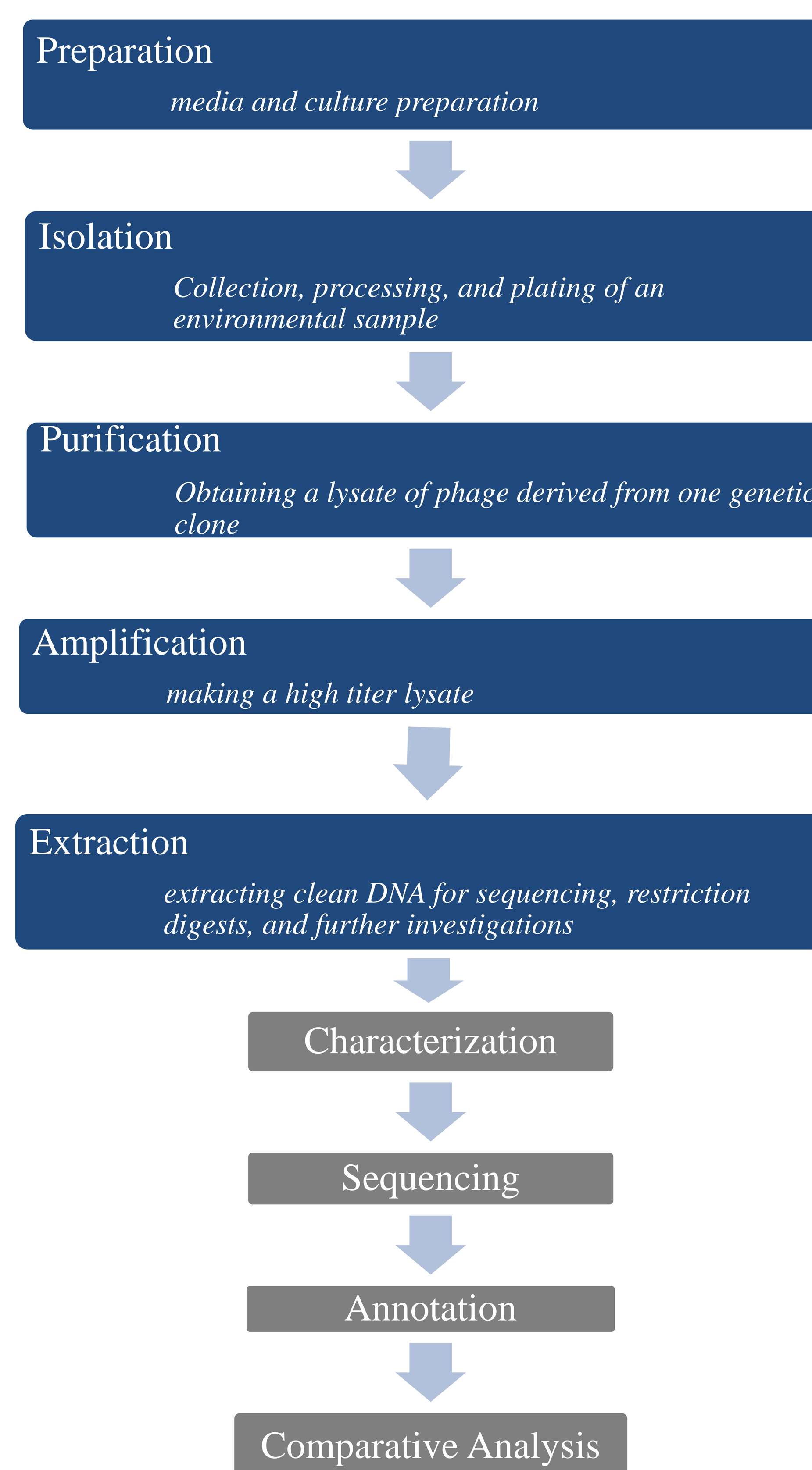


Figure 2. Kalon1 empirical high titer tests. A. After conducting a spot titer test and assuming that there would be 3000 plaques/plate, 4.5 µL of a 10^{-3} dilution was calculated to be an optimal high titer lysate value for the Kalon1 plaque picked using a glass Pasteur pipette. **B.** Empirically tested 2.3 µL of the undiluted, micropipette tip plaque. But clearly, it was not the optimal value, thus I proceeded with the plaque picked with the glass Pasteur pipette.

5. Extraction

To prepare for DNA extraction, the amplified phage sample was harvested and concentrated. The buffer from the 30-plate amplification was centrifuged to remove agar and cell debris and stirred until NaCl and polyethylene glycol (PEG 8000) were in the solution. The phage was then re-suspended in which phage buffer was added to the phage pellets. The final step prior to extraction of nucleic acids is DNase/RNase treatment of viral lysates, the purpose of which was to clean the phage lysate.



Results

Figure 3. Environmental Sample Collection Sites. A. Map of the JCVI Rockville campus, where 5 samples were collected. Collection sites are denoted using a red marker. Out of these five locations, samples from sites 3, 7, and 8, contain confirmed phage. **B.** Image of collection site 3, this was under a willow tree and was very wet. **C.** Image of collection site 5S in Clarksburg. The sample was taken below the layer of mulch and was moderately moist. **D.** Image of collection site 5E in Gaithersburg. The location had an abundance of moss in the area surrounding it and was moldy due to recent rain. **E.** Image of collection site 6E in Gaithersburg. The location was moist and covered in moss.

Although Kalon1 was able to grow using the LB media, such was not the case for phages from other samples. Out of nine samples, six displayed multiple plaques with 7H9 media but nothing when plated on the LB media. Conversely, three samples displayed plaques on both 7H9 and LB media. However, these samples displayed slightly more plaques with 7H9 than with LB.

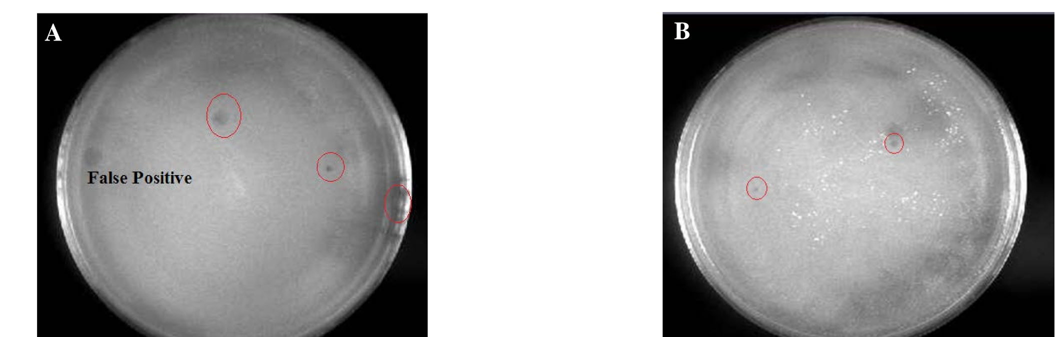


Figure 4. Sample 5E on both 7H9 and LB media. A. Environmental sample 5E plated on 7H9 media, it has three plaques which are all confirmed to contain phages. **B.** The same environmental sample, 5E, was also plated on LB media, it has two plaques. Both plaques are confirmed to contain phages.

The reason for more phages growing on 7H9 media is because it is chemical defined and contains a variety of inorganic salts, sodium citrate, and vitamins. Inorganic salts provide substances essential for the growth of *Mycobacteria*. Sodium citrate provides citrate ions in the medium, which holds inorganic cations in solution. Additionally, Middlebrook ADC Growth Supplement is then added to the culture and top agar, which supplies energy from dextrose and protects *Mycobacteria* from toxic agents.

Conclusion

We have recently concluded that the reason the 7H9 culture grows overnight is because it is contaminated. For this reason, we will be testing with carbenicillin, an additive to prevent other bacteria from growing in our culture.

With this research, we have shown that there are several alternative methods that improve the results obtained. These methods will greatly accelerate and enhance the efficiency by which we can isolate and cultivate phage. In the future, we will continue to work with Kalon1 to characterize, sequence, and annotate it. Additionally, we will work to compile our own booklet of phagehunting protocols, as the publicly available methods are convoluted and hold serious flaws in their organization.

From a broader perspective, we were able to establish phagehunting methods that can be used in MDR-TB testing when working with the non-pathogenic *M. smegmatis*.

Acknowledgements

I would like to thank everyone who took time out of their busy schedules to help me with this great learning experience. I would like to especially thank Nacyra Assad-Garcia and Lauren Oldfield for helping me immensely throughout this entire process, and Dr. Fouts for his invaluable guidance.

References

1. Centers for Disease C & Prevention (2006) Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 55(11):301-305.
2. Organization WH (2000) Anti-tuberculosis drug resistance in the world: prevalence and trends. Anti-tuberculosis drug resistance in the world: prevalence and trends, (WHO).