Isolation of *Enterobacter aerogenes* and *Micrococcus luteus* Bacteriophages from Environmental Samples

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Introduction The goal of this project is to eventually identify how phages interact with the ecology of the local rivers. The first step of the study is to isolate bacteriophages from an environmental sample.

Bacteriophages, or phages, are types of viruses that are capable of infecting bacteria. They also cause the bacterial host to lyse, or die by breaking the cell membrane, and spread to other bacteria. According to the paper "Phages in Nature" by Clokie et al, "Bacteriophages or phages are the most abundant organisms in the biosphere…" (2011). According to the paper, phages were first discovered in 1915 by William Twort. Then in 1917, Felix d'Herelle discovered they had the potential to kill bacteria. They have been studied for disease treatment in European countries like Georgia but not as much in other countries due to the availability of antibiotics. Phages work by attaching to the bacteria and inserting the phages genetic material. This first step is where the two know life cycles diverge. According to chapter six section 3 of the textbook *Microbiology (OpenStax)* the life cycles are called Lytic and Lysogenic (Libtexts). Lytic phages enter the bacterium, use the bacterium's ribosomes to create multiple copies of itself, and cause the cell to lyse releasing the phages. For lysogenic phages, the phages infect the bacterium and integrate their genetic material into that of the bacterium. This can cause the phages to not reproduce right away but cause the bacterium to reproduce the phage genome as part of its genome during cell division. At a later date the phage can start reproducing and eventually cause the cell to lyse. When they are grown with bacteria on a agar plate, they form plaques or clear spots on the plate.

For this experiment, two bacteria were used for the hosts for phages, *Enterobacter aerogenes* and *Micrococcus luteus. Enterobacter aerogenes* was chosen because it was present in some water samples collected for the experiment and *Micrococcus luteus* was chosen because it is a soil bacterium related to *Arthrobacter* bacteria. According to a report by Davin-Regli and Pages, *Enterobacter aerogenes* are gram-negative bacteria that can be an opportunistic pathogen. It is also closely related to *Klebsiella* bacteria (2015). According to a paper by Li et al, there are very few varieties of bacteriophages for *Enterobacter aerogenes*, specifically that there were only two identified at the time of writing in 2016 with the study covering a new novel phage on the bacteria (2016). According to the Wickham Laboratories fact sheet for *Micrococcus luteus*, it is a gram-positive/gram-variable bacteria that can be found in soil, dust human skin and foods. When it grows, it grows to be bright yellow (2019). According to a paper by Hillis, Hulse, and Sheridan, it is possible to isolate bacteriophages for *Micrococcus luteus* from dairy cattle feces and collect plaques (2018).

Materials and Methods for *Enterobacter aerogenes*: Water Sample Isolation

These are the methods used when isolating bacteriophages from a water sample. For these methods, *Enterobacter aerogenes* was the main bacterial species used, but other bacteria were screened such as *Escherichia coli, Bacillus subtilis,* and *Bacillus cereus.* The basic procedures for this section were adapted from the protocols at The Actinobacteriophage Database at phagedb.org (n.d.).

Media Preparation. For this isolation method; tryptic soy agar (TSA), tryptic soy broth (TSB), eosin methylene blue agar (EMB), and a phage buffer were prepared. Two concentrations of TSA were prepared. One was a 4% concentration and was used to make TSA plates. The other solution was 0.7% concentration and was used for top agar. The 0.7% solution was aliquoted in 4.5 ml increments for easy use after sterilization. For the TSB, a concentration of 3% was prepared and aliquoted in 9 ml increments for easy use after sterilization. The EMB was prepared with a concentration of 3.74% and used for EMB plates. The phage buffer was prepared by combining 20ml of .5M Tris solution at a pH of 7.5, 10ml of 1M $MgSO_4$, 4g NaCl, and 970 ml diH_2O. All media, after prepared, was autoclaved in a liquid cycle.

Sample Collection. Samples were collected along some of the local rivers (San Juan River and Animas River). Samples consisted of mostly water, little soil or plant life. GPS coordinates for collection spots are listed in the results. Pictures of the collection sight are in Figures 1-3.

GPS Coordinates for Sample Collection

Sample Name	GPS Coordinates
Water Sample	36.7359890, -108.1687260
Soil Sample 1	36.771085, -108.167478
Soil Sample 2	36.772467, -108.172336

Figure 1 This is a picture of the water one of the samples was collected from. Photo taken by researcher.

Figure 2 This is another photo of the collection site of the water sample. Photo taken by researcher.

Figure 3 This is a picture of the area around the collection site for the water sample Photo taken by researcher.

Bacterial Culture Preparation. Using some of the water from the samples, the water was spread across a TSA and an EMB plate using a Lazy-L Spreader. They were then incubated overnight at room temperature. After one day observations were made about the present bacteria. Example plates are seen in Figures 4-6

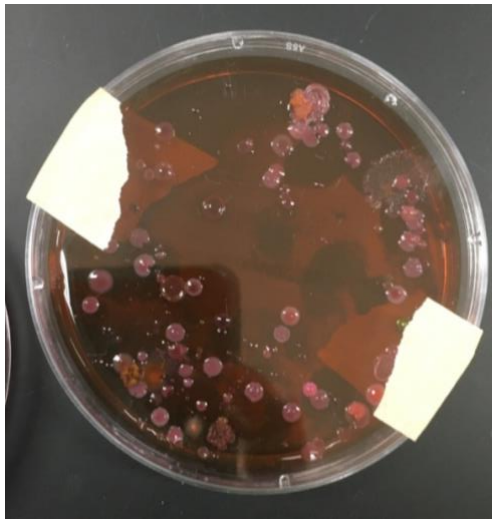

Figure 4 This is a photo of direct bacterial growth from the water samples. This is on EMB. Photo taken by researcher.

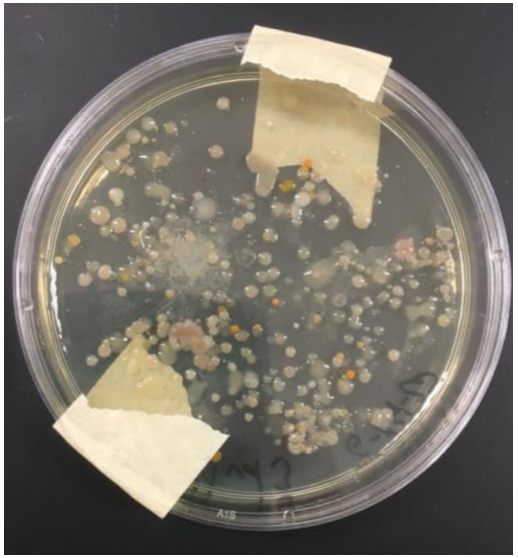

Figure 5 This is a photo of direct bacterial growth from the water samples. This was on TSA. Photo taken by researcher.

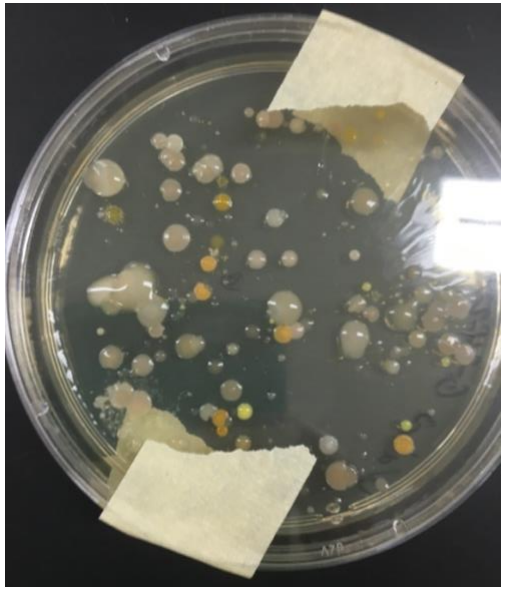

Figure 6 This is a photo of direct bacterial growth from the water samples. This was grown on a TSA plate.

Photo taken by researcher.

They were left another day in order to identify if any other colonies grow. Some of the most common colonies with similar morphology were stained with a gram stain to better identify what bacteria is present. *Enterobacter aerogenes* was one of these bacteria. Once positively identified, another colony was picked to use for a streak plate. After 24 to 48 hours, the plates are removed and verified that there are a single colony morphology present. If so, a colony is picked and mixed into TSB and grown for 24 to 48 hours until used. If not, another streak test is done with a single colony.

Initial Screening. Once bacteria are positively identified, the initial screening can be done. Using a sterile syringe with a Luer-Lock tip, 1ml of the water is pulled up. Then attach a .22µl syringe filter and filter .25µl of the sample into a microcentrifuge tube. Then add .125ml of a prepared *Enterobacter aerogenes* culture to the tube. Let the tube sit for roughly ten minutes to allow any phage particles to start infecting the bacteria. During this, heat the top agar tube until the agar is melted. Let the agar cool until it is easy enough to hold and add the

phage/bacteria solution. Mix with a sterile pipette. Once mixed, the top agar was poured immediately. These plates were left to grow for 24 hours. After the day, the plates were checked for plaques and left for another day if needed. Examples of plates after this time are in Figures 7-9.

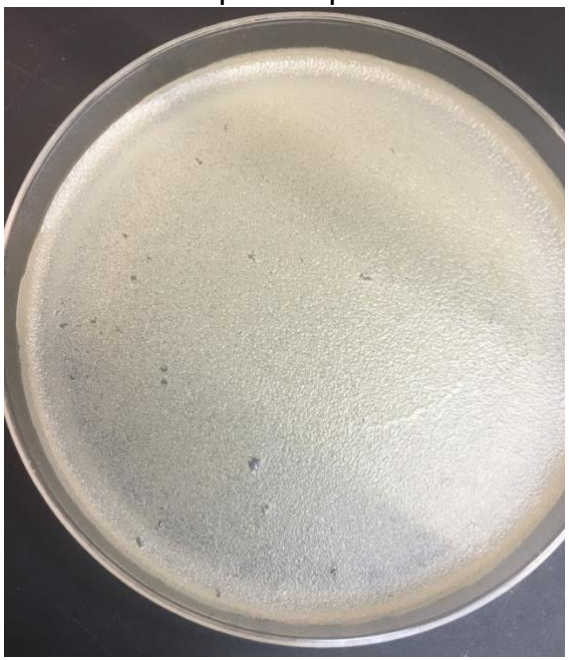

Figure 7 This is a photo of one of the direct isolation tests. Some potential plaques can be seen, but most clear spots are chunks of agar. Photo taken by researcher.

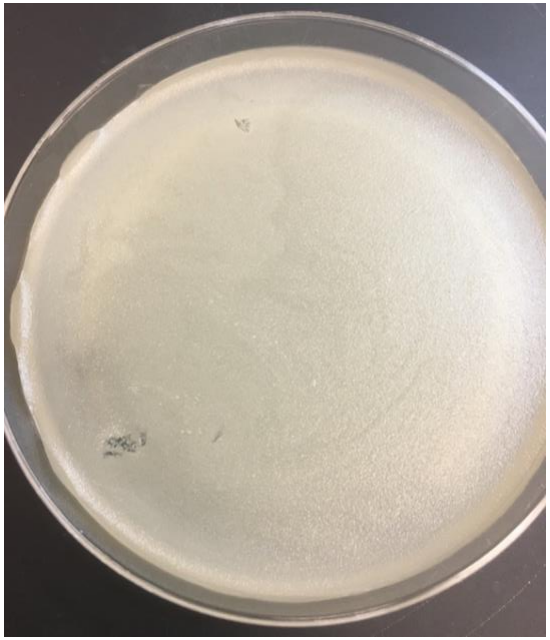

Figure 8 This is a photo of a direct isolation trial. The spots where the lawn appears to have moved are from preparations for a spot test. A spot test was done, but the results were negative. Photo taken by researcher.

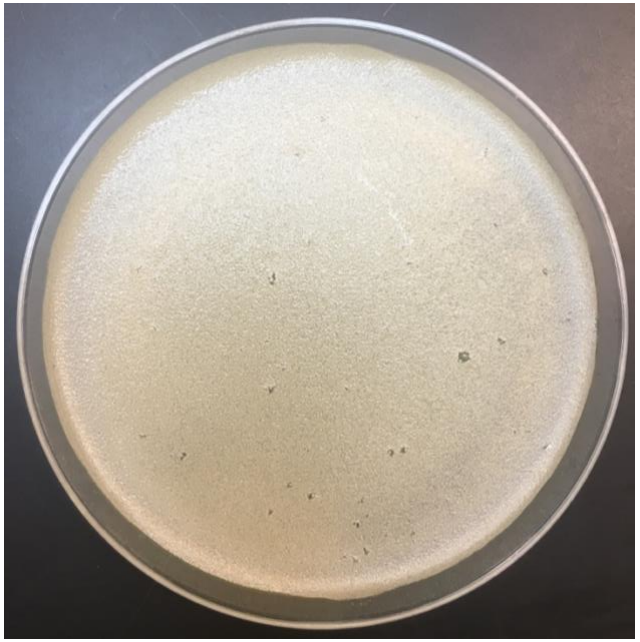

Figure 9 This is a photo of direct isolation method. No plaques were found, just chunks of agar. Photo taken by researcher.

Spot Tests. Using any plates from the initial screen that had any spots that may be plaques were screened. Using a tip for a p200 or a p20 micropipette, the plaque was picked. Once picked, the plaque was mixed with 100µl of phage buffer in a microcentrifuge tube. Prepare a TSA plate by dividing into equal sections based on how many plaques that are going to be screened plus one for a control. The control consists of just the 100µl of phage buffer. Then the TSA top agar is melted and cooled. Once easy to touch, mix .5ml of the Enterobacter aerogenes culture into the agar and pour on the TSA plate. Once the top agar has solidified to the plate, pull up some of the

phage buffer with the plaque and place a drop on the top agar. Let the phage buffer absorb and incubate for 24 hours. Check to see if the spot with the phage buffer is clear.

Materials and Methods for *Micrococcus luteus*: Soil Sample Isolation

For the *Micrococcus luteus* procedures, the procedures were adapted from the procedures presented by Cross et al. Along with *Micrococcus luteus, Streptomyces albus* was screened once, but there were not enough trials with it to give any results.

Media Preparation For these procedures, Luria broth media was used. For the plates, a 4% concentration of LB top agar was prepare using premixed agar powder. For LB broth, 5g of Tryptone, 5g NaCl, and 2.5g Yeast Extract are combined with 500ml of water. The top agar was made by preparing LB broth as described above and adding 3.5g agarose powder. Once combined, the top agar was aliquoted into 9ml tubes. A new phage buffer was prepared for these trials. It was a 68mM NaCl, 10mM MgSO4, and 10mM Tris solution. These solutions were autoclaved before use. The final solution prepared was a 2M CaCl2 solution.

Bacterial Culture Preparation Using a plate from a streak test with individual colonies, collect a colony with a sterile loop and swirl into a sterile culture flask with 25ml of the LB broth. Place in a shaker incubator overnight at 25º C and 225 rpm. If the broth is cloudy the next day or if a mass has collected at the bottom, it is ready to use. If not, leave the culture for another day. Once the bacteria have grown and appear to be ready to use, measure the concentration using the OD600 on a Smart Spec Spectrophotometer.

Smart Spec Results

Sample	Date	Absorbance	Concentration
M. luteus Sample 1	7/23/20	.456 AU	$2.28e^8$ cell/ml
M. luteus Sample 2	7/23/20	.885 AU	$4.42e^8$ cell/ml
M. luteus Sample 1	7/24/20	.520 AU	$2.60e^8$ cell/ml

M. luteus Sample 2	7/24/20	.959 AU	$4.80e^{8}$ cell/ml
M. luteus	8/3/20	3.623 AU	$1.81e^{9}$ cell/ml
S. albus	8/3/20	2.564 AU	$1.28e^{9}$ cell/ml

Sample Collection For this method of isolation, soil samples are used instead of the water samples above. GPS Coordinates were also collected.

Initial Screening First, add between 200 and 400g of the soil sample to a large beaker. Add 400ml of the phage buffer and mix. Let the particulates settle and filter the supernatant into a graduated cylinder until the total filtrate is 200 ml. Add 2g tryptone, 2g NaCl, and 1g Yeast extract to the filtrate and stir until combined. Using a sterile 60ml syringe with a luer-lock tip, pull up 50ml of the filtrate/LB broth solution and attach a .22µm filter and expunge into a sterile culture flask. This is done until there are three flasks with filtrate. Add varying amounts of the 2M CaCl2 solutions because phages survive at different concentrations. To each flash, add 2.5ml of the M. luteus culture. Place flasks in the shaking incubator at 25ºC and 225 rpm overnight. These are the enriched samples and can be seen in Figure 10.

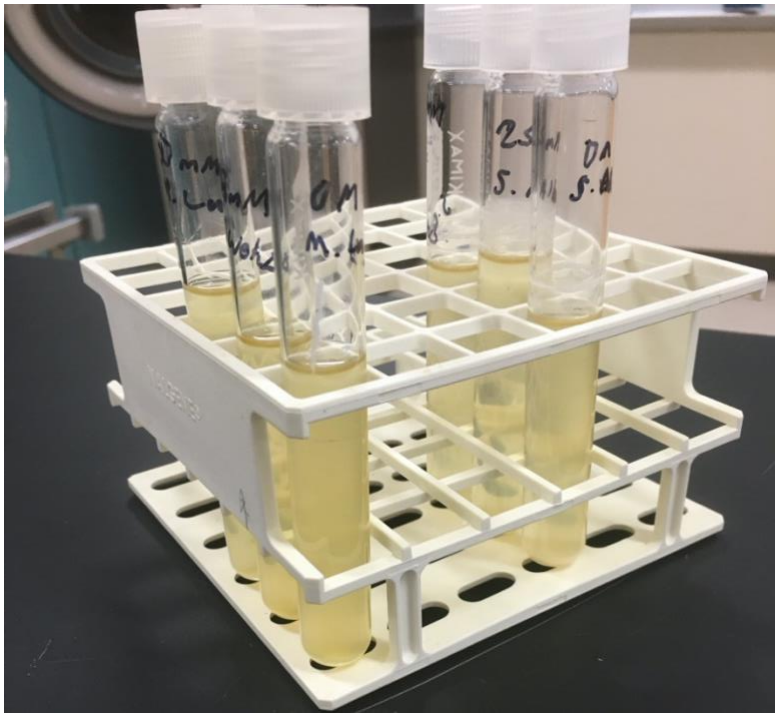

Figure 10 This is a photo of the culture tubes for the enriched isolation with M. luteus. Photo taken by researcher.

The next day, add .5ml of the bacterial culture to a sterile microcentrifuge tube. Add 250µl of the enrichment culture. Heat the top agar in the microwave until fully melted. Add the contents of the microcentrifuge tube and mix. Pour

onto a LB plate and let fully solidify. Place in a room temperature incubator overnight. Examples of plates from this trial can be seen in Figures 11-13

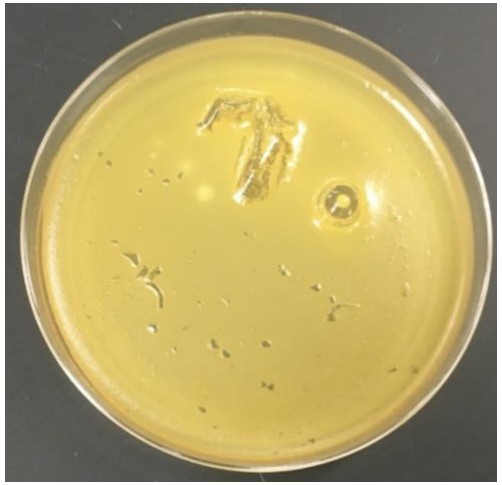

Figure 11 This is a photo of the isolation test with M. luteus. Because of the size of the agar chunks, it was easy to tell there were no plaques on this plate. Photo taken by researcher.

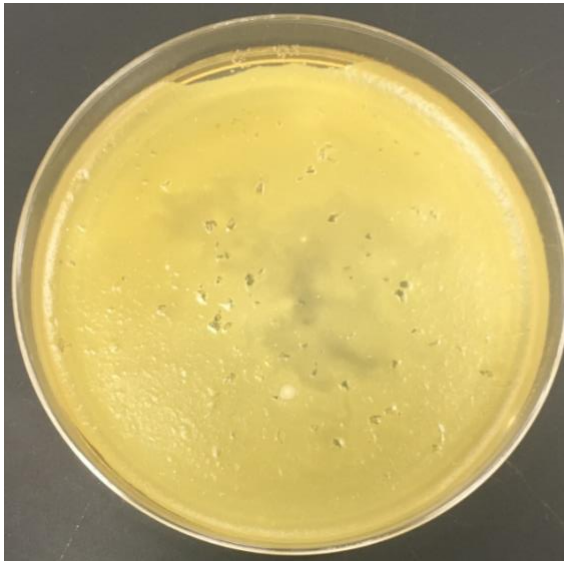

Figure 12 This is a photo of the isolation test with M. luteus. Because there were more smaller chunks, it was harder to tell if there were plaques. Photo taken by researcher.

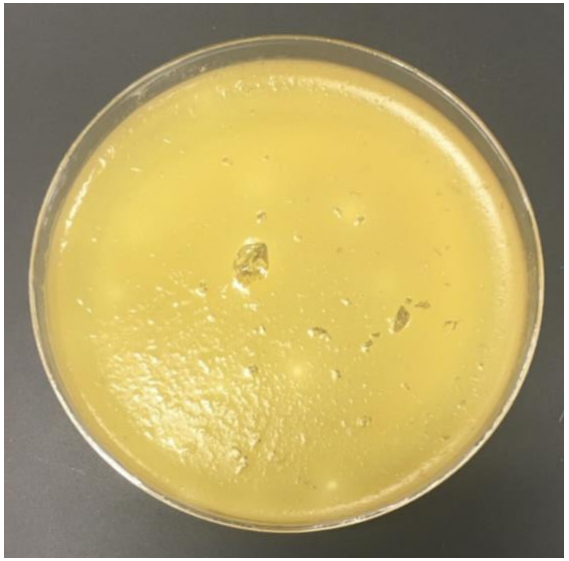

Figure 13 This is a photo of the isolation test with M. luteus. While there were smaller chunks, they were easy to identify because they protruded from the plate. No plaques were identified. Photo taken by researcher.

Results and Discussion The samples did not test positive during the initial isolation. Due to issues with top agars, it became hard to distinguish between what was a plaque and what was solidified agar. For the plates that had potential plaques and were tested with a spot test, none came back positive.

Conclusion While none of the samples screened returned possible plaques, some possible reasons for the lack of results were identified. One possible reason may have been with the top agar mixture. Most of the trials where the top agars were used, both LB top agar and TSA top agars, formed chunks when poured onto the plate. Sometimes, these chunks were large and easily identified, but more often than not the chunks were small. These chunks, when the bacterial lawn formed, were sometimes hard to distinguish from plaques. However, it was verified with spot tests that some of the possible spots were just agar. These agar chunks may have made it harder to identify the plaques. Another problem may have been the bacteria screened. It would have been better to screen more bacteria species to increase the chances a specific bacterium would have a potential plaque to test.

For future trials, more time would be spent working with top agars to minimize chunks for an easier time identifying plaques. Also, some trials will be done melting the top agars and then storing them in a 40-50ºC incubator to keep them melted. According to most literature, top agars stay melted at that temperature. This might be able to alleviate some problems with the top agars. Other than that, more trials need to be done.

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