Characterization and Isolation of Bacteriophages

by

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Abstract

Attempts to isolate *Arthrobacter* phages were made by extracting phages from soil samples taken from sewage drain, Blind Brook river, and Van Cortlandt Park. Strains of *Arthrobacter* sp. ATCC 21022 were cultured on LB agar or Luria broth for experiments of phage infection. Bacteriophages were not detected on plates containing mixture of soil sample diluted with phage buffer and top agar. Plaques were formed on plates containing mixture of soil sample dilated in phage buffer, top agar and 2.0 M $CaCl_2$ concentrations. As a result, we conclude that phages form at different $CaCl_2$ concentrations. A single type of phage was isolated from the Blind Brook river soil sample due to the constant plaque morphology.

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Introduction

Bacteriophages are viruses that infect and replicate within bacteria. They are natural enemies to bacteria and can undergo mechanisms to fight bacterial infections in humans (Rao and Lalitha, 2015). Phages replicate within bacteria following the injection of their genome into its cytoplasm. They are unable to replicate themselves in the absence of a host, depending on host metabolism to provide organic material and machinery necessary for their replication. The replication of phage particles during the infection of hosts allows phages to exchange DNA within the host's genome through recombination, generating diversity as a result. Phages are associated with nearly all known bacterium, thus are found in diverse environments ranging from soil to oceans (Duerkop *et al*, 2014).

Phage diversity

Bacteriophages are found wherever bacteria exist, with an estimate of about more than 10^{31} on the planet, making them the most abundant biological entities on earth. They are found either associated with their bacterial host or in large numbers as free virions in the environment (Duerkop *et al*, 2014). Phages are distributed in locations that are populated by bacterial hosts such as soil or sea water. Phages can undergo far more environmental stress than bacteria (Rao and Lalitha, 2015).

One of the largest natural sources for phages is soil, where counts of up to 2×10^{9} virions per ml have been recorded (Ashelford *et al*, 2003). Bacteriophages in soil is rarely detected unless the soil is nutritionally amended and incubated (Casida and Liu, 1974). This included incubations of soil with added toxins like nicotine salts for periods of over one year, giving rise to phages capable of only infecting *A. globiformis.* A technique resembling direct plating was used in past studies which gave rise to several phages while still having a low success rate by the

researchers. This enrichment technique uses a filtered extract free of contaminating bacteria as the base for indicating bacteria growth. By removing soil bacteria first, target phages are not hindered by competition with native soil bacteria present in initial soil samples. This technique has resulted in dozens of unique phages from several different soil types and even produced different types of phage from the same enriched soil sample isolate (Cross et al, 2015).

Densities of about 9 x 10^8 virions per ml have also been found at the microbial mats surface in sea water. The first phage was isolated from the marine environment and it is believed that marine phages are the most diverse with a Shannon index of 7-8 for water. The Shannon index is a measure of diversity and it increases as both the richness and evenness of the community increases. Suspended particles in the aquatic environment with high densities of bacteria have shown to be hot spots for phage production with a viral abundance estimate of 10^5 to 10^{11} per ml. There is decrease in viral density moving from shallow to deep waters, indicating wider host range of deep-sea viruses (Rao and Lalitha, 2015). Phages are at an average of 10-fold more abundant than their bacterial hosts (Ashelford *et al,* 2012).

Phage structure

Phages are structurally composed of closely related families that are distinguished by their tail morphology. Their basic structure consists of an icosahedral head with a tail, an icosahedral head without a tail or a filamentous form. Phages belong to the *Myoviridae;* long contractile tails, dsDNA, and an icosahedral symmetrical head, *Siphoviridae;* long non-contractile tails, dsDNA and an icosahedral capsid, or *Podoviridae*; short non-contractile tails, dsDNA and an icosahedral capsid (Rao and Lalitha, 2015).

Phage lifecycle

In order to reproduce, phages utilize the intercellular machinery of host cells. Their replication strategies can either be lytic or lysogenic; including stages of adsorption, DNA injection and replication, viron production, and release. The lytic cycle results in the lysis of the bacterial host following the infection of the phage in the bacterial cell. Other phages enter the lysogenic cycle, where their genome integrates with host DNA resulting in replication of phage nucleic acid within host genes for several generations without major consequences to the cell. Lysogeny is commonly referred to as the cycle in which phages undergo lytic and lysogenic lifestyles. It occurs when phages that are maintained in the lysogenic cycle revert to lytic cycle by releasing their daughter phage particles into the bacterial cell. Phages that develop both of these cycles are known as temperate phages (Rao and Lalitha, 2015).

Phage as a therapy for disease

Phage therapy involves the use of phages as bioagents for the treatment of bacterial infectious diseases. Evidence on the effectiveness of phage therapy against bacterial infectious diseases has been collected, along with reports on the appropriate administration of phages used to treat these lethal diseases caused by gram-negative and gram-positive bacteria. The sustained misuse of antibiotics has caused pathogenic bacteria to develop resistance against antimicrobial drugs. Phage therapy has been used before the application of penicillin (the first antibiotic) as a treatment method against bacterial infections. Phage therapy was abandoned with the arrival of the antibiotic era, however, the evolution of bacterial resistance to multi-drug has motivated researchers to re-evaluate phage therapy for infectious diseases that are incurable by chemotherapy (Matsuzaki *et al*, 2005).

Phage therapy has many advantages over chemotherapy; it is effective against pathogenic bacteria that show multidrug-resistant because it induces bacteriolysis completely different from antibiotics; it has high specificity for target bacteria therefore the use of infection with microbes doesn't occur; it responds quickly to phage-resistant mutants because the phages themselves are able to mutate; developing a phage system is cheaper than developing an antibiotic; and lastly, phages aren't able to affect eukaryotic cells so there are no common side effects (Matsuzaki *et al*, 2005).

Phage aquaculture

Phages are believed to be important in controlling infections in aquatic environments, such as, fish diseases. Bacterial diseases present a severe threat to world fisheries production (Rao and Lalitha, 2015). As the host fish organisms live in aqueous media, they are in close and continuous physiological contact with the pathogens. The use of phage therapy to control disease in aquaculture environments has been demonstrated. In previous studies, isolated phages with lytic activity against *Vibrio harveyi* were applied as phage therapy against vibriosis to improve the survival of *Penaeus monodon larvae* (Pereira *et al*, 2011). The success of phage therapy to control pathogenic bacteria of fish depends mostly on viral survival and viability in culture water of fish-farming plants. Although there is data available on the mechanisms and rates of mortality or decrease of infectivity of phages in aquatic environments, there is little information on the longevity of their survival.

Lytic phages that are capable of lysing bacterial pathogens as an alternative for controlling bacterial infections in aquatic food animals can be safe and effective. They are capable of controlling bacterial infections while at the same time evading phage resistance. Lysogenic phages can present an issue of enhancing the virulence of pathogens, infecting aquatic

food animals and thereby threatening aquaculture. It was reported that two bacteriophages belonging to *Myorividae* and *Siphoviridae* are able to change the phenotype of *V. harveyi* isolates from non-virulent to virulent, causing bacteriosis in shrimp hatcheries and culture farms (Rao and Lalitha, 2015).

Renibacterium salmoninarum

Renibacterium salmoninarum is the causative agent of bacterial kidney disease in both wild and farmed salmonid fishes. It is strongly gram positive, short rod-shaped, and often occurs in pairs. Non-encapsulated, non-motile, having absent endospores and aerobic. This species occurs among populations of salmonid fishes in North America, Europe, and Asia. *R. salmoninarum* is slow growing and very fastidious, having growth requirements of up to 5 weeks (Daly and Stevensons, 1985) on peptone yeast extract agar supplemented with cysteine and serum (KDM2) (Austin *et al*, 1983).

Bacterial kidney disease

R. salmoninarum produces a slowly developing chronic infection characterized by gray-white, enlarged necrotic abscesses in the kidney (Sanders and Fryer, 1980). The disease can be transmitted directly by the feeding of raw viscera from infected fish or horizontally from infected fish sharing the same water supply (Elliot *et al*, 1989). It is the only bacterial disease of fishes that has been shown to be transmitted vertically by intraovum infection during egg maturation. Although *R. salmoninarum* has shown affinity for kidney tissues, other organs are also infected (the bacterium becomes systemic by the time clinical signs are shown). BKD is chronic and mortality occurs often in 6- to 12-month-old juvenile salmonids and young adults. A definitive characteristic of infection is the survival and multiplication of the bacteria inside phagocytic cells (macrophages, monocytes, and possibly polymorphonuclear leukocytes). The survival of the

pathogen limits the use of therapeutic measures, making it one of the most difficult bacterial fish diseases to control (Elliot *et al,* 1989).

The most recorded outbreaks of BKD have occurred in fish cultured facilities with loses as high as 80% in stocks of Pacific salmon and 40% in Atlantic salmon (Wiens, 2008). There are currently no efficient vaccines and antibiotics that have eliminated the pathogen in the Pacific salmon. The Atlantic salmon, however, has shown greater resistance and immunization with live *Arthrobacter davidaneili* (Salonius *et al,* 2005).

Arthrobacter

Arthrobacter have shown to consist of a considerable proportion of the bacteria isolated from soil (Brown *et al,* 1978). Environmental strains of *Arthrobacter* are commonly used since this group constitutes an important portion of indigenous microflora in soil. *Arthrobacter* are nutritionally diverse, having some isolates capable of degrading various pesticides. They produce amino acids which are used in industrial fermentations, perform bioconversion of steroidic compounds, and have a major role in cheese manufacturing (they produce flavoring agents and form the surface microflora of various cheeses). Bacteriophages are useful tools for studying *Arthrobacter* genetics because they can be used in the transfer of genetic information (Trautwetter, 1988). The ubiquity of *Arthrobacter* species in soil environments offers a vast number and diversity of phages capable of being isolated from this species of host bacteria (Cross et al, 2015).

Genome sequences of Renibacterium salmoninarum compared to Arthrobacter

R. salmoninarum is believed to be evolved from the *Arthrobacter* genus, via genome reduction. While *R. salmoninarum* is a fastidious pathogen of the salmonid fish, *Arthrobacter* constituents are soil habitants and are known for their resistance to environmental stress. The

similarities of *Arthrobacter* and *R. salmoninarum* serve as model for pathogen evolution because of the inability of *R.* salmoninarum to grow for extended periods outside the salmonid host, and *Arthrobacter* members inability to colonize any host species (Wiens *et al,* 2008).

Arthrobacter davidanieli has shown significant cross-protection and immunization in Atlantic salmon. Its ability to protect against BKD is considered to be a function of the genetic relationship between *Arthrobacter* and *R. salmoninarum.* The genome sequencing of *R. salmoninarum* has demonstrated the pathogen evolved via genomic reduction and horizontal gene acquisition from members of the *Arthrobacter* genus (Wiens *et al,* 2008). Phylogenetic analysis of common genes and whole-genome alignment identified *Arthrobacter* sp. as the closest known relative of *R. salmoninarum*. The sequences of TC1 and FB24 genomes belonging to *Arthrobacter* and the *R. salmoninarum* chromosome share 1,562 protein ORF clusters. The *R. salmoninarum* genome is 1.44 Mb smaller than the TC1 chromosome and 1.55 Mb smaller than the FB24 chromosome and has lower G+C content, suggesting that significant genome reduction occurred from the common ancestor. *A. davidanieli* is closely related to *R. salmoninarum* as they share more than 97% of 16 sRNA sequence homology (Salonius *et al,* 2005).

Materials and Methods

Preparation of Arthrobacter A culture of *Arthrobacter* sp. ATCC 21022 (obtained from the American Type Culture Collection) was used for this study. It was routinely cultured on LB agar or Luria broth. Cultures of *Arthrobacter* bacteria were made for experiments of phage infection. Single colonies were chosen and then streaked on LB agar plates. It took about 2-3 days to obtain *Arthrobacter* colonies incubated at 30º C. Broth cultures of *Arthrobacter* were made.

Collection of phages from soil Four soil samples were taken from different locations. The following measurements of soil were collected: 288g of soil from a sewer drain at Purchase College; 151g near a log and 127g near a tree from Van Cortlandt Park in the Bronx, NY; 284g from Blind Brook river in Purchase, NY. Phage buffer (68 mM NaCl, 10 mM $MgSO_4$, 10 mM Tris-Cl) adjusted to pH 7, added to the soil and mixed in a large flask. To reach the final desired concentration, 2.0 M $CaCl_2$ was added. Phage buffer and soil mix is centrifuged. Lennox Luria Bertaini (LB) powder was added and mixed to make a 2% solution consisting of 1g LB powder in 50 mL soil extract. The solution was passed through a 0.45 μm filter to obtain a sterile filtrate and stored overnight. Twenty mL portions of the filtered extract were aliquot in to different culture flasks. These methods were based on those used by Cross *et al.* (2015).

Phage isolation About 5 mL of bacteria culture was added to each of the flasks and shaken at 250 rpm for 24 hours in a shaking incubator at a temperature of 30° C. $CaCl_2$ was added to 4 flasks and were labeled accordingly. After the 24hr incubation period, the enrichment flasks were diluted in phage buffer. Culture tubes with 0.5 mL of late exponential/early stationary phase bacteria culture, different amounts of the enrichment culture and its dilutions. $CaCl_2$ was added to only to the culture tubes labeled "$CaCl_2$" to make the final concentration equal to the concentration in the original enrichment flask. 4.5 mL of Lennox Luria-Bertani Broth (LB) top agar (4.5 mL) was mixed in the culture tube and poured onto an LB agar plate. Top agar is LB agar with 0.8 % agar rather than the traditional agar concentration of 1.5%. The mixture was distributed evenly across the plate. The top agar solidified for approximately 15 minutes. Aliquots of 250 mLs of top agar were made and placed in a 60° C water bath (to keep in liquid state). Finally, plates were inverted and incubated at 30° C overnight.

Phage purification After the incubation of plates, plaque formation was checked. Desired phages were purified from clear isolated plaques using the streak plaque method. The bacteria and phage solution were plated with the top agar. Once the LB agar plate was streaked, 0.5 mLs of *Arthrobacter* and 4.5 mLs of LB top agar was applied and spread evenly across the plate. The plate was inverted and incubated overnight. Streak plaque technique was repeated to ensure a pure phage species was isolated. Once the desired isolated plaque was grown, it was picked with a micropipette tip and re-suspended in 100 μL PB. This solution was diluted to the 10^{-8} dilution by passing 20 μL of the sample in 180 μL of PB. Dilutions of 10 μL were added to 0.5 mL of *Arthrobacter* in a culture tube for 5-10 minutes at (24°C) and plated by the mixing of the infected bacteria with 4.5 mL of molten LB top agar with original $CaCl_2$ concentrations. The phage serial dilution needed to make a web pattern using the traditional plaque titer assay was determined. 5 mL of *Arthrobacter* culture was added to a sterile 50 mL culture flask. One hundred μL of the dilution which gave a web pattern to the *Arthrobacter* culture was added in the culture flask, allowing the phages to infect the bacterial cells for 5-10 minutes. It was then mixed with molten LB top agar containing initial $CaCl_2$ concentrations. 5mL of this mixture was plated onto 10 fresh LB plates then solidified and incubated at 30 °C overnight. 5 mL of PB was added to the web pattern plates and stored overnight at 4 °C. The phage lysate was filtered through a 0.22 μm syringe filter and stored at 4°C.

Results and Discussion

Attempts to isolate bacteriophage by plating soil samples near parks, sewage, and river, on soil isolate were made. We were unable to isolate phages on the plates from soil samples around the park, the sewage or the river. The plates with soil samples around the park or the

sewage containing $CaCl_2$ enrichment were also unsuccessful. However, we were able to isolate phages from the Blink Brook enriched with $CaCl_2$ concentrations. Enrichment methods consist of removing all soil bacteria from the soil extracted phage samples by filtration through a filter. The sterile filtered lysate containing phage particles are diluted in 2x LB broth with the desire bacteria used as the phage host. This enrichment procedure eliminates contaminating soil bacteria competing for growth with the host bacteria during the enrichment incubation time period. This allows the soil extracted phage to only infect the desired host bacteria and therefore maximizing the growth potential of the host bacteria and amplification of the host-specific phages. The river sample enriched with $CaCl_2$ concentrations yield over 100 plaques on the agar plate. Phage isolates show physiological differences on parameters like calcium ion concentrations and temperature (Cross *et al*, 2015).

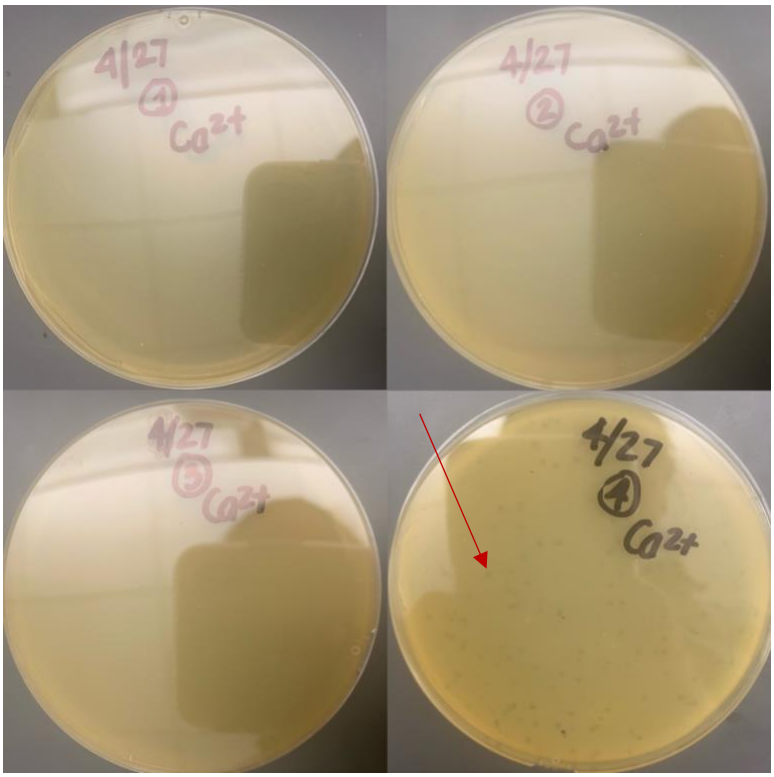

Figure 1. Agar plates containing mixture of soil samples diluted with phage buffer with $CaCl_2$ concentrations. Plates numbered 1 through 3 show *Arthrobacter* as it was in the top agar, as a result gave the agar a "yellow" color. Plate number 4 showed plaques (see arrow).

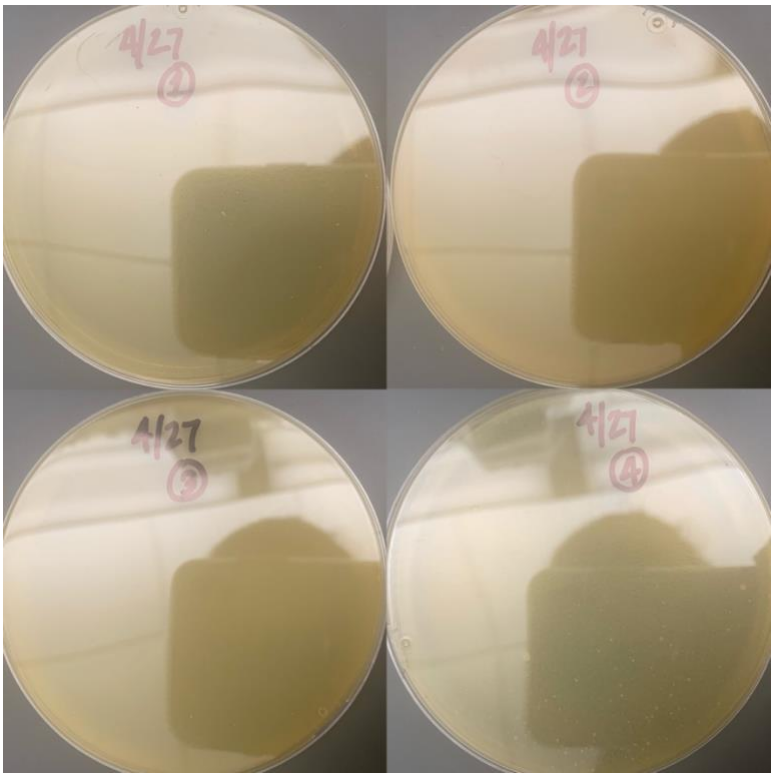

Figure 2. Agar plates containing mixture of soil sample extract diluted in phage buffer. Plates numbered 1 through 4 had no appearance of plaques, however, shows *Arthrobacter* as it was in the top agar.

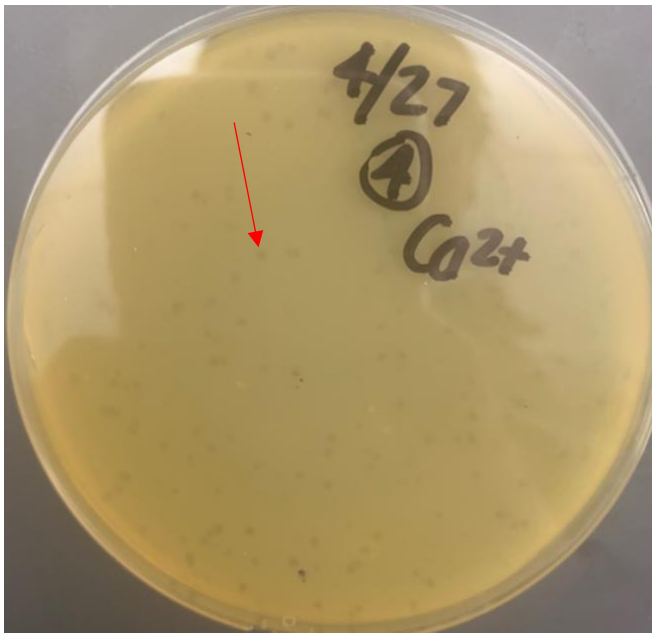

Figure 3. Agar plate containing mixture of soil extract diluted in phage buffer with $CaCl_2$ concentrations. Phage morphology in size and appearance is constant throughout the plate.

The phages formed plaques on the plate (see Figure 3) that were consistent in size and appearance. The similarities in plaque morphology correlated with similarities in host range and antigenic characteristics (Brown *et al*, 1978). The similarity in the plaque morphology of the phages also indicate one type of phage isolated from the river sample concentration. Plaque size ranged as small as 1-2mm. After a few days of incubation, phage size and appearance remained the same.

Since we were able to isolate phages from concentrated samples of soil near Blind Brook river, further attempts should be made to isolate *Arthrobacter* phage from the other soil samples. Collecting a larger soil mass may be useful in obtaining phages from the soil. For example, soil samples collected from different locations around the park only weighed 151g and 127g, as compared to the sewage drain and the river which weighed 288g and 284g. Furthermore, we

were unable to isolate phages from the soil extract diluted in phage buffer. None of the plates without $CaCl_2$ concentrations showed signs of bacterial growth or plaques. Isolation of phages using $CaCl_2$ concentrations has proven to be successful. We were unsuccessful in isolating phages during the winter, but other students have had great success isolating *Arthrobacter* phages from Purchase College during the summer. Perhaps there is a seasonality when there are more phages present in the soil (Daly, personal communication).

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