Fall 12-16-2023

Isolation and Characterization of Bacteriophages from Fish tank Samples

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Isolation and Characterization of Bacteriophages from Fish Tanks

By

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Presented to the Faculty of the Graduate School of

Stephen F. Austin State University

In Partial Fulfillment

Of the Requirements

For the Degree of

Master of Science

STEPHEN F. AUSTIN STATE UNIVERSITY

December 2023

Isolation and Characterization of Bacteriophages from Fish Tanks

By

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ABSTRACT

Bacteriophages (phages) are viral obligate intracellular parasites that infect bacteria. Research has been carried out on alternative treatment plans for bacterial infections due to the increase in antibiotic resistance. Phages have been proposed as an alternative to antibiotics. This research focused on the isolation and characterization of bacteria from small aquariums. A total of 42 bacterial isolates were isolated from seven different fish tank water samples and were tested to determine if they possessed phages. Spot plaque assay revealed presence of lytic phage for only one of the isolated bacterial strains, which was determined to belong to *Enterobacteriaceae* family based on the 16S ribosomal RNA sequencing. The phage isolated in spot plaque assay was successfully propagated in liquid bacterial culture. TEM showed that the isolated phage had a complex capsid and according to its morphology probably belongs to the family *Myoviridae.* Analysis of phage's genetic material indicated that it is a DNA phage with its highest DNA sequence similarity with previously described phages of bacteria from *Enterobacteriaceae*. Further analysis is required to determine if this phage belongs to a previously described phage species or not and if it has potential in the treatment of enterobacterial infections.

ACKNOWLEDGEMENTS

First, I want to thank the Department of Biology who facilitated the completion of and provided assistance and resources. I would like to thank my original thesis advisor Dr. Zeljko Radulovic who guided me throughout my entire project from my proposal to my thesis defense, as well as Dr. Dan Bennett who became my advisor at the end of the project. Throughout this project, Dr. Radulovic has been an integral part, from guiding me through my data collection to helping me prepare for my proposal and thesis defense. As such I would like to give a heartfelt thanks to him. I would also like to thank Dr. Josephine Taylor, who was there for me when I needed her throughout my project research. I would also like to thank Dr. Li Ma, whose suggestions on how to make my thesis better helped move this project forward.

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INTRODUCTION

Bacteriophages, also known as phages, are viral obligate intracellular parasites that infect bacteria. Like other viruses, phages exist outside the host cell as infectious particles known as virions. There is a huge diversity in the structure of phage virions, but all of them have at least two components: (1) capsid, a protein coat that encloses (2) nucleic acid, which is the genetic material of the virus. Virions attach to a bacterial host cell and inject their nucleic acid into the host cell cytoplasm. This is the first step in the phage replication process. Phages use the host cell machinery to replicate. In this process phages can kill the host cell (lytic cycle) or incorporate their genome into the host's genome and replicate along with the host's genome (lysogenic cycle). Like other viruses, phages are usually species-specific and only infect specific bacteria or specific strains of a bacterial species. Most of the known bacteria species have described phages which makes them a very diverse group of viruses. There, however, have been only 6,000 described phages so far with at least 100 additions every year (Ackermann, 2011). Phages are important because they can be used in a variety of different biotechnological processes, in research, and as therapeutics due to their predation on bacteria. Phages have been suggested as an alternative to antibiotics due to the increase in antibiotic resistance. The myriad of uses for phages shows a need for the discovery and characterization of new phages from different samples. Common samples used for phage isolation involve

human saliva, ocean water samples, sewage samples, etc. (Khan et al., 2002). Our attention, however, was focused on small aquariums due to the increase in antibiotic resistance of the bacteria found in them. Thus, we were interested in the isolation and characterization of phages present in small aquariums due to their potential use in the treatment of bacterial infections.

Objectives

The first part of this research was the isolation of phages from small aquarium water samples. The second part of this research was the characterization of the isolated phages.

LITERATURE REVIEW

Structure of Phage Virion

Phage virions have a huge diversity in their shapes, sizes, and nucleic acids. Phage virion shapes are determined by the type of capsids they possess. Most phages have an icosahedral capsid with a tail, which can be long and contractile or short noncontractile (Figure. 1). The icosahedral part of a capsid is made of protein subunits, known as capsomeres, which are assembled in such a way that they form 20 equilateral triangles connected in an icosahedron. This part of the capsid is known as the head, and it encloses nucleic acid. At one pole of the icosahedral part of a capsid there is a disc-like radially symmetrical protein structure known as the collar. This is the place where the tail sheath is attached to the icosahedral part of a capsid. In addition, many bacteriophages contain short protein filaments that project outwards from collar, known as whiskers. Both the collar and whiskers play a role in contraction of the tail sheath. The tail sheath is responsible for nucleic acid injection into a host cell. This is usually associated with tail sheath contraction due to the doubling of the subunits per annulus (the rings found in the tail sheath responsible for contraction). At the distal part of the tail sheath there is a base plate, a structure required for the adsorption process. The tail fibers are attached to the base plate, and they are specific to each phage and its corresponding bacteria.

When the tail fibers are in contact with the bacterium the base plate, which contains lysozyme, degrades a portion of the cell envelope of the host bacterium. Spikes play a role in the connection of the phage with the bacterial surface in the process of nucleic acid injection. The contraction of the sheath thus ejects the phage nucleic acid from the capsid through the tail sheath and into the host cell (Anderson, 2021).

Around 96% of phages have the capsid structure as described above and double stranded DNA as genetic material. However, there is a huge diversity in capsid structures and types of nucleic acids among the remaining 4% of described phages. Some phages have icosahedral capsids without tails. A small number of phages have filamentous, polyhedral, or pleomorphic capsids. Also, there is a growing group of identified phages that contain a lipid envelope that encloses the capsid (Mäntynen et al., 2019). Capsid size for the different phages has not been precisely determined. However, the diameter for known tailed phage capsid is in the range of 40 nm to 180nm (Lee et al., 2022).

The nucleic acid housed in the capsid can be either double stranded or single stranded DNA or RNA (dsDNA, ssDNA, dsRNA, or ssRNA). In addition, nucleic acids in phage capsids can be in linear or circular forms (Ackermann, 2011). The tailed phage genomes range from 5 to 735 kilobase pairs, and their overall genome size is dependent on the type of capsids that enclose them (Lee et al., 2022). In non-tailed phages, which are the less common phages, the nucleic acids present in the capsids are ssDNA, dsDNA, or ssRNA with small genome sizes (Dion et al., 2020).

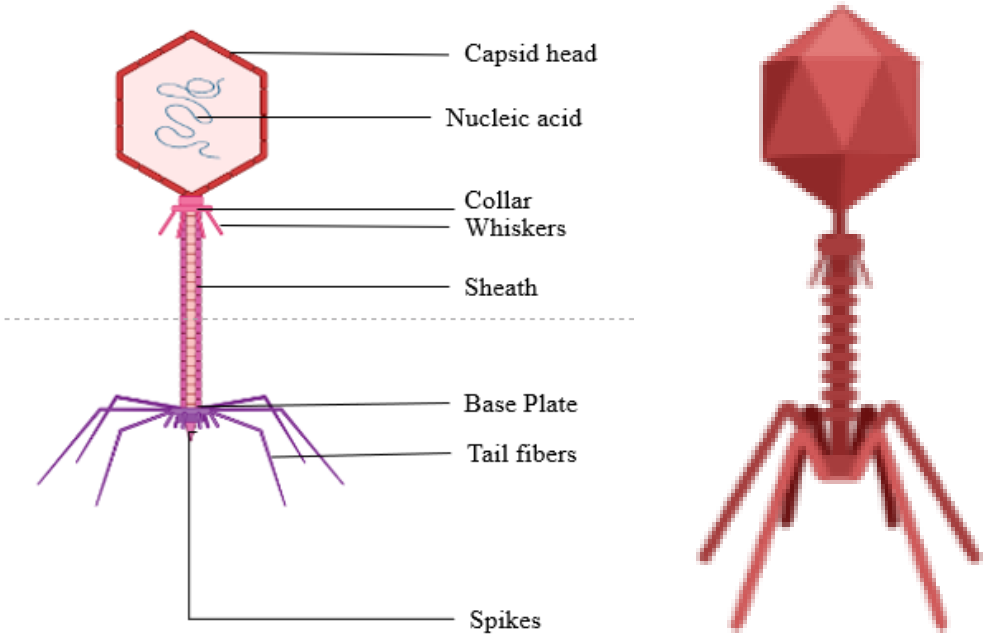

Figure 1: Structure of a tailed icosahedral bacteriophage (Sapkota, 2020).

Phage Taxonomy

According to the International Committee on Taxonomy of Viruses (ICTV) bacteriophages are classified into ten families with species definitions for phages still being discussed. Three families of phages namely *Myoviridae*, *Siphoviridae*, and *Podoviridae* make up over 96% of described phages and belong to the order Caudovirales, which includes phages with tailed icosahedral capsids and dsDNA (Ackermann, 2011). The remaining seven families, namely *Microviridae*, *Corticoviridae*, *Tectiviridae*, *Leviviridae*, *Cystoviridae*, *Inoviridae*, and *Plasmaviridae*, differ significantly in the type of nucleic acid they contain, as well as in the shape and size of

their capsids, and are classified into different orders of viruses (Ackermann, 2011). Virions of the various families are shown in Figure 2.

Family *Myoviridae* has over 1312 described genera of phages with an icosahedral capsid containing linear dsDNA and a contractile tail (Ackermann, 2011). The capsid has an approximate diameter of 50-110 nm and is made up of 152 capsomers enclosing genetic material. The contractile tail is 16-20 nm in diameter and 80-455 nm in length. Members of this family are virulent and infect enteric bacteria by injecting their nucleic acid into the bacteria host cell. The size of genomes is between 17.0-33.6 kb with at least 49 protein encoding genes. A well described member of this family is the Enterobacteria phage T4 (Lavigne and Ceyssens, 2011).

Family *Podoviridae* has over 771 described genera of phages with members of this family being non-enveloped, having linear dsDNA, and an icosahedral capsid with a T=7 symmetry (Lavigne and Kropinski, 2009). Virions of this family have a short non-contractile tail 20 nm long and 8 nm diameter. The icosahedral part of capsid enclosing genetic material is between 59-63 nm in diameter and made from 72 capsomers. Members of this family infect enterobacteria, but some phage genera replicate in gram-positive bacteria if their nucleic acid is artificially injected into a bacterial host cell. They can be either lytic or lysogenic in nature, with the lytic cycle being more common. The length of the genomes is between 40- 45 kb with about 55 protein encoding genes. A well described member of this family is the *Escherichia coli* T7 phage (Lavigne and Kropinski, 2009).

Family *Siphoviridae* has over 3262 genera of phages with members of this family being non-enveloped, having linear dsDNA, an icosahedral capsid with a T=7 symmetry, and a long non-contractile tail (Hendrix et al., 2011). The tail is between 65 to 570 nm in length, and 7 to 10nm in diameter. The capsid enclosing genetic material is about 60 nm in diameter and consists of 72 capsomers. Members of this family infect enterobacteria, via generalized transduction- this is the transduction in which any region of host genome of appropriate size could be packaged mistakenly into the phage. They can be either lytic or lysogenic in nature but are more likely to be lytic. The length of the genomes is between 4.85 to 121kb with 6 to 15 protein encoding genes. A well described member of this family is the Enterobacteria lambda (λ) phage (Hendrix et al., 2011).

Family *Microviridae* has over 38 genera of phages with members of this family being non-enveloped, having positive sense circular ssDNA molecules with a T=1 icosahedral symmetry capsid and 12 knoblike spikes on its vertices (Cherwa and Fane, 2011). Depending on the genus, virions are about 30 nm in diameter. Members of this family infect enterobacteria, intracellular parasitic bacteria, and mollicutes when introduced to them. These phages use rolling circle replication for their genome's replication. The size of genomes is between 4.4 kb and 6.1 kb with 12-13 protein encoding genes. A well described member of this family is the Enterobacteria phage ΦX174 (Cherwa and Fane, 2011).

Family *Corticoviridae* has only one described species of icosahedral internal membrane-containing virulent virus with circular dsDNA (Oksanen, 2017). Virions for

this family have a diameter of about 57 nm. Interestingly the phage Pseudoalteromonas virus PM2 is the only member of this family that has been recognized and is the only phage with a lipid membrane present on its virions. This virus infects *Pseudoaltermonas* bacteria via the presence of spikes on the surface of the phage. This phage uses rolling circle replication for its genome replication and which is initiated by P12, a virus-encoded protein. The size of the genome is about 10.1 kb with 21 protein encoding genes (Oksanen, 2017).

Family *Tectiviridae* has only one recognized genus, and thus the family description correlates to the genus' description (Oksanen and Bamford, 2009). The *Tectiviridae* viruses have their linear dsDNA genome with a protein-rich internal membrane enclosed in an icosahedral capsid. Virions for this family measure about 66 nm from each side with the capsid possessing flexible spikes 20 nm in length protruding from the virion vertices-the point at which each side of the capsid meet. Members of this family infect Enterobacteria via injection of genetic material into bacterial host cells. Interestingly replication is protein-primed and proceeds via strand displacement. The size of the genome is about 14.9 kb with 27 protein encoding genes. A well described member of this family is *Enterobacteria* phage PRD_1 (Oksanen and Bamford, 2009).

Family *Leviviridae* has over 38 genera of phages with members of this family being non-enveloped having a linear positive sense ssRNA as genetic material, and an icosahedral capsid with symmetry T=3 (Olsthoorn and, Van Duin 2011). The virions for this family are spherical in nature and have a diameter of about 26 nm. Members of this

family infect Enterobacteria species of *Caulobacter*, *Pseudoomonas*, *Acinetobacter*, and many more Gram-negative bacteria through their pili. It is interesting that these phages replicate via a virus-encoded replicase which uses host translation factors as cofactors. The size of genomes is between 3.5-3.6 kb with 4 protein encoding genes. A well described member of this family is *Enterobacteria* phage MS2 (Olsthoorn and, Van Duin 2011).

Family *Cystoviridae* has 1 described genus and 7 described species of enveloped phages with tri-segmented dsRNA (Poranen et al., 2017). Enveloped virions are spherical and about 85 nm in diameter with the envelope surrounding an isometric nucleocapsid, about 58 nm in diameter, and spikes protruding from the virion's surface. Members of this family infect Gram-negative bacteria, mostly *Pseudomonas* species, through their pili. The size of the genome is between 12.7-15.0 kb with the virions containing three segments of linear, dsDNA with each segment having protein encoding genes. A well described member of this family is *Pseudomonas* ϕ6 phage (Poranen et al., 2017).

Family *Inoviridae* belongs to the order Tubulavirales and includes more than 20 genera and more than 25 described species of flexible filamentous phages with circular positive-sense ssDNA (Knezevic et al., 2021). Depending on the genus, virions are 0.6-2.5 µm in length and 6-10 nm in diameter. Members of this family infect gram-negative bacteria through their pili. It is interesting that these phages use rolling circle replication for their genome replication. The size of genomes is between 5.5-10.6 kb with 7-15

protein encoding genes. A well described member of this family is *Escherichia coli* M13 phage (Knezevic et al., 2021).

Family *Plasmaviridae* has at least 5 genera of phages with slightly pleomorphic, enveloped virions which are 50 nm to 125 nm in diameter and contain circular, supercoiled dsDNA molecules (Krupovic, 2018). Members of this family infect *Acholeplasma* sp. upon entering a host's cell. Plasmaviruses are released from the infected cell via budding without causing cell lysis. The size of the genome is about 12 kb with 4 protein encoding genes. A well described member of this family is *Acholeplasma* virus L2 phage (Krupovic, 2018).

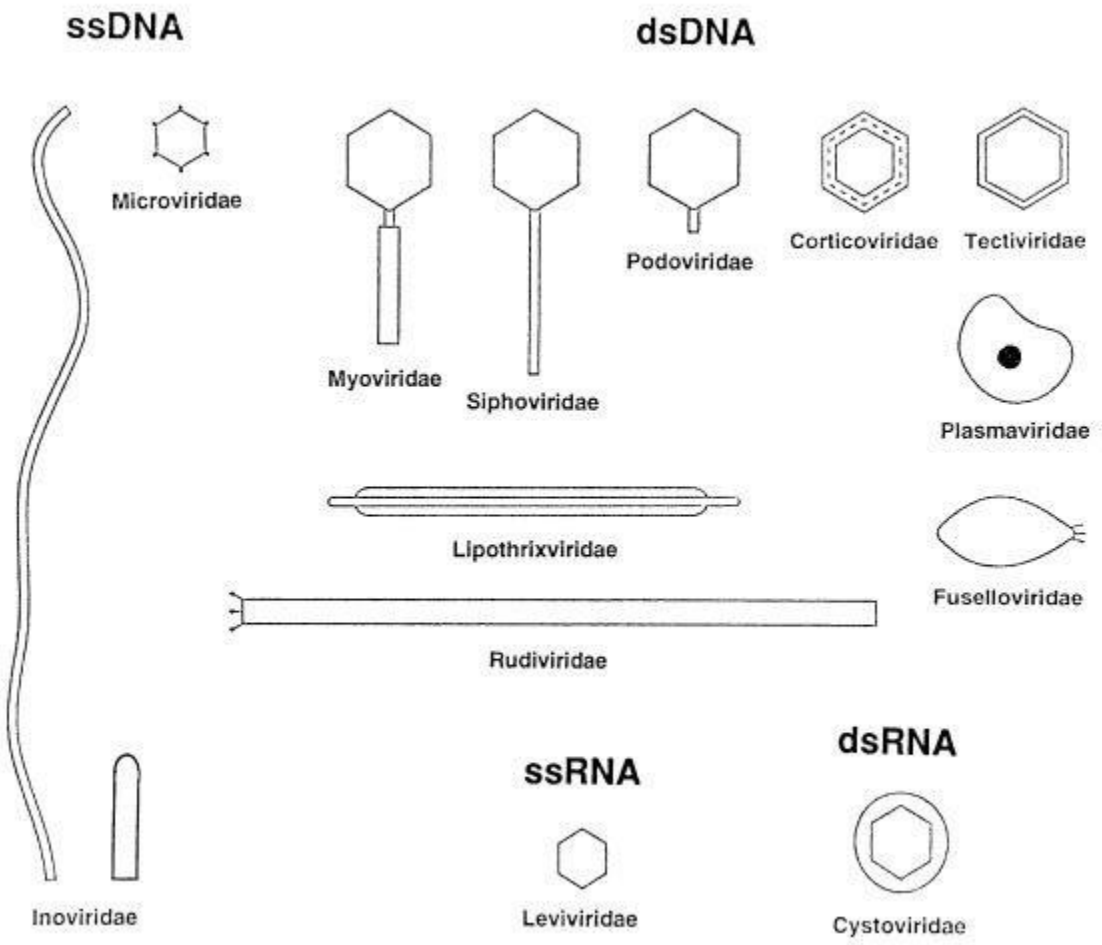

Figure 2: Virion structures of the various characterized phage families. (Ackermann, 2011).

Replication of Phages

Replication of phages, like other viruses, can be described as progressing through five stages, namely: attachment or adsorption, genome entry, synthesis of phage proteins and genomes, assembly or maturation, and release. Typical replication of a phage is shown in Figure 3.

In the attachment phase, phage virions collide with bacterial host cells. Using spike glycoproteins, present on the tail of phages with a complex head-tail capsid structure, the phage attaches to the cell surface by recognizing a specific receptor. Different phages recognize different receptor molecules on the surface of bacterial cells, which are commonly proteins, polysaccharides, lipopolysaccharides, or carbohydrate moieties (Bertozzi Silva et al., 2016). Phages that infect gram-negative bacteria usually bind to the surface lipopolysaccharides, outer membrane proteins, or proteins of pili and flagella (Bertozzi Silva et al., 2016). On the other hand, phages that infect gram-positive bacteria use peptidoglycan or other polysaccharide components of the cell as receptors (Bertozzi Silva et al., 2016). The absence of a particular cell surface receptor that is required for the attachment of a phage means that the bacterium is resistant to the phage.

After the phage attachment to the host cell, the nucleic acid is delivered into it. This process varies between different phages. DNA phages with a head-tail capsid structure inject their DNA into the host cell through the bacterial envelope. This process is well described for T4 phage, which uses the enzyme lysozyme at the tip of its tail to degrade a portion of the peptidoglycan cell wall (Anderson et al., 2021). Then the tail contracts and injects the nucleic acid into the host cell, while the capsid is left outside the cell. RNA phages with simple icosahedral capsids and envelopes use different strategies to penetrate the bacterial cell envelope and release their genetic material in the cytoplasm of the bacterial cell. In a multistep process they combine fusion of their envelope with the bacterial outer membrane, enzymatic activity related to degradation of the peptidoglycan

layer in the bacterial cell wall, and an endocytosis-like process followed by uncoating of viral RNA in the cytoplasm of the bacterial cell (Callanan et al., 2018).

With the presence of phage nucleic acid in the host cell, phage genes begin to be transcribed and translated, using machinery and resources of the host cell. Among the first phage proteins produced in infected bacterial cells are commonly nucleases that specifically degrade bacterial DNA and, in that way, prioritize use of phage genetic information and synthesis of viral proteins. Also, in this group are phage proteins needed for phage nucleic acid replication. There is a variety of strategies for nucleic acid replication used by different phages, including rolling circle replication used by most phages with circular DNA, as well as activity of RNA-dependent RNA polymerase and reverse transcriptase in replication of phages with dsRNA and ssRNA, respectively (Callanan et al., 2018). Some phages that do not destroy the host cell's DNA, but encode other proteins which are responsible for the modification of a subunit of the host cell's RNA polymerase that prevents the host genes from being expressed (Anderson et al., 2021). Late proteins are the proteins produced towards the end of infection and are structural proteins, responsible for the formation of the capsid and other protein components of a virion.

The production of copies of the phage genome and its other structural components leads to the assembly of these components into new phage virions. Usually, the icosahedral part of the capsid is assembled first, the nucleic acid is then packed into the phage head, and subsequently the tail with spikes is attached to the phage head. Some

phage virion components self-assemble, while the icosahedral part of capsid usually needs scaffolding proteins for assembly.

During the late phase of infection, the lysozyme is synthesized. This enzyme degrades the bacterial cell wall, causing lysis. The lysed cell thus releases previously assembled phage virions (Anderson et al., 2021). The *Cystoviridae* family, which is one of two phages that possesses a lipid envelope, also has a lytic lifecycle. *Pseudomonas* phage phi6 acquires a lipid-protein envelope around its nucleocapsid which is derived from the phospholipid of the host cytoplasmic membrane. During host cell lysis, the phi6 phage is released with its own lipid-protein envelope (Lyytinen et al., 2019).

Lytic and Lysogenic Cycle

The phage replication cycle that ends with lysis of a host bacterial cell is known as a lytic cycle. Some phages have an alternative replication strategy which does not cause lysis of the host bacterial cell and is known as the lysogenic cycle. The replication of phage nucleic acid in the lysogenic cycle occurs simultaneously with bacterial host cell nucleic acid. Phage attachment and genome entry for the lysogenic replication occurs as in the lytic cycle replication. These two processes differ in that the phage DNA is incorporated into the bacterial chromosomal DNA during a lysogenic cycle, so that when the host cell undergoes replication, the phage genome does too. In the lytic cycle the virions are virulent and cause the death of the host cell. In the lysogenic cycle the incorporated phage DNA, also known as the prophage, is non-virulent, and replicates together with host's cell chromosomal DNA to be inherited by new daughter cells

without causing host cell death (Sapkota, 2020). Phages that enter a lysogenic cycle and do not cause death of infected bacterial cell are also known as temperate phages. In times of high energy supply to the cell, the lysogenic cycle is the preferred mode of infection, but when the energy supply is low the lytic cycle is the common mode of infection (Taylor, 2014). The lysogenic cycle is shown in Figure 4.

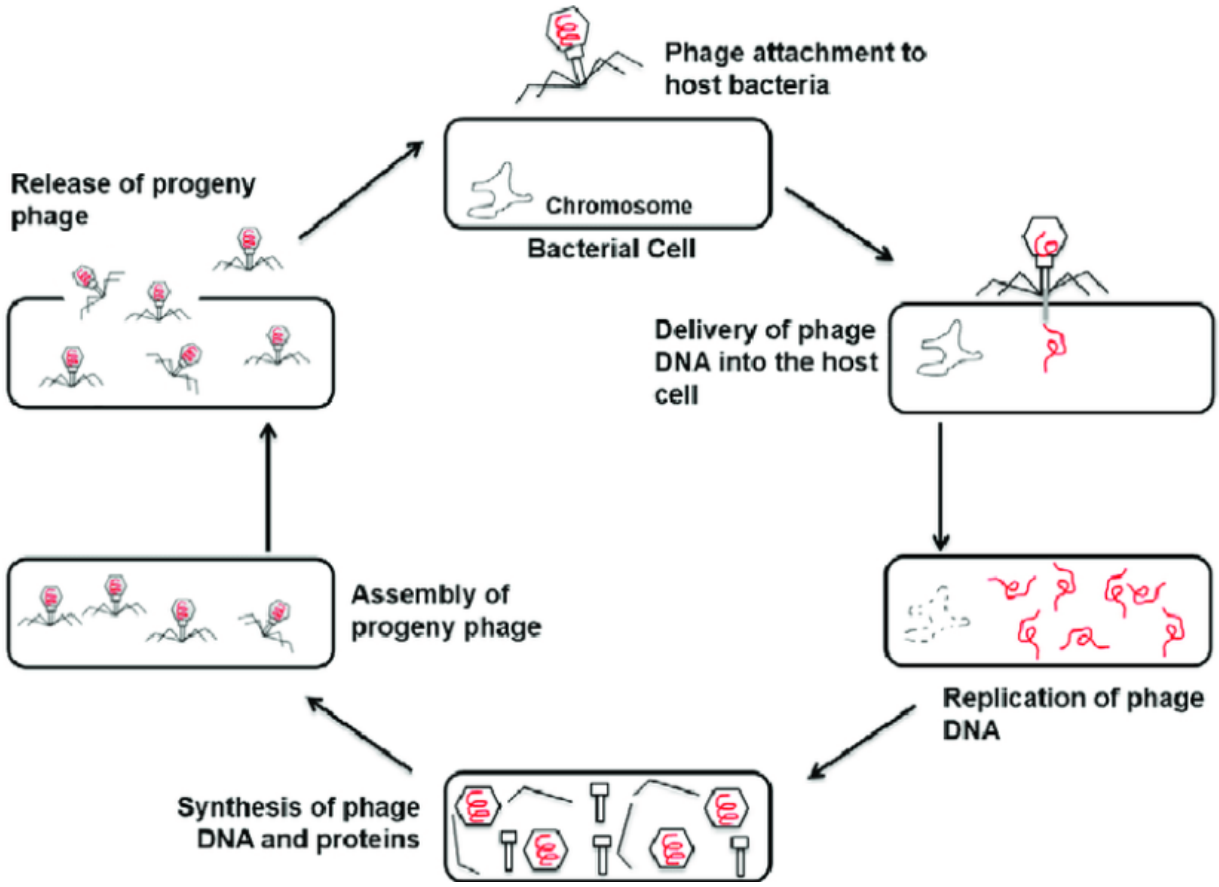

Figure 3**:** Replication of lytic bacteriophages (Anderson et al., 2021).

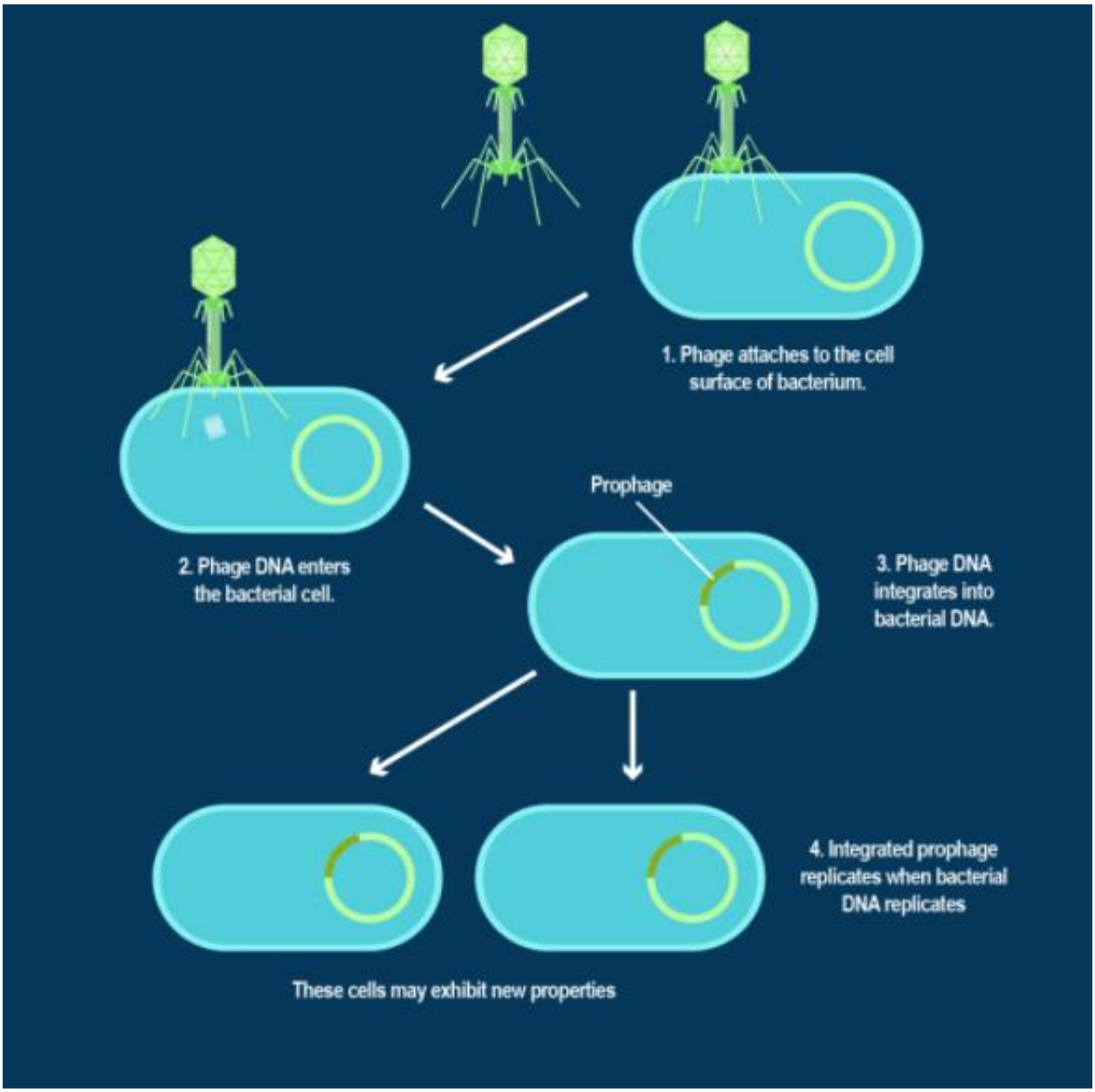

Figure 4: The lysogenic cycle of a phage (Google Image, 2020).

The prophage can stay in the host genome indefinitely due to the presence of a repressor protein that prevents the excision of the prophage. The repressor protein is

inactivated when the host cell undergoes either physical or chemical stress. This leads to the excision of the prophage from the host genome and allows the phage DNA to enter the lytic cycle in a process known as phage induction.

Lysogenic conversion is the process in which the bacteria host's cell phenotype changes due to expression of prophage genes. Phages can confer virulence factors to the bacteria they infect by undergoing lysogenic conversion. In the lysogenic cycle, the integration of the phage genome into the bacterial genome confers the genes necessary for virion production and additional genes called morons. The expression of morons improves the survivability of the bacterial host and the phage integrated into the bacterial genome. The conversion of nontoxic *Corynebacterium diphtheria* to its virulent strain or the transfer of toxin encoding genes from a toxic *Streptococcus* strain to non-toxic strains are examples of bacteria that gain their virulence from phages integrated into their genome. This allows phages to affect the evolution of a bacterial population. Up to 20% of the bacterial genome originates from phages (Schroven et al., 2020).

Importance of Phages

In the early 20th century before the introduction of antibiotics, phages were thought to be useful in the treatment of bacterial infections because they infect and kill bacteria (Haq et al., 2012). This strategy was discarded due to the discovery of antibiotics, which were highly effective and cost efficient. However, with the overuse of antibiotics in subsequent years and the increase in antibiotic resistance, phages are again being considered as potential antibacterial agents for antibiotic resistant strains (Haq et

al., 2012). The biotechnological uses of phages include their use in phage therapy, food safety, and bacteria control in aquaculture (Rodríguez-Rubio et al., 2016).

Using phages in the treatment of bacterial infections has a long history. This was first proposed by Fe'lix d'He'relle in 1917 who made use of phages to treat a child with disentheria (Orlova, 2012). Phage therapy was shelved due to antibiotic development. In recent years phage therapy has been revisited due to the increase in antibiotic resistance in bacteria. Phages have been used in the treatment of plants, animals, and humans with varying degrees of success (Orlova, 2012). The use of bacteriophages has been extensively studied over the past decade and used in the treatment of some gastrointestinal infections and human wounds in former Soviet Union countries and other parts of Eastern Europe (Richards, 2014). Phages are host specific and prevent damage to the surrounding microbiome of host. This is because bacterial cells have receptors specific for different phages, and thus phages are not able to infect other types of bacteria without the receptors. They are self-limiting i.e., they will not propagate in the absence of a host. This is because phages require a living organism to propagate, and in their absence are incapable of replicating. Phages evolve with the bacteria they infect when the bacteria develop resistance to them (Orlova, 2012). There are also some problems with using phages in the treatment of bacterial infections. Phages can spread to every organ in the body but dissipate very quickly when the immune system clears them from the body i.e., they cause an antibody response *in vivo.* Also, they are not always lytic but can also be lysogenic in nature under certain physiological conditions. As a result, phages can

transfer virulence to the bacteria they infect due to the lysogenic conversion process (Orlova, 2012).

Phages can be used in food safety protocols. New research into the inactivation of food bacteria, such as *Salmonella, Listeria,* and *Campylobacter,* has led to the development of technological means to combat these bacteria using phages (Rodríguez-Rubio et al., 2016). For a specific technology to be used against foodborne pathogens, it must be harmless to humans, animals, and the environment. Lytic proteins such as endolysins produced by phages have been approved for use in the USA as bio preservatives and as tools to detect specific pathogenic bacteria and lyse them (Rodríguez-Rubio et al., 2016). Lytic proteins produced by phages have been approved for use in livestock due to their narrow range of activity i.e., they are specific, and this allows the phage to target pathogenic bacteria and not affect the surrounding microbiota (Rodríguez-Rubio et al., 2016).

Phages can be used for bacterial control in aquaculture. Due to the ever-increasing demand for fish and shellfish that cannot be met via natural harvesting, extensive aquaculture production has blossomed (Richards, 2014). The extensive culturing of marine and freshwater organisms, however, has been met with challenges, namely from the myriad of bacterial pathogens that infect them. These pathogens can spread through feed, water, aerosols, contaminated surfaces, and from animal to animal. These pathogens are opportunistic in nature and are often undetectable until the fish or shrimp are stressed. These pathogens were treated extensively with antibiotics, but with

the overuse of antibiotics, antibiotic resistant strains of these pathogens started to proliferate (Richards, 2014). Hence, bacteriophages are a viable alternative for treating antibiotic-resistant bacteria strains in aquacultures. Recently Phage Biotech Ltd. has been developing a phage treatment for the pathogen *Vibrio harveyi* in shrimp. Phages specific to the pathogens found in fish and shrimp have been isolated, with their potential use in treating the bacteria being studied (Gon Choudhury et al., 2017). Some of the bacterial pathogens in fish and shrimp that phages have been isolated from, include *Aeromonas hydrophila, Vibrio harveyi, Lactococcus garvieae, Streptococcus iniae, Pseudomonas plecoglossicida*, *Edwardsiella tarda* etc. (Gon Choudhury et al., 2017). The first reported case of the use of bacteriophage for the treatment of *A. hydrophila* and *E. tarda* was made by Wu and Chao (Wu, 1982). Carrias et al. (2011), isolated a phage belonging to the family *Siphoviridae* from the bacterium *Edwardsiella ictaluri* causal pathogen of enteric septicemia, from a catfish aquaculture pond (Oliveira et al., 2012). A total of three different phages, namely ϕeiAU, ϕeiDWF (which were isolated in a study by Timothy Welsh), and ϕMSLS (which was isolated from an aquaculture pond water sample) have been isolated *E. ictaluri* (Carrias et al., 2011). These phages were isolated in different parts of the world, but they share some genomic characteristics. These phages encode two enzymes namely an endolysin which degrades the bacteria peptidoglycan cell wall, and a holin enzyme which makes the cell membrane permeable (Carrias et al., 2011). Subsequent genomic analysis of the interaction between the phage and the bacterium in a

controlled setting revealed the phage to be a virulent phage lacking the ability for lysogeny (Oliveira et al., 2012).

Fish which are kept as pets are placed in a fish tank which can be a source of pathogenic bacterial infections (Vanderzwalmen et al., 2022). These bacteria can be introduced into the fish tank through tap water or items added to the tank. These bacteria include *Pseudomonas* spp., *Aeromonas* spp., and some gram-negative bacteria that can cause systemic aquatic infections (Vanderzwalmen et al., 2022). Bacterial infections are treated using antibiotics, but due to the increase in antibiotic resistant bacteria in aquaculture, an alternative to antibiotics, control with phages, has been proposed. The goal of this research was to isolate and characterize bacteriophages found in fish tank samples. This was done to determine if the bacteria isolated from fish tanks have a coexisting relationship with the bacteriophages.

METHODS

Sample Collection and Processing

Bacteria Isolation

A 50 ml water sample was collected from each of the seven fish tanks housing tropical fishes and appropriate decoration with the water temperature was maintained at approximately 27℃. Four samples were donated from households, two were collected from fish tanks in the Department of Biology at Stephen F. Austin State University, and one was collected from Petsense in Nacogdoches, TX, where a single water source supplied multiple tanks. A portion of the fish tank water samples was used for isolation of bacteria. Luria-Bertani (LB) and Nutrient (N) media were used as broth and agar for isolation of bacteria. Between 25-100 µl of the water samples was inoculated and spread on LB and N agar plates using L-shaped spreaders. These plates were incubated at 27℃ and allowed to grow overnight. Cultures were visually inspected to identify different colony types in each sample. At least two individual colonies per each identified colony type per sample were isolated by inoculation and propagated in their respective liquid media (LB broth or N broth). After incubation at 27℃ overnight with shaking at 200 rpm, bacterial cultures were used for glycerol stock preparation by mixing 500 µl of the bacterial culture and 500 µl of 30% glycerol. Glycerol stocks were immediately stored at -80℃.

Filtration

The remaining fish tank water samples were used for the isolation of phages. The water samples were aseptically filtered using 0.22 μm filters aseptically. The filtrate was stored at 4℃ until further use as the source of phages.

Phage Isolation

A spot assay was used for phage isolation from fish tank water samples. Fresh liquid cultures of isolated bacteria were prepared. First, from glycerol stocks bacteria were streaked on the appropriate agar plate and incubated at 27℃ overnight. The next day an individual colony from the plate was inoculated in 10 ml of liquid media and incubated overnight at 27℃ with shaking at 200 rpm. Then 100 μl of overnight bacterial culture was added to 3 ml of pre-melted 0.6% soft agar (LB or N) cooled to 50℃ and mixed by gently vortexing the tube. The mix was poured and evenly spread on a prewarmed and dried agar plate (LB or N agar plates) and allowed to solidify. Between 200-300 μl of filtrate was pipetted onto the individual plates and incubated overnight at 27℃. Plates were checked for the presence of plaques, which were signs of phage presence.

Propagation and Storage of Isolated Phages

Plates with plaques were sealed with paraffin and stored at 4℃ to be a primary source of isolated phages. Propagation of isolated phages was performed in liquid bacterial culture. A fresh bacterial culture was prepared as previously described. A piece of soft agar from the plaque was introduced into 10 mL of bacterial culture grown in a 20

ml conical tube and incubated 6-8 hours at 27℃ with shaking at 200 rpm. Simultaneously, a control tube with a bacterial culture to which the phage was not introduced was incubated. A spectrophotometer was used to measure absorbance at 600 nm. Values for cultures with phages were compared to the controls to determine if phages replicated and caused bacterial lysis. Lysates were stored at 4℃ to be used as a secondary source of isolated phages in procedures for their characterization.

Phage Burst Size Titer Assay

A phage burst size titer assay was done to determine the number of phage particles released during the process of lysis. A fresh bacteria culture was grown in 20 ml of LB medium until OD600 reached 0.4-0.5. Ten ml of the bacterial culture was aliquoted into 15 ml tubes, and centrifuged at 4,000xg for 10 mins at 4°C. The supernatant was decanted, with the pellet resuspended in 1 mL SM buffer. Then 0.9 ml of the bacterial culture suspension was aliquoted into a microcentrifuge tube with 0.1 mL of phage lysate to make sure the number of phages was 10 or more times more than the number of bacterial cells. The microcentrifuge tubes were incubated at 27°C for 5 mins, and later centrifuged at 16,000xg for 2 mins with the supernatant decanted. The pellet was resuspended in 1 mL SM buffer and centrifugated again at 16, 000xg for 2 mins with the supernatant being decanted. The pellet was resuspended in 10 mL of LB medium and incubated at 27°C with shaking at 200 rpm. Samples were collected with and without chloroform at 10-min intervals for 80 mins and stored at 4°C. Chloroform was used to prevent bacterial growth. For the samples without chloroform, 500 µl of the resuspended

sample in 10 ml LB medium was aliquoted at each time interval. For the samples with chloroform, 450 µl of the resuspended sample in 10 ml LB medium was aliquoted at each time interval with 50 µl chloroform and vortexed thoroughly. The microcentrifuge tubes were centrifuged for 5 mins at 5000xg, and 300 µl of the supernatant was aliquoted into a sterile tube.

Determination of Phage Titers in Lysates

Phage tittering was performed to determine the number of phages present in a lysate. A 10x serial dilution of lysate was prepared. Nine sterile microcentrifuge tubes were labelled with the dilution factor required, i.e., 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{c} (control). A total of 900 µl of appropriate liquid media was aseptically aliquoted into each tube, and 100 µl of the lysate was added to the 10^{-1} tube and vortexed briefly to mix. Then, 100 µl of the mixture was taken from the 10^{-1} tube and added to the 10^{-2} tube and vortexed, before 100 µl of this mixture was taken to be introduced in the 10^{-3} tube. This process is then repeated for preparation of each of the listed dilutions. The 10^{c} was the control tube and contained only liquid media. This tube served as the negative control to ensure that the liquid media was not contaminated.

Using a labelled sterile microcentrifuge tube, 100 µl of fresh pure bacterial culture was aliquoted and mixed with 100 µl of diluted phage. The mixture was allowed to sit for 5-10 minutes, aspirated using a new pipette tip, and added to 3 ml of melted appropriate 0.6% soft agar cooled to 50℃. After brief vortexing the mixture was poured on LB agar plates that were prewarmed and dried, and evenly spread before left on a horizontal

surface until the soft agar solidified. Plates were inverted and incubated at 27℃ overnight. The plates were checked the next day and dilution with 100-200 separate plaques was counted and used as the number of plaque forming units (PFU).

Phage titer was calculated as follows:

Phage titer (PFU/ ml) = plaque count / (dilution factor x aliquot volume (ml))

Phage Characterization

Determination of Phage Genetic Material

This process was done to extract and determine the type of nucleic acid in isolated phages. Cell lysate was used as the starting material for extraction of phage nucleic acid. Lysate was filtered through 0.22 μm filters to remove cell debris. Residual bacterial DNA and RNA was removed by incubating the lysate with DNase I and RNase A (Qiagen). A total of 450 μl of filtered lysate was mixed with 50 μl of 10x DNase I buffer, 1 μl DNase I (10 mg/ml), and 1 μl RNase A (100 mg/ml) for 90 minutes at 37℃ without shaking. To deactivate DNase I and RNase A, 20 μl of 0.5 M EDTA was added to the mixture. Then, phage protein capsid was digested by the addition of 1.25 μl of proteinase K (20 mg/ ml) and incubation at 56℃ for 1 hr 30 min without shaking.

Phage nucleic acid was purified using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocol. Briefly, a total of 200 μl of lysate treated with DNase I, RNase A, and proteinase K as previously described was added to 200 μl of AL buffer and vortexed thoroughly. The mixture was incubated for 10 min at 70℃, and then 200 μl of absolute EtOH was added and mixed by vortexing. The mixture was transferred to a 2

ml DNeasy Mini spin column positioned on a collection tube provided by the kit and centrifuged for 1 min at 6000xg. The DNeasy Mini spin column was placed in a new 2 ml collection tube and its flowthrough was discarded. Two washing steps were performed by adding 500 µl of buffer AW2 to the spin column and centrifugation for 1 min at 6,000xg for the first step, and for 3 mins at 20,000xg for the second washing step. The flow-through was discarded with the collection tube and the DNeasy Mini spin column was placed in a new collection tube and centrifuged for an extra 1 min at 20,000xg to prevent carryover of ethanol. At the end, the DNeasy Mini spin column was placed in a sterile 1.5 ml microcentrifuge tube and 30 µl of AE Buffer was aliquoted directly onto the DNeasy membrane. The tube was incubated at room temperature for 1 min and centrifuged for 1 min at 6,000xg to elute phage nucleic acid. The sample was stored at -80°C until it was needed for further analysis.

To determine if isolated genetic material of phages was DNA or RNA, 10 µl of isolated phage nucleic acid was aliquoted into 3 microcentrifuge tubes and labeled as control, DNase I treated, and RNase treated. The control sample had only the purified phage nucleic acid, while the DNase treated sample tube had purified phage nucleic acid treated with 2µl DNAse buffer, and 1µl DNAse I, and the RNase treated sample tube had purified phage nucleic acid treated with 1µl RNase. The samples were incubated without shaking for 1.5hr at 27°C. The samples were then run on a 1% agarose gel at 100V for 30 minutes with the first well holding a 5µl DNA ladder placed in lane M. Products of digestions, as well as the control sample, were resolved by 1% agarose gel

electrophoresis and visualized under UV light. Comparison of products of reactions with DNase I and RNase A resolved on the agarose gel with the control sample showed which enzyme digested extracted nucleic acid and revealed if isolated phage genetic material was DNA or RNA.

Morphological Characterization of Isolated Phages

A transmission electron microscope (TEM) was used to view the structural components of the phage and estimate the size of virions. To characterize the different phage samples and their structure a negative stain of the viral particles was performed. A 5 μl droplet of the lysate with phages was placed on an EM support film overlying a 300-mesh copper grid and allowed to dry. Then a 5 μl droplet of 2% uranyl acetate was placed on the grid for 5 mins, after which the excess was wicked away. Sanples were examined with a Tecnai 12 transmission electron microscope operating at 80 keV. Five-megapixel images were captured using an AMT NanoSprint 500sCMOS sidemount CCD camera and measurements were performed using the camera software. The negative stain filled in depressions around the edges of the virions so that their outline and form were revealed.

Direct PCR Ribotyping of Bacteria

Bacterial species infected by isolated phages were identified through bacterial ribotyping. A portion of the bacterial 16S rRNA gene was amplified through colony PCR using 2xGoTaq Green Master Mix (Promega) and standard 16S rRNA primers such as the universal primers 905F (5’-TGAAACTYAAAGGAATTG-3′), and 1492R (5′-

GGTTACCTTGTTACGACTT-3) (Wang et al., 2014). The PCR program had a template as shown in Table 1. The amplified DNA sequence was run on a 1.5% agarose gel and the band of the appropriate size was excised. DNA from gel pieces was extracted using Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions. The excised gel was placed in a 1.5ml microcentrifuge tube for the isolation of the nucleic acid fragment. The weight of an empty microcentrifuge tube and the weight of the tube with the excised gel were measured. The weight of the empty tube was subtracted from the tube with the excised gel. Membrane binding solution was added to the tube with the excised gel at a ratio of 10µl of binding solution to 10mg of agarose gel slice. The tube was vortexed and incubated for 10 mins or until the gel fully dissolved. The extracted nucleic acid was purified. The dissolved gel slice was transferred into one SV Minicolumn tube placed in a collection tube. The SV Minicolumn assembly is centrifuged at 16,000x g for 1 min in a microcentrifuge, and the flow through was discarded from the collection tube. The column was washed using 700µl of Membrane Washing Solution which contained 95% ethanol. The SV Minicolumn assembly was centrifuged at 16,000x g for 1 min, and the flow through was discarded. The wash was repeated with 500µl of Membrane Washing Solution, the SV Minicolumn assembly was centrifuged at 16,000x g for 1 min, and the flow through was discarded. The SV Minicolumn was allowed to airdry to remove residual ethanol, and after drying was transferred to a new collection tube. Then 50µl of Nuclease-Free Water was added to the Minicolumn assembly at the center of the column membrane without touching it and

incubated for 1 min. The Minicolumn assembly was centrifuged at 16,000x g for 1 min, and the SV Minicolumn was discarded. The purity of the purified PCR product was tested using a spectrophotometer. The purified PCR products were submitted for Sanger sequencing using Eurofins Genomics sequencing services. Sequences were submitted for nucleotide BLAST analysis against the NCBI GenBank database and bacteria species were identified based on the best similarity match.

Table 1. PCR conditions for direct ribotyping of bacteria

Stage	Temperature	Duration	Number of Cycles
Initial denaturation	94°C	5 minutes	1x
Denaturation	94°C	50 seconds	25x
Annealing	53°C	50 seconds	
Extension	72°C	50 seconds	
Final extension	72°C	7 minutes	1x

Molecular Characterization of Isolated Phage

To genetically characterize isolated phages, a portion of the phage nucleic acid was amplified using PCR. The reaction consisted of a total of 50µL of GoTaq mix, working primer solution, phage DNA, and nuclease free water were placed in five PCR tubes and run in a thermocycler. The PCR program had a template as shown in Table 2. The samples were then run on a 1% agarose gel at 100V for 30 minutes with the first well holding a 5µl DNA ladder placed in lane M. The PCR product was extracted from the gel

and purified using the Gel purification protocol mentioned above. The purified nucleic acid was sent for Sanger sequencing using primers P1, P2, and OPL5. Obtained sequences were deposited in the NCBI GenBank.

Table 2. PCR conditions for amplification of phage nucleic acid fragments.

Stage	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	5 minutes	1x
Denaturation	95°C	30 seconds	35x
Annealing	45°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	7 minutes	1x

RESULTS

Isolation of Bacterial Host Strains and Bacteriophages

Among the seven fish tanks sampled, 42 morphologically distinct bacterial colonies were identified on LB and nutrient agar plates after inoculation of collected water samples (Table 2, Figure 5).

Table 3. Number of morphologically distinct bacterial colonies identified from collected fish tank water samples grown on LB and nutrient agar plates.

Sample ID	Number of colonies on LB agar plates	Number of colonies on nutrient agar plates	Total number
Samples collected from households			
ZR-1	2	2	4
ZR-2	4	4	8
ATCW	2	2	4
Samples collected from fish tanks at SFA Department of Biology			
BIO Office	5	5	10
BIO Lab	5	6	11
Sample collected from pet store			
PET	3	2	5
		Total number of colonies	42

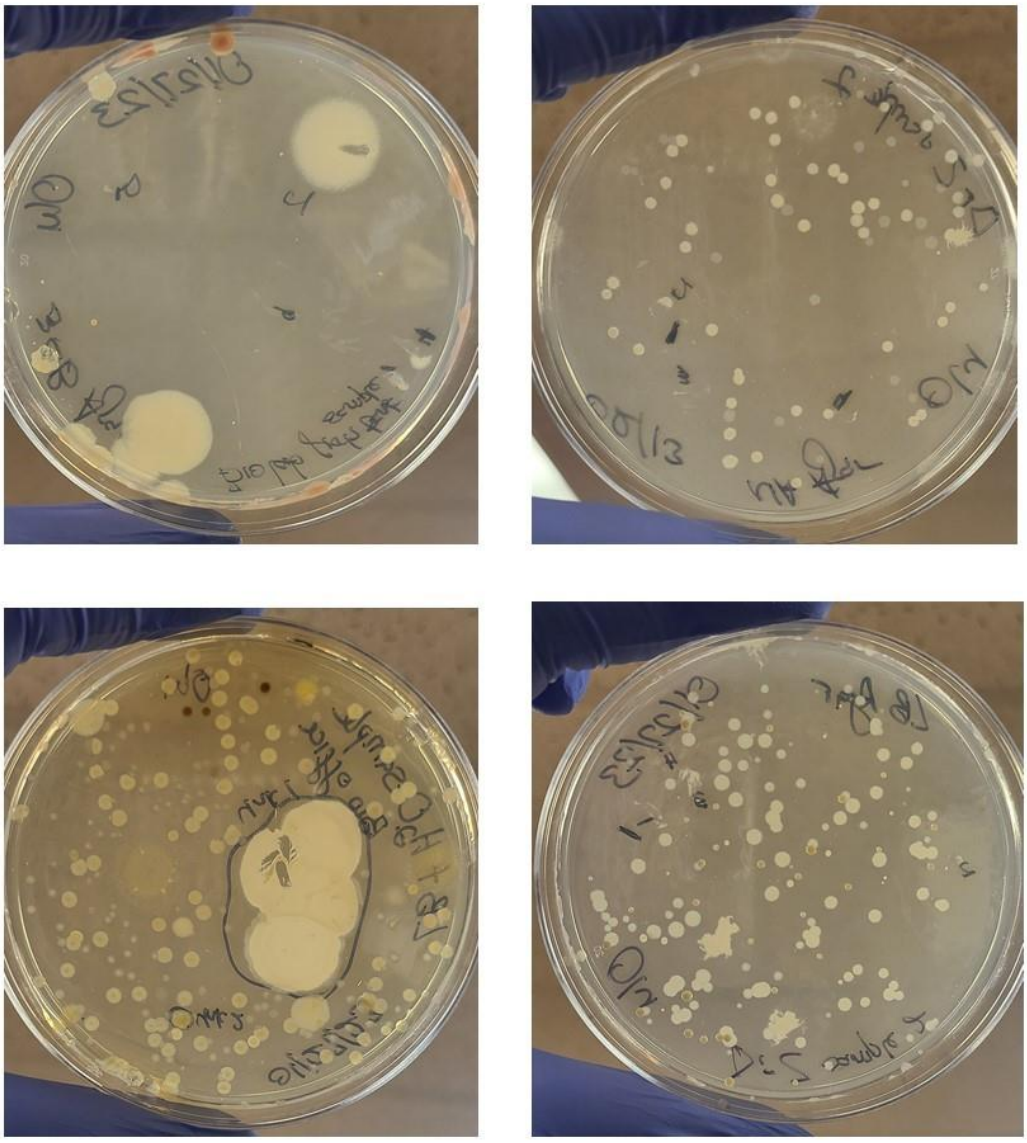

Figure 5. Agar plates with bacterial colonies grown from collected aquarium samples.

Each of the 42 bacterial isolates was used in a spot plaque assay to isolate lytic phages. After incubation, plaques were identified in only one LB agar plate with bacterial isolate ATCW-2 (Figure 6), revealing the presence of lytic phages in this sample.

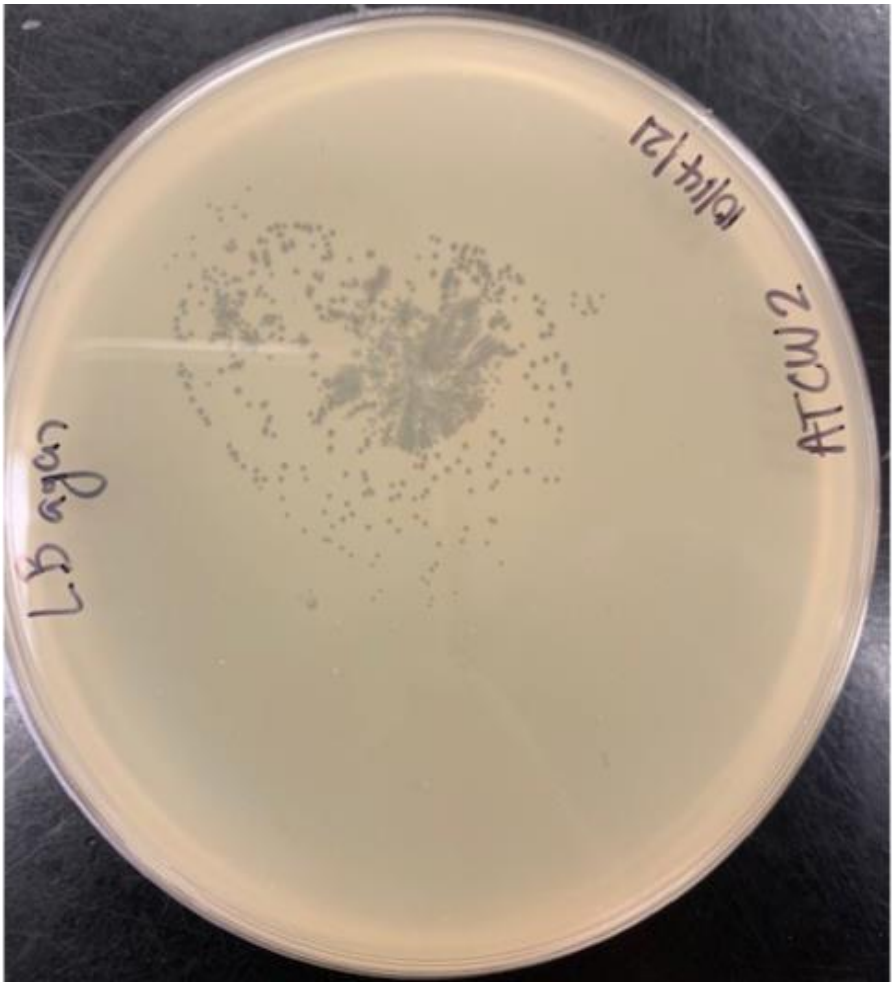

Figure 6. LB agar plate after spot plaque assay with plaques.

Propagation and Storage of Isolated Phages

As seen in Figure 7, the control tubes were cloudy indicating bacterial growth over time, while the plaque inoculated tube were clearer. This difference was confirmed

with spectrophotometer as shown in Table 3. The optical density of the control tube increased over time, while that of the plaque inoculated tubes did not.

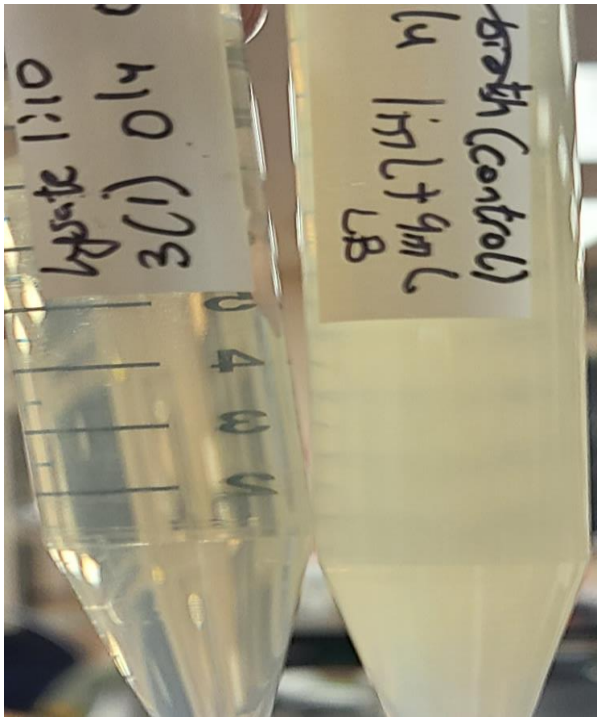

Figure 7. Control bacteria (right) and bacteria inoculated with plaque (left) to form lysates after 8hours.

Table 4. Optical density (OD) 600nm of control bacteria culture and bacteria inoculated with plaque at two-hour intervals.

Time (hours)	Control (OD)	Plaque Isolate and Bacteria (1) Optical Density	Plaque Isolate and Bacteria (2) Optical Density	Plaque Isolate and Bacteria (3) Optical Density
0 hours	0.0335	0.0593	0.0342	0.0335
2 hours	0.2006	0.1877	0.1735	0.1530
4 hours	0.6047	0.1662	0.1777	0.1328
6 hours	0.8656	0.1306	0.1306	0.1017
8 hours	0.8936	0.0901	0.1008	0.0933

Table 5. Optical density (OD) 600nm of control bacteria culture and the average of bacteria inoculated with plaque at two-hour intervals.

Time (hours)	Control (OD)	Plaque Isolate and Bacteria Average
0 hours	0.0335	0.0423
2 hours	0.2006	0.1714
4 hours	0.6047	0.1589
6 hours	0.8656	0.1210
8 hours	0.8936	0.0947

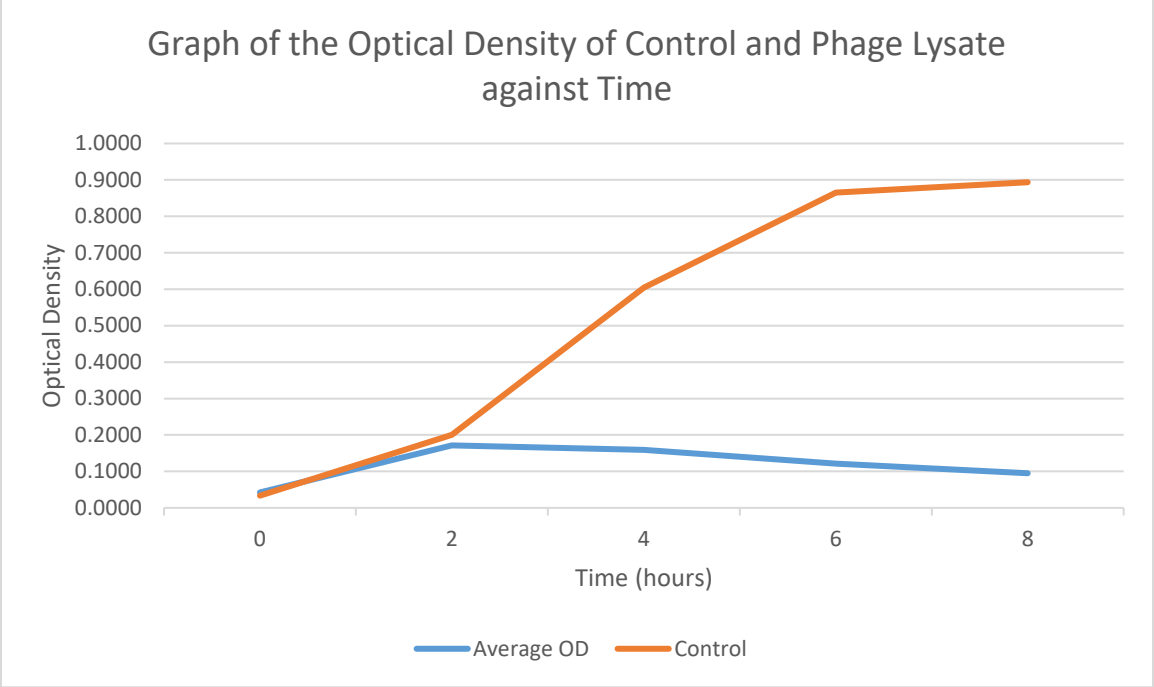

Figure 8. Optical Density of the control bacteria culture and the average of bacteria inoculated with plaque at two-hour intervals.

Burst Titer Assay

The phage titers inside and outside the ACTW-2 bacteria were calculated to be 44000, and 7000 respectively and the total phage count is 51000 after 10 mins and repeated for 80 mins at 10 mins intervals as shown in Table 5. Figure 10 shows the relationship between phage titers inside and outside the ACTW-2 bacteria.

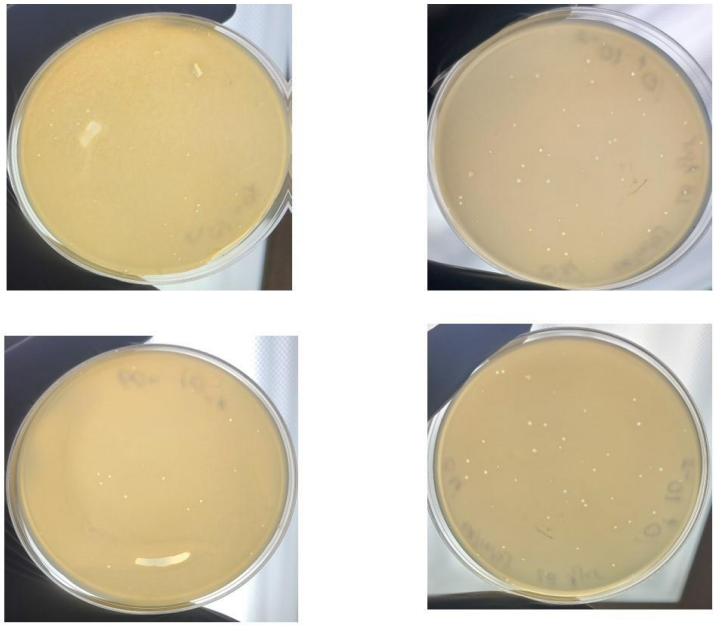

Figure 9. LB-top agar plate after serial dilutions of phages found inside and outside the bacteria cell.

Table 6. Number of phages counted inside and outside of the isolated bacteria host cell, and the total at time points 10 minutes to 80 minutes.

Minutes	Phage count inside bacteria cells	Phage count outside bacteria cells	Dilutions	Phage Titer (inside bacterial cells)	Phage Titer (outside bacterial cells)	Total
10 mins	44	7	10^{-2}	4.4×10^{4}	7×10^{3}	5.1×10^{4}
20 mins	34	33	10^{-2}, 10^{-3}	3.4×10^{4}	3.3×10^{5}	3.64×10^{5}

Table 6 Continued

30 mins	21	24	10^{-4}	2.1×10^{6}	2.4×10^{6}	4.5×10^{6}
40 mins	41	15	10^{-2}, 10^{-3}	4.1×10^{4}	1.5×10^{5}	1.91×10^{5}
50 mins	30	73	10^{-4}	3.0×10^{6}	7.3×10^{6}	1.03×10^{7}
60 mins	50	18	10^{-4}	5.0×10^{6}	1.8×10^{6}	6.8×10^{6}
70 mins	9	13	10^{-6}	9×10^{7}	1.3×10^{8}	2.2×10^{8}
80 mins	22	78	10^{-6}	2.2×10^{8}	7.8×10^{8}	1×10^{9}

Table 7. Log_{10} of the number of phages counted inside and outside of the isolated bacteria host cell, and the total at time points 10 minutes to 80 minutes.

Time (mins)	Phage count inside the bacteria cell (log_{10})	Phage count outside the bacteria cell (log_{10})	Total phage count (log_{10})
10 mins	4.64	3.85	4.71
20 mins	4.53	5.52	5.56
30 mins	6.32	6.38	6.65
40 mins	4.61	5.18	5.28
50 mins	6.48	6.86	7.01
60 mins	6.70	6.26	6.83
70 mins	7.95	8.11	8.34
80 mins	8.34	8.89	9.00

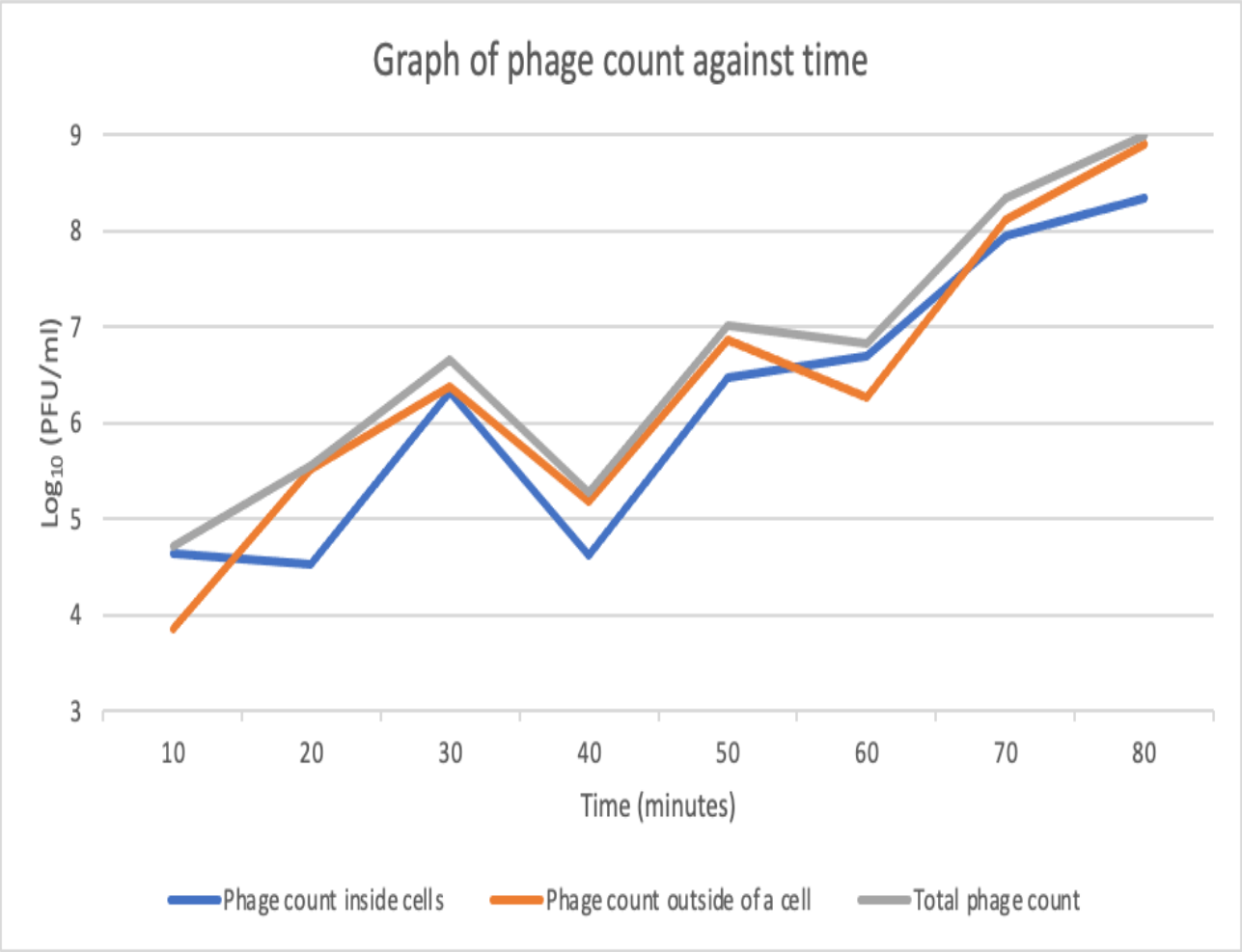

Figure 10. Number of phages found inside and outside the bacteria host cells and the total present. The phage count is plotted against time in 10 minutes interval for 80 minutes.

Morphology of Isolated Phage

Transmission electron microscopy revealed that the isolated phage had a complex capsid as seen in (Figure 11) composed of an icosahedral capsid and a helical tail. The capsid had a diameter of approximately 200 nm, and an approximate length of 260 nm. The helical tail had a diameter of approximately 60 nm, and an approximate length of 240 nm.

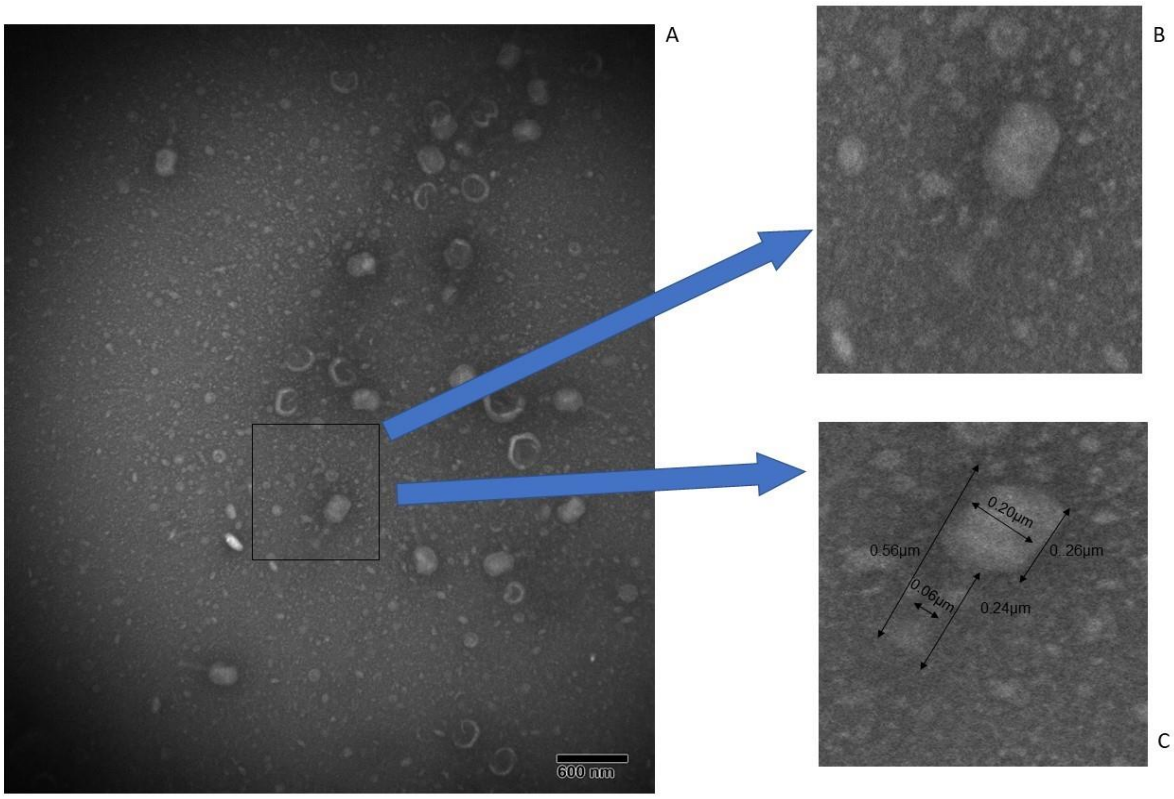

Figure 11A, B, and C. TEM imaging of a complex capsid viewed at 400,000X magnification.

Characterization of Bacteria

Following the DNA extraction protocol, the result of the Sanger sequencing showed that the DNA fragment of the ACTW-2 isolate had a high degree of similarity with several different taxa as shown in Table 8.

Table 8. GenBank blast results for the amplified 16S rRNA gene of ACTW-2 isolate.

Sample	Most Similar Hit	Query Cover	%Identity
ACTW-2	*Leclercia adecarboxylata*	98%	99.48%
	Enterobacter sp.	98%	99.30%
	Uncultured *Serratia sp.*	98%	99.30%
	Leclercia sp.	98%	99.30%
	Enterobacter asburiae	98%	99.30%
	Enterobacter cancerogenus	98%	99.30%
	Enterobacter sichuanensis	98%	99.30%
	Enterobacter mori	98%	99.30%
	Enterobacter chengduensis	98%	99.30%
	Enterobacter rogenkampii	98%	99.30%

Determination of Phage Genetic Material

As described in the materials and methods sections, products of digestion of phage nucleic acid were resolved on an agarose gel and the results are presented in Figure 12, a where high molecular weight band, representing the genome of the isolated phage,

is present in the control sample and the sample treated with RNase A, while it is absent in the sample treated with DNase I. This indicates that the isolated phage contains DNA as genetic material, since the DNase I enzyme digested genomic nucleic acid in the sample.

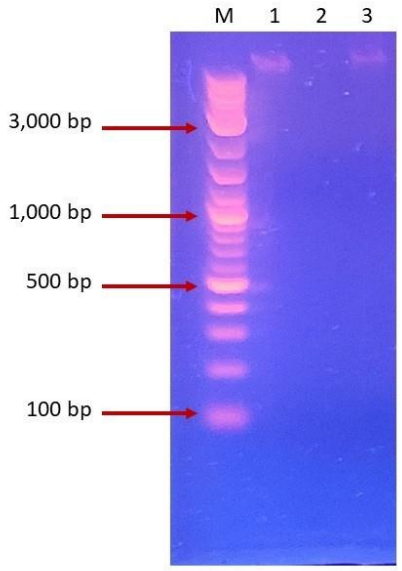

Figure 12. Products of digestion of isolated phage nucleic acid with nucleases. resolved on 1% agarose gel: lane M- marker; lane 1- control phage nucleic acid that was not digested; lane 2- products of digestion of phage nucleic acid with DNase; lane 3- products of digestion of phage nucleic acid with RNase.

PCR Amplification of a Portion of Identified Phage DNA

A portion of the phage DNA was successfully amplified. It was approximately 450 bp long as seen in Figure 13. The sample was extracted, purified, and sent for sequencing.

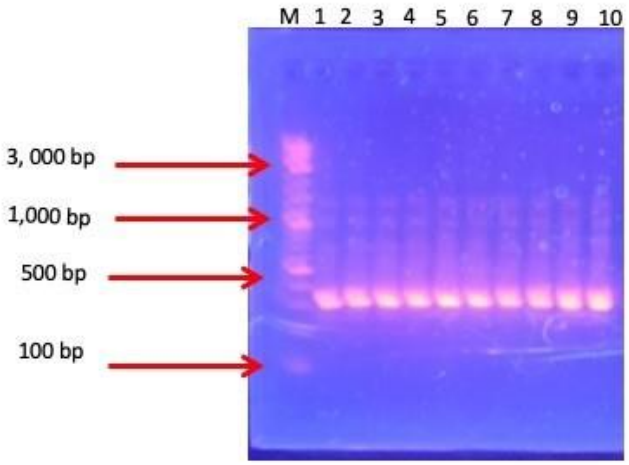

Figure 13. Products of PCR amplification of a fragment of phage DNA.

Twenty-five µL of amplified DNA was resolved on 1% agarose gel; M- marker; lane 1-10: phage DNA.

Molecular Characterization of Phage

Sequences obtained using primers P1 and P2 were compared to the NCBI database as shown in Table 9 and 10. Similarity of the phage sequence to samples from known phages of Enterobacter was 92% or higher.

Table 9. GenBank blast sequence alignment of phage fragment amplified using primer P1.

Sample primer	Most similar hit	Query cover	% Identity
P1 Forward primer	Enterobacter phage ENC9	92%	95.45%
	Enterobacter phage vB_EcIM_Q7622	92%	94.08%
	Enterobacter phage Entb_45	92%	93.38%
	Enterobacter phage vB_EcIM_CIP9	92%	93.01%
	Kosakonia phage 305	92%	92.33%
	Enterobacter phage vB_EhoM-IME523	92%	99.99%
	Caudoviricetes sp.	51%	77.91%

Table 10. GenBank blast sequence alignment of phage fragment amplified using primer P2.

Sample primer	Most similar hit	Query cover	% Identity
P2 Reverse Primer	Enterobacter phage Entb_45	94%	98.25%
	Enterobacter phage ENC9	94%	98.25%

Table 10 Continued

	Kosakonia phage 305	94%	98.25%
	Enterobacter phage vB_EcIM_Q7622	96%	95.55%
	Enterobacter phage vB_EhoM-IME523	93%	93.29%
	Enterobacter phage vB_EcIM_CIP9	96%	92.12%

DISCUSSION

Bacteria are ubiquitous and, as such, can be found in and around fish. Thus, they are found as part of the microflora of fish and in the sediment and excrement found in fish tanks (Austin, 2006). Most are opportunistic in nature but can become pathogenic when the opportunity arises. Examples of these opportunistic bacteria taxa include *Aeromonas, Acinetobacter, Escherichia, Serratia,* and *Enterobacter* etc (Das et al., 2019).

The bacteria isolated in this study had a 99.48% identity match with *Leclercia adecarboxylata*, and 99.30% identity match with *Enterobacter* sp. and uncultured *Serratia* as shown in Table 6. These bacteria are Gram negative pathogens that are found in the gills and skin of freshwater fish and are opportunistic in nature (Klūga et al., 2019). *Leclercia adecarboxylata* bateria is often seen in aquatic environments (Klūga et al., 2019). *Enterobacter* spp. are prevalent commensal bacteria present in the intestines of fish, as well as in soil, and water (Davin-Regli et al., 2019). The *Serratia* genus is also often isolated in fish intestines and can cause high mortality in fish (Klūga et al., 2019). Thus, the bacteria isolated in this study likely belong to either of the mentioned taxa. To determine which taxa the bacteria, belong to the phage blast search result helps narrow it down. This is because phages are specific to a particular bacterium.

The isolated bacteria ACTW-2 was shown to undergo lysis as shown in (Figure 8) after 8 hours. After 2-hours following inoculation, the bacteria continued to grow as shown by the OD600 of the control and lysate tube at 0.0335-0.2006, and 0.0423-0.1714. This shows that the bacteria continued to replicate during this period, but the bacteria growth rate decreased after 8 hours as shown by the OD600 of the lysate tube 0.0947, and the control tube growing further as shown by its OD600 of 0.8936. This shows that as bacteria growth continues in the control tube without interruption, the bacteria in the lysate tube undergoes lysis and hence its OD600 is lesser than the control tube.

Phage burst size assay was done to determine the amount of phage that is found inside and outside the bacteria when it undergoes replication after being infected with phage nucleic acid (Bolger-Munro et al., 2013). As shown in Table 5, the burst size of the phage increases as the infection progresses-at 10-mins the phage count inside and outside the bacterial cell was 4.64, and 3.85 respectively with the total phage count being 4.71. The burst size increased after the 80-min period with the phage count inside and outside the bacterial cell being 8.34, and 8.89 respectively with the total phage count being 9.00 as shown in Graph 1. This coincides with the hypothesis postulated by Ellis and Delbrück "that burst size increases as a function of time, and the longer the incubation time the smaller the bursts will be compared to bursts that took a longer incubation time" (Bolger-Munro et al., 2013).

The morphology of the isolated and characterized phage is consistent with that of the family *Myoviridae,* having a complex capsid composed of an icosahedral head and a

contractile helical tail (Lavigne and Ceyssens, 2011). Previously characterized phages in this family have reported tail lengths and diameters of 80-455 nm and 16-20 nm respectively (Lavigne and Ceyssens, 2011). With an icosahedral head capsid size of 200 x 260 nm and a tail size of 60 x 240 nm, the phage described here appears to fall outside of the reported size range. It should be noted, however, that the TEM manufacturer's specifications for magnification, on which all measurements are based, may vary by 10% and still be within an acceptable range, and that a high degree of accuracy requires the inclusion of a standard of known size alongside the TEM specimen to be measured (Bozzola and Russell, 1999). Confirmation of particle size using a TEM serviced and maintained for the purposes of detailed analytical analysis would be required to fully characterized the morphology of the phage isolated in this research. The most extensively studied phage in the family *Myoviridae* is the T4 phage. The T4 phage infects *E. coli* bacteria and has a capsid with a diameter of 80-100 nm and a tail that is 200 nm long (Orlova, 2012).

The phage nucleic acid extracted from the phage capsid was determined to be DNA because it was degraded by DNase during the experiment. As shown in Figure 10, this also supports the fact that this phage belongs to the family *Myoviridae*. This is because the phages belonging to this family have DNA as their genetic material, a complex capsid with an icosahedral capsid and a contractile tail (Lavigne and Ceyssens, 2011). This phage does not belong to the *Podoviridae* or the *Siphoviridae* family because these families have non contractile short and long tail respectively (Ackermann, 2011).

The *Myoviridae* also are known to infect Gram negative enteric bacteria which include *Enterobacter* spp.

The sequencing results of the amplified phage DNA fragment shows that the phage has a high degree of similarity to previously isolated pathogens of the genus *Enterobacter* (Table 8 and 9). This information lends support to the identification of the bacterial host to be a species of *Enterobacter*.

This study was successful in isolating and characterizing a phage found in a small aquarium. Bacteria in this study were obtained from all seven aquariums examined, with 42 isolates tested for the presence of phage. Of the isolated bacteria, a single isolate, ACTW-2, was found to belong to the taxon *Enterobacter.* This genus had an accompanyin*g* phage with a portion of its genetic material having a high degree of similarity with previously characterized phages of *Enterobacter.* This information would be useful in further research to determine if the isolated phage can be used in the treatment of *Enterobacter* infections, as it has been shown to cause lysis of a species within this genus of bacteria.

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