ISOLATION OF A BACTERIOPHAGE FROM SEWAGE SLUDGE AND CHARACTERIZATION OF ITS BACTERIAL HOST CELL

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Abstract

In light of an increased interest in the use of phage as a bacterial control agent, this study was conducted to determine if phage could be used to remove fecal coliforms from aquatic environments. A suspension was prepared from sewage sludge and tested for the presence of phage. Potential bacterial host strains were isolated from sewage sludge and brine water prepared from seawater collected from Hampton Beach, Hampton NH. One phage was identified in the viral suspension when grown in the presence of a bacteria (RD^2-2) isolated from sewage sludge. None of the brine water bacterial isolates were host for this phage. RD^2-2 was tentatively identified using BIOLOG, fermentation tests (lactose, sucrose, dextrose, inositol, and trehalose), the methyl red and Vogues Proskauer tests. Results from BIOLOG and the biochemical tests indicate that the isolated host cell is similar to the enteric bacterium Citrobacter freundii. *Preliminary data suggests that the phage was unable to infect its host when incubated under brine water conditions.*

INTRODUCTION

Viruses are considered obligate intracellular parasites requiring a specific host cell for its replication (Carlton, 1999; Mayer, 2005). Bacteriophage (phage) are viruses that exclusively target and reproduce within bacterial cells. Generally, phage attach to the surface of their host cell by specialized structures called tail fibers (Figure One). Once attached, the bacteriophage injects its nucleic acid into the bacterium. Using the host cells replication, translation, and transcription machinery, the viral nucleic acid is replicated and incorporated into its protein capsid. The escape of mature viruses from the host cell places stress on the plasma membrane resulting in the eventual death of the bacterium.

The host specificity of viruses offer an enticing technology for fighting infections caused by bacteria or for the treatment of environments contaminated with pathogenic bacteria. Research into potential use of viral therapy is limited, but studies have shown success using this technology to treat infections in livestock, plants, aqua-cultured fish and humans (Sulakvelidze and Burrow, 2005; Sulakvelidze and Kutter, 2005). The use of phage in the treatment of bacterial infections is an attractive alternative to existing therapies (example, antibiotics), because unlike broad-spectrum antibiotics phage target a particular host and are unlikely to illicit resistance in untargeted bacterial strains (Sulakvelidze and Kutter, 2005). Also, unlike chemical therapeutic agents, phage are not susceptible to the onset of bacterial resistance because they have the ability to evolve with their host (Sulakvelidze and Kutter, 2005).

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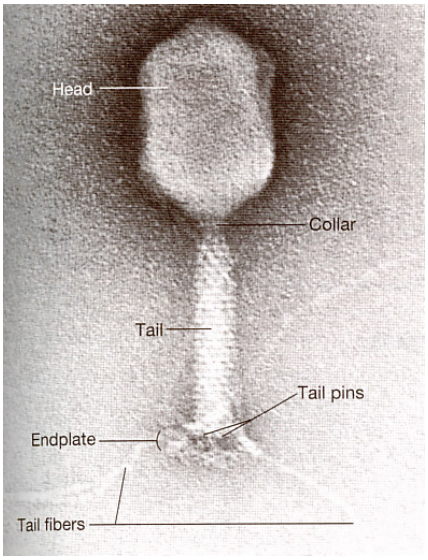

Figure 1: Basic Structure of Bacteriophage (Madigan and Martinko, 2006).

Enteric bacteria are normal inhabitants of the intestines of humans and other animals (Davis, 2005) but are often isolated from aquatic ecosystems after sewage has been introduced into the environment. Sewage contains high numbers of potentially very pathogenic enteric bacteria known as fecal coliforms. Coliforms are characterized as gram-negative, facultative anaerobic bacteria that ferment lactose within 48 h at 35°C. Examples of fecal coliforms include *Escherichia coli* and *Enterobacter aerogenes*. In their natural habitat enteric bacteria are typically harmless but they can produce severe disease symptoms when ingested by susceptible individuals, particularly young individuals and individuals with weakened immune systems (Davis, 2005). Aquatic environments contaminated with enteric bacteria posses a potentially serious threat to wildlife and human health. After hurricane Katrina, high numbers of enteric bacteria were isolated from Lake Pontchartrain leaving this popular recreational lake a potential hazard. This prompted the current investigation looking at the potential of phage therapy to restore this lake to its natural state.

The objectives of this study were to isolate phage from sewage sludge, identify its enteric bacterial host(s), and examine its potential to use as a technology to remove these bacteria from recreational and potable water sources without causing harm to natural nonpathogenic bacterial assemblages.

MATERIAL AND METHODS

Isolation of Bacteria from Seawater

Approximately 200 L of seawater were collected from Hampton Beach, Hampton, NH. Brine water was prepared by combining 60% seawater and 40% distilled water to approximate salinity conditions found in Lake Pontchartrain (4.0 ppt) (USGS, 2002). Brine water was stored in a 210 L aquarium tank and held at room temperature for the duration of the project. Bacterial strains were isolated from the brine water by streaking brine water samples onto either Tryptic Soy agar (TS) or Tryptic Soy agar made with brine water (TSBW) using a sterile cotton-tipped swab. Inoculated agar plates were incubated for 24 h at room temperature. To ensure purity of the culture, after incubation, individual and distinct colonies were

identified and inoculated onto either TS or TSBW agar plates using the streak plate technique. After 24 h incubation at room temperature, a well-isolated colony was transferred to either TS or TSBW agar slants. Prior to phage assays and identification tests, 18-24 h broth cultures were prepared by aseptically transferring a loopful of bacteria from the agar slant to either TS or TSBW broth. Inoculated broth tubes were incubated room temperature while shaking at approximately 150 rpm.

Isolation of Potential Bacterial Enteric Host Strains from Activated Sewage Sludge

Two containers (2 liters) of sewage sludge were obtained from the waste water treatment facility located in Nashua, NH. Bacteria were isolated from the sewage sludge by plating 100 µl of sludge onto either TS or TSBW agar. After 24 h incubation at room temperature, pure cultures were obtained using the streak plate technique, and working broth cultures were prepared as previously described for bacterial isolation from brine water samples.

Preparation of Viral Suspension

A viral suspension was prepared from sewage sludge. Sludge (10 ml) were transferred to a sterile 25 ml centrifuge tube and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was aseptically transferred to a sterile 15 ml tube without disturbing the pellet. A viral suspension was prepared by aseptically filtering the supernatant through a 0.8 µm pore sized cellulose filter to remove particulates, followed by filtration through a 0.45 µm pore sized filter to remove bacterial cells and cellular debris.

Viral Isolation

The Phage Assay (Cappuccino and Sherman, 2001) was used to determine if phage were present in the sewage sludge suspension. Underlay "hard" TS and TSBW agar were prepared as previously described. Overlay "soft" TS and TSBW agar were prepared by adding half of the amount of agar used for "hard" underlay agar. Sterile underlay agar was poured into sterile petri dishes and left to harden. After the underlay agar had solidified, 1ml of sewage sludge suspension and 3 drops of a 24 h broth culture were added to 3.0 ml soft agar, vortexed, then poured on top of the underlay agar. Soft overlay was allowed to harden before incubation of plates at room temperature. After 24 h incubation, the plates were checked for plaques (clearing zones) within the bacterial lawn, indicating the presence of phage (Figure 2). The phage assay was repeated twice using all the bacterial strains isolated from brine water and sewage sludge.

When plaques were identified, a pure suspension was prepared by carefully removing a portion of the plaque using a sterile pipette tip and transferring the plaque to 10 ml sterile TS or TSBW. The broth was vortexed to free viral particles from the agar and residual cells were removed by aseptically transferring the broth to a sterile 25 ml centrifuge tube and centrifuging at 5000 rpm for 5 min. The supernatant was aseptically transferred to a sterile 15 ml tube and stored at 5°C.

The Phage Assay was repeated using isolated bacterial strains and the stored viral suspensions to ensure presence of phage, determination of phage numbers, and assess host specificity. Serial dilutions (10^{0}-10^{-9}) of viral filtrate were prepared. As previously described, 1.0 ml of viral dilutions and 3 drops of a 24 h bacterial broth culture were added to soft agar (TS or TSSW) and poured on top of the hard agar. Plates were incubated for 24 h and were examined for plaques.

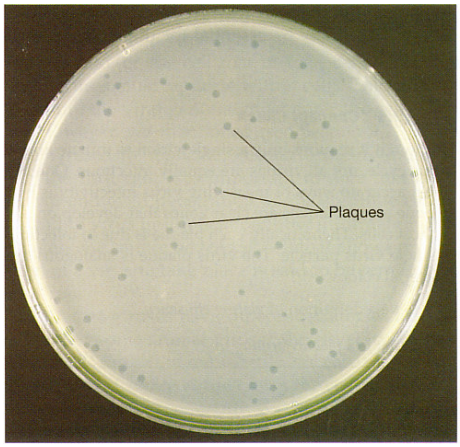

Figure 2: Phage Assay and Plaque Formation (Madigan and Martinko, 2006).

Identification of Bacterial Host Strains

BIOLOG

Bacterial isolates that were found to be host cells for phage were tentatively identified using BIOLOG system (BIOLOG, 2006). The BIOLOG system differentiates and identifies bacteria based on differences in their ability to metabolize 96 different biochemical substrates. The Gram stain, catalase or oxidase tests (catalase-Gram positive or oxidase-Gram negative) were performed on 24 h bacterial broth cultures. Based on these tests, the appropriate BIOLOG plate was inoculated with a 150 µl of a 24 h bacterial broth culture. After the plates were incubated for 24 h at room temperature, utilizable carbon sources were identified and scored giving a characteristic pattern for the individual isolate. Results were keyed into BIOLOG's computer program that automatically cross-referenced the resulting patterns to an extensive library of identified bacterial species based on the same biochemical tests. Using cluster analysis, potential matches for the unknown were determined by the BIOLOG program.

Fermentation, Methyl Red, and Vogues-Proskauer Tests

To support strain identification by BIOLOG, fermentation, methyl red, and Vogues-Proskauer tests were preformed. Three-loopfuls of 24 h pure broth cultures were inoculated into fermentation broth containing lactose, dextrose, sucrose, inositol, or trehalose as sole carbon source. Durham tubes were placed into the test tubes in the inverted position to trap gas (CO_2, H_2), potential end products of fermentation. All fermentation tubes contained the pH indicator phenol red. Acid production from the fermentation of the sole carbon source was reported when the media changed color from red (pH 7.4) to yellow (pH < 6.0). After 48 h incubation at 35°C, inoculated fermentation tubes were scored on the basis of acid production (as indicated by a color change from red to yellow) and/or gas production (as indicated by gas bubbles trapped in the inverted Durham tube).

The Methyl Red test (Cappuccino and Sherman, 2001) was used to determine the host cell's ability to oxidize glucose with production of a high concentration of acid end products. Methyl red broth was inoculated with 24 h broth test cultures. Tubes were incubated for 24 h at 35 °C. After incubation 3-4 drops of methyl red indicator (red pH 7.0) were applied to the methyl red tubes. A change in the color of

the medium from amber to red (pH 4) was scored positive, and a color change from amber to yellow (pH 6) was scored as negative.

Vogues-Proskauer test was used to determine the test organism's ability to produce non-acidic or neutral end products from the fermentation of glucose Cappuccino and Sherman, 2001). Vogues-Proskauer media tubes were inoculated with 24 h broth test cultures and incubated at 35 °C for 24 h. After incubation, 10 drops of Barritt's A (α-naphthol, 5.0 g; ethanol, 95.0 ml) solution followed by 10 drops of Barritt's B solution (KOH, 40.0 g; creatine, 0.30 g; dH_2O, 100.0 ml) were added to the test tubes. The tubes were shaken every 3-4 minutes for 15 minutes. A positive test was indicated by a color change of the media from amber to rose.

Enteric bacteria (*Enterobacter cloacae, Enterobacter aerogenes,* and *Citrobacter freundii*) obtained from the Rivier College Culture Collection were used as control organisms for fermentation, methyl red, and Vogues-Proskauer tests. All manipulations and preparations of control isolates were prepared as described for host cell isolates.

RESULTS

Isolation of Bacteria from Seawater and from Sludge

Six bacterial strains were isolated from seawater and six bacterial isolates were isolated from sewage sludge. All twelve isolates were determined to be Gram negative. Both cocci and bacilli were represented (Table 1).

Table 1: Gram Reaction, Cell Morphology, and Cell Arrangement of Seawater and Sludge Isolates.

Bacterial Isolate	Gram Stain	Cell Morphology	Cell Arrangement
Brine Water			
RD^2-1BW	Negative	Coccus	Diplo
RD^2-2BW	Negative	Coccus	Staphylo
RD^2-3BW	Negative	Rod	Single
RD^2-4BW	Negative	Rod	Diplo
RD^2-5BW	Negative	Rod	Single
RD^2-6BW	Negative	Coccus	Diplo
Sludge			
RD^2-1	Negative	Rod	Single
RD^2-2	Negative	Rod	Diplo
RD^2-3	Negative	Coccus	Diplo
RD^2-4	Negative	Rod	Single
RD^2-5	Negative	Coccus	Single
RD^2-6	Negative	Rods	Chains

Phage Assay

One phage was isolated when plated with bacterial cultures cultured from sludge. Isolate RD^2-2 served as host for this phage. Viral plaques were not produced when RD^2-2 and phage were incubated on brine water agar suggesting an inhibitory effect of the brine water on viral infection. The phage assay was

performed on brine water isolates to ensure that natural seawater populations of bacteria would not serve as host to phage. Results obtained in this study showed that there were no phages present in the sewage suspension that infected any of the brine water bacteria isolated in this study.

Tentative Identification of Host Cell RD^2-2

Results from BIOLOG tentatively identified RD^2-2 as *Enterobacter arogenes* (96.5% similarity) or *Citrobacter youngae* (97.8% similarity). Results from the fermentation, methyl red and Vogues Proskauer tests showed that bacterial isolate RD^2-2 responded similarly to *Citrobacter freundii* (Table 2).

Table 2: Results of Fermentation, Methyl Red, and Vogues-Proskauer Tests.

Test	*Enterobacter cloacae*	*Enterobacter aerogenes*	*Citrobacter freundii*	RD^2-2
Fermentation				
Inositol	[+]/-	+/-	+/-	+/-
Trehalose	+/-	+/+	+/+	+/+
Lactose	[+]/-	+/-	+/-	+/-
Sucrose	+/-	+/+	+/+	+/+
Dextrose	+/-	+/+	+/+	+/+
Methyl Red	-	-	+	+
Vogues-Proskauer	+	+	-	-

* Fermentation Test: Acid/Gas reactions; + = positive; [+] = partial positive; - = negative

DISCUSSION

The presence of coliform bacteria in aquatic environments indicates that the environment has been contaminated with fecal material originating from humans or other animals. After hurricane Katrina, to alleviate flooding problems in the city of New Orleans, billions of gallons of polluted water containing potentially dangerous levels of fecal coliforms, were pumped into Lake Pontchartrain leaving the lake in an unhealthy state (Daily, 2005). The bacterial host for the phage isolated in this study was found to be similar to *Citrobacter freudii*, a common enteric bacteria belonging to the family E*nterobacteriaceae*. *C. freudii* is commonly found in sewage and has been associated with nosocomial infections in the urinary, respiratory, and biliary tracts of debilitated hospital patients (Tortora et al., 2006). This organism represents an increased health risk because an important aspect of this organism's physiology is its ability to resist the affects of antibiotics typically prescribed to treat the infections it causes. Resistance is due to an inducible chromosomally encoded cephalosporinase that can inactivate cephamycins and cephalosporins (Kim and Lim, 2005).

Typically, when high numbers of coliforms are found in recreational or potable water sources, usage must be restricted to prevent a human or animal health crisis. The restoration of the environment

to a healthy state is dependent on local climate conditions and removal of coliforms from aquatic environments can take extended periods of time. The use of phage therapy for the removal of pathogenic coliforms from polluted water could offer a fast and relatively inexpensive technology. Phage therapy would be a safe way to treat contaminated water because phage are specific to their prokaryotic host cell and will not infect non-host cells. Unlike enteric bacteria, it is believed that if phage were ingested there would be little adverse affects on human health. Although the phage isolated in this study has not been identified, a common phage that is known to infect *Citrobacter* sp. is the temperate phage Mu (Guttman et al., 2005). Unlike many other viruses, this phage has a broad host range that includes *E. coli*, *Salmonella*, and *Erwinia* (plant pathogens). The potential for this phage to infect and kill several potentially harmful enteric bacteria without the application of other phage further supports its potential as a control agent. However, in order for this phage to be used as a control agent in Lake Pontchartrain it would have to be able to infect its host under brine water conditions. Results from this study suggest that the infection of the host by this phage is inhibited under brine water conditions.

Future studies on the RD^2-2 phage will focus on testing the phage on other members of the *Enterobacteriaceae* family for susceptibility. Studies will also focus on phage survivability in brackish environments to further assess its potential for future applications in contaminated brine water treatment.

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