TEM of Bacteriophages Found in Marine Sources

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Abstract

We describe here a project to illustrate the diversity and abundance of marine bacteriophages undertaken by two high school students participating in The Scripps Research Institute's High School Student Research Education Program. The students were interns in the Automated Molecular Imaging group over the summer of 2007, during which time they acquired high magnification transmission electron microscopy images of bacteriophage filtered from samples collected from nearby marine waters. The basic protocols for sample collection, grid preparation, and electron microscopy imaging are described in this manuscript along with some of the images of the bacteriophages.

Introduction

A bacteriophage, often abbreviated to simply "phage", is a type of virus that infects bacteria. Phages reproduce by transferring their genetic material into a bacterial host where it is replicated to produce new phages that then typically leave the host via lysis. Over 95% of the bacteriophages described to date are classified as Caudovirales, which consist of a head region, composed of a protein shell surrounding genetic material, and a tail, which can sometimes resemble a syringe, that helps to attach the phage to the bacterium and transfer the genetic material into the host.

Viruses are understood to be the most abundant biological entity in the world's oceans and as such have a major impact on all aspects of life on Earth (Suttle, 2005). The abundance of phages in aquatic environments is thought to control the population of bacteria and influence genetic exchange (Bergh et al., 1989). Bacteriophages exhibit an enormous range of genetic diversity and are thus thought to harbor a large number of novel genes (Breitbart, 2002; Breitbart, 2004; Breitbart et al., 2004). Through the constant exchange of genomic material between phages within the host bacteria, phages have had a major impact on evolution.

Transmission electron microscopy has played an important role in the development of our understanding of both the abundance and the diversity of marine phages (Bergh et al., 1989). Although other techniques, such as Epifluorescence microscopy (Hennes and Suttle, 1995) and gene sequencing (Rohwer, 2000), are now more commonly used for these purposes, TEM remains the only method by which the exquisite structure of individual viruses may be visualized in detail. The techniques required are relatively straightforward and the study described is well within the reach of a scientifically inclined high school student with access to an electron microscopy lab. The goal of the simple experiment described here was to examine and compare two samples of marine waters in terms of the abundance and diversity of the phage population in each. While a much larger statistical sampling would be required for a quantitative comparison, the results amply illustrate the enormous range and diversity of the phage populations of our oceans.

Methods and Materials

Sample collection: Water samples were collected from two marine sources as indicated in figure 1. At each location at least 2 samples of approximately 12 ml of water were collected into sterile conical plastic test tubes and then transferred to an ice bucket to help preserve the specimens. To remove impurities and larger entities, 10ml of each water sample was filtered through a 0.45µm filter using a 60mL syringe and transferred to a new test tube. When in use, the samples were always stored or transferred in an ice bucket to maintain preservation and were subsequently stored for further use in a cold room (4°C).

Grid preparation: Substrates used to support the specimen for TEM imaging consisted of 400 mesh copper grids covered with a plastic coat and a layer of carbon. The grids were first cleaned in a ultrasonic bath for two minutes in a glass beaker with 20mL acetone. After the first cleaning, the acetone was removed and replaced

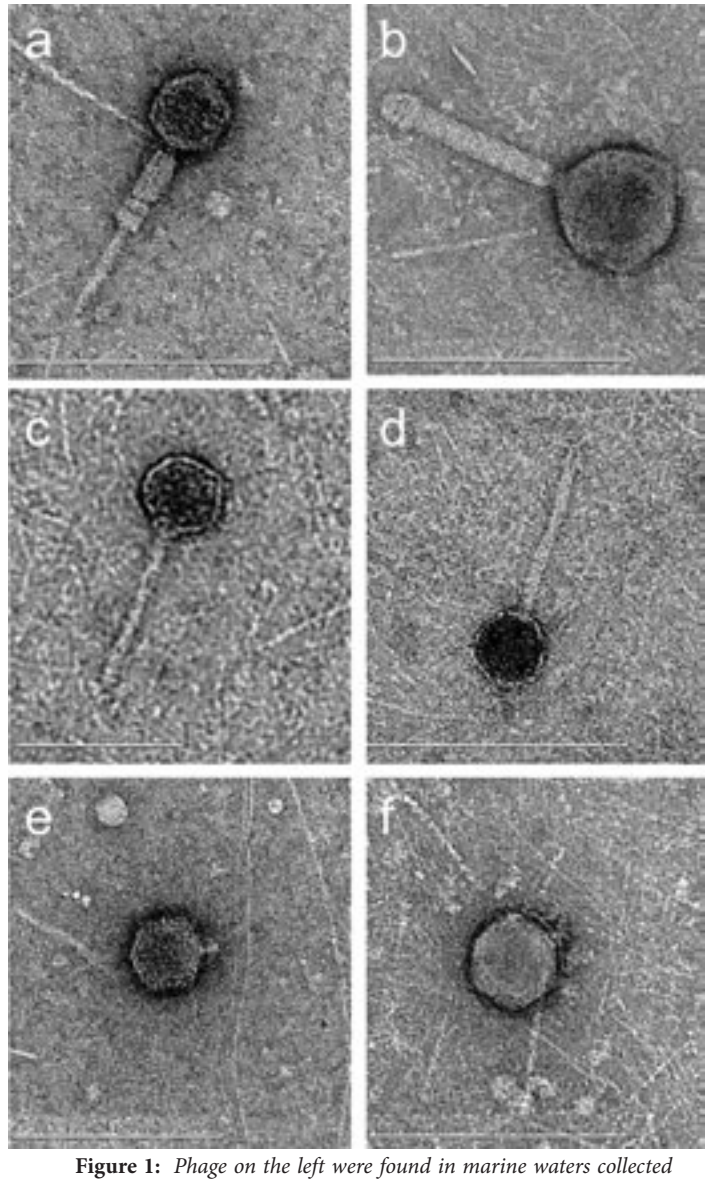

Figure 1: *Phage on the left were found in marine waters collected from the Torrey Pines marsh land in Del Mar, and those on the right from Imperial Beach, San Diego. Top row: Myoviridae, both images acquired at a nominal magnification of 52000x. Middle row: Siphoviridae, image on the left acquired at a magnification of 67000x, image on the right at 52000x. Bottom row: Podoviridae, image on the left acquired at a magnification of 42000x, image on the right at 52000x.*

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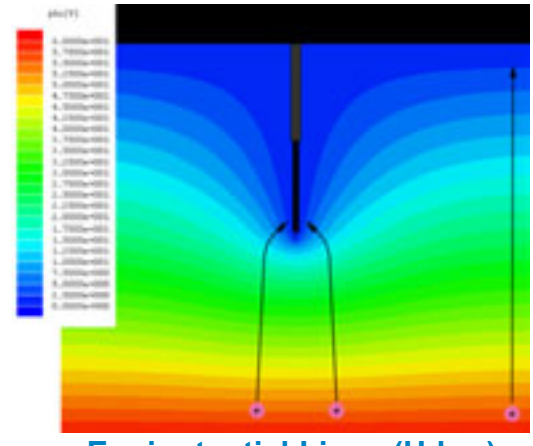

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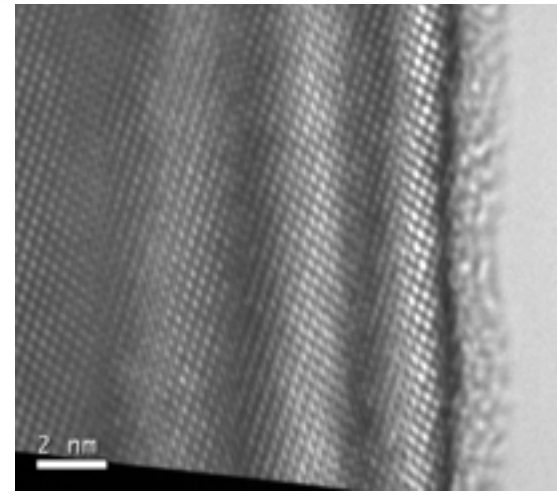

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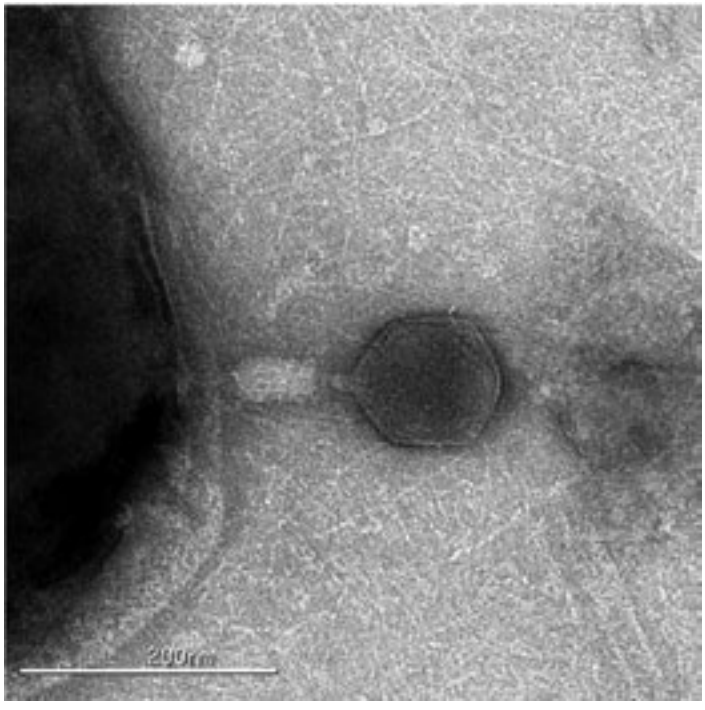

Figure 2: *Image of a phage infecting a bacterium.*

with 20mL of deionized water, and sonicated for an additional two minutes. The grids were then removed and left to dry in a Petri dish containing filter paper. A plastic coating of 1% nitrocellulose in amyl acetate was placed on the lustrous side of the clean grids to provide support for the carbon coating. The protocol used was to fill a Petri dish with deionized water past the brim to a positive meniscus. Next, two drops of 1% nitrocellulose in amyl acetate were placed onto the surface of the water. When a plastic layer became visible, clean grids were placed lustrous side down onto the surface of the water. A small sheet of Parafilm was then used to pick up the coated grids by placing the sheet on the surface of the water and sandwiching the grids between the Parafilm and plastic layer of 1% nitrocellulose in amyl acetate. The grids were subsequently coated with a layer of carbon using a carbon evaporator (Edwards Auto 306 Turbo) to provide strength and improve conductivity. Grids were then stored in a desiccator to await further use. Prior to specimen application, the grids were processed in a Gatan Solaris plasma cleaner for 20 sec at 25W power rating, using an $Ar/0_2$ gas mixture to create a clean, hydrophilic surface. Plasma cleaning of the grids is an essential step in the specimen preparation process as without this step very few phage adhered to the surface of the grid.

Ultracentrifugation was used to concentrate the phage onto the surface of the grids following the protocol described in (Bergh *et al.*, 1989). A single grid, carbon side up, and 200µL of sea water were placed into a centrifuge tube and spun at 47,000 rpm, (100,000g), for 90 minutes, at a temperature of 4°C. The grid was then removed and stained using 4µL of 1% uranyl acetate. For the staining process, three 200µL beads of water were set up on to the surface of a Parafilm-covered Petri dish. After removing each grid from its centrifuge tube, it was fastened to the tip of a pair of tweezers and mixed into each water bead to wash the sample. Immediately following this procedure, 4µL of 1% uranyl acetate was pipetted onto each sample grid. After a few seconds, folded Kimwipes were used to blot off the excess stain solution and the grid was allowed to dry before being stored.

Electron Microscopy: The grids were examined in a Tecnai G-2 Spirit transmission electron microscope (FEI Co.) equipped with a 2Kx2K CCD camera (TVIPS F224). The entire grid was initially examined at a low magnification (~500x) to identify potentially well-stained areas. These target areas were searched at higher magnifications (3900x-5800x) to find the phage, which were then centered in the field of view and a digital image was acquired using the Leginon software (Suloway *et al.*, 2005) at magnifications of 30,000x (pixel size 0.45nm at specimen) or 67,000x magnification (pixel size 0.21nm at specimen).

Results

We classified the phages collected from the two marine sources into three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Ackermann, 2001). The *Myoviridae* family consists of phage with a relatively thick double-layered tail (Figure 1a and b) which is thought to contract during the process of injecting its genetic material into the host bacterium using a syringe-like action. The *Siphoviridae* family posses a longer, thinner, flexible non-contractile tail (Fig. 1c and d), the end of which most likely contains adsorption structures that attach to receptors on the surface of bacteria. Finally, the *Podoviridae* family utilizes a shorter non-contractile tail that contains enzymatically-active spikes, essentially chewing their way through the cell's outer membrane down to the bacterial receptors (Fig. 1e and f).

The phages we identified ranged in size from ~50 to ~700nm; *Siphoviridae* were between 150-650nm; *Myoviridae* from 175-380nm; and *Podoviridae* from 50-100nm. The number and distribution of the samples observed from the two marine sources is summarized in Table 1. However, given the limited sample size of the observations these numbers are only qualitative in nature and have no statistical significance.

During the course of the project we observed several other interesting structures. These included the image shown in Figure 2, which we interpret as a phage in the process of infecting a bacte-

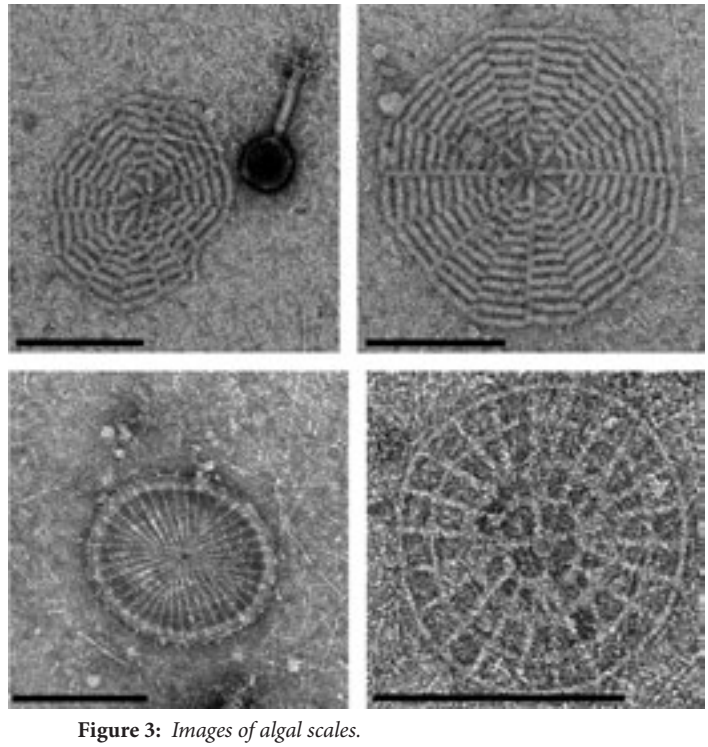

Figure 3: *Images of algal scales.*

rium, and a large number of images that are interpreted to be algal scales (see Figure 3).

Table 1: Distribution Of Phage Types Found in Two Different Samples of Seawater

Sample	Total number of phages	Total number of images	% of each class
Torrey Pines Marshland	19	120	Myoviridae: 37% Siphoviridae: 47% Podoviridae: 16%
Imperial Beach	66	119	Myoviridae: 61% Siphoviridae: 32% Podoviridae: 7%

Discussion

The TSRI High School Student Research Education program provides students the opportunity to explore science in many different ways. The summer internship program is preceded by spring tutorials and supplemented by a seminar series intended to provide a broad view of the opportunities in science. The lab experience provided first hand knowledge of specimen preparation and the use of an electron microscope, and also provided the opportunity to plan, execute and report on an experimental protocol. The students placed high value on the experience of learning how to interact in a collaborative work environment. Overall the students found the experience rewarding and of benefit in preparing for future college careers as well in deciding whether to pursue a career in science. ■

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