Exploration of the phage population in bacterial mats of the Sippewisset salt marsh

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Abstract

The importance of phages in aquatic systems has been studied extensively and is reviewed in several recent articles [1, 2, 3, 4]. Although many studies have addressed the impact of viruses in the ocean, little is known about their ecology in other habitats. The aim of this project was to examine the presence and the diversity of phages in the bacterial mats of the Sippewisset marsh. For this purpose, the four most upper layers, upper green, purple, lower green and black, of a bacterial mat from the Sippewisset salt marsh were separated and bacteria and phages extracted by suspending the mat in sterile seawater. Using the DNA stain SYBR Green 1, phages were easily detectable in each of the layers, and their concentration was determined by direct counting of the fluorescently labeled particles. Transmission Electron Microscopy could confirm the presence of phages in the bacterial mat, but in spite of the relatively high concentration of phages that was determined by direct counts only a few phage particles could be found. This discrepancy is likely to be due to the sample preparation method that was used. Six different strains of purple non-sulfur bacteria isolated from the Sippewisset marsh during the Microbial Diversity Course were used for the attempt to isolate phage-host pairs, but a successful infection could not be detected.

Materials and Methods

Sample preparation

An approximately 20x30cm sample of a bacterial mat from the Sippewisset salt marsh was taken (Figure 1). The four most upper layers of the mat were separated by carefully scraping off material from the surface, resulting in the four samples:

1. Upper green layer (400mL)
2. Purple layer (250mL)
3. Lower green layer (100mL)
4. Black layer (200mL)

Each of the samples was suspended in an equal amount of sterile seawater and, slowly shaking, incubated for 20h at RT. The supernatant of each of the samples was decanted and filtered in three subsequent steps using hydrophilic Durapore filter discs (Millipore Corporation, Bedford, MA) with poresizes 5.0μm, 0.45μm and 0.22μm. 10mL samples were taken from each filtration step. As a control, 1L of surface seawater from a site close to the bacterial mat-sampling site was prepared accordingly.

Bacteria and phage counts

The concentration of bacteria and phages in the different layers of the bacterial mat were determined using SYBR Green 1 staining, according to a previously published protocol [5, see also http://www.usc.edu/dept/LAS/biosci/faculty/documents/fuhrman_SYBR_protocol.pdf] with the following modifications. The final dilution of SYBR Green 1 used was 1:50 instead of 1:400 since this allowed multiple microscopic examination and multiple freezing-thawing cycles without fading of the samples. The amount of antifade solution was reduced from 40 to 10μL to decrease smearing of the sample under the cover slip. The filtrates

<5.0μm and <0.22μm were used for counting bacteria and phages, respectively. 500μl of a diluted sample was filtered in each case; the dilution was varied until a monolayer of particles on the filter was obtained. Visualization of the slides was carried out using an Axioplan 2 microscope (Zeiss, Germany) with a green emission filter. Counts of the fluorescently labeled particles was obtained using MetaMorph 4.6 Imaging Software (Universal Imaging Corporation, Downingtown PA), the counts of five microscopic fields was averaged. The concentrations of bacteria and phages were determined with the following equation:

$$\frac{\#\text{ of particles}}{\text{mL}} = \frac{\#\text{ of particles} * \text{filter area} * \text{dilution factor}}{\text{filtered volume} * \text{image area}}$$

Filtered volume = 0.5mL

Image area = 175 * 140 μm = 24500 μm^2 (determined with stage micrometer)

Filter area = $r^2 * \pi$ = 491 mm^2

T7 Coliphage (kindly provided by Jen Hughes, Microbial Diversity Course member) was used as a positive control, sterile seawater was used as a negative control, and, in addition to that, some of the samples were stained before and after autoclaving for 20 minutes at 121 °C.

Transmission Electron Microscopy (TEM)

Two different sample sets were used for TEM. For the first set, 10mL of the <0.22μm fraction of the four samples was concentrated 100x using Centricon Plus-20 filter columns with a cut-off of 30,000 kDa (Millipore Corporation, Bedford, MA). The second set of samples consisted of the unconcentrated <5.0μm fraction of the four samples. A drop of the sample was brought onto specimen grids (Formvar Carbon Support Film on 300 mesh Copper grids,

Electron Microscopy Sciences, Fort Washington, PA) and left on the grid for 1 to 5 minutes. After carefully wiping the sample off with a filter paper, the sample was negatively stained. A freshly centrifuged 2% uranylacetate solution was pipetted onto the grid and, after an incubation period of 40 seconds, carefully wiped off with a filter paper. After a drying period of about one hour, the samples were visualized with a High Resolution Electron Microscope EM 10C/CR (Zeiss, Germany) at 80,000 kV with a magnification of up to 40,000x at the Central Microscopy Facility of the MBL.

Plaque assay

For the isolation of virus-host pairs, the following strategy was used. Six different isolates of purple non-sulfur bacteria isolated from the Sippewisset marsh (kindly provided by Kirk Harris, Microbial Diversity Course member) were grown up over-night under aerobic conditions in liquid Low Chloride Medium containing 10mM succinate (LCM succinate, see course medium recipes). The four <0.22μm fractions were concentrated 10x using Centricon Plus-20 filter columns with a cut-off of 30,000 kDa. 100μL of the concentrate and the original sample were each mixed ith 300μL of each of the bacterial cultures and incubated at RT for 20 minutes. 4 mL of melted LCM topagar (LCM succinate medium with 1.0g/L gelatin, 50g/L glycerol and 4g/L low melting agarose) were added, the tube inverted and the mixture poured onto a LCM succinate agar plate. The plates were examined for the formation of plaques after a two-day incubation period. As a positive control the virus solution was omitted, the medium with no additions served as a negative control.

Results and Discussion

Concentration of bacteria and phages in the bacterial mat of the Sippewisset marsh

Figures 2A and B show typical examples of computer images of SYBR Green I stained samples A) after filtration through a 5.0μm filter and B) after three subsequent filtration steps through 5.0μm, 0.45μm and 0.22μm filters. The following table contains the concentrations of bacteria and phages of the different layers of a bacterial mat sample from the Sippewisset salt marsh and the surface seawater control, all of which were determined using the computer images.

Layer	Bacteria / mL	Phages / mL	Ratio
Upper green layer	$2.6 \times 10^{7*}$	2.8×10^{8}	11*
Purple layer	6.9×10^{7}	1.6×10^{8}	2
Lower green layer	9.2×10^{7}	1.7×10^{8}	2
Black layer	10.0×10^{7}	0.5×10^{8}	1
Surface seawater	0.5×10^{7}	0.5×10^{8}	10

***includes only bacteria smaller than 5.0μm, many Cyanobacteria are likely to be excluded in these counts which may give a 'false' ratio**

Whereas bacteria could be easily identified based on their morphology, it was difficult to say whether the little fluorescent dots in the pictures are indeed phages and not abiotic particles present in the sample. A couple of controls were carried out to make sure that the calculated concentrations were not based on staining artifacts. As a positive control, a T7 coliphage lysate was included, which resulted in a picture similar to the <0.22μm fraction pictures. No stained particles could be observed in the negative control, which consisted of sterile water that was used for dilution of the samples. A <0.22μm fraction sample as

well as a <5.0μm sample was stained with SYBR Green I before and after autoclaving for 20 minutes at 121°C. Autoclaving resulted in complete disappearance of stained particles in the <0.22μm fraction and left only a few diffuse stained particles in the <5.0μm sample. Although this is not an absolute proof that the counted small particles are indeed phages, it at least is strong evidence supporting it.

Since the calculated concentrations of bacteria and phages are based on a single sampling, the above shown numbers should not been taken as absolute numbers. But, nevertheless, the determined concentrations show that a considerable number of phages are present in the bacterial mat. There seems to be a decrease of the number of phages with depth, and also a significant difference between the ratio of phages to bacteria in the mat sample when compared to the seawater sample. Future studies have to confirm these observations.

Transmission Electron Microscopy (TEM)

Only a few phages (all icosahedron shapes with tails, no pictures shown) could be observed using TEM, although the concentration of phages should have been 10^{10}/mL for the 100x concentrated samples and 10^{8}/mL for the unconcentrated samples. Most likely this was due to the sample preparation procedure, which could be improved by using spincolumns that are specifically designed for holding an EM grid. The number of particles absorbing to the grid is greatly increased by the centrifugation step, which would certainly help to get more conclusive results. In summary, TEM confirmed the presence of phages in the samples, but the initial goal; the confirmation of the determined concentrations and also comparison of phage populations in the different layers of the bacterial mat could not be accomplished.

Plaque assay

Six different, uncharacterized purple nonsulfur isolates were tested as potential hosts for phages present in the <0.22μm fractions of the four separated layers of the bacterial mat. No plaque formation could be observed. In all cases, bacterial cultures grew as lawns in the toplayer of the plates and no growth was observed in the negative control. Since host-phage associations are usually very specific, the isolation of host-phage pair(s) is difficult; it is therefore not surprising that the experiment was not successful.

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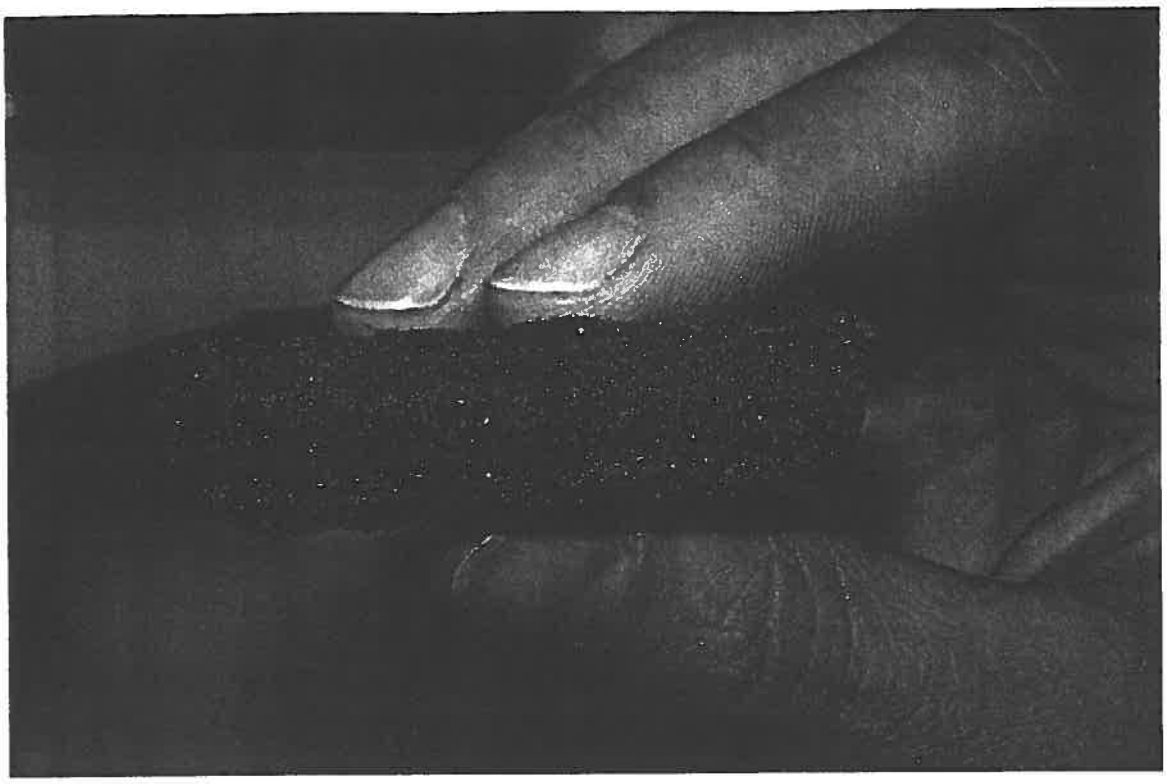

Figure 1: Bacterial mat from the Sippewisset salt marsh used as a sample for the isolation of bacteriophages.