Analysis of marine viral assemblages

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ABSTRACT

Viruses are the numerically dominant microbes in every oceanic environment from sea surface to sediments. One liter of surface seawater from a typical mesotrophic area contains 10^{10} viruses, about an order of magnitude greater than the number of bacteria. While total counts of viruses are becoming easier to make, we still know very little about the viruses that comprise a given assemblage. Infectivity assays are extremely useful and still the best way to assay for infectious viruses for any particular host. However, this approach requires that each potential host organism be cultured making it impractical, if not impossible, to completely characterize natural assemblages. Morphological studies have been enlightening, but are time consuming and difficult to do quantitatively. Here we report on a fingerprinting approach to characterize natural viral assemblages. In this approach the viruses are concentrated, and intact viral genomes separated based on their size using pulsed-field gel electrophoresis. The number of distinguishable bands produced provides a minimum estimate of the number of different viruses, while band position and staining intensity reveal the genome size distribution within the assemblage. With this technique we have detected spatial and temporal differences, as well as many similarities, in viral assemblages among a variety of marine habitats. Current efforts are directed toward combining this technique with other methods of fractionation and sequence analysis to allow both morphological and genetic description of uncultivated marine viruses. Direct investigation of dominant or particularly widespread viruses may ultimately provide clues as to which marine organisms contribute most to the viral pool, and which organisms are likely to be significantly influenced by viral mortality.

Introduction

Viruses are thought to have been co-evolving and diversifying along with their hosts from the very beginnings of life on earth [14]. They are ubiquitous parasites in all environments infecting virtually every living organism from the simplest prokaryotes to the most complex multicellular plants and animals. Although viruses of all marine organisms could contribute to the free viral pool, it seems likely that the marine virioplankton is comprised predominantly of viruses infecting prokaryotes [15]. Infection rates of eukaryotic phytoplankton and the microzooplankton are not well known [19], but viruses infecting these organisms are readily detected [2, 20] suggesting that they too could be significant contributors to the total viral pool. Despite all that has been learned recently about the distribution, abundance, production and decay rates of viruses [8], we still know surprisingly little about the actual composition of natural assemblages and how that

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composition varies over space and time. This is mainly due to limitations of the techniques currently employed, which are either too specific or too general, to provide the information necessary to answer this particular question about viral assemblages.

The most general information is obtained by epifluorescence microscopy [18]. This technique is quickly replacing transmission electron microscopy (TEM) for virus counts because of its speed and simplicity. However, since viruses are below the resolution of light microscopy, this technique provides only a total count of virus-like particles. In contrast, it is possible to resolve a great deal of morphological detail by TEM [7]. Unfortunately, the uncertainty in ascribing fine morphological detail to any individual virus, and the need to characterize hundreds of viruses per sample, makes quantitative, morphological characterization of natural viral assemblages difficult. Such studies have therefore had to classify viruses into a limited number (3 to 10) of broadly defined groups based on capsid size and presence or absence of a tail [4, 10, 22].

Plaque and most-probable-number assays [18] are very specific assays that detect only the infectious viruses for a particular host. This data is not obtainable by any other means. However, since each potential host must be maintained in culture, the number of host organisms that can be screened at any one time by these methods is quite limited. Characterization of an entire viral assemblage is simply not feasible by such an approach and the results are necessarily biased by the choice of host organisms to be screened. Thus, while infectivity assays are essential tools for the study of individual viruses, they are not suited for comprehensive characterization of complex assemblages.

Molecular biological techniques are now being successfully applied for analysis of specific groups of indigenous marine viruses. Initial studies have focused on the characterization of different viruses isolated against a particular host [5, 12]. More recently, PCR amplification followed by cloning and sequencing [3], or denaturing gradient gel electrophoresis [9], has been employed to examine the diversity within particular viral groups. One advantage of these molecular approaches is that the level of specificity can be adjusted by careful selection of the target sequence and/or the use of degenerate probes or primers. However, due to their extreme variability, there is no universal target sequence for viruses comparable to the rRNA genes used for classification of all other living things. The diversity found even within specific groups of viruses suggests that the overall genetic diversity of viruses in seawater is extraordinarily high. The application of different techniques, or combinations of techniques, which can encompass the full range of genetic diversity would be a useful complement to the more specific molecular methods.

One characteristic of viruses which varies over a wide range and that is readily determined is the genome size. Reported viral genomes range from only a few kilobases (kb) to several hundred thousand kb [6] suggesting that the frequency distribution of genome sizes might be used to characterize any given viral assemblage. Previous analyses of aquatic viral DNA have used conventional electrophoresis [1, 13] which lacks the resolution necessary to distinguish most viral genomes. Pulsed-field gel electrophoresis (PFGE) provides much better resolution over the full range of intact viral genome sizes and has been reported for the analysis of bacteriophage communities in sheep rumen [21]. This approach is now just beginning to be applied in aquatic microbial ecology [17, 23]. In this communication we describe the application of this technique in the marine

environment and illustrate the information obtainable, as well as potential applications, to aquatic viral ecology.

Materials and Methods

Sample collection and concentration

Surface seawater samples were collected with Niskin bottles from two stations (ADR1 and ADR2) in the Gulf of Trieste, Italy, and by hand submersion of a polycarbonate carboy from Scripps Pier, San Diego, CA. Samples (5-10 l) were pressure filtered through 0.2 µm pore-size capsule filters (Gelman) then concentrated to 100-150 ml using 100,000 nominal molecular weight cut-off (MWCO) membrane (200 cm^2) in a Benchmark® vortex flow filtration system (Membrex). A sample (3 l) from Monterey Bay, CA, USA was collected with a Niskin bottle, filtered through a syringe filter with a peristaltic pump (Sterivex, Millipore) and concentrated on a 30K MWCO spiral wound ultrafiltration membrane (Amicon) to ca. 150 ml final volume. Primary concentrates were stored refrigerated with 0.01% sodium azide as a preservative to prevent bacterial growth. Primary concentrates were further concentrated using 50K MWCO centrifugal ultrafiltration devices (Centricon Plus-20; Amicon).

PFGE

For samples from the Gulf of Trieste and Scripps Pier, viruses were pelleted by ultracentrifugation for 2 hr at 30,000 rpm and 20°C in a Beckman SW60 rotor. DNA was recovered from the pellets by addition of 0.5X TBE and heating to 60 °C for 10-15 min. For the Monterey Bay concentrate, an aliquot was rinsed three times with 0.5X TBE by dilution and reconcentration in a 50K MWCO centrifugal ultrafiltration unit (Microcon 50; Amicon). The recovered concentrate in 0.5X TBE was heated to 60°C for 10-15 min. DNA in each sample was quantified by fluorometry with PicoGreen (Molecular Probes). DNA samples were diluted to 45 µl with sterile H_2O and 5 µl of loading buffer added. A lambda HindIII restriction digest, a lambda ladder, and a nominal 5 kb ladder (BioRad) were used as molecular weight markers. Bacteriophages Ø21/SP50, Ø14/SP67 and Ø21/SP43 were isolated from Scripps Pier (Steward, unpublished data). Gels were run at 14°C, 6V/cm and a 120° included angle for 16 hr with a 1-6 sec switch time on CHEF DR II or DR III PFGE units (BioRad).

Analysis

Gels were stained with SYBR Green I (Molecular Probes), and photographed with Polaroid film or scanned with a laser fluorometer (Molecular Dynamics). Digital images were obtained from Polaroids using a flat bed reflectance scanner (DuoScan, AGFA). Analysis of digital images was performed using RFLPScan software (Scanalytics) to quantify band position and intensity.

Results and Discussion

Separation of viral genomes of three marine bacteriophage isolates, as well as DNA from several natural samples by PFGE, is shown in Fig. 1. Viral DNA in natural samples is only operationally defined as that which passes a 0.2 µm filter, is retained on a 30,000 or 100,000 MWCO membrane and has a sedimentation coefficient greater than 100 S. Tests on other samples have demonstrated that most or all of the DNA in this fraction is in a

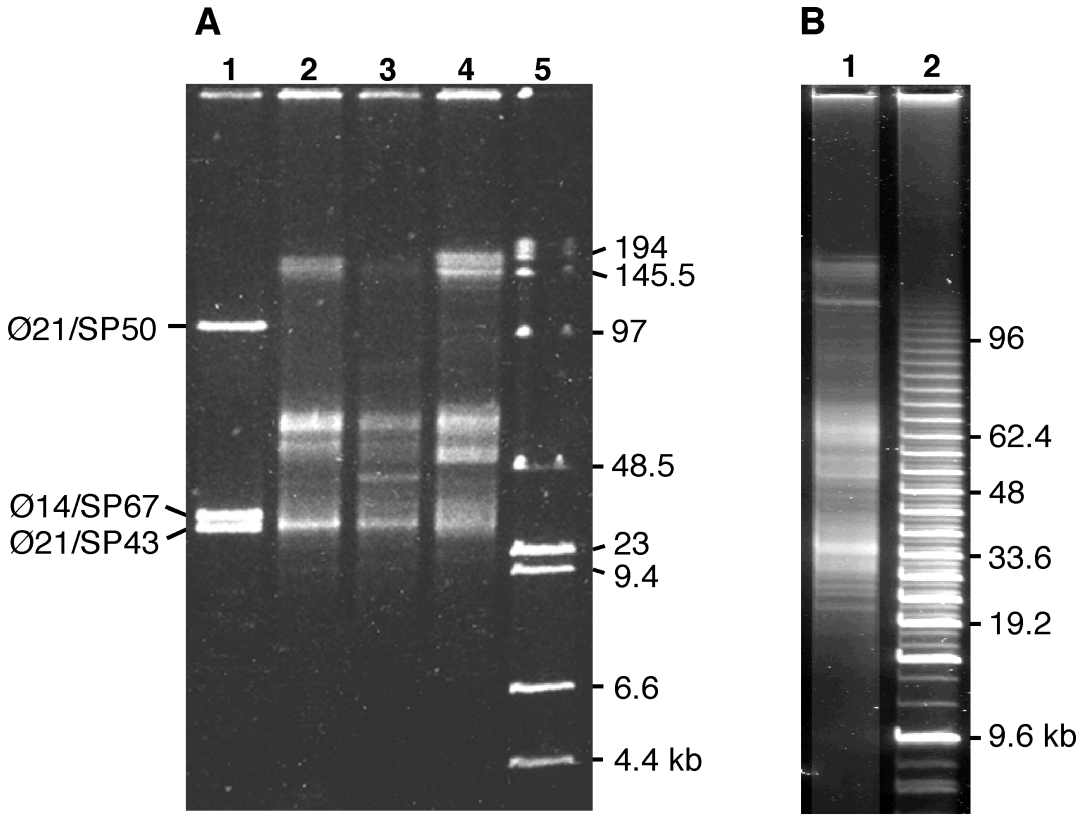

Fig. 1 DNA banding patterns obtained by PFGE. (A) Gel showing banding patterns of DNA from a mixture of three marine bacteriophage isolates (lane 1) as well as viral concentrates from natural seawater samples collected at Gulf of Trieste stations ADR1 (lane 2) and ADR2 (lane 3) and at Scripps Pier (lane 4). The molecular weight marker was a mixture of a lambda ladder and a lambda/HindIII restriction digest (lane 5). (B) Gel showing banding pattern of DNA from a viral concentrate collected in Monterey Bay (lane 1) and a molecular weight ladder with 4.8 kb steps (lane 2).

form which also behaves like viruses in terms of DNase susceptibility and buoyant density (unpublished data). However, the presence of non-viral DNA which behaves like viral DNA and falls in the same size range, has not been conclusively ruled out [11]. Therefore, this DNA will be referred to as virus-like genomes (VLGs).

Separation of a mixture of three phage isolates in a single lane demonstrates the ability of PFGE to discriminate between viruses having different genome sizes (Fig. 1A). Different DNA banding patterns for three different natural seawater samples run on the same gel implies differences in viral community composition. In these samples between 14 to 18 bands could be readily distinguished per sample providing a minimum estimate of diversity. It is quite likely that much of the genetic diversity in a sample is not resolved by this method since many different viruses can have the same or very similar-sized genomes. Nevertheless, the resulting fingerprint is a relatively quick and convenient way of quantitatively characterizing a complex viral assemblage with finer resolution than is practical by TEM. Even higher diversity was detected in a sample from Monterey Bay (Fig. 1B) in which at least 35 bands could be resolved. Some of this apparent higher diversity may be due to the improved resolution afforded by direct fluorescent scanning.

The molecular weight and DNA content of each band was determined by image analysis and comparison with standards. From the molecular weight and DNA content, the number of putative genome copies was then calculated. The percent of the total detected VLGs present in each band is presented as a histogram in Fig. 2. Although differences are evident, the overall similarity in distributions is also striking. The distributions are essentially bimodal with peaks in VLGs occurring around 30 and 60 kb, although one sample also shows an additional peak at 45 kb. Other minor peaks are distinguishable in the Monterey Bay sample. From the mode of these distributions, the most common size of VLG was found to be 30-35 kb, while the mean size for the assemblages ranged from 45 to 56 kb.

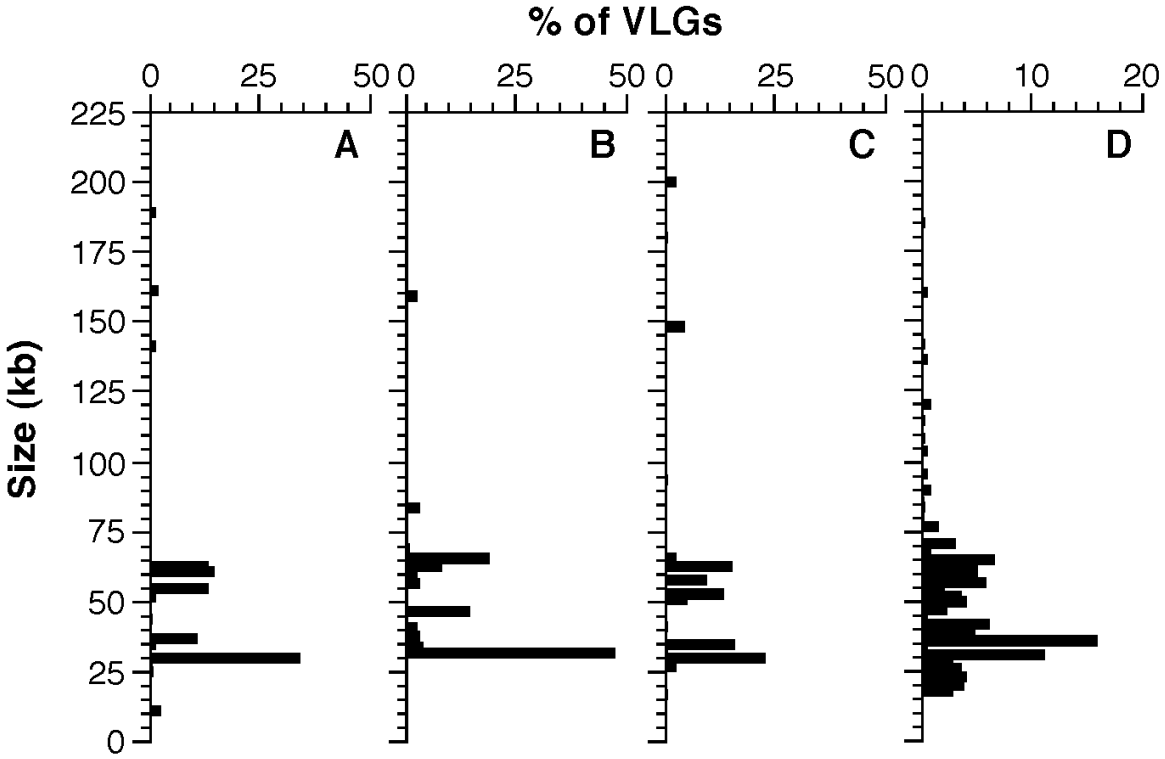

Fig. 2. Histograms showing percent of virus-like genomes (out of approximately 10^9 total detected) in different size classes for each of the four seawater samples in Fig. 1: (A) ADR1, (B) ADR2, (C) Scripps Pier, and (D) Monterey Bay.

An advantage of this technique over PCR-based fingerprints is the avoidance of potential amplification biases. However, it should be kept in mind that there is potential for some biased losses during sample preparation. In particular, filtration can result in variable losses in total virus numbers [16]. Whether this significantly alters the relative community composition has yet to be determined. PFGE fingerprints may ultimately aid in answering this question. Although the results presented have not specifically addressed the potential occurrence of RNA vs. DNA and single vs. double stranded viral genomes, all of these genome types are amenable to fingerprinting by this type of approach. An attractive feature of this fingerprinting technique is that entire viral genomes can be excised from the gels for further analyses such as PCR amplification, cloning, and/or sequencing. In this way, the potential diversity hidden within any particular band can be further explored, and bands of ecological interest (e.g., those suggesting particularly

dominant or cosmopolitan viruses) can be identified and targeted for future ecological studies [24]. This fingerprinting approach should thus prove complementary to existing techniques for investigating aquatic viral ecology.

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