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Pulsed-field gel electrophoresis analysis of virus assemblages present in a hypersaline environment

Summary A method for analyzing virus assemblages in aquatic environments was developed and used for studying the highest-salinity ponds (from 13.4 to 35% salinity) from a multi-pond solar saltern in Alicante, Spain. The protocol consisted of a series of concentration and purification steps including tangential flow filtration and ultracentrifugation, followed by the preparation of total viral nucleic acids that were subsequently separated by pulsed-field gel electrophoresis. For every sample analyzed, a characteristic DNA pattern was obtained, whose complexity was related to viral diversity. The comparison of our results with a similar analysis carried out with marine virus assemblages shows that, as expected, the viral diversity corresponding to the analyzed hypersaline environment is considerably lower than that of a marine environment.

Key words Virus assemblages · Halophage · Haloarchaea · Pulsed-field gel

Introduction

The discovery that free viruses are abundant in the plankton in most marine environments [3, 6, 12] has led to an increasing interest in the study of marine viruses. Most studies on virus assemblages, however, have been carried out by electron microscopy [5, 11], which provides little information about virus identity, since morphology is not a very informative trait. To understand the complexity of marine virus assemblages, it is necessary to know both their composition in natural environments and the relationship between the diversity of viruses and that of their hosts (prokaryotic diversity in many cases). Recently, other authors [18, 20] have applied pulsed-field gel electrophoresis (PFGE) to the study of virus assemblages in marine environments. A similar approach was used in the study of ruminal bacteriophage populations [7, 19]. PFGE allows separation of the viral genomes according to their size. The band pattern obtained from a given community, therefore, provides a fingerprint of its viral assemblage. The number of bands is a minimal estimation of the number of different viruses, and the band patterns of different samples can be compared. This application of PFGE, however, is still in its infancy and requires testing in different environments.

We have developed a protocol for the analysis of the viral assemblages present in high-salinity ponds from a multi-pond

solar saltern. Multi-pond solar salterns consist of a series of shallow ponds connected in a sequence of increasingly saline brines, used for the commercial production of salt from seawater. During evaporation of seawater, sequential precipitation of calcium carbonate, calcium sulfate and, finally, sodium chloride occurs. The microbiology of this system has been extensively studied by both molecular and culture-based techniques [2, 8, 15, 16].

Over 30% salinity, the domain of NaCl saturated waters occurs, in which few primary producers (at least based on chlorophyll *a* determinations) are detected. The abundant (up to 10⁸ cells/ml) halophilic archaeal populations found here, with doubling times established as somewhere between 2 and 50 days, probably live on the allochthonous organic matter carried over from ponds of lower salinities [6, 10]. Prokaryotic diversity decreases to the point that the most saline ponds were described as almost monospecific cultures of halophilic archaea [13]. More recent molecular studies have also revealed an extremely low prokaryotic diversity in these ponds [2].

Prokaryotic and viral abundances along the salinity gradient in a multi-pond solar saltern from Alicante (Spain) have been measured [6, 10]. These authors found that total counts of prokaryotes increased with salinity from 10⁷ cells/ml in the less saline pond (3.8% NaCl) up to 10⁸ cells/ml in the most saline pond (37%), whereas abundance of virus-like particles (VLP) increased from 4×10⁸ up to 2×10⁹ VLP per ml in the same range

of salinities. It has been suggested that viruses might play a major role in the decline of halophilic archaeal communities in the hypersaline Dead Sea [9]. In the solar saltern from Alicante [6], however, viruses were not found to exert a strong control over the prokaryotic abundance and growth rate.

Although prokaryotic and VLP abundance were significantly correlated in the solar saltern from Alicante, whether there is a correlation between viral and prokaryotic diversities remains unknown. If such a correlation occurred, one could expect a decrease in viral diversity along the salinity gradient. To ascertain whether this was the case, our approach was to explore viral diversity by studying the genome size distribution of the viral assemblage in different ponds. Our protocol consisted of several steps of virus purification and concentration and the final preparation of intact nucleic acids from the viral assemblage that were subsequently separated by pulsed field gel electrophoresis.

Materials and methods

Sampling Samples were collected on January (JA), April (AP) and July (JU) 1996 from the multi-pond saltern "Braç del Port" located in Santa Pola (Alicante, Spain, 38°12' N, 0°36' W). Three ponds were sampled: two concentration ponds (CO-72 and CO-108) and a crystallizer (CR-30). A total of six samples were taken: CR30-JA, CR30-AP, CO108-AP, CR30-JU, CO108-JU and CO72-JU. All the samples were taken a few centimeters below the water surface using 10-liter acid-washed polypropylene bottles. The corners of the square ponds were avoided, since the wind accumulates organic matter scums and debris in those areas. Salinity was measured with a hand refractometer (Atago S-28).

Virus purification and concentration The protocols used for virus concentration included removal of eukaryotic and prokaryotic cells, and concentration of viruses by tangential flow filtration (TFF) [1] and ultracentrifugation. In the first tested protocol, 20 liters of water from the solar salterns was filtered through Millipore-AP20 filters (3 µm effective pore size) using positive filtration pressure, to remove eukaryotic cells. Prokaryotic cells were additionally removed by TFF with a Pellicon System (Millipore) provided with a 0.45 µm filter cassette (HVMP0000C5). Viruses contained in the cell-free filtrate were concentrated by TFF in the same system with a 100,000 Da filter cassette (PTHK00005). The virus containing retentate was subjected to ultracentrifugation (45,000 rpm, 6 hours) using a TFF 70 rotor in a Beckman XL80 ultracentrifuge. The pellet was resuspended in 25% artificial sea water (NaCl 195 g/l, MgCl₂·6H₂O 34.6 g/l, MgSO₄·7H₂O 49.5 g/l, CaCl₂ 0.73 g/l, KCl 5 g/l, NaHCO₃ 0.17 g/l, NaBr 0.67 g/l, [14]) and filtered through a disposable 0.45 µm filter (Sartorius) coupled to a 10 ml syringe, to eliminate any remaining cells. This virus suspension was kept at 4°C until needed.

Three modifications to the basic protocol were tested: **Modification A:** The water sample, free of eukaryotic cells, was passed through a 142 mm Millipore 0.22 µm-pore-size GV Durapore filter using positive filtration pressure rather than TFF through the 0.45 µm filter cassette. **Modification B:** a 10,000 Da (PTGC00005) cassette was used for the concentration of virus by TFF instead of one of 100,000 Da (PTHK00005). **Modification C:** the ultracentrifugation time was raised from 6 to 24 hours.

Enumeration of viruses by TEM For every step in the concentration protocol an aliquot was taken and fixed, and the viral abundance was determined by transmission electron microscopy (TEM) [3, 4, 6].

Isolation and manipulation of viral DNA DNA for pulsed-field gel electrophoresis (PFGE) [17] was prepared using the following protocol: the virus pellet obtained in the final ultracentrifugation step was resuspended in 25% artificial seawater. 1 ml of this suspension was mixed with 1 ml of 1.6% agarose (pulsed field certified, BioRad) in sterilized distilled water, distributed in 100 µl moulds and allowed to solidify for 30 min at room temperature. The agarose plugs, containing the virus suspension, were removed from the moulds and incubated in ESP (0.5 M EDTA, pH 9, 1% N-laurylsarcosine, 1 mg/ml proteinase K) overnight at 50°C. Once the virus capsids had been disrupted by the ESP treatment, the agarose plugs were stored in ESP at 4°C until electrophoresis could be carried out. Before enzymatic treatment (see below) the plugs were extensively washed with TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) to eliminate completely the ESP.

Viral DNA was used for PFGE either in a Pharmacia LKB Navigator or in a BioRad Chef-DRII, using 1% LE agarose (FMC) and 0.5× TBE buffer. Running conditions were modified to separate optimally the different DNA bands. Lambda ladder and Low Range PFGE marker (New England Biolabs) were used as size standards. Viral DNA was treated with RNase and DNase by incubating the agarose plugs, previously washed with TE, in a solution containing 1 µg/ml of DNase or RNase. Incubations were carried out at 37°C for 30 minutes.

Viral DNA included in agarose plugs was treated with restriction enzymes (15). Fifteen different restriction enzymes were assayed: *ClaI*, *DraI*, *BclI*, *Sau3AI*, *XbaI*, *HaeIII*, *RsaI*, *KpnI*, *EcoRI*, *BglI*, *BamHI*, *PstI*, *SalI*, *HindIII*, *SfiI* (New England Biolabs). Agarose plugs containing viral DNA were washed with TE buffer and the DNA was purified with Gene Clean Spin Kit (BIO101 Inc., California) according to the manufacturer's recommendations. Comparison of DNA concentration among different DNA samples was carried out by agarose gel electrophoresis and ethidium bromide staining. Equivalent amounts of DNA (from 5 to 25 µl in a total volume of 100 µl) of each sample were applied into a positively charged nylon membrane (Hybond, Amersham). DNA was immobilized using a UV crosslinker (Hybond, Amersham) following the

manufacturer's recommendations. Viral DNA obtained from CR-30 JA was purified with Gene Clean Spin Kit (BIO 101 Inc., California) labeled with digoxigenine following the manufacturer's protocol (Boehringer Mannheim) and hybridized against the dot blot membrane obtained as described above. Hybridization was carried out as recommended by the manufacturer for maximum stringency conditions.

Results

Virus concentration and purification Viral abundance throughout the different purification steps and morphology were determined by TEM (Fig. 1). The virus fraction recovered at the end of the process presented the same morphologies found in the original water samples. Most viruses observed by TEM had a head diameter ranging from 50 to 90 nm. At least two morphologies were found: icosahedral (head diameter around 60 nm) (Fig. 1A) and lemon shaped viruses (head diameter around 90 nm) (Fig. 1B).

The variations in VLP concentrations throughout the different steps in the assayed concentration protocol and the total amount of viruses recovered in every step were obtained multiplying the virus concentration by the total volume of the sample at the considered step in the protocol. A summary of these results is shown in Table 1. In every step of the "basic" protocol, from the removal of the eukaryotic cells by filtration to the ultracentrifugation of the virus suspension, there was a considerable loss of viruses. When, to remove prokaryotic cells, we replaced TFF through 0.45 μm filter cassette by filtration through 0.22 μm filters (which we have called modification A), the virus recovery efficiency for this step increased. However, this modification slows down appreciably the concentration process because it takes around 2 hours to filter 1 liter of water from the most saline ponds, even when eukaryotic cells have been previously removed. Another critical step in the protocol, involving very high virus losses, was the concentration with TFF using 100,000 Da filters. When the 100,000 Da filter cassette was changed to one of 10,000 Da (modification B), the recovery did not improve. On the contrary, the recovery efficiency for this step decreased. As expected, ultracentrifugation was also critical. Increasing the ultracentrifugation time from 6 to 24 hours (modification C) yielded a significantly higher recovery of viruses.

Analysis of viral DNA Treatment of viral nucleic acids with RNase-free DNase yielded total digestion of the sample whereas treatment with DNase-free RNase produced no degradation (data not shown). These results indicated that the viral nucleic acids isolated with our protocol were DNA.

Figure 2 shows some examples of DNA band patterns obtained by PFGE for the analyzed samples. Size ranged from 20 to more than 300 kb. To separate the different bands in each sample, several PFGE conditions (pulse time, electric field

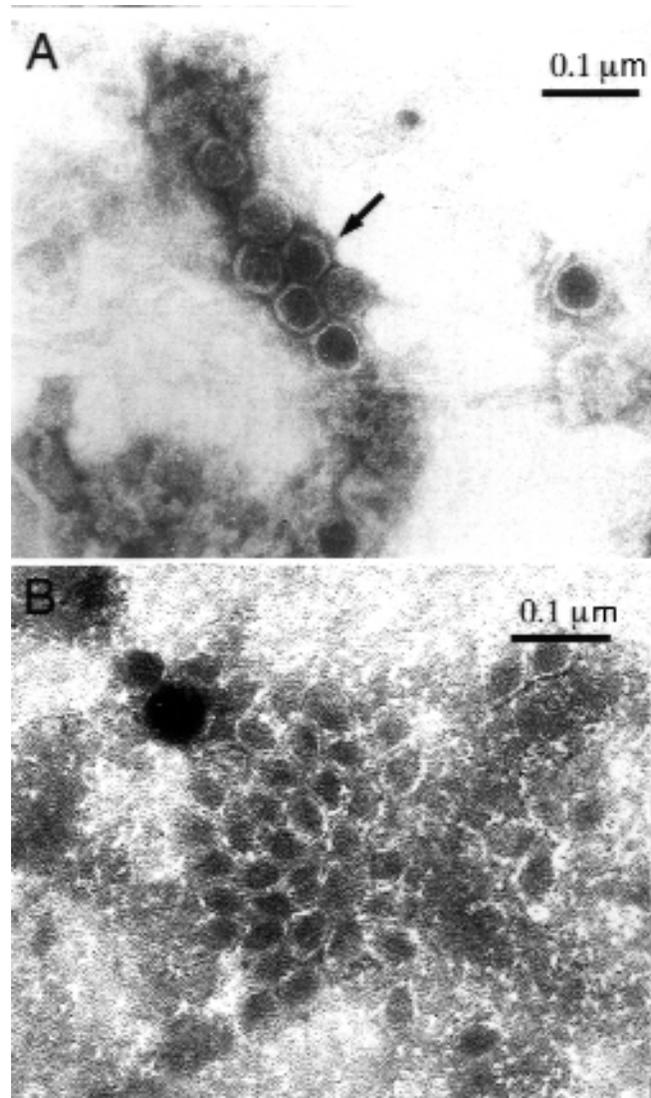


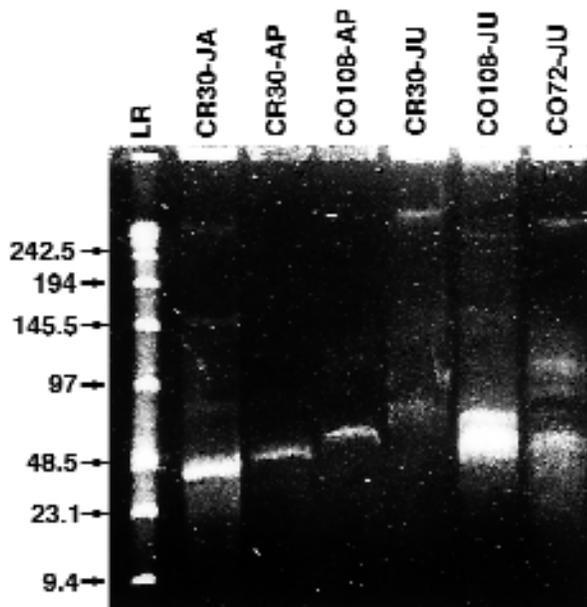
Fig. 1 Micrographs showing virus-like particles after the purification-concentration protocol. (A) Sample CR30-JA indicating the icosahedral virus morphology (arrow, head diameter around 60 nm). (B) Sample CO72-JU

strength, run duration) were assayed. The best separation was achieved under the following conditions: 6 V/cm, 16 hours, 14°C, and a pulse ramp from 1 to 15 s.

For a given sample, the band pattern was very reproducible (data not shown), even when the sample was stored at 4°C for months before being treated for PFGE analysis. This result indicates that the patterns obtained are not influenced by the preparation of DNA. For all samples, several discrete DNA bands (1 to 8) of different intensities were separated by PFGE (Table 2). In most cases, the most intense bands had an apparent size of around 40 kb. It cannot be ascertained whether these bands were unique and, therefore, corresponded to the most abundant viral populations or whether there were different bands with very similar molecular weights.

Table 1 Comparison of the assayed protocols for purification and concentration of viruses

Sample	Initial volume (liters)	Final volume (ml)	Modification to the basic protocol	Initial viral abundance (10 ¹⁰ VLP/ml)	Final viral abundance (10 ¹⁰ VLP/ml)
CR30-JA	20	4	(I/N)	0.054	3.62
CR30-AP	12	5	B, C	0.339	1.68
CO108-AP	12	5	B	0.260	3.64
CR30-JU	15	5	A, C	0.160	6.24
CO108-JU	15	5	A, C	0.219	4.39
CO72-JU	15	5	A, C	0.211	6.73

**Fig. 2** Pulsed-field gel electrophoresis separation of viral DNA from different samples. Running conditions: 6 V/cm, 16 hours, and a pulse ramp from 1 to 15 seconds. LR: Low-Range PFGE molecular marker (sizes in kb). Notation as in Table 1

Viral DNA was resistant to 8 of the 15 restriction enzymes assayed. In some cases, the endonuclease treatment yielded a DNA smear (Fig. 3A, restriction with *DraI*; Fig. 3B, restriction with *BclI*; Fig. 3C, restriction with *ClaI*), most likely indicating that restriction target was present in the DNA. In another case (Fig. 3A, restriction with *BclI*) the digestion yielded discrete new bands: at least two restriction products of about 105 and 110 kb.

When viral DNA from CR30-JA was hybridized against viral DNA from the rest of the samples, the strongest hybridization signals were obtained for DNAs purified from the same pond, whereas the signals for the DNAs obtained from the rest of the ponds were appreciably weaker (Fig. 4).

Discussion

The viral DNA size range found corresponds to that of the halophages studied so far [9]. Size range for halophage genomes has been reported to be from 29.4 to 230 kb, whereas we found DNA species with an apparent size ranging from 25 to about 300 kb. Note that we use the term "apparent size", because more studies are needed to ascertain whether the DNA

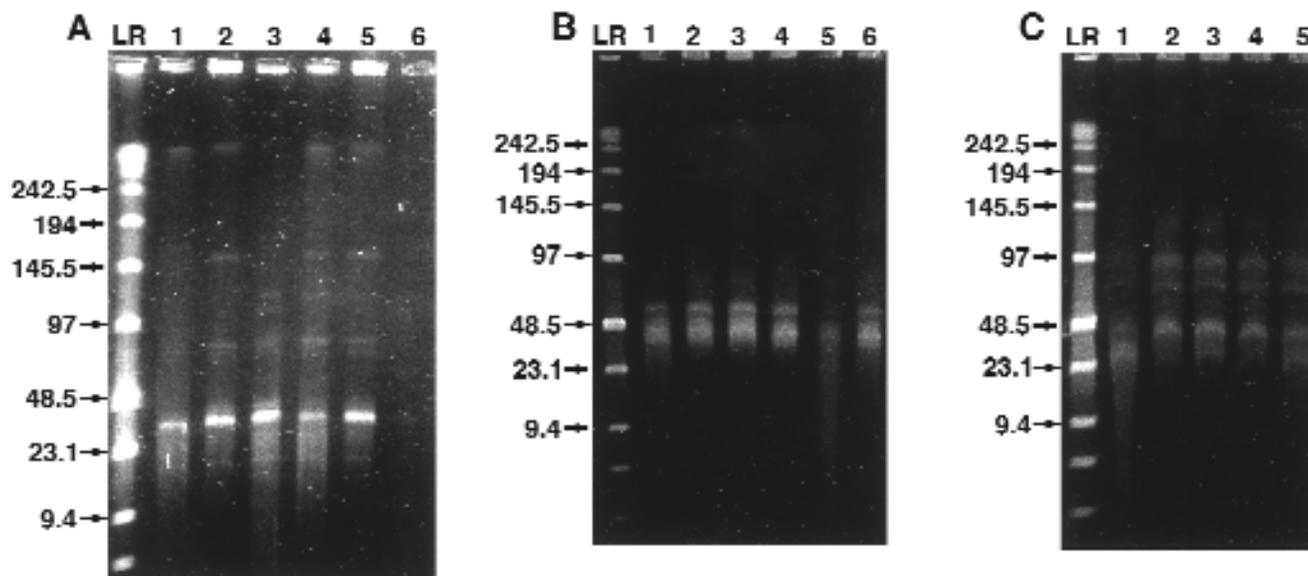
**Fig. 3** Restriction analysis of different viral DNA samples. (A) DNA from CR30-JA digested with: *DraI* (1), undigested (2), *BclI* (3), *ClaI* (4), undigested (5), *Sau3AI* (6). (B) DNA from CO108-JU digested with: *ClaI* (1), undigested (2), *HaeIII* (3), undigested (4), *BclI* (5), *XbaI* (6). (C) DNA from CO72-JU digested with: *ClaI* (1), undigested (2), *HaeIII* (3), undigested (4), *BclI* (5). LR: Low-Range PFGE molecular marker (sizes in kb)

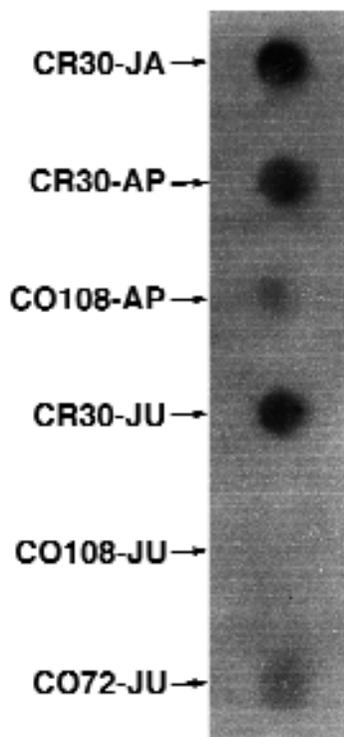
Table 2 Minimal number of bands in the viral DNA patterns from different samples

Sample	Salinity (‰)	Minimal number of DNA bands
CR30-JA	350	8
CR30-AP	316	1
CO108-AP	190	2
CR30-JU	350	3
CO72-JU	230–240 ^a	8
CO108-JU	134	7

^aThe salinity of this pond was not measured but had been previously registered [8].

molecules we separated by PFGE were circular or linear. Our results, however, suggest they were linear, since their electrophoretic mobility was always proportional to that of the linear size standards used in our experiments. This range of values was slightly wider than the ranges found by other authors [18, 20]. The minimal number of DNA bands observed for every sample analyzed is shown in Table 2. These values are just an estimation since in many cases (see Fig. 2) we observed fuzzy rather than clear DNA bands. Most likely, these fuzzy bands corresponded to a group of viruses with genomes of similar but unequal sizes. The minimal number of bands found in the crystallizer samples was similar to the number of different morphologies detected by TEM in this pond: icosahedral with head 20–30 nm, icosahedral with head 40–60 nm and lemon-shaped. These morphologies could correspond to more than one virus. The band patterns obtained for the different ponds, as well as hybridization analysis of viral DNA extracted from the different ponds, indicate that different ponds harbor different viral assemblages, which is consistent with the variation of the prokaryotic assemblage along the salinity gradient [15]. Although changes in the number of bands along the gradient were small, our results are consistent with the expected trend of decreasing bands with increasing salinity, when samples concentrated with the same protocol are compared. When the optimal protocol was used in samples from July, fewer bands were retrieved from the crystallizer (CR30-JU) than from the concentrators (CO108-JU and CO72-JU) (see Table 2).

PFGE has been used to analyze natural variability and diurnal fluctuations of the bacteriophage populations of the rumen [19]. In this case, the DNA pattern obtained was less sharp than the ones we show, probably due both to the large number of virus (ca. 10^{10} /ml) and to the fact that diversity in ruminal fluid was wider than in the salterns. The composition of Chesapeake Bay viroplankton has also been studied through the analysis of PFGE patterns [20]. These authors found that PFGE fingerprints contained an average of 11 bands, ranging from 7 to 16 bands. Stewart and Azam [18] found 14 to 35 bands in four marine samples. These values are significantly higher than the ones we found in the salterns (from 1 to 8 bands). Therefore, assuming that the number of bands in the PFGE pattern reflects viral diversity, our results indicate that the viroplankton in the high-salinity ponds is less diverse than that from other marine environments, such as Chesapeake Bay,

**Fig. 4** Autoradiogram of CR30-JA digoxigenine labeled viral DNA hybridized with viral DNA samples from different ponds

the Gulf of Trieste and Monterey Bay. This fact is in accordance with the low prokaryotic diversity found in these highly-saline ponds [15]. The main drawback of the technique is the number of viruses lost in the different concentration steps. Our study, however, shows PFGE to be a useful technique to study viral composition in a wide range of aquatic environments.

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