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ORIGINAL PAPER

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Virioplankton community structure along a salinity gradient in a solar saltern

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Abstract The virioplankton community structure along a salinity gradient from near seawater (40‰) to saturated sodium chloride brine (370‰) in a solar saltern was investigated by pulsed-field gel electrophoresis. Viral populations with genome sizes varying from 10 kb to 533 kb were detected. The viral community structure changed along the salinity gradient. Cluster analysis of the viral genome-banding pattern resulted in two main clusters. The virioplankton diversity within the samples with salinity from 40‰ to 150‰ was on the same cluster of a cladogram. The other group consisted of virioplankton from samples with salinity above 220‰. The virioplankton diversity in the different samples was calculated using the Shannon index. The diversity index demonstrated an increase in diversity in the samples along the gradient from 40‰ to 150‰ salinity, followed by a decrease in the diversity index along the rest of the salinity gradient. These results demonstrate how viral diversity changes from habitats that are considered one of the most common (seawater) to habitats that are extreme in salt concentrations (saturated sodium brine). The diversity index was highest in the environments that lie in between the most extreme and the most common.

Keywords Diversity · Pulsed-field gel electrophoresis · Salinity gradient · Solar saltern · Virioplankton

Introduction

Solar salterns are used for commercial production of salt from seawater and provide a range of environments with different salinities, from that of seawater up to sodium chloride saturation. During evaporation of seawater, sequential precipitation of calcium carbonate, calcium sulfate, and finally sodium chloride occurs. As water evaporates and salinity increases, water is pumped or fed by gravity to the next ponds. The different ponds in the solar salterns are in this way under constant conditions, enabling the microbial populations to reach equilibrium in each pond (Rodríguez-Valera 1988).

Limited species diversity, high cell abundance, and short food chains make the system simple and presumably easier to analyze than a more diverse marine environment (Pedrós-Alió et al. 2000). Several studies have been carried out in the Bras del Port solar saltern in Santa Pola (Spain) (Rodríguez-Valera et al. 1981, 1985; Benlloch et al. 1996; Guixa-Boixareu et al. 1996; Casamayor et al. 2000). These studies have shown that the increase in salinity along the gradient is accompanied by a decrease in prokaryotic diversity, from the marine biota to the dense populations of halophilic Archaea found in the crystallizers with salinity above 300‰ (Benlloch et al. 1995; Antón et al. 1999; Rodríguez-Valera et al. 1999; Antón et al. 2000; Casamayor et al. 2000; Oren 2002). However, it has recently become clear that Bacteria may also contribute to the aerobic heterotrophic prokaryotic community at the highest salt concentrations (Antón et al. 2000; Oren and Rodríguez-Valera 2001).

We still know little about factors responsible for the death of halophilic Archaea and Bacteria in their natural environment. Protozoa have never been encountered in large numbers, if at all, in saltern crystallizer ponds (Oren 2002). Death by lysis due to bacteriophages, however, may occur (Guixa-Boixareu et al. 1996; Pedrós-Alió et al. 2000). Investigating the number of virus-like particles along the salinity gradient in two solar salterns showed that the number increased with increasing salinity, from 10^7 to 10^9 virus particles ml^{-1} (Guixa-Boixareu et al. 1996). In the hypersaline Dead Sea as well, virus particle numbers higher than bacterial numbers by a factor of 0.9 to 9.5 have been reported (Oren et al. 1997).

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Compared to prokaryotic diversity, knowledge about the virioplankton community structure in these ponds is scarce. Virus-like particles along the salinity gradient in two solar salterns have been characterized by shape using electron microscopy (Guixa-Boixareu et al. 1996). The viral abundance along the salinity range from 134‰ to 370‰ has also been investigated using pulsed-field gel electrophoresis (PFGE), demonstrating that the number of dominant viral populations in a hypersaline environment is considerably lower compared to a marine environment (Diez et al. 2000). In the present study, we investigate the viral community assemblage from seawater (40‰) up to sodium chloride precipitation (370‰) in a solar saltern, using PFGE. Samples were collected twice, with a 6-day interval, to test the short-time stability of the virioplankton community structure in the saltern systems.

Materials and methods

Water samples of 500 ml were collected from the multipond marine solar saltern "Bras del Port" located in Santa Pola (Alicante, Spain). For analysis of the virioplankton community structure along a salinity gradient, samples were taken from eight ponds (40, 54, 80, 110, 150, 220, 316, and 370‰). The same sites were sampled 6 days later to investigate the short-time stability of the virioplankton community in the solar saltern.

Larger particles were removed from the sample by low speed centrifugation (Beckman J2-HS, swing-out centrifuge) at 7,500 rpm for 30 min at 4 °C. The supernatant was decanted and the procedure repeated once. This step was performed to avoid clogging the tangential flow module used in the concentration step and to remove most of the bacteria from the samples. The 500-ml sample was then concentrated down to 35 ml using the Vivaflow 200 (Vivascience, Lincon, UK) tangential flow module, with a molecular weight cutoff of 100,000 daltons, following the manufacturer's procedure. The virus particles in the 35-ml sample were subsequently concentrated by ultracentrifugation (Beckman L8-M with SW-28 rotor) for 2 h at 28,000 rpm at 10 °C. The virus pellet was resuspended and incubated overnight in 200 μl SM buffer [0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, 0.005% (w/v) glycerol, pH 8.0] (Wommack et al. 1999) at 4 °C.

Equal volumes of virus concentrate and molten 1.5% InCert agarose (FMC, Rockland, Me.) were mixed and dispensed into plug molds. One sample was separated into three plugs, and each plug represented viruses found in a total volume of 167-ml sample. After the gel had solidified, plugs were punched out from the molds into a small volume of buffer (250 mM EDTA, 1% SDS) containing 1 mg/ml Proteinase K. The plugs were incubated in the dark at 30 °C overnight. The Proteinase K digestion buffer was decanted and the plugs were washed three times, 30 min each, in TE buffer (10 mM Tris-Base; 1 mM EDTA, pH 8.0). The virioplankton agarose plugs were stored at 4 °C in TE 20:50 (20 mM Tris, 50 mM EDTA, pH 8.0).

Plugs containing phage lambda concatamers (Bio-Rad, Richmond, Calif.) and *Hin*dIII-digested lambda fragments (Promega, Madison, Wis.) served as molecular weight markers. These and the viral plugs were placed into wells of a 1% SeaKem GTG agarose gel (FMC, Rockland, Me.) in 1X TBE gel buffer (90 mM Tris-borate, and 1 mM EDTA, pH 8.0) with an overlay of molten 1% agarose. Three gels were run on each sample using a Bio-Rad DR-II CHEF Cell (Bio-Rad, Richmond, Calif.) electrophoresis unit operating at 6 V cm^{-1}, with different pulse ramps (1–10 s, 8–30 s, and 20–40 s), at 14 °C for 22 h in 0.5× TBE tank buffer (45 mM Tris-borate, and 1 mM EDTA, pH 8.0). After electrophoresis, the gels were stained for 30 min in SYBR green I (Molecular Probes Inc., Eugene, Ore.) according to the manufacturer's instructions and digitally scanned for fluorescence using a laser fluoroimager (Fuji Film, FLA2000). The band patterns, resulting from the different virus-like genomes, were analyzed using a computer program (GEL2 K, Svein Norland, Department of Microbiology, UoB, Norway). The intensity of a peak/band is calculated as the integrated pixel value (in the peak) above its background. By comparing the intensity and position of each band with the signal of a standard with known amount of DNA and a given molecular size, the relative number of virus-like genomes with different molecular sizes can be estimated. Using these numbers, the Shannon diversity index was calculated (Shannon 1948).

The dendrograms of the viral populations from the different ponds were constructed from a binary matrix of similarity values, using a distance calculation algorithm based on absence or presence of bands (Svein Norland, Department of Microbiology, University of Bergen, Norway). Clustering was based on the simple matching algorithm, while the dendrogram was drawn applying the complete-link method.

Results and discussion

The virioplankton PFGE banding patterns from the solar salterns revealed virus-like genomes ranging in size from 10 kb to 533 kb (Fig. 1). All the samples from the ponds with salinity gradients ranging from 40‰ to 220‰ contained virus-like genomes with sizes from 32 kb to 340 kb (Fig. 1). In addition, some virus-like genomes with sizes of 412, 440, 485, and 533 kb were detected in some of these samples. The ponds with salinities higher than 220‰ contained virus-like genomes with sizes ranging from 10 kb to 189 kb. Two subpopulations with virus-like genomes of 10 kb and 17 kb were detected in these samples only. The most dominant virus-like population detected in the hypersaline samples (> 150‰ salinity) had genome sizes of 32 kb and 63 kb. This corresponds with the reported genome sizes of hitherto described haloviruses, where all are double-stranded DNA viruses with an average genome length of 50 kb (Tang et al. 2002). However, it should be noted that only about 13 viruses of halobacteria have been reported so far, and just a few of these have been studied in more than preliminary detail (Tang et al. 2002).

The clustering analysis resulted in a dendrogram consisting of two main groups (I and II) (Fig. 2). Samples from ponds with salinities lower than 150‰ clustered together in one main group (I), while samples with salinities higher than 220‰ clustered together in the other group (II). Recent studies of viral populations have revealed dynamic viral, bacterial, and phytoplankton communities in seawater, indicating a close linkage between algal, bacterial, and viral populations (Castberg et al. 2001; Larsen et al. 2001; Øvreås et al. 2003). The changes in the virioplankton community structure seen in this study might thus reflect changes in both the prokaryotic and eukaryotic community structures. The sequential changes in the phytoplankton group along the salinity gradient in Bras del Port have been thoroughly described (Rodríguez-Valera 1988; Pedrós-Alió et al. 2000). They all show the disappearance of diatoms as salinity reaches 200‰ and

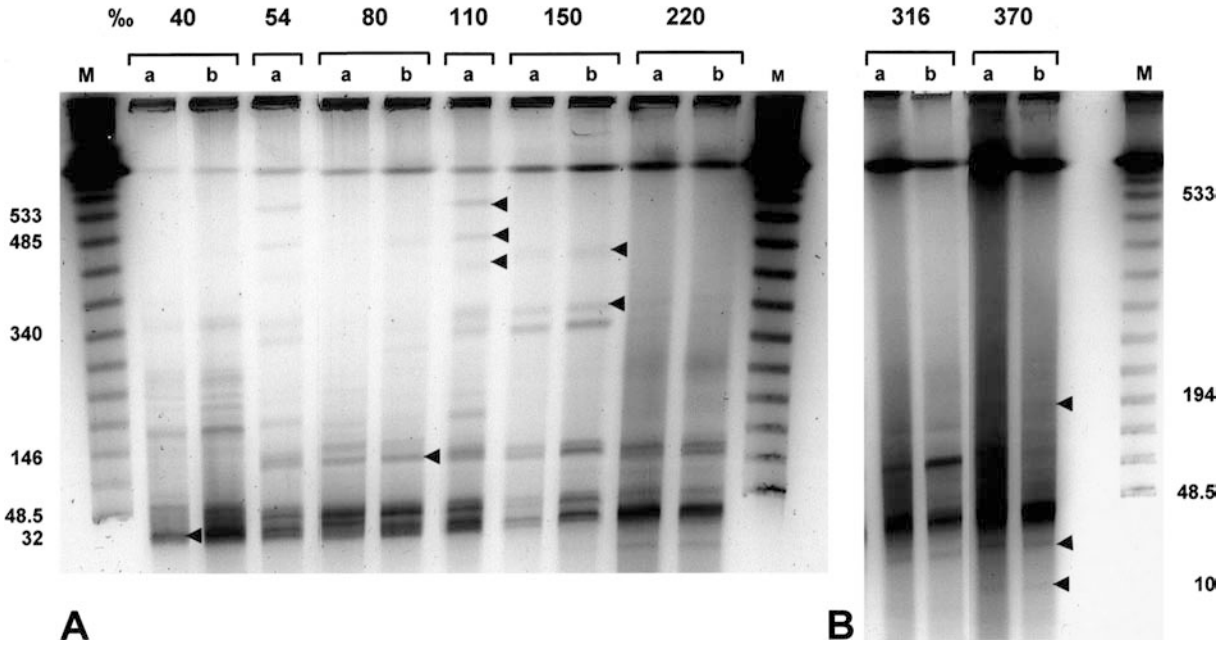

Fig. 1 PFGE profiles showing variations in the viral diversity along the salinity gradient from 40‰ to 220‰ **(A)** and 31.6‰ to 370‰ **(B)** in a solar saltern. **A** Samples taken 18 May 1999, **B** samples taken 6 days later. M: Molecular size standard, λ-phage concatamers. Numbers indicate genome size in kb. Bands of specific interest are marked with *arrows*

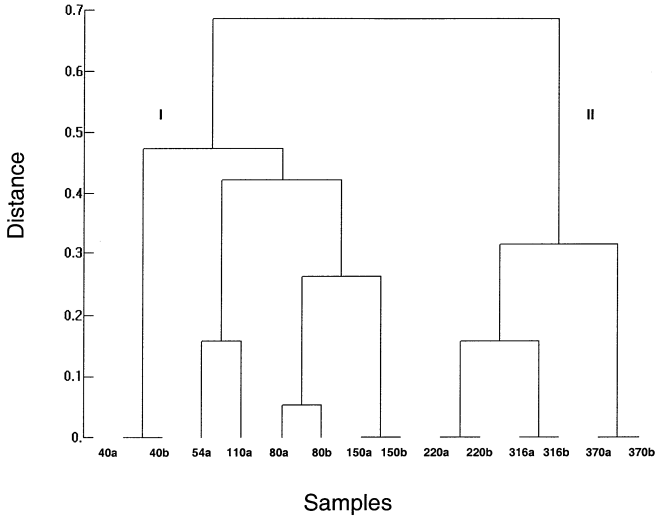

Fig. 2 Dendrogram showing the results of the cluster analysis performed on the PFGE viral banding pattern from samples with salinity ranging from 40‰ to 370‰. I and II refer to the two main groups formed by the cluster analysis. Clustering is based on the simple matching algorithm, while the dendrogram is drawn applying the complete-link method. All the *a* samples were collected 18 May 1999, while the *b* samples were taken 6 days later

predominance of cyanobacteria around 100‰. Between 150‰ and 300‰ salinity, the population of *Dunaliella* increases, with a peak around 250‰. Changes in culturable fractions of the prokaryotes at Bras del Port also have been studied in detail (Rodríguez-Valera et al. 1981). These studies show that halophilic bacteria are most predominant above 100‰ salinity. Moderate halophilic bacteria are the most abundant in the 100–200‰ salinity range, while extreme halophiles are most predominant above 250‰ salinity. Presence of both extreme halophilic Bacteria and Archaea in the crystallizers with salinity from 300‰ to 370‰ have been demonstrated by in situ hybridization (Antón et al. 1999, 2000). The changes seen in the total prokaryotic community structure at Bras del Port correspond to the changes seen in our study. The near-seawater samples, with a salinity of 40‰, comprised one of two subgroups in cluster I. This shows that even a small increase in salinity, from 40‰ to 54‰, results in a substantial change in the virioplankton community structure (Fig. 2). In the range from 54‰ to 150‰ salinity, several populations of virus-like genomes occurred (Fig. 1). The change in community structure, placing the 40‰ sample and the 54‰ sample in different clusters, may reflect changes in the prokaryotic community structure, where cyanobacteria increase in abundance, together with moderate halophilic bacteria (Rodríguez-Valera 1988; Pedrós-Alió et al. 2000). Between 150‰ and 220‰ salinity there is a marked change in the virioplankton community structure, as populations with virus-like genomes over 189 kb disappear, whereas virus-like genomes with sizes of 10 kb and 17 kb appear. In these ponds extreme halophilic prokaryotes and Archaea become more predominant (Rodríguez-Valera 1988; Antón et al. 1999, 2000).

Stability in the virioplankton community in the solar salterns was demonstrated by repeated sampling. No differences were seen in the samples collected from the same ponds 6 days later (Figs. 1, 2). The solar salterns have been reported to be a very stable environment, as each pond is considered to be at equilibrium and the biota consists of a well-adapted and established

community (Pedrós-Alió et al. 2000). The salt concentration in each pond is kept relatively high, and the microbial community densities are generally high. Although salterns are superficially similar all over the world, they do differ with respect to nutrient status and retention time of the water, depending on climatic conditions (Javor 1983).

The value of the Shannon diversity index increased along the salinity gradient from 40‰ to 150‰ and decreased again as the salt concentration increased further (Fig. 3). The highest value was found for the sample from the pond with 150‰ salinity (2.313). This may seem contradictory, as the highest numbers of different viral genome bands were found in the sample with 110‰ salinity. The reason for this might be that the Shannon index, in addition to the number of different species, also takes into account the population size distribution. The lowest value of the diversity index was found in the pond with highest salinity (1.043). Samples collected in the same pond 6 days later showed similar banding patterns; however, a variation was seen in the intensity of each band. This resulted in some differences in the diversity index of samples collected from the same pond (Fig. 3). There are difficulties in evaluating diversity by comparing number and density of bands for calculating indices and performing cluster analysis. Different viruses might have the same genome size and therefore might be indistinguishable on a PFGE gel. Another important issue is the difficulty of precise determination of the different genome sizes. To reduce this uncertainty, each sample was run three times, with focus on separation in different genome size ranges. By doing this it was possible to more correctly estimate the sizes of the different viral-like genomes. Another problem with this approach is that absence of bands does not mean that virus-like genomes with these sizes are totally absent from the sample. Using PFGE for viral diversity studies, there will be a threshold level where the bands no longer can be detected on the gel. The limit of detection will vary as a function of genome size, where viruses with larger genome sizes will result in a band with higher density compared to a virus with smaller genome size. The genome size will also affect the signal intensity in a band and hence the gel analysis. The viral-like genome sizes were taken into consideration in this study when calculating the diversity index. With all these limitations, it should be noted that the diversity indices presented in this study give only a rough estimate of the viral diversity; small changes in the community might not be noted.

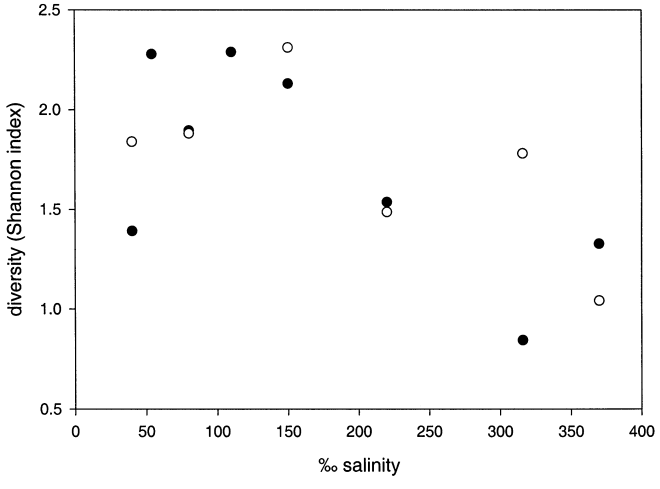

Fig. 3 Relationship between salinity and the Shannon diversity index. The diversity index is based on the number of different genomes and their abundance. Samples taken 18 May 1999 (●), samples taken 6 days later (○)

On average eight bands were detected in the samples on the PFGE gels. The lowest number of bands (4) was detected in the samples with highest salt concentrations, while the highest number of different bands (12) was found in the sample from the pond with 110‰ salinity (Fig. 1). The number of bands varied between 4 and 12, and the average number was 8 bands. This number is higher compared to the results of Diez et al. (2000), who reported a range of 1 to 8 bands, with an average of 5 bands. In their study, however, they only investigated the salinity range 134–350‰, revealing a decrease in the number of bands with increasing salinity. A similar decrease was seen in our study as the number of bands decreased from 12 in the sample with 150‰ salinity to 4 in the sample with 370‰ salinity. When using PFGE for investigating the virioplankton community assemblage in a seawater mesocosm, the number of bands ranged from 5 to 16, with an average of 11 bands (Castberg et al. 2001). In Chesapeake Bay as well, the virioplankton community was more diverse than the solar saltern systems, with an average of 11 bands (Wommack et al. 1999). All these results show that, overall, the number of bands in the solar salterns is lower compared to the marine environment; however, the number of virioplankton subpopulations present in the solar salterns ranging from 40‰ to 150‰ salinity harbor nearly as many virus-like genomes as the marine environment.

The increase seen in the diversity of the viral population in samples with salinity ranging from 40‰ to 150‰ probably reflects a diversity increase in the prokaryotic and eukaryotic community (Pedrós-Alió et al. 2000). Above 150‰ salinity, the prokaryotic and eukaryotic communities become less diverse, as salt intolerance leads to a reduction in diversity (Oren 1994, 2002; Casamayor et al. 2000; Pedrós-Alió et al. 2000). Likewise, the reduction in the viral diversity along the gradient from 150‰ to 370‰ salinity probably reflects a corresponding decrease in the host community.

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