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ORIGINAL ARTICLE

Metatranscriptomic analysis of extremely halophilic viral communities

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Hypersaline environments harbour the highest number of viruses reported for aquatic environments. In crystallizer ponds from solar salterns, haloviruses coexist with extremely halophilic *Archaea* and *Bacteria* and present a high diversity although little is known about their activity. In this work, we analyzed the viral expression in one crystallizer using a metatranscriptomic approach in which clones from a metaviromic library were immobilized in a microarray and used as probes against total mRNA extracted from the hypersaline community. This approach has two advantages: (i) it overcomes the fact that there is no straightforward, unambiguous way to extract viral mRNA from bulk mRNAs and (ii) it makes the sequencing of all mRNAs unnecessary. Transcriptomic data indicated that the halovirus assemblage was highly active at the time of sampling and the viral groups with the highest expression levels were those related to high GC content haloarchaea and *Salinibacter* representatives, which are minor components in the environment. Moreover, the changes in the viral expression pattern and in the numbers of free viral particles were analyzed after submitting the samples to two stress conditions: ultraviolet-radiation and dilution. Results showed that *Archaea* were more sensitive than *Bacteria* to these stress conditions. The overexpression in the predicted archaeal virus fraction raised and the total numbers of free viruses increased. Furthermore, we identified some very closely related viral clones, displaying single-nucleotide polymorphisms, which were expressed only under certain conditions. These clones could be part of very closely related virus genomes for which we propose the term 'ecoviriotypes'.

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Introduction

Viruses from hypersaline environments are one of the most abundant biological entities in aquatic systems (Guixa-Boixareu *et al.*, 1996). Although some halophages have been characterized so far by culture-dependent methods (Dyall-Smith *et al.*, 2003, www.haloarchaea.com and references therein), little is known about their diversity and activity in natural samples (NSs). Previous studies on haloviruses in NSs using transmission electron microscopy revealed that the numbers of virus-like particles (VLPs) increased along the salinity gradient and their abundance was correlated with the numbers of cells (Guixa-Boixareu *et al.*, 1996). Pulsed-field gel electrophoresis was also applied to analyze the size of viral genomes in hypersaline environments (Díez *et al.*, 2000; Jiang *et al.*, 2003; Sandaa *et al.*, 2003; Santos *et al.*, 2007) showing that the viral assemblage changes from low to high salinity ponds, with genome 'populations' between 10 and 533 kb. Metagenomic analyses (Santos *et al.*, 2007, 2010) of the CR30 crystallizer from the multipond solar salterns of Santa Pola (Alicante, Spain) revealed that the halophilic viral assemblage was highly diverse as indicated by the analyses of their sequences (referred here as halophilic viral sequences, HVSs) which were classified according to their dinucleotide frequency and GC content (Santos *et al.*, 2010). Genomic sequences from the extremely halophilic *Haloquadratum walsbyi, Salinibacter ruber* (*Archaea* and *Bacteria*, respectively, which coexist with haloviruses in the

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crystallizer pond) and some high GC content haloarchaea, such as *Halobacterium salinarum*, *Haloarcula marismortui* and *Natronomonas pharaonis,* were also included in the analysis. The resulting classification scheme grouped all the sequences into five different clusters (HVS-1 to HVS-5, Figure 1a). The genomic sequences of high GC haloarchaea, *S. ruber*, and *Hqr. walsbyi* were grouped within the clusters HVS-1, HVS-2 and HVS-4, respectively, which included HVS of similar GC content and dinucleotide frequencies. According to this grouping schema, we suggested that the high GC content clusters HVS-1 and HVS-2 could correspond to viruses that might infect, respectively, high GC haloarchaea and *Salinibacter* representatives, while the low GC content cluster HVS-4 would include viruses infecting *Haloquadratum* lineages (Santos *et al.*, 2010).

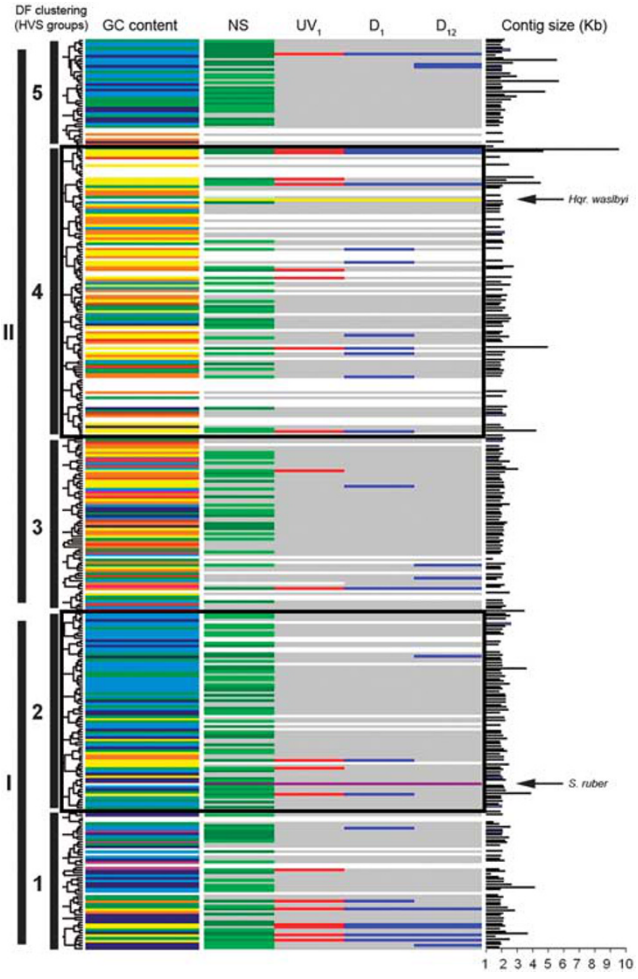

Figure 1 Schematic representation showing the expression and overexpression of viral contigs. Clustering of HVSs is based on dinucleotide frequency analysis (DF). The GC content of each viral contig is also shown in colours (according to the rainbow spectrum, from low to high GC; for example, yellow is 45–50% GC and dark blue is 60–65%, as in Santos *et al.*, 2010). The column labelled as NS shows the expression of viral contigs in the sample taken in May 2007, where contigs highly expressed are coloured in light green and contigs very highly expressed are indicated in dark green. Contigs marked in red in column UV1 are the contigs overexpressed 1 h after the UV-radiation treatment. Contigs marked in blue in columns D1 and D12 are the contigs overexpressed 1 and 12 h after the application of the osmotic shock. Black bars indicate the size of each contig, in kb. Arrows in panels show the position of *Hqr. walsbyi* and *S. ruber* genomes, according to their dinucleotide frequency (their GC content is also indicated by yellow and purple lines, respectively).

Once the viral 'community' was analyzed by the above mentioned metagenomic study, we took a metatranscriptomic approach using microarray technology to detect and quantify the expression of viral genes. This technology has been applied in the field of microbial ecology to study the composition of microbial communities using ribosomal RNA (rRNA)-targeted probes (for example, Garrido *et al.*, 2008) and the expression of selected genes (Parro *et al.*, 2007; Moreno-Paz *et al.*, 2010), which have provided information about the functional relevance of the diversity in a given ecosystem. In viruses, as there are no 'universal markers', microarrays have been basically used as a tool for studies within virus families (probes designed for the genes from one viral strain can be used to study their presence or absence in related viral genomes (Willson *et al.*, 2005; Allen and Wilson, 2006; Chou *et al.*, 2006; Allen *et al.*, 2006, 2007; Webster *et al.*, 2009)) and analyses of the viral dynamics in the infant gut using metaviromic DNA as probes (Breitbart *et al.*, 2008). Recently, Snyder *et al.* (2010) used microarrays designed based on CRISPR spacer sequences to identify viruses in hot springs. The use of microarrays for detecting viral expression in natural communities is, however, very scarce. To the best of our knowledge, the only examples on such an approach are the works by Kunin *et al.* (2008) who monitored a sludge bioreactor at three time points spanning 3 months using expression arrays constructed from predicted genes from both phage and bacterial metagenomes, and the use of microarrays to assess the diversity and activity of viruses related to infections in humans, allowing the detection of both known and novel pathogenic viral variants (Wang *et al.*, 2002; Kistler *et al.*, 2007).

Viral activity in natural communities has been traditionally measured using a few techniques. The counting of infected cells by transmission electron microscopy is direct evidence that viruses infect prokaryotes. Viral activity can also be measured by calculating the rate of production of viruses using different techniques: by measuring tritiated thymidine incorporation into viral DNA, by incorporation of fluorescently labelled viruses added into a sample, by inhibiting viral production and comparing with a non-inhibited control or, finally, by diluting the sample before directly counting virus production (Fuhrman, 1999; Wilhelm *et al.*, 2002; Weinbauer, 2004). Viral decay rates can also be analyzed by several techniques, for example, incubating NSs after adding a toxic agent against the cellular fraction (Wommack and Colwell, 2000). However, these techniques consider the whole viral

assemblage as a 'black box' and do not provide information regarding the expression of different genotypes within the viral assemblage.

In this work, we have constructed microarrays containing clones from the previously characterized metavirome of the above mentioned CR30 crystallizer (Santos *et al.*, 2010), which were used as probes for hybridization against complementary DNAs (cDNAs) obtained from total environmental RNAs. The same NSs were used for constructing the viral metagenomic libraries analyzed in Santos *et al.* (2010), and for the microarray construction, nucleic acid extractions and stress experiments described here. Thus, we were able to identify viral transcripts from the bulk mRNAs and ascertain which components of the viral community were active under different conditions at the time of sampling. In addition, we analyzed the viral overexpression when the NSs were submitted to two stress conditions (ultraviolet (UV)-radiation and dilution). Changes in the composition of the stressed prokaryotic communities were monitored by denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH), while changes in the numbers of free viral particles were detected by SYBR-green staining. Our results indicated that the viral halophilic 'community' that inhabits the crystallizers was highly active at the time of sampling and that stress treatments had different effects on the prokaryotic and viral assemblages.

Materials and methods

Sampling

Hypersaline water samples (named 'NS') were collected in May 2007 and May 2009 from a crystallizer (CR30) located in the multipond solar saltern 'Bras del Port' (Santa Pola, Alicante, Spain, 38°12′N, 0°36′W). The samples were taken a few centimetres below the surface using acid-washed polypropylene bottles. Salinity was measured *in situ* with a hand refractometer (Sper Scientific, Scottsdale, AZ, USA).

Microarray construction

Purification of the viral particles contained in 2 l of the NS taken in 2007 and viral DNA extraction, cloning and PCR amplification of viral inserts using vector primers were carried out as described in Santos *et al.* (2010). Purified PCR products were dried in a Speed Vac Concentrator (Savant, Thermo Fisher Scientific, Waltham, MA, USA), resuspended at 50–200 ng μl^{-1} in microSpotting Solution Plus 1 × (Arrayit Corp., Sunnyvale, CA, USA) and used for the viral microarray construction. Spotting was carried out with the MicroGrid-TAS II Arrayer (Genomic Solutions, Huntingdon, UK) at 22 °C and 50–60% relative humidity on epoxy-substrate slides (Arrayit Corp.) according to the manufacturer's instructions. PCR products of 16 S rRNA genes from *Hqr. walsbyi*, *S. ruber* and *Halomonas* sp. were included as hybridization controls.

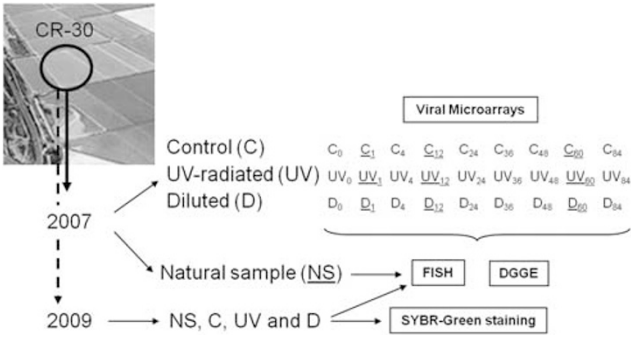

Figure 2 Experimental setting. NSs taken in May 2007 and May 2009 were analyzed by FISH and used for stress experiments. In both cases, subsamples were submitted to UV-radiation (UV) and dilution (D) and changes in the prokaryotic communities were monitored by FISH during 84 h, comparing with not-stressed subsamples used as controls (C). Subsamples C, UV and D from May 2007 were also analyzed by DGGE in all the sampling time points. Expression and overexpression of viral genes in the NS sample and after the treatments (1, 12 and 60 h after the shocks) were analyzed only in the samples from May 2007 by using microarrays. Samples from May 2009 were also used to study the changes in the free viral fraction by SYBR-green staining of viral particles.

Stress experiments

A scheme of the experimental design is shown in Figure 2. Samples were submitted to two different stress conditions: (i) UV-radiation (30-min exposure in a TELSTAR AV-100 cabin flux, operating at 40 W and $\lambda = 254$ nm; 'UV' samples) and (ii) dilution (by adding 1 volume of sterile mQ water; 'D' samples). Each stress experiment was carried out in duplicate. Water samples not submitted to stress were used as controls ('C'). All the samples were incubated in the laboratory and aliquots were taken for fixation and nucleic acid extraction at different times after the application of the shocks: C_0, UV_0 and D_0 ($t = 0$ h); C_1, UV_1 and D_1 ($t = 1$ h after the treatments); C_4, UV_4 and D_4 ($t = 4$ h); C_{12}, UV_{12} and D_{12} ($t = 12$ h); C_{24}, UV_{24} and D_{24} ($t = 24$ h); C_{36}, UV_{36} and D_{36} ($t = 36$ h); C_{48}, UV_{48} and D_{48} ($t = 48$ h); C_{60}, UV_{60} and D_{60} ($t = 60$ h); C_{84}, UV_{84} and D_{84} ($t = 84$ h). The time elapsed between the collection of NS and the beginning of the stress experiments was 2 h.

DAPI counts and FISH

NS, C, UV and D samples were fixed with formaldehyde (7% final concentration; Sigma-Aldrich, Steinhem, Germany). Hybridization, 4,6-diamidino-2-phenylindole (DAPI) staining and microscopy were carried out as described in Antón *et al.*, 1999. For every sample, at least one filter was analyzed, and 500 DAPI-stained cells were counted. Probes EUB338 and Arc915 were used for counting *Bacteria* (Amann *et al.*, 1990) *Archaea* (Stahl and Amann, 1991), respectively. Cells were visualized and counted in

an epifluorescence microscope (Leica, type DM4000B, Vashaw Scientifics Inc., Norcross, GA, USA).

Nucleic acids extraction and RNA amplification
Ten ml of NS, C, UV and D samples from 2007 were filtered through 0.22 μm pore-size GV filters (Durapore, Millipore, Billerica, MA, USA) to retain cells and the viral fraction inside cells (that contains actively replicating viruses). Filters were stored at −70 °C until the extraction was carried out. Filters were cut into small pieces, mixed with 500 μl of TE (Tris-Hcl 10 mM, EDTA 1 mM, pH 8.0) with lysozyme (final concentration: 40 μg μl^{-1}) and incubated for 5 min at room temperature before nucleic acid extraction with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). An aliquot of each sample was used for electrophoresis onto 1% LE agarose gels (FMC products, Rockland, MF, USA) and PCR amplification (see below). The rest of each extract was digested with RNase-free DNase DNA-free (Ambion, Austin, TX, USA) at 37 °C for 1 h PCR amplifications of 16S rRNA genes with several amounts of digested samples (1–5 μl) were performed in order to confirm that genomic DNA was absent from the samples.

The amount and quality of each RNA preparation was checked with the 2100 Bioanalyzer (Agilent Technologies, Van Nuys, CA, USA). Aliquots of 250–350 ng of total viral RNA were amplified through a method based on T7 RNA polymerase linear amplification as previously described (Moreno-Paz and Parro, 2006) using the MessageAmp II aRNA Kit (Ambion).

PCR amplification of 16S rRNA genes
DNA from the microbial fraction in each sample was extracted as described above and used for PCR amplification of 16S rRNA genes by using universal DGGE primers for *Bacteria* (Muyzer *et al*., 1993) and *Archaea* (Muyzer *et al*., 1996) with the conditions previously reported in Muyzer *et al*., (1993), adding a final step at 72 °C for 30 min (Janse *et al*., 2004). Reaction mixtures contained 100 ng of DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 200 μM of each dNTP, 1 U of Taq DNA polymerase (Invitrogen, Leek, The Netherlands) and 0.5 pmol of each oligonucleotide primer, in a total volume of 50 μl. To eliminate heteroduplexes, PCR products were diluted 10-fold and used as templates for a low-cycle-number reamplification using fresh reaction mixture, as described by Thompson *et al* (2002). To check for quality, 5 μl of the amplification products were loaded into 1% LE agarose gels (FMC).

Denaturing gradient gel electrophoresis
DGGE was carried out with a Dcode System (Bio-Rad, Hercules, CA, USA). In all the samples, 500 ng of PCR products were separated by electrophoresis at 100 V for 16 h in a linear gradient of denaturing agents from 45% to 65% (where 100% of denaturing agents was 7 M urea and 40% deionized formamide) in a 6% acrylamide-bysacrylamide (37.5:1) gel. After electrophoresis, the gels were stained with SYBR-green and the bands were cut, re-amplified, and checked again by DGGE to ascertain that they corresponded to single bands with the expected mobility compared with the NS. The nucleotide sequences of the bands were determined using the Big Dye Terminator Cycle Sequencing kit ABI PRISM 310 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Partial 16S rRNA gene sequences were compared with reference sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

cDNA labelling and microarray hybridization
Around 2 μg of total amplified RNA (see above) were labelled by cDNA synthesis with the CyScribe cDNA direct labelling Kit, using Cy3- or Cy5-labeled dUTP (GE Healthcare, Waukesha, WI, USA) and random hexamers, following the manufacturer's instructions. Printed arrays were denatured and pre-hybridized at 42 °C in pre-hybridization buffer (saline-sodium citrate 5 × solution, 0.1 mg ml^{-1} herring sperm denatured-DNA, 0.1% sodium dodecyl sulphate and 0.1% bovine serum albumin) for 1 h, and then hybridized against environmental labelled cDNAs. Two types of hybridizations were carried out: single hybridizations (using NS or *Leptospirillum ferrooxidans* labelled cDNAs, see Supplementary Material) and double hybridizations, in which labelled cDNAs from control and stressed samples were simultaneously hybridized in a microarray (C_1/UV_1, C_1/D_1, C_{12}/UV_{12}, C_{12}/D_{12}, C_{60}/UV_{60} and C_{60}/D_{60}). Hybridizations were performed overnight at 50 °C in HybIt hybridization solution (Arrayit Corp.). Washes were carried out with saline-sodium citrate 2 × buffer and SDS 0.1% for 5 min, saline-sodium citrate 2 × buffer for 5 min, and 0.2 × saline-sodium citrate for 5 min.

Scanning and data analysis
Hybridized arrays were scanned for Cy3 and Cy5 dyes in a GenePix 4100A Scanner (Axon Instruments Inc., Foster City, CA, USA). The scanned images were saved as 16-bit greyscale tagged image file format and analyzed by quantifying the fluorescence intensity of each spot using GenePix Pro v.6.0 software (Axon Instruments Inc.). The local background signal was subtracted automatically from the hybridization signal for each spot. Microarray hybridization results were analyzed with Genepix pro v.6.0 software (Axon Instruments Inc.) and were normalized by Global normalization using the global median of log intensity ratios (Yang *et al*., 2001).

SYBR-green staining of viral particles
One ml from the NS taken in May 2009 and from subsamples submitted to UV-radiation and dilution were centrifuged for 5 min at 15 700 g (Heraeus

Labofuge 400R, DJB Labcare Ltd, Buckinghamshire, UK). The supernatant was fixed with 0.02 μm Anodisc 25 filters (Whatman Int. Ltd, Maidstone, UK) filtered formaldehyde (4% final concentration) for 15–30 min, at room temperature. In all, 10 μl from the fixed samples were filtered using 0.02 μm Anodisc 25 filters and stained with SYBR-green as described in Noble and Fuhrman (1998). Viral particles were then visualized and counted in an epifluorescence microscope (Leica, type DM4000B).

Results

Metatranscriptome analysis of viral assemblages in the NS

The salinity of the NS taken in May 2007 was 32% and the DAPI and FISH counts (Figure 3) were in the range of those from other crystallizer samples analyzed around the world (Rosselló-Mora *et al.*, 2003; Elevi-Bardavid *et al.*, 2007; Antón *et al.*, 2008). The dominant group was *Archaea*, whose representatives accounted for up to 77% of the DAPI counts, while members of *Bacteria* accounted for up to 17.6%.

Total RNA from the sample was extracted, amplified, reverse-transcribed and labelled as described above and then hybridized against the viral microarray that had been constructed with the metaviromic shotgun clones from the same sample. Up to 340 spots out of 596 showed fluorescence above nine times the background fluorescence and therefore were selected for the analysis (see Supplementary Material). As the viral nucleic acids used for the microarray had been previously sequenced, assembled and annotated (Santos *et al.*, 2010), the expression in the spots could be correlated with the expression in their corresponding contigs (Figure 1, section NS). Larger contigs were formed by a higher number of clones, thus representing viruses that were more abundant in the environment. However, the level of expression detected was not always directly related to the size of the contig being expressed (Figure 1, section contig size). In general, larger contigs showed the highest fluorescent signal, indicating a high expression level of the corresponding genes. However, some small contigs, which could be related to less abundant viruses, were also very highly expressed in the NS.

The percentage of the contigs with an expression above nine times the background fluorescence included in each group was: 65% and 69% in the HVS-1 and HVS-2 groups (which included the sequences of high GC content haloarchaea and *S. ruber*, respectively), 67% for HVS-3, 48% for HVS-4 (that included the sequence of *Hqr. walsbyi*) and 72% in the HVS-5 group. In general, the contigs that show expression are expressed along their whole length. Table 1 shows a list of the predicted open reading frames in these contigs. Putative methyltransferases, terminases, membrane and regulation proteins were expressed in all groups, as well as many hypothetical and conserved hypothetical proteins.

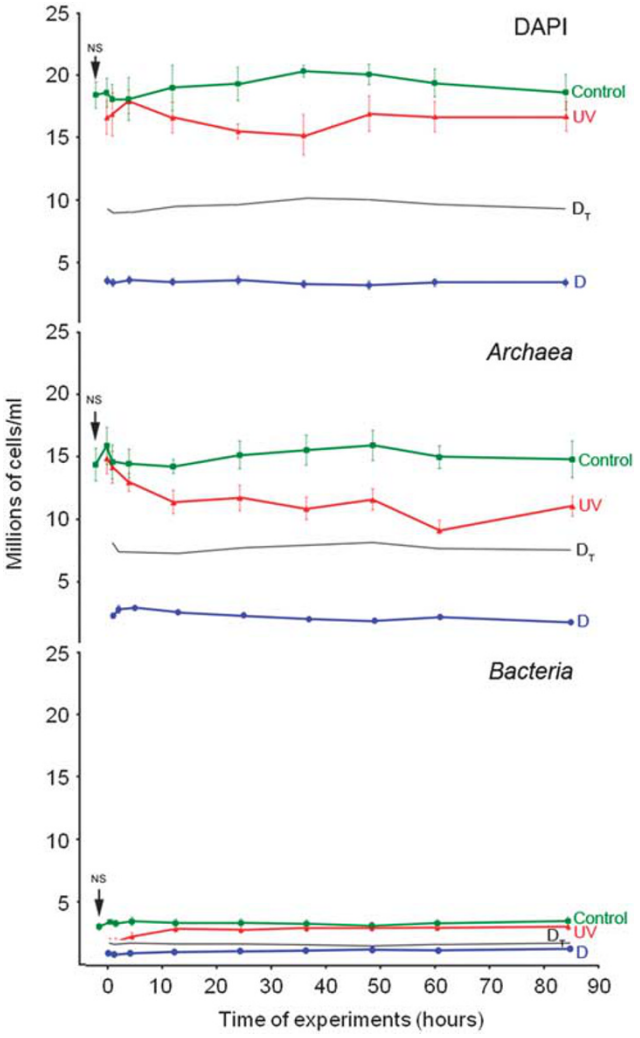

Figure 3 Changes in the number of total cells (DAPI), *Archaea* and *Bacteria* with time after the application of the stress treatments, compared with values in the NS taken in May 2007. Green lines are the values of the control samples. In red (UV), the number of cells after the UV-radiation treatment. In blue (D), the number of cells under osmotic shock. Black lines (D_T) represent the theoretical values after the dilution of the sample without considering lysis.

Metatranscritome analysis of viral assemblages under stress conditions

The viral microarrays were used for double hybridizations in which UV and D samples were compared with the C samples (Figure 1, sections UV_1, D_1 and D_{12}). Changes in the prokaryotic community caused by these stress conditions were monitored by FISH and DGGE, as explained above.

Stability of the control sample. The sample used as a control was highly stable throughout the stress experiments; both DAPI and FISH counts (Figure 3, green lines) in the C sample did not change

Table 1 List of predicted ORFs in contigs expressed in the NS

HVS group	*Contigs*	*Predicted ORFs*
HVS-1	022, 030, 058, 110, 141, 151, 198, 225, 246	HP
	078, 209	CHP
	126, 152	CHP, HP
	008, 113	HP, Zn-finger
	012	Zn-finger, HP, CHP
	029	RHH protein, CHP
	038	HP, terminase
	042	CHP, Zn-finger
	061	Transcription factor, HP
	070	RHH protein, integrase
	096	HP, envelope phage protein
	140	HP, transposase
	178	Replicative ATPase
	193	Integrase
	208	HP, methyltransferase
HVS-2	014, 024, 027, 041, 065, 106, 120, 128, 138, 162, 179, 194, 200, 202, 218, 228, 242, 247	HP
	055, 088, 107	Membrane, HP
	066, 090, 237	Membrane
	067, 199	CHP
	127, 129	HP, CHP
	163, 190	HP, methyltransferase
	011	Terminase, HP
	049	Membrane, CHP, tat-signal protein
	059	Zn-finger, CHP
	062	Prohead peptidase, capsid structure
	069	HP, phosphohydrolase
	072	Membrane, CHP
	104	Terminase
	123	SsDNA-binding protein, HTH-protein, HP
	213	Phage mu-like
	214	HP, transcriptional regulator
	261	Deoxyribosyltransferase
HVS-3	017, 031, 144, 176, 221, 226, 248	HP
	171, 116	Membrane, HP
	236, 251	Membrane
	044	MCM protein (DNA replication initiator)
	060	Replicative DNA helicase (intein endonuclease)
	076	HNH endonuclease, HP
	080	HP, ssDNA-binding protein
	084	HTH phage repressor (peptidase)
	114	LAGLIDADG endonuclease
	130	CHP
	137	Methyltransferase, phosphoadenosine reductase
	150	Ssl1-like protein (transcription factor)
	227	Hypothetical DUF protein
	249	HNH endonuclease, membrane
HVS-4	006, 036, 047, 050, 063, 098, 118, 149	HP
	005, 035, 074	HP, CHP
	001	HP, capsid, methyltransferase
	004	HP, CHP, terminase, methyltransferase
	007	Methyltransferase, nuclease, HP
	009	HP, traslocation domain protein
	019	Terminase, HP
	043	HP, transposase
	068	Lamgl protein (glucanase)
	113	HP, homeodomain protein
	125	Membrane, HP
	158	WD40/YVTN repeat-like domain
	165	HP, mRNA autoregulator
	174	Arginine *N*-succinyltransferase
HVS-5	083, 131, 132, 142, 164, 169, 173, 216, 255	HP
	002, 015	HP, CHP, membrane
	026, 182	Phage DNA primase
	160, 266	Membrane
	075	HP, membrane
	081	Hypothetical ParB nuclease-like protein
	111	Terminase
	124	Replicative DNA-dependent ATPase
	167	HP, CHP
	212	CHP

Abbreviations: CHP, conserved hypothetical protein; DUF, domain of unknown function; HP, hypothetical protein; HTH, helix-turn-helix domain; HVS, halophilic viral sequence; NS, natural sample; ORF, open reading frame; RHH, ribbon-helix-helix domain; ssDNA, single-stranded DNA.

significantly with time, and differences in DAPI were no higher than 11% in all the analyzed points. Between 93% and 100% of total cell counts could be assigned by FISH to either *Archaea*, which always dominated the community, or *Bacteria*. Both archaeal and bacterial DGGE profiles (Figures 4a and b) in the sample did not show any change over the course of the experiment. Sixteen bands were excised from the gel and sequenced and, as in previous studies (Benlloch *et al.*, 2002; Casamayor *et al.*, 2002; Gomariz *et al.*, unpublished), the sequences obtained matched in databases with cultured and uncultured members of *Halobacteriaceae* and *Bacteroidetes* (see Supplementary Table 1).

A double hybridization with C_1 and NS showed (Supplementary Material) that during the time elapsed between the collection of the sample and 1 h after the treatments there was no overexpression of viral genes.

Changes under UV-radiation treatment. Figure 3 (red lines) shows the variation in the numbers of DAPI, *Archaea* and *Bacteria* following the UV treatment. Just after the shock there was a remarkable reduction in the number of *Bacteria* (43.42%), probably due to lysis, compared with the effect on the archaeal populations (6.42% reduction).

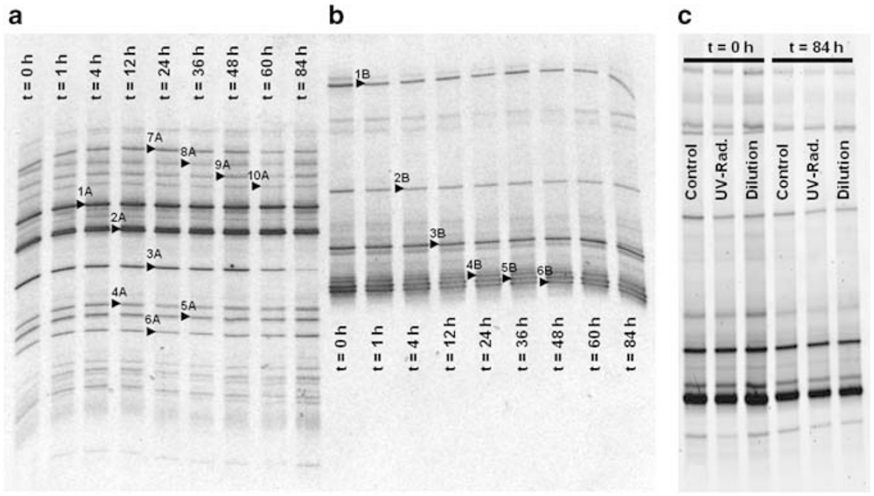

Figure 4 DGGE profiles from archaeal (**a**) and bacterial (**b**) 16S rRNA gene amplification products in the samples used as controls. Each lane corresponds to different sampling times in the experiment (in hours). In (**c**), comparison among the bacterial assemblages in the control, diluted and UV-radiated samples in two different sampling times. Sequences 1, 2 and 10, in (**a**), were related to the species *Hqr. walsbyi*. The rest of the bands in (**a**) were associated to uncultured *Halobacteriaceae* environmental clones. Sequences from band 3 were related to the species *S. ruber*. The rest of the sequences in (**b**) were associated to uncultured *Salinibacter* (bands 2, 4-6) and *Bacteroidetes* (band 1) clones.

However, after 1 h, *Archaea* started to decrease and numbers of *Bacteria* increased.

When 16S rRNA genes from UV samples were amplified for monitoring by DGGE changes in archaeal and bacterial diversity, the same patterns as in the control were obtained (Figure 4c), thus indicating that the 'species' composition of the crystallizer remained stable. Further analysis would be needed to ascertain if the treatments produced changes in the microdiversity of the sample, as DGGE profiles only provide information on diversity patterns based on a partial amplification of the 16S rRNA genes.

Up to 114 spots in the array showed overexpression in the sample UV_1 when compared with sample C_1 while no overexpression was detected in UV_{12} and UV_{60} when compared with their corresponding controls. As with the NS, a correlation among the overexpressed spots with their corresponding contigs was established (Figure 1, section UV_1). A list of contigs overexpressed after radiation with their respective open reading frames is shown in Table 2.

In the UV_1 sample, the groups showing the highest numbers of overexpressed contigs were HVS-1 and HVS-4 (15% and 12% of the contigs in each group, respectively), while in the groups HVS-2, HVS-3 and HVS-5, only 4.6%, 3.3% and 2.7% of the contigs, respectively, were overexpressed with respect to the C_1 sample. As mentioned above, besides viral sequences, HVS-1 includes high GC haoarchaeal sequences and HVS-4 includes the genomic sequence of *Hqr. walsbyi* (Santos *et al.*, 2010). Thus, HVS groups that displayed the strongest response to UV treatment were those containing viruses related to *Archaea*, which could explain the continuous decrease in the archaeal numbers observed once the stress disappeared, contrasting with the recovery in bacterial counts detected in the same period (although the initial effect of UV radiation on bacterial numbers seemed more dramatic).

Changes under dilution treatment. Dilution of the sample (Figure 3, blue lines) produced a very marked decrease in *Archaea* numbers. *Bacteria* seemed more resistant to dilution and numbers started to increase after 1 h. DGGE profiles after dilution showed the same patterns as the control and UV-radiated samples (Figure 4c).

One hour after the dilution of the sample, up to 134 spots in the microarray showed overexpression with respect to the undiluted sample. After 11 h (in D_{12}), 42 spots (some of them different from the ones expressed at $t = 1$ h) were overexpressed. At 60 h after the dilution of the sample, no viral overexpression could be detected. The correlation between the overexpressed spots and the corresponding contigs is shown in Figure 1, sections D_1–D_{12}, and Table 2.

The groups with the highest number of overexpressed contigs in D samples were also HVS-1 and HVS-4 (15% of contigs in each group, after 1 h; 13% and 4.5% of the contigs, respectively, after 12 h). Thus, as observed with the UV treatment, HVS groups containing archaeal sequences showed the strongest response to the dilution treatment. This, again, could be related to the significant decrease in archaeal numbers.

Changes in the viral counts of stressed samples. Numbers of free viruses in the NS and subsamples C, UV and D were analyzed by SYBR-green staining in the sample taken in May 2009. This sample had 34% salinity and contained $1.52 \times 10^9 \pm 1.45 \times 10^8$ VLPs ml^{-1} and $3.46 \times 10^7 \pm 3.25 \times 10^6$ cells ml^{-1}.

Table 2 Viral contigs overexpressed (+) after UV-radiation and dilution treatments, compared with expression in NS

Group	*Contig*	*Predicted ORFs*	*Expression NS*	*Overexpression (+)*		
				UV_1	D_1	D_{12}
HVS-1	012	CHP, HP, Zn-finger proteins	VH	+	+	+
	030	HPs	VH	+	+	P
	039	CHP, ribbon-helix protein	H	—	P	—
	042	CHP, Zn-finger proteins	VH	+	+	+
	073	HPs	—[a]	+	+	+
	152	CHP, HP	VH	+	+	+
	193	Integrase	VH	—	—	+
	225	HPs	H	+	+	—
	250	CHP	H	+	—	—
HVS-2	011	Terminase, HP	VH	P	P	—
	106	HPs	VH	+	+	—
	129	CHP, HP	VH	—	—	P
	199	CHP	H	+	—	—
HVS-3	017	HPs	H	+	—	—
	060	Replicative DNA helicase	H	—	—	+
	100	HNH endonuclease, HP	—	—	+	—
	177	Phage capsid protein, HP	—	—	—	+
	226	HP	VH	+	+	+
HVS-4	001	HP, methyltransferase, capsid protein	VH	+	+	P
	005	CHP, HP	VH	P	+	P
	006	HPs	H	P	P	P
	007	Methyltransferase, nuclease, HP	P	P	P	—
	009	HP, translocation protein	VH	P	—	—
	010	Terminase	—	—	P	—
	019	Terminase, HP	H	P	—	—
	068	LamGL protein (glucanase)	H	—	P	—
	113	HP, homeodomain	H	+	—	—
	165	HP, mRNA autoregulator	H	—	+	—
	191	HP, membrane protein	H	—	+	—
	241	HPs	—	—	+	—
HVS-5	021	Integrase, HP	H	—	—	+
	159	HP	—	—	—	+
	167	HP, CHP	H	+	+	+

Abbreviations: CHP, conserved hypothetical protein; H, contigs highly expressed in NS; HNH, conserved amino acid sequence in certain endonucleases; HP, hypothetical protein; HVS, halophilic viral sequence; ORF, open reading frame; NS, natural sample; P, the contig was partially expressed; VH, contigs very highly expressed in NS; UV, ultraviolet.
[a]Absence of expression and/or overexpression.

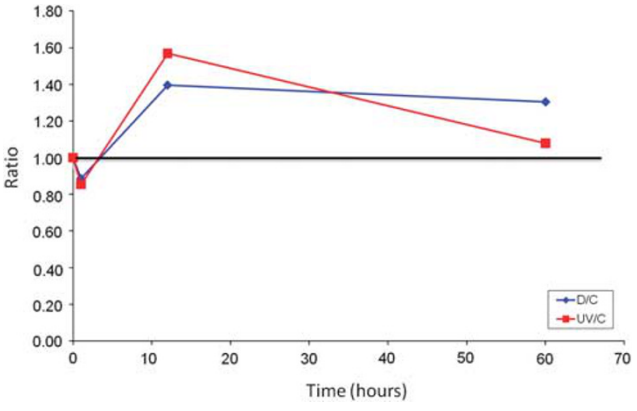

Figure 5 Changes in the numbers of the viral fraction in the May 2009 sample under stress conditions (UV radiation, in red; dilution, in blue) divided by changes in the control sample.

Archaea and *Bacteria* accounted for 79.35% and 10.31% of the DAPI counts, respectively. FISH analyses indicated that changes in the prokaryotic community because of stress treatments were similar to those detected in the experiment carried out in 2007 (see Supplementary Figure 1).

The number of VLPs present in the C sample decreased after 60 h, from 1.52×10^9 to 8.40×10^8. In both UV and D samples, a reduction in the number of VLPs was observed 1 h after the treatments, when compared with their corresponding C subsamples (Figure 5). After 12 h, the number of VLPs increased, being slightly higher in the UV sample. At 60 h after the shocks, the free VLPs in the radiated sample decreased while diluted samples maintained the levels reached previously. An explanation for the initial decrease in the number of free viruses could

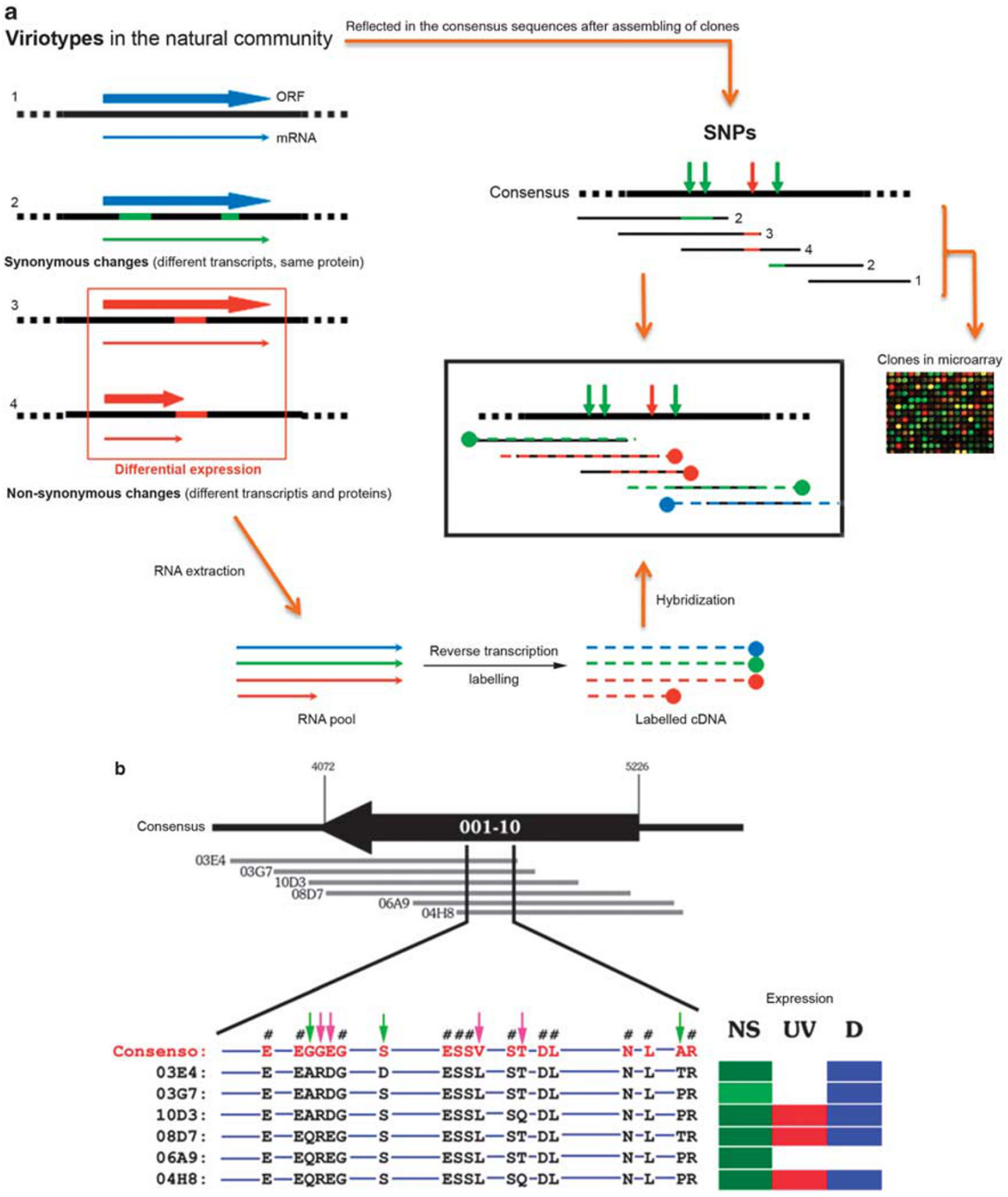

Figure 6 Ecoviriotypes. (**a**) Theoretical scheme showing how nearly identical viruses (viriotypes) with non-synonymous changes can respond differentially to environmental changes. This differential response can be detected in the expression of the overlapping clones from a given contig. (**b**) Differential expression of 'ecoviriotypes'. The overlapping clones (grey lines) that form a given contig present SNPs that can be translated in neutral–non-synonymous changes (pink arrows) and non-neutral–non-synonymous changes (green arrows). Symbol #indicates synonymous changes. Blue lines indicate 100% identity in amino acid and nucleotides among clones.

be that capsids were disrupted because of high radiation and osmotic shock. However, part of the viral particles (located either inside or outside the cells) might be activated because of the treatments (or the hosts made more susceptible), favouring the release of new viruses and their subsequent increase.

The observed increase in free VLPs numbers was in agreement with the high levels of overexpression observed in the metatranscriptomic analyses. Indeed, both techniques indicate a more persistent effect of the dilution since viral numbers did not decrease along the treatment and viral overexpression was detected also 12 h after diluting the sample.

Expression of 'ecoviriotypes'

The characterization of the CR30 metavirome (Santos *et al.*, 2010) revealed the existence of a

significant number of single-nucleotide polymorphisms (SNPs) in some viral contigs. The presence of SNPs corresponded to a very high mutation frequency (7.65×10^{-3} substitutions per nucleotide) that reflected a high microdiversity of closely related haloviruses. Around 40% of these SNPs corresponded to non-synonymous changes that could have a phenotypic effect on the corresponding viriotypes. We hypothesize that certain viriotypes of the community, because of these non-synonymous mutations, could code for transcripts varying in nucleotide composition and length (Figure 6a), which finally result in different proteins from a given homologous open reading frame. Differences in the function/efficiency (fitness) among such polymorphic proteins would determine the expression of a given viriotype under a given environmental condition. The analysis of the metatranscriptomic data could then reveal these differences in the expression of clones (that is, spots in the microarray) covering the same polymorphic open reading frames (Figure 6a). For this analysis we only considered the clones that were expressed under at least one of the assayed conditions because the absence of expression in a clone under all the conditions would indicate an absence of DNA in the microarray (if other clones in the same contig were expressed).

With these premises, we analyzed the differences in the expression of the 49 polymorphic contigs (an example is shown in Figure 6b). Out of the 49 contigs, 24 showed expression only in the NS while 12 were expressed and/or overexpressed in two or three analyzed conditions, thus showing differential expression (the remaining 13 contigs were not expressed under any conditions or were wholly expressed in the three conditions assayed). Up to 67% of these differentially expressed contigs had consensus sequences displaying both synonymous and non-synonymous changes. If we consider that the non-synonymous changes reflect viral proteins that could be differentially expressed (the amino acidic composition in each clone was not identical), the transcripts with a given number of SNPs would not hybridize well with their polymorphic spots (Figures 6a and b). On the basis of these observations, we suggest the term 'ecoviriotype' to refer to these different but very closely related viral genotypes that could respond differentially to changes in the environmental parameters.

However, the remaining 33% of the contigs displaying differential expression showed only synonymous changes. In these cases, the differences in the nucleotide sequence of the clones that form a given contig could also be affecting the hybridization efficiency of the viral transcripts. These clones could not be considered ecoviriotypes because the observed differential expression would not be due to differential responses to environmental conditions, but to a differential efficiency of the hybridization between target and probes in the microarray.

Discussion

This work presents the use of microarrays as a tool to carry out metatranscriptomic analyses in viral communities. This technique makes it unnecessary to carry out the complete sequencing of all the transcripts present in the sample and the *in silico* search for viral messengers in the total RNA extracts.

The CR30 crystallizer showed a high viral expression at the time of sampling (60% of the metaviromic contigs were expressed) although the expression was not homogeneous for all the contigs. Groups HVS-1, HVS-2 and HVS-5 had the highest percentages of expressed contigs. Given that sequences from HVS-1 and -2 groups could correspond, respectively, to viruses infecting high GC content haloarchaea and *S. ruber* representatives (Santos *et al.*, 2010), these viruses could be responsible for the lower numbers that their hosts reach in the CR30 sample compared with the high abundance of *Hqr. walsbyi* (Antón *et al.*, 1999).

It is well known that *Hqr. walsbyi* dominates the prokaryotic community in many (but not all) crystallizers analyzed around the world (Antón *et al.*, 1999; Bolhuis *et al.*, 2004; Burns *et al.*, 2004; Legault *et al.*, 2006; Maturrano *et al.*, 2006; Baati *et al.*, 2008; Mutlu *et al.*, 2008), but little is known about the reasons for this phenomenon. One could argue that, besides phage control, the dominance of *Haloquadratum* over *Salinibacter* could be due either to competition for resources, different nutrient requirements, or halocin activity, among other reasons. When archaeal populations are reduced by the stress conditions used in this work (see below), *Bacteria* do not seem to obtain any benefit and reach the same numbers they had in the NS. Thus, competition with *Archaea* for specific resources does not seem to be controlling bacterial populations.

Haloquadratum populations are considered to be highly versatile metabolically and very tolerant to high concentrations of magnesium (Cuadros-Orellana *et al.*, 2007). In addition, it seems that different lineages of *Haloquadratum* harbour a large variety of surface proteins (Cuadros-Orellana *et al.*, 2007; Rodríguez-Valera *et al.*, 2009) that could mediate phage processes and confer resistance to viral infection in a significant proportion of their natural strains. Given the high abundance of their hosts, viruses affecting *Haloquadratum* representatives should be the most abundant viruses in the system, which is supported by the fact that largest contigs in the metavirome (those formed by the largest number of clones) are included in the HVS-4 group, which also contains the *Hqr. walsbyi* genome. However, this is not the most highly expressed (that is, active) group, a fact in agreement with the idea that 'abundance alone does not tell how active the viruses may be in infecting hosts' (Fuhrman and Schwalbach, 2003). Although, in our case, most of the contigs probably do not correspond to very

minor components of the community, given the amount of analyzed sequences, our findings somehow contrasts with the 'Bank model' proposed by Breitbart and Rohwer (2006) that assumes that 'only the most abundant viruses are active'. In the 'Bank model', different hosts become dominant and their viruses move from the 'bank' into the active fraction when the environment changes. Data reported for years from the CR30 crystallizer show that *Haloquadratum* members always dominate the prokaryotic community (Antón *et al*., 1999; Bolhuis *et al*., 2006). Our results indicate that their dominance could be related to resistance/evasion mechanisms to viral infection and are in agreement with the experiments carried out with marine samples by Bouvier and del Giorgio (2007), who proposed a scenario of bacterial–viral interactions where the dominant bacterial phylotypes in the system might have a lower susceptibility to viral infection because of the development of resistance or by intrinsic metabolic activity, in a density-independent form. In principle, this would contradict the density-dependent 'kill the winner' hypothesis, in which it is assumed that 'viruses should preferentially infect the most common hosts'. In the 'kill the winner' model, proposed by Thingstad and Lignell (1997) and revised by Winter *et al*. (2010), the 'winner' refers to the most active population in the community. In the CR30 crystallizer, *Hqr. walsbyi* would be the 'winner', which would dominate the system not only because of their metabolical adaptability but also because of their survival strategies when faced with a viral attack. Once again, it seems that a semantic issue is at the heart of this discussion, because the winner can be either the dominant species (*Hqr. walsbyi* in our case) or the most abundant lineage within a given species (one specific *Hqr. walsbyi* strain, below what would be considered a 'population'). Indeed, DGGE patterns display a diversity of phylotypes within the *Haloquadratum* assemblage, in agreement with the vast pan-genome reported for this species (Legault *et al*., 2006). Such a wide range of microdiversity has been reported for other prokaryotes inhabiting this low diversity environment, such as *Halorubrum* spp. isolates (Papke *et al*., 2004) and different *S. ruber* strains (Peña *et al*., 2010).

Electromagnetic radiation is known to inactivate prokaryotic viruses, which are particularly vulnerable because of the absence of DNA reparation mechanisms. Sunlight, particularly UV radiation, can be very important for viral inactivation in the upper water column (Suttle and Chen, 1992; Murray and Jackson, 1993), as the inactivated viruses are unable to infect sensitive hosts. Since solar salterns represent ecosystems submitted to high solar radiation, the UV treatment could give information about the strategies for the activity and survey of halophages in the crystallizers. In addition, the dilution of brines is a common phenomenon after rainfalls and it is known that decreasing the salinity of the medium increases the burst size (Dyall-Smith *et al*., 2003). High salinities inhibit the absorption of some haloviruses that infect *Halobacterium salinarum*, favouring high cell densities. However, when the salinity is reduced, viruses are stimulated to reproduce and exit cells quickly given that cells become unstable because of the change in the osmotic pressure (Dyall-Smith *et al*., 2003).

Our results showed that both stress conditions increased the expression of some viral groups and the number of viruses found in the sample. In addition, they affected differentially the behaviour of archaeal and bacterial populations. Putative archaeal viruses also showed more activity under stress, which in fact implies that the decrease in the archaeal populations could be due to both the treatments and the increase in the expression of archaeal viruses. In any case, neither of these factors were affecting the rank of 'species' in the samples, at least at the level of resolution provided by the analysis of DGGE patterns.

The question now is to what extent viruses may select prokaryotic lineages within a given phylotype under the assayed conditions. Indeed, this strain-specific phage susceptibility has been previously reported for members of the species *S. ruber* (Peña *et al*., 2010) and proposed for members of the *Haloquadratum* assemblage (Rodríguez-Valera *et al*., 2009). The replacement of closely related strains by the effect of viral activity within a temporarily stable community has been recently proposed for solar salterns in San Diego (Rodriguez-Brito *et al*., 2010). These observations are coherent with the constant-diversity dynamics model (Rodríguez-Valera *et al*., 2009) according to which the diversity of prokaryotic communities is preserved by phage predation.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)