Extremophiles (2014) 18:195–206
DOI 10.1007/s00792-013-0599-4

ORIGINAL PAPER

Molecular characterization of pHRDV1, a new virus-like mobile genetic element closely related to pleomorphic viruses in haloarchaea

Shaoxing Chen · Chuanming Wang · Jian-Ping Xu · Zhu L. Yang

Received: 24 April 2013 / Accepted: 14 November 2013 / Published online: 29 December 2013

Abstract A novel haloarchaeal plasmid, pHRDV1 (13,053 bp), was isolated from the haloarchaeal isolate *Halorubrum* sp. T3. Molecular and bioinformatics analyses showed that this element is a double-stranded circular DNA molecule containing two putative transcripts with opposite directions. The amino acid sequences of six of the nineteen predicted open reading frames were similar to those found in haloarchaeal pleomorphic viruses, such as *Halorubrum* pleomorphic virus 3 and *Halogeometricum* pleomorphic virus 1. There was also a strong conservation in gene order between the plasmid and these viruses. All three conserved viral proteins (VPs), which are characteristic of haloarchaeal pleomorphic viruses VP3, VP4 and VP8, were found in pHRDV1. Furthermore, a typical repressor-operator system similar to haloarchaeal myovirus φCh1, was found on the genome of pHRDV1. However, no viral particles were detected in the supernatants of *Halorubrum* sp. T3, either in the presence or absence of mitomycin C. These results imply that plasmid pHRDV1 is a distinctive virus-like mobile genetic element that harbors some unique properties that make it different from all of the known haloarchaeal plasmids or viruses.

Keywords *Halorubrum* · Hypersaline environment · Mobile genetic elements · Horizontal gene transfer

Communicated by S. Albers.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-013-0599-4) contains supplementary material, which is available to authorized users.

S. Chen · Z. L. Yang (✉)
Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, People's Republic of China
e-mail: fungi@mail.kib.ac.cn

S. Chen
e-mail: haloarchaea1979@gmail.com

S. Chen · C. Wang
University Key Laboratory of Crop High Quality and High Effective Cultivation and Safety Control in Yunnan Province, Honghe University, Mengzi, People's Republic of China
e-mail: wcm_biology2@126.com

J.-P. Xu
Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada

Introduction

Hypersaline environments, such as salt lakes, saltern crystallizer ponds, solar salterns, salt-pan sediments, and soda lakes, are found in many parts of the world (Chen et al. 2012a). These environments typically have a salinity ranging from 1.5 M to saturation (6.15 M), but many haloarchaea can live in such environments (Atanasova et al. 2012). Studies of the cultivated (Sabet et al. 2009) and uncultivated (Maturrano et al. 2006) haloarchaea from hypersaline environments indicate that the most common species belong to the genera *Halorubrum*, *Haloarcula*, *Haloferax*, *Haloquadratum* or *Halobacterium* (Benlloch et al. 2001; Oren 2002). Lateral gene transfers (LGT) frequently occur between haloarchaea and other microorganism and between different kinds of haloarchaea (Rhodes et al. 2011; Chen et al. 2012a). Mobile genetic elements (MGEs) consisting of viruses, plasmids, and associated elements facilitate the horizontal transfer of genes that promotes the evolution and adaptation of microbial communities (Sobecky and Hazen 2009).

Many species of haloarchaea that flourish in hypersaline environments are rich in plasmids (Zhou et al. 2007). Direct

electron microscopy studies of hypersaline environments have shown that they also have high concentrations of virus-like particles (VLPs) (10^7–10^9 per milliliter) (Guixa-Boixereu 1996; Oren et al. 1997; Dyall-Smith et al. 2003; Sime-Ngando et al. 2011). To date, approximately 60 virulent or temperate haloarchaeal viruses have been described (Atanasova et al. 2012). These include some well-documented viruses with detailed molecular characteristics, such as φH (Schnabel et al. 1982), His1 (Bath and Dyall-Smith 1998), φCh1 (Klein et al. 2002), HF2 (Tang et al. 2002), SH1 (Bamford et al. 2005; Porter et al. 2005), His2 (Bath et al. 2006), HRPV-1 (Pietilä et al. 2009), HHPV-1 (Roine et al. 2010), SNJ1 (Zhang et al. 2012), HRPV-2, HRPV-3, HRPV-6, and HGPV-1 (Pietilä et al. 2012; Atanasova et al. 2012; Senčilo et al. 2012). As viruses are the only predators of microbes in such environments (Pedrós-Alió et al. 2000), they represent a major force in the evolution of haloarchaea and the biogeochemistry of hypersaline environments (Guixa-Boixereu 1996).

Plasmids are MGEs that are commonly found in haloarchaea, and there are now many examples where genes carried on these plasmids have been shown to be closely related to those of haloviruses. A previously reported "plasmid" called pHH205 (Ye et al. 2003) was found to be closely related to halovirus SH1, which is distantly related to the icosahedral virus P23-77 of *Thermus thermophilus* (Jalasvuori et al. 2009). Subsequently, pHH205 was reclassified as a halovirus named SNJ1, as it can be induced from the plasmid pHH205 by mitomycin C (MMC) (Mei et al. 2007). There are not many genes on the genome of pHH205 that resemble genes of known viruses. However, the well-documented haloarchaeal plasmid pHK2 (Holmes et al. 1995; Roine et al. 2010) and thermococcal plasmid pAMT11 (Gonnet et al. 2011) are different from "plasmid" pHH205 in that they contain virus-like genetic elements, but cannot be induced by MMC to become viruses. In addition, half of the genes on the genome of PAV1 (*Pyrococcus abyssi* virus 1) show a high homology with pTN2-like plasmids (Krupovic et al. 2013). Furthermore, the Sulfolobus plasmid pSSVx (a hybrid between a plasmid and a fusellovirus from *Sulfolobus*) contained three open reading frames (ORFs), two of which show high sequence similarity to a tandem of ORFs in fusellovirus genomes, which enable pSSVx to use the packaging system of the viral helpers for spreading (Arnold et al. 1999). These observations suggest that there are many robust connections between plasmids and viruses (virulent or temperate).

To better understand the in situ plasmid-mediated HGT in hypersaline microbial communities, information on the transcriptome, host range, and genetic composition of the newly isolated virus-like mobile element was examined. Here, we report the isolation and molecular characterizations of a novel haloarchaeal plasmid, pHRDV1, isolated from *Halorubrum* sp. T3. The copy number, nucleic acid type, transcriptome under MMC induction or not, and replication origin of the plasmid pHRDV1 were analyzed. Our analyses determined that plasmid pHRDV1 is similar to two viruses, *Halorubrum* pleomorphic virus 3 (HRPV-3) and *Halogeometricum* pleomorphic virus 1 (HGPV-1), according to the amino acid sequences of predicted ORFs and in gene order. However, pHRDV1 showed no lytic ability and could not produce mature viral particles. These characteristics make it similar to other virus-like extrachromosomal genetic elements such as pHK2 or pAMT11. Our comparative genomic analysis of this virus-like mobile genetic element enriches our understanding of the relationships among plasmids, viruses, and proviral elements.

Materials and methods

Media and growth conditions

Halorubrum sp. T3 isolated from a halite deposit from the Yuanyongjing Salt Mine (latitude 25°9′31.6″, longitude 101°54′16.9″), Yunnan, Southwestern China is the host strain of the haloarchaeal plasmid pHRDV1. A modified growth medium (MGM) was used to culture all of the haloarchaeal strains analyzed in this study (Supplementary Table S1). The medium and culture conditions are described in the online HaloHandbook (http://www.haloarchaea.com/resources/halohandbook/index.html). Luria–Bertani (LB) medium was used to culture *Escherichia coli* strain DH5α for propagating recombinant plasmids (Sambrook and Russell 2001). Agar plates were made by adding 1.5 % (w/v) agar powder to liquid media for the MGM or LB media. All of the strains were grown aerobically at 37 °C.

Isolation and cloning of pHRDV1

Haloarchaeal plasmid pHRDV1 was isolated from *Halorubrum* sp. T3 (Supplementary Table S1) during its exponential growth phase using the alkaline lysis method (Sambrook and Russell 2001). The plasmid pHRDV1 was purified with a phenol: chloroform: isoamyl alcohol (25:24:1) extraction precipitated with equal volumes of isopropanol in the presence of 0.2 M NaAc and washed with 75 % (v/v) ethanol twice. Seventeen restriction endonuclease enzymes (TaKaRa, Dalian, China) (*Nco* I, *Sma* I, *Sac* I, *Bam*H I, *Kpn* I, *Not* I *Sph* I, *Pvu* II, *Hind* III, *Xba* I, *Hae* III, *Bgl* II, *Eco*R V, *Sca* I, *Nde* I, *Pst* I, *Sal* I) were used to construct a restriction genome map of pHRDV1. However, only two (*Kpn*I and *Bam*HI) of the twelve enzymes were chosen to digest pHRDV1 and to generate fragments for cloning. All of the fragments were

purified with a gel extraction kit (OMEGA) and cloned into the plasmid vector pUC18 (TaKaRa) for sequencing.

Sequence and analysis of plasmid pHRDV1

Recombinant plasmids for sequencing were constructed by inserting digested and purified DNA fragments (*Kpn*I and *Bam*HI) into cloning vector pUC18 that had been digested with the same restriction enzymes. The genomic sequence of the plasmid pHRDV1 was determined by primer walking. The primers used for the PCR and sequencing are listed in Supplementary Table S2. All of the sequencing was carried out using BigDye ver 3.1 and analyzed on an ABI 3730 (Applied Biosystems). The preliminary genomic sequence of the plasmid pHRDV1 was verified with PCR products. The primers and pairs of primers used for sequence verification are listed in Supplementary Tables S3 and S4, respectively.

The purified plasmid was digested with DNaseI (1 U of RNase-Free DNaseI, 1 μg^{-1} of genomic DNA) and RNase A (1 $\mu g/1\ \mu g^{-1}$ of genomic DNA), respectively. Mung Bean Nuclease (MBN) and S1 Nuclease digests were processed in 20 µl reactions for 1 h at +37 °C using 0.5, 5, or 10 U of per 1 µg of genomic DNA (Roine et al. 2010). The plasmid pUC18 was used as a dsDNA form control (all of the enzymes used here were brought from TaKaRa, Dalian, China).

The completed sequence of pHRDV1 was annotated using Glimmer 1.0 (Delcher et al. 1999) and GeneMark.hmm (Lukashin and Borodovsky 1998). The homologous nucleotide and protein sequences in the databases were identified using the BLASTN and BLASTP tools (Altschul et al. 1990), respectively, which are available from the National Center for Biotechnology Information (NCBI). The pairwise comparison of the conserved protein sequences was determined using the EMBOSS Needle tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The properties of the identified proteins and the products of the putative ORFs were analyzed with the expert protein analysis system (ExPASy) proteomics tools (http://www.expasy.org/proteomics). The isoelectric points and molecular masses were determined using the Compute pI/MW tool (Bjellqvist et al. 1993). The signal sequences for viral protein (VP)-like proteins were predicted using Signal P (version 3.0) (Bendtsen et al. 2004) and Tat-Find Servers (Rose et al. 2002). The conserved protein signature sequences were determined using Inter-Pro-Scan (Hunter et al. 2009). The putative trans-membrane regions of the proteins were additionally determined using combined predictions from the TMHMM (Sonnhammer et al. 1998) and TM-Pred tools (Hofmann and Stoffel 1993). The coiled-coil regions were predicted using the Coil tool (Lupas et al. 1991). Multiple sequence alignments were generated using CLUSTALW (Larkin et al. 2007). The putative replication origin was predicted based on a nucleotide disparity analysis using the Z-CURVE bio-software under the Z' component algorithm (Zhang and Zhang 2005).

Determination of the copy number of the plasmid pHRDV1

To estimate the ratio of the plasmid pHRDV1's copy number to that of its chromosome, real-time quantitative PCR (Rotor-Gene 6000, Corbett Research, Australia) was conducted. Cells harvested from 1.5 ml cell suspensions were lysed and resuspended in 100 µl 8 % (w/v) NaCl, to which 400 µl distilled water was added. After this, the total DNA from *Halorubrum* sp. T3 was prepared as previously described (Charbonnier et al. 1992). The single copy genes *fhd* (fumarate hydratase class II) on the chromosome of the host strain T3 (Chen et al. 2012b) and *orf19* on the genome of pHRDV1 were chosen to determine the relative copy numbers. The specific primers and profiles used in the real-time quantitative PCR are listed in Supplementary Table S5. The reagent used in the quantitative PCR was Fast-start Universal SYB Green Master (Rox) Kit (Roche, USA) (three repeats). All of the reports generated by Rotor-Gene 6000 were analyzed using Software 6.0 (Corbett Research, Australia).

Testing for the production of virus by *Halorubrum* sp. T3

Cultures of *Halorubrum* sp. T3 were grown to the exponential phase (Supplementary Figure S1) and MMC added to a final concentration of 1 µM. After a week, the cells and debris were removed by centrifugation (Beckman Coulter optima l-100xp, 8000×g, 20 min, 4 °C) and supernatants were collected. To concentrate any virus particles present in the supernatants, they were treated with polyethylene glycol (PEG6000, 10 % w/v final concentration, 15 m at room temperature), and the precipitates collected by centrifugation (Beckman Coulter optima l-100xp, 152000×g, 40 min, 4 °C), and resuspended in 18 % MGM. These preparations were examined for the presence of virus particles by transmission electron microscopy (HITACHI HF-3300, Japan) following the method described by Pietilä et al. (2009). In addition, the presence of virus proteins was detected by 12 % SDS-polyacrylamide gel electrophoresis (PAGE) combined with the staining of Coomassie Bright Blue R250 (Sambrook and Russell 2001).

To detect the presence of pHRDV1 sequences in the PEG-precipitated culture supernatants, DNA was extracted using phenol-chloroform, collected by ethanol precipitation, resuspended in TE buffer (10 mM, pH 8.0), and used as template in PCR (Roine et al. 2010; Senčilo et al. 2012). The primer pairs (from *orf1* to *orf19*) and the PCR procedure used for the detection of the virus are listed in Supplementary Table S6.

Host range test

The DNA analysis revealed that some of the predicted genes on the genome of pHRDV1 shared a high similarity with those of *Halorubrum* pleomorphic viruses. The haloarchaeal strains, shown in Supplementary Table S1, were tested for their susceptibility to infection by plasmid pHRDV1. The supernatants of *Halorubrum* sp. T3 with or without MMC induction were plated with different haloarchaeal strains using 18 % MGM. After incubation at 37 °C for two weeks, the plates were checked for plaque formation (Roine et al. 2010).

Transcriptome of plasmid pHRDV1

Halorubrum sp. T3, the host strain of the plasmid pHRDV1, was induced with MMC as described. After 2, 4, 12, and 24 h of induction, the total RNA was isolated with TRizol reagent according to the manufacturer's instructions (CWBIO, China). The total RNA extracted from *Halorubrum* sp. T3 without MMC induction was used as a reference. The amount of total RNAs used as template of reverse transcription (1 μg total RNAs for each reaction) was determined by the turbidity and volume. Complementary DNA (cDNA) was generated by reverse transcription PCR (RT-PCR) using the total RNA as a template and the reverse primers of each ORF (Supplementary Table S6) as the reverse transcriptional primer. The reverse transcriptase M-MLV (RNase H^-) (TaKaRa) was used to generate the cDNA. The transcriptional pattern of 19 ORFs on the genome of plasmid pHRDV1 was determined by PCR amplification using cDNA as the template and ORF-specific primer pairs for each ORF (Supplementary Table S6).

Nucleotide sequence accession number

The completed genome nucleotide sequence of pHRDV1 was deposited in GenBank under the accession number of KC545797. The accession number of the 16S rRNA gene for *Halorubrum* sp. T3 is JQ936845. The accession numbers of the other 16S rRNA genes for *Halorubrum* sp. are listed in Supplementary Table S1.

Results

Phylogenetic analyses of the host strain of plasmid pHRDV1

Thirty-eight haloarchaeal strains were isolated from salt mines or saline soils from Jiangsu, Hainan, and Yunnan provinces (China) (Table S1). The haloarchaeal strains were identified according to their 16S rRNA gene sequences. A neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showed that the strain T3 was closely related (>99 %) to *Halorubrum sodomense* ATCC 33733 (D13379) (Supplementary Figure S2). Accordingly, the host strain was designated as *Halorubrum* sp. T3. An extrachromosomal genetic element was obtained from *Halorubrum* sp. T3 (Fig. 1a), and was designated pHRDV1.

Genome of pHRDV1 is a double-stranded circular DNA molecule

After agarose gel electrophoresis, plasmid pHRDV1 displayed two distinct bands (supercoiled and nicked plasmid migration) (Fig. 1a), both of which could be linearized by endonuclease *Eco*RI (Fig. 1b). These results suggest that pHRDV1 is a circular double-stranded DNA molecule. Further analysis was carried out using different nucleases at different concentrations. We found that pHRDV1 could be degraded by DNaseI, but was resistant to RNaseA, confirming that it is a DNA molecule (Fig. 1c). In addition,

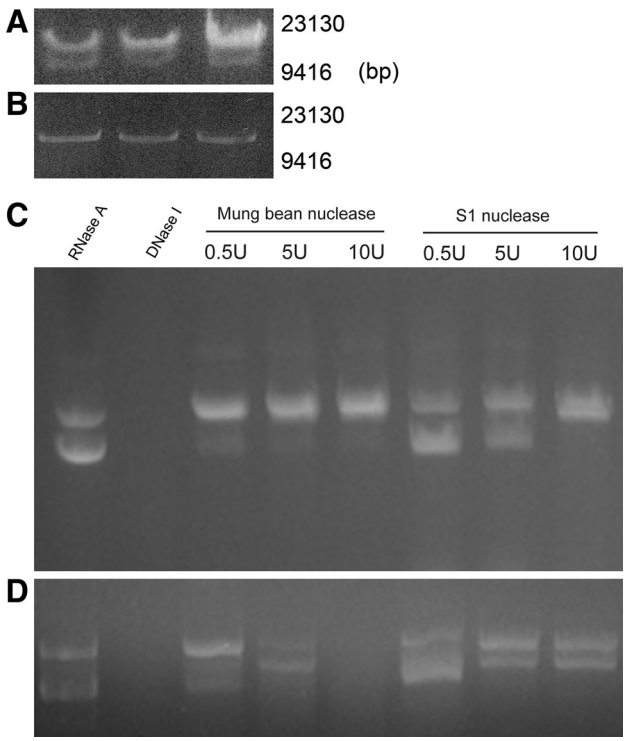

Fig. 1 Determination of the nucleic acid type of the plasmid pHRDV1. **a** Plasmid pHRDV1 extracted from haloarchaeal strain T3 using the alkaline lysis method. **b** Plasmid pHRDV1 digested with endonuclease *Eco*R I. **c** Plasmid pHRDV1 digested with RNase A, DNase I, Mung bean nuclease, and S1 nuclease. **d** The plasmid pUC18 was used as a positive control for the double-stranded DNA of a circular plasmid. It was treated with the same group of nucleases as in (**c**)

a wide range of Mung Bean Nuclease (MBN) was used, ranging from concentrations digesting only ssDNA (0.5 U 1 μg^{-1} of DNA) to concentrations that could act on nicked dsDNA (5 U 1 μg^{-1} of DNA) and dsDNA (10 U 1 μg^{-1} of DNA). The S1 nuclease was different from that of MBN, even at a high concentrations (10 U 1 μg^{-1} of DNA) it did not degrade the dsDNA of pHRDV1 (Fig. 1c). This confirms that pHRDV1 is a circular double-stranded DNA molecule.

Plasmid pHRDV1 is related to haloviruses The sequenced pHRDV1's genome was 13,053 bp in size and the GC percentage was 66.4 %, encoding 19 putative ORFs (Fig. 2a). The ORFs were organized into two different transcriptional directions, with most of the similarly oriented ORFs being closely spaced or overlapping at stop and start codons (Table S7). The close spacing of ORFs indicated that each of the two groups of ORFs may be transcribed as single units, which would predict transcripts of 5.5 and 7.5 Kb (Fig. 2b). Many overlaps were found among those 19 ORFs, for example, ORF2 and ORF3, ORF4 and ORF5, ORF6 and ORF7, ORF9 and ORF10, ORF11 and ORF12, ORF12 and ORF13, ORF18 and ORF19 (Table S7).

To identify the homologous sequences and predict the functions of the putative proteins of the plasmid pHRDV1, their translated amino acid sequences were compared to those in the public sequence databases using the BLASTP method (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 1). Five of the nineteen predicted genes had no significant similarity with any proteins in the existing databases. Another five ORFs had significant similarities with hypothetical proteins with unknown functions. The remaining

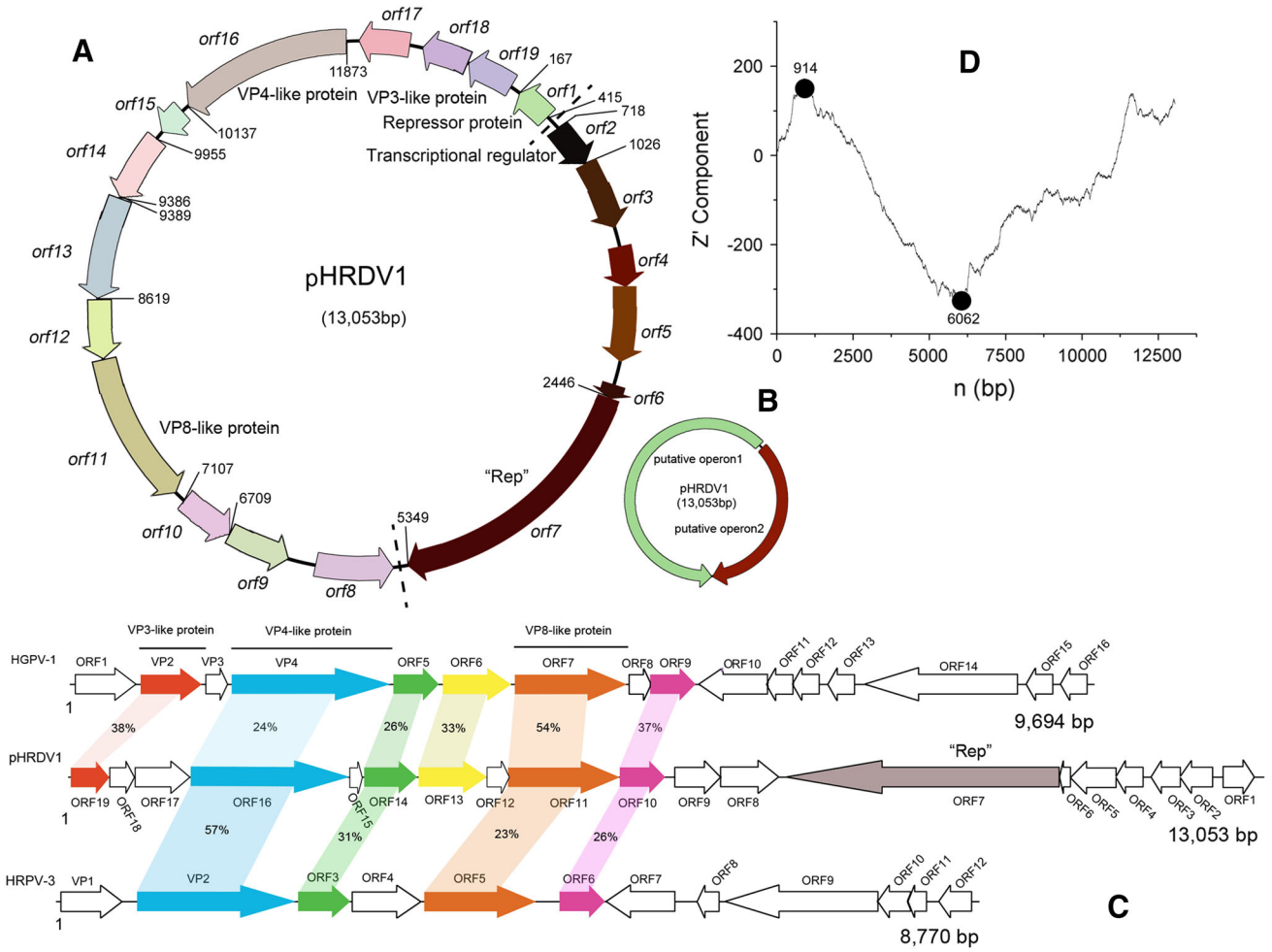

Fig. 2 Organization of the pHRDV1 genome. **a** The genome map of pHRDV1 including nineteen predicted ORFs (*external*) with identified and proposed functional assignment (*internal*). **b** The pHRDV1 genome was organized into two different transcriptional directions (two putative transcripts) located on opposite strands: transcript 1 was 7.5 kb (in a counterclockwise direction) and transcript 2 was 5.5 kb (in a clockwise direction). **c** The alignment of the genomes of HGPV-1, pHRDV1, and HRPV-3. The identities (in percentage) of the homologous translated ORFs or identified products. The predicted genes encoding VP3-like, VP4-like, and VP8-like protein homologues are marked below the alignment of the genomes (**d**). The origin of replication was predicted by Z-CURVE based on the nucleotide sequence of the pHRDV1 genome

Table 1 General features of the predicted genes (ORFs) of the plasmid pHRDV1

ORFs	Similarity	Total length/ Alignment length (aa)	Identity (%)	E-value	Reference	Proposed function/ features	RBS/distance
*orf*1	phage PhiH1 repressor protein [*Halorubrum lacusprofundi* ATCC 49239]	83/78	47	2e−14	YP_002564588	Repressor protein	CGGAG/-16
	phage PhiH1 repressor protein [*Halobiforma lacisalsi* AJ5]	83/78	46	1e−13	ZP_09950204		
	PhiH1 repressor-like protein [*Halobacterium* sp. NRC-1]	83/78	46	2e−13	NP_395857		
*orf*2	transcriptional regulator, PadR-like family [*Halorubrum lacusprofundi* ATCC 49239]	103/95	34	5e−04	YP_002567434	DNA binding domain-containing protein	GGAG/-14
*orf*3	hypothetical protein HlacAJ_13886 [*Halobiforma lacisalsi* AJ5]	120/98	42	2e−04	ZP_09948837		GAGGG/-12
*orf*4							
*orf*5	hypothetical protein Hbor_28950 [*Halogeometricum borinquense* DSM 11551]	159/151	53	8e−39	YP_004037879		CGGGA/-13
	hypothetical protein HFX_0928 [*Haloferax mediterranei* ATCC 33500]	159/151	50	2e−36	YP_006348635		
	hypothetical protein HFX_1288 [*Haloferax mediterranei* ATCC 33500]	159/151	48	5e−35	YP_006348987		
*orf*6							
*orf*7	hypothetical protein HacjB3_05670 [*Halalkalicoccus jeotgali* B3]	968/744	31	1e−59	YP_003736317	Replication protein	CGGAG/-36
	hypothetical protein pHV2p2 [Plasmid pHV2]	968/398	33	2e−42	NP_040403		
	pNRC100 replication protein H-like [*Halobacterium salinarum*]	968/504	30	3e−36	YP_001688242		
	plasmid replication protein, RepH [*Natrialba* phage PhiCh1]	968/621	29	2e−34	NP_665972		
	plasmid replication protein RepH [*Halorubrum lacusprofundi* ATCC 49239]	968/486	24	2e−07	YP_002567426		
	plasmid replication protein RepH [*Halobacterium* sp. NRC-1]	968/479	23	7e−05	NP_045956		
*orf*8	hypothetical protein HVO_1695 [*Haloferax volcanii* DS2]	217/218	36	2e−25	YP_003535739		GGAG/-25
	hypothetical protein HFX_1787 [*Haloferax mediterranei* ATCC 33500]	217/209	34	1e−23	YP_006349478		
*orf*9	hypothetical protein HlacAJ_20459 [*Halobiforma lacisalsi* AJ5]	131/94	37	1e−06	ZP_09950132	Putative conserved domains	GGCG/-17
	hypothetical protein Halxa_0284 [*Halopiger xanaduensis* SH-6]	131/100	36	1e−06	YP_004586141		

Table 1 continued

ORFs	Similarity	Total length/ Alignment length (aa)	Identity (%)	E-value	Reference	Proposed function/ features	RBS/distance
*orf*10	ORF9 [*Halogeometricum* pleomorphic virus 1]	133/105	37	6e−10	AFD04030		CGGGG/-40
	ORF6 [*Halorubrum* pleomorphic virus 3]	133/122	26	0.034	AFD04005		
*orf*11	ORF7 [*Halogeometricum* pleomorphic virus 1]	369/369	54	1e−125	AFD04028		GGGG/-9
	VP8 [*Halorubrum* pleomorphic virus 1]	369/236	30	9e−05	YP_002791893		
*orf*12							
*orf*13	ORF6 [*Halogeometricum* pleomorphic virus 1]	257/219	33	1e−19	AFD04027		GGCG/-37
	ORF7 [*Halorubrum* pleomorphic virus 1]	257/253	24	0.018	ACO54902		
*orf*14	ORF5 [*Halogeometricum* pleomorphic virus 1]	190/197	26	7e−07	AFD04026		GGAG/-49
	ORF3 [*Halorubrum* pleomorphic virus 3]	190/195	31	0.001	AFD04002		
*orf*15							
*orf*16	VP5 precursor [*Halorubrum* pleomorphic virus 2]	579/568	53	0.0	AFD03989		GAGGA/-12
	VP5 precursor [*Halorubrum* pleomorphic virus 6]	570/579	52	0.0	AFD04016		
	VP2 precursor [*Halorubrum* pleomorphic virus 3]	579/495	57	2e−177	AFD04001		
	VP4 [*Haloarcula hispanica* pleomorphic virus 1]	579/499	24	2e−14	YP_003412003		
	VP1 [His2 virus]	579/324	24	0.001	YP_529659		
*orf*17							
*orf*18	hypothetical protein BSU31380 [*Bacillus subtilis* subsp. subtilis str.168]	88/57	40	1e−04	NP_391016	Putative conserved domains	GTGGA/-10
*orf*19	VP2 [*Halogeometricum* pleomorphic virus 1]	115/113	38	4e−14	AFD04023		GGCG/-7
	hypothetical protein HVO_0268 [*Haloferax volcanii* DS2]	115/113	40	5e−12	YP_003534346		

RBS Ribosome binding site

nine predicted protein-coding genes were closely related to known viral structural or regulatory proteins (Table 1). The ribosomal binding sites of those 14 predicted genes are shown in Table 1.

The properties of the predicted polypeptides are shown in Supplementary Table S7. The genome organization and protein homology analyses showed that plasmid pHRDV1 is closely related to the recently isolated and characterized pleomorphic dsDNA viruses HRPV-3 and HGPV-1 (Senčilo et al. 2012) (Fig. 2c). On the one hand, the ORF11, ORF16 and ORF19 of pHRDV1 are similar to VP8, VP4 and VP3-like protein, the characteristic proteins of group II *Halorubrum* pleomorphic virus. However, plasmid pHRDV1 encodes a replication protein, which is absent among the group II *Halorubrum* pleomorphic viruses. On the other hand, the ORF11 and ORF16, except ORF19, show a relative low similarity with VP8 and VP4-like protein, the characteristic proteins of group I *Halorubrum* pleomorphic viruses. However, both of the pHRDV1 and group I *Halorubrum* pleomorphic virus carry genes encoding a replication protein (Table 1).

The highest identity (57 %) was found between the predicted protein of pHRDV1 *orf*16 and the VP2 precursor of HRPV-3. Although the plasmid pHRDV1 is closely related to some *Halorubrum* pleomorphic viruses, its *orf*19 showed a relatively high similarity with the VP2 of HGPV-1 in the genus *Halogeometricum*. When both gene orientation and amino acid sequence similarity were considered, pHRDV1 was most closely related to HGPV-1 (Fig. 2c).

The *Z*-curve method has been used in many different areas of genome research, including replication origin identification. The peak at 914 bp or the trough at 6062 bp on the *Z*-curve might represent the replication origin region of the nucleotide sequence (Fig. 2d). The predicted replication origin region at the *Z*-curve minimum (6062 bp) is located at 713 bp downstream of the putative replication gene (*orf*7) of plasmid pHRDV1.

Plasmid pHRDV1 contains a characteristic high copy number

The total DNA extracted from the host strain T3 was used as a template for the real-time PCR amplification. The amplification efficiency using primers *orf*19F/*orf*19R and *fhd*F/*fhd*R is shown in Supplementary Figure S3. The relative amounts of available templates for *orf*19 (on pHRDV1 genome) and *fhd* (on host strain T3's genome) amplification were calculated according to a universal formula based on the Cts (cycle thresholds) of gene *orf*19 (Figure S6, A) and *fhd* (Figure S5, B). Taking the genome of the host strain (*fhd*) as one copy, the copies of the plasmid pHRDV1 genome (*orf*19) were estimated as 56 ($\Delta Ct = -5.8$, $2^{-\Delta Ct} = 56$) (Figure S5).

No evidence of virus production by *Halorubrum* sp. T3

The strains listed in Supplementary Table S1 were prepared as lawn host cells for screening lytic viruses. With or without MMC induction, supernatants of strain T3 did not make any clear plaques on lawn plates of these strains. Furthermore, the PEG-concentrated supernatants of strain T3 cultures were examined for the presence of virus particles by electron microscopy (TEM), gel electrophoresis of proteins, and PCR amplification of pHRDV1 DNA (see "Materials and methods"). No evidence of virus production was detected by any of these methods, either from the supernatants of cells treated with or without MMC. Primers used for PCR amplification are listed in supplementary Table S6.

Transcriptional pattern of pHRDV1 under MMC induction

Total RNA was extracted from strain T3 cells that had been grown in the presence or absence of MMC (Fig. S4), and reverse-transcribed to produce cDNA using ORF-specific primers (Materials and methods and Table S6). The resulting cDNAs were then used in PCRs to detect transcripts covering the annotated ORFs of pHRDV1 (Fig. 3). The 19 ORFs varied in their transcriptional levels. As shown in Fig. 3a, transcription across all 19 predicted ORFs of pHRDV1 could be detected in strain T3. All PCR products were of the expected size (for the primer pairs used), and control experiments confirmed that no products were observed if RNA was not added to the RT reaction mix, or if the RNA was first treated with RNase A. Although the method used does not allow accurate and quantitative comparison of transcript levels, the experimental conditions were kept as constant as possible. This is supported by the transcription of ORF7 (encoding RepH), which showed a consistently strong RT-PCR band in all samples (panels a–e). In comparison, the levels of other transcripts appeared to be affected by MMC treatment. For example, the level of transcription across *orf*8 and *orf*13 appeared to decrease after 2 h of MMC treatment, but after 24 h the level of *orf*13 appeared to return to that of untreated cells.

Discussion

The host strain of the plasmid pHRDV1, *Halorubrum* sp. T3, was isolated from a halite deposit from the Yuanyongjing Salt Mine, Yunnan, China; it is closely related to *Halorubrum sodomense* ATCC 33733 (supplementary Figure S2). While other members of the genus *Halorubrum* are known to carry plasmids, the only one that has been sequenced is pZMX101 from *Halorubrum saccharovorum*

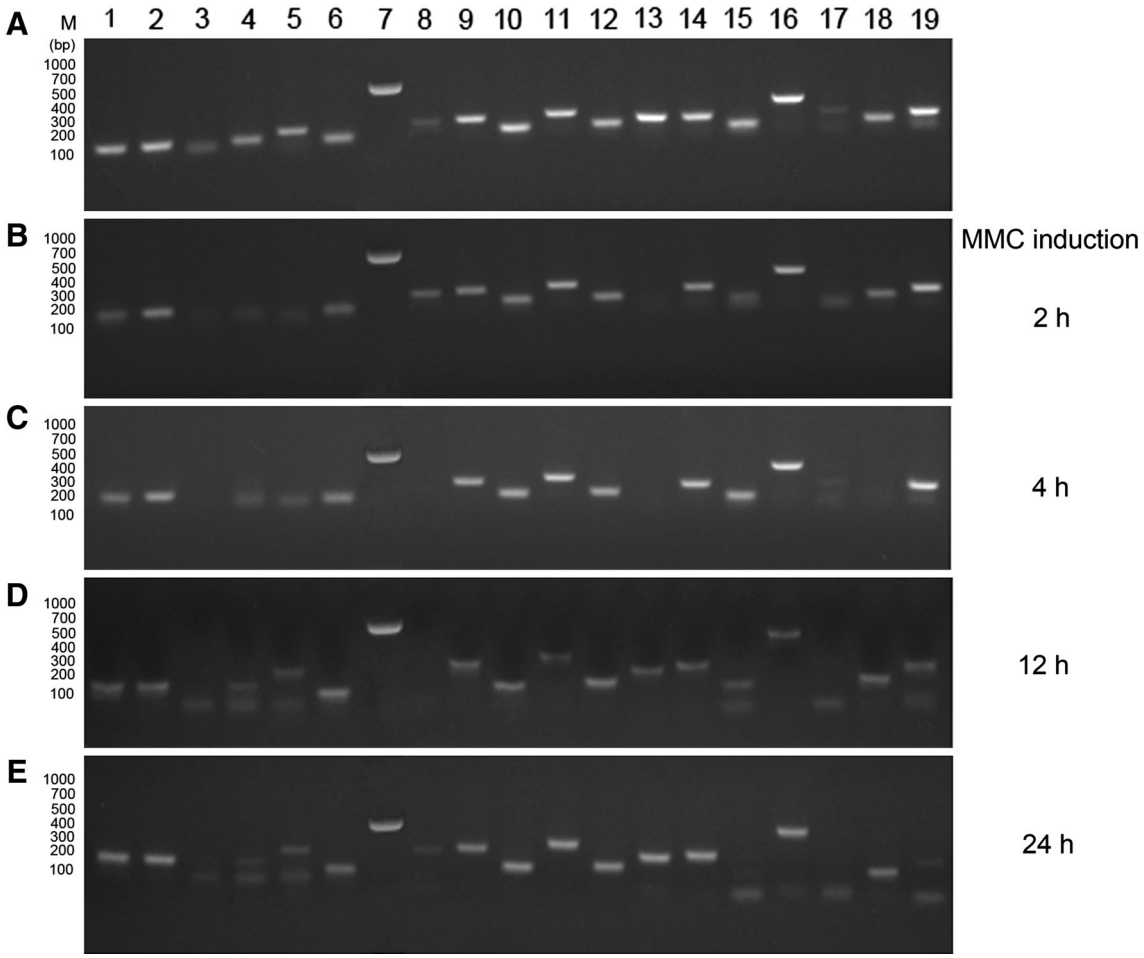

Fig. 3 The transcriptional patterns of plasmid pHRDV1. The total RNAs were extracted from strain T3 under both the condition of MMC induction and no MMC induction (Figure S4). The series of numbers on the top bar from 1 to 19 represent the primer pairs of *orf1* to *orf19*. The reverse primers of each *orf* were used for the reverse transcription. The duration of the MMC induction is shown to the *right* of the gel. **a** Transcriptome of plasmid pHRDV1 without MMC induction. Transcriptomes of plasmid pHRDV1 induced with 1 μM (final concentration) of MMC for 2, 4, 12, and 24 h, are shown in (**b**), (**c**), (**d**), and (**e**), respectively. The DNA molecular weight standard (M) is shown to the *left* of the gel

(Zhou et al. 2007). The plasmid pHRDV1 (13, 053 bp) differs from the plasmid pZMX101 (3, 918 bp) and other haloarchaeal plasmids at the level of its gene or protein amino acid sequence. Like most other haloarchaeal plasmids, pHRDV1 is also a double-stranded circular DNA molecule (Fig. 1), encoding proteins with unknown functions. However, the copy number of the plasmid pHRDV1 is higher than that of the average haloarchaeal plasmid ($\geq$10), and similar to some haloviruses (Figure S5). Furthermore, the predicted *orf7* of the plasmid pHRDV1 has a relatively high similarity (30 % amino acid sequence identity) with the replication protein H (RepH) of the haloarchaeal plasmid pNRC100 (Ng and DasSarma 1993) (Table 1), suggesting that the plasmids pHRDV1 and pNRC100 share a similar replication mode.

Our sequence comparisons showed that six predicted ORFs on the genome of plasmid pHRDV1 had high sequence identities with one or more structural proteins in seven haloarchaeal pleomorphic viruses, that is, His2 (Bath et al. 2006), HRPV-1 (Pietilä et al. 2009), HHPV-1 (Roine et al. 2010), HRPV-2, HRPV-3, HRPV-6, and HGPV-1 (Senčilo et al. 2012). These haloarchaeal pleomorphic viruses can be classified into three subgroups: the first group (subgroup I) includes HHPV-1, HRPV-6, HRPV-2, and HRPV-1; the second group (subgroup II) includes HRPV-3 and HGPV-1; and the third group (subgroup III) includes only His2 (Senčilo et al. 2012). The plasmid pHK2, isolated from haloarchaeal species *Haloferax lucentense*, is homologous to subgroup I haloarchaeal pleomorphic viruses (Holmes et al. 1995), whereas the plasmid pHRDV1 is homologous to subgroup II haloarchaeal pleomorphic viruses (including HRPV-3 and HGPV-1). Subgroup II haloarchaeal pleomorphic viruses are distinct from the plasmid pHRDV1 because HRPV-3 and HGPV-1

lack the replication protein (Rep) that is found on the genome of the plasmid pHRDV1. The highest degree of conservation and the preliminary phylogenetic analyses of these proteins suggest that they share similar evolutionary histories (Senčilo et al. 2012). The encoded regions of all three characteristic proteins of subgroup II haloarchaeal pleomorphic viruses were found in the plasmid pHRDV1 genome; they were designated as *orf*19, *orf*16, and *orf*11 (Fig. 2c). *Orf*16, on the genome of plasmid pHRDV1, shows their conserved nature: it contains a typical twin-arginine translocation (Tat) signal sequence, two transmembrane domains, and a coiled-coil region. Similar conserved domains have been found in all of the VP4-like proteins with a receptor recognition function. Although the amino acid sequences of the plasmid pHRDV1's gene products showed a relatively low similarity with the subgroup I haloarchaeal pleomorphic viruses (including HHPV-1, HRPV-6, and HRPV-2), each of them contains a typical replication protein gene.

Surprisingly, *orf*11 of pHK2 (Roine et al. 2010) and *orf*1 (−) of plasmid pHRDV1 (Fig. 2) share a high sequence similarity with the phage φH1 repressor-like protein of *Halorubrum lacusprofundi* (accession YP_002564588, $E = 3\times10^{-13}$). In the genome of pHRDV1, the *orf*2 (+) and *orf*1 (−) showed a 34 % amino acid sequence identity with a member of the padR-like transcriptional regulator family and a 47 % amino acid sequence identity with the phage φH1 repressor protein in *Halorubrum lacusprofundi*, respectively (Table 1). The properties of the putative gene products and the gene order of *orf*1 (−) and *orf*2 (+) closely resemble the repressor-operator system of the haloarchaeal temperate virus, φCh1, which are composed of *orf*48 (−) and *orf*49 (+) (Iro et al. 2007) (Supplementary Table S8). Plasmids and viruses, the two main parts of MGEs, are closely related, and both of their properties are important in HGT (Sobecky and Hazen 2009).

Although further experiments will be needed to determine the exact nature of transcription and regulation (by MMC) of pHRDV1 genes in strain T3 cells, it is obvious that plasmid pHRDV1, like pHK2, appears to be a chimeric DNA molecule, containing virus-like and chromosomal regions, showed no evidence of virus production, which implies that those two haloarchaeal plasmids share the similar origin and replication mechanism. Another typical chimeric DNA is represented by the *Sulfolobus* plasmid pSSVx, which contains three ORFs that are closely related to those of fuselloviruses. Sequences of these virus-related ORFs enable pSSVx to use the packaging system of the viral helpers for spreading (Arnold et al. 1999). A fusellovirus SSV4 and a pRN-like plasmid pXZ1 were co-isolated from a single strain of *Sulfolobus*. Furthermore, the integrase gene of fusellovirus (SSV4) has been transferred to the pRN-like plasmid (pXZ1), which made the pXZ1 to be an active mobile genetic element (Peng 2008). For pHRDV1, the virus-like genetic elements on its genome may come from *Halorubrum* pleomorphic virus, which are widely distributed in the hypersaline environment.

Hypersaline environments, which are typically harsh niches, contain considerable numbers of haloviruses (Kukkaro and Bamford 2009; Atanasova et al. 2012). To survive in these difficult conditions, most haloarchaea have evolved an adaptive immune system comprised of CRISPR-Cas system (clustered regularly interspaced palindromic repeats/CRISPR-associated proteins) (Li et al. 2013). However, the host strain of the plasmid pHRDV1 does not have any CRISPR-*cas* architectures on its genome. (Chen et al. 2012b). Instead, *Halorubrum* sp.T3 may gain some conserved viral protein genes through LGT, and the insertion of these proteins into its plasmid (Fig. 2) allows it to resist viral infection. A similar process occurs in the widely used DNA vaccines composed of a partial viral structure and self-replication genes. LGT events occur in haloarchaea with a high frequency (Chen et al. 2012a; Nelson-Sathi et al. 2012). The new forms of MGEs such as plasmid pHRDV1 may endow their host strains with a wide ecological adaptability, allowing them to flourish in harsh environments.

In conclusion, plasmid pHRDV1 is a peculiar chimera comprised of viral and chromosomal components (Table 1). The chromosomal part is responsible for its replication, whereas the function of the viral part is unknown. One hypothesis is that the viral function benefits the host cell by preventing viral infection. However, based on the available data, plasmid pHRDV1 is not a virus. This study expands our understanding of the diversity and potential functions of virus-like MGEs in haloarchaea. However, more research is needed to better understand their unique natures and their huge influence on their environments.

Acknowledgments We thank Dr. Ming Ding from Yunnan Academy of Agricultural Science for the technical assistance of transmission electron microscopic observation, and Dr. Zhi-Xiong Xie from Wuhan University and Dr. Zhi-Wei Zhao from Yunnan University for providing constructive suggestions. We also thank Dr. Elina Roine from University of Helsinki for giving helpful discussions. This work was supported by the CAS/SAFEA International Partnership Program for Creative Research Teams and the Hundred Talents Program of the Chinese Academy of Sciences (2010–2012).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arnold HP, She Q, Phan H, Stedman K, Prangishvili D, Holz I, Kristjansson JK, Garrett R, Zillig W (1999) The genetic element pSSVx of the extremely thermophilic crenarchaeon Sulfolobus is

a hybrid between a plasmid and a virus. Mol Microbiol 34:217–226

Atanasova NS, Roine E, Oren A, Bamford DH, Oksanen HM (2012) Global network of specific virus-host interactions in hypersaline environments. Environ Microbiol 14:426–440

Bamford DH, Ravantti JJ, Rönnholm G, Laurinavicius S, Kukkaro P, Dyall-Smith M, Somerharju P, Kalkkinen N, Bamford JK (2005) Constituents of SH1, a novel lipid-containing virus infecting the halophilic euryarchaeon *Haloarcula hispanica*. J Virol 79:9097–9107

Bath C, Dyall-Smith ML (1998) His1, an archaeal virus of the *Fuselloviridae* family that infects *Haloarcula hispanica*. J Virol 72:9392–9395

Bath C, Cukalac T, Porter K, Dyall-Smith ML (2006) His1 and His2 are distantly related, spindle-shaped haloviruses belonging to the novel virus group, *Salterprovirus*. Virology 350:228–239

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: signalP 3.0. J Mol Biol 340:783–795

Benlloch S, Acinas SG, Antón J, López-López A, Luz SP, Rodríguez-Valera F (2001) Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. Microb Ecol 41:12–19

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, Frutiger S, Hochstrasser D (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14:1023–1031

Charbonnier F, Erauso G, Barbeyron T, Prieur D, Forterre P (1992) Evidence that a plasmid from a hyperthermophilic archaebacterium is relaxed at physiological temperatures. J Bacteriol 174:6103–6108

Chen S, Tulloss RE, Liu Y, Feng B, Zhao Z, Yang ZL (2012a) Lateral gene transfer occurring in haloarchaea: an interpretative imitation study. World J Microbiol Biotechnol 28:2913–2918

Chen S, Wang C, Zhao Z, Yang ZL (2012b) Genome sequence of *Halorubrum* sp. strain T3, an extremely halophilic archaeon harboring a virus-like element. J Bacteriol 194:6608–6609

Delcher AL, Harmon D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27:4636–4641

Dyall-Smith M, Tang SL, Bath C (2003) Haloarchaeal viruses: how diverse are they? Res Microbiol 154:309–313

Gonnet M, Erauso G, Prieur D, Le Romancer M (2011) pAMT11, a novel plasmid isolated from a *Thermococcus* sp. strain closely related to the virus-like integrated element TKV1 of the *Thermococcus kodakaraensis* genome. Res Microbiol 162:132–143

Guixa-Boixereu N (1996) Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. Aquat Microb Ecol 11:215–227

Hofmann K, Stoffel W (1993) TM-base: a database of membrane spanning protein segments. Biol Chem Hoppe Seyler 374:166

Holmes ML, Pfeifer F, Dyall-Smith ML (1995) Analysis of the halobacterial plasmid pHK2 minimal replicon. Gene 153:117–121

Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Das U, Daugherty L, Duquenne L, Finn RD, Gough J, Haft D, Hulo N, Kahn D, Kelly E, Laugraud A, Letunic I, Lonsdale D, Lopez R, Madera M, Maslen J, McAnulla C, McDowall J, Mistry J, Mitchell A, Mulder N, Natale D, Orengo C, Quinn AF, Selengut JD, Sigrist CJ, Thimma M, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2009) InterPro: the integrative protein signature database. Nucleic Acids Res 37:D211–D215

Iro M, Klein R, Gálos B, Baranyi U, Rössler N, Witte A (2007) The lysogenic region of virus phiCh1: identification of a repressor-operator system and determination of its activity in halophilic Archaea. Extremophiles 11:383–396

Jalasvuori M, Jaatinen ST, Laurinavicius S, Ahola-Iivarinen E, Kalkkinen N, Bamford DH, Bamford JK (2009) The closest relatives of icosahedral viruses of thermophilic bacteria are among viruses and plasmids of the halophilic archaea. J Virol 83:9388–9397

Klein R, Baranyi U, Rössler N, Greineder B, Scholz H, Witte A (2002) *Natrialba magadii* virus phiCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon. Mol Microbiol 45:851–863

Krupovic M, Gonnet M, Hania WB, Forterre P, Erauso G (2013) Insights into dynamics of mobile genetic elements in hyperthermophilic environments from five new *Thermococcus* plasmids. PLoS One 8:e49044

Kukkaro P, Bamford DH (2009) Virus-host interactions in environments with a wide range of ionic strengths. Environ Microbiol Rep 1:71–77

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Li M, Liu H, Han J, Liu J, Wang R, Zhao D, Zhou J, Xiang H (2013) Characterization of CRISPR RNA biogenesis and Cas6 cleavage-mediated inhibition of a provirus in the haloarchaeon *Haloferax mediterranei*. J Bacteriol 195:867–875

Lukashin AV, Borodovsky M (1998) GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res 26:1107–1115

Lupas A, van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. Science 252:1162–1164

Maturrano L, Santos F, Rosselló-Mora R, Antón J (2006) Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. Appl Environ Microbiol 72:3887–3895

Mei Y, Chen J, Sun D, Chen D, Yang Y, Shen P, Chen X (2007) Induction and preliminary characterization of a novel halophage SNJ1 from lysogenic *Natrinema* sp. F5. Can J Microbiol 53:1106–1110

Nelson-Sathi S, Dagan T, Landan G, Janssen A, Steel M, McInerney JO, Deppenmeier U, Martin WF (2012) Acquisition of 1,000 eubacterial genes physiologically transformed a methanogen at the origin of Haloarchaea. Proc Natl Acad Sci USA 109:20537–20542

Ng WL, DasSarma S (1993) Minimal replication origin of the 200-kilobase *Halobacterium* plasmid pNRC100. J Bacteriol 175:4584–4596

Oren A (2002) Molecular ecology of extremely halophilic Archaea and bacteria. FEMS Microbiol Ecol 39:1–7

Oren A, Bratbak G, Heldal M (1997) Occurrence of virus-like particles in the Dead Sea. Extremophiles 1:143–149

Pedrós-Alió C, Calderón-Paz JI, MacLean MH, Medina G, Marrasé C, Gasol JM, Guixa-Boixereu N (2000) The microbial food web along salinity gradients. FEMS Microbiol Ecol 32:143–155

Peng X (2008) Evidence for the horizontal transfer of an integrase gene from a fusellovirus to a pRN-like plasmid within a single strain of *Sulfolobus* and the implications for plasmid survival. Microbiology 154:383–391

Pietilä MK, Roine E, Paulin L, Kalkkinen N, Bamford DH (2009) An ssDNA virus infecting archaea: a new lineage of viruses with a membrane envelope. Mol Microbiol 72:307–319

Pietilä MK, Atanasova NS, Manole V, Liljeroos L, Butcher SJ, Oksanen HM, Bamford DH (2012) Virion architecture unifies globally distributed pleolipoviruses infecting halophilic archaea. J Virol 86:5067–5079

Porter K, Kukkaro P, Bamford JK, Bath C, Kivelä HM, Dyall-Smith ML, Bamford DH (2005) SH1: a novel, spherical halovirus

isolated from an Australian hypersaline lake. Virology 335:22–33

Rhodes ME, Spear JR, Oren A, House CH (2011) Differences in lateral gene transfer in hypersaline versus thermal environments. BMC Evol Biol 11:199

Roine E, Kukkaro P, Paulin L, Laurinavicius S, Domanska A, Somerharju P, Bamford DH (2010) New, closely related haloarchaeal viral elements with different nucleic acid types. J Virol 84:3682–3689

Rose RW, Brüser T, Kissinger JC, Pohlschröder M (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol 45:943–950

Sabet S, Diallo L, Hays L, Jung W, Dillon JG (2009) Characterization of halophiles isolated from solar salterns in Baja California, Mexico. Extremophiles 13:643–656

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Schnabel H, Zillig W, Pfäffle M, Schnabel R, Michel H, Delius H (1982) *Halobacterium halobium* phage φH. EMBO J 1:87–92

Senčilo A, Paulin L, Kellner S, Helm M, Roine E (2012) Related haloarchaeal pleomorphic viruses contain different genome types. Nucleic Acids Res 40:5523–5534

Sime-Ngando T, Lucas S, Robin A, Tucker KP, Colombet J, Bettarel Y, Desmond E, Gribaldo S, Forterre P, Breitbart M, Prangishvili D (2011) Diversity of virus-host systems in hypersaline Lake Retba, Senegal. Environ Microbiol 13:1956–1972

Sobecky PA, Hazen TH (2009) Horizontal gene transfer and mobile genetic elements in marine systems. Methods Mol Biol 532:435–453

Sonnhammer EL, von Heijne G, Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 6:175–182

Tang SL, Nuttall S, Ngui K, Fisher C, Lopez P, Dyall-Smith M (2002) HF2: a double-stranded DNA tailed haloarchaeal virus with a mosaic genome. Mol Microbiol 44:283–296

Ye X, Ou J, Ni L, Shi W, Shen P (2003) Characterization of a novel plasmid from extremely halophilic archaea: nucleotide sequence and function analysis. FEMS Microbiol Lett 221:53–57

Zhang R, Zhang CT (2005) Identification of replication origins in archaeal genomes based on the Z-curve method. Archaea 1:335–346

Zhang Z, Liu Y, Wang S, Yang D, Cheng Y, Hu J, Chen J, Mei Y, Shen P, Bamford DH, Chen X (2012) Temperate membrane-containing halophilic archaeal virus SNJ1 has a circular dsDNA genome identical to that of plasmid pHH205. Virology 434:233–241

Zhou L, Zhou M, Sun C, Xiang H, Tan H (2007) Genetic analysis of a novel plasmid pZMX101 from *Halorubrum saccharovorum*: determination of the minimal replicon and comparison with the related haloarchaeal plasmid pSCM201. FEMS Microbiol Lett 270:104–108