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# Extremely halophilic pleomorphic archaeal virus HRPV9 extends the diversity of pleolipoviruses with integrases

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**Abstract**

Certain pleomorphic archaeal viruses are highly infectious even at saturated salt. These viruses belong to the genus *Betapleolipovirus* of the recently described archaeal virus family *Pleolipoviridae*. Pleolipoviruses comprise single-stranded or double-stranded, circular or linear DNA genomes that share countless homologs among various archaeal genetic elements. Here we describe a new extremely halophilic betapleolipovirus, *Halorubrum* pleomorphic virus 9 (HRPV9), which has an integrase gene. We also identified new genes encoding minor pleolipoviral structural proteins. The studies on HRPV9 enhance our knowledge on pleolipoviruses, especially their reciprocal relatedness and relation to certain archaeal plasmids, proviruses and membrane vesicles.

**Keywords:** Archaeal virus; pleomorphic virus; halophilic; hypersaline environment

## 1. Introduction

High numbers of prokaryotic viruses have been observed in hypersaline environments throughout the world. Most of these viruses infect extremely halophilic archaea, although halophilic bacteriophages are also abundant. Although research on archaeal viruses has escalated during recent years, it still lags behind compared to the knowledge on bacteriophages and eukaryotic viruses. Archaeal viruses are well-known for their unique morphologies and orphan genes [1, 2]. However, most of the described viruses that infect halophilic archaea resemble tailed bacteriophages [3, 4]. The other virus morphotype that has frequently been encountered during virus isolations from hypersaline environments, are the pleomorphic viruses of the *Pleolipoviridae* family. [5]. The currently described eight virus species are specific for halophilic archaeal hosts. In addition, pleolipoviruses have many qualities similar to membrane vesicles and non-viral mobile genetic elements [1, 6].

The *Pleolipoviridae* family contains three genera: *Alphapleolipovirus*, *Betapleolipovirus* and *Gammapleolipovirus* [5]. The type species, HRPV-1, as well as HRPV-2, HRPV-3, and HRPV-6 all infect different archaeal strains belonging to the genus *Halorubrum* [5]. Both the viruses and their hosts originate from geographically distant hypersaline environments. The other members of the *Pleolipoviridae* family infect archaeal strains from the genera *Haloarcula* or *Halogeometricum* [5]. Double-stranded DNA genomes of betapleolipoviruses contain frequent single-stranded discontinuities and replicate by an unknown mechanism, being the least understood among pleolipoviruses [7]. Another specific feature of betapleolipoviruses is their requirement for extremely high NaCl concentrations. Betapleolipoviruses HHPV3 and HHPV4 require over 3 M NaCl, while HGPV-1 and HRPV-3 can tolerate also lower salinities [8, 9]. All betapleolipoviruses maintain their infectivity even at saturated NaCl concentrations. While some

icosahedral tailed haloviruses are reversibly inactivated by low NaCl concentrations [10], in the case of pleolipoviruses, the inactivation is irreversible [9].

*Halorubrum* pleomorphic virus 9 (HRPV9) was originally isolated during a study on halocins, which are antimicrobials produced by halophilic archaea [11]. Here we describe the morphology, life cycle and genomic features of the virus, as well as study the genetic relatedness to the newly identified archaeal proviruses. The characterization of HRPV9 broadens the knowledge on betapleolipoviruses, especially those containing a viral integrase.

## 2. Materials and methods

### *2.1 Virion stability, host range and life cycle*

The archaeal strains used in this study (Table S1) were aerobically grown according to the Halohandbook (http://www.haloarchaea.com/resources/halohandbook/) and as described in [11]. HRPV9 was isolated from a turbid plaque observed on *Halorubrum* sp. SS5-4 lawn inoculated with culture supernatant of *Halorubrum* sp. B2-2 (the original isolation name was B2-2/SS5-4) [11]. Host range of HRPV9 was examined by a spot-on-lawn test [12] using haloarchaeal strains (Table S1). *Halorubrum* sp. SS5-4 was used as a host for HRPV9 in temperature tests and two repeats of NaCl sensitivity test. *Halorubrum* sp. SS7-4 was used as the host in all other tests due to its better stability as a virus host over time compared to the strain SS5-4.

Stability of agar stocks stored at 4°C (prepared as described in [12]) was examined by plaque assay for a two-month period. Stability of HRPV9 infectivity at different ionic strengths was studied by diluting virus agar stock 1:1000 in 18% salt water (SW), pH 7.2 (Halohandbook http://www.haloarchaea.com/resources/halohandbook/) and excluding one compound ($MgCl_2 \times 6H_20$, $MgSO_4 \times 7H_2O$, KCl, $CaCl_2$ or NaCl) at a time or by changing the molarity of NaCl (0-5.0 M). Infectivity was measured by plaque assay after 3 h, 24 h, and 7 d incubation at 37°C. Stability was assayed similarly in 50 mM Tris-HCl, pH 7.2. Infectivity at different temperatures was tested by incubating agar stock for 30 min at 4°C, and 20-80°C (with intervals of 10 degrees).

Adsorption and life cycle of HRPV9 using SS7-4 strain were analyzed as described in [9] with the following exceptions. For adsorption test, cells were collected when $OD_{550}$ was 0.8, concentrated tenfold, and infected using a multiplicity of infection (MOI) of 0.1 at 37°C. For life cycle, cells where infected when $OD_{550}$ was 1.0 and infective centers were assayed immediately

post infection (p.i.), at 0.5 and 1 h p.i. Cells were washed at 1.5 h p.i. after which $OD_{550}$, free progeny viruses, infective centers, and viable cells were monitored.

### *2.2 Analysis of HRPV9 virions*

Virus particles were precipitated from agar stocks using 10% polyethylene glycol 6000 and aggregates were removed [13]. Viruses were purified by subsequent rate-zonal (sucrose) and equilibrium (CsCl) centrifugation as described in [13], using Sorvall TH641 rotor (rate-zonal centrifugation time was 2 h 40 min; density of CsCl solution was 1.35 g $ml^{-1}$, and CsCl centrifugation temperature was 20°C). After purification in CsCl, particles were diluted 1:1 to 18% SW devoid of NaCl and concentrated by differential centrifugation (Sorvall Ti50 rotor, 30000 rpm, 2 h, 15°C). Protein and lipid analyses on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to [13]. Lipids were extracted from HRPV9, the host SS7-4, and *Haloarcula hispanica* and analyzed by thin layer chromatography as described in [9] with the exception that after ammonium molybdate staining, the silica plates were incubated at 130°C for 5 min.

For transmission electron microscopy (TEM) analyses, the purified particles were negatively stained with 3% uranyl acetate, pH 4.5 on copper pioloform coated grids and visualized (JEOL 1400, 80 kV, Electron Microscopy Unit, HiLIFE-Institute of Biotechnology, University of Helsinki). The particle diameter was calculated as the average of 13 particles visualized by TEM.

For mass spectrometry (MS), HRPV9 protein bands were cut from the polyacrylamide gel and processed as described in [9]. Liquid chromatography and tandem MS analysis were carried out according to [9] with the following exceptions. Samples were digested by adding either 0.75 µg trypsin (Sequencing Grade Modified Trypsin, V5111, Promega) or 0.75 µg Asp-N (New

England BioLabs) and incubated overnight at 37°C. Only full-tryptic or Asp-N peptides with a maximum of one missed cleavage were considered in the analysis of MS scans.

### *2.3 Genome sequencing and annotation*

For nucleic acid extraction, particles (obtained after rate-zonal centrifugation) in 18% SW were diluted 1:4 in 20 mM Tris-HCl, pH 7.2 and treated with 1% (w/v) SDS and 100 µg/ml proteinase K (Thermo Scientific) in the presence of 1 mM EDTA for an hour at 37°C. Purified nucleic acid was treated with nucleases and restriction enzymes according to manufacturers' instructions.

For sequencing, pure DNA was fragmented by sonication (Bioruptor NGS, Diagenode), and a standard Illumina TruSeq library was constructed and analyzed on an MiSeq instrument using MISeq reagent kit v3 (600 cycles; Sequencing and Genomics Laboratory, HiLIFE-Institute of Biotechnology, University of Helsinki). The paired-end sequences were trimmed using Cutadapt [14] and assembled with SPAdes [15]. The sequence (GenBank Acc. No KY965934) was analyzed as summarized in Table S2. Phylogenomic analyses (Genome BLAST Distance Phylogeny, GBDP) of virus genome sequences were performed using VICTOR online tool at https://victor.dsmz.de [16].

## 3. Results and discussion

HRPV9 infected only *Halorubrum* sp. SS7-4 in addition to isolation host *Halorubrum* sp. SS5-4 (same efficiency of plating; Table S1). The agar stock titer (on average ~$2\times10^{11}$ pfu/ml) remained unchanged throughout two months at 4°C, but incubation at 60°C and above, resulted in the loss of infectivity. For all the tested salts, only the absence of NaCl reduced virus titer to $3.3\times10^{9}$ pfu/ml after 7 d incubation. The virus was stable even at 5.0 M NaCl, as after 7 d incubation, the titer was $1.3\times10^{11}$ pfu/ml. Infectivity dropped over time in 50 mM Tris-HCl to $6.5\times10^{9}$ (3 h), $6.4\times10^{8}$ (24 h), and $9\times10^{3}$ pfu/ml (7 d). These results indicate that HRPV9 is stable from relatively low (0.5 M) to almost saturated NaCl concentrations, which seems to be characteristic for betapleolipoviruses [9].

Viruses adsorbed efficiently to the SS7-4 cells, as 60% of the particles were adsorbed at 2 h p.i (Fig. 1A). The adsorption rate constant calculated for 30 min p.i. was $8.5\times10^{-11}$ ml/min. Similarly to other pleolipoviruses [9, 13], HRPV9 infection cycle is non-lytic and persistent with a continuous, high production of progeny virus particles (Fig. 1B). However, the numbers of viable cells were substantially lower in the infected culture (Fig. 1C), as also reported for other betapleolipoviruses, such as HHPV3 and HRPV-3 [9, 13]. The observed high numbers of infective centers (Fig. S1) indicate that the majority of the cells were infected. Two-step virus purification yielded approximately 10% of highly pure infective particles. SDS-PAGE analysis followed by MS analysis showed that the composition of HRPV9 structural proteins is typical for pleolipoviruses (Fig. 1D, Fig. S2) [13]. HRPV9 has one spike protein (VP14) and two membrane proteins (VP11 and VP12), similarly to HGPV-1 [13]. VP19, a putative NTPase, as well as VP16 and VP21 were identified as structural proteins (see also below, Fig. 2). The lipid analysis of the virion and its host (Fig. 1D) indicated non-selective lipid acquisition, which is characteristic for

pleolipoviruses [13]. The particles had pleomorphic morphology with a ~57 nm diameter (Fig. 1E).

HRPV9 genome is a circular dsDNA molecule of 16,159 bp and contains 28 predicted ORFs (Fig. S3, Table S3). Most of the predicted proteins (45-584 amino acids) had low calculated pI (Table S3). A conserved block of ORFs or genes characteristic for the members of the family *Pleolipoviridae* was found in HRPV9 genome (Fig. 2). HRPV9 VP14 displays similarity to spike proteins found in all pleolipoviruses (Table S3). HRPV9 VP12 is similar to the membrane proteins found in pleolipoviruses HHPV3, HHPV4, HGPV-1, and His2 (23-27 % amino acid similarity; similar gene locus), suggesting that VP12 is an internal membrane-associated protein. Similarly, HRPV9 VP11 is predicted to be an internal membrane protein, based on the sequence similarities to other pleolipoviruses (Table S3, Fig. 2). Both proteins VP11 and VP12 contain predicted transmembrane helices. In addition to HRPV9, betapleolipoviruses HGPV-1 and HHPV3, and HHPV4 have two internal membrane protein types [8, 9, 13]. VP19 identified as a structural protein, contains a P-loop NTPase domain proposing that it is a putative NTPase. Previously, the gene encoding this protein has only been identified for HRPV-1, the type species of *Pleolipoviridae* [17]. In addition to VP19, the minor structural proteins VP16 and VP21 are also encoded by the conserved pleolipoviral gene block (Fig. 2). HRPV9 is the first pleolipovirus for which these genes have been identified, and the corresponding, collinear ORFs are found in other pleolipoviruses as well (Fig. 2). Putative protein 23 of HRPV9 has a predicted winged helix-turn-helix DNA binding domain found also from its counterparts in some other betapleolipoviruses [7]. In addition, some HRPV9 ORFs were similar to those of other archaeal or bacterial viruses. Interestingly, BLAST search with HRPV9 ORF25 as a query retrieved hits to

environmental halophilic bacteriophage genomes assembled from metagenomic data obtained from a solar saltern in Santa Pola, Alicante, Spain (Table S3).

Based on BLAST hits and predicted conserved domains (Table S4), HRPV9 ORF1 and ORF5 encode a putative integrase and PhiH1-like repressor, respectively. Thus, from 12 currently isolated pleolipoviruses, only SNJ2 [18], HHPV4 [8], and HRPV9 have an integrase and PhiH1-like repressor genes (Fig. 2). HRPV9 integrase is ~45-48 % identical to those of SNJ2 and HHPV4, but shares over 99% identity to the phage integrases/site-specific recombinases found in the genomes of *Halorubrum terrestre* (WP_007344666.1), *Hrr. coriense* (WP_006113931.1), *Hrr. ezzemoulense* (WP_094583293.1), *Hrr.* sp. SD626R (WP_092566421.1), and *Hrr. halodurans* (WP_094531550.1). In addition, a number of related putative proviruses with integrase genes have been found in the genomes of halophilic archaea or as haloarchaeal plasmids [9, 18]. Recently, it has been shown that SNJ2 integrase is critical for virus replication [6]. Moreover, SNJ2-type integrases are suggested to form a novel family within the tyrosine recombinase superfamily [6]. HRPV9 integrase contains a conserved catalytic pentad of $R_I$...K…$H_{II}XXR_{II}$...Y (where X is any residue) typical for tyrosine recombinases, including SNJ2-type ones [19] (blue in Fig. S4). A distinctive feature of SNJ2-type integrases is that the other two conserved sites, i.e. $E/D_I$ and $H/W_{III}$, are substituted with G/A and A/V residues, respectively [6]. The same substitutions are found in HHPV4 integrase (Fig. S4), suggesting that it also belongs to the SNJ2-type family. Interestingly, HRPV9 integrase also contains G at the $E/D_I$ site, but M at the $H/W_{III}$ site (red in Fig. S4). Same substitution (M) is found also in almost identical integrases of *Halorubrum* species mentioned above (Fig. S4). Whether these integrases represent a distinct subgroup remains to be investigated. SNJ2 encodes two accessory proteins necessary for efficient integration into the chromosome [6]. However, no homologs are found in

HRPV9 nor HHPV4 [8]. In these viruses, the putative integrases may be defective, or their functions are not dependent on any such SNJ2-like accessory proteins.

We propose classifying HRPV9 into the genus *Betapleolipovirus* of the family *Pleolipoviridae*. In the conserved block of pleolipovirus genes, amino acid identities between HRPV9 and other pleolipoviral proteins are quite low, which is typical for pleolipoviruses, except for the very closely related viruses HHPV3 [9] and HHPV4 [8]. However, high (up to 99%) amino acid identities are found between HRPV9 and putative proviruses in the genomes of halophilic archaeal strains. In addition to the previously known putative proviruses related to betapleolipoviruses [9, 18], here we have detected eleven more such proviruses (Fig. S5).

Using the complete genome sequences of pleolipoviruses, phylogenomic GBDP tree analyses had high support values at the nucleotide (68%; Fig. 3A) and amino acid (80%; Fig. 3B) levels, and OPTSIL clustering suggested that all these viruses are their own species. However, at the genus level, OPTSIL clustering at the nucleotide sequence level yielded nine genera (squares in Fig. 3A), while amino acid sequence level analysis suggested four genera (squares in Fig. 3B), where HRPV9 clusters together with betapleolipoviruses. At the family level, OPTSIL clustering resulted into two families (one containing His2 and the other one for all other pleolipoviruses) or to a single family, when nucleotide and amino acid sequences were analyzed, respectively. Thus, the grouping based on the whole viral proteome is consistent with the current classification of the family *Pleolipoviridae* in the International Committee on Virus Taxonomy.

## Conflict of interest

The authors declare no conflict of interest.

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## Legends to figures

**Figure 1**. Virus life cycle and virion components. 1A Adsorption of HRPV9 to *Halorubrum* sp. SS7-4 cells. 1B Virus life cycle. Growth curves of uninfected (white circles) and infected (black circles) SS7-4 cultures and numbers of free progeny viruses (grey bars). 1C Number of viable cells in the uninfected (white circles) and infected (black circles) cultures. 1D. Virion protein profile on SDS-PAGE gel stained with (1.) Coomassie Blue (VP14, spike protein; VP19, NTPase; VP16 and VP21, minor structural proteins; VP11 and VP12 membrane-associated proteins), black arrow heads indicate the positions of VP16 and VP21 and white arrow head indicates the position of VP21, identified by MS. (2) Sudan Black. Positions of lipids are marked with asterisks. (3.-5.) Thin layer chromatogram of the extracted lipids of (3.) *Har. hispanica*, (4.) HRPV9, and (5.) *Halorubrum* sp. SS7-4. Positions of *Har. hispanica* lipid species are indicated. 1E TEM micrograph of HRPV9 viruses stained with 3% uranyl acetate. Scale bar 100 nm.

**Figure 2.** The genomes of HRPV9 and betapleolipoviruses. ORFs or genes are shown as arrows and their numbers are marked inside the arrows. Similar ORFs are in the same colors, and the percentages of amino acid identity between HRPV9 and betapleolipoviral ORF or gene products are shown in blue. ORFs or genes that belong to the conserved block of pleolipoviral ORFs or genes are highlighted with black outlines. Genes that encode structural proteins are marked with asterisks.

**Figure 3.** Phylogenomic GBDP trees for pleolipoviruses. 3A Nucleotide and 3B amino acid level sequence analyses. Viruses that currently belong (or are proposed to belong) to the same genus in the family *Pleolipoviridae* are marked with the same colors (see color codes). Viruses suggested to belong to the same genus based on OPTSIL clustering are united by squares.

1 **Figures**

2

3 **Figure 1.** Virus life cycle and virion components.

4 **Figure 2.** The genomes of HRPV9 and betapleolipoviruses.

5 **Figure 3.** Phylogenomic GBDP trees for pleolipoviruses

**Supplementary material for on-line submission**

**Figure S1.** Infective centers in the HRPV9 life cycle.

**Figure S2.** Identification of HRPV9 virion proteins by mass spectrometry.

**Figure S3.** HRPV9 genome treated with restriction endonucleases.

**Figure S4.** HRPV9 integrase.

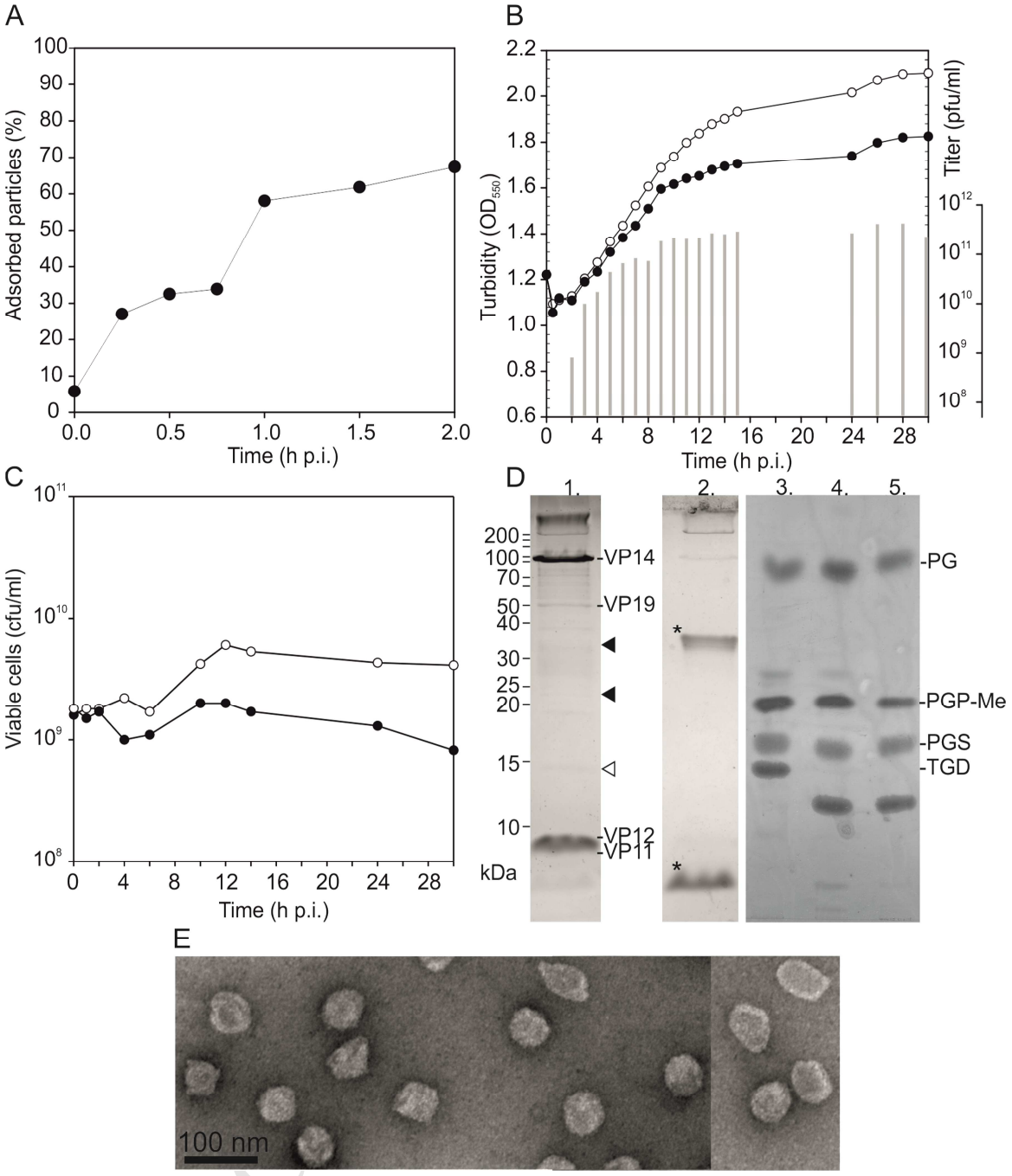
**Figure S5.** HRPV9-related putative proviruses.

**Table S1.** Archaeal strains used in this study.

**Table S2.** Bioinformatic tools used for HRPV9 genome annotation

**Table S3.** HRPV9 predicted ORFs and identified genes.

**Table S4.** Conserved domains predicted in HRPV9 (putative) proteins.


A
Adsorbed particles (%)
Time (h p.i.)
B
Turbidity (OD550)
Titer (pfu/ml)
Time (h p.i.)
C
Viable cells (cfu/ml)
Time (h p.i.)
D
1.
2.
3.
4.
5.
kDa
VP14
VP19
VP12
VP11
PG
PGP-Me
PGS
TGD
E
100 nm