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Article

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Posted Date: October 15th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7669873/v1>

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Additional Declarations: There is **NO** Competing Interest.

Version of Record: A version of this preprint was published at Communications Biology on September 24th, 2026. See the published version at <https://doi.org/10.1038/s42003-026-11061-7>.

Tale of Two viruses: Two coinfecting archaeal viruses provide insights into virus-virus interactions

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Authors Contributions

L.Q., C.M., and S.E. conceived and led the study and performed primary writing of the manuscript. T.A.S. performed phylogenetic and network analysis of viruses, and the co-occurrence analyses. G.D. and M.L. did the proteomics analysis of the purified viral proteins by mass spectrometry. F.A.V. provided the genome assembly of *Hrr*. TLS-6. J.K. was involved in the supervision of L.Q. during first isolation of the viruses. M.C and M.E.F provided the host strain *Hrr*. TLS-6. All authors participated in the analysis and interpretation of the data and contributed to the writing of the manuscript.

Competing Interest Statement:

The authors declare no competing interests.

Keywords: spindle-shaped virus, pleolipovirus, archaea, virus-virus interactions, mutualism, halophiles, extremophiles, central Andes

Abstract

Upon characterization of a novel spindle-shaped virus, Tebenquiche spindle-shaped Virus 1 (Tebi-SV1), isolated from *Halorubrum* strain TLS-6, we detected the presence of a second virus, Tebenquiche pleomorphic Virus 1 (Tebi-PV1), that coinfects host cells. Gene sharing network analysis revealed several genomes related to Tebi-SV1 in public databases, most of which also co-occur with pleolipoviruses in their host genomes. Attempts to separate the two viruses remained unsuccessful, suggesting a mutualistic relationship between Tebi-SV1 and Tebi-PV1. Virus abundances seem to be regulated through a shared operator sequence allowing Tebi-PV1 to keep Tebi-SV1 copy numbers low without excluding Tebi-SV1 from the host genome. Additionally, Tebi-PV1 encodes for a type-4-pilin, which we detected by proteomics, and likely plays a role in regulating the interactions between the host and viruses. Our findings highlight the complexity of virus-virus interactions and that residential viruses are important regulators of virus-host interactions.

Introduction

Virus coinfections occur in various environments and play significant roles in viral evolution and ecology across all three domains of life. A recent study showed that up to 35% of infected bacterial cells in a single-cell genomic dataset and nearly 50% of microbial genomes from public archaeal and bacterial datasets contained more than one virus^{1,2}. Virus-virus interactions have been shown to influence viral infectivity, alter the host range and lifecycle, alter host physiology, and facilitate genetic exchange²⁻⁸. However, only a few studies describe virus coinfections in archaea.

In one study, two proviruses in *Aeropyrum pernix*, unclassified spindle-shaped virus, APSV1, and guttavirus, APOV1, were simultaneously induced with no apparent cell lysis under suboptimal oxic conditions⁹⁻¹¹. In another example, rudivirus SIRV3 and bicaudavirus SMV1 were stably maintained in *Sulfolobus islandicus* REY15A for nearly two weeks¹². Interestingly, in neither case the infection has detrimental effects on the host.

The majority of virus-virus interactions studied in prokaryotes describe antagonistic relationships between coinfecting viruses. Viruses can exhibit mechanisms driving superinfection exclusion, where one virus prevents replication or injection of a superinfecting virus to maintain control over the host organism's resources. This phenomenon is well studied in bacteriophages^{13,14}, and has also been described more recently in archaeal viruses¹⁵⁻¹⁸. Archaeal viruses and other mobile genetic elements (MGEs) were shown to carry mini-CRISPR arrays as a distinct superinfection exclusion mechanism^{17,19}. Induction of CRISPR spacer acquisition against coinfecting viruses and a plasmid was described for SMV1^{20,21}. Coinfection experiments with bicaudaviruses SMV1 and STSV2 triggered the CRISPR adaptation of the host against STSV2, which resulted in exclusion of STSV2 while SMV1 persisted in the host²¹.

Studies on cooperative coinfections - where the presence of one entity ensures the success of another - are limited, but those that explore this phenomenon mainly describe subviral agents such as satellite viruses and helper viruses²²⁻²⁵. Increased infection rates have been described for bacteriophage $\Phi 6$, upon coinfection of cells infected with the same virus²⁶, and coinfection of different virus variants can lead to recombinant viral genomes²⁷. The majority of synergistic interactions have been observed between viruses and plasmids, with viruses supporting the distribution of plasmids²⁸⁻³¹, and plasmids supporting viral infection³². Coinfections with different MGEs have rarely been described in Archaea, with most studies involving spindle-shaped viruses. In the hyperthermophilic archaeon *Thermococcus prieurii*, spindle-shaped virus TPV1 coexists with two plasmids pTP1 and pTP2, without antagonistic effects within the same host³³. A synergistic relationship has been described in a *Sulfolobus islandicus* strain where a small plasmid-virus hybrid, pSSVx, is packaged in a spindle-shaped virion and requires co-transfection with a helper spindle-shaped virus, SSV1 or SSV2, to proliferate successfully²⁸. These studies explore coinfection dynamics in temperate and hyperthermophilic environments, but the dynamics of virus-virus interactions in hypersaline environments remain largely unexplored. In halophilic archaea, only one example of coinfection has been investigated. Several studies examined the relationship between SNJ1 (sphaerolipovirus) and SNJ2 (pleolipovirus), coinfecting *Natrinema* sp. J7^{16,34}. Most recently it was shown that SNJ2 provides the receptor for SNJ1, while SNJ1 supports the replication of SNJ2 providing evidence for a mutualistic interaction between the two viruses³⁵.

In this study, we use a *Halorubrum* strain isolated from Laguna Tebenquiche, in Salar de Atacama, Chile³⁶. Laguna Tebenquiche is a hypersaline lagoon at 2000 m above sea level, exposed to extreme solar radiation and enriched in volcanic-derived arsenic (up to 5 mg/L), lithium (~145–445 mg/L), and boron (~158–543 mg/L)³⁶⁻³⁸. To date, no viruses have been isolated from Laguna Tebenquiche. Notably, a correlation between increased salinities and the prevalence of spindle-shaped viruses has been observed³⁹⁻⁴³. Despite their abundance in hypersaline systems, only two halophilic spindle-shaped viruses, His1 and LSV-48N, have been described to date^{44,45}. His1 is currently the sole member of the virus family *Halospiviridae*, and shares little to no sequence homology to other spindle-shaped virus families⁴⁶, but the structure of the major capsid protein (MCP) is similar to that of other spindle-shaped viruses⁴⁷.

Here we report the co-isolation and characterization of a novel spindle-shaped virus, Tebi-SV1, and a novel pleomorphic virus, Tebi-PV1, from the same environmental isolate *Halorubrum* sp. TLS-6³⁶. Our study reveals that Tebi-SV1 belongs to a new family of spindle-shaped viruses and seems to be mutually regulated by the coinfecting virus, Tebi-PV1.

Results

Discovery of virus-like particles in the supernatant of an isolated Halorubrum strain

While attempting to grow a colony of strain *Halorubrum* sp. TLS-6 (*Hrr.* TLS-6)³⁶ in liquid media, we observed a deviation from its typical growth behavior; the culture failed to reach exponential phase and did not exceed an OD_{600 nm} of 0.08. This indicated severe growth inhibition of the culture. Analysis of the supernatant of the culture by transmission electron microscopy (TEM) revealed the presence of spindle-shaped virus-like particles (VLPs) of approximately 120 nm in length and 60 nm in diameter (Figure 1A). We also observed VLPs with an elongated tubular morphology of approximately 200 nm in length (Figure 1B-C), which we suggest could represent particles of the same virus with the genetic material ejected, as described for the spindle-shaped virus His1⁴⁸. The concentrated culture supernatant was purified on an Iodixanol density gradient, resulting in three bands (Figure 1E). Each band was examined by TEM, and high-throughput sequencing was performed on total DNA extracted from the second band, which contained the most intact spindle-shaped VLPs. The first band contained mainly cellular debris and membranous vesicles, while the third band contained mostly elongated spindle-shaped VLPs.

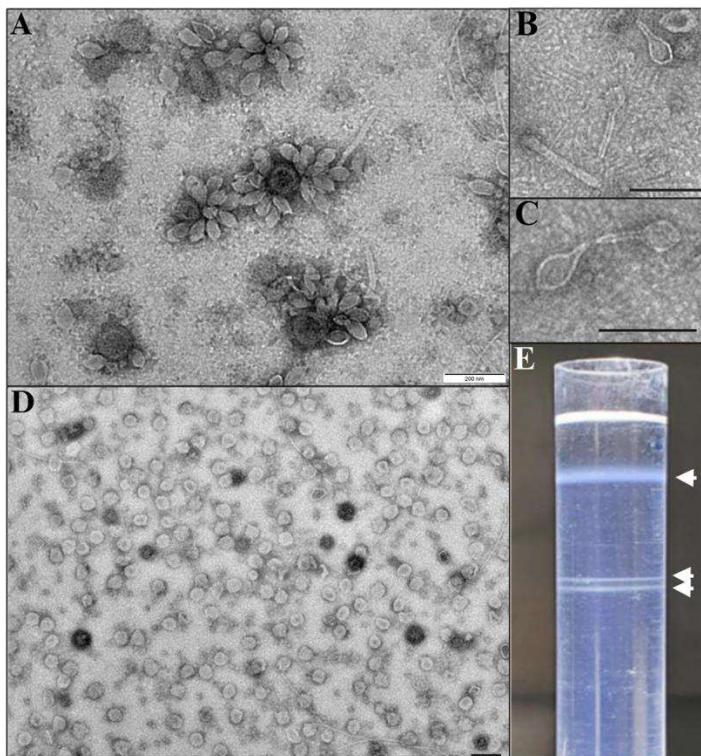


Figure 1: Electron micrographs of Tebi-SV1 and Tebi-PV1. (A) Electron micrograph of the first virus preparation (second band of Iodixanol gradient in E) from *Hrr.* TLS-6 supernatant dominated by Tebi-SV1 particles (B-C). Tebi-SV1 particles exhibiting pleomorphism with elongated tails and in a tubular form (size bars are 100 nm) from band 3 of Iodixanol gradient (E). (D) Electron micrographs of supernatant enriched with Tebi-PV1 particles. (E) 40-25 % Iodixanol density gradient of the first preparation from *Hrr.* TLS-6 supernatant.

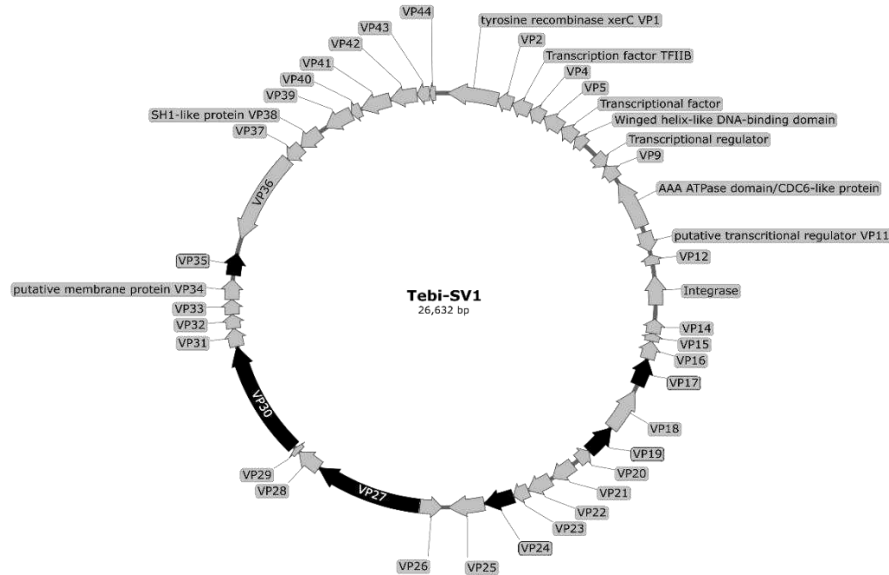
Sequencing of total DNA from purified virus particles reveals two viral genomes

High-throughput Illumina sequencing of total DNA and subsequent assembly of reads revealed two distinct circular contigs of 26.6 kbp and 15.4 kbp. The larger contig (26.6 kb) encodes for 44 open reading frames (ORFs) (Figure 2A), with an average GC content of 62.8% (GC content of host chromosome being 69.8%) and a median coverage depth of 12,000. Based on TEM observations of the band containing mainly spindle-shaped particles, we infer that this contig corresponds to the spindle-shaped virus. We named the virus Tebi-SV1, Tebi for Laguna Tebenquiche (place of isolation), and SV for spindle-shaped virus. The smaller contig (15.4 kb) encodes for 26 ORFs, with a GC content of 62.4% and a median coverage of 100. Both contigs were detected in the genome of the host *Hrr* TLS-6, Tebi-SV1, as an integrated provirus with a mean coverage of ~ 550 (~420 for the entire host genome contig) and the smaller contig as a circular extrachromosomal element (mean coverage ~2730).

The smaller 15.4 kbp contig displays the highest similarity (52% nucleotide sequence pairwise identity) to Halorubrum pleomorphic virus 9 (HRPV9)⁴⁹. It encodes for the conserved set of proteins characteristic of members of the *Pleolipoviridae* family, including the spike protein (VP12 [viral protein 12]) and an internal membrane protein (VP9) (Figure 2B). VP1 encodes for an integrase, while VP4 and VP21 encode for transcriptional regulators. VP4 contains an ArsR-like helix-turn-helix domain (IPR011991), which is found in metal (including arsenic) regulated repressors⁵⁰. VP16 encodes for an NTPase. Notably, VP6 encodes for an archaeal type-IV pilin-like protein (T4P), and VP19 shows significant similarity to a proteosome beta subunit-like protein. Both VP7 and VP8 show high similarity (HMM and tertiary structure) to antitoxin proteins (ParD-like and VapB-like, respectively). Gene sharing network and phylogenetic analyses reveal that it belongs to the *Betapleolipovirus* genus⁵¹ (Figure 3 & Supplementary Figure 1). We concluded that this genome represents a pleolipovirus, and named it Tebi-PV1.

The genome of Tebi-PV1 was found as a single contig, with a higher coverage of ~2730 (Tebi-SV1 mean coverage of ~550), in the sequencing data from the host genome³⁶, and with 100% identity to the genome isolated from purified supernatant. While Tebi-PV1 does encode for an integrase (VP1), it remains to be elucidated if Tebi-PV1 also can integrate into the host chromosome.

A



B

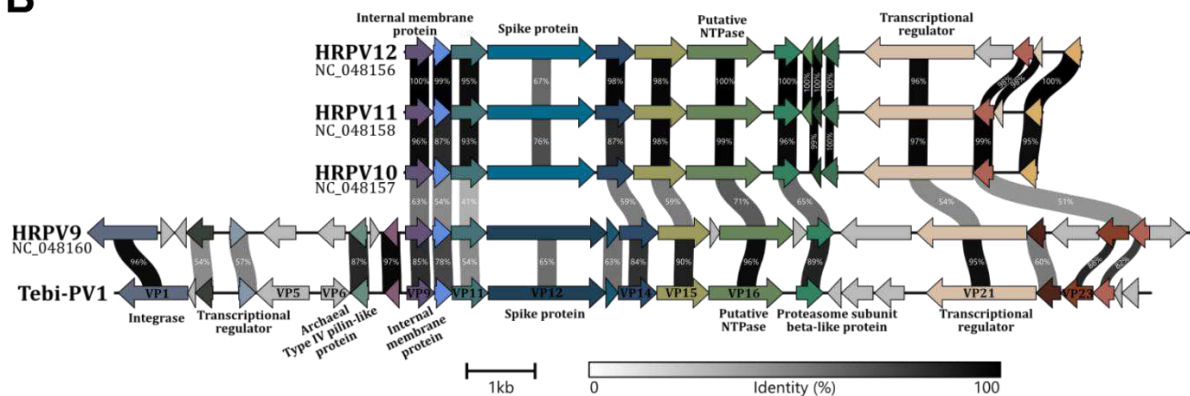


Figure 2: Genomic characteristics of Tebi-SV1 and Tebi-PV1. (A) Genome map (SnapGene software (www.snapgene.com)) of Tebi-SV1 with functional annotations. Proteins detected in purified virus particles are shown in black (B). Tebi-PV1 genome comparison with closest pleolipovirus relatives. Homologous genes are shown in the same colors, with percent identity represented vertically between genes ⁵².

The spindle-shaped virus Tebi-SV1 belongs to the newly proposed family Almyrocytroviridae

The genome of Tebi-SV1 exhibited no significant similarity at the nucleotide or amino acid level to any characterized virus isolate or genome from the IMG/VR database ⁵³. However, homologs to multiple hypothetical proteins of Tebi-SV1 were found in public databases in *Halorubrum* sp. LN27, a strain isolated from Dingyuan Salt Mine in China ⁵⁴, suggesting a similar virus may be integrated into the genome. Further inspection revealed an average nucleotide identity (ANI) of 82% between the putative provirus in LN27 (referred to as SSV_LN27) and Tebi-SV1, which is well below the typical threshold used to delineate species in viral genomics ⁵⁵. Genome-based phylogeny and classification analyses using VICTOR (Figure 3) suggest that Tebi-SV1, as well as SSV_LN27 and a proposed plasmid from the IMPGR database (IMPGR_plasmid_2904944409_000002), together with the spindle-shaped

viruses described by Turgeman-Grott *et al.*⁴⁵, belong to a new virus family, which we name *Almyrocitroviridae* (Almyro- for salty, and -citro for lemon).

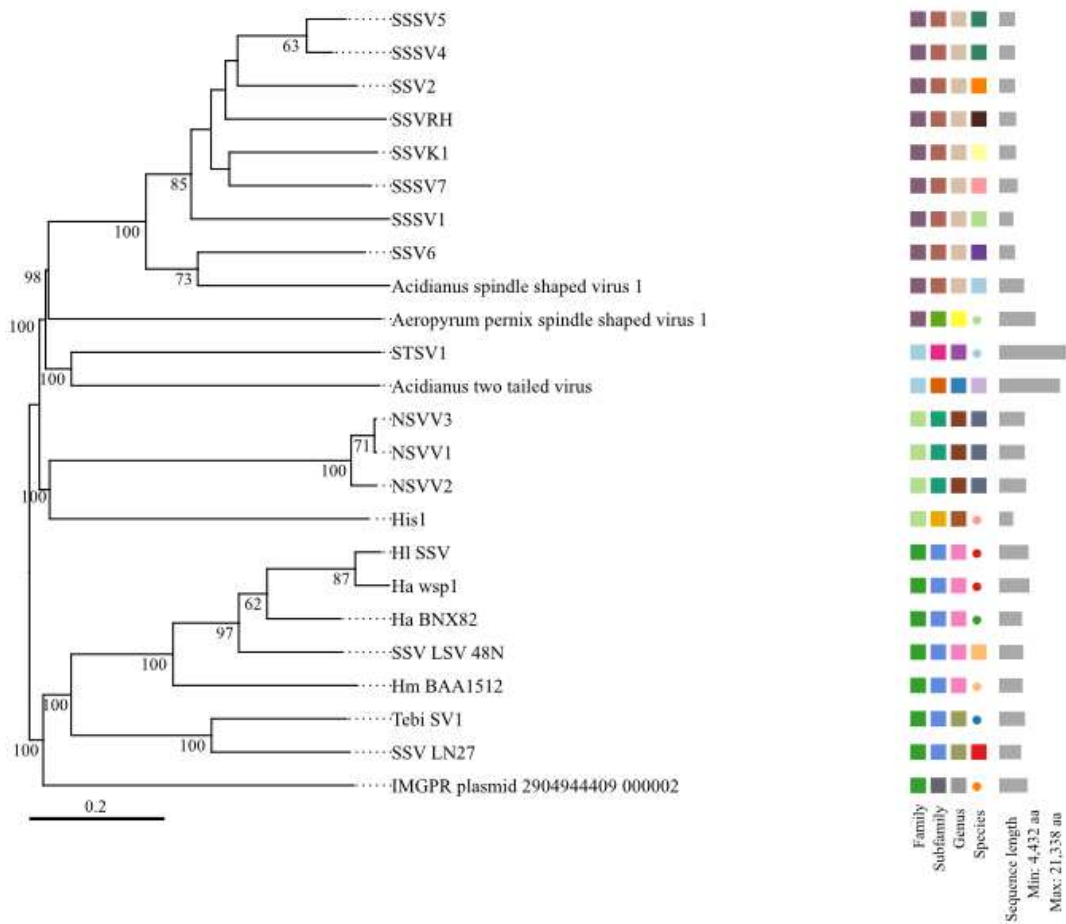


Figure 3: Genome-based phylogeny classification analyses. VICTOR tree using the predicted protein sequences of Tebi-SV1, SSV_LN27, IMGPR_plasmid_2904944409_000002, together with the spindle-shaped viruses described by Turgeman-Grott *et al.*⁴⁵, and other previously described spindle-shaped viruses (of families *Halspiviridae*, *Thaspiviridae*, *Fuselloviridae*, and *Bicausaviridae*) using the D6 formula.

Additionally, gene-sharing network analysis for these virus-like elements supported that these subsets of genomes form two separate groups of distantly related species (Supplementary Figure 1 and Supplementary Table 1), which suggests that they correspond to at least two different genera, with one comprised by the LSV-48N virus and its relatives and a second genus including Tebi-SV1 and SSV-LN27. However, sparse or no connections between members of the two groups were observed (Supplementary Figures 1 and 2), and often the genes shared correspond to small ORFs of unknown function with low identity values (Supplementary Figure 2). This clustering pattern, along with the lack of recognizable syntenic genome organization (i.e., absence of similarly organized structural or replication modules), suggests limited to absent gene flow and horizontal gene transfer between the groups, consistent with a distant evolutionary relationship and likely different evolutionary trajectories.

The majority of the 44 predicted gene products on the Tebi-SV1 genome are hypothetical proteins, and even when using predicted tertiary structures, only a few could be annotated. VP1 is a predicted tyrosine recombinase, and VP3 is a putative transcription initiation factor (TFIIB). VP6 and VP8 are predicted to be transcriptional regulators (helix-turn-helix), and VP7 contains a winged helix-like DNA-binding domain. VP10 is a CDC6-like protein, VP13 is a second tyrosine recombinase, and VP29 is a large IG-like domain containing protein with a transmembrane and a sugar binding domain (Figure 2A).

Tebi-SV1 is found integrated as a provirus in the host chromosome next to an alanine tRNA gene with the integrase (VP1) at the downstream boundary of the provirus and VP44 next to the tRNA. A 13 bp stretch that is found twice upstream and once downstream of Tebi-SV1 in the host genome, and that is only detected once in the circular virus genome, is likely used for circularization. The genome isolated from purified Tebi-SV1 particles shares 99.99% identity with the provirus. We detected two single nucleotide polymorphisms (SNP) on the virus genome from this preparation when compared to the integrated virus in the assembled host genome. A deletion of base A at position 3248 (variant frequency 34%), that is missing in the assembled virus genome when compared to the integrated virus in the host genome, and the deletion of base G directly downstream (variant frequency 44%). Both SNPs potentially influence the promotor region of the downstream gene (VP8). The SNPs are not detectable in re-sequencing data from the host (mean coverage 319).

Separation attempts of the two viruses remain unsuccessful

The first virus preparation from growth retarded *Hrr*. TLS-6 cultures showed a high Tebi-SV1 to Tebi-PV1 ratio of 1,200:1 (based on sequencing coverage) in the gradient band subjected to sequencing. However, such a strong growth inhibition was not reproducible in subsequent cultures, neither when grown from colonies from the same plate, when grown from the original glycerol stock, nor upon reinfection with viral particles isolated from the supernatant of lysed cultures. Genome copy number per mL ratios between Tebi-PV1 and Tebi-SV1 always averaged at about 1760:1, respectively. Attempts to induce Tebi-SV1 production revealed that Tebi-PV1 particles are particularly abundant in the supernatant of cultures grown in DBCM2+ media (high nutrient medium), and Tebi-SV1 particles are enriched by over 50-fold in CDM media (low nutrient medium) supplemented with 1 mM PO₄, in comparison with DBCM2+ (p-value of 4.89E-11) (Figure 4 and Supplementary Table 2). However, none of the conditions tested resulted in virus preparation with only one of the two viruses. All single colonies (~20) isolated from *Hrr*. TLS-6 cultures, after incubation at 28 °C, 37 °C, and 50 °C, maintained both viruses (data not shown). Physical separation with various density gradients also failed to separate the viruses (Supplementary Figure 3, Supplementary Table 3). Both viruses were always detected in all gradient bands tested, with one exception that was not reproducible.

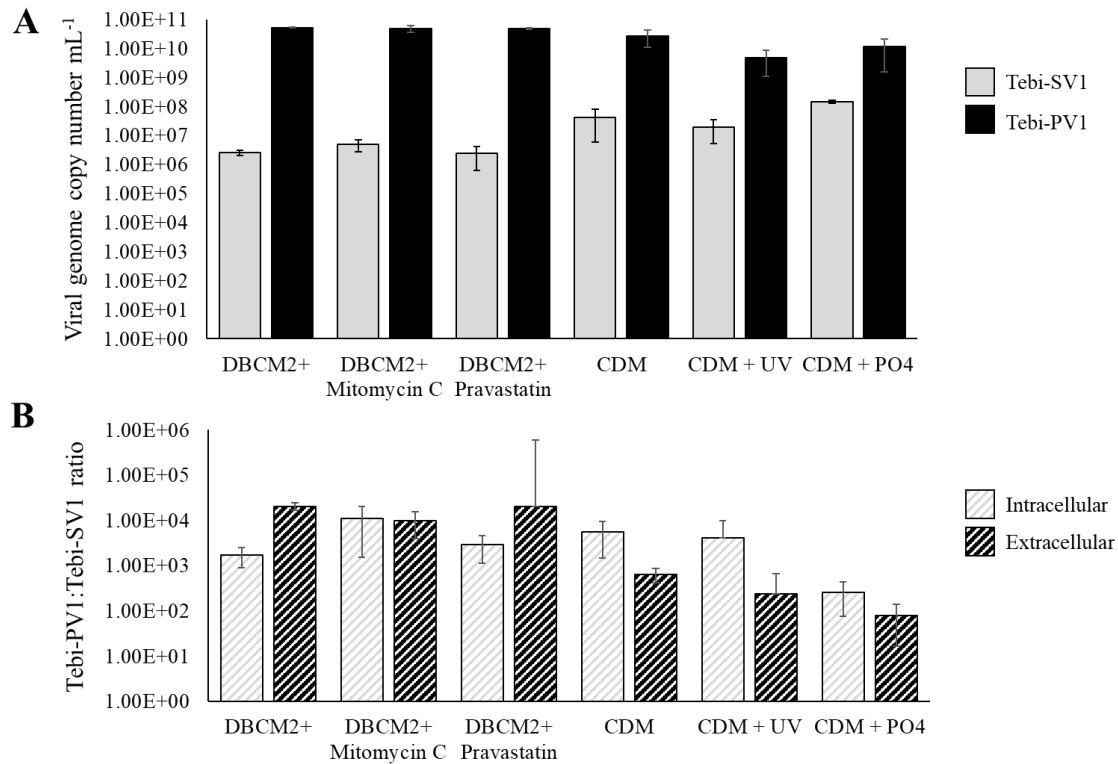


Figure 4: Determination of virus ratios under different growth conditions by qPCR. (A) Viral genome copy number of Tebi-SV1 and Tebi-PV1 per ml supernatant was quantified from supernatant of *Hrr. TLS-6* in differently treated cultures. (B) Intracellular viral genome copy number ratios (Tebi-PV1:Tebi-SV1) for A. (C) Average virus genome copy number per ng of DNA from cells treated with different temperatures (intracellular). Mitomycin C and Pravastatin treatment was done in DBCM2+ medium. UV treatment and supplementation with PO₄ was done in CDM medium. Error bars represent the standard deviation of n=3 biological replicates (A-C). Statistical analysis is summarized in Supplementary Table 2.

Finally, neither protease nor chloroform treatment resulted in the removal of Tebi-SV1 or Tebi-PV1. Protease treatment did not destroy Tebi-SV1 particles, but reduced infectivity in *Hrr. lacusprofundi* significantly by ~1.8 fold. Tebi-PV1 showed to be less sensitive to protease treatment with a ~1-fold reduction of intracellular genome copy numbers (Figure 5). Tebi-SV1 virions were sensitive to chloroform treatment with a >2-fold reduction, indicating the presence of lipids; however, Tebi-PV1 was not sensitive to chloroform (Figure 5).

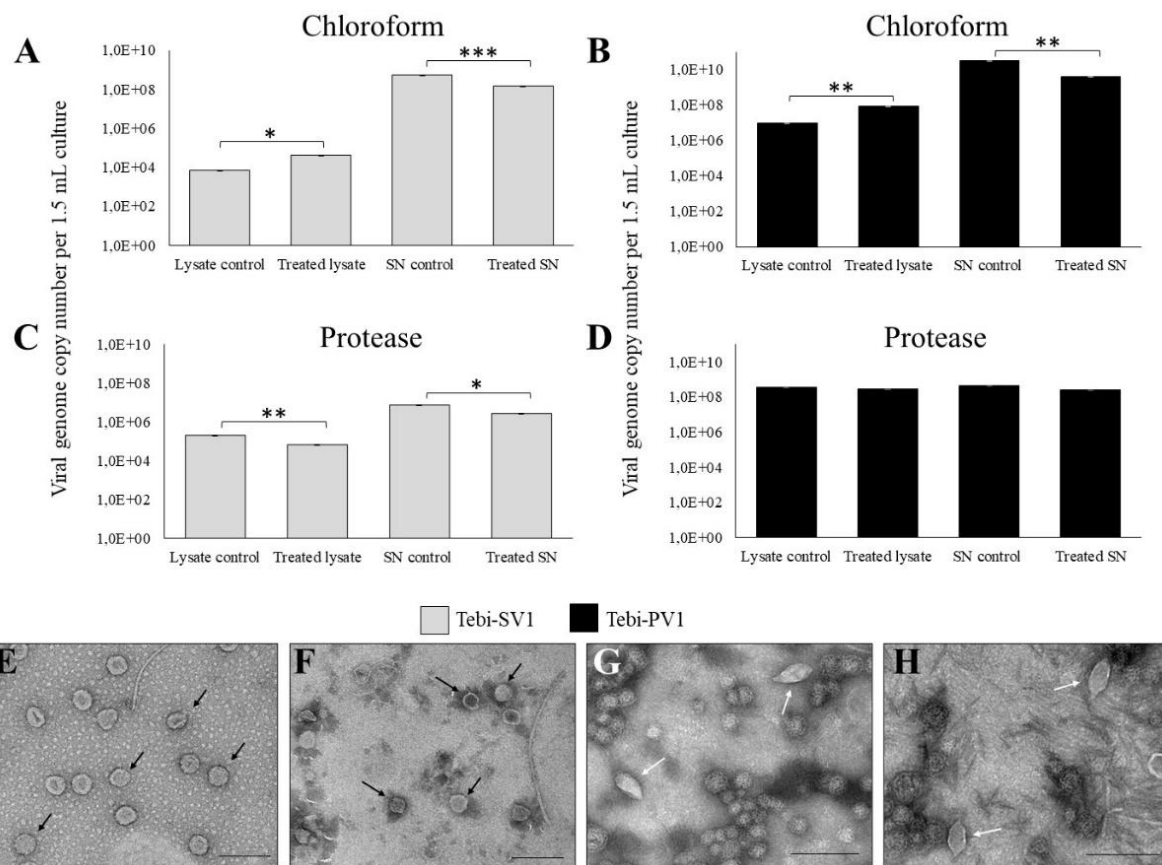


Figure 5: Proteinase K and chloroform treatment of virus preparations. Proteinase K (7.5 mg/ml) and chloroform (1:4 chloroform to sample ratio) treated virus preparations from TLS-6 supernatant and TLS-6 cell lysate were used to infect *Hrr. lacusprofundi*. Virus activity was compared by determining intracellular viral genome copy numbers by qPCR. Intracellular genome copy number of Tebi-SV1 (A) and Tebi-PV1 (B) per 1.5 mL culture infected with chloroform treated and untreated (control) viral preparations. Intracellular genome copy number of Tebi-SV1 (C) and Tebi-PV1 (D) per 1.5 mL culture infected with proteinase K treated and untreated (control) viral preparations. Error bars represent the standard deviation of n=3 biological replicates, and statistical significance is indicated with the following significance codes: '*' for p-value ≤ 0.05 , '**' for p-value < 0.01 , and '***' for p-value < 0.001 . Lysate – virus particles isolated from disrupted cells. SN – virus particles isolated from culture supernatants. All experiments were carried out in biological triplicates. (E, G) Electron micrograph of untreated viral preparation. (F) Electron micrograph of the chloroform treated viral preparation with black arrows denoting Tebi-PV1 virions. (H) Electron micrograph of the protease treated viral preparation with white arrows denoting Tebi-SV1 virions. Scale bars are 200 nm.

Host range determination indicates attachment and initial virus replication in a broad range of host organisms

To find a virus free host and further attempt to separate Tebi-SV1 and Tebi-PV1, we infected seven different haloarchaeal strains with a total virus genome number to host cell ratio of approximately 2.5 (Tebi-PV1:Tebi-SV1 ratio ~28:1). After allowing initial adsorption in a small volume without shaking (see methods), we detected the genomes of both Tebi-SV1 and Tebi-PV1 by qPCR intracellularly in all

strains (*Hrr. lacusprofundi*, *Haloferax mediterranei* and *Haloferax volcanii* at 17 hpi; *Halobacterium salinarum* and *Haloarcula japonica*, at 16 hpi; *Haloquadratum walsbyi* and *Haloterrigena turkmenica* at 8 hpi) (Figure 6 D-I, Supplementary Figure 5). The cells were transferred into fresh media (50 mL cultures) to follow the growth behavior (Figure 6 A-C, Supplementary Figure 4). Only minor or no effects of the infection on growth were detected for all tested strains. A slight growth inhibition was observed initially in *Hbt. salinarum* and later in *Hrr. lacusprofundi*, and a slight increase in growth was observed initially in *Hfx. volcanii*, *Hq. walsbyi*, and *Ht. turkmenica* (Figure 6 A-C, Supplementary Figure 4). While most strains had significantly higher intracellular genome copy numbers of Tebi-PV1 (Supplementary Figure 5), none of the strains tested showed detection of only one of the two viruses after the initial adsorption time. The ratios of Tebi-PV1 to Tebi-SV1 genomes varied across the different strains, from ~5 in *Hbt. salinarum* to ~210 in *Hqt. walsbyi* (Supplementary Figure 5), indicating different efficiencies of infection for the two viruses across the different strains. Once the cultures reached late exponential phase, we tested for the genomes of both Tebi-SV1 and Tebi-PV1 by qPCR in the supernatant and detected both viruses in the supernatant of all tested strains (Figure 6 A-C, Supplementary Figure 4), indicative of virus particle production. Additionally, total virus genome copy numbers (sum of intracellular and extracellular genome copy numbers) of Tebi-SV1 were higher than the total number of virus genomes added for infection in *Hfx. volcanii*, *Hfx. mediterranei* and *H. japonica*. Total numbers of Tebi-PV1 increased in *Hfx. mediterranei* and *H. japonica*.

Cultures of five organisms were diluted into fresh media to OD_{600nm} of ~0.05 and allowed to continue again to the late exponential phase (Figure 6 A-C, Supplementary Figure 4). In all strains, the intracellular virus number decreased over the 209h monitoring timeframe. Only in *Hfx. volcanii*, intracellular genome copies of Tebi-SV1 became undetectable (209 hpi), however, Tebi-SV1 was still detectable in culture supernatants (1.09E+05/1.5 ml at 209 hpi).

Intracellular virus genome-to-host genome ratios were determined for three of the strains (Figure 6 D-I). In all tested strains, the viral genome copy number remained below the host chromosome copy number and decreased over time, indicating that not all cells were infected, though haloarchaea can contain several copies of their chromosome per cell^{56,57}. It also remains to be determined whether there is a small number of infected cells with a high virus-to-host ratio, or a higher number of infected cells with low virus-to-host ratios in these populations.

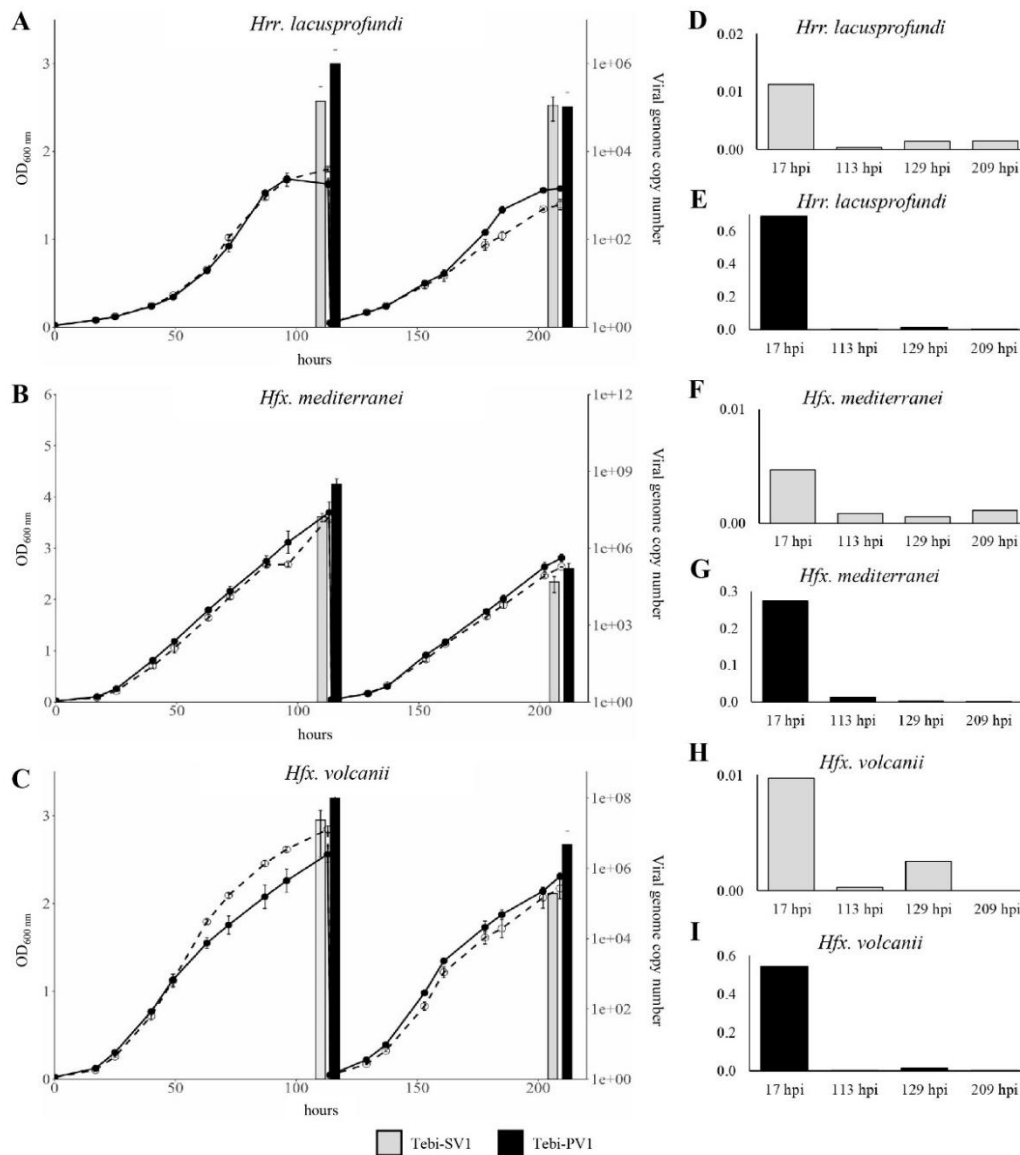


Figure 6: Determination of the host range: (A-C) Growth curves of infected (broken line) and uninfected (solid line) of three tested host organisms. After reaching stationary phase, cultures were diluted to a starting OD_{600nm} of 0.05 and allowed to regrow without additional virus added. Error bars represent the standard deviation of biological triplicates in all curves. Grey bars represent the number of Tebi-SV1 genomes per 1.5 mL culture, and black bars represent Tebi-PV1 genomes per 1.5 mL culture detected in the supernatant at 117 and 209 hpi. Error bars represent the standard deviation of biological triplicates. (D-I) Intracellular virus-to-host genome copy number was detected at different time points during the growth curves presented in A-C. The number of Tebi-SV1 genomes, Tebi-PV1 genomes, and host chromosome copy numbers were determined by qPCR in cells of 1.5 mL culture in biological triplicates. The ratio was determined by dividing the average of Tebi-SV1/Tebi-PV1 genome copy numbers by the average of the host chromosome numbers. Any value is >1, indicates that the number of Tebi-SV1 or Tebi-PV1 genome copy numbers are higher than the host chromosome copy numbers. Grey bars represent Tebi-SV1 and black bars represent Tebi-PV1.

Co-occurrence of Tebi-SV-like relatives and Pleolipoviruses in other haloarchaea and a shared genomic sequence hint towards an interdependency

When searching for Tebi-SV1-like relatives in public databases, we found that several Tebi-SV1-like elements co-occurred with pleolipovirus-like proviruses in the same host genomes (Supplementary Table 4). In five out of seven organisms containing genomes of Tebi-SV1 relatives, we could identify a complete pleolipovirus genome in the same host genome. However, these pleolipovirus genomes vary in length, and do not all share a particular taxonomic affiliation at the genus level (Supplementary table 4).

To identify a potential genomic interdependency between Tebi-SV1 and Tebi-PV1 we searched for similarities between the two genomes. Similarities between Tebi-PV1 and Tebi-SV1 covered mostly small proteins with unknown function, and the integrases (VP1 in both genomes) (Supplementary Figure 6). However, both viruses share two short stretches of sequence (27bp and 41 bp, interspaced by 32bp in TebiSV1 and 46bp in Tebi-PV1) starting 125bp (Tebi-PV1) and 182bp upstream (Tebi-SV1) of genes that encode putative transcriptional regulators (Tebi-SV1 VP8 and Tebi-PV1 VP4, 33% aa sequence identity). Tebi-SV1 VP8 shows similarities with a MarR family transcriptional regulator (HMM comparison, E-value 0.000159) or a PhiH1 repressor-like protein (HMM comparison, E-value 0.00014), and Tebi-PV1 VP4 shows similarities with a PhiH1 repressor-like protein (HMM comparison, E-value 0.0000077), containing an ArsR-like domain.

The only significant sequence similarity we detected in all of the other virus pairs was between the integrases (LSV-48N, BNX82) and a winged helix-turn-helix domain-containing protein (SSV_LN27) (Supplementary Figure 6).

Protein composition of virus particles

Protein analysis via SDS-PAGE of Tebi-SV1 enriched virus preparations appear very distinct from Tebi-PV1 enriched preparations (Supplementary Figure 7). Mass spectrometry (MS) was performed on both samples to determine the protein composition of the two virus particles (Supplementary table 5). Six proteins were detected for Tebi-SV1: VP17, VP19, VP24, VP27, VP30, and VP35. VP17 and VP24, both hypothetical proteins, contain predicted transmembrane domains. VP19 shows structural similarities to HEPN toxins (RNase). VP27 had the highest relative abundance of the six proteins detected (Supplementary table 5), but does not show similarity to other annotated proteins, and no confident structural model could be retrieved. VP30 was the second most abundant of the six viral proteins detected in the proteome. The N-terminal region of VP30 (AlphaFold 3 model ⁵⁸ aligns with carbohydrate binding domains of various proteins, suggesting that VP30 could interact with carbohydrates. VP35, 151 amino acids in length, has no homologs on NCBI, but is the only protein detected that shares a similar hydrophobicity profile with capsid proteins of other spindle-shaped viruses (Supplementary Figure 8). The MCPs of all so far identified spindle-shaped viruses share

structural similarity and are smaller than 200 amino acids in length^{47,59}. To further investigate the putative structural role of VP35 as the potential major capsid protein of Tebi-SV1 we used the predicted tertiary structure for comparisons. It was shown that the MCP of spindle-shaped virus SMV1 is processed at the N-terminus⁴⁷. Therefore, we gradually removed the N-terminal amino acids of VP34 and modeled the structure using AlphaFold 3, with the removal of 43 aa resulting in the structure with the best score (pTM=0.64). Structural alignment using DALI, revealed minor similarities with the MCP of APBV1, SMV1 and SSV19 (Z-scores 2.3, 3.7 and 3.1, respectively). A PDB search of the structure of the full-length protein (AlphaFold 3, pTM=0.49) with DALI revealed similarities with membrane associated proteins. Best hit (Z-score 5.1) was VIPP1 (Vesicle-inducing protein in plastids 1), a protein that is involved in membrane binding and remodeling⁶⁰. However, VP35 is a small protein and the modeled structure is very simple, leading to a number of nonspecific hits. Therefore, additional experiments are needed to verify whether it could be the MCP of Tebi-SV1.

For Tebi-PV1, four proteins were detected with MS (Supplementary Table 5). The spike protein VP12 was present in multiple bands of the SDS-PAGE. It was particularly enriched in the 95 kDa (3A) and 130-150 kDa (2A) bands (representing 28% and 33.8% of the total protein abundance, respectively). Additionally, an archaeal Type IV pilin-like protein (VP6), a putative NTPase (VP16), and a hypothetical protein (VP15) were detected (Supplementary Table 5). All detected proteins have homologs in other pleolipoviruses (Figure 1B), and were detected in pleolipovirus particles previously^{49,61}, except VP6, the archaeal T4P-like protein.

We also detected a number of host proteins in the samples, many of which were hypothetical proteins (Supplementary table 5). Annotated proteins detected included a significant number of membrane-associated proteins, such as components of ABC transporters, and other proteins previously detected in EVs of haloarchaea^{62,63}. We suggest these host proteins derive from EVs and cellular appendages that co-purify with the virus particles, as also observed by TEM.

Infectious virus particles can be isolated from lysed cells

Due to the low detection of Tebi-SV1 in culture supernatants by TEM, despite various induction efforts, we assumed that Tebi-SV1 virus particles possibly accumulate within the cell, or at the cell surface^{45,64}, without being released. Therefore, we manually lysed the host cells and indeed retrieved Tebi-SV1 particles from the cellular lysate, as observed by TEM and qPCR (Supplementary Figure 9). Analysis of the genome copy numbers by qPCR revealed that both Tebi-SV1 and Tebi-PV1 genomes are present in these preparations, even after DNase treatment, indicating that both genomes are enclosed in particles or associated with structures protecting them from degradation. Additionally, using virus particles obtained from the lysate preparations resulted in infections of *Hrr. lacusprofundi* (Figure 5), demonstrating that the particles are infectious. Notably, the Tebi-PV1:Tebi-SV1 ratio was significantly

different in these preparations, with an average of 1:10 (in contrast to 1: 4.07E+04) and we subsequently used these preparations as ‘Tebi-SV1 enriched’ preparations.

To test whether infectious particles are bound to the S-layer on the cell surface of *Hrr*. TLS-6 cells, we first removed the S-layer from the cells by depleting the cells of $MgCl_2$ ⁶⁵, before lysing the cells by sonication. Approximately 50% of Tebi-SV1 protected genomes were detected in the S-layer preparations, and nearly equivalent amounts were also detected in the lysate preparations. While some virions were indeed detected in the lysate preparation, significantly (p-value of 0.0000021) higher titers of Tebi-PV1 were detected from the isolated S-layer preparation (Figure 7). Upon examining the preparations with TEM, we observed numerous free spindle-shaped virus particles in the S-layer and lysate preparations, while Tebi-PV1 virions were continuously observed attached to cellular pili-like structures (Figure 7). The manually lysed preparations treated with protease showed no significant change in infectivity for Tebi-PV1 particles, but showed a significant reduction in infectivity of Tebi-SV1 particles, as detected for particles isolated from the supernatant (Figure 5).

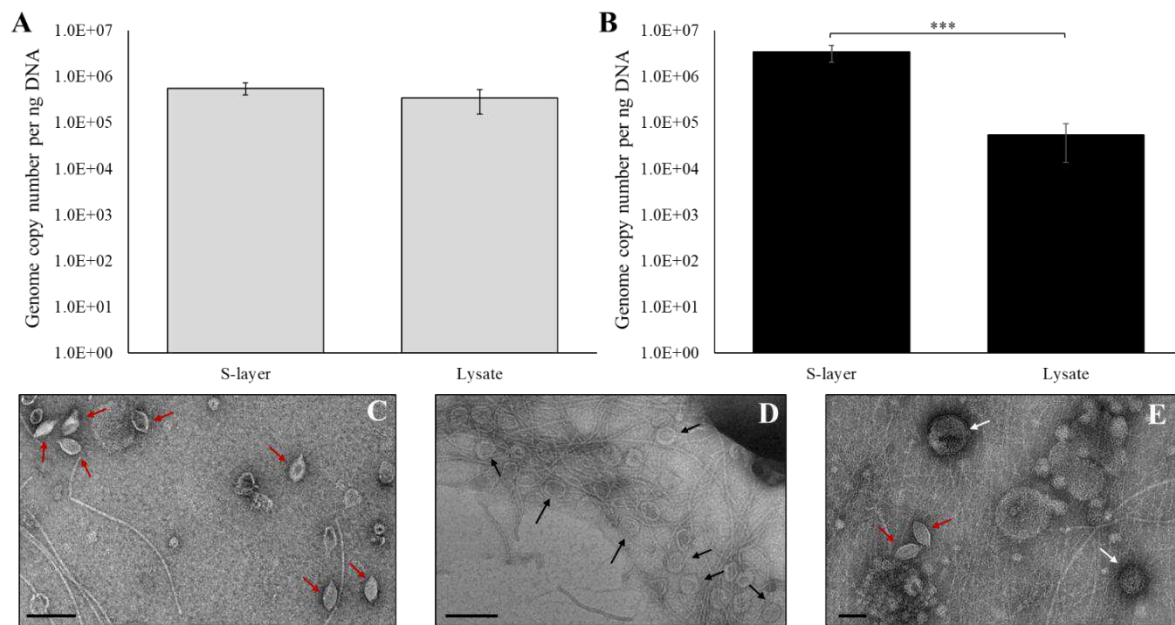


Figure 7: Isolation of virus particles from cellular lysates. Viral genome copy number per ng of DNA is shown from the S-layer fraction and cellular lysate fraction for Tebi-SV1 (A) and Tebi-PV1 (B). Bars represent the average across biological triplicates, error bars represent the standard deviation of n=3 biological replicates, and statistical significance is indicated with the following significance codes: ‘*’ for p-value ≤ 0.05, ‘**’ for p-value < 0.01, and ‘***’ for p-value < 0.001. Negatively stained electron micrographs of the S-layer fraction (C-D) with spindle-shaped virus particles indicated with red arrows and pleomorphic virus particles indicated with black arrows (scale bar 200 nm). (E) Electron micrograph of cellular lysate preparation with spindle-shaped virus particles indicated with red arrows EVs indicated by white arrows (scale bar 100 nm).

Discussion

Here we describe the discovery of two active viruses hidden in a haloarchaeal strain, *Halorubrum* TLS-6, isolated from a hypersaline lagoon in the Chilean central Andes³⁶. When growing this strain from a single colony, we observed strong growth retardation and detected a spindle-shaped virus in the culture supernatant. Sequencing analyses revealed a second virus, a pleolipovirus. We propose the names Tebi-SV1 (Tebenquiche spindle-shaped virus 1) and Tebi-PV1 (Tebenquiche pleolipovirus 1).

Tebi-SV1 belongs to a new virus family and likely evolved by recombination with another virus-like element

Tebi-SV1 lacks sequence similarity with the previously isolated spindle-shaped virus His1 from a hypersaline environment, or with any other characterized viruses or published virus genomes, highlighting that only its isolation allowed identification of this genetic element as a virus. Genome-based phylogeny classification analyses place Tebi-SV1 into a new virus family, *Almyrocitroviridae*, together with a predicted provirus in *Halorubrum* strain LN27 (SSV-LN27), a proposed plasmid (IMGPR_plasmid_2904944409_000002)⁵³, which likely represents a virus, and a group of spindle-shaped viruses recently discovered⁴⁵. However, only SSV-LN27 exhibits significant sequence similarities, classifying it into the same genus as Tebi-SV1.

The genome of Tebi-SV1 contains two integrases, VP1 and VP13, both belonging to the family of tyrosine recombinases. Integrases of this family have been described for both pleolipoviruses⁶⁶ and fuselloviruses (a family of spindle-shaped viruses)^{67,68}. Tebi-SV1 also encodes for a Cdc6-like protein (VP10) and three predicted helix-turn-helix transcriptional regulators (VP6, VP7 and VP8). A recent study determined that an Orc1/Cdc6, along with a hypothetical protein and a winged helix-turn-helix DNA binding protein, control the lysogeny-induction switch of pleolipovirus, SNJ2⁶⁹, and possibly the tailed virus MSTV1⁷⁰. We suggest that a similar mechanism may control the life cycle of Tebi-SV1.

Proteomics analysis revealed that six Tebi-SV1 proteins were present in virus particle preparations. Four of the proteins (VP17, VP24, VP27, and VP30) contain predicted transmembrane domains, indicative of a lipid layer within the virus particle. VP19, detected in the viral preparations, shows similarities to HEPN toxin proteins, which are RNases targeting tRNAs and thereby inhibiting translation⁷¹. The HEPN toxin is inactivated by MNT (minimal nucleotidyltransferase) through covalent di-AMPylation. An MNT antitoxin could not be identified on the Tebi-SV1 genome; however, VP18 (not detected in virus particles) contains a P-loop NTPase fold, indicative of NTP binding. We suggest that VP18 could be the antitoxin, but may inactivate VP19 in a different manner than MNT antitoxins. VP19 and VP18 might function as an addiction module responsible for the maintenance of Tebi-SV1 in *Hrr. TLS-6*. The packaging of VP19 into the virus particle could ensure the timely transcription of the virus genome after injection into a new host cell. Alternatively, HEPN-domain

containing proteins have been shown to be involved in phage defense in bacteria ⁷², and VP19 could be involved in superinfection exclusion.

Based on the predicted structural characteristics of protein VP35, which was also detected in viral preparations, and the similarities it shares with the major capsid proteins (MCPs) of other spindle-shaped viruses, we suggest that this could be the MCP of Tebi-SV1. It was detected in several size fractions larger than its predicted size (~17.5 kDa), suggesting post-translational modification (lipid modification) or the formation of strong multimers. Post-translational modifications may hinder the detection by MS ⁴⁷, which could be the reason why it was not the most abundant protein in virus preparations.

Considering that all structural proteins of Tebi-SV1 were detected only downstream of the second integrase (VP13), we assume that this region (VP13-VP44) is the core region of Tebi-SV1 and suggest that the upstream region containing another integrase might derive from another mobile genetic element. The Cdc6-based lysis-lysogeny mechanism has only been described for other viruses; therefore, we hypothesize that VP1 through VP12 may have been acquired from another virus rather than a plasmid. Horizontal gene transfer and recombination events have been described for other spindle-shaped viruses, such as PAV1 and *Sulfolobus* spindle-shaped viruses ^{73,74}. The integrases, one transcriptional regulator, and the Cdc6 protein are shared between Tebi-SV1 and some of its relatives ⁴⁵. This indicates that the proposed recombination event, of a Tebi-SV1-like virus with another MGE, may have occurred early in the evolution of these spindle-shaped viruses.

The pleolipovirus Tebi-PV1 dominates virus preparation from Hrr. TLS-6 and modifies host pili

Tebi-PV1 exhibits the characteristic core genes shared by all members of the *Pleolipoviridae* family, as well as the commonly shared genes among the *Betapleolipovirus* genus ⁴⁹. MS analysis of virus preparations detected the spike protein along with other structural proteins that were detected in pleolipovirus particles previously ^{49,61}. Interestingly, VP6, a T4P-like protein, was also detected in virus preparations. Host pilin and archaellum proteins were detected in the same virus preparation, suggesting that cell appendages co-purify with the viruses and VP6 could be involved in forming pilin structures at the host cell surface, as recently described for the pleolipovirus SNJ2 ³⁵. Additionally, the knockout of proviruses (including two pleolipoviruses) in *Hfx. volcanii*, led to an increased expression of motility related genes, including pili genes, and a significant increase in motility ⁶. Therefore, we conclude that pleolipoviruses target host pilin formation, both by changing the composition of the pili and by downregulating pili formation. These mechanisms likely evolved to prevent infection with the same virus and/or with other viruses; however, T4P modification by a pleolipovirus has also been shown to promote infection with a co-infecting virus ³⁵. Virions of Tebi-PV1 were often observed in interaction with host cell appendages (Supplementary Figure 10). This may indicate that some pleolipoviruses use pili for host cell attachment, as it has been reported for several other archaeal viruses ^{18,75–77}.

Virus preparations from TLS-6 were usually highly dominated by Tebi-PV1 (average genome copy number ratio 1760 Tebi-PV1:1 Tebi-SV1). Changing of growth conditions and attempts to induce Tebi-SV1, caused only minor changes to this ratio, except in CDM media supplemented with phosphate, where the difference is significant (average ratio ~80:1) (Supplementary table 2). Tebi-PV1 was detected as a circular extrachromosomal element in assemblies from host sequencing data ³⁶, exhibiting a significantly higher coverage (~7-fold) than the main chromosome, while Tebi-SV1 was detected as an integrated element with slightly higher coverage (~1.4-fold). Therefore, we assume that Tebi-PV1 is constantly replicating, while Tebi-SV1 is only induced under specific conditions, leading to an overrepresentation of Tebi-PV1 in virus preparations.

Host range assessment suggests a receptor for both viruses that is available amongst a broad range of haloarchaea

With the aim of finding a host strain that allows the propagation of only one of the two viruses, and a virus free host for both viruses, we tested seven different species of haloarchaea (representing six different genera) for their susceptibility to Tebi-SV1 and Tebi-PV1 infection. While none of the infected strains showed any significant growth defects, we could detect both viruses intracellularly and in culture supernatants across all tested haloarchaea by qPCR. Varying Tebi-PV1:Tebi-SV1 genome ratios in the different strains indicated different infection efficiencies of each virus in the different species. Tebi-SV1 showed the highest infection efficiency in *Hbt. salinarium* (ratio added was 30:1 and ratio detected 17 hpi was 5:1), while Tebi-PV1 was more successful in all other tested strains. Virus-to-host genome ratios were often far below 1 for the strains tested, which is likely due to the low virus-to-host genome ratios used for infection, and long-term cultures revealed the gradual decrease of both Tebi-SV1 and Tebi-PV1 genomes. Therefore, we conclude that both viruses can infect a wide range of haloarchaea (across genus level), can replicate within and egress from those host organisms initially. However, Tebi-PV1 and Tebi-SV1 only infected a small portion of the cells, and Tebi-SV1 was excluded from *Hfx. volcanii* during the second growth cycle (Figure 6), and also excluded during subsequent transfers from the other strains (data not shown). While *Hrr.* TLS-6 exhibits only restriction-modification systems (RM-systems) as potential virus defense systems; the majority of the other tested species contain either CRISPR systems or BREX systems, which could be responsible for defense against Tebi-PV1 and Tebi-SV1 (Supplementary Table 6). With exceptions, it is typical for pleolipoviruses of the family *Pleolipoviridae* and spindle-shaped viruses of families *Thaspiviridae*, *Fuselloviridae*, and *Halspiviridae* to exhibit a narrow host range ^{44,64,78}. We show that together, Tebi-SV1 and Tebi-PV1 exhibit a broad host range and can infect across six haloarchaeal genera. This broad host range suggests that both viruses use a receptor that is highly conserved across genera. Whether both viruses use the same receptor needs to be determined.

Chronic or lytic? – the life cycle of Tebi-SV1 remains obscure

Nearly all spindle-shaped viruses described so far exclusively carry out chronic infections, where cell lysis does not occur^{33,64,79–82}. One exception is the Acidianus two-tailed virus (ATV) which is described as causing cell lysis⁸³. Highest titres of Tebi-SV1 were obtained from a culture, grown from a single colony, that showed a very strong growth inhibition. Subsequent cultivations of different colonies of the same strain or the original stock showed growth behaviour similar to other *Halorubrum* strains (reaching OD600 of ~1.8), while producing only a small amount of spindle-shaped virus particles. With the lack of a virus-free strain, we were unable to judge the growth differences between an uninfected culture and a culture producing low amounts of Tebi-SV1. However, considering that only low titers of Tebi-SV1 are produced, the influence on the growth behavior of the entire host population might be minor. Chronic virus infections rarely cause such significant growth deficiencies observed for the first culture producing high amounts of Tebi-SV1^{61,84}. Therefore, we suggest that Tebi-SV1 may cause a lytic infection as described for ATV⁸³. Supporting this, we were able to recover infectious Tebi-SV1 particles from the cellular lysate following mechanical lysis of the host cells. Approximately half of Tebi-SV1 virions recovered from lysates were detected in S-layer preparations, suggesting that many particles accumulate on the cell surface or may be trapped between the S-layer and the cell membrane, an observation consistent with previous reports^{45,64}. The remaining virions were isolated by spheroplast lysis, indicating that Tebi-SV1 virions can accumulate intracellularly. The isolation of infectious Tebi-PV1 particles from lysed spheroplasts was puzzling, though the Tebi-PV1:Tebi SV1 ratio was significantly lower than in virus preparations from culture supernatants. Considering that pleolipoviruses are presumably released by budding through the host cell membrane, we did not expect to isolate active virus particles by cell lysis. We assume that Tebi-PV1 particles may have been attached to pili (Supplementary Figure 10), which are not affected by S-layer removal but disrupted during cell lysis, or that Tebi-PV1 particles might have been bound to a fraction of the S-layer that is membrane anchored and not removed by MgCl₂ depletion⁶⁵. Alternatively, Tebi-PV1 genomes could be enclosed in spontaneously formed membrane vesicles upon cell lysis, which subsequently fuse with a host cell. Transformation of naked pleolipovirus genomic DNA was shown to be sufficient for successful infection¹⁵.

Several proteins detected in virions of Tebi-SV1 contain predicted transmembrane domains, and virus particles isolated from both the culture supernatant and the cell lysate were sensitive to chloroform treatment. We hypothesize that Tebi-SV1 particles contain lipids, as reported for other spindle-shaped viruses^{47,80–82,85,86}. However, the presence of lipids still does not allow us to conclude the life cycle of Tebi-SV1, because lipids can be acquired both intracellular^{87–89} or by budding through the host cell membrane⁹⁰. In summary, there is strong evidence that Tebi-SV1 release has a notable effect on host growth. However, we cannot infer whether this growth inhibition is caused by cell lysis. Strong growth

retardation has also been described for some chronic viruses⁸⁴, or could be caused by virus induced cell death or dormancy^{79,91}.

Interactions between Tebi-SV1 and Tebi-PV1 suggest a mutual regulation through a shared operator sequence

While virus preparations from *Hrr*. TLS-6 always contained both Tebi-SV1 and Tebi-PV1 particles; there was a clear tendency that the abundance of one virus changed with the abundance of the other virus. The first virus preparation from *Hrr*. TLS-6 had a ratio of 1200 Tebi-SV1 genomes per Tebi-PV1 genome, while in any following preparations this ratio was inverted, with 1760 Tebi-PV1 per Tebi-SV1 genome. When attempting to induce a higher production of Tebi-SV1 particles, an increase in Tebi-SV1 abundance also correlated with a decrease in Tebi-PV1 abundance (e.g., CDM medium versus CDM+PO4 and DBCM2 media versus CDM media, Figure 4). We concluded that the activity of the viruses is at least partially regulated by virus-virus interactions.

While we could not identify growth conditions that would reproduce the high titers of Tebi-SV1 particles obtained in the first virus preparation of *Hrr*. TLS-6, we were able to identify a genomic variation of Tebi-SV1 particles produced from this culture. Sequencing of the virus genome from the first culture revealed two SNPs in the Tebi-SV1 genome, which we could not identify on the integrated Tebi-SV1 genome when re-sequencing the host genome in subsequent cultures. The SNPs are located in the intergenic region upstream of VP8, a transcriptional regulator. Interestingly, this region partially shares 100% sequence identity with the intergenic region upstream of VP4 on the Tebi-PV1 genome. Both ORFs VP4 (Tebi-PV1) and VP8 (Tebi-SV1) show similarities to PhiH-like repressors, and we suggest that the shared region represents the operators. Considering that a mutation in the putative operator of VP8 led to a significantly increased activity of Tebi-SV1 with low Tebi-PV1 titers, we propose that the viruses co-regulate each other through this shared operator sequence. Tebi-SV1 is integrated into the host genome and usually represented in significantly lower numbers than Tebi-PV1, suggesting that Tebi-PV1 VP8 helps maintain Tebi-SV1 in its lysogenic state. Similar virus-virus regulatory mechanisms have been observed in other archaeal systems. In *Hfx. volcanii*, for example, the provirus Halfvol3 and virus HFPV-1 were both shown to influence each other's transcription and replication. HFPV-1 massively downregulated the integrase and a transcriptional regulator (similar to PhiH-like repressors) in the Halfvol3 provirus region upon infection, and in a Halfvol3-free mutant, HFPV-1 replicated significantly more¹⁵. In *Halobacterium halobium* cells, a homolog to PhiH1 repressor protein gives the cells immunity to PhiH1 virus^{92,93}.

The proposed regulation between Tebi-SV1 and Tebi-PV1 does not seem to represent a strict superinfection exclusion mechanism as previously described for virus-virus coinfections of archaea, where one virus completely prevents infection of the other^{21,34,94}. Instead, the data suggests a more subtle form of mutual regulation, allowing both viruses to co-exist while modulating each other's

activity. Similar to the recent discovery of the mutual interaction between SNJ1 and SNJ2³⁵, Tebi-SV1 and Tebi-PV1 may be dependent on each other. Infection of other haloarchaea with the mixture of the two viruses always led to a coinfection with both viruses, suggesting that a coinfection could be crucial for both viruses to enter new host cells and replicate.

Further supporting a functional link, we found that four out of six host genomes containing Tebi-SV1-like viruses also harbored pleolipovirus-related sequences. This raises the possibility that the successful maintenance and replication of certain spindle-shaped viruses may depend on coinfection with a compatible pleolipovirus.

Conclusion

In this study, we isolated and characterized a new spindle-shaped virus (Tebi-SV1) that co-exists with a pleolipovirus in the same host strain. We were only able to identify the spindle-shaped virus (Tebi-SV1) because we visualized the culture supernatant with TEM. Relying solely on genome analysis would not have allowed for the identification of this MGE as an actual virus, due to the unique structure and sequence of the virus genome. This nicely demonstrates that isolation is often still a crucial method for identifying viruses, especially when working with archaeal viruses. We uncovered that the coinfecting pleolipovirus, Tebi-PV1, likely regulates the activity of Tebi-SV1 through a predicted repressor protein, without excluding Tebi-SV1 from the host. Additionally, Tebi-PV1 encodes for T4P which we detected by proteomics and may also play an important role in both virus-host and virus-virus interactions. Finally, coinfection with both viruses appeared to be crucial to allow the infection of other haloarchaea. Considering other recent findings on the interactions of pleolipoviruses with coinfecting viruses and their prevalence in haloarchaea genomes, we propose that pleolipoviruses play a particularly important role in virus-host and virus-virus interactions in haloarchaea. This example of mutual regulation portrays the importance of understanding such complex virus-virus interactions that may occur across all domains of life.

Experimental Procedures

Strains and cultivation conditions

All haloarchaeal strains used in this study are summarized in Supplementary Table 7. *Halorubrum* sp. strain TLS-6 (TLS-6) was isolated from a microbial mat from Laguna Tebenquiche, Chile³⁶. TLS-6 was cultivated either in nutrient rich media, modified DBCM2⁹⁵, or alternatively, nutrient deficient media, modified CDM⁹⁶ supplemented with 1 mM phosphate (K_2HPO_4). All *Halorubrum* strains were cultured aerobically with constant agitation of 120 rpm at 28 °C unless otherwise stated. All strains were inoculated from glycerol stocks (in media with 15% glycerol stored at -80°C) and grown in approximately 5 mL of the corresponding media and temperatures (Supplementary Table 7) until an optical density at 600 nm (OD_{600}) of ~0.5. The cultures were diluted to OD_{600} = 0.05 in fresh media and

regrown to an OD₆₀₀ of approximately 1. Dilution to OD₆₀₀ = 0.05 was repeated once more in fresh media and cultures were grown to exponential growth phase before used for all experimental procedures described. Conditions tested to enrich either Tebi-PV1 or Tebi-SV1 included UV treatment, antibiotics, and PO₄ supplementation (1mM K₂HPO₄). UV treatment was performed in early exponential growth phase using a UV crosslinker from Biometra™ at 0.05 J of UV radiation for 30 seconds. Antibiotics Mitomycin C (15 µg/mL) and Pravastatin (1 µg/mL) were added in early exponential phase.

Isolation and purification of viruses

Hrr. TLS-6 was initially cultivated from a single colony (CDM agar) in 50 mL CDM⁹⁶ supplemented with 1 mM K₂HPO₄ and with and without 11 µM arsenic (As (V)). The cultures did not reach exponential phase, but were, regardless, diluted into 500 mL and grown for ~30 days. Cells were removed by filtration with 0.45 µm pore size followed by 0.22 µm pore size filters. Subsequently, the flow through was ultra-centrifuged (41,000 rpm for 2 h, 4 °C, fixed angle 70.1 Ti rotor, Beckman & Coulter). The resulting pellets were re-suspended in DBCM2 salt solution (180 g/L NaCl, 25 g/L MgCl₂, 29 g/L MgSO₄·7 H₂O, 5.8 g/L KCl). For genomic DNA extraction, the preparation was syringe filtered (0.22 µm pore size) and treated with 50 µg/mL RNase A (16 units, ThermoFisher™) and 50 µg/mL DNase I (16 units, NEB) at 37 °C for 2 hours. The virus preparation was then loaded onto an Iodixanol (25 % - 40 % OptiPrep) gradient that was pre-layered and allowed to linearize overnight (38,000 rpm for 20 h, 4 °C, SW41 Ti Swinging-Bucket rotor, Beckman & Coulter). Three resulting bands were extracted with a syringe, and virus-like particles (VLPs) were observed under the TEM. DNA was extracted from all bands (see Preparation and analysis of viral genomes), and DNA from the second band was used for genome sequencing.

For Tebi-SV1 enriched preparations, TLS-6 was grown in 1 L of modified CDM supplemented with 1 mM phosphate. Cells were collected *via* centrifugation (4500 x g, 45 min) and resuspended in 6 mL of DBCM2 salt solution. Cells were harvested *via* centrifugation (10,000 x g, 10 min) and washed once more with fresh buffer to remove any free virus particles. After centrifugation (10,000 x g, 10 min), the cells were resuspended and incubated in spheroplasting solution (1 M NaCl, 27 mM KCl, 50 mM Tris-HCl, 15% sucrose) and 0.5 M EDTA, pH 8, at room temperature (RT) for 10 min. Samples were sonicated in an ice water bath (Bandelin Sonorex TK52) for 25 min. Cell debris was removed by centrifugation (15,800 x g, 30 min at 4 °C) and filtration with 0.45 µm pore size followed by 0.22 µm pore size filters. The lysate was collected by ultracentrifugation (41,000 rpm for 2 h, 4 °C in Beckman 70.1 Ti rotor), the pellet was dissolved in 1 mL DBCM2 salt solution, and then sterile filtered once more with 0.22 µm pore size. For Tebi-PV enriched preparations, TLS-6 was grown in nutrient rich media (DBCM2). Cultures were harvested in stationary phase by centrifugation (4,500 x g, 45 min, 4 °C) to pellet the cells. The supernatant was filtered (0.22 µm pore size), and virus particles were collected by ultracentrifugation (as above). The re-suspended (1 ml DBCM2 salt solution) pellets were

filtered once more (0.22 µm pore size) to a final volume of 1 mL. DNA extraction was performed on 20 µL of DNase I treated concentrated supernatant and screened for both viruses by PCR or qPCR (see *Virus quantification by qPCR*). Virus preparations were tested for host contamination both by PCR and by incubation in medium for several days before further use.

Genome sequencing and genome analysis

DNA was extracted using a genomic DNA extraction kit (ISOLATE II PCR & Gel Kit, Bioline®). Library preparation (FS DNA Library, NEBNext® Ultra™) and sequencing Illumina HiSeq3000, 2 x 150 bp, 1 Gigabase were performed at the Max Planck-Genome-Centre (Cologne, Germany). The reads were quality trimmed using Cutadapt⁹⁷ minimum quality of 30, and a minimum length of 50 (-q 30, -m 50). Assembler metaplasmidSPAdes⁹⁸ was used to perform assembly of high-quality trimmed reads. Sequence similarity was assessed using BLASTn⁹⁹, e-value < 10⁻⁵, word size 28, and compared to public databases NCBI, Nucleotide Selection, and RefSeqProteins¹⁰⁰. Open reading frames were predicted using Prodigal¹⁰¹. InterProScan¹⁰² was used to detect putative conserved domains and other protein signatures. Manual annotations of the two resulting contigs were compared and refined by IMG/VR¹⁰³ NCBI BLASTx, InterProScan, HHblits^{99,102,104}, Uniclust¹⁰⁵, and Glimmer¹⁰⁶ results. The Mauve alignment algorithm and EasyFig BLAST tool were used to identify similar viral and host genomes for whole-genome alignments¹⁰⁷. Accession numbers for the genomes of Tebi-SV1 and Tebi-PV1 are PX315771 and PX315772, respectively. The *Halorubrum* TLS-6 genome sequencing reads (SRR12122876, SRA) were assembled with SPAdes v.3.11.0 (default parameters) into 48 contigs (~1.6 Mbp, ~1.2 Mbp, ~101kbp, 15.2kbp, 2.2 kbp, 1.8kbp, 1.5kbp and 41 contigs below 1kbp)¹⁰⁸. Resequencing of the TLS-6 genome was performed as described above. To identify mutations in the Tebi-SV1 and Tebi-PV1 genomes, the reads (available under BioProject accession number PRJNA1322139) were trimmed and mapped to the assembled genome of TLS-6 using Geneious mapper (Geneious Prime® 2025.0.3, low sensitivity, default parameters), and the ‘find variations’ feature (default settings).

Phylogenic analysis and identification of relatives

Network analyses were performed using vConTACT2¹⁰⁹ with the Viral RefSeq-archaea database (version 211) and the IMGPR database¹¹⁰. The analysis utilized BLASTP as the relationship mode (—rel-mode BLASTP) with an e-value threshold of 10⁻³ to determine significant sequence similarities. The analysis revealed the close relationship of proposed plasmid IMGPR_plasmid_2904944409_000002 with Tebi-SV1. The resulting network structures were visualized using the R packages ggplot2 and Network^{111,112}, allowing for the graphical representation of gene-sharing patterns. Phylogenetic analysis and whole-genome comparisons were conducted using VICTOR¹¹³, considering D0, D4, and D6 formulas of predicted gene sequences for clustering and taxonomic assignment. Clustering results were reconciled with Victor analysis to indicate the

acceptable thresholds of gene-sharing at the approximate genus level. The average nucleotide identity (ANI) threshold (95%) used to delineate species in viral genomics was taken from ⁵⁵.

Protein content analyses of virus particles

Purified virus preparations were supplemented with 1X sample buffer (0.2 g SDS, 1 mg Bromphenol Blue, 0.78 mL glycerol, 0.2 mL 0.5 M Tris pH 6.8, and 0.155 g DTT per 10 mL) and Urea (3.3M). The protein mixture was heat denatured for ~10 min at 95 °C. Viral proteins were separated by Tris-Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gel electrophoresis (12% acrylamide in resolving gel). Gels were stained with fast Coomassie staining (50% ethanol, 10% acetic acid, 0.5% wt/vol Coomassie Blue). In-gel digestion of excised bands or lanes was performed as described in ¹¹⁴. Protein content analyses of gel separated samples was performed using mass spectrometry (MS) as described in ¹¹⁵. For protein identification, we generated a custom database by predicting open reading frames from the host genome (Prodigal ¹⁰¹) and manually curating the viral genome as described in *Viral genome sequencing and genome analysis*. Additionally, we included protein sequences predicted the cRAP protein sequence database (<http://www.thegpm.org/crap>) of common laboratory contaminants. Searches of the MS/MS spectra against this database were performed with the Sequest HT node in Proteome Discoverer vs. 2.5 (Thermo Fisher Scientific). False discovery rates (FDRs) for peptide spectral matches (PSMs) were calculated and filtered using the Percolator Node in Proteome Discoverer. The percolator was run with a maximum delta Cn 0.05, a strict target FDR of 0.01, and a relaxed target FDR of 0.05, and validation based on q-value. The Protein FDR Validator Node in Proteome Discoverer was used to calculate q-values for inferred proteins based on the results from a search against a target-decoy database. Proteins with a q-value of <0.01 were categorized as high-confidence identifications and proteins with a q-value of <0.01 were categorized as medium-confidence identifications.

Chloroform and Protease treatment of viral preparations

Triplicates of cell lysate preparation or supernatant virus preparation from TLS-6 cultured in CDM + 1 mM K₂HPO₄ (see *Isolation and purification of viruses*) were diluted with 2 parts of water to preserve the activity of Proteinase K. A final concentration of 7.5 µg/ml of Proteinase K, or the equivalent volume of H₂O were added and samples were incubated for 4 h at 37 °C with occasional inversion to mix. The proteinase K was inactivated with PMSF (2 mM final concentration). Unprotected DNA was digested with DNase I after adding 1x final concentration DNase buffer for 2 h at 37 °C (New England Biolabs). The salinity was adjusted with DBCM2 salt solution, and the entire sample was used to infect *Hrr. lacusprofundi* cells (~5E+05 cells). Fresh media was added to reach a final volume of 1.5 mL, and samples were left to adsorb for ~16 h at room temperature. Cells were then harvested at 4,500 x g for 45 min. and the cell pellets were washed twice with DBCM2 salt solution (10,000 x g for 10 min). DNA was extracted from cell pellets, and internalized virus genomes were detected *via* qPCR.

Triplicates of cell lysate preparation or supernatant virus preparation from TLS-6 cultured in CDM + 1 mM K₂HPO₄ (see *Isolation and purification of viruses*) were exposed to chloroform (manufacturer) in a 1:4 (solvent to virus) volume ratio and incubated with constant agitation for 20 min at RT. The samples were centrifuged (13,500 x g, 3 min) and the watery upper layer was collected in a fresh tube. For untreated controls, the same volume of the same virus preparation was used; however, no chloroform was added. Unprotected DNA was digested with DNase I after adding 1x final concentration DNase buffer (2 h at 37 °C). An equal volume of fresh DBCM2+ media was added to the sample (final volume ~1.5 mL). *Hrr. lacusprofundi* cells were infected (as above) with chloroform treated and control virus preparations. Infected cells were harvested, washed, and screened for internalized virus genomes with qPCR (as above).

Infectivity and host range assessment

Seven different haloarchaeal hosts were tested in biological triplicates (Figure 6 & Supplementary Figure 4). All strains were grown in 2 sequential cultures as described above, and infected in the exponential phase with a total MOI of approximately 2.5 (0.09 for Tebi-SV1 and 2.5 for Tebi-PV1, total virus genome copy number added per culture: 1.44E+08 Tebi-SV1 and 4.18E+9 Tebi-PV1). Host cell number was calculated with 1.65E+9 in 50ml at OD₆₀₀ 0.05, using cell numbers determined for *Hrr. lacusprofundi*²⁷. Notably, these numbers can differ slightly for the other organisms. After incubation overnight at RT (17 hours for *Hrr. lacusprofundi*, *Haloferax mediterranei* and *Haloferax volcanii*; 16 hours for *Halobacterium salinarum* and *Haloarcula japonica*; 8 hours for *Haloquadratum walsbyi* and *Haloterrigena turkmenica*) for optimal adsorption, cells were pelleted and washed twice to remove any unbound virus remaining in the supernatant (10,000 x g, 10 min and re-suspended in 500 µL medium). Cells were transferred into 7 ml fresh medium and incubated as described. Optical density at 600 nm (OD_{600nm}) was used to monitor growth kinetics (Figure 6 and Supplementary Figure 4). Samples (1.5 mL) were taken at different time points post infection (indicated in the figures) where hours post-infection (hpi) specify the time after the virus was mixed with host cells for adsorption. Cells from 1.5 ml of culture were harvested by centrifugation (10,000 x g, 10 min), washed twice with fresh media (to remove any free viruses), and screened for infection with Tebi-SV1 and Tebi-PV1 with specific qPCR probes (Supplementary table 8). The supernatant was filtered twice with 0.22 µm syringe filters, and virus particles were harvested by ultracentrifugation (41,000 rpm for 2 h, 4 °C, fixed angle 70.1 Ti rotor, Beckman & Coulter). The pellets were resuspended in 100 µL of DBCM2 salt solution and treated with DNase I before DNA extraction and quantification by qPCR.

Virus quantification by qPCR

DNA extractions were performed with an Isolate II Genomic DNA Kit (Bioline, London. UK) according to the manufacturer's instructions. Virus genome copy numbers were determined in technical triplicate for each biological replicate as described previously^{27,116}. The primer sequences, annealing

temperatures, and concentrations of all primers used are listed in Supplementary Table 8. Host and virus genome copy numbers in all samples were quantified as previously described¹¹⁶. The means from the various conditions in Figure 4 were compared with two-sided, Student's t-test (Supplementary Table 2).

Negative-stain Transmission electron microscopy

Five µL of a viral preparation was adsorbed onto a carbon-coated copper grid for 3 minutes. The sample was dipped into a 5 µL droplet of ddH₂O before negative staining with 2% uranyl acetate for 45 seconds. Imaging was performed at 200 kV using a JEOL JEM-2100 Plus electron microscope.

Data Availability

The genomes of Tebi-SV1 and Tebi-PV1 are available under accession numbers PX315771 and PX315772, respectively. Raw reads for the re-sequencing of the TLS-6 genome are available under BioProject accession number PRJNA1322139. Mass spectrometry data are under submission to PRIDE, they are currently available for review under this link <https://nextcloud.mpi-bremen.de/index.php/s/m2wx8JLQCB595eS> (Password: TQ6Kpvu7a2).

Acknowledgments

We thank the Max Planck-Genome-Centre Cologne (Cologne, Germany) for assistance with DNA sequencing, and Daniela Thies and Ingrid Kunze (MPI for Marine Microbiology, Bremen, Germany) for experimental support. We are grateful to Dominik Lücking for valuable advice on genome analysis. We also acknowledge the Max Planck Institute for Marine Microbiology and the Max Planck Society for their continuous support. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Project-ID 465087098 – SPP 2330 to S.E.

References

1. Roux, S. *et al.* Ecology and evolution of viruses infecting uncultivated SUP05 bacteria as revealed by single-cell- and meta-genomics. *eLife* **3**, e03125 (2014).
2. Roux, S., Hallam, S. J., Woyke, T. & Sullivan, M. B. Viral dark matter and virus–host interactions resolved from publicly available microbial genomes. *eLife* **4**, e08490 (2015).
3. *Bacteriophage Ecology: Population Growth, Evolution, and Impact of Bacterial Viruses.* (Cambridge University Press, Cambridge ; New York, 2008).
4. Casjens, S. Prophages and bacterial genomics: what have we learned so far? *Mol. Microbiol.* **49**, 277–300 (2003).

5. Díaz-Muñoz, S. L. Viral coinfection is shaped by host ecology and virus–virus interactions across diverse microbial taxa and environments. *Virus Evol.* **3**, vex011 (2017).
6. Di Cianni, N. *et al.* Provirus deletion from *Haloferax volcanii* affects motility, stress resistance, and CRISPR RNA expression. *microLife* **6**, uqaf008 (2025).
7. Hendrix, R. W., Smith, M. C. M., Burns, R. N., Ford, M. E. & Hatfull, G. F. Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. *Proc. Natl. Acad. Sci.* **96**, 2192–2197 (1999).
8. Kumar, N., Sharma, S., Barua, S., Tripathi, B. N. & Rouse, B. T. Virological and Immunological Outcomes of Coinfections. *Clin. Microbiol. Rev.* **31**, 10.1128/cmr.00111-17 (2018).
9. Krupovic, M., Quemin, E. R. J., Bamford, D. H., Forterre, P. & Prangishvili, D. Unification of the Globally Distributed Spindle-Shaped Viruses of the Archaea. *J. Virol.* **88**, 2354–2358 (2014).
10. Mochizuki, T., Sako, Y. & Prangishvili, D. Provirus Induction in Hyperthermophilic Archaea: Characterization of *Aeropyrum pernix* Spindle-Shaped Virus 1 and *Aeropyrum pernix* Ovoid Virus 1 †. *J. Bacteriol.* **193**, 5412–5419 (2011).
11. Prangishvili, D., Mochizuki, T. & Krupovic, M. ICTV virus taxonomy profile: Guttaviridae. *J. Gen. Virol.* **99**, (2018).
12. Papathanasiou, P. *et al.* Stable maintenance of the rudivirus SIRV3 in a carrier state in *Sulfolobus islandicus* despite activation of the CRISPR-Cas immune response by a second virus SMV1. *RNA Biol.* **16**, 557–565 (2019).
13. Abedon, S. T. Prophages Preventing Phage SuperinfectionSuperinfections. in *Bacteriophages as Drivers of Evolution: An Evolutionary Ecological Perspective* (ed. Abedon, S. T.) 179–191 (Springer International Publishing, Cham, 2022). doi:10.1007/978-3-030-94309-7_16.
14. Bertani, G. STUDIES ON LYSOGENESIS III: Superinfection of Lysogenic *Shigella dysenteriae* with Temperate Mutants of the Carried Phage. *J. Bacteriol.* **67**, 696–707 (1954).
15. Alarcón-Schumacher, T., Naor, A., Gophna, U. & Erdmann, S. Isolation of a virus causing a chronic infection in the archaeal model organism *Haloferax volcanii* reveals antiviral activities of a provirus. *Proc. Natl. Acad. Sci.* **119**, e2205037119 (2022).

- 788 16. Chen, B. *et al.* ORF4 of the Temperate Archaeal Virus SNJ1 Governs the Lysis-Lysogeny Switch
789 and Superinfection Immunity. *J. Virol.* **94**, (2020).
- 790 17. Medvedeva, S. *et al.* Virus-borne mini-CRISPR arrays are involved in interviral conflicts. *Nat.*
791 *Commun.* **10**, 5204 (2019).
- 792 18. Quemin, E. R. J. *et al.* First Insights into the Entry Process of Hyperthermophilic Archaeal
793 Viruses. *J. Virol.* **87**, 13379–13385 (2013).
- 794 19. Krupovic, M. *et al.* Integrated mobile genetic elements in Thaumarchaeota. *Environ. Microbiol.*
795 **21**, 2056–2078 (2019).
- 796 20. Erdmann, S., Shah, S. A. & Garrett, R. A. SMV1 virus-induced CRISPR spacer acquisition from
797 the conjugative plasmid pMGB1 in *Sulfolobus solfataricus* P2. *Biochem. Soc. Trans.* **41**, 1449–
798 1458 (2013).
- 799 21. Erdmann, S., Le Moine Bauer, S. & Garrett, R. A. Inter-viral conflicts that exploit host CRISPR
800 immune systems of *ulfolobus*. *Mol. Microbiol.* **91**, 900–917 (2014).
- 801 22. Contursi, P., Fusco, S., Cannio, R. & She, Q. Molecular biology of fuselloviruses and their
802 satellites. *Extremophiles* **18**, 473–489 (2014).
- 803 23. deCarvalho, T. *et al.* Simultaneous entry as an adaptation to virulence in a novel satellite-helper
804 system infecting *Streptomyces* species. *ISME J.* **17**, 2381–2388 (2023).
- 805 24. Eppley, J. M., Biller, S. J., Luo, E., Burger, A. & DeLong, E. F. Marine viral particles reveal an
806 expansive repertoire of phage-parasitizing mobile elements. *Proc. Natl. Acad. Sci. U. S. A.* **119**,
807 (2022).
- 808 25. La Scola, B. *et al.* The virophage as a unique parasite of the giant mimivirus. *Nature* **455**, 100–
809 104 (2008).
- 810 26. Joseph, S. B., Hanley, K. A., Chao, L. & Burch, C. L. Coinfection rates in $\phi 6$ bacteriophage are
811 enhanced by virus-induced changes in host cells. *Evol. Appl.* **2**, 24–31 (2009).
- 812 27. Mercier, C., Thies, D., Zhong, L., Raftery, M. J. & Erdmann, S. Characterization of an archaeal
813 virus-host system reveals massive genomic rearrangements in a laboratory strain. *Front.*
814 *Microbiol.* **14**, (2023).

28. Arnold, H. P. *et al.* The genetic element pSSVx of the extremely thermophilic crenarchaeon *Sulfolobus* is a hybrid between a plasmid and a virus. *Mol. Microbiol.* **34**, 217–226 (1999).
29. Lossouarn, J. *et al.* An abyssal mobilome: Viruses, plasmids and vesicles from deep-sea hydrothermal vents. *Res. Microbiol.* **166**, 742–752 (2015).
30. Orbach, M. J. & Jackson, E. N. Transfer of chimeric plasmids among *Salmonella typhimurium* strains by P22 transduction. *J. Bacteriol.* **149**, 985–994 (1982).
31. Valero-Rello, A., López-Sanz, M., Quevedo-Olmos, A., Sorokin, A. & Ayora, S. Molecular Mechanisms That Contribute to Horizontal Transfer of Plasmids by the Bacteriophage SPP1. *Front. Microbiol.* **8**, (2017).
32. Rumnieks, J. & Tars, K. Diversity of pili-specific bacteriophages: genome sequence of IncM plasmid-dependent RNA phage M. *BMC Microbiol.* **12**, 277 (2012).
33. Gorlas, A., Krupovic, M., Forterre, P. & Geslin, C. Living side by side with a virus: Characterization of two novel plasmids from *Thermococcus prieurii*, a host for the spindle-shaped virus TPV1. *Appl. Environ. Microbiol.* **79**, 3822–3828 (2013).
34. Liu, Y. *et al.* Identification and characterization of SNJ2, the first temperate pleolipovirus integrating into the genome of the SNJ1-lysogenic archaeal strain. *Mol. Microbiol.* **98**, 1002–1020 (2015).
35. Xiang, J. *et al.* A type IV pili-mediated mutualism between two co-resident temperate archaeal viruses and their host. *Cell Rep.* **44**, (2025).
36. Saona, L. A. *et al.* Phosphate-Arsenic Interactions in Halophilic Microorganisms of the Microbial Mat from Laguna Tebenquiche: from the Microenvironment to the Genomes. *Microb. Ecol.* (2021) doi:10.1007/s00248-020-01673-9.
37. Farías, M. E. *et al.* Characterization of bacterial diversity associated with microbial mats, gypsum evaporites and carbonate microbialites in thalassic wetlands: Tebenquiche and La Brava, Salar de Atacama, Chile. *Extremophiles* **18**, 311–329 (2014).
38. Fernández, A. B., Visscher, P. T., Rasuk, M. C., Contreras Leiva, M. & Farías, M. E. Prokaryotic Diversity at the Hypersaline Laguna Tebenquiche in the Salar de Atacama, Chile. in *Microbial*

Ecosystems in Central Andes Extreme Environments 141–152 (Springer International Publishing, 2020). doi:10.1007/978-3-030-36192-1_10.

39. Eissler, Y., Dorador, C., Kieft, B., Molina, V. & Hengst, M. Virus and potential host microbes from viral-enriched metagenomic characterization in the high-altitude wetland, Salar de Huasco, Chile. *Microorganisms* **8**, 1–15 (2020).
40. Guixa-B, N., Calderon-Pazl, J. I. & Pedros-, C. *Viral Lysis and Bacterivory as Prokaryotic Loss Factors along a Salinity Gradient*. vol. 1 215–227 (1996).
41. Oren, A., Bratbak, G. & Heldal, M. Occurrence of virus-like particles in the Dead Sea. *Extremophiles* **1**, 143–149 (1997).
42. Santos, F. *et al.* Culture-Independent Approaches for Studying Viruses from Hypersaline Environments. *Appl. Environ. Microbiol.* **78**, 1635 (2012).
43. Sime-Ngando, T. *et al.* Diversity of virus-host systems in hypersaline Lake Retba, Senegal. *Environ. Microbiol.* **13**, 1956–1972 (2011).
44. Bath, C. & Dyal-Smith, M. L. His1, an Archaeal Virus of the Fuselloviridae Family That Infects *Haloarcula hispanica*. *J. Virol.* **72**, 9392–9395 (1998).
45. Turgeman-Grott, I. *et al.* A new archaeal virus that suppresses the transcription of host immunity genes. Preprint at <https://doi.org/10.1101/2024.02.12.579488> (2024).
46. Adriaenssens, E. M. *et al.* Taxonomy of prokaryotic viruses: 2018-2019 update from the ICTV Bacterial and Archaeal Viruses Subcommittee. *Arch. Virol.* **165**, 1253–1260 (2020).
47. Wang, F. *et al.* Spindle-shaped archaeal viruses evolved from rod-shaped ancestors to package a larger genome. *Cell* 1–11 (2022) doi:10.1016/j.cell.2022.02.019.
48. Hanhijärvi, K. J., Ziedaite, G., Pietilä, M. K., Hæggström, E. & Bamford, D. H. DNA Ejection from an Archaeal Virus—A Single-Molecule Approach. *Biophys. J.* **104**, 2264–2272 (2013).
49. Atanasova, N. S., Demina, T. A., Krishnam Rajan Shanthi, S. N. V., Oksanen, H. M. & Bamford, D. H. Extremely halophilic pleomorphic archaeal virus HRPV9 extends the diversity of pleolipoviruses with integrases. *Res. Microbiol.* **169**, 500–504 (2018).
50. Bairoch, A. A possible mechanism for metal-ion induced DNA-protein dissociation in a family of prokaryotic transcriptional regulators. *Nucleic Acids Res.* **21**, 2515 (1993).

870 51. Bamford, D. H. *et al.* ICTV Virus Taxonomy Profile: Pleolipoviridae. *J. Gen. Virol.* **98**, 2916–
871 2917 (2017).

872 52. Sullivan, M. J., Petty, N. K. & Beatson, S. A. Easyfig: a genome comparison visualizer.
873 *Bioinformatics* **27**, 1009–1010 (2011).

874 53. Paez-Espino, D. *et al.* IMG/VR v.2.0: An integrated data management and analysis system for
875 cultivated and environmental viral genomes. *Nucleic Acids Res.* **47**, D678–D686 (2019).

876 54. Chen, S. *et al.* VOLN27B: A New Head-Tailed Halovirus Isolated from an Underground Salt
877 Crystal and Infecting Halorubrum. *Archaea* **2021**, (2021).

878 55. Roux, S., Emerson, J. B., Eloie-Fadrosch, E. A. & Sullivan, M. B. Benchmarking viromics: an in
879 silico evaluation of metagenome-enabled estimates of viral community composition and diversity.
880 *PeerJ* **5**, e3817 (2017).

881 56. Ludt, K. & Soppa, J. Polyploidy in halophilic archaea: regulation, evolutionary advantages, and
882 gene conversion. *Biochem. Soc. Trans.* **47**, 933–944 (2019).

883 57. Soppa, J. Non-equivalent genomes in polyploid prokaryotes. *Nat. Microbiol.* **7**, 186–188 (2022).

884 58. Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3.
885 *Nature* **630**, 493–500 (2024).

886 59. Ni, Y., Xu, T., Yan, S., Chen, L. & Wang, Y. Hiding in plain sight: The discovery of complete
887 genomes of 11 hypothetical spindle-shaped viruses that putatively infect mesophilic ammonia-
888 oxidizing archaea. *Environ. Microbiol. Rep.* **16**, e13230 (2024).

889 60. Gupta, T. K. *et al.* Structural basis for VIPP1 oligomerization and maintenance of thylakoid
890 membrane integrity. *Cell* **184**, 3643–3659.e23 (2021).

891 61. Pietila, M. K. *et al.* Virion Architecture Unifies Globally Distributed Pleolipoviruses Infecting
892 Halophilic Archaea. *J. Virol.* **86**, 5067–5079 (2012).

893 62. Erdmann, S., Tschitschko, B., Zhong, L., Raftery, M. J. & Cavicchioli, R. A plasmid from an
894 Antarctic haloarchaeon uses specialized membrane vesicles to disseminate and infect plasmid-
895 free cells. *Nat. Microbiol.* **2**, 1446–1455 (2017).

896 63. Mills, J. *et al.* Extracellular vesicle formation in Euryarchaeota is driven by a small GTPase. *Proc.*
897 *Natl. Acad. Sci.* **121**, e2311321121 (2024).

898 64. Jong-Geol Kima *et al.* Spindle-shaped viruses infect marine ammonia-oxidizing thaumarchaea.
899 *PNAS* **116**, 15645–15650 (2019).

900 65. Kandiba, L., Guan, Z. & Eichler, J. Lipid modification gives rise to two distinct *Haloferax volcanii*
901 S-layer glycoprotein populations. *Biochim. Biophys. Acta BBA - Biomembr.* **1828**, 938–943
902 (2013).

903 66. Wang, J. *et al.* A novel family of tyrosine integrases encoded by the temperate pleolipovirus SNJ2.
904 *Nucleic Acids Res.* **46**, 2521–2536 (2018).

905 67. Muskhelishvili, G., Palm, P. & Zillig, W. SSV1-encoded site-specific recombination system in
906 *Sulfolobus shibatae*. *MGG Mol. Gen. Genet.* **237**, 334–342 (1993).

907 68. Zhan, Z., Zhou, J. & Huang, L. Site-Specific Recombination by SSV2 Integrase: Substrate
908 Requirement and Domain Functions. *J. Virol.* **89**, 10934–10944 (2015).

909 69. Chen, Z. *et al.* A virus-borne DNA damage signaling pathway controls the lysogeny-induction
910 switch in a group of temperate pleolipoviruses. *Nucleic Acids Res.* **51**, 3270–3287 (2023).

911 70. Baquero, D. P. *et al.* Stable coexistence between an archaeal virus and the dominant methanogen
912 of the human gut. *Nat. Commun.* **15**, 7702 (2024).

913 71. Songailiene, I. *et al.* HEPN-MNT Toxin-Antitoxin System: The HEPN Ribonuclease Is
914 Neutralized by OligoAMPylation. *Mol. Cell* **80**, 955-970.e7 (2020).

915 72. Rostøl, J. T., Quiles-Puchalt, N., Iturbe-Sanz, P., Lasa, Í. & Penadés, J. R. Bacteriophages avoid
916 autoimmunity from cognate immune systems as an intrinsic part of their life cycles. *Nat.*
917 *Microbiol.* **9**, 1312–1324 (2024).

918 73. Geslin, C. *et al.* Analysis of the first genome of a hyperthermophilic marine virus-like particle,
919 PAV1, isolated from *Pyrococcus abyssi*. *J. Bacteriol.* **189**, 4510–4519 (2007).

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

923 75. Hartman, R. *et al.* The Molecular Mechanism of Cellular Attachment for an Archaeal Virus.
924 *Structure* **27**, 1634-1646.e3 (2019).

925 76. Rowland, E. F., Bautista, M. A., Zhang, C. & Whitaker, R. J. Surface resistance to SSVs and
926 SIRVs in pilin deletions of *Sulfolobus islandicus*. *Mol. Microbiol.* **113**, 718–727 (2020).

927 77. Wang, F. *et al.* The structures of two archaeal type IV pili illuminate evolutionary relationships.
928 *Nat. Commun.* **11**, 3424 (2020).

929 78. Sensevdi, E. R., Sourrouille, Z. A. & Quax, T. E. Host range and cell recognition of archaeal
930 viruses. *Curr. Opin. Microbiol.* **77**, 102423 (2024).

931 79. Dewerff, S. J., Zhang, C., Schneider, J. & Whitaker, R. J. Intraspecific antagonism through viral
932 toxin encoded by chronic *Sulfolobus* spindle-shaped virus. *Philos. Trans. R. Soc. B Biol. Sci.* **377**,
933 (2022).

934 80. Pietilä, M. K., Atanasova, N. S., Oksanen, H. M. & Bamford, D. H. Modified coat protein forms
935 the flexible spindle-shaped virion of haloarchaeal virus His1. *Environ. Microbiol.* **15**, 1674–1686
936 (2013).

937 81. Uldahl, K. B. *et al.* Life Cycle Characterization of *Sulfolobus* Monocaudavirus 1, an
938 Extremophilic Spindle-Shaped Virus with Extracellular Tail Development. *J. Virol.* **90**, 5693–
939 5699 (2016).

940 82. Xiang, X. *et al.* *Sulfolobus tengchongensis* Spindle-Shaped Virus STSV1: Virus-Host Interactions
941 and Genomic Features. *J. Virol.* **79**, 8677–8686 (2005).

942 83. Prangishvili, D. *et al.* Structural and Genomic Properties of the Hyperthermophilic Archaeal Virus
943 ATV with an Extracellular Stage of the Reproductive Cycle. *J. Mol. Biol.* **359**, 1203–1216 (2006).

944 84. Svirskaitė, J., Oksanen, H. M., Daugelavičius, R. & Bamford, D. H. Monitoring physiological
945 changes in haloarchaeal cell during virus release. *Viruses* **8**, (2016).

946 85. Geslin, C. *et al.* PAV1, the first virus-like particle isolated from a hyperthermophilic
947 euryarchaeote, ‘*Pyrococcus abyssi*’. *J. Bacteriol.* **185**, 3888–3894 (2003).

948 86. Quemin, E. R. J. *et al.* *Sulfolobus* Spindle-Shaped Virus 1 Contains Glycosylated Capsid Proteins,
949 a Cellular Chromatin Protein, and Host-Derived Lipids. *J. Virol.* **89**, 11681–11691 (2015).

950 87. Brumfield, S. K. *et al.* Particle Assembly and Ultrastructural Features Associated with Replication
951 of the Lytic Archaeal Virus *Sulfolobus* Turreted Icosahedral Virus. *J. Virol.* **83**, 5964–5970
952 (2009).

953 88. Fu, C. & Johnson, J. E. Structure and cell biology of archaeal virus STIV. *Curr. Opin. Virol.* **2**,
954 122–127 (2012).

955 89. Jäälinoja, H. T. *et al.* Structure and host-cell interaction of SH1, a membrane-containing,
956 halophilic euryarchaeal virus. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 8008–8013 (2008).

957 90. Quemin, E. R. J. *et al.* Eukaryotic-like virus budding in archaea. *mBio* **7**, (2016).

958 91. Bautista, M. A., Zhang, C. & Whitaker, R. J. Virus-Induced Dormancy in the Archaeon
959 *Sulfolobus islandicus*. *mBio* **6**, e02565-14 (2015).

960 92. Dyall-Smith, M., Pfeifer, F., Witte, A., Oesterhelt, D. & Pfeiffer, F. Complete Genome Sequence
961 of the Model Halovirus PhiH1 (ΦH1). *Genes* **9**, 493 (2018).

962 93. Ken, R. & Hackett, N. R. Halobacterium halobium strains lysogenic for phage phi H contain a
963 protein resembling coliphage repressors. *J. Bacteriol.* **173**, 955–960 (1991).

964 94. DeWerff, S. J., Bautista, M. A., Pauly, M., Zhang, C. & Whitaker, R. J. Killer Archaea: Virus-
965 Mediated Antagonism to CRISPR-Immune Populations Results in Emergent Virus-Host
966 Mutualism. *mBio* **11**, 10.1128/mbio.00404-20 (2020).

967 95. Gebhard, L. J., Duggin, I. & Erdmann, S. Improving the genetic system for Halorubrum
968 lacusprofundi to allow in-frame deletions. *Front. Microbiol.* **14**, 1095621 (2023).

969 96. Nuttall, S. *et al.* The Halohandbook. *Media* 1–144 (2008).

970 97. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
971 *EMBnet.journal* **17**, 10–12 (2011).

972 98. Antipov, D., Raiko, M., Lapidus, A. & Pevzner, P. A. Plasmid detection and assembly in genomic
973 and metagenomic data sets. *Genome Res.* **29**, 961–968 (2019).

974 99. Camacho, C. *et al.* BLAST+: Architecture and applications. *BMC Bioinformatics* **10**, 1–9 (2009).

975 100. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search
976 tool. *J. Mol. Biol.* **215**, 403–410 (1990).

977 101. Hyatt, D. *et al.* Prodigal: Prokaryotic gene recognition and translation initiation site identification.
978 *BMC Bioinformatics* **11**, 119 (2010).

979 102. Jones, P. *et al.* InterProScan 5: Genome-scale protein function classification. *Bioinformatics* **30**,
980 1236–1240 (2014).

103. Chen, I. M. A. *et al.* The IMG/M data management and analysis system v.6.0: New tools and advanced capabilities. *Nucleic Acids Res.* **49**, D751–D763 (2021).
104. Gabler, F. *et al.* Protein Sequence Analysis Using the MPI Bioinformatics Toolkit. *Curr. Protoc. Bioinforma.* **72**, e108 (2020).
105. Mirdita, M. *et al.* Uniclust databases of clustered and deeply annotated protein sequences and alignments. *Nucleic Acids Res.* **45**, D170–D176 (2017).
106. Kelley, D. R., Liu, B., Delcher, A. L., Pop, M. & Salzberg, S. L. Gene prediction with Glimmer for metagenomic sequences augmented by classification and clustering. *Nucleic Acids Res.* **40**, (2012).
107. Sullivan, M. J., Petty, N. K. & Beatson, S. A. Easyfig: A genome comparison visualizer. *Bioinformatics* **27**, 1009–1010 (2011).
108. Bankevich, A. *et al.* SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **19**, 455–477 (2012).
109. Bin Jang, H. *et al.* Taxonomic assignment of uncultivated prokaryotic virus genomes is enabled by gene-sharing networks. *Nat. Biotechnol.* **37**, 632–639 (2019).
110. Camargo, A. P. *et al.* IMG/PR: a database of plasmids from genomes and metagenomes with rich annotations and metadata. *Nucleic Acids Res.* **52**, D164–D173 (2024).
111. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verl. N. Y.* (2009).
112. Butts, C. T. network : A Package for Managing Relational Data in R. **24**, (2008).
113. Meier-Kolthoff, J. P. & Göker, M. VICTOR: genome-based phylogeny and classification of prokaryotic viruses. *Bioinforma. Oxf. Engl.* **33**, 3396–3404 (2017).
114. Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856–2860 (2006).
115. Kleiner, M. *et al.* Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use. *Proc. Natl. Acad. Sci.* **109**, E1173–E1182 (2012).
116. Gebhard, L. J., Vershinin, Z., Alarcón-Schumacher, T., Eichler, J. & Erdmann, S. Influence of N-Glycosylation on Virus–Host Interactions in Halorubrum lacusprofundi. *Viruses* **15**, 1469 (2023).

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