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TPV1, the first virus isolated from the hyperthermophilic genus *Thermococcus*

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Summary

We describe a novel virus, TPV1 (*Thermococcus prieurii* virus 1), which was discovered in a hyperthermophilic euryarchaeote isolated from a deep-sea hydrothermal chimney sample collected at a depth of 2700 m at the East Pacific Rise. TPV1 is the first virus isolated and characterized from the hyperthermophilic euryarchaeal genus *Thermococcus*. TPV1 particles have a lemon-shaped morphology (140 nm × 80 nm) similar to the structures previously reported for *Fuselloviruses* and for the unclassified virus-like particle PAV1 (*Pyrococcus abyssi* virus 1). The infection with TPV1 does not cause host lysis and viral replication can be induced by UV irradiation. TPV1 contains a double-stranded circular DNA of 21.5 kb, which is also present in high copy number in a free form in the host cell. The TPV1 genome encompasses 28 predicted genes; the protein sequences encoded in 16 of these genes show no significant similarity to proteins in public databases. Proteins predicted to be involved in genome replication were identified as well as transcriptional regulators. TPV1 encodes also a predicted integrase of the tyrosine recombinase family. The only two genes that are homologous between TPV1 and PAV1 are TPV1-22 and TPV1-23, which encode proteins containing a concanavalin A-like lectin/glucanase domain that might be involved in virus–host recognition.

Introduction

Since the discovery of *Archaea*, the third domain of life, by Woese and Fox (1977), extensive studies have been carried out on their extra-chromosomal genetic elements (viruses and plasmids). To date about 50 archaeal viruses have been described in detail. Archaeal viruses have been detected in such extreme habitats as hypersaline environments (Porter *et al*., 2007), hot and acidic springs (Rachel *et al*., 2002), and hydrothermal deep-sea vents (Geslin *et al*., 2003a,b). The majority of archaeal viruses was isolated from hot terrestrial and coastal environments and infects members of the phylum *Crenarchaeota* (one

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of the two major archaeal phyla), in particular the genera *Sulfolobus*, *Thermoproteus*, *Acidianus, Pyrobaculum, Stygiolobus* and *Aeropyrum*. These viruses show unexpected diversity at the morphological and genomic levels and have been classified into eight new viral families (reviewed by Prangishvili *et al.*, 2006; Mochizuki *et al.*, 2010).

The second major archaeal phylum, *Euryarchaeota*, includes methanogens, halophiles, thermophilic and extremely acidophilic microorganisms of the order *Thermoplasmatales* and hyperthermophilic microorganisms of the orders *Thermococcales* and *Archaeoglobales* and show much greater physiological and metabolical diversity than *Crenarchaeota*. Conversely, viruses described to date in *Euryarchaeota* are much less diverse than viruses of *Crenarchaeota*. Most of the isolated euryarchaeal viruses have been isolated from mesophilic organisms and display morphologies similar to those of head-and-tail bacteriophages. The few viruses isolated from methanogens such as 'ΨM1-like viruses' belong to the *Siphoviridae* family (Pfister *et al.*, 1998). Haloarchaeal viruses such as phiCH1, phiH, HF2, BJ1, belong to the *Myoviridae* or *Siphoviridae* families (Schnabel *et al.*, 1982; Nuttall and Dyall-Smith, 1993; Witte *et al.*, 1997; Pagaling *et al.*, 2007), except for His 1 and His 2, which infect *Haloarcula hispanica* and are classified in a novel virus group the *Salterprovirus* (Bath *et al.*, 2006). His 1 and His 2 are lytic lemon-shaped haloviruses with linear dsDNA genomes of 14.4 kb and 16 kb respectively. Another exception is HRPV-1, a non-lytic pleomorphic halovirus, which is the first characterized archaeal virus with a single-stranded DNA genome (Pietilä *et al.*, 2009).

No virus has been so far isolated from thermophilic or hyperthermophilic euryarchaea of the orders *Thermoplasmatales* and *Archaeoglobales*, and only one virus-like particle (VLP) has been described from the order *Thermococcales* (Geslin *et al.*, 2003a). *Thermococcales* is one of the predominant groups of hyperthermophilic microorganisms isolated from deep-sea hydrothermal vents (Prieur *et al.*, 2006). These ecosystems represent one of the most extreme environments on Earth. They are characterized by strong physicochemical gradients such as temperature from 2°C to more than 350°C and high hydrostatic pressure, lack of solar energy and prevalence of chemosynthesis. The order *Thermococcales* is represented by three genera: *Thermococcus*, *Pyrococcus* and *Paleococcus* (Stetter, 1986; Achenbach-Richter, 1988; Takai *et al.*, 2000), and includes obligate anaerobic, sulfur-metabolizing hyperthermophiles.

The widespread Thermococcales have become popular model organisms for studies on microbial adaptation to extreme temperature and ionizing radiation, DNA replication mechanisms, metabolism, phylogeny and genome evolution (Prieur *et al.*, 2004; Leigh *et al.*, 2011; Soler *et al.*, 2011). Accordingly, intensive effort has been made to sequence diverse genomes of members of this order, resulting in the availability of seven genomes of *Thermococcus* (*T. kodakaraensis, T. gammatolerans, T. onnurineus, T. sibiricus, T. barophilus, Thermococcus* sp. AM4, *Thermococcus* sp. 4557) and five genomes of *Pyrococcus* (*P. horikoshii*, *P. furiosus*, *P. abyssi, P. yayanosii, Pyrococcus* sp. NA2).

The availability of all these data makes *Thermococcales* a good model to explore the hyperthermophilic euryarchaeal virome that currently remains largely uncharacterized. Several proviruses have been detected in genomes of *Thermococcales* (TKV1 to TKV4 in *T.*

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kodakaraensis (Fukui *et al*., 2005) (TGV1 and TGV2 in *T. gammatolerans*) (Zivanovic *et al*., 2009) (PHV1 in *P. horikoshii* genome) (Soler *et al*., 2010). To investigate the diversity of VLPs from deep-sea vents, a systematic search was carried out on samples collected in various geographically distant hydrothermal sites located on the East Pacific Rise (EPR 9° N and 13° N) and Mid-Atlantic Ridge (MAR 36° N and 37° N). This study revealed striking similarity between VLP morphologies in deep-sea and terrestrial hot environments (Geslin *et al*., 2003b).

All this effort notwithstanding PAV1, isolated from *P. abyssi* (Geslin *et al*., 2003a), is the only VLP described in *Thermococcales*. The PAV1 virion is lemon-shaped (120 nm × 80 nm) and is morphologically similar to the members (SSV1-7, SSVk1, SSVrh, ASV1) of the *Fuselloviridae* family (Palm *et al*., 1991; Schleper *et al*., 1992; Stedman *et al*., 2003; Wiedenheft *et al*., 2004; Peng, 2008; Redder *et al*., 2009) and *Salterprovirus* genus (His1 and His2) (Bath *et al*., 2006). The viral particle persists in the host strain in a stable carrier state. The genome of PAV1 consists of a double-stranded circular DNA of 18 kb and is also present at a high copy number in a free form in the host cell. The genome of PAV1 displays unique genetic features and does not show affinity to any known virus family.

Isolation of new viruses from *Thermococcales* is the first step required to characterize viral diversity in deep-sea hydrothermal environments and eventually the role of viruses in these extreme ecosystems.

Here we report the characterization of a virus, *Thermococcus prieurii* virus 1 (TPV1), from a new species of *Thermococcus* isolated from a hydrothermal deep-sea vent. To date TPV1 and PAV1 are the only viruses isolated from described marine hyperthermophilic archaea (Euryarchaeota). TPV1 is the first virus isolated and described from the hyperthermophilic *Thermococcus* genus. We show that TPV1 is UV inducible and causes a growth retardation of species of *Thermococcus*, in contrast to PAV1 for which infectivity has not been shown. These properties make TPV1 a promising element to study host–virus interactions in *Thermococcales*. We further present the results of analysis of the complete sequence of TPV1 genome (21.5 kb), which shows very limited similarity to PAV1 indicating that TPV1 might become a founding member of a new virus family.

Results

Viral morphology

Particles of TPV1 were lemon-shaped, about 140 nm long and 80 nm wide, with a 15 nm – tail terminated by caudal fibres (Fig. 1A), which are likely to participate in virus adsorption on the host cells. Clusters of virus particles forming rosette-like aggregates were also observed (Fig. 1B).

In $CsCl_2$ solution, TPV1 had a density of 1.35 g ml^{-1} and in iodixanol solution, TPV1 had a density of 1.20 g ml^{-1}. TPV1 particles seemed not to be sensitive to purification procedures, e.g. high-speed centrifugation or suspension in iodixanol or $CsCl_2$ solutions, indicating that they were osmotically resistant. Infrequently virus particles appeared to be flexible and a few elongated forms were observed indicating plasticity of the particle shape (Fig. 1C).

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The structural stability of TPV1 was assessed by exposing purified virions to organic solvents, detergents and proteolytic degradation. The viral particles appear to be sensitive to chloroform (25% wt/vol) because only spherical particles were observed in treated samples (Fig. 1D). The particles were also disrupted by treatment with 0.3% Triton X-100 (data not shown) as well as by incubation with 0.1% sodium dodecyl sulfate (SDS) (data not shown). Treatment with proteinase K (1 mg ml^{-1}) resulted in complete destruction of virions (Fig. 1E). These preliminary results suggest that TPV1 is covered by an envelope composed of proteins and lipids. The purified virions were tolerant to all temperatures (4°C–85°C) and pH (4–11) tested (Fig. 1F). Thus, TPV1 seems to be psychro/thermotolerant and stable under acidic and basic conditions.

Virus–host relationship

Viruses appeared to be released continuously and spontaneously in the extracellular medium throughout the growth of *T. prieurii* (Fig. 2). Due to the lack of a plaque assay for *Thermococcales*, quantification of TPV1 was performed by epifluorescence microscopy. The viral count reached its maximum after 18 h of incubation (2×10^8 virus particles ml^{-1}). During growth of the infected host, there was neither a decrease of cellular density nor formation of cell debris. This observation indicated that viral production was not accompanied by lysis of the host cells. Attempts were made to increase the viral production by induction using different chemical or physical treatments and physiological stresses. Induction was never observed under any stress condition except for UV. UV irradiation of growing host cells induced the production and release of TPV1. The viral production began almost immediately after UV irradiation, followed by an increase of the viral titre, which reached about 10^{11} virus particles ml^{-1} 15 h after induction. UV exposure for 1 h was required to obtain the higher production of TPV1. A significant decrease in host cell counts and concomitant increase in cell debris were observed after 9 h incubation post irradiation (Fig. 2). Despite the presence of cell debris, no extensive lysis of the host was detected suggesting that TPV1 is a temperate virus.

Figure S1 shows many viruses protruding through the envelope, obviously during extrusion. Protrusions of particles through the cell envelope were an indication of viral production and were never observed in unirradiated cultures (Fig. S1).

Host range

The host range of TPV1 was tested in liquid medium by adding iodixanol purified viruses to growing cultures of potential hosts: *Thermococcus siculi*, *T. barophilus*, *T. gorgonarius*, *T. kodakaraensis, T. celer*, *T. pacificus* and *T. fumicolans*. TPV1 production in infected cultures was monitored by epifluorescence microscopy. Of the potential host strains tested, TPV1 was able to cause, in liquid medium, a substantial delay of *T. barophilus*, *T. celer, T. kodakaraensis* and *T. gorgonarius* growth during approximately 20 h after infection (Figs 3 and S2).

To confirm that the viral production occurred inside the infected host, aliquots of *T. barophilus* infected with TPV1 were collected after 1 h and 12 h of incubation. Total DNA was extracted, and using specific primers, a viral DNA fragment of 600 bp was amplified by

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PCR from the DNA extracted after 12 h of incubation but not after 1 h of incubation (Fig. S3).

Viral genome

Three extra-chromosomal elements were isolated from *Thermococcus prieurii* by the alkaline lysis method (Fig. S4A) (A. Gorlas and C. Geslin, unpublished). We suspected that one of these elements could be the free form of the TPV1 genome. To test this hypothesis, the viral genome was extracted from purified particles and was compared with cccDNAs (covalently closed circular DNA) isolated from the host strain (Fig. S4B). The same viral band was found in both cases. Samples of viral DNA extracted from the virions were heated for 10 min at 70°C and chilled on ice before loading on gels to determine if the viral DNA was in fact not circular in the virions, but rather linear with cohesive ends that cause circularization. No change in the band pattern was observed (Fig. S4C). Nucleic acid extracted from purified virus particles was digested by a number of type II restriction enzymes and was not sensitive to RNase. We conclude that the viral genome is a double-stranded, circular DNA that is not affected by Dcm or Dam-like methylation. The genome size was estimated at 21.5 kb (Fig. S4D).

The copy number of the episomal form of the viral genome in the host was evaluated by real-time PCR. A viral fragment of 160 bp and 160 bp of the 16S rDNA of the host were amplified by qPCR. There was a linear correlation between the threshold cycle (ct) and the initial quantity of DNA. The standard curves (for viral and chromosomal fragments) showed slopes of −3.27 and −3.13, respectively, indicating high effectiveness of PCR. The assays for viral and host DNA showed a strong correlation (R^2 0.98). The result indicated an intracellular copy number of TPV1 genome of approximately 20 copies per host chromosome (Figs S5 and S11).

The circular, dsDNA genome of TPV1 was totally sequenced using a combination of overlapping clone library and primer walking.

Genome analysis of TPV1

The complete nucleotide sequence of the TPV1 genome was determined on both strands as outlined in *Experimental procedures* with a minimum threefold coverage. The genome size of TPV1 determined by sequencing is 21 592 bp. Sequence analysis in the six possible frames resulted in the prediction of 28 protein-coding genes each of which encoded a putative protein product of at least 62 amino acids; collectively, these predicted genes covered 90.6% of the genome sequence (Fig. 4). The positions and main features of the predicted genes are listed in Table 1. The majority of the putative genes (24) have the same orientation and four are present on the complementary strand. The overall G + C content of the TPV1 genome (49.9%) is comparable with the overall G + C content of the host genome (53.6%) (e.g. A. Gorlas, unpublished). All predicted coding regions were preceded by putative ribosome-binding sites (see Table S1 for details). The predominant start codon is ATG (85.5 %) and three ORFs start from GTG.

In an attempt to identify the origin of replication, a cumulative GC skew analysis was performed (Fig. S6). However, the results of this analysis failed to clearly pinpoint the

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origin. One inflection point, between positions 19 160 and 21 080 bp, was evident, indicating a change in the base composition bias. A region containing AT-rich repeats was detected at positions 19 770 to 19 850 bp, and the DNA sequence between positions 20 675 and 21 592 bp contained the largest intergenic region, 917 bp. Furthermore, helical stability analysis (Huang and Kowalski, 2003) indicates that positions 21 241 bp to 21 341 bp correspond to the lowest helical stability region, suggesting that this region contains a DNA unwinding element (DUE).

To identify homologous relationships and predict functions of the putative proteins of TPV1, their amino acid sequences were compared with those in the public sequence databases using the PSI-BLAST and HHPred methods (Table 1). For more than half of the predicted proteins (16 out of 28), no significant similarity with any sequences in the public databases was detected. However, six of these 'ORFans' encode predicted membrane proteins (Table 1). Notably, genes encoding the predicted integral membrane proteins form a cluster in the middle of the TPV1 genome suggesting that these proteins are coexpressed and might form distinct, membrane-spanning complexes in TPV1 virions.

The TPV1-2 gene encodes a predicted helicase of the minichromosome maintenance (MCM) family within the AAA+ superfamily of P-loop ATPases (Koonin, 1993; Iyer *et al.*, 2004). The closest homologue of this TPV1 protein was found in the provirus TKV4 (TK1351), with 35% identity (Krupovic and Bamford, 2008). The MCM protein of TPV1 possesses all the conserved motifs that are known to be required for the ATPase and helicase activities (Walters and Chong, 2010) (Fig. S7). Phylogenetic tree analysis placed the TPV1 MCM protein into a strongly supported group of proviral and archaeal proteins from *Thermococcales* (Fig. S8). The MCM proteins are so-called licensing factors that are involved in the initiation of DNA replication in all archaea and eukaryotes (Sakakibara *et al.*, 2009), and a similar role in viral genome replication can be inferred for TPV1-2.

TPV1-20 encodes another predicted AAA+ superfamily ATPase (Iyer *et al.*, 2004) that has no closely related homologues but, in terms of size and spacing of conserved motifs implicated in ATPase activity resembles, ATPases encoded by several viruses (e.g. SSV1) and plasmids of hyperthermophilic *Crenarchaeota* (Table 1 and Fig. S10). These proteins are in some cases described as DnaA-like but their similarity to DnaA, the protein involved in DNA replication initiation in all bacteria, is remote.

TPV1-6 encodes a predicted archaeal-type Holliday junction resolvase (Aravind *et al.*, 2000), with the closest homologue is encoded in the TGV1 provirus integrated in the genome of *T. gammatolerans* (Zivanovic *et al.*, 2009). TPV1-28 encodes another predicted nuclease that belongs to the RecB family (Aravind *et al.*, 1999), with the closest homologue present in the genome of *Pyrococcus* sp. NA2 (Lee *et al.*, 2011).

TPV1-10 encodes an integrase (SSV-type) that belongs to the tyrosine recombinase family. The closest homologue of this protein is present in the TKV2 provirus (Fukui *et al.*, 2005), and more distant homologues are found in numerous archaeal viruses and integrated virus-like elements. The multiple alignment of SSV-type integrase sequences and TPV1-10 shows

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the conservation of the tyrosine recombinase superfamily catalytic site motif R … **K**XXR … Y (She *et al.*, 2004) (Fig. S9).

Two genes of TPV1 encode predicted helix–turn–helix transcription regulators, one of the IclR family (TPV1-8) and the other of the SpoVT/AbrB family (TPV1-28). Highly conserved homologues of both these proteins are present in the TKV3 genome (Table 1). SpoVT/AbrB-like transcriptional regulators have also been detected in the genomes of two crenarchaeal viruses, STSV1 and SIFV (Prangishvili *et al.*, 2006; Krupovic and Bamford, 2008), and in the pT26-2 plasmid from *Thermococcus* sp. (Soler *et al.*, 2010). These transcriptional regulators seem to represent a distinct family of SpoVT/AbrB-like protein that is specific to archeoviruses.

On the complementary strand, TPV1-27 encodes a C2H2-type Zn-finger protein that is highly similar to small proteins from *Pyrococcus* sp. and two *Archaeoglobus* species. The phylogenetic spread of this protein family is unusual in that, except for TPV1 and three archaeal species, highly conserved homologues are present only in eukaryotes. This protein potentially could be an additional regulator of TPV1 gene expression.

The domain architectures of the predicted proteins encoded by tandem genes TPV1-22 and TPV1-23 are similar and include, respectively, two and three predicted transmembrane segments, in the C-terminal portion and a concanavalin A like lectin/glucanase (LamGL) domain in the N-terminal portion. These predicted proteins are homologous to two proteins (ORF676 and ORF678) of PAV1 (Geslin *et al.*, 2007). Similarities with LamGL domain-containing protein encoded in the genomes of crenarchaeal lipothrixviruses (AFV3, AFV6 and AFV8) were also detected. Many concanavalin A-like domains are contained in proteins involved in cell recognition and adhesion (Crennell *et al.*, 1994; Tisi *et al.*, 2000).

Discussion

Approximately 40 archaeal species of the order *Thermococcales* have been described (Prieur *et al.*, 2006) but despite the prominence of this euryarchaeal order in marine thermophilic ecosystems, little is known about their viruses. It is unclear whether this lack of described viruses is due to the low abundance of viruses in *Thermococcales* or insufficient screening, or both. An unexpected morphological diversity of VLPs was observed in high temperature enrichment cultures from deep-sea hydrothermal samples where *Thermococcales* are well represented. Among the viral morphotypes observed, the lemon-shaped type prevailed (Geslin *et al.*, 2003b). Thus, it seems likely that viruses are as common in *Thermococcales* as they are in hyperthermophilic Crenarchaeota, and the main reason for the current lack of isolated viral forms could be insufficient experimental effort. A major goal of future research will be to combine omics and cultural approaches mimicking as close as possible environmental conditions (i.e. a multiplicity of physical and chemical gradients) to explore the virome in deep-sea hydrothermal environments.

Only one viral particle, PAV1 (*Pyrococcus abyssi* virus 1), has been described from hyperthermophilic *Euryarchaeota* (Geslin *et al.*, 2003a; 2007). Here, we discuss the characterization of the second lemon-shaped virus (TPV1: *Thermococcus prieurii* virus 1)

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discovered in a hyperthermophilic *Euryarchaeota* isolated from a deep-sea hydrothermal vent.

TPV1, which is not yet classified, shows morphological similarities with *Fuselloviruses* (Peng, 2008), His1/His2 of the *Salterprovirus* group (Bath *et al.*, 2006) and to the unclassified PAV1 (Geslin *et al.*, 2003a). This lemon morphology is commonly observed among archaeal viruses isolated from the environments inhabited by hyperthermophilic, acidophilic and halophilic archaea (Zillig *et al.*, 1994; Oren *et al.*, 1997; Rice *et al.*, 2001; Rachel *et al.*, 2002). With regard to the taxonomic status of the viruses of *Thermococcales*, TPV1 was found to infect a *Thermococcus* strain and PAV1 a *Pyrococcus* strain; these strains were isolated from two distinct hydrothermal deep-sea vents, respectively, in the North East-Pacific Rise and in the North Fiji Basin. The two euryarchaeal viruses, PAV1 and TPV1, have similar lemon-shaped morphologies but share only two homologous genes detectable at the sequence level. The lectin domain-containing proteins encoded by these paralogous genes are exposed on the surface of the enveloped virus and are likely to be involved in host cell recognition. These two viruses might become prototypes for two novel viral families or possibly two genera within the same family.

TPV1 causes growth retardation of several species of *Thermococcus* like *T. barophilus*, *T. celer*, *T. gorgonarius* and *T. kodakaraensis* isolated respectively from a hydrothermal vent site on the Mid-Atlantic Ridge (Marteinsson *et al.*, 1999), from solfataric marine water in Italy (Zillig *et al.*, 1983), from a New Zealand submarine hot vent (Miroshnichenko *et al.*, 1998) and from a solfatara in Japan. The fact that TPV1 presents a wide host range with a broad geographic distribution and delays the growth of *T. kodakaraensis*, which is a hyperthermophilic model organism for genetic studies, makes TPV1 a promising tool – to study virus–host interactions in *Thermococcales*.

TPV1 is released during all phases of host growth. Virions extrude from cells without causing host lysis. In contrast to PAV1, TPV1 viral production can be induced with UV irradiation, and this production is accompanied by a decrease in cell density. In UV-induced cultures, viral titre peaked at > 10^{11} particles ml^{-1}. It is notable that TPV1 is UV inducible whereas PAV1 is not although the implications of this difference for the biology of virus–host interactions remain unclear. It will be important to address this question in follow-up studies, and one approach would involve characterization of the transcription cycle of the UV inducible TPV1 by Northern analysis. Such analysis has been performed for SSV1, and it has been shown that SSV1 exhibits temporal regulation of transcription upon UV irradiation and that the cycle starts with a small UV-specific transcript (Fröls *et al.*, 2007).

Little is known about the modes of entry or release of archaeal viruses (Bize *et al.*, 2009). Attempts to analyse the processes of TPV1 adsorption and extrusion by visualization of thin sections by electron microscopy have, thus far, been unsuccessful. Like many archaea, *T. prieurii* has only a thin, glycoprotein surface layer (S-layer) protecting the cell membrane, which means that release of virions does not necessarily require breakdown of the cell wall and subsequent cell lysis. TPV1 seems to extrude without cells disruption, so it should have a release mechanism similar to that of *Fuselloviruses*. Indeed, similarly to PAV1 or *Fuselloviruses*, TPV1 appears to be a non-lytic virus. The host cells produce virions

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constitutively, consistent with an equilibrium existing between viral replication and host cell multiplication. Moreover, these viruses persist in cells in a stable state and are not lost during continuous growth of infected cell cultures (Prangishvili and Garrett, 2005).

In contrast to PAV1, TPV1 is likely to integrate into the host chromosome. Viral integration is a process widely shared in viruses present in the three domains of life. In the extreme environments, proviruses could contribute to the fitness of the host strains whereas for the virus, the integrated state represents a means to avoid the harsh conditions of these ecosystems (Williamson *et al.*, 2008). The genome of TPV1 contains a gene encoding a tyrosine integrase homologous to the integrases of *Fuselloviruses* and putative proviruses (TKV, TGV, PHV) as well as the plasmid pT26-2 isolated from *Thermococcales* (Fukui *et al.*, 2005; Zivanovic *et al.*, 2009; Soler *et al.*, 2010). A characteristic feature of the SSV-type integrase is that the gene is partitioned into longer Int (C) and shorter Int (N) fragments after integration in the host chromosome (She *et al.*, 2004). Integration of *Fuselloviruses* occurs within specific tRNA genes (for example: $tRNA^{Arg}$ for SSV1, $tRNA^{gly}$ for SSV2 and SSV7, $tRNA^{glu}$ for SSV4) (Redder *et al.*, 2009). In the case of TPV1, additional analyses remain to be done in order to identify potential viral integration into the host chromosome.

All archaeal viruses described so far possess dsDNA genomes, with the sole exception of HRPV-1, a recently isolated haloarchaeal virus with a ssDNA genome (Pietilä *et al.*, 2009). The 21.5 kb dsDNA genome of TPV1 is packaged into viral particles and an episomal form is present in the host cells. The genome of TPV1 is circular like the genomes of PAV1 and HRPV-1. All other known euryarchaeal viruses have a linear genome (Dyall-Smith *et al.*, 2003; Porter *et al.*, 2005).

Functions and/or biochemical activities were assigned to approximately half of the predicted TPV1 proteins on the basis of homology to functionally characterized proteins, conservation of functional motifs and prediction of structural features. A substantial fraction of TPV1 genes (eight out of 28) encodes predicted membrane proteins that most likely are components of the viral envelope. In contrast, the identity of the major capsid protein of TPV1 remains unknown; detailed searches indicate that TPV1 does not encode a homologue of the icosahedral capsid protein found in TKV4 (Krupovic and Bamford, 2008). The TPV1 genome encodes several proteins implicated in genome replication, such as a minichromosome maintenance protein (TPV1-2). MCM proteins are DNA-dependent ATPases involved in the initiation of archaeal and eukaryal DNA replication (Sakakibara *et al.*, 2009; Krupovic *et al.*, 2010). MCM proteins have been identified in one *Bacillus* phage genome (Krupovic and Bamford, 2008), in one halovirus BJ1 (Pagaling *et al.*, 2007) and in the provirus TKV4 (Krupovic and Bamford, 2008). Phylogenetic analysis clearly shows the common origin of the MCM proteins of TPV1 and TKV4. The TPV1-20 encodes a putative AAA+ superfamily ATPase that has no highly conserved homologues, but in size and location of conserved motifs, resembles predicted ATPases encoded by many crenarchaeal viruses. Thus, TPV1 encodes an unusual combination of two ATPases that are both likely to be involved in genome replication initiation. Involvement in genome replication and/or recombination and repair is predicted also for the Holliday junction resolvase (TPV1-6) and the RecB-family nuclease (TPV1-26) and encoded in the TPV1 genome.

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Genomic alignment shows that TPV1 shares more homologous genes with autonomous genetic elements (proviruses and plasmids) from *Thermococcus* species than with PAV1, the only other known virus for hyperthermophilic euryarchaea (Fig. 5). On the whole, the genomes of all these elements show a patchy pattern of gene sharing, resembling that revealed by genomic comparison of Crenarchaeal viruses (Prangishvili *et al.*, 2006). The SSV-type integrase that is missing only in PAV1 seems to be a signature of viruses and virus-like elements of hyperthermophilic euryarchaea and could be used as a marker to explore the viral diversity in the *Thermococcales* order. Moreover, the frequent occurrence of homologous resolvases and transcription regulators is notable as well (Fig. 5).

Although horizontal gene transfer occurs in *Thermococcales* (Soler *et al.*, 2011), the mechanisms of gene disper sion and its frequency remain unknown. Nevertheless, it is evident that the identified genetic elements including viruses, proviruses and plasmids share a common pool of genes and probably play an important role in the evolution of *Thermococcales*.

Experimental procedures

Thermococcales strains

Thermococcus celer JCM (Japan Collection of Microorganisms) 8558^T, *T. siculi* DSM (Deutsche Sammlung von Mikroorganismen) 12349^T, *T. barophilus* DSM 11836^T, *T. kodakaraensis* JCM 12380^T, *Thermococcus gorgonarius* DSM 10395^T, *Thermococcus pacificus* DSM 10394^T, *Thermococcus fumicolans* DSM 12820^T were used as reference strains and provided by the Brittany culture collection.

The new species *T. prieurii* strain Bio-pl-0405IT2 (JCM16307^T = LMM3069) is the host of the virus TPV1, and carries also two other extra-chromosomal elements in a free form in the cytoplasm. *Thermococcus prieurii* was isolated from a hydrothermal chimney sample collected from the East Pacific Rise at 2700 m depth on the ‘Sarah Spring’ area (7°25′24 S, 107°47′66 W).

Culture conditions

Thermococcales strains were cultured in Ravot medium as previously described (Geslin *et al.*, 2003a) with minor modifications as described in Supporting information.

Isolation of TPV1

A total of 150 novel hyperthermophilic and anaerobic microorganisms isolated from deep-sea hydrothermal vents in the East Pacific Rise were screened for genetic elements.

From these 150 isolates, cccDNA were extracted by the alkaline lysis method allowing us to detect presence of extra- chromosomal DNA, in free form in the host cytoplasm. From positive samples, epifluorescence microscopy was performed and isolates scored positive were checked for the presence of virus particles by transmission electron microscopy. One of them, the new species *T. prieurii* was discovered to be the host of TPV1.

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Purification of TPV1

The viral suspension was purified by ultracentrifugation in a linear Iodixanol gradient [OptiPrep, 30–45% diluted in a buffer (10 mM Tris-HCL, 100 mM NaCl, 5 mM $CaCl_2$)] at 180 000 *g* for 6 h (Beckman Optima LE-80 K 70.1Ti rotor). Following ultracentrifugation, the opaque virus band was recovered and stored at 4°C until use.

TPV1 particles were also purified by centrifugation in a CsCl buoyant density gradient (Beckman Optima LE-80 K 70.1Ti rotor) at 180 000 *g* for 6 h. Fractions containing the nucleic acids were detected at 254 nm and collected using a density gradient fractionator (model 185, ISCO). Fractions were then dialysed by using nitrocellulose filters (MF Membrane Filter, 0.025 μm, Millipore) placed above a large volume of buffer given above. Viral solutions dialysed were recovered on the surface of the filter.

Virus counts by epifluoresence microscopy

The virus counts were determined by epifluorescence microscopy according to Noble and Fuhrman (Noble and Fuhrman, 1998) and as described in supporting information. At 1000 × magnification 20–100 positive particles were counted in each of 20 random fields. The average number of viruses per millitre was calculated according to Suttle (Suttle and Fuhrman, 2010).

Transmission electronic microscopy

A droplet of a viral solution dialysed and resuspended in a buffer (10 mM Tris-HCL, 100 mM NaCl, 5 mM $CaCl_2$), was adsorbed onto a carbon-coated copper grid for 2 min. After removed the excess of liquid, the sample was negatively stained with 2% uranyl acetate for 45 s, as described by Geslin and colleagues (2003a). Specimens were examined using a JEOL electron microscope, JEM 100 CX II.

Virus sensitivity and stability

All assays were performed with viral suspensions purified by ultracentrifugation in CsCl, dialysed and resuspended in the buffer containing 10 mM Tris-HCL, 100 mM NaCl, 5 mM $CaCl_2$.

A suspension of purified TPV1 was exposed to chloroform (25 % wt/vol) for 1, 2 and 5 min at room temperature with constant agitation. TPV1 was also exposed to 0.3% (wt/vol) Triton X-100 for 1 and 3 min at room temperature, and to 0.1 % (wt/vol) SDS for 3 min at 50°C. TPV1 was also incubated with proteinase K (1 mg ml^{-1}) for 1 h at 56°C in a reaction buffer containing 10 mM Tris (pH 7.8), 5 mM EDTA and 0.5% SDS.

Finally purified viruses were incubated at different temperatures (4°C, 20°C, 37°C, 50°C, 75°C and 85°C for 1 and 15 h in the buffer used to resuspend TPV1) and different pH (4 and 11.5 for 1 h and 15 h) in acid or basic buffer. To determine viral stability, all samples obtained from the different conditions tested were examined under a transmission electron microscope (JEM 100 CX II).

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Induction assays

Attempts were made to increase the viral production by induction using different chemical or physical treatments that damage DNA (antibiotics, UV) and exposure of the *T. prieurii* host cells to physiological stresses (aerobiosis, hydrostatic pressure, sulfur depletion and freezing). In this part only the UV induction is described, informations about the other treatments are given in the Supporting information.

All UV experiments were carried out in an anaerobic chamber under 93% N_2 – 7% H_2 atmosphere and in semidarkness to avoid possible photoreactivation. A culture in a mid-exponential-phase (10^8 cells ml^{-1}), was transferred in glass Petri dishes by paying attention to avoid the presence of sulfur. UV irradiation (254 nm) was performed with an ultraviolet lamp (typical small short wave UV light, model UVGL-25, UVP, Upland, CA, emitting essentially monochromatic 254-nm UV at a maximal intensity less than 750 μW cm^{-2}) at a distance of 3 cm from the cell suspension at different exposure times: 15 min, 30 min, 1 h and 2 h. After irradiation, the cell suspension was placed in sterile vials containing elemental sulfur (1%), and incubated at 85°C. Aliquots (8 ml) were collected 4, 8 and 15 h after UV treatment to determine the optimal UV exposure time. When the optimal condition was established, aliquots (2 ml) were collected every 3 h. A nonirradiated culture, treated identically, was used as control.

Aliquots were collected to determine the microbial growth by direct cell counting by using a phase-contrast microscope and a modified Thoma chamber (depth 10 μm). The viral counts were estimated by epifluorescence microscopy as previously described. All the induction assays were performed in duplicate.

Host range

Despite several attempts, we never obtained a cured *T. prieurii* strain. TPV1 is very stable in its host, as it has never been lost after many transfers in subcultures and was found in all colony clones checked. Reference marine strains belonging to the *Thermococcales* order were screened for extra-chromosomal genetic elements and strains scored negative were used as potential hosts.

Infection tests were done in liquid culture. Potential host cells were cultured in 50 ml of Ravot medium at 85°C. 10 μl of a viral suspension purified by ultracentrifugation in a linear Iodixanol gradient (10^6 viruses) were added 3 h after the inoculation (at 1%) of the host cells and incubations were continued at 85°C. Aliquots were collected every 2 or 3 h until 27 h. The microbial growth and the viral abundance were determinated as previously described. Controls were performed to verify that iodixanol and the other buffers used did not present an inhibitory effect on the host growth.

To confirm that the viral production occurred inside the infected host, aliquots of *T. barophilus* infected with TPV1 were collected after 1 and 12 h of incubation and total DNA were extracted. A PCR using forward (5′-GGC GAT ATT TAC CTC GTC ATC-3′) and reverse (5′-ATG GGC GCA ACA TTC AAC-3′) primers specific to TPV1 was performed. The reaction was performed in a volume of 25 μl containing 50 ng template, 10 mM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 1 × buffer and 1 U polymerase (Taq Core, Qiagen).

The PCR products were purified by PCR QIAquick (Qiagen) kit and deposited on an agarose gel electrophoresis.

Total DNA, cccDNA and viral DNA extraction

Total DNA from *T. prieurii* was prepared as previously described (Charbonnier *et al.*, 1992; Geslin *et al.*, 2003a).

cccDNA (Covalently Closed Circular DNA) was extracted from cells in exponential growth phase, by the alkaline lysis method (Birnboim and Doly, 1979; Geslin *et al.*, 2003a).

Viral DNA was extracted from purified viral fractions. After a treatment of the viral fraction with DNase I (final concentration 50 μg ml^{-1}) and RNase I (final concentration 100 μg ml^{-1}), the viral DNA was extracted by the same procedure than that used to obtain total DNA as previously described.

Determination of the nucleotide sequence

Purified TPV1 DNA was completely digested with HindIII and SmaI respectively and all the fragments obtained were cloned in the corresponding sites of pUC18 to obtain an overlapping clone library of TPV1 genome. Sequencing reactions were carried out with the kit 'BigDye Terminator' (Applied Biosystems) and analysed at the 'Platforme Biogenouest' (Roscoff, France, http://www.sb-roscoff.fr/plateformes-techniques/genomique-sbr.html) on an ABI prismTM 3100 GA. Each insert was sequenced from both ends using the M13 forward and M13 reverse primers. Gaps in the sequence were filled by using specific primers directly for sequencing on library clones (GATC Biotech AG, Konstanz – Germany). The sequences were trimmed and assembled using the SeqMan, Lasergene 8.0 program (DNASTAR, Madison, USA) with both strands completely sequenced and with a threefold coverage.

Sequence analysis and annotation

Glimmer (Delcher *et al.*, 1999) and RBS finder (Suzek *et al.*, 2001) were used to predict protein-coding genes. The amino acid sequence of each predicted protein was searched against the NCBI non-redundant protein database (Altschul *et al.*, 1997), and compared with the database of known protein structures using the HHPred program (Söding *et al.*, 2005). Membrane-spanning region in ORFs were predicted using the TMHMM program (Krogh *et al.*, 2001). Multiple amino acid sequence alignments were constructed using the MUSCLE program (Edgar, 2004). Maximum likelihood phylogenetic trees were constructed using the FastTree program (Price *et al.*, 2010). Cumulative GC skew analysis was performed using GC Skew Tool (http://bioinformatics.upmc.edu/SKEW/ index.html). It was calculated according to the following formula: $\Sigma(G-C)/(G+C)$, using a sliding window of twenty nucleotides.

Analysis of the Helical stability is computed using the nearest-neighbour-thermodynamics algorithm (Huang and Kowalski, 2003) using the web-based program WEB-THERMODYN (http://wings.buffalo.edu/gsa/dna/dk/WEBTHERMODYN/).

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Nucleotide sequence accession number

The sequence data described here have been deposited in GenBank under Accession No. JQ010983.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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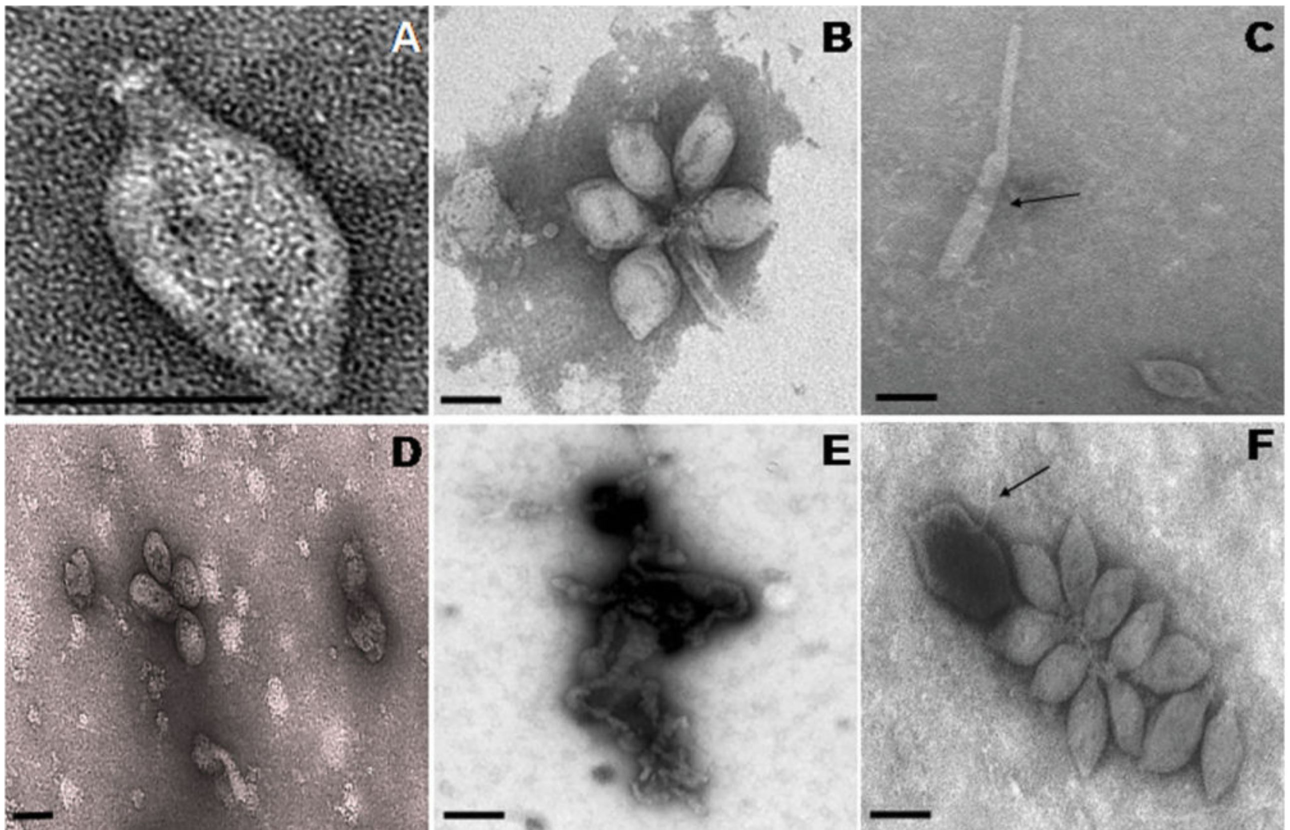

Fig. 1.

A. Electron micrographs of TPV1 negatively stained with 2% uranyl acetate. Lemon shaped particle with a tail terminated by fibres.

B. Clusters of virus particles forming rosette-like aggregates.

C. Elongated virus particles (arrow).

D. Effect of exposure to chloroform on virus particle structure.

E. Disrupted virions caused by exposure to proteinase K.

F. Effect of acidic pH on virions structure (arrow). Scale bars represent 120 nm.

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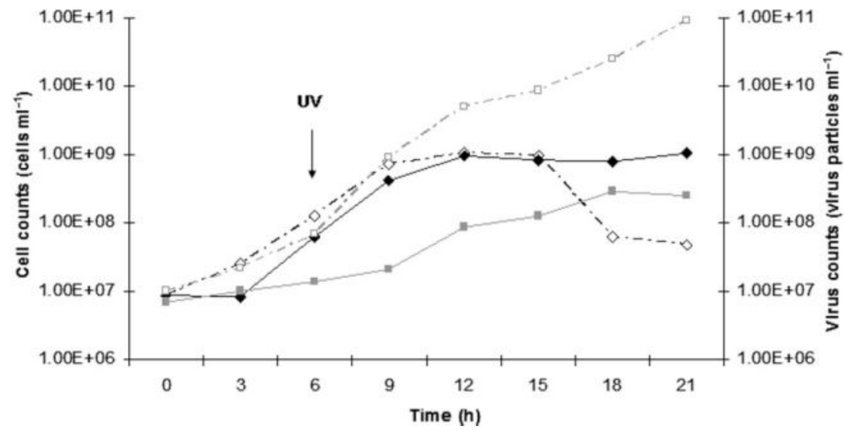

Fig. 2.

Typical growth curve (black diamonds) of the host and production of TPV1 (grey squares). UV induction. Cultures were grown and irradiated for 1 h (white diamonds) and the virus counts were estimated by epifluorescence microscopy (white squares).

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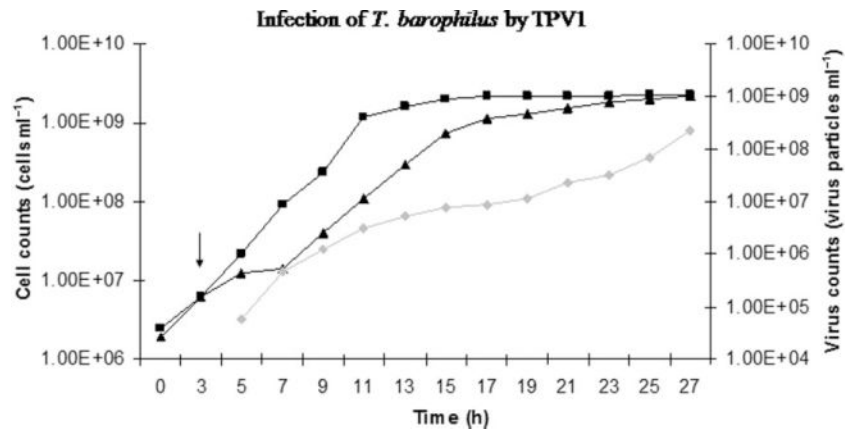

Fig. 3.

Infection of *T. barophilus* by TPV1. Squares: cells of *T. barophilus* non-infected. Triangles: cells of *T. barophilus* infected with TPV1. Diamonds: free TPV1 virions. The arrow indicates the addition of TPV1 virions in the culture.

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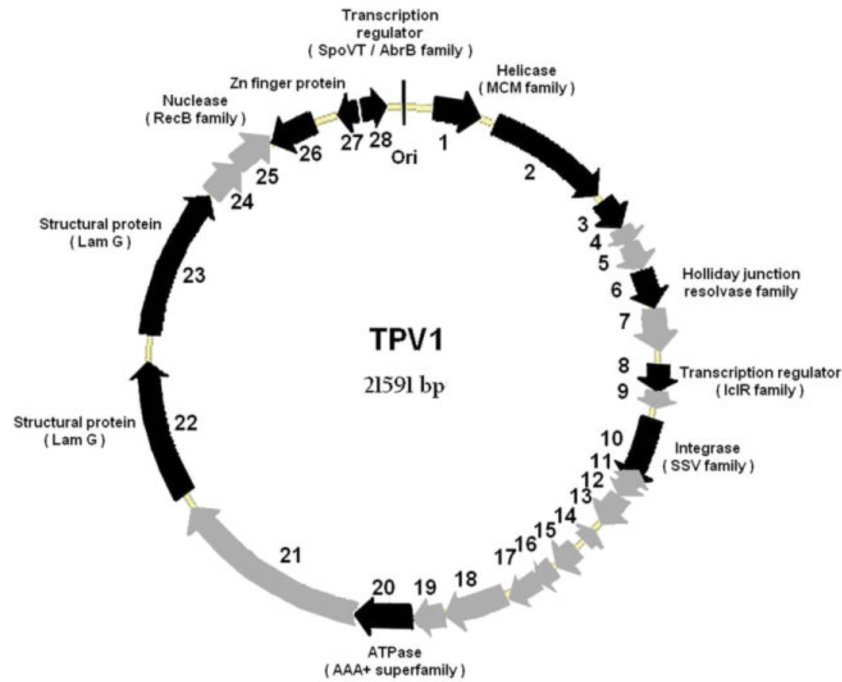

Fig. 4.

TPV1 genome map. Predicted genes are presented by thick arrows; grey arrows indicate ORFs with no similarity or unassigned function and black arrows indicate either conserved hypothetical ORFs or ORFs with an attributed function. The approximate location of the origin (Ori) of replication is also indicated.

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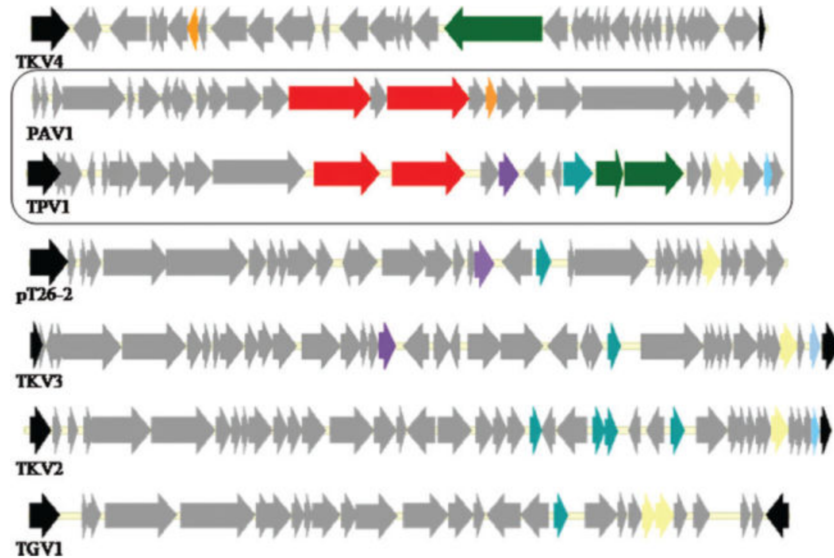

Fig. 5.

Alignment of the genomes of TKV2-4, pT26-2, TGV1, PAV1 and TPV1, with a focus on genes present in the TPV1 and/or PAV1 genomes, and shared with other euryarchaeal genes are a colour coded as follows: integrase in black, the LamG domain protein in red, the MCM protein in green, transcription regulator SpoVT/AbrB in dark blue and the resolvase in yellow. Orange, light blue and mauve ORFs represent genes encoding uncharacterized conserved proteins.

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Table 1

General features of the predicted genes (ORFs) of TPV1 virus.

Predicted gene	Predicted protein size (aa)	Closest homologue: e-value, % identity, taxonomy	Homologues: taxonomic range	Conserved motifs/domains and predicted function(s)	Comments
TPV1-1	200	TK_1361. 2e-45; 44% TKV4 (*T. kodakaraensis*)	TKV4 (*T. kodakaraensis*) only	None	N-terminal part of the MCM2-like helicase in TKV4 but a separate protein in TPV1
TPV1-2	561	TK_1361. 7e-88; 36% TKV4 (*T. kodakaraensis*)	All archaea and eukaryotes	All conserved motifs of the MCM family within the AAA+ ATPase superfamily. MCM2-like helicase involved in DNA replication initiation (licensing factor).	C-terminal part of the MCM2-like helicase in TKV4 but a separate protein in TPV1; contains all elements required for licensing factor activity
TPV1-3	136	None	None	MutL-transducer domain (e-value 1.7e-03, RPS-BLAST); possible involvement in replication, interaction with TPV1-2.	In DNA gyrase, involved in transduction of structural change from ATP-binding site to breakage-rejoining Toprim domain
TPV1-4	83	None	None	None	
TPV1-5	133	TGAM_0671. 1e-47; 61% TGV1 (*T. gammatolerans*)	*T. gammatolerans* provirus and *T. barophilus* plasmid	None	
TPV1-6	174	TGAM_0672. 8e-119; 94% TGV1 (*T. gammatolerans*)	Most archaea, some bacteria, some archaeal and bacterial viruses	Archaeal-type Holliday junction resolvase, all catalytic motifs conserved	Member of a distinct family of Holliday junction conserved in *Thermococcales* and *Archaeoglobales*
TPV1-7	187	None	None	None	
TPV1-8	117	TK0576/TK0420. 1e-54; 81% TKV3/TKV2 (*T. kodakaraensis*)	Many archaea and bacteria	Winged helix–turn–helix domain; transcription regulator of the IclR family	
TPV1-9	71	None	None	Predicted integral membrane protein	
TPV1-10	318	TK0381. 9e-66; 45% TKV2 (*T. kodakaraensis*)	Many archaeal viruses and proviruses integrated in archaeal genomes	SSV1-type integrase	
TPV1-11 (–)	87	None	None	None	
TPV1-12	119	None	None	Predicted integral membrane protein	
TPV1-13	136	None	None	Predicted integral membrane protein	
TPV1-14 (–)	62	None	None	None	
TPV1-15	112	None	None	Predicted integral membrane protein	
TPV1-16	89	None	None	Predicted integral membrane protein	

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Predicted gene	Predicted protein size (aa)	Closest homologue: e-value, % identity, taxonomy	Homologues: taxonomic range	Conserved motifs/domains and predicted function(s)	Comments
TPV1-17	152	None	None	None	
TPV1-18	285	None	None	Predicted integral membrane protein	
TPV1-19	147	None	None	None	
TPV1-20	258	MA2591. 0.026; 26% *Methanosarcina acetivorans*	Distant homologues in all Archaea, bacteria and eukaryotes, some archaeal viruses	A distinct AAA+ superfamily ATPase; all motifs characteristic of active ATPases conserved	AAA+ ATPases with low sequence similarity but similar size and arrangement of conserved motifs found in several crenarchaeal viruses (Fig. S10)
TPV1-21	884	None	None	Predicted non-globular protein	Large non-globular proteins are common among bacteriophage tail subunits
TPV1-22	623	PAV1_ORF676. 7e-15; 23% PAV1	Distant homologues in many Archaea, bacteria and eukaryotes	Concanavalin A-lectin/glucanase domain (jelly roll fold); two predicted transmembrane helices	Potential role in adsorption
TPV1-23	688	PAV1_ORF678. 3e-91; 36%	Distant homologues in many Archaea, bacteria and eukaryotes	Concanavalin A-lectin/glucanase domain (jelly roll fold); three predicted transmembrane helices	Potential role in adsorption
TPV1-24	183	None	None	None	
TPV1-25	182	PNA2_1322. 0.002; 34% *Pyrococcus sp* (predicted provirus)	TKV3 (TK_0597) and other putative proviruses in *Thermococcales*	None	
TPV1-26 (–)	234	PNA2_0680. 7e-30; 37% *Pyrococcus* sp.	Distant homologues in most archaea and bacteria	A distinct subfamily of RecB family nucleases; restriction-like endonuclease superfamily motifs	
TPV1-27 (–)	83	Arcve_1816. 6e-18; 71% *Archaeoglobus veneficus*	*Archaeoglobus veneficus, A. fulgidus, Pyrococcus* sp.; more distant homologues in all eukaryotes	C2H2 Zn finger	
TPV1-28	106	PNA2_0681. 6e-12; 33% *Pyrococcus* sp.	Most Archaea (including TKV2/3), many bacteria	Helix–turn–helix domain; transcription regulator of the SpoVT/AbrB family	