A novel Sulfolobus non-conjugative extrachromosomal genetic element capable of integration into the host genome and spreading in the presence of a fusellovirus

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Abstract

An integrative non-conjugative extrachromosomal genetic element, denoted as pSSVi, has been isolated from a Sulfolobus solfataricus P2 strain and was characterized. This genetic element is a double-stranded DNA of 5740 bp in size and contains eight open reading frames (ORFs). It resembles members of the pRN plasmid family in genome organization but shows only weak similarity to the latter in conserved regions. pSSVi has a \textit{copG} gene similar to that of a pRN plasmid, encodes a large replication protein which, unlike a typical pRN RepA, contains no polymerase/primase domain, and lacks the \textit{plrA} gene. Interestingly, pSSVi encodes an SSV-type integrase which probably catalyzes the integration of its genome into a specific site (a tRNAArg gene) in the \textit{S. solfataricus} P2 genome. Like pSSVx, pSSVi can be packaged into a spindle-like viral particle and spread with the help of SSV1 or SSV2. In addition, both SSV1 and SSV2 appeared to replicate more efficiently in the presence of pSSVi. Given the versatile genetic abilities, pSSVi appears to be well suited for a role in horizontal gene transfer.

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Introduction

Analysis of sequenced archaeal genomes has revealed the widespread presence of integrated genetic elements. Based on the integrases that they encode, these genetic elements fall into two groups: the SSV type and pNOB8 type (She et al., 2004). The integrase of the Sulfolobus spindle-shaped virus SSV1, a prototype of the SSV-type integrases, has been characterized (Serre et al., 2002; She et al., 2004). SSV1 integrates into the downstream half of a tRNAArg gene in the host genome, generating a 44-bp direct repeat at the integration borders of the provirus. The integrase gene is partitioned upon integration since the attP site is located within the \textit{int} gene. Therefore, the integrated provirus was bordered by a smaller N-terminal and a larger C-terminal integrase fragments (Schleper et al., 1992). Integrated genetic elements harboring the partitioned \textit{int} gene of the SSV type have been identified in \textit{Sulfolobus solfataricus} P2, \textit{Sulfolobus acidocaldarius}, \textit{Sulfolobus tokodaii}, \textit{Pyrococcus} sp. OT3 and \textit{Aeropyrum pernix} (Kawarabayasi et al., 1999, 2001; Makino et al., 1999; Peng et al., 2000; She et al., 2001a, 2002, 2004). Another type of archaeal integrases is represented by the putative integrase of the \textit{Sulfolobus} conjugative plasmid pNOB8 (ORF439) (She et al., 1998). The pNOB8 plasmid has recently been shown to integrate site specifically into the \textit{S. solfataricus} P2 genome and the plasmid-encoded integrase is presumably responsible for the integration (She et al., 2004). The pNOB8-type integrated genetic elements contain an intact integrase gene with the integration borders flanked by a direct repeat. A tRNA gene, serving as the integration site, precedes the \textit{int} gene in the same orientation. The
putative pNOB8-type integrated genetic elements have been found in 14 of 18 available sequenced archaeal genomes (She et al., 2004).

Several of the integrated genetic elements (pXQ1, pST1 and pST3) appear to have originated from members of the pRN family, which includes non-conjugative plasmids such as pRN1, pRN2, pHEN7, pDL10 and pSSVx (Arnold et al., 1999; Keeling et al., 1996; Kletzin et al., 1999; Peng et al., 2000; She et al., 2004). These plasmids share a large conserved region containing three ORFs (repA, copG and plrA) (Peng et al., 2000). RepA, CopG and PlrA from pRN1 have been biochemically characterized. RepA, a putative replication initiator, is a novel DNA replication protein harboring primase, helicase, DNA polymerase and ATPase activities (Lipps et al., 2003). Crystallographic studies have identified the N-terminal part of the protein as a primase/polymerase domain (Lipps et al., 2004). The C-terminal domain resembles a helicase of superfamily III (Lipps et al., 2003). The copG gene, located upstream of repA, encodes a putative copy number control protein. CopG binds to its own promoter, presumably down-regulating the transcription of its own gene and the downstream gene repA (Lipps et al., 2001b). PlrA binds to two neighboring binding sites upstream of its coding gene and is a possible plasmid regulatory protein (Lipps et al., 2001a). Homologues of both copG and plrA have also been found in most known Sulfolobus conjugative plasmids including pNOB8, pING1, pKEF9, pARN3, pHVE14 and pSOG1 (Eraso et al., 2006; Greve et al., 2004; Schleper et al., 1995; She et al., 1998; Stedman et al., 2000). The integrated genetic elements of the pRN plasmid origin all encode at least two putative replication proteins characteristic of the pRN family (RepA and CopG) (She et al., 2004).

Unlike all other members of the pRN family, pSSVx is a hybrid between a pRN plasmid and a fusellovirus. It spreads as a virus with either SSV2 or SSV1 as a helper. Since a tandem array of two ORFs in pSSVx is homologous to a similar tandem array of two ORFs in SSV1 and SSV2, but is absent from pRN1 and pRN2, it has been speculated that the sequences of these ORFs are essential for the packaging and spreading of pSSVx (Arnold et al., 1999).

In this article, we report the isolation and characterization of pSSVx, a novel Sulfolobus non-conjugated plasmid capable of both integrating into the host genome using an SSV-type mechanism and spreading as a virus satellite in the presence of SSV1 or SSV2. The availability of this versatile genetic element will not only permit analyses of viral–host interactions and interactions between the helper and the satellite viruses but also facilitate the construction of a spreading and integrative vector.

Results

Identification of pSSVx

The initial clue of the presence of pSSVx was obtained during our studies of the transformation of a S. solfataricus P2 strain, generously provided by K. Stedman (Portland, Oregon, USA), by electroporation with the SSV2 DNA prepared from Sulfolobus islandicus REY31A. When the extrachromosomal DNAs isolated from the transformed cells were analyzed by agarose gel electrophoresis, a fast-migrating, major DNA band, in addition to those expected for SSV2, was found (Fig. 1A). Cleavage of the DNAs from the transformed cells and the pure SSV2 DNA with Xbal yielded four and three fragments, respectively (Fig. 1B, lanes 2 and 6). The additional fragment in the digest of the DNAs from the transformed cells was about 5–6 kb in length. Digestion of the DNAs from the transformed cells with Smal, which occurs once in the SSV2 genome, produced two fragments, which added up to ∼5.5 kb, in addition to the linearized SSV2 DNA molecule (Fig. 1B, lanes 1 and 5). These results suggest the presence of a plasmid or viral genome of ∼5.5 kb in the transformed cells. Electron microscopic examination revealed lemon-shaped virus-like particles (VLPs) of two distinct sizes in the culture supernatant of the transformed cells (Fig. 1C). The larger VLP was identical to SSV2 in size and shape, and the smaller one (60 × 40 nm)
presumably represented a novel virus particle containing the ~5.5 kb genome. In agreement with this observation, both SSV2 DNA and the smaller episomal DNA were extracted from a mixture of the two VLPs. The smaller VLP is denoted as pSSVi. We later found that pSSVi originated from a genetic element integrated into the genome of the *S. solfataricus* P2 strain used in the experiment (see below). None of our other *S. solfataricus* P2 stocks contained pSSVi. To avoid confusion, we designate the *S. solfataricus* P2 strain from which pSSVi was first isolated as *S. solfataricus* P2 (pSSVi).

To test if pSSVi was able to infect a *Sulfolobus* host, we sought to obtain a preparation of pSSVi particles free of SSV2 by using conventional methods such as plaque purification. However, these attempts were unsuccessful. We then co-infected *S. solfataricus* P2 strain containing no pSSVi with a mixture of SSV2 and pSSVi and allowed the infected cells to grow at 80 °C. Both SSV2 and pSSVi DNAs were found in large amounts in the infected cells, and the two types of VLPs identical to those shown in Fig. 1C were observed in the supernatant of the culture. In addition to the *S. solfataricus* strain P2, *S. solfataricus* P1 and *S. islandicus* REY15A could also be co-infected by pSSVi and SSV2.

### Genomic analysis

The complete pSSVi genome contains 5740 bp with a G+C content of 39.0%, which is close to those of pRN1 (37.3%), pSSVx (38.7%), SSV1 (39.7%) and SSV2 (38.5%). A total of eight ORFs were identified in the pSSVi genome (Table 1). The genomic structure of pSSVi is similar to that found in members of the pRN plasmid family including the integrated genetic elements in the *Sulfolobus* genomes (e.g., pSA2 from *S. acidocaldarius* DSM 639, pST1 from *S. tokodaii* str. 7 and pXQ1 from *S. solfataricus* P2) (Fig. 2). However, most of the pSSVi ORFs showed low sequence similarity to those of the pRN plasmids. The apparent exception is pSSVi ORF57, which shares 47%/63% sequence identity/similarity with CopG of pRN1. CopG is a putative copy number control protein, which binds to the inverted repeats in the promoter of its own gene and down-regulate the transcription of *copG* as well as the downstream *repA* gene (Lipps et al., 2001b). The inverted repeats also exist upstream of ORF57 in pSSVi. Therefore, ORF57 appears to serve the role of controlling the copy number of pSSVi DNA.

ORF735, located downstream of ORF 57, is the largest ORF in the pSSVi genome. Intriguingly, ORF735 contains no primase/polymerase domain in the N-terminal portion, which has been found to be conserved among RepA proteins encoded by most pRN plasmids, and shows weak similarity to RepA of pXQ1 and pDL10 in the C-terminal part (from amino acid residue 610) including a region conserved in the helicase III superfamily. Notably, the protein is 23/38% (29/51% for residues 108 to 598) identical/similar to pSA2 ORF684, which also lacks the N-terminal primase/polymerase domain. ORF735 has two Walker A motifs located at positions 259 and 537, respectively. It contains an AAA+ ATPase domain (residues ~200 to ~500) and an HTH domain (residues ~460 to ~570), suggesting that ORF735 is an ATPase capable of binding to double-stranded DNA. Based on these data, we speculate that ORF735 is a novel replication protein. Interestingly, the middle portion of ORF735 (residue 211 to 555) is 49% similar to A460 of SSV K1, while the C-terminal part of the protein (~50 amino acid residues) displays 88% similarity to ORFe78 located downstream of A460. These results hint at the possibility that the two proteins encoded by SSV K1 are involved in viral DNA replication.

ORF57 and ORF735 are separated by ORF121. This arrangement is similar to that found in pSSVx (Fig. 2). ORF121 shares little sequence homology with ORFs in plasmids of the pRN family including pSSVx but is 42/59% identical/similar to a putative protein (ORF117) encoded by pSA2. ORF87 of pSSVi is 35/42% identical/similar to ORF D of pHEN7, whose function is unknown. ORF c56 is highly similar to ORF c68 of pSSVx (42/53% identity/similarity). Homologues of ORF c56 have only been found in pSSVx (ORF c68), pSA2 (ORF c58) and pST1 (ORF c60) so far. It is worth noting that ORF c56 and its homologues as well as similarly located ORFs in pRN2 and pHEN7 are all oriented in a direction opposite to most of the genes in the genomes.

pSSVi also displays a number of unique features. Most interestingly, pSSVi encodes a putative integrase (ORF336). This is the first report of a free non-conjugative *Sulfolobus* genetic element that encodes an integrase. Sequence analysis indicates that ORF336 belongs to the tyrosine recombinase family which utilizes a catalytic tyrosine to attack the DNA backbone during cleavage (Argos et al., 1986). The enzyme has the active site, RXXR…Y, which is characteristic of SSV-type integrases (Fig. 3). Based on sequence comparison, ORF336 is more closely related to SSV1 integrase than to SSV2 integrase. Upon integration, the pSSVi genome is bordered by the partitioned integrase gene (see below). pSSVi also encodes ORF182 and ORF c150, which have no significant matches in public databases. Furthermore, pSSVi lacks *plrA*, which is conserved among all known non-conjugated *Sulfolobus* plasmids except for pORA1 from *Sulfolobus neoezealandi*-

### Table 1

<table>
<thead>
<tr>
<th>ORF</th>
<th>Annotation</th>
<th>Homologues (% identity/similarity)</th>
</tr>
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<tbody>
<tr>
<td>ORF 336</td>
<td>Integrate</td>
<td>SSV1 integrase (71/83)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pXQ1 integrase (64/79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSV2 integrase (38/57)</td>
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<tr>
<td></td>
<td></td>
<td>SSV K1 integrase (36/57)</td>
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<tr>
<td>ORF 735</td>
<td>Replication</td>
<td>pSA2 ORF 684 (aa108-598 29/51)</td>
</tr>
<tr>
<td>ORF 57</td>
<td>CopG</td>
<td>pRN1 CopG (47/63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pHEN7 CopG (22/40)</td>
</tr>
<tr>
<td>ORF 87</td>
<td>Hypothetical</td>
<td>pHEN7 ORF D (35/42)</td>
</tr>
<tr>
<td>ORF c56</td>
<td>Hypothetical</td>
<td>pST1 ORF 80 (43/54)</td>
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<td></td>
<td>pSSVx ORF D (35/42)</td>
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<tr>
<td>ORF c68</td>
<td>Hypothetical</td>
<td>pSA2 ORF c58 (69/85)</td>
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<tr>
<td></td>
<td></td>
<td>pSSVx ORF c68 (42/53)</td>
</tr>
<tr>
<td>ORF c150</td>
<td>Hypothetical</td>
<td>pSA2 ORF 117 (aa20–120 42/59)</td>
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Our inability to purify pSSVi away from SSV2 points to the possibility that the former depended on the latter for its ability to propagate. To understand their relationships, we purified the pSSVi DNA by gel electrophoresis, and transformed the *S. solfataricus* P2 strain containing no pSSVi with pSSVi DNA and SSV2 DNA separately or in combination. We found that cells transformed with pSSVi DNA alone grew as well as the untransformed control (Fig. 4A), and the pSSVi DNA in the cells was undetectable by agarose gel electrophoresis. The growth of cells transformed with SSV2 DNA alone was only slightly inhibited when the viral DNA replication became apparent towards the end of the exponential growth phase. Interestingly, a strong growth inhibition was observed in cells transformed with both pSSVi and SSV2 DNA. The pSSVi and SSV2 DNAs accumulated to high levels in the cells when the growth inhibition occurred. Replication of both genetic elements appeared to proceed in parallel and peaked at the same time. The level of SSV2 DNA in the cells co-transformed with both pSSVi and SSV2 DNAs was, on average, over three folds higher than that in the cells transformed with SSV2 DNA alone, as measured by Southern hybridization and subsequent quantification by Phosphorimager (Fig. 4C). The stimulation of SSV2 DNA replication depended specifically on the presence of...
pSSVi since this effect was not observed when pSSVi DNA was replaced with pUC18 DNA in the assay. SSV1 DNA replication was enhanced in a quantitatively similar fashion in the presence of pSSVi (date not shown). Furthermore, the interaction between pSSVi and a fusellovirus occurred not only in \textit{S. solfataricus} P2 but also in \textit{S. solfataricus} P1 and \textit{S. islandicus} REY15A (date not shown).

We also examined the culture supernatants of the cells transformed either with SSV2 DNA alone or with a mixture of SSV2 and pSSVi DNAs under electron microscope. As expected, both SSV2 and pSSVi particles were observed in the culture transformed with the two DNAs whereas only SSV2 virions were found in the culture transformed with SSV2 DNA. In the co-transformed culture, the ratio of the two types of particles appeared to be consistent with the ratio of the two DNAs. Based on these data, we conclude that pSSVi spreads as a satellite virus in the presence of the helper virus SSV1 or SSV2, and the helper virus replicates more efficiently in the presence of pSSVi.

To determine if pSSVi was stably maintained in the host cell in the absence of a helper virus, we transformed \textit{S. solfataricus} P2 with pSSVi DNA. The pSSVi DNA in the transformed cells became undetectable by agarose gel electrophoresis after the first subculture. To follow the pSSVi DNA after it was introduced into the host cell, we designed a sensitive PCR assay which involved the use of a pair of primers flanking the site of integration in the \textit{int} gene of pSSVi (Fig. 5A). Successful amplification of the target pSSVi DNA fragment by PCR with the primers would indicate the presence of free plasmid DNA in a sample. By using this assay, we found that free pSSVi DNA remained detectable after the third subculture (Fig. 5B), but was not detected after the eighth subculture, in the transformed \textit{S. solfataricus} P2 cells in the absence of a helper virus. We infer from these results that the free pSSVi DNA is not stably maintained in the host in the absence of a helper.

\textbf{pSSVi integration}

Genomic sequencing revealed that pSSVi encoded a putative integrase. This raised a possibility that the viral DNA was capable of integrating into the host genome. To test this possibility, we first identified a putative pSSVi \textit{attP} site by searching the pSSVi sequence with the conserved \textit{attP} sequences of the SSV-type integrases. As shown in Fig. 6, the \textit{attP} site of pSSVi is very similar to that of SSV1. A subsequent sequence homology search in the \textit{S. solfataricus} P2 genome using the putative \textit{attP} site of pSSVi led to the identification of 10 potential sites of integration. One of the sequences, which was the downstream portion of the tRNA\textsubscript{Arg} gene (tRNA31 in the \textit{S. solfataricus} P2 genome), matched perfectly with the \textit{attP} site and the others had one or two mismatches. Using a PCR-based integration assay (Fig. 5A), we examined the viral integration at these sites. We found that, when total DNA from \textit{S. solfataricus} P2 cells transformed with both pSSVi and SSV2 was used as the template, PCR reactions produced a specific product only with the primer pair designed for the perfectly matched site. Integration of pSSVi at this site was verified by sequencing of the PCR product and Southern hybridization (Fig. 7). Based on these results, we conclude that pSSVi DNA is capable of integrating site specifically into the tRNA\textsubscript{Arg} gene in the \textit{S. solfataricus} P2 genome. It is worth noting that pSSVi resembles SSV1 in employing an arginyl tRNA gene as the site for integration, but the two arginyl tRNA genes are different.

Since pSSVi was initially isolated from \textit{S. solfataricus} P2 (pSSVi), we were interested in determining if it contained the...
genetic element by using the PCR assays. Our PCR-based assays for free pSSVi revealed no free plasmid in the strain. However, by using the PCR-based integration assays, we found that this strain contained the genetic element integrated into the host genome (Fig. 8). Furthermore, we found that *S. solfataricus* P2 (pSSVi), when infected with SSV2, accumulated both pSSVi and SSV2 DNAs in the cells, and released both SSV2 and pSSVi particles into the culture supernatant. It appears that the integrated pSSVi DNA became excised from the host genome and actively replicated upon introduction of SSV2 DNA into the host cells. The presence of the pSSVi sequence in *S. solfataricus* P2 (pSSVi) was unexpected since it was not found in the sequenced P2 genome (She et al., 2001b). To clarify the issue, we tested our *S. solfataricus* P2 stocks from different sources.

Fig. 4. Physiological interactions between pSSVi and SSV2 in *S. solfataricus*. (A) Growth of *S. solfataricus* P2 transformed with pSSVi DNA and/or SSV2 DNA. *S. solfataricus* cells were transformed with indicated viral DNA(s) (200–300 ng) by electroporation. The transformed cells were incubated at 80 °C with shaking. Samples were taken at various times and the cell density of each sample was determined. ◯, *S. solfataricus* culture which was not transformed; ■, culture transformed with pSSVi DNA; △, culture transformed with SSV2 DNA; ●, culture transformed with both pSSVi and SSV2 DNAs. (B) Replication of pSSVi and SSV2 DNAs in *S. solfataricus* P2 cells. Viral DNAs were isolated from samples taken at specified times from the growing culture transformed with pSSVi DNA and/or SSV2 DNA, and digested with *Nco*I and *Bsp*EI. The restriction enzyme digests were subjected to electrophoresis in 0.8% agarose. Lane M, size markers (from top to bottom): 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5 and 1.0 kb; lanes 1–6, SSV2-transformed culture sampled at 36, 48, 60, 72, 84 and 100 h, respectively; lanes 7–12, pSSVi/SSV2-transformed culture sampled at 36, 48, 60, 72, 84 and 100 h, respectively. The optical densities of the samples at 600 nm in lanes 1–12 were 0.61, 0.80, 1.01, 1.15, 1.25, 1.30, 0.58, 0.72, 0.86, 1.00, 1.08 and 1.11, respectively. (C) Quantification of pSSVi and SSV2 DNAs in the transformed *S. solfataricus* P2 cells. pSSVi and SSV2 DNAs were isolated from the transformed *S. solfataricus* P2 cells, digested and electrophoresed in agarose gel as described in the legend to panel B. The DNAs were then subjected to Southern hybridization. After hybridization, the membrane was analyzed by using the Phosphorimager.

Fig. 5. Detection of the free form of pSSVi in *S. solfataricus* P2 cells transformed with the viral DNA. (A) A diagram showing the integration of pSSVi DNA into the host genome. The position and orientation of the two primers for the PCR detection of the free pSSVi DNA are indicated by solid arrows on the circular map of the plasmid, whereas those of the primers for the detection of pSSVi integration shown by dotted arrows on the linear representation of the integrated genome. The oligonucleotide used as the probe in the detection of pSSVi integration by Southern hybridization is indicated by an arrow above the linear sketch of the genome shown at the bottom. (B) Analysis of the free pSSVi DNA by PCR. *S. solfataricus* P2 cells were transformed with pSSVi DNA by electroporation. After the third subculture, cells were collected, and DNA was isolated from the cells. A PCR assay using the specific primers was carried out to detect the presence of free pSSVi in the DNA. Lane M, size markers (from top to bottom): 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5 and 1.0 kb; lane 1, pSSVi DNA as the template; lane 2, DNA from pSSVi-transformed cells as the template; lane 3, the *S. solfataricus* P2 genomic DNA as the template.
for the presence of the pSSVi sequence. None of our other *S. solfataricus* P2 stocks or *S. islandicus* strains contained pSSVi.

**Discussion**

We have isolated a novel extrachromosomal genetic element, denoted pSSVi, from *S. solfataricus* P2 (pSSVi). pSSVi has a covalently closed circular double-stranded DNA genome of 5740 bp in size, and is capable of integrating site specifically into the host *Sulfolobus* genome and spreading in the presence of the helper virus SSV1 or SSV2. Interestingly, despite its similarity to the pRN plasmids in genomic organization, pSSVi shares significant similarity with the latter in only one ORF (*copG*). It is distinctly different from known pRN plasmids in several aspects. First, pSSVi encodes an SSV-type integrase (ORF336). The pSSVi integrase appears to be functional since, when a pSSVi-based shuttle vector harboring *pyr*EF was transformed into a pyrimidine auxotrophic *Sulfolobus* strain, integration of the vector into the host chromosome was observed in Southern hybridization experiments (to be published elsewhere). Furthermore, the recombinant pSSVi integrase produced in *E. coli* was active in *in vitro* cleavage assays (unpublished results). Second, pSSVi lacks the *plrA* gene, which is the most conserved ORF of the pRN plasmid family. PlrA binds specifically to its own promoter region in an *in vitro* assay and is believed to be important for plasmid replication (Lipps et al., 2001a). None of the integrated pRN plasmids encodes PlrA, an observation which has been taken to infer that the protein is responsible for the loss of the free form of these plasmids (She et al., 2004). However, PlrA is apparently not essential for efficient replication of the plasmid since the intracellular level of free pSSVi DNA increased drastically in the presence of the helper virus. Third, pSSVi encodes a putative replication protein (ORF735) without a typical primase/polymerase domain. There is a striking difference between RepA from pSSVi and those from most pRN1 plasmids in amino acid sequence. Most known pRN RepAs possess a primase/polymerase domain. By contrast, the pSSVi protein has two Walker A motifs but contains no primase/polymerase domain. The C-terminal part of the protein (starting from amino acid residue 610) exhibits weak similarities to pyrimidine auxotrophic *Sulfobolus* strain, integration of the vector into the host chromosome was observed in Southern hybridization experiments (to be published elsewhere).

![Fig. 6. Sequence alignment of the *attP* sites of pSSVi and its related genetic elements.](image)

![Fig. 7. Detection of pSSVi integration into the genome of *S. solfataricus* P2 by PCR (A) and Southern hybridization (B). *S. solfataricus* P2 cells were transformed with SSV2 and pSSVi DNAs by electroporation. After incubation at 80 °C with shaking, total DNA from the transformed cells was isolated. Viral integration was determined by PCR with a pair of specific primers using the isolated DNA as the template. For Southern hybridization, the total DNA was digested with *Bgl*II, and the restriction digest was separated by electrophoresis in 0.8% agarose. Hybridization was performed using a radiolabeled oligonucleotide as the probe. (A) Lane M, size markers (from top to bottom): 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75 and 0.5 kb; lane S, the PCR product. (B) Total DNA from the transformed cells digested with *Bgl*II. The optical densities of the samples at 600 nm in lanes 1–5 were 0.64, 1.01, 1.32, 1.53 and 1.12, respectively. A band of 4.6 kb in size resulting from pSSVi integration is indicated by an arrow.](image)

![Fig. 8. Detection of the presence of integrated pSSVi in various *S. solfataricus* P2 stocks by PCR. Total DNA, isolated from *S. solfataricus* cells, was used as the template in the PCR-based integration assays. DNA from *S. solfataricus* P2 cells transformed with a mixture of SSV2 and pSSVi was used as a positive control. PCR products were subjected to electrophoresis in 0.8% agarose. Lane M, size markers (from top to bottom): 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1 and 0.75 kb; lane 1, *S. solfataricus* P2 transformed with SSV2 and pSSVi DNA; lane 2, *S. solfataricus* P2 (pSSVi); lane 3, *S. solfataricus* P2.](image)
helicase III. A similar RepA has also been found in pSA2. RepA from pRN1 has been extensively characterized. The protein has multiple enzyme activities including primase, DNA polymerase and helicase (Lipps et al., 2003, 2004). The recombinant form of the pSSVi RepA showed no DNA polymerase or primase activity (unpublished results). However, the protein is an active DNA helicase. Therefore, it is speculated that pSSVi RepA may serve to unwind DNA helix and recruit the host DNA replication machinery for steps following DNA unwinding in plasmid DNA replication.

Recently, several cryptic plasmids have been isolated from *S. neozealandicus* (pTAU4, pORA1 and pTIK4) and *S. solfataricus* IT3 (pIT3) (Greve et al., 2005; Prato et al., 2006). Plasmids pORA1, pTIK4 and pIT3 encode RepA proteins clearly related to those of the pRN plasmids, while pTAU4 encodes a MCM. Like pSSVi, both pORA1 and pIT3 lack *plrA*. It appears that the loss of the free form of the integrated pRN plasmids and the instability of pSSVi may not be related to the absence of *plrA* in the genomes of these genetic elements.

Our results suggest that pSSVi is capable of spreading in the presence of the helper virus SSV1 or SSV2, presumably in a similar fashion to pSSVx. A tandem array of two ORFs in pSSVx, ORF154 and ORF288, was proposed to contribute to the packaging and spreading of the satellite virus, processes that depended on the presence of SSV1 (Arnold et al., 1999). However, no homologues of the two ORFs were found in pSSVi, suggesting that the two proteins may not serve the proposed role. Sequence comparison between pSSVi and pSSVx showed that they shared a single homologue (ORF c56 in pSSVi and ORF c68 in pSSVx) that was not present in pRN family plasmids that lack the ability to spread as a satellite virus in the presence of a fusellovirus. The possibility exists that this ORF is involved in viral packaging and spreading. However, it is more likely that a specific sequence feature shared by pSSVi and pSSVx, and not a protein encoded by them, initiates the packaging of these satellite viruses by the helper virus.

Interestingly, the interactions between pSSVi and SSV1/SSV2 appear to be more complex than the dependence of the former on the latter for packaging. SSV1/SSV2 and pSSVi appear to replicate more efficiently when they were both present in the host cells than alone. The high copy number of free pSSVi (~200 molecules per cell) in the host cell found in the presence of SSV1/SSV2 may not be due solely to the gained ability to spread since the free form of pSSVi would otherwise not be lost so rapidly in the absence of a helper. On the other hand, SSV1 or SSV2 replication was also significantly enhanced in the presence of pSSVi. The mechanistic basis for the mutually beneficial effect of the two viruses on each other remains to be understood.

As an integrative plasmid with the potential to spread, pSSVi is the most versatile genetic element that has been isolated from archaea so far. Our results show that pSSVi is not stably maintained in the host cells as a free plasmid in the absence of the helper virus. One may speculate that, once free pSSVi is lost, pSSVi integrated into the host genome becomes trapped due to the loss of a functional integrase. The integrated pSSVi may lose the potential to be excised as a result of mutation at the attachment site and can no longer be induced even in the presence of a helper virus. Therefore, genetic elements such as pSSVi may facilitate gene capture and serve an important role in horizontal gene transfer in *Sulfolobus*.

**Materials and methods**

**Growth of *Sulfolobus* strains**

*S. solfataricus* P2 (pSSVi) was a gift from K. Stedman, who obtained the strain from the culture collection of W. Zillig (personal communication). *S. solfataricus* P2 was obtained from Dennis Grogan (Cincinnati, Ohio, USA) and the German Collections of Microorganisms and Cell Cultures (Braunschweig, Germany). *S. solfataricus* P1 was kindly provided by K. Stedman. *Sulfolobus shibatae* 51178 was purchased from the American Type Culture Collection (Rockville, MD, USA). *S. islandicus* strain REY15A was isolated from an enrichment culture prepared from a sample collected in a solfataric field in Iceland (Contursi et al., 2006). *S. islandicus* REY31A was derived from REY15/4, the natural host of SSV2 and pSSVx, by curing pSSVx (Contursi et al., 2006). All strains were grown aerobically at 80 °C with shaking in Zillig’s medium (Zillig et al., 2006) supplemented with 0.2% sucrose and 0.05% yeast extract and adjusted to an initial pH of ~3.1.

**Transformation**

*Sulfolobus* cells were transformed by electroporation as described previously (Schleper et al., 1992) with modifications. *Sulfolobus* cells were grown to an OD600 of 0.25–0.3, centrifuged, washed three times with ice-cold 20 mM sucrose and resuspended in the same solution to an OD600 of 60–75. The cells in a 50-μl aliquot were transformed with indicated viral DNA(s) (200–300 ng) by electroporation at 1.5 kV. The cell suspension was mixed immediately with 1 ml of the supplemented Zillig’s medium pre-warmed at 80 °C. After incubation for 1 h at 80 °C without shaking, an aliquot (950 μl) of the sample was added to 50 ml of the pre-warmed medium. The culture was subsequently incubated at 80 °C with shaking.

**DNA isolation**

To isolate pSSVi DNA, *S. solfataricus* P2 transformed with a mixture of pSSVi and SSV2 DNAs was grown to the late exponential phase. The cells were harvested by centrifugation at 7000×g for 15 min at 4 °C. The viral DNAs were isolated by using the alkaline lysis method (Birnboim and Doly, 1979), digested with *NcoI*, which linearized SSV2 DNA at a unique site, and subsequently subjected to electrophoresis in agarose. The band containing the pSSVi DNA was sliced and the pSSVi DNA was recovered from the gel slice by using a Qiagen DNA purification kit.

To isolate SSV1 or SSV2 DNAs, *S. shibatae* 51178 or *S. islandicus* REY31A was grown to an OD600 of 0.8 at 80 °C. The
viral DNA was isolated from the cells by using the alkaline lysis method (Birnboim and Doly, 1979). Total DNA from *Sulfolobus* cells was isolated as described previously (Xiang et al., 2005).

**Virus purification**

To prepare a mixture of SSV2 and pSSVi particles, *S. solfataricus* P2 cells transformed with SSV2/pSSVi DNAs were grown. After centrifugation at 7000×g for 15 min at 4 °C, NaCl and polyethylene glycol 6000 were added to the supernatant to final concentrations of 1 M and 10% (w/v), respectively, to precipitate virus particles. The precipitate was collected by centrifugation at 25,000×g for 20 min at 4 °C. Virus particles were resuspended in ddH₂O and purified by centrifugation at 125,000×g for 24 h at 20 °C in a CsCl density gradient (0.40 g/ml). Virus particles were recovered, dialyzed against ddH₂O and stored at 4 °C. SSV2 particles were prepared from *S. islandicus* REY31A in a similar fashion.

**Southern hybridization**

DNA samples were subjected to electrophoresis in 0.8% agarose, and the resolved DNA species transferred onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotech). Southern hybridization was performed according to the procedure of Sambrook et al. (1989). The probes for SSV2 Southern hybridization was performed according to the procedure of Sambrook et al. (1989). The probes for SSV2 and pSSVi DNAs were prepared by labeling the corresponding DNAs with [α-³²P]dCTP using a Random Primer DNA Labeling Kit (TaKaRa). After hybridization, the membrane was exposed to X-ray film.

For the detection of the free form of pSSVi in the host cell, we used a similar procedure with the same probes as described above. The presence of the free form of pSSVi in the host cell was detected by PCR with a pair of primers: 5′-TCAGAGAGG-GACGTAGAA (p3) and 5′-CGGTAGACCGCATAGACA (p4), which flank the pSSVi attachment site (attP). Total DNA (20 ng) from *Sulfolobus* cells was used as the template. PCR reactions were performed for 30 cycles with 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C for each cycle.

**Electron microscopy**

Samples were stained for 3 min with 2% (wt/vol) uranyl acetate and observed under an electron microscope at 75keV (Hitachi H-600A).

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**References**


