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Characterization of the *Sulfolobus* host–SSV2 virus interaction

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Abstract The *Sulfolobus* spindle virus, SSV2, encodes a tyrosine integrase which furthers provirus formation in host chromosomes. Consistently with the prediction made during sequence analysis, integration was found to occur in the downstream half of the tRNA^{Gly} (CCC) gene. In this paper we report the findings of a comparative study of SSV2 physiology in the natural host, *Sulfolobus islandicus* REY15/4, versus the foreign host, *Sulfolobus solfataricus*, and provide evidence of differently regulated SSV2 life cycles in the two hosts. In fact, whereas a significant induction of SSV2 replication takes place during the growth of the natural host REY15/4, the cellular content of SSV2 DNA remains fairly low throughout the incubation of the foreign host. The accumulation of episomal DNA in the former case cannot be traced to decreased packaging activity because of a simultaneous increase in the virus titre in the medium. In addition, the interaction between SSV2 and its natural host is characterized by the concurrence of host

growth inhibition and the induction of viral DNA replication. When this virus–host interaction was investigated using *S. islandicus* REY15A, a strain which is closely related to the natural host, it was found that the SSV2 replication process was induced in the same way as in the natural host REY15/4.

Keywords Archaeal virus · Integration · *Sulfolobus* · Replication induction · Growth regulation

Introduction

The first spindle-shaped virus was discovered in *Sulfolobus shibatae* B12, a crenarchaeon isolated from a sulphurous hot spring in Beppu, Japan (Grogan et al. 1990; Martin et al. 1984; Yeats et al. 1982). The virus was named SSV1 and has been characterized in greater detail. SSV1 virus production can be induced in both the natural and foreign hosts by UV irradiation or treatment with mitomycin C (Reiter et al. 1987a, b; Schleper et al. 1992). All transcripts in the viral genome have been mapped and their promoters and terminators have been determined (Palm et al. 1991; Reiter et al. 1987b, 1988). The SSV1 genome is stable in three forms: (1) the viral genome packaged in virus particles which is positively supercoiled (Nadal et al. 1986); (2) the episomal genome in the *Sulfolobus* cells which includes positively and negatively super-coiled and relaxed double-stranded DNAs (Nadal et al. 1986; Prangishvili et al. 2001; Snyder et al. 2003); (3) a provirus is present in the chromosomes of both the natural host, *Sulfolobus shibatae* B12, and the foreign host, *Sulfolobus solfataricus* P1 (Reiter and Palm 1990; Reiter et al. 1989; Schleper et al. 1992).

The functions of four SSV1 open reading frames (ORFs) have so far been identified based on functional analysis. The ORF codings of the three structural proteins VP1, VP2 and VP3 have been identified by sequencing the purified coat proteins (Reiter et al. 1987a). The fourth ORF encodes a tyrosine integrase

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(Esposito and Scocca 1997; Nunes-Duby et al. 1998) of the SSV-type, which facilitates the recombination between the virus and archaeal host attachment sites, *attP* and *attA* (Muskhelishvili et al. 1993; Serre et al. 2002) whereas the remaining SSV ORFs show little or no significant sequence similarity to any sequence with a known function in public databases.

A few more SSV viruses have been isolated from different locations in the world, forming a novel *Fuselloviridae* virus family (Arnold et al. 1999b). These include: (1) SSV2 and pSSVx, i.e. the helper and satellite viruses carried by *Sulfolobus islandicus* REY15/4, which were originally isolated from a solfataric hot spring in Reykjanes, Iceland (Arnold et al. 1999a; Stedman et al. 2003); (2) SSV RH, which was obtained from an enrichment culture sampled in the Ragged Hills region of the Norris Geyser basin in Yellowstone National Park, United States (Snyder et al. 2003; Wiedenheft et al. 2004) and (3) SSV K1, which was isolated in the Geyser Valley region of the Uzhno-Kamchatsky National Park in the Kamchatka peninsula, Russia (Snyder et al. 2003; Wiedenheft et al. 2004). Genome comparison has revealed that these four SSV viruses are similar in size and gene organization (Wiedenheft et al. 2004). Sequence and orientation similarity has been observed in the ORFs of one half of the genome, but not in those of the other half (Palm et al. 1991; Stedman et al. 2003; Wiedenheft et al. 2004).

Comparative genomics analysis with a selected set of archaeal viruses revealed that these viruses are interrelated albeit they exhibit very different morphotypes and belong to different virus families (Prangishvili et al. 2006a). Among SSV viruses, only SSV1 was included for that analysis. It has been revealed that SSV1 encodes ORFs orthologous to those present in the genomes of *Acidianus* ATV (Prangishvili et al. 2006b) and AFV1 (Bettstetter et al. 2003), as well as *Sulfolobus* SIRV1 (Blum et al. 2001; Peng et al. 2001) and STSV1 (Xiang et al. 2005) viruses, all of which belong to crenarchaeal viruses. This allows that Prangishvili et al. (2006a) postulated that these viruses share a common origin and their morphotypic diversity reflects their fast evolution. Moreover, archaeal viral genomes usually contain genes that are likely acquired from their hosts, and vice versa (Prangishvili et al. 2006a; She et al. 2004).

Another striking finding among *Sulfolobus* virus studies has been the identification of the first archaeal helper and satellite virus system: *Sulfolobus* viruses SSV2 and pSSVx. In this system, SSV2 acts as an ordinary virus and a helper virus to pSSVx, while pSSVx is a satellite virus assumed to generate virus particles thanks to the packaging mechanisms of SSV2 (Arnold et al. 1999a; Stedman et al. 2003). At sequence level, pSSVx genome contains the putative minimal replicon of the *Sulfolobus* pRN plasmid family (pRN1, pRN2, pHEN7), the *Acidianus ambivalens* plasmid pDL10 (Garrett et al. 2004), and several integrated plasmids which are found in *Sulfolobus* genomes (She et al. 2001a, 2002, 2004).

Here we report the characterization of the specific interactions between the SSV2 virus and its *Sulfolobus* hosts and provide evidence that SSV2 replication activity is reversely related to the growth rate of the natural host.

Materials and methods

Enzymes and chemicals

Restriction and modification enzymes were purchased from New England Biolabs or Amersham Biosciences. Synthetic oligonucleotides were purchased from TAG Copenhagen. Radioactive materials were obtained from Amersham Biosciences.

Strains used in this study

Sulfolobus islandicus REY15/4 and related *Sulfolobus* enrichment cultures were kindly provided by Dr. Wolfram Zillig (Zillig et al. 1998, 1994).

Sulfolobus islandicus REY31A is a pSSVx-cured strain isolated from REY15/4. The plasmid curation was done by growing REY15/4 continuously for approx. 100 generation times at an optical density below 0.4 (OD₆₀₀). Thereafter, six single colonies were isolated via Gelrite plating and purified by re-streaking on plate for three times. Since pSSVx was hardly detected at low optical density values in REY15/4 cells, the REY31 isolates, were carefully checked for the absence of pSSVx by polymerase chain reactions (PCRs) and Southern analysis over all the growth phases.

Sulfolobus islandicus REY15A is virus-free isolate obtained by Gelrite plating of the same enrichment culture from which the REY15/4 strain had been isolated. Six single clones (REY15A-F) were isolated and purified as single colonies as described for REY31A. They were then tested for the absence of extrachromosomal DNA and for the incapability in conferring growth inhibition to *S. solfataricus* P2 strain.

Sulfolobus solfataricus P2 (DSM1617) was purchased from the German Collections of Microorganisms and Cell Cultures.

Virus-harboring *S. solfataricus* P2 (SSV2-P2) and *S. islandicus* REY15A (SSV2-REY15A) strains were obtained as following: 1–2 µl of the REY15/4 supernatant containing SSV2 and pSSVx viruses were spotted onto the soft layer of a Gelrite plate seeded with *S. solfataricus* P2 or *S. islandicus* REY15A; the plates were incubated at 80°C for 2–3 days and turbid halos (plaques) resulting from the inhibition of host growth by SSV2 appeared; the virus-infected cells present in the plaques were extracted and revitalized in liquid medium. Finally, single colonies of SSV2-P2 and SSV2-REY15A were isolated and purified as described for REY31A.

Characterization of *S. islandicus* strains

Comparison of the *S. islandicus* REY strains/isolates were conducted by cloning and sequencing the gene codings of 16S RNA, MCM (mini-chromosomal maintenance protein) and the orotate phosphoribosyltransferase and orotidine-5'-monophosphate decarboxylase (*pyrEF*). The primers designed for PCR cloning of the above genes were listed in Table 1. PCR reaction was conducted as described for detecting SSV2 integration (see below) except that the annealing temperature was set at 55°C. The amplified PCR products were first purified by means of the QIAGEN PCR Purification Kit and then sequenced using PCR primers and additional internal sequencing primers. DNA sequencing was carried out via dye terminator chemistry and MegaBACE 1000 (Molecular Dynamics/Amersham). The sequences obtained were analysed using Sequencher 4.2 (Gene Codes Corp).

Media and growth conditions

Sulfolobus were colonized on 0.8% gelrite plates as described by Zillig et al. (1994). For liquid cultivation, the basal salt solution was supplemented with 0.1% tryptone, 0.05% yeast extract and 0.2% sucrose (w/v); its pH was adjusted to 3.5 using concentrated H₂SO₄. Incubation was conducted in a 100 or 250 ml Erlenmeyer flask with a long neck, using the Innova 3100 water bath shaker (New Brunswick Scientific Corp). The incubation temperature was 80°C, and the shaking rate 150 rpm.

To determine the number of viable cells in *Sulfolobus* cultures, samples were taken at different OD₆₀₀ values and used for making tenfold dilution series. The diluted

samples were then mixed with 5 ml of the above medium containing 0.2% Gelrite and poured onto prewarmed Gelrite plates. Colonies were counted up after 5 days of incubation at 80°C.

SSV plaque assay

The virus titre of a culture was determined by means of plaque assay using *S. solfataricus* P2 as an indicator strain according to Schleper et al. (1992). The plates were incubated for 2–3 days at 80°C and examined for the appearance of the turbid halos which the inhibition of host growth by SSV2 virus generates.

DNA isolation and characterization of the SSV2 integration into the genome of *S. islandicus* REY15/4

The Plasmid DNAs of SSV2 and pSSVx were prepared from *Sulfolobus* cells using the QIAGEN Spin Miniprep kit. Total DNAs were isolated from 10–15 ml of *Sulfolobus* cultures. Summing up, cell pellets were re-suspended in 400 µl of the lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA and 0.2% N-Lauroyl Sarcosine) and incubated at 55°C in the presence of proteinase K (380 µg/ml) for 2 h. To extract the samples we used phenol twice, phenol-chloroform once and, finally, chloroform. Thereafter, the DNAs were precipitated using ethanol.

To characterize the integration of SSV2 into the *Sulfolobus* genome, total DNA was digested using *SalI*, *EcoRV*, *BglII*, *HindIII* or *BamHI* as restriction enzymes and the resulting DNA fragments were analysed by Southern blot and hybridization, according to the standard procedures (Sambrook and Russell 2001). Two adjacent similar-size fragments (*BglII*–*BglII* and *BglII*–*SalI*) encompassing each a 4,689 bp sequence of the SSV2 genome were obtained by digesting with *SalI* and *BglII* and used as probe. Thereafter, the Random Prime DNA Labelling Kit (Roche Applied Science) was used to label these DNA fragments with radioactive [α -³²P]dATP as described by the manufacturer. Unincorporated [α -³²P]dATP was removed from the labelled probe by gel filtration, using the Nick Columns (Amersham Biosciences).

Identification of the SSV2 integration site by PCR

The primers designed for the PCR identification of SSV2 integration are listed in Table 1. The PCR reactions contained ca. 100 ng of the template DNA, 1 U of Taq DNA polymerase, 1 µM of each primer, 1 µM of dNTP and the buffer needed (final concentration of Mg²⁺ was 1.5 mM) for 30 cycles of amplification (60 s at 95°C, 60 s at the appropriate annealing temperature and 60 s at 72°C) with initial denaturation and final elongation for 5 min at 95 and 72°C, respectively. The amplified

Table 1 Primers used in this study

Name	Sequence
16SArc21F	5'-TTCCGGTTGATCCYGCCGG-3'
16SUni1492R	5'-GGTTACCTTGTTACGACTT-3'
SulMCMfwd	5'-TAGAAMTTAGAAAAMTAAGAAGTA-3'
SulMCMrev	5'-TCTCATKATATTTATTGCTCTTTC-3'
Sulpyr-fwd	5'-GTACTWGGCTCAAAGAATGCTA-3'
Sulpyr-rev	5'-GATATAGCTGARGCTACAATAGAT-3'
Sul16Sp03	5'-GTAAGATTCCAGGCGTTGACTCCA-3'
Sul16Sp04	5'-CCTGGGGAGTACGGTCGCAAGA-3'
SulMCM-p03	5'-MTTGAAGAMGATTTAGTTGAT-3'
SulMCM-p04	5'-GATAGAGTWGCMATTCATGARGCA-3'
SulMCM-p05	5'-GTYTGYTGTTCATTGTCYTCATGW-3'
Sulpyr-p03	5'-TCGTACATTAGAACTCGATGT-3'
Sulpyr-p04	5'-ATAAGTTAAATCTGTTGTTGGA-3'
Sulpyr-p05	5'-GAAACTACGTGTCTTAATCTCACA-3'
Sulpyr-p06	5'-TACTCTACTTTTCAACATTCTTCA-3'
SSV2attAp01	5'-GTGTTCTACCTTTTCCACAGTC-3'
SSV2attA'p02	5'-TGGGTACGTCATTTATTGATCT-3'
SSV2attP'p03	5'-GCTTTTATGCAGTTATTGCTTT-3'
SSV2attPp04	5'-GGGAAATGATTAGATGGTCTTC-3'
SSV2Seq2p05	5'-ACTCTTAATATACCCACTGTGGG-3'
SSV2Seq2'p06	5'-GTATATAACCCCTTGGCTTCTTGTT-3'

products were analysed by means of Agarose gel electrophoresis and photographed.

Estimation of cellular SSV2 and pSSVx content during host growth

To visualize SSV2 and pSSVx DNA accumulation during the growth of host cells, total DNAs were extracted from the samples collected at different incubation stages (as indicated in the individual experiments) from the cultures of *S. islandicus* REY15/4, REY31A, SSV2-P2 and SSV2-REY15A. Total DNAs were isolated from the same amount of the cells (normalized by OD₆₀₀) using the procedure described above. Similar amount of DNAs (estimated by OD₂₆₀ and ethidium bromide fluorescence) was used for restriction digestion and Southern blot analysis.

Normalization of the loaded DNAs was performed using the *alba* gene (SSO0962, one copy per genome) or the correspondent PCR product amplified from the genome of *S. islandicus* for the DNAs of infected *S. solfataricus* and *S. islandicus* REY15/4, respectively.

To determine the SSV2 and pSSVx copies per chromosome, three different sequences, i.e. the *alba* gene, the above mentioned 4.69-kb SSV2 (or the coding region of *vp3* gene) and a *HindIII* 1.7-kb pSSVx fragments, were ³²P-radiolabelled by random priming, yielding to a specific activity of ca. 10⁹ cpm/μg DNA. The same amounts of the radiolabelled probes (1 × 10⁶ cpm/ml for each) were mixed and used for Southern hybridization, according to the standard procedure (Sambrook and Russell 2001).

For the experiments illustrated in Figs. 4 and 7, the host–SSV2 hybrid fragment derived from SSV2 integration into the host genome (one copy per genome) was used for normalization in place of *alba* gene.

Since only one copy of the *alba* gene (or the host–SSV2 hybrid fragment) is present in *Sulfolobus* genomes, SSV2 and pSSVx copy numbers were determined by the ratios of extrachromosomal-derived hybridization signals to chromosomal-derived ones which were quantified using a phosphorimager and the Quantity One 4.2 software (Bio-Rad).

SSV2 copy number has been determined for the samples of REY31A, REY15/4 and SSV2-harboursing *S. solfataricus* from at least three independent growth experiments.

Results

Site-specific SSV2 integration into host chromosomes

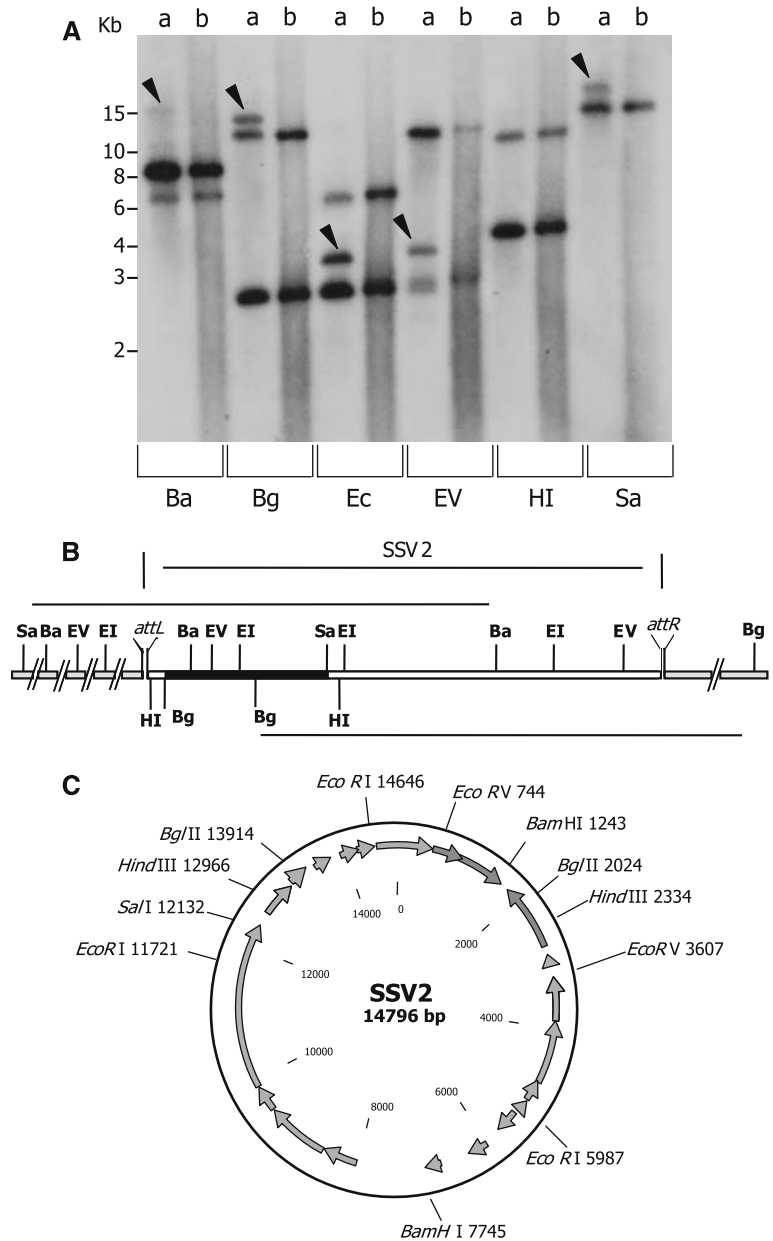
The *Sulfolobus* SSV2 virus encodes a tyrosine integrase (Esposito and Scocca 1997; Nunes-Duby et al. 1998) of the SSV1 integrase family (She et al. 2002; Stedman et al. 2003). To establish if SSV2 generates provirus in

the natural host, the episomal and the genomic DNAs of the REY15/4 strain were prepared from an exponentially growing culture and analysed by Southern hybridization using the 4.7 kb SSV2 fragment as a probe (see [Materials and methods](#)). As expected, in REY15/4 cells the SSV2 genome was found to be present in an episomal form. However, the fact that the SSV probe detected an additional host chromosome fragment (see arrowheads in Fig. 1a) indicated that SSV2 generates provirus in the natural host. The host–virus hybrid fragments which correspond to the provirus and were obtained by digestion with *EcoRI*, *EcoRV* and *SalI* contain the left attachment site, *attL*, while the only host–virus hybrid fragment from the digestion of *BglII* contains the right attachment site, *attR* (Fig. 1b). Because of the probe used, *HindIII* digestion does not allow the detection of the host–virus hybrid fragments. When the membrane used was hybridized with a pSSVx-specific probe we found that pSSVx was only present in an episomal form and did not integrate into the host chromosome. This result is consistent with the fact that pSSVx does not encode an integrase.

It has been reported that the putative SSV2 *attP* site is located within its *int* gene (Stedman et al. 2003) as for the SSV1 virus. While searching the complete genome of the *S. solfataricus* P2 (She et al. 2001a, b) with the sequence of SSV2 *attP* region using the BlastN tool (Altschul et al. 1990), we found two sequence matches denoted as *attA* and *seq2*, each overlapping a tRNA^{Gly} gene in the downstream halves of the tRNA genes. The only mismatch was in the anticodons (Fig. 2a). Hence we were interested in establishing if the two tRNA^{Gly} genes would act as *attA* sites for SSV2 in the *S. solfataricus* genome.

A PCR approach was employed to establish the integration site of SSV2 into *S. solfataricus* P2 chromosome. Three pairs of PCR primers were designed for use in identifying the putative host–virus hybrid fragments of the SSV2 provirus in each tRNA^{Gly} gene ([Materials and methods](#), Table 1). When genomic DNA prepared from two single clones of SSV2–P2 cells was used as a template, host–virus hybrid attachment sites (*attL* and *attR*) of the predicted sizes were amplified at the tRNA^{Gly} (CCC), but not at the “seq2” site (Fig. 3). Identical results were obtained with the other six clones analysed (not shown). These results suggest that the tRNA^{Gly} (CCC) gene serves as the only *attA* for SSV2 in the *S. solfataricus* chromosome. SSV2 integration into the *S. solfataricus* genome was also investigated using the Southern hybridization procedure and the same probe, as described for SSV2 integration into the natural host. Our finding was that the sizes of the integrated fragments were those predicted assuming the tRNA^{Gly} (CCC) gene to be the attachment site (Fig. 2c), thus confirming that SSV2 integrates only at one site in the *S. solfataricus* genome. The SSV2 integration model is shown in Fig. 2b.

Fig. 1 Identification of the SSV2 provirus in the natural host. **a** Southern analysis of the SSV2 integration. Genomic DNA (“a” lanes) and plasmid DNA (“b” lanes) prepared from an exponentially growing culture of REY15/4 were digested using the restriction enzymes indicated *below*. The sizes of DNA molecular markers (kb) are reported on the *left*. *Arrowheads* appear beside host–virus hybrid fragments of the SSV2 provirus. **b** An illustration of host–virus hybrid fragments. Host–virus hybrid fragments containing the attachment site *attL* or *attR* are illustrated by *lines*. The 4.69 kb SSV2 fragment used as a probe for Southern hybridization is filled *black*. Restriction sites are schematically indicated on both SSV2 and host genomes. *attL* and *attR*: left and right attachment sites of the provirus. Restriction enzymes used: Bg, *Bgl*II; EI, *Eco*RI, EV, *Eco*RV; HI, *Hind*III; Sa, *Sal*I. **c** Restriction map of SSV2 genome. The positions of the restriction enzymes used for the analysis of the SSV2 integration into the genome of REY15/4 are indicated



Accumulation of SSV2 and pSSVx DNA during the growth of *S. islandicus* REY15/4

At an early stage of our experiment, we prepared episomal SSV2 DNA using approximately equal amounts of *S. islandicus* REY15/4 cells from different cultivation batches. In some experiments, we obtained a large yield of episomal SSV2 and pSSVx DNAs; in others, a fairly small amount of plasmid DNAs. To account for this finding, we prepared episomal and total DNA using REY15/4 cells at different optical densities. We used Southern hybridization and the 4.69-kb SSV2 as well as the *Hind*III 1.7-kb pSSVx fragments as probes (see Material and methods), to identify the episomal SSV2 and pSSVx. Figure 4 shows that whereas in early growth phases (0.5–0.9, OD_{600}) episomal SSV2 and pSSVx DNA was produced at a low rate, large amounts of

SSV2 and pSSVx DNA accumulated at maximum cell density (1.25–1.3 OD_{600}). This suggests that there was an induction of SSV2 and pSSVx replication during the growth of the natural host.

Furthermore, multiple topological forms of the SSV2 and pSSVx episomal genomes were observed after the induction of viral replication (Fig. 4, 1.25–1.3 OD_{600}), consistent to the results obtained from the SSV1 studies (Nadal et al. 1986).

Characterizing the SSV2 and pSSVx replication induction process

A typical growth curve of *S. islandicus* REY15/4 is reported in Fig. 5a and sample taking indicated. In a basal medium which contained tryptone, yeast extracts and

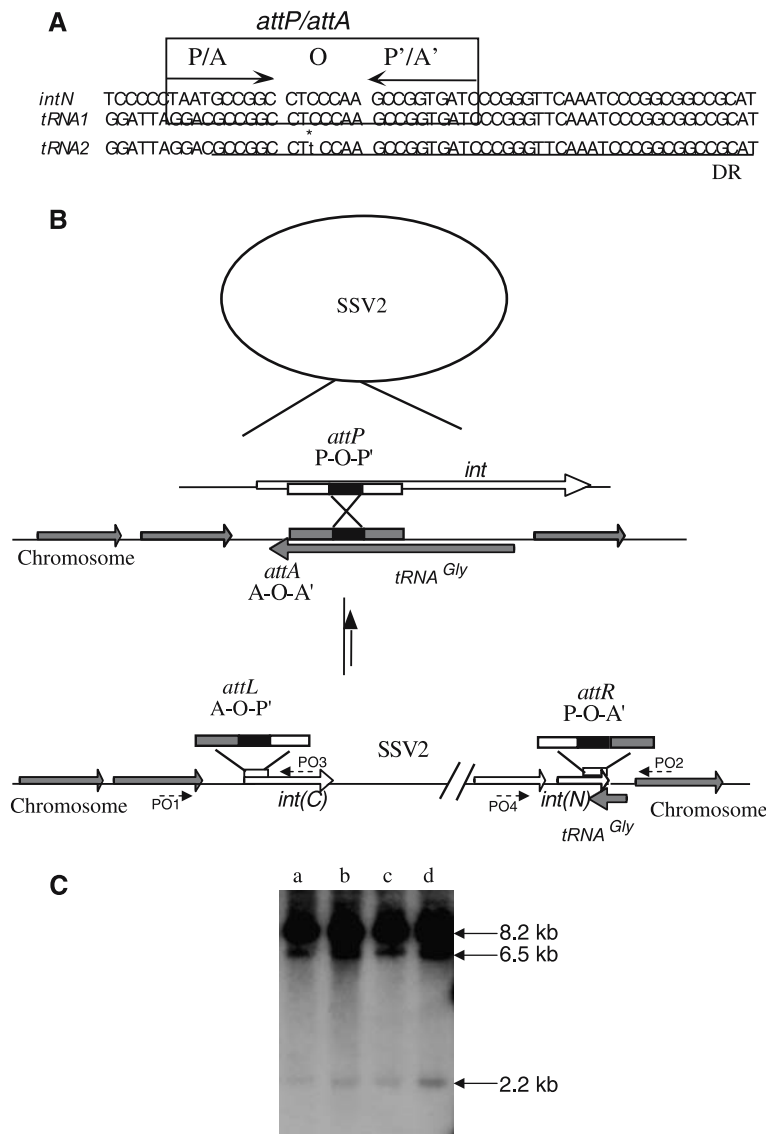


Fig. 2 SSV2 integration in the foreign host. **a** Composition of the SSV2 integration sites. The boxed motifs correspond to the minimal attachment sites identified for the SSV1 integrase in an in vitro assay (25). *attP* (P-O-P'): virus integration attachment site; *attA* (A-O-A'): host integration attachment site. Lines with arrows indicate imperfect inverted repeats (A, A', P and P') which constitute putative binding sites for the integrase. "O" indicates the overlap region where DNA recombination occurs. The *tRNA2* sequence with only one mismatch to the *attA* site, *tRNA^{Gly}* (UCC), has been included for comparison. In the report of the PCR assay this site is named "seq2" (see [Materials and methods](#)). DR bordering the provirus is underlined. DR direct repeat, *tRNA1* *tRNA^{Gly}* (CCC), *tRNA2* *tRNA^{Gly}* (UCC). **b** A graphic presentation of SSV2 integration/excision. Unfilled ORFs are those of the SSV2 genome, shaded ORFs are those of the *S. solfataricus* P2

genome. The composition of all SSV2 attachment sites is illustrated. The non-filled box represents P or P'; the inverted repeat of the *attP*, box filled black indicates the overlap region "O", and the shaded box represents A or A', the inverted repeat of the *attA*; *int* is the integrase gene, *attL* (A-O-P') and *attR* (P-O-A') are the left and right host-virus hybrid attachment sites, respectively. The positions of the PO1 and PO2 primers on the host chromosome and of PO3 and PO4 primers on the SSV2 genome are schematically indicated. **c** Southern analysis of the SSV2 integration into the genome of *S. solfataricus*. Genomic DNAs prepared from four independent SSV2-infected *S. solfataricus* clones were digested with *Bam*HI. The 8.2 and 6.5 kb fragments correspond to the episomal SSV2, whereas the 2.2 kb sizes is the host-virus hybrid fragment of the SSV2 provirus

sucrose, REY15/4 grew in a generation time of about 4.5 h as long as the OD₆₀₀ value was below 0.4. This growth rate slowed down slightly at higher OD₆₀₀ values and stopped altogether when the OD₆₀₀ value rose to 1.3 approx. Interestingly, the optical density of the cultures remained fairly constant for at least 20 h and no

apparent cell lysis occurred in that period. In contrast, the pH of the culture changed very little at cell densities (OD₆₀₀) below 1.3; thereafter it gradually increased to pH 5.5 in 20 h (Fig. 5a).

To shed further light on the increase in SSV2 and pSSVx observed during the growth of the natural REY

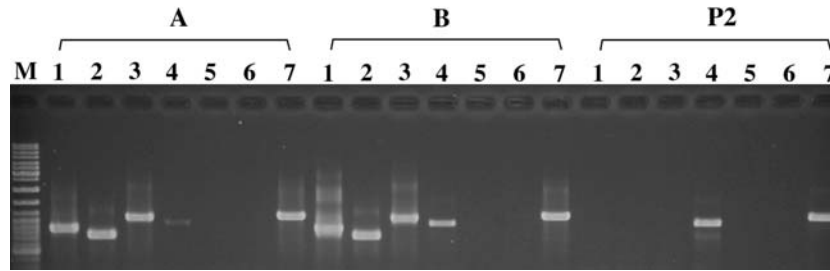


Fig. 3 PCR amplification of SSV2 integration attachment sites. Total DNAs were prepared from two independent SSV2-containing *S. solfataricus* clones (A and B) and an uninfected *S. solfataricus* P2 (P2) and used as templates for PCR. For each sample, PCR products containing *attP* (the SSV2 viral integration site, 912-bp) and the host integration site *attA* (968-bp) or a sequence motif which is very similar to *attA* (named seq2) were

loaded in lanes 1, 4 and 7, respectively; lanes 2 and 3 are the PCR amplifications of the virus–host hybrid fragments of *attL* and *attR*, with the sizes of 722 and 1,158-bp, respectively; Lanes 5, 6 are PCR amplifications for the virus–host hybrid fragments at the seq2 site (predicted to be 1,195 and 843 bp, respectively). M: DNA kb ladder. Identical results were obtained with the other six SSV2-containing *S. solfataricus* clones analysed

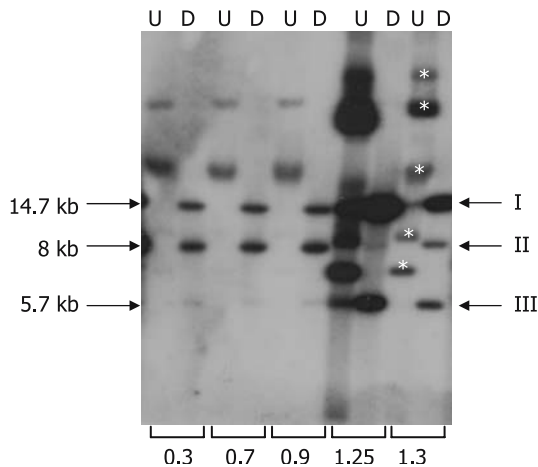


Fig. 4 Content of the virus DNAs in REY15/4 cells of different optical densities. The OD_{600} values of the *S. islandicus* REY15/4 cultures used for preparing the genomic DNA are reported at the bottom. Lanes labelled with “D” indicate the DNA samples digested with *ClaI* restriction enzyme (one cut for both SSV2 and pSSVx genomes). Lanes marked with “U” indicate undigested total DNAs. The linear SSV2 and pSSVx are indicated. “I” indicates the linearized episomal SSV2 (14.7 kb), “II” indicates the integrated SSV2 (8 kb) and “III” indicates the linearized episomal pSSVx (5.7 kb). The amount of total DNAs at 1.25 and 1.3 OD_{600} values was respectively ten and fivefolds lower, than that used for samples at 0.3–0.9 OD_{600} . The asterisks indicate the multiple topological forms of the SSV2 and pSSVx episomal genomes observed after the induction of viral replication

host we collected REY15/4 cell samples at 2-h intervals during incubation (see [Materials and methods](#)). Over the first 24 incubation hours, no episomal SSV2 and pSSVx bands were visible in the total DNAs prepared from rapidly growing REY15/4 cells; thereafter, episomal SSV2 and pSSVx DNA accumulated rapidly reaching a maximum within 4 h, i.e. between the 26th and 30th incubation hours (Fig. 5b). This indicates that the increase of SSV2 and pSSVx must be traced to the induction of SSV2 and pSSVx replication which sets in 4 h after the growth of host cells came to a stop.

The extent of SSV2 and pSSVx replication induction was determined via Southern blot from three independent

cultures and the results of a typical experiment are shown in Fig. 6. Since there is only one copy of *alba* gene in *S. islandicus* REY15/4 genome, the amount of SSV2 was measured at every growth stage as the ratio episomal/chromosomal radioactive signals via a phosphorimager (see [Materials and methods](#)). The episomal SSV2 was produced at a rate of just one copy per chromosome approx during the exponential growth phase and a 50-fold increase (on average) occurred within 4 h upon the growth stop (Fig. 6b).

The amount of pSSVx was under the limit of Southern blot detection between the 16th and 26th hours, therefore a precise evaluation of pSSVx increase was not possible. However, the pSSVx genome was induced in a growth-dependence fashion, similarly to SSV2. At the latest sample-taking points analysed (30th–32nd hours, Fig. 6) the estimated copy number was ca. 2–5 copies per cell. Moreover, after repeating this experiment several times, we found that the copy number of pSSVx in a rapidly growing REY15/4 varied from undetectable to detectable by Southern analysis. Nevertheless, there is a general trend of increase of pSSVx copies upon viral replication induction (Figs. 4, 5b, 6).

It was previously shown (Arnold et al. 1999a; Stedman et al. 2003) that SSV2 replication is not dependent on pSSVx in *Sulfolobus solfataricus* P1 cells. We were interested in establishing if pSSVx contributes to the SSV2 replication induction observed in the natural host REY15/4. To obtain a pSSVx-free strain for investigation, a REY15/4 culture was left to grow continuously for approx. 100 generation times at an optical density below OD_{600} 0.4. Thereafter, we obtained single colonies via Gelrite plating and one of these, REY31A, was used to investigate the physiology of SSV2 replication, after checking carefully the absence of pSSVx (see [Materials and methods](#)).

Figure 7a shows that the growth characteristics of REY31A were similar to REY15/4. Moreover the analysis of SSV2 DNA content performed by quantification of the radioactive signals after Southern blot, revealed that the induction of SSV2 takes place in

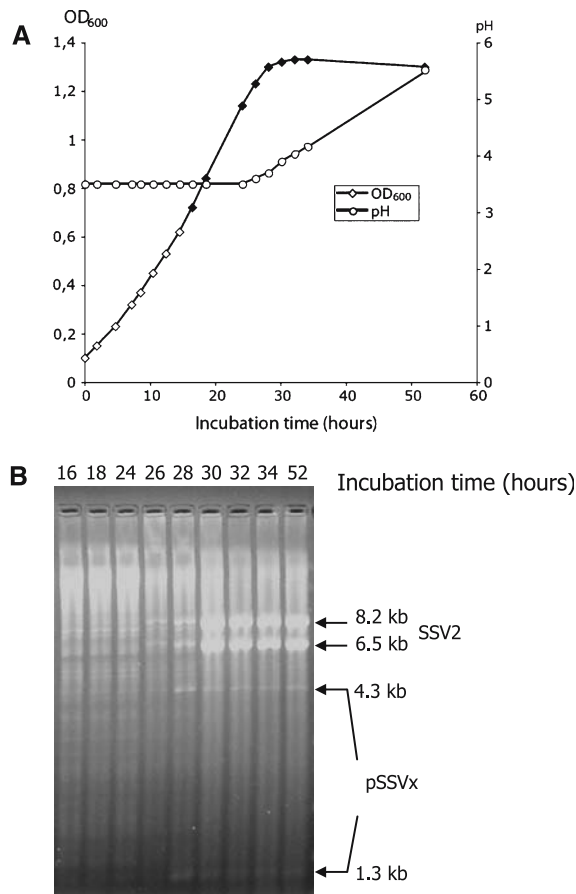


Fig. 5 Induction kinetics of SSV2 and pSSVx replication. **a** Growth characteristics of the *S. islandicus* REY15/4. During incubation, the OD₆₀₀ (on the first Y axis) and the pH (on the second Y axis) of the culture were measured and plotted versus the incubation time. At the same time, samples were also taken for preparing total DNA. **b** Relative content of the episomal SSV2 and pSSVx genomes in the REY15/4 cells during incubation. The genomic DNA was prepared from REY15/4 samples indicated by *solid cycles*, digested with *Bam*HI, and analysed by agarose gel electrophoresis. The agarose gel was stained with ethidium bromide and a picture was taken using a Kodak system. Two *Bam*HI fragments of the episomal SSV2 and pSSVx are indicated with their sizes

REY31A cells with the same modality and at the same extent described for REY15/4 cells (Fig. 7b). These findings indicate that the presence of pSSVx is dispensable to the SSV2 replication induction process.

To establish if SSV2-determined growth inhibition is a reversible process, we carried out a dilution experiment, i.e. we released the REY15/4 host cells into fresh medium at a 10–20:1 ratio after the virus induction process had set in. The growth rates and quantities of SSV2 DNA in the diluted cultures were estimated as described in *Materials and methods*. Our results showed that host cells grow exponentially after a lag phase of varying length (Fig. 8a) and that thereafter the average SSV2 cellular content reverts to ca. one copy per chromosome, as shown in the Southern blot of Fig. 8b.

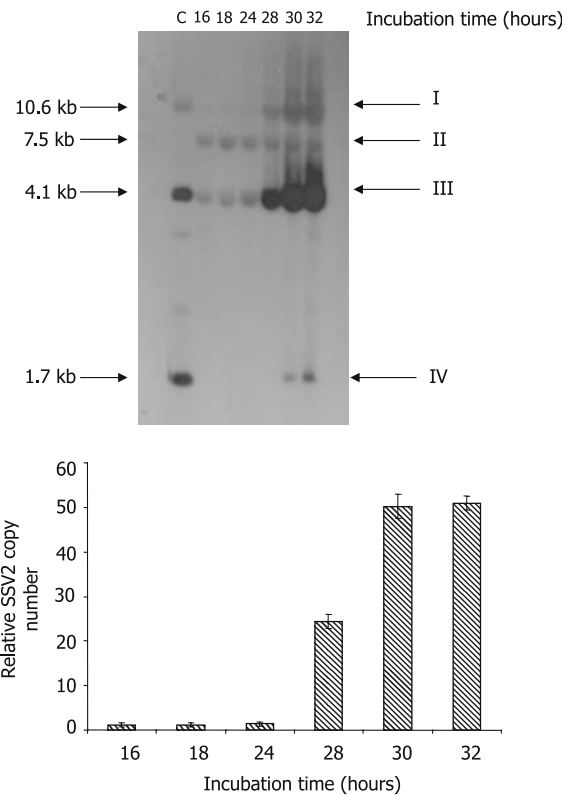


Fig. 6 Estimate of the SSV2 induction level in REY15/4 cells. **a** Total DNAs of REY15/4 *Sulfolobus* cultures were prepared at the indicated incubation times and analysed by *Hind*III restriction digestion and Southern hybridization. Lane C was loaded with *Hind*III digests of SSV2 and pSSVx episomal DNAs, as positive controls. A mix composed of ³²P-radiolabelled *alba* gene (SSO0962 amplified from the *Sulfolobus* REY15/4 genome), the *Bgl*II and *Sal*I fragments of the SSV2 genome and a *Hind*III-pSSVx 1,7 kb fragment (see *Materials and methods*), were used as probes. The sizes of the radioactive signals of SSV2 (I and III), pSSVx (IV) and the chromosomal restriction fragment containing the *alba* gene (II) are reported on the left. The SSV2 probe used does not allow the detection of the integrated SSV2 host–virus fragment (see Fig. 1a, b). **b** The SSV2 copy number in REY 15/4 cells was calculated as the ratio of the radioactivity counts (cpm) of the SSV2 hybridized signal at a specific growth stage to that of the *alba* gene (see *Materials and methods*). The graphic bars show the increase of SSV2 copy number and the error-bars represent the standard deviations for five independent experiments

Virus packaging capacity during host growth

To decide if SSV2 DNA accumulation was caused by the lower virus packaging capacity of the host, we also measured the SSV2 virus titre of the REY15/4 culture. The virus particle titre was found to be fairly low ($2\text{--}4 \times 10^3$ cfu/ml) in the exponential growth phase but rose tenfold after the host had entered the late exponential growth-phase (see below). Thereafter, it remained approx. 1×10^5 until the latest sample-taking points. These results indicate that when SSV2 replication is induced a concurrent, consistent packaging and extrusion of the virus particles occurs and that the fact that free episomal SSV2 DNA accumulates in the cytoplasm must be traced back, not to any reduced viral DNA packaging

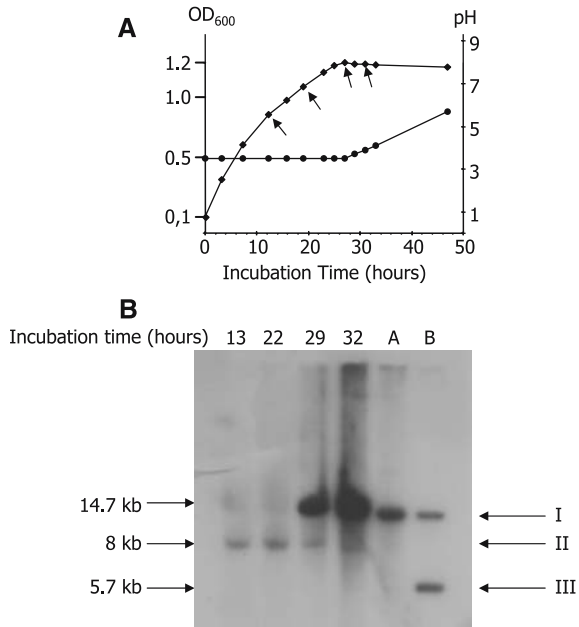


Fig. 7 Analysis of the growth characteristics of REY31A and evaluation of SSV2 induction level. **a** Growth characteristics of the *S. islandicus* REY31A. OD_{600} and pH values of the culture were measured and plotted versus the incubation time. **b** Total DNAs from equal amounts of REY31A cells (normalized by OD_{600}) were prepared at the incubation times reported at the top and indicated by arrows on the growth curve of panel **a**. DNAs were digested with *Cla* I (one single cut for both SSV2 and pSSVx genomes) and the probes used are the SSV2 4,69 kb and a pSSVx-*Hind*III 1,7 kb restriction fragments, this latter included to demonstrate the absence of pSSVx. The size of SSV2 (I), pSSVx (III) and the host-virus hybrid fragment (II) are indicated on the left. *Cla*I digests of purified SSV2 (lane A) and SSV2 together with pSSVx (lane B) were loaded onto the same membrane as positive control for hybridization. The radioactive signal of the host-virus hybrid fragment was used to normalise the amount of the DNA loaded and to determine the SSV2 amount at every growth stage (see [Materials and methods](#))

and/or exportation capacity, but rather to a higher replication rate of SSV2.

SSV2 replication in *S. solfataricus* P2

To investigate host-virus interaction between SSV2 and a foreign host, an SSV2 infected *S. solfataricus* P2 culture was revitalized in liquid medium from areas of inhibition growth plaques (see [Materials and methods](#)). As it is shown in Fig. 9a cells grew up with a generation time of about 9–10 h after a lag of ca. 20 h and reach the maximum value of ca. 0.6 OD_{600} . However, when single colonies obtained by this liquid culture after two subsequent cycles of streaking and tooth-picking were made to grow in parallel with uninfected cultures, the virus-containing and virus-free strains showed the same growth characteristics throughout cultivation, indicating that once established in a foreign host SSV2 does not affect the growth of this host. SSV2-P2 single clones

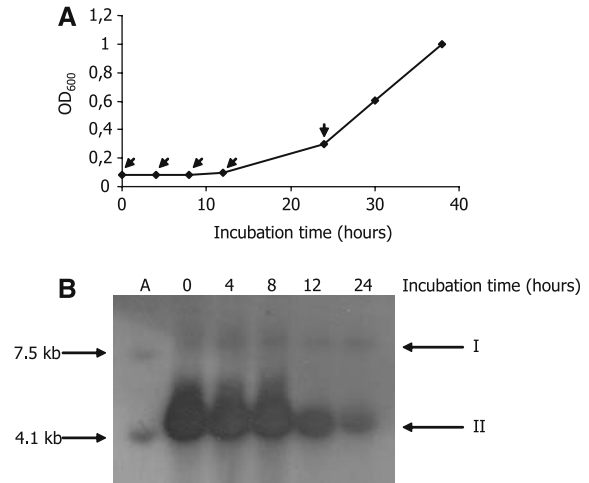


Fig. 8 The SSV2 induction process is reversible. **a** *S. islandicus* REY15/4 cells were diluted in fresh medium at a 10–20:1 ratio after the virus induction process had set in. OD_{600} values were plotted versus the incubation time. Samples taken for preparing total DNAs are indicated by arrows. **b** Total DNAs from equal amounts of REY15/4 cells (normalized by OD_{600}) were digested with *Hind*III and the membrane hybridized with the coding region of the *vp3* gene (13,652–13,930 bp on the SSV2 genome) and the REY 15/4 *alba* gene, as probes. The sizes of the radioactive signals of the chromosomal restriction fragment containing the *alba* gene (I) and the episomal SSV2 (II) are reported on the left. Lane A: Total DNA from REY15/4 cells, before the SSV2 replication had set in. Lanes 1–5: Total DNAs from REY15/4 cells once SSV2 replication induction had set in and harvested at different incubation hours after dilution into fresh medium (see arrows in **a**). A progressive decrease of episomal SSV2 content occurs within 24 h after dilution of SSV2-induced REY15/4 cells into fresh medium

isolated from the plate, did not contain the pSSVx (not shown).

Southern blot analysis performed on total DNAs of a SSV2-P2 isolate, using the *vp3* coding region of SSV2 genome and the *alba* gene as probes, showed that the SSV2 content in infected *S. solfataricus* cells remained fairly constant throughout the growth. This demonstrates that SSV2 replication correlates with the growth rate of *S. solfataricus* and that the replication induction observed in the natural host does not occur in the foreign host (Fig. 9b).

The virus particles production, determined throughout SSV2-P2 cultivation, starts during the exponential growth, reaching its maximum peak (1.1×10^5 cfu/ml) before cells approach to the stationary phase and eventually decreases.

The mechanisms governing the induction of SSV2 replication

Compared to virus-free *Sulfolobus* cultures, for REY15/4 cells (Fig. 5a) we always observed a sudden halt in growth and a substantial difference in final optical density values (typically 1.3 vs. 2.5, OD_{600}), i.e. a sign pointing to growth inhibition. Since growth inhibition

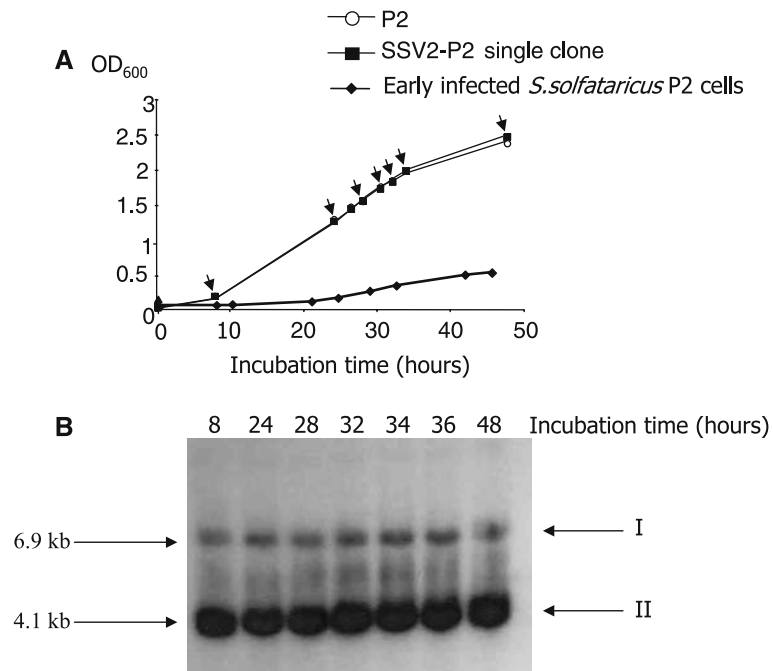


Fig. 9 Characterising *S. solfataricus* P2 cells containing SSV2 virus. **a** Comparison among growth rates of uninfected *S. solfataricus* P2 (filled circle), early infected *S. solfataricus* P2 cells (filled diamond) and a single clone of SSV2-harboured P2 (filled square). **b** Southern blot analysis of total DNAs extracted from a SSV2-containing *S. solfataricus* single clone at the incubation hours indicated in (a). DNAs were digested with *Hind*III and the

membrane hybridized with the coding region of the *vp3* gene (13,652–13,930 bp on SSV2 genome) and the *alba* gene (SSO0962), as probes. The size of the host genome fragment containing the *alba* gene (I) and SSV2 radioactive signal (II) are indicated on the left. The *Hind*III digestion and the probe used do not allow the detection of the integrated SSV2 host–virus fragment (see Fig. 1a, b)

coincided with the onset of the viral DNA replication induction, we resolved to investigate the mechanisms governing these two processes and the relations between them. For this purpose we isolated a *S. islandicus* strain (which is closely related to the REY15/4 strain) and obtained virus-harboured strain (SSV2-REY15A) as described in [Materials and methods](#). Figure 10 shows that the three SSV2-REY15A cultures grew in the same manner as the REY15/4 strain. However, in the infected cultures, growth came to a stop at 1.3 (OD₆₀₀), whereas the uninfected REY15A culture continued to grow for 20 h reaching cell density of 2.0–2.5 (OD₆₀₀). Analysing the total DNA in the SSV2-infected REY15A cells during growth, we found that SSV2 replication sets in within 4 h after the end of cell growth, as in the natural host of SSV2.

To establish whether the uninfected REY15A was actively growing beyond 1.3 OD₆₀₀, when we observed the sudden halt of SSV2-REY15A cells, we took nine cell samples between 1.1 and 2.2 OD₆₀₀, determined the colony formation units (CFU) by Gelrite plating (see [Materials and methods](#)) and found a linear relationship between the CFUs and OD₆₀₀ values of REY15A cells (not shown). Moreover, the CFU/OD₆₀₀ fell into the range of $7.29 \pm 0.66 \times 10^8$ indicating that the *Sulfolobus* cells were still able to divide when the OD₆₀₀ of a REY15A culture increased up to 2.2 OD₆₀₀. Thus, the sudden halt of the optical density increase of SSV2-REY15A cultures reaching 1.3 OD₆₀₀, reflected growth

inhibition occurring in actively dividing culture at a growth stage corresponding to an exponential growth phase.

Since REY15A and REY15/4 are very closely related to each other, we inferred that the same would be applicable to the natural host REY15/4, i.e. growth inhibition occurred at a late exponential growth phase and REY15/4 cells would continue to grow and reach a higher cell density level (as for REY15A) if it was cured of SSV2.

Discussion

SSV provirus formation is furthered by virus-encoded integrases of the tyrosine integrase family (Esposito and Scocca 1997; Nunes-Duby et al. 1998). Like most integrative viruses, bacteriophages and plasmids, the *Sulfolobus* virus SSV2 integrates into host chromosomes at the host integration attachment sites located at the 3' end of the tRNA (CCC) gene (Campbell 1992; Reiter et al. 1989; She et al. 2002; Williams 2002). SSV2 integration into host chromosomes is highly sequence-specific: integration occurs only at the sequence-identical *attA* sites of *S. islandicus* REY15/4 and *S. solfataricus* P2, and it does not occur at the “seq2” site of the tRNA^{Gly} (UCC) gene of the latter because of a sequence mismatch. In comparison, SSV1 integration into the genome of *S. solfataricus* did occur

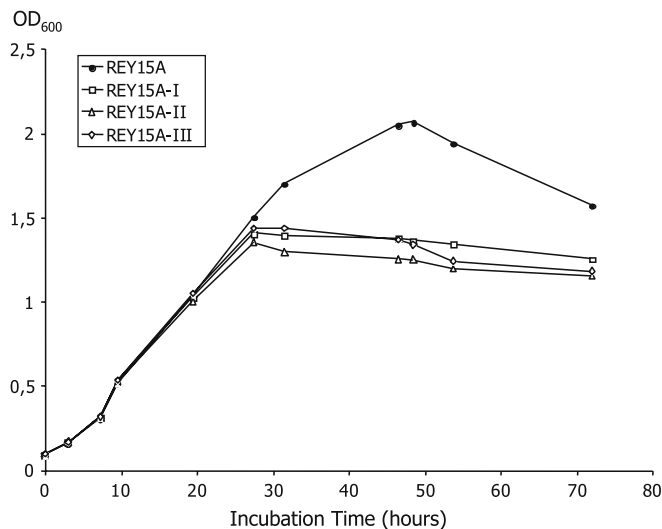


Fig. 10 Coupling of host growth inhibition and SSV2 replication induction in REY15A. *S. islandicus* REY15A and its three SSV2-containing strains, REY15A-I, II, III were grown in a TYS medium. The results were the same in three experiments carried out in succession

at the sequence of the foreign host that exhibited one mismatch versus *attA* of the natural host (Schleper et al. 1992) although it turned out that a sequence-identical SSV1 *attA* site is absent from the complete genome of *S. solfataricus* P2 (She et al. 2001b). Apparently, it remains to be studied whether SSV2 would integrate into the “seq2” site when the perfectly matched target is removed from the foreign host. Thus, further comparative studies of all known SSV integration systems will help shed light on the factors underlying the site-specificity of this viral integration.

Infecting *Sulfolobus* species with SSV viruses always retards host growth during which a large amount of virus particles has been produced, leading to the formation of the inhibition spots (plaques) on solid medium. *Sulfolobus* SSV2 virus is no exception to this rule (Stedman et al. 2003). However, once settle in the foreign *S. solfataricus* host, SSV2 no longer inhibits host growth and this is in strict contrast to what is seen in early infected cells. Neither does SSV2 genome exceed over a few copies per cell throughout cultivation. Consequently, there is a major difference in growth rates between the early SSV2-infecting culture revitalized from a plaque and SSV2-harbouring strain of *S. solfataricus*: the former showed a greatly retarded growth and the latter exhibited a similar rate of growth as an uninfected control. This suggests that a series of interactions between SSV2 and its foreign host will eventually lead to a co-existence harmony between them as seen for the SSV2-carrying *S. solfataricus* strain. It is thus very interesting to investigate whether this conversion of host response is a general scheme for the host defence of *S. solfataricus* to viral infection and to study the molecular mechanisms governing it.

Another interesting feature of SSV2–host interaction lies on the fact that SSV2 DNA replication is characterized by a physiological induction in the natural host. Viral genome copy number increases up to 50-fold within 4 h. We have shown that growth inhibition of the host concurs with the virus replication induction and the inhibition takes place at a late exponential growth phase. Moreover, the inhibition effect is a reversible. Upon being released into a fresh medium, the SSV2-induced *Sulfolobus* culture resumes their exponential growth and host regains control over SSV2 replication. Whereas it appears that the replication induction must have been turned on by an unknown internal signal(s), either originated from virus or host, it is not clear if the growth inhibition constitutes part of host self-defence mechanism or it is conferred by a signal related to the SSV2 replication induction and/or another SSV2 signal. Nevertheless we suggest that SSV2–host interaction may perform a dual function: (1) ‘freezing’ host metabolism into an inactive state under unfavourable or detrimental environmental conditions; (2) enabling the cells to react more quickly to changes in their ecological niches and, thus, to ‘resume’ their metabolic activity when favourable conditions are restored in the environment. This points to a physiological role of the SSV2 virus in host survival and to a mutually beneficial interaction (like those of some bacteriophage–host systems), which increase the host’s metabolic activity and/or environmental fitness (Ackermann and DuBow 1987; Weinbauer 2004). Moreover, this kind of interaction is specific to REY strains but not to *S. solfataricus*, indicating that the specific interaction of SSV2 with its natural host is a prerequisite to initiate the growth-phase regulated virus replication process. Clearly, there is interesting molecular biology behind these phenomena and further investigation should reveal mechanisms governing host–virus interactions as well as co-evolution of viruses and their hosts in archaea.

In addition to the SSV viruses, another temperate archaeal virus, *Acidiamus* two-tailed virus (ATV), has recently been described (Prangishvili et al. 2006b). As for the SSV1 virus, ATV infection of the cells growing under optimal conditions produced lysogenization and the lysogeny can be interrupted by adding mitomycin C or exposing to ultraviolet radiation. Furthermore, ATV infection can also be induced by cold-shock (Prangishvili et al. 2006b). Nevertheless, the induction of SSV1 and ATV involves only external factor(s), and by contrast SSV2 replication induction is induced by endogenous signal(s). Like most crenarchaeal viruses, the induction of SSV2 replication does not lead to the lysis of host cells. By contrast, the ATV virus causes host lysis under environmental stress, constituting the first example of lytic virus ever described for crenarchaea (Prangishvili et al. 2006b). Thus, these viruses provide models for studying mechanisms and evolution of virus infection in archaea.

Sulfolobus virus study was initiated to identify suitable genetic elements for constructing host-vector systems and SSV1 virus has been successfully used for this

purpose. Several genetic systems have been reported including most recent developments in gene reporter, protein expression and gene expression systems (Jonuscheit et al. 2003; Albers et al. 2006; Lubelska et al. 2006). Thus, one of the next targets in archaeal virus study is to conduct systematic characterization on selected host–virus systems using genome microarray and genetic knockout as well as genetic complementation technologies to reveal principles of archaeal molecular biology.

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