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Genetic Requirements for the Function of the Archaeal Virus SSV1 in *Sulfolobus solfataricus*: Construction and Testing of Viral Shuttle Vectors

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ABSTRACT

Directed open reading frame (ORF) disruption and a serial selection technique in *Escherichia coli* and the extremely thermophilic archaeon *Sulfolobus solfataricus* allowed the identification of otherwise cryptic crucial and noncrucial viral open reading frames in the genome of the archaeal virus SSV1. It showed that the 15.5-kbp viral genome can incorporate a 2.96-kbp insertion without loss of viral function and package this DNA properly into infectious virus particles. The selection technique, based on the preferential binding of ethidium bromide to relaxed DNA and the resulting inhibition of endonuclease cleavage to generate a pool of mostly singly cut molecules, should be generally applicable. A fully functional viral shuttle vector for *S. solfataricus* and *E. coli* was made. This vector spreads efficiently through infected cultures of *S. solfataricus*, its replication is induced by UV irradiation, it forms infectious virus particles, and it is stable at high copy number in both *S. solfataricus* and *E. coli.* The classification of otherwise unidentifiable ORFs in SSV1 facilitates genetic analysis of this virus, and the shuttle vector should be useful for the development of genetic systems for Crenarchaeota.

ALL known viruses of the extremely thermophilic Crenarchaeota, one of the kingdoms in the domain Archaea, are morphologically unique, and their genomes show very little similarity to other sequences in the public databases (reviewed in Palm *et al.* 1991; Stolt and Zillig 1995; Zillig *et al.* 1998). Thus, these viruses could provide novel useful thermostable enzymes, particularly enzymes of nucleic acid synthesis and modification. Additionally, these viruses open a window into the fundamental cellular processes of the still poorly understood Crenarchaeota.

The complete genomic sequence of the fusellovirus SSV1 (*Sulfolobus shibatae* virus 1) was the first to be determined for a virus of this kingdom (Palm *et al.* 1991). SSV1 both specifically integrates into its host genome and is present in the cell as a circular episome (Yeats *et al.* 1982). Its proliferation is also induced by ultraviolet (UV) irradiation (Martin *et al.* 1984). SSV1 served as the model system for fundamental studies of transcription in the Archaea (*e.g.*, Reiter *et al.* 1987b). However, apart from the viral integrase (Muskhelishvili *et al.* 1993) and the viral structural proteins (Reiter *et al.* 1987a), the activities of the proteins encoded by the other open reading frames (ORFs) have not been determined, although two of them have putative homologues (Koonin 1992; Muskhelishvili 1994). While first found in *S. shibatae* (Yeats *et al.* 1982; Grogan *et al.* 1990), SSV1 was recently shown to infect *S. solfataricus* (Schleper *et al.* 1992).

S. solfataricus has become one of the best-studied extremely thermophilic organisms for several reasons: optimal growth at 80°, a pH optimum of 3, heterotrophy, ease of plating, aerobiosis (Zillig *et al.* 1994), and the more than 30 plasmids and viruses that have been found in Sulfolobus isolates (reviewed in Zillig *et al.* 1996, 1998). The complete genome of *S. solfataricus* is also being determined (Sensen *et al.* 1996, 1998). However, fundamental understanding of the genetics of Sulfolobus has lagged behind biochemical characterization of certain gene products due to the absence of a stable transformation system and methods for specific gene disruption. Complete comprehension of the data from the genome sequence also requires such a system. Recently some progress has been made in this field (Elferink *et al.* 1996; Aravalli and Garrett 1997; Cannio *et al.* 1998), but the usefulness of these vectors is limited, particularly due to plasmid instability and low copy number.

Vectors based on SSV1, on the other hand, are attractive, because the SSV1 genome is present at relatively high copy number in Sulfolobus as a circular covalently closed plasmid (Yeats *et al.* 1982) and *S. solfataricus* can be readily transformed with this DNA (Schleper *et al.* 1992). It is also present in infected cells as an integrated copy in the genome and thus should be stably maintained. In fact, cells containing the virus could not be cured of it (Reiter and Palm 1990; C. Schleper, unpublished data).

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TABLE 1

Strains and plasmids

	Comments	Reference
Strain		
S. solfataricus P1	Wild type	DSM1616
S. solfataricus PH1	*lacS*	Schleper *et al.* (1994)
S. solfataricus P2	Wild type	DSM1617
S. solfataricus GΘ	MT3 derivative	Cannio *et al.* (1998)
S. shibatae B12	Original SSV1 host	Grogan *et al.* (1990)
S. acidocaldarius	Wild type	DSM639
E. coli DH5α	F^- (*φ80dlacZΔM15*) *supE44 hsdR17 thi-1 recA1 endA1 deoR gyrA96 relA1* Δ(*lacZYA-argF*) U169	Woodcock *et al.* (1989)
Plasmids		
pBluescript II	Cloning plasmids (SK^+, KS^+)	Stratagene
pC32	Entire SSV1 genome in *Bam*HI site of pBR325	Yeats *et al.* (1982)
$pC32^{-Sal}$	pC32 with *Sal*I site in pBR325 deleted	H. P. Arnold, personal communication
pKMSD2	*Apa*I-*Sal*I fragment of SSV1 genome in pBluescript II SK+	This work
pKMSD4	Entire SSVI genome with a point mutant to introduce a *Bgl*II site in the putative SSV1 origin in $pC32^{-Sal}$	This work
pKMSD48	pBluescript II SK+ in SSV1 (*Sau*3AI selection)	This work
pKMSD54	pBluescript II KS+ in *Pst*I-cut SSV1 SK[a]	This work
pKMSD55	pBluescript II KS+ in *Pst*I-cut SSV1 KS[a]	This work
pKMSD59	pBluescript II KS+ in *Nhe*I-cut SSV1 SK[a]	This work
pKMSD60	pBluescript II KS+ in *Nhe*I-cut SSV1 KS[a]	This work

[a] Orientation of restriction endonuclease sites in the pBluescript II KS+ polylinker relative to the transcription of SSV1 transcript T5.

As a first step in the development of molecular genetics for Sulfolobus and to genetically dissect the functions of SSV1, this article reports the identification of a number of crucial ORFs in SSV1. Via a genetic selection technique one nonessential ORF was identified, allowing the construction of recombinant viral shuttle vectors and the identification of an additional nonessential ORF. These vectors replicate stably and at high copy number in both *E. coli* and *S. solfataricus.* Along with previous work in the field (Elferink *et al.* 1996; Aravalli and Garrett 1997; Cannio *et al.* 1998), this report provides valuable tools for the development of badly needed genetic systems for extremely thermophilic Archaea.

MATERIALS AND METHODS

Plasmids and strains: See Table 1.

Cell growth: Sulfolobus strains were grown in liquid culture at pH 3.2 with moderate shaking at 80° in long-necked Erlenmeyer flasks. The liquid medium used was similar to that of Brock *et al.* (1972) and contained 0.1% yeast extract (Difco, Detroit) and 0.2% sucrose (Merck, St. Louis) as carbon source. Solid media were made by addition of Gelrite (Kelco) to medium at a final concentration of 0.6% w/v. Soft layers for overlays were made by the addition of Gelrite to 0.2%. Nearly 100% plating efficiency of single colonies was obtained by spreading 100 μl of diluted cultures (generally diluted 10^{-5} in medium) on plates containing 0.2% tryptone (Difco) as a sole carbon source together with 250 μl of melted soft layer also containing tryptone as a sole carbon source. Colonies were visible after 5 days of incubation at 80°. *E. coli* cells were grown in Luria-Bertani medium (LB) with 200 μg/ml ampicillin, either in liquid culture or on a solid surface (1.5% agar, Difco).

DNA isolation: Viral DNA was isolated from Sulfolobus cells essentially by the alkaline lysis method of Birnboim and Doly (1979), followed by three extractions with 1 volume phenol, 1 volume phenol:chloroform:isoamyl alcohol (25:24:1), and 1 volume chloroform. DNA was isolated from virus particles according to Schleper *et al.* (1992). Chromosomal DNA was isolated by lysis of the cells with Triton X-100 and *N*-lauryl sarcosine (final concentrations of 0.06 and 0.8%, respectively). Briefly, 1.5 ml of late logarithmically growing cells (OD_{600nm} = ∼0.7) were centrifuged for 2 min in a microcentrifuge. The cell pellet was resuspended in 250 μl of TEN (10 mm Tris/HCl, 1 mm EDTA, 150 mm NaCl, pH 8.0). A total of 250 μl of TENST (TEN plus 0.12% Triton X-100 and 1.6% *N*-lauryl sarcosine) was added, and the mixture was incubated for 30 min at room temperature. A total of 500 μl of a mixture of phenol (saturated with TE), chloroform, isoamyl alcohol (25:24:1 v/v) was added and mixed by vortexing, and the phases were separated by centrifugation for 10 min in a microcentrifuge at maximum rpm. The aqueous phase was extracted two more times, and then DNA was precipitated by the addition of 2.5 volumes of ethanol. The precipitate was briefly washed with 70% ethanol, dried, and dissolved in 15 μl TE containing 2.5 μg/ml DNase-free RNase (Boehringer Mannheim, Mannheim, Germany). Generally 5 μl of this genomic DNA was used for restriction endonucleolytic analysis. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Kraft *et al.* (1988) or by using an ion exchange column (Tip 100, QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.

Sulfolobus transformation: All strains of Sulfolobus were transformed by electroporation essentially as described in Schleper *et al.* (1992). Immediately after electroporation (1500 V, 400 Ω, 25 μF, 1-mm cell, Bio-Rad, Richmond, CA),

the cells were diluted with 1 ml of hot (80°) medium and incubated for 1 hr at 80° before dilution into prewarmed medium or plating.

Testing for virus function: Plaque tests were performed as in Schleper *et al.* (1992), except that 2–3 ml of soft layer overlay was used. Turbid plaques were observed after incubation of plates at 80° for 2 days. As a rapid screen for virus production, 0.5 μl of growing cultures or aliquots from single colonies were spotted onto a soft layer seeded with *S. solfataricus* P1 or PH1. These plates were incubated at 80° for 1–2 days, and turbid halos were observed around the spots of cultures that were producing virus. Ultraviolet induction of virus production was performed as described in Martin *et al.* (1984).

Electron microscopy: Samples from growing cultures or supernatants from low-speed centrifugation (6000 rpm Heraeus Christ Minifuge II) were applied to carbon-coated grids, stained for 15 sec with 2% uranyl acetate, and observed with a Phillips CM-10 transmission electron microscope at 120 keV.

General molecular biology techniques and materials: Restriction endonucleases, T4 DNA ligase, and Klenow fragment were purchased from M.B.I. Fermantas, Boehringer Mannheim, or New England Biolabs (Beverly, MA). Standard molecular biology techniques were performed as in Sambrook *et al.* (1989). Chemicals were from Sigma, Merck, or Fluka (Buchs, Switzerland) and were analytical grade or higher.

Specific disruption of individual open reading frames: ORFs c792, b115, e51, e178, and d335 were disrupted by completely cleaving SSV1 viral DNA with *Spe*I, *Sau*I, *Pst*I, *Nhe*I, and *Xho*I, respectively, and ligated to similarly digested pBluescript II KS+ plasmid (digested with *Xba*I to ligate to *Nhe*I-cut SSV1 DNA) that had been treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions. In complementary experiments, plasmid C32 (the complete SSV1 genome in the *Bam*HI site of pBR325; Yeats *et al.* 1982) was digested to completion with the restriction endonucleases *Xho*I, *Bst*EII, *Spe*I, and *Sau*I individually. The overhanging ends thus generated were filled in with the Klenow fragment of DNA polymerase. The linear DNA was then treated with T4 DNA ligase and transformed into *E. coli.* Constructs that were no longer cleaved by the respective restriction endonucleases were cut with *Bam*HI to remove the pBR325 DNA and transformed into *S. solfataricus.* Open reading frame b129 was disrupted by cutting SSV1 viral DNA with *Nru*I, the linear DNA isolated by agarose gel electrophoresis, and ligated by T4 ligase to pBluescript II SK+ (Stratagene, La Jolla, CA) that had been linearized by cleavage with *Eco*RV. All plasmid constructions were confirmed by restriction digestion of plasmids prepared from *E. coli* before transformation into *S. solfataricus.*

Disruption of the putative origin of replication of SSV1: PCR was performed on plasmid pKMSD2, which contained the *Apa*I-*Sal*I fragment of SSV1 (Figure 1) cloned into similarly digested pBluescript II SK+. The "left" portion of the insert was amplified and a *Bgl*II site was introduced by using primers T3 (5′-AATTAACCCGTCACTAAAGGG-3′) and *Bgl*-II-SSV1-comp (5′-CGCATTGAAGA**T**CTTCTAAAATC-3′), where the *Bgl*II recognition site is underlined and the mutagenic nucleotide is in boldface. In a separate reaction, to amplify the "right" portion of the insert and to introduce the other half of the *Bgl*II site, the T7 (5′-GTAATACGACTCACTATAGGGC-3′) and the *Bgl*-II-SSV1 (5′-GATTTTAGAAGA**T**CTTCAATGCG-3′) primers were used. The 3.7-kbp and 400-bp fragments thus generated were isolated from agarose gels and mixed, and further PCR was performed using the T3 and T7 primers to generate a full-length insert containing the *Bgl*II site. The resulting 4.1-kbp PCR fragment was cut with *Apa*I and *Sal*I and ligated into plasmid $C32^{-Sal}$ (plasmid C32 cut with *Sal*I and religated to delete one *Sal*I site from the vector; H. P. Arnold, personal communication) to give plasmid pKMSD4. Correct constructs were found in *E. coli* and tested for virus function by cleavage of pKMSD4 with *Bam*HI and transformation into *S. solfataricus.* To determine if this region would tolerate insertions of exogenous DNA, pBluescript II SK+ was inserted into the *Bgl*II site in both orientations. This was performed by cutting pKMSD4 with *Bam*HI to remove the pBR325 DNA and *Bgl*II and ligating the 11.5-kbp band and the 4-kbp bands to pBluescript II SK+ that had been digested with *Bam*HI. The correct constructs were screened in *E. coli* and transformed into *S. solfataricus.*

Partial *Sau*3AI digestion using ethidium bromide: To find optimal conditions for partial digestion, SSV1 viral DNA was incubated with increasing amounts of ethidium bromide (10–200 μg/ml final concentration) in 1 × *Sau*3AI buffer [100 mm NaCl, 10 mm bis-tris-propane HCl, 10 mm $MgCl_2$, 1 mm dithiothreitol, pH 7.0 (New England Biolabs) plus 100 μg/ml bovine serum albumin] with 1 unit of *Sau*3AI (New England Biolabs). The mixture was incubated for 1 hr at 37°. The proteins were extracted with phenol, and the DNA was precipitated and analyzed by agarose gel electrophoresis. When the optimal ethidium bromide concentration was found (this varied from viral DNA preparation to preparation, generally ~50 μg/ml), the same procedure was repeated in multiple tubes in the same volume as the screening experiments. Linear singly cut DNA was identified by examination of ethidium bromide-stained gels with long-wavelength UV light. The DNA was eluted from the gel using adsorption to diatomaceous earth (Prep-A-Gene kit, Bio-Rad) according to the manufacturer's instructions.

Serial selection of a functional shuttle vector and identification of important open reading frames: Partially digested linear DNA of SSV1 was ligated to dephosphorylated and *Bam*HI-digested pBluescript II SK+ (Stratagene) with T4 DNA ligase overnight at 16°. The ligation mixture was transformed into *E. coli* DH5α by electroporation (1.5 kV, 25 μF, 800 Ω, 1-mm cells, Bio-Rad). After electroporation, 800 μl of the electroporation mix was diluted to 25 ml of LB containing 200 μg/ml ampicillin. After overnight culture at 37°, plasmid DNA was prepared by the method of Kraft *et al.* (1988). This plasmid DNA mixture was directly electroporated into *S. solfataricus* PH1 or P1. After 4 days of liquid culture at 80°, extrachromosomal DNA was prepared (Birnboim and Doly 1979; see above) and analyzed by agarose gel electrophoresis.

RESULTS

DNA isolated from *E. coli* transformed *S. solfataricus*: To facilitate the analysis of open reading frames of SSV1, it was important to show that transformation of *S. solfataricus* is possible with SSV1 DNA isolated from *E. coli.* When *S. solfataricus* was transformed with *E. coli* plasmid $C32^{-Sal}$ (the complete SSV1 genome cloned in pBR325; see materials and methods), no replication of viral DNA was observed nor were the cultures capable of forming plaques on lawns of sensitive strains (data not shown). However, when the *E. coli* plasmid DNA was cut out of the plasmid before electroporation virus production was observed (data not shown). This experiment demonstrated that, unlike *S. acidocaldarius* (Aagaard *et al.* 1996), *S. solfataricus* can be efficiently transformed by SSV1 DNA isolated from *E. coli* without modification.

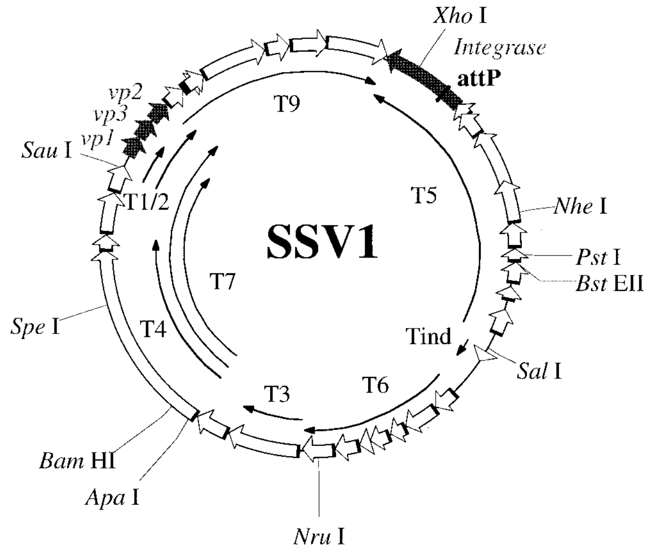

Figure 1.—Map of SSV1. Transcripts are marked with thin arrows. Open reading frames are marked with wide arrows. The four identified genes, *vp1*, *vp3*, *vp2*, and the viral integrase, are labeled and highlighted in gray. The viral attachment site is also marked (attP). All restriction endonuclease sites used in this study are listed. Data adapted from Palm *et al.* (1991) with permission.

TABLE 2

Analysis SSV1 ORFs

ORF (gene)	Modification	Functional virus
d335[a]	*Xho*I insertion/disruption	N
e178	*Sau*3AI insertion[b]	Y
	*Nhe*I insertion	
e51	*Pst*I insertion	Y
e96	*Bst*EII disruption	N
f112	*Sau*3AI insertion	(N)
a132	Four *Sau*3AI insertions	(N)
c80	*Sau*3AI insertion	(N)
c102b	*Sau*3AI insertion	(N)
b129	*Nru*I insertion	N
a291	Two *Sau*3AI insertions	(N)
c792	Two *Sau*3AI insertions	N
	*Spe*I insertion/disruption	
	*Bam*HI insertion	
b78	*Sau*3AI insertion	(N)
c166	*Sau*3AI insertion	(N)
b115	*Sau*I insertion	(N)
vp2[c]	*Sau*3AI insertion	(N)
b277	*Sau*3AI insertion	(N)
a153	*Sau*3AI insertion	(N)
a251[d]	*Sau*3AI insertion	(N)

All ORFs except those marked had no known function. Y, construct made a fully functional virus; N, construct did not make a functional virus; (N), construct was not found in Sulfolobus after serial selection and presumably did not make functional virus.

[a] Viral integrase gene.

[b] The second *Sau*3AI site was not found in the selection; see discussion.

[c] Viral DNA-binding protein.

[d] Reported to be homologous to DnaA by Koonin (1992).

The region near the T_{ind} transcript was crucial for virus function: Like plasmid $C32^{-Sal}$, plasmid pKMSD4, which contains a point mutation introduced *in vitro*, was unable to transform *S. solfataricus* unless the pBR325 portion of the DNA was separated from the plasmid. The point mutation in pKMSD4 created a unique *Bgl*II restriction site and was made outside of all open reading frames in SSV1 (Palm *et al.* 1991) in order not to disrupt any of them; see Figure 5. Plasmids made by the insertion of pBluescript II SK+ into the *Bgl*II restriction site in the mutated SSV1 genome (isolated from pKMSD4) were stable in *E. coli* but, like $C32^{-Sal}$, did not replicate or produce infectious virus particles in *S. solfataricus.* The trivial explanation that SSV1 could not tolerate the pBluescript II SK+ DNA was ruled out by later experiments (see below).

ORFs shown by site-directed disruption to be essential for virus function: The pBR325 DNA insertions in plasmids C32, $C32^{-Sal}$, and pKMSD4 were all in the unique *Bam*HI site of the largest ORF in SSV1, c792 (Figure 1). Because these plasmids did not function as viruses in *S. solfataricus*, it appeared that ORF c792 was indispensable for virus function. A plasmid formed by insertion of pBluescript II SK+ DNA near the C-terminal end of ORF c792, at the *Spe*I site, also did not function as a virus (Figure 1). Insertions in the viral integrase gene, ORF d335 (*Xho*I), and ORF b129 (*Nru*I) also proved to be nonfunctional (Table 2). These data were supported and the list of essential ORFs was extended to ORFs b115 and e96 by disruption of unique restriction sites in the SSV1 genome (see materials and methods; Figure 1; Table 2). These latter experiments demonstrated that insertion of plasmid DNA *per se* did not disrupt viral function, but that the ORFs themselves could not tolerate even small changes. The *Sau*I cleavage site overlaps with the major promoter for the SSV1 structural genes, *vp1*, *vp3*, and *vp2*. This promoter was probably required for virus formation and was possibly inactivated by the disruption of the *Sau*I site.

SSV1 tolerated the insertion of 2.96 kbp of DNA and the ColEI origin of replication: To make a pool of singly cut SSV1 genomes for screening of more ORFs, virus DNA was cleaved with *Sau*3AI in the presence of excess amounts of ethidium bromide. The ethidium bromide intercalated into linear DNA to a much greater extent than into supercoiled DNA (Osterlund *et al.* 1982) and thus blocked further cleavage by the restriction enzyme (Parker *et al.* 1977). Therefore a heterogeneous population of mostly singly cleaved DNA molecules was generated (Figure 2), presumably at every *Sau*3AI site. These DNAs were separated from smaller fragments on an agarose gel, isolated, and cloned into the pBluescript II SK+ plasmid that had been cut with *Bam*HI. The mixture (Figure 2) was transformed into

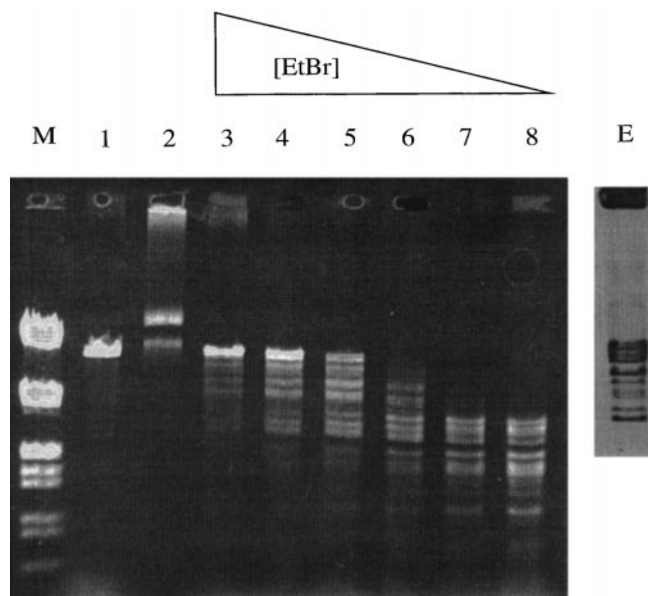

Figure 2.—Partial *Sau*3AI digest of SSV1. Lane M, lambda DNA cleaved with *Eco*RI and *Hin*dIII as size standards. Lane 1, *Bam*HI-digested SSV1 DNA. Lane 2, uncut SSV1 DNA isolated from infected *S. solfataricus* P1 cells. Lanes 3–8, *Sau*3AI-digested SSV1 DNA with decreasing concentrations of ethidium bromide: 80, 70, 60, 50, 40, and 30 μg/ml, respectively. Lane E, DNA prepared from *E. coli* that had been transformed with ligation products of completely digested pBluescript II SK+ DNA and SSV1 DNA partially digested with *Sau*3AI (see lane 3). This is the DNA used for selection of a shuttle vector by transformation into *S. solfataricus.*

E. coli that was then grown overnight in selective medium to select for plasmids containing an *E. coli* origin and antibiotic resistance. Plasmid DNA was prepared, and the DNA was then electroporated into *S. solfataricus.* A single DNA species that was 2.96 kbp larger than the wild-type SSV1 genome was observed in transformed cells (data not shown) and the cells themselves produced turbid plaques on lawns of uninfected *S. solfataricus.* The SSV1 virus incorporated extra DNA into its genome and still remained infectious.

By partial digestion and selection, 1 ORF was not necessary for virus function whereas 11 others appear to have been: The *Sau*3AI site in which the pBluescript II SK+ DNA was integrated was identified by restriction endonuclease digestion of the selected DNA. This site, at nucleotide 3147, was in ORF e178, which therefore must not be critical for virus function. Assuming that the pool of DNA used for the selection contained all possible inserts into *Sau*3AI sites, insertions that did not pass the selection were nonviable. Therefore, 11 additional ORFs, a251, f112, a132, c80, c102b, a291, b78, c166, b277, a153, and the viral DNA-binding protein gene, *vp2*, were potentially essential for virus function (Table 2).

Construction of four additional shuttle vectors: The result of the *Sau*3AI selection was confirmed by complete digestion of SSV1 DNA with the *Nhe*I restriction endonuclease, which also cleaves in ORF e178, and ligation of pBluescript II KS+ DNA into the site in both orientations (see Figure 1). The resulting constructs, pKMSD59 and pKMSD60, also replicated in *S. solfataricus*, indicating that neither orientation nor exact location of the inserted DNA was critical. The nearby ORF e51 was then disrupted by cleavage of SSV1 with *Pst*I and insertion of pBluescript II KS+ DNA in both orientations. The resulting constructs, pKMSD54 and pKMSD55, also replicated in *S. solfataricus* and formed infectious virus particles. All of these specific constructs appeared to be equally stable and have a similar copy number to pKMSD48 (see below).

Plasmid pKMSD48 was a true shuttle vector and replicated stably in both *S. solfataricus* and *E. coli*: The plasmid DNA selected by the *Sau*3AI protocol described above was transformed into *E. coli*, and a single antibiotic-resistant clone was isolated. DNA from this single clone, named plasmid pKMSD48, was retransformed into *S. solfataricus*, and eight independent plaques were investigated. All of the DNAs from these plaques were apparently identical to the original isolate from *S. solfataricus* and *E. coli* (data not shown). Additionally, eight independent *E. coli* clones were picked from a transformation with pKMSD48 DNA. The restriction endonuclease cleavage pattern of each DNA was identical to that of the original DNA (data not shown). A long-term growth experiment was also carried out with transformed *S. solfataricus.* Single clones of *S. solfataricus* containing pKMSD48 were grown under standard conditions (see materials and methods) until they reached an optical density of 0.7. Samples were taken every 2 days, the DNA from the cells was analyzed, and the cultures were diluted 1:50 in the same medium. No change in the DNA was observed even after 3 wk (∼100 generations) of continuous growth.

Plasmid pKMSD48 spread efficiently through a culture after transformation: To check transformation efficiency, *S. solfataricus* was transformed with varying amounts of the plasmid pKMSD48 (10–500 ng) and then checked for plaque-forming units (pfu). Single colonies were also screened for virus production (see materials and methods). One hr after electroporation there were very few transformants, 1 pfu/2.5×10^6 cells, corresponding to the usual transformation frequency of 10^{-5} (Schleper *et al.* 1992). After 1 day of culture the amount increased to 6 pfu/10^4 cells. After 2 days the pfu's had increased to 1.5/cell, after which the pfu/cell remained relatively constant. There was no difference noted at different DNA concentrations.

Plasmid pKMSD48 is UV inducible: The replication of SSV1 was induced by irradiation with ultraviolet light (Martin *et al.* 1984). Therefore *S. solfataricus* cells that had been infected with pKMSD48 were irradiated with ultraviolet light as in Martin *et al.* (1984). The total genomic and extrachromosomal DNAs were then ana-

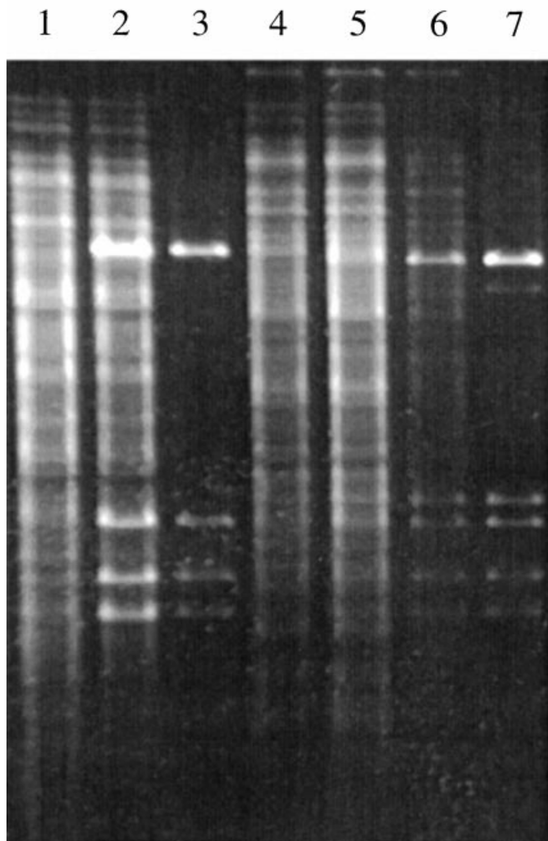

Figure 3.—UV induction of shuttle vector production. Total Sulfolobus DNAs were made as described in materials and methods and cleaved with *Eco*RI before separation on a 0.6% agarose gel. Lane 1, *S. shibatae* B12 (original host of SSV1). Lane 2, *S. shibatae* B12 after UV induction. Lane 3, purified SSV1 DNA. Lane 4, *S. solfataricus* PH1. Lane 5, *S. solfataricus* PH1 infected with pKMSD48. Lane 6, *S. solfataricus* PH1 infected with pKMSD48 after UV induction. Lane 7, purified pKMSD48 DNA.

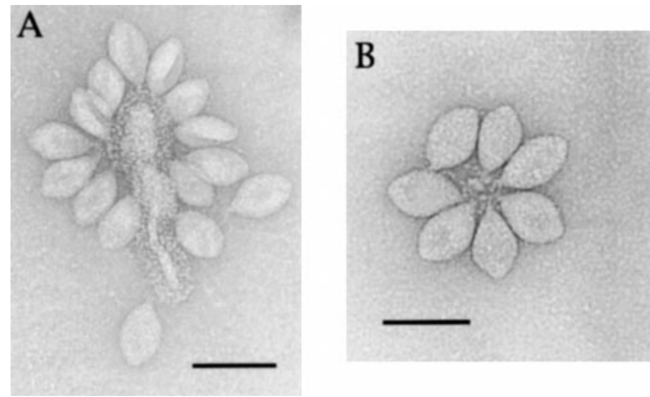

Figure 4.—Transmission electron micrographs of virus particles. Samples are negatively stained with 2% uranyl acetate. (A) Wild-type SSV1 particles attached to a membrane fragment. (B) pKMSD48 virus particles attached by their short tail fibers to either each other or a small membrane fragment. Bars, 100 μm.

lyzed by agarose gel electrophoresis (Figure 3). An ~10-fold induction was observed for both wild-type SSV1 and pKMSD48.

Plasmids pKMSD4, pKMSD48, and pKMSD54 make virus particles: Both cell-free supernatants of infected cultures of *S. solfataricus* and the cultures themselves, either with or without ultraviolet irradiation, were examined by electron microscopy. *S. solfataricus* cells infected with either linearized pKMSD4 without *E. coli* plasmid DNA (see above) or the shuttle vectors pKMSD48 or pKMSD54 formed virus particles that are very similar to the wild-type virus (Figure 4; data not shown).

Plasmid pKMSD48 was present in *S. solfataricus* at high copy number: By comparing the intensity of the pKMSD48 DNA bands in restriction endonuclease digestions of total DNA from infected cells to that in uninfected cells (see Figure 3), it was estimated that there are as many as 20–40 copies of the viral DNA in single cells. Large quantities of DNA were easily isolated even from transformed Sulfolobus cells that had not been treated with UV irradiation (Zillig *et al.* 1998). This copy number was considerably higher than the estimated 1–2 copies per cell reported by Cannio *et al.* (1998) and Aravalli and Garrett (1997).

Plasmid pKMSD48 infected many *S. solfataricus* strains, but not *S. acidocaldarius*: In addition to infecting the strains that were mainly used in this study, *S. solfataricus* P1 and PH1 (*S. solfataricus*, *lacS*$^{-}$; Schleper *et al.* 1994), the pKMSD48 construct also infected *S. solfataricus* P2 (DSM 1617) and the *S. solfataricus* MT3 derivative GΘ (Cannio *et al.* 1998; data not shown). However, no transformation was seen with *S. acidocaldarius* (DSM 639) even when the pKMSD48 DNA was fully methylated with *Hae*III methylase to protect it from the endogenous endonuclease activity of *S. acidocaldarius* (Prangishvili *et al.* 1985). Neither PCR nor Southern hybridization analysis could detect pKMSD48 DNA in *S. acidocaldarius* even after 2 days of growth (data not shown).

DISCUSSION

SSV1 ORFs crucial for virus function: In the absence of significant homology to sequences or motifs present in the public databases (Palm *et al.* 1991; K. M. Stedman, unpublished observation) direct analysis of the ORFs present in the viral genome is the only way to determine their function. Previously, the viral capsid proteins, VP1 and VP3, and the DNA-binding protein VP2 were identified (Reiter *et al.* 1987a). After sequencing of the SSV1 genome, the viral integrase gene was identified (Palm *et al.* 1991) and extensively characterized *in vitro* (Muskhelishvili *et al.* 1993; Muskhelishvili 1994). In this article it is demonstrated that this gene is also required for virus function. The largest ORF in the virus, c792, has no obvious motifs or homologies. Nonetheless, this gene is crucial for virus function. In addition to ORF c792 and the viral integrase, ORFs

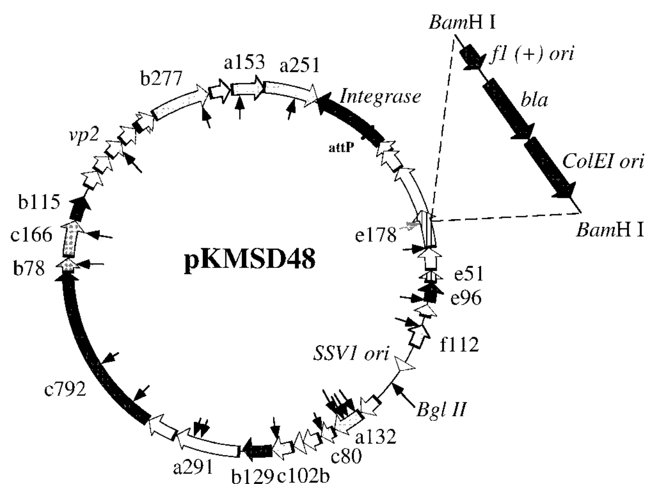

Figure 5.—Map of the shuttle vector pKMSD48 with open reading frame analysis. All open reading frames analyzed are labeled. Apparently essential genes are shaded in dark gray. Possibly essential genes are shaded in light gray. Nonessential genes are vertically striped. The viral integrase gene is labeled and the genomic insertion point is shown (attP). The *Bgl*II site introduced by site-directed mutagenesis is italicized. Cleavage sites for *Sau*3AI are marked by small arrows inside the map; the *Sau*3AI site selected by the serial genetic selection to form pKMSD48 is shown in light gray. The β-lactamase gene, *bla*, and *E. coli* plasmid replication origin, *ColEI ori*, thus introduced are noted as solid arrows. The putative SSV1 ori is also listed.

b115, b129, and e96 were shown to be essential for virus function by directed ORF disruption (Table 2). The partial digestion and serial selection results suggest that 11 additional ORFs are required, because only one construct survived the selection (see Table 2). However, it is distinctly possible that the pool from which pKMSD48 was selected did not contain all possible *Sau*3AI inserts. One would expect to also find the inverse orientation of the pBluescript II SK+ plasmid in the *Sau*3AI site at nucleotide 3147 or an insertion at the *Sau*3AI site at the beginning of ORF e178 (see Figure 5). Therefore the number of apparently essential ORFs identified here must be considered as an upper limit.

The apparent requirement for so many ORFs may also be due to polar effects of insertions and disruptions on other ORFs, although this does not appear to be true for the essential viral integrase in the shuttle vectors reported here (see below). On the other hand, SSV1 may require all but the two nonessential ORFs identified in this study. Only more in-depth genetic characterization of this and other viruses of extremely thermophilic crenarchaeotes will allow an answer to this question.

Evidence for origin of viral replication near the T_{ind} promoter: DNA replication in Archaea is poorly understood. The DNA polymerases and accessory proteins appear to be either eukaryotic-like (reviewed in Edgell and Doolittle 1997) or unique (Ishino *et al.* 1998). However, there are some recent data indicating that plasmid partitioning in Archaea is bacterial in nature (She *et al.* 1998). Replication origins in plasmids, viruses, or chromosomes of archaea have not yet been unambiguously identified, except recently in the plasmid pGT5 from *Pyrococcus abyssi* (Marsin and Forterre 1998). It was speculated that the region near the UV-inducible transcript, T_{ind}, contained the origin of replication for SSV1, due to numerous direct and inverted repeats in the DNA sequence and the absence of major transcripts or ORFs (Palm *et al.* 1991). This region has also recently been used as a replicon for a low copy number recombinant vector for the *S. solfataricus* strain GΘ (Cannio *et al.* 1998). Although this region of the genome can accommodate a point mutation (see Figure 5), its inability to tolerate the insertion of 2.96 kbp of DNA provides further support for its putative function as an origin of replication.

The virus particle can accommodate large inserts into its DNA and retain infectivity: Because SSV1 virus particles can have a variety of sizes and are also very large in comparison to the size of the virus genome (Martin *et al.* 1984), the assumption can be made that virus particles can accept insertions of foreign DNA as long as essential virus functions are not disrupted. Virus particles are formed by extrusion through the membrane (Martin *et al.* 1984), rather like enveloped eukaryotic viruses (D'Halluin 1995) and filamentous bacteriophage (reviewed in Russel *et al.* 1997), potentially allowing for variability in the size of DNA packaged. Here it is shown that the virus particle can accommodate 18,506 bp, almost 20% more DNA than the wild-type genome (Figure 4). The virus particle size may correspond to the size of the DNA packaged (H. P. Arnold, personal communication; K. M. Stedman, unpublished results).

SSV1 transcript T5 encodes both the nonessential ORFs and also the essential viral integrase gene: The inserted *E. coli* plasmid DNA selected in the partial digestion protocol was in ORF e178, which lies within transcript T5 of the viral genome as does ORF e51, which was later found to be nonessential (Figure 1; Reiter *et al.* 1987b). Transcript T5 also encodes the viral integrase, which appears to be essential for viral function. Therefore, either transcription must take place from a cryptic archaeal promoter in the *E. coli* plasmid sequence or the entire insert is transcribed in Sulfolobus as part of the T5 transcript. The former is probably the case because there are nine potential Sulfolobus promoters (Hain *et al.* 1992) and two transcription terminators in pBluescript II (Reiter *et al.* 1988).

The recombinant virus is stable: The construct that contains both SSV1 and pBluescript DNA is completely stable in both *E. coli* and *S. solfataricus.* This is in contrast to previous reports of vectors for Sulfolobus (Aagaard *et al.* 1996; Elferink *et al.* 1996; Aravalli and Garrett 1997) and even the extremely halophilic archaeon *Halobacterium halobium* (Pfeifer and Blaseio 1990). The stability in Sulfolobus may be due to selective pressure

for maintenance of viral function or due to integration of the virus into the host genome (K. M. Stedman, unpublished results). The stability of the shuttle vectors reported here in *E. coli* is surprising, because previously described shuttle vectors were reported to be unstable in *E. coli* at high copy number (Aagaard *et al.* 1996; Aravalli and Garrett 1997). The archaeal plasmids pRN1 (Keeling *et al.* 1996) and pGT5 (Erauso *et al.* 1996) used as the basis for these shuttle vectors are possibly unstable in *E. coli* at high copy number.

Molecular genetics in Crenarchaeota using pKMSD48: As a high copy number and inducible vector, pKMSD48 should prove useful for *in vivo* experiments. Transcription from the T_{ind} promoter can be controlled by UV irradiation (Reiter *et al.* 1987b) and this promoter could potentially be used for regulated expression or overexpression of genes in *S. solfataricus.* This vector should prove invaluable for the analysis of the genomic sequence of *S. solfataricus* P2, which should be available in the near future (Sensen *et al.* 1998). Expression and complementation studies using this shuttle vector are underway.

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