

A spreadable, non-integrative and high copy number shuttle vector for *Sulfolobus solfataricus* based on the genetic element pSSVx from *Sulfolobus islandicus*

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ABSTRACT

The pSSVx genetic element from *Sulfolobus islandicus* REY15/4 is a hybrid between a plasmid and a fusellovirus, able to be maintained in non-integrative form and to spread when the helper SSV2 virus is present in the cells. In this work, the satellite virus was engineered to obtain an *Escherichia coli*–*Sulfolobus solfataricus* shuttle vector for gene transfer and expression in *S.solfataricus* by fusing site-specifically the pSSVx chromosome with an *E.coli* plasmid replicon and the ampicillin resistance gene. The pSSVx-based vector was proven functional like the parental virus, namely it was able to spread efficiently through infected *S.solfataricus* cells. Moreover, the hybrid plasmid stably transformed *S.solfataricus* and propagated with no rearrangement, recombination or integration into the host chromosome. The high copy number of the artificial genetic element was found comparable with that calculated for the wild-type pSSVx in the new host cells, with no need of genetic markers for vector maintenance in the cells and for transformant enrichment.

The newly constructed vector was also shown to be an efficient cloning vehicle for the expression of passenger genes in *S.solfataricus*. In fact, a derivative plasmid carrying an expression cassette of the lacS gene encoding the β -glycosidase from *S.solfataricus* under the control of the *Sulfolobus* chaperonine (thermosome tf55) heat shock promoter was also able to drive the expression of a functional enzyme. Complementation of the β -galactosidase deficiency in a deletion mutant strain of *S.solfataricus* demonstrated that lacS gene was an efficient marker for selection of single transformants on solid minimal lactose medium.

INTRODUCTION

Host/virus interaction modes have provided windows to study microbial diversity (1) as well as genetic processes at the molecular level, in particular for prokaryotes, and hence have helped in clarifying the physiological mechanisms, the dependence on the specific biochemical environment and evolution of their host cells (2,3).

Very few viruses have been identified from Archaea (4) as compared with Bacteria and Eukarya and detailed description has been reported for those from hyperthermophilic archaea (5,6) with representatives that replicate in the genus *Sulfolobus* being the majority within the kingdom Crenarchaeota (6–8). To date, the *Fuselloviridae* are the most widespread on earth in the *Sulfolobus* genus with viruses sharing similar morphology as well as DNA genome size and organization (9–12).

Sulfolobus spindle-shaped virus 1 (SSV1) is the best studied member of this family and demonstrated to be temperate both in *Sulfolobus shibatae* and in non-natural but related *Sulfolobus* hosts, such as *Sulfolobus solfataricus* (13,14); infection, integration of DNA into the host chromosome and production of virions cause apparently no phenotype change but a significant growth retardation of the host cells which can be visualized as turbid plaques around propagation foci on plated lawns of indicator host cells (14–18).

More recently, another fusellovirus, SSV2 from *Sulfolobus islandicus* strain 15/4 was isolated, characterized and its complete genomic sequence determined. SSV2 shares with SSV1 similar morphology, replication and DNA size (19). The overall genome architecture is conserved but the low similarity in the sequences should be responsible for the higher copy number and the lack of a strong ultraviolet induction of episomal SSV2 DNA and particle production, as well as for the different integration of the SSV2 genome which occurs into the host chromosome at the site of a glycyl tRNA instead of arginyl-tRNA (12).

S.islandicus REY15/4 harbors also a small plasmid, pSSVx, assigned to the pRN family (20,21) of *Sulfolobales* plasmids; pSSVx is also capable of spreading in the cell cultures of

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S.solfataricus but only in the presence of either SSV2 or SSV1, necessary as helpers (9). In fact, pSSVx contains two open reading frames showing high-sequence similarity to a tandem of ORFs in both SSV1 and SSV2 genomes; the proteins encoded by these ORFs are probably necessary for specific recognition of the pSSVx DNA but need viral helper components for capsid formation and packaging (9,19).

In general, the choice of *S.solfataricus* as a model for fundamental understanding of the genetics of extremely thermophilic archaea is due to growth conditions operatively non-prohibitive (22) and capability of maintaining and propagating either natural or genetically modified extrachromosomal DNAs (23,24) from other sources. The complete genome of *S.solfataricus* has also been determined (25), the biochemical characterization of many gene products obtained (26) and the development of post-genomics tools such as proteomics and metabolic pathway reconstruction recently attempted (27,28). Some progress has been made to develop stable transformation (29–32), specific gene disruption methods (33) as well as overexpression of foreign and homologous genes (34); nevertheless none of the systems described so far has been proven efficient for reproducibility and stability of gene cloning and protein expression levels in *Sulfolobus*, probably due, in most cases, to low transformation efficiencies, inefficient selection and/or instability of the vectors in the host as well as changes in the ratio episomal versus integrative forms occurring in the cell during replication of virus-derived constructs (35).

In this study, a genetic system for *Sulfolobus* was developed that is based on the satellite virus pSSVx from *S. islandicus* 15/4. The different recombinant *Escherichia coli*–*Sulfolobus solfataricus* shuttle vectors constructed retained the wild-type capability to replicate at high copy-number and to spread in cell cultures in the presence of its helper virus SSV2. *Sulfolobus* transformants were demonstrated to be stable and propagate the pSSVx derived plasmids in a reproducible and constant fashion without any rearrangement, recombination or integration into the chromosome.

Moreover, stable complementation of a β -galactosidase mutant of *S.solfataricus* previously isolated and characterized in our laboratory (32) and reproducible gene expression levels were also obtained by introducing the β -galactosidase gene (*lacS*) as a reporter under the control of a strong and heat-inducible promoter into the shuttle vector.

MATERIALS AND METHODS

Growth of *Sulfolobus* strains and isolation of SSV2-infected *S.solfataricus* G0W and P2

S.solfataricus strains P2 (DSM 1617), G0 (23) and the derivative mutant G0W [$\Delta lacS$, (32)] as well as *S.islandicus* REY 15/4 (22) were grown at 75 or 80°C in glycine buffered Brock's medium (36) with 0.1% tryptone, 0.05% yeast extract and 0.2% sucrose at pH 3.2. For electroporation and plaque assays, cells were grown with phosphate buffered medium N.182 (M182) suggested by the DSMZ Catalogue of strains containing 0.1% glucose. For isolation of independent clones, medium M182 contained only 0.25% lactose and no other nutrient. The optical density of liquid cultures was

monitored at 600 nm. For solid media, gellan gum (Gelrite, Sigma) was added to a final concentration of 0.8% (0.35% for overlays), and MgCl₂ and CaCl₂ were added to 10.0 and 3.0 mM, respectively.

The *S.solfataricus* strains G0W and P2 transfected with the SSV2 and pSSVx were extracted from zones of growth inhibition (plaques) formed on indicator lawns around spots of the *S.islandicus* REY 15/4 culture supernatants (2–6 μ l for each spot), as described by Arnold *et al.* (9). Clones cured selectively for pSSVx were obtained by isolation of single colonies formed on plates by the cells extracted from plaques and revitalized in liquid cultures.

Viral DNA isolation and plasmid constructions

Extrachromosomal SSV2 and pSSVx DNAs from both *S.islandicus* REY 15/4 and transfectants of *S.solfataricus* strains were performed with Qiaprep Spin Miniprep kit (Qiagen) following the standard procedure suggested by the manufacturer for *E.coli* cells.

Plasmid pSSVrt was constructed by cloning the pSSVx DNA linearized at the position 2812 with AflIII and modified with Klenow DNA polymerase into the SmaI site of the pUC19 *E.coli* vector. Linearization was obtained by AflIII partial digestion in the presence of ethidium bromide using a protocol already described for the SSV1 virus DNA linearized with Sau3AI (37). Clones with insertion at the specific position were selected by restriction analysis of the resulting plasmid collection. A size reduced derivative of pSSVrt was also constructed; the polycloning sequence (between the AatII and EcoRI sites) of the pUC28 vector was inserted into a 1812 bp AatII/AflIII DNA fragment from pUC18 after suitable modification of incompatible ends. The pSSVx sequence was excised from the pSSVrt plasmid with SacI and PstI and inserted into the same sites of the minimal plasmid obtained to produce the pMSSV vector. An expression cassette of the *lacS* gene (38) was PCR amplified by the vector pMJ03 (35) and inserted between the XhoI and PstI sites of pMSSV generating the expression vector pMSSV*lacS*. Excision of the *E.coli* minimal plasmid was obtained by digestion of pMSSV*lacS* (1.0 μ g) with SacI and purification of the pSSVx/*lacS* moiety from agarose gel. The DNA was re-circularized by ligation in a final volume of 5.0 μ l, diluted in water to 20 ng/ μ l final concentration, and 2 μ l were used for electroporation.

Transformation procedure and analyses of *Sulfolobus* transformants

S.solfataricus cells of SSV2 lysogenes were grown up to mid-logarithmic phase (0.3–0.45 OD₆₀₀), harvested by centrifugation and repeatedly washed in 20 mM sucrose as described previously by Schleper *et al.* (14). Aliquots of 10¹⁰ cells/ml (50 μ l) were mixed with 1 μ l DNA (10–100 ng/ μ l), incubated for 1 min on ice and transferred to chilled plastic cuvettes with an electrode gap of 0.1 cm (BioRad). High voltage electroporation (25 μ F) was performed with a BioRad Gene Pulser Xcell™ at a field strength of 1.5 kV/cm and 400 Ω resistance; two successive shock pulses were applied to competent cells producing pulse length of ~10.0 and 9.8 ms, respectively. Immediately after electroporation cell mixtures were diluted with 1 ml of medium M182 containing 0.1%

glucose, transferred to glass vials and incubated for 3 h at 75 or 80°C. After suitable scale-up, 5–15 ml aliquots of the cultures (the volumes varying in order to withdraw the same number of cells per aliquot) were harvested at increasing cell density for DNA extractions. For monitoring propagation of the recombinant satellite virus, extrachromosomal DNA mini preparations and plaque assays were performed. Transformants were stored at –80°C in 15% glycerol stocks.

For Southern blot analysis, ~2 µg of total cellular DNAs, extracted according to Arnold *et al.* (9), and 5 ng of pMSSVlacS plasmid purified from *E.coli*, were cut with HindIII and BglII, and electrophoresed in a 0.8% agarose gel; DNA digests were blotted and hybridized according to standard procedures (39). The probe was prepared by cutting out and purifying a HindIII restriction fragment from the pMSSVlacS vector encompassing the lacS gene and a portion (up to –302) of the tf55α 5' flanking region. This restriction DNA fragment was randomly labeled using the random prime DNA labeling kit (Boehringer Mannheim).

β-galactosidase complementation and isolation of mixed and single transformants

pMSSVlacS transformed cells were tested for β-galactosidase activity; 1 ml aliquots of cultures were centrifuged and cell pellets overlaid with a X-Gal solution (2 mg/ml in phosphate buffered medium) and incubated for 15 min at 75°C for blue color development. *In situ* assays were performed on the same cultures seeded on plates and grown as circular colonized areas, as previously described (32). Supernatants of cultures grown up to 0.5, 1.0 and 1.3 OD₆₀₀ were checked for plaque formation as already described and analyzed by X-Gal staining of plaques formed on continuous lawns of the *S.solfataricus* G0W strain. For infection in liquid culture, 400 µl supernatants from pMSSVlacS transformants were added to a 20 ml culture of cells transfected only with SSV2 and grown up to 0.5 OD₆₀₀. After incubation under shaking for 48 h, cells were diluted 1:50, grown up to 0.36 and 1.0 OD₆₀₀ and tested for β-galactosidase activity as already described.

Single transformants were selected either on rich (M182, glucose 0.1%) or on minimal (lactose 0.25% as the only nutrient) solid media, by plating or by streaking of electroporated cells propagated until they showed positive staining with X-Gal and diluted after they had reached different cell densities (0.3–1.0 OD₆₀₀). Single clones formed after 2 weeks of incubation at 75°C were stained with X-Gal (incubation at 75°C for 3–4 h for color development on lactose plates), picked and resuspended in 100 µl lactose minimal medium and seeded as spots onto fresh rich (M182) medium plates. After *in situ* X-Gal test on plates, cell spots were transferred to M182 medium containing glucose, propagated up to early stationary phase (1.0 OD₆₀₀) and analyzed for extrachromosomal DNA content as described above.

β-galactosidase enzyme activity

β-galactosidase activity of transformants was visualized and quantified as follows. Crude extracts were prepared by a freeze thaw technique, suspension of the cells in 10 mM Tris–HCl buffer, pH 8.0, placing at 80°C for 10 min, then at 50°C for 5 min. This procedure was repeated four times,

and the extract was spun for 30 min at ~10 000g. The supernatant was either assayed immediately or stored at –80°C before testing. Protein concentrations of the crude cell extracts were determined by the Bradford assay (Bio-Rad). Detection of the β-galactosidase in protein extracts was performed on 10% acrylamide SDS–PAGE gels after electrophoretic separation and extensive washing in 10 mM Tris–HCl buffer, pH 8.0. The specific enzyme band was visualized incubating the gel 30 min at 75°C in the same buffer containing 2 mg/ml X-gal.

The β-galactosidase assay procedure followed essentially the protocol of Pisani *et al.* (40). A sample of 10 µl extract was transferred to a preheated (75°C) quartz cuvette containing 990 µl assay buffer (2.8 mM ONPG in 50 mM sodium phosphate buffer, pH 6.5). The ONPG hydrolysis reaction was followed spectrophotometrically at 75°C by measuring the increase in absorbance at 405 nm in a Beckman spectrophotometer with heatable cuvettes. One unit was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of ONPG min⁻¹ at 75°C with a molar absorption coefficient of 3100 M⁻¹ cm⁻¹ at 405 nm for ONP.

RESULTS

Transfection and isolation of *Sulfolobus solfataricus* SSV2 transfectants

The strains P2 and G0W, a β-glycosidase defective mutant isolated earlier (32), were tested for susceptibility to infection by SSV2 and pSSVx viruses produced by *S.islandicus* REY15/4. Supernatants of REY15/4 cultures generated growth inhibition halos on continuous lawns of both *S.solfataricus* strains (Figure 1A), similar to those observed for the strain P1 (9); like for SSV1 virus (37), plaques did not form on *Sulfolobus acidocaldarius* cells, namely no infection occurred. Cells extracted from areas of these plaques and revitalized in liquid cultures contained extrachromosomal DNA indistinguishable from that isolated from REY15/4, indicating the presence and active replication of both virus and satellite elements (Figure 1B). From this culture, clones infected only by the SSV2 virus, namely cured for the pSSVx, were isolated as single colonies on plates after suitable dilutions, as described for the P1 strain by Arnold *et al.* (9). The absence of the satellite virus was confirmed by restriction analysis of extrachromosomal DNA (Figure 1C) and Southern hybridization (data not shown).

Shuttle vector construction

A sequence analysis on the pSSVx DNA was performed in order to locate regions that could be manipulated without affecting DNA replication and particle proliferation/spreading, and thus representing candidate targets for site-specific insertion of foreign DNA sequences. A 200 bp segment was identified which contains the tail-to-tail intergenic region between the ORFs C68 and 288. The segment shows archaeal transcription termination signals (41) and tendency to form hairpin loops as for rho-independent termination mechanisms (42,43). Moreover, it has an AflIII site (cut at the position 2812 on the pSSVx genome), useful for cloning, since it is situated in the 3' direction beyond the ORF288

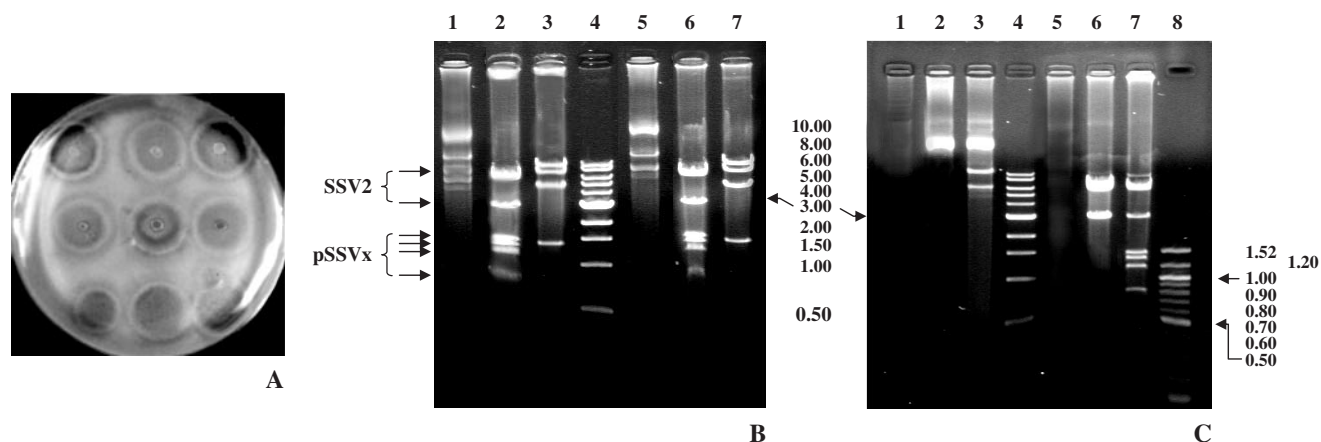


Figure 1. Transfection of *S. solfataricus* G0W with conditioned medium of *S. islandicus* REY15/4. The spontaneous mutant G0W lacking the β -glycosidase activity was first tested for infection with both SSV2 and pSSVx by formation of growth inhibition areas in cell lawns on plate. Virus plaques of SSV2 and pSSVx were obtained by spotting aliquots of REY 15/4 supernatants (2 μ l for the three plaques in the first row, 4 μ l for those in the second row and 6 μ l for those in the third row) onto a continuous indicator lawn of the G0W cells (A). (B) Extrachromosomal DNAs (uncut in lanes 1 and 5), extracted from both the natural source *S. islandicus* (lanes 1–3) and the infected *S. solfataricus* (lanes 5–7), propagated after extraction from the plaques and revitalization, was cut with EcoRI (lanes 2 and 6) and BamHI (lanes 3 and 7). Episomal DNAs were confirmed to be identical and to contain both SSV2 and pSSVx DNAs, as demonstrated by the two distinct EcoRI restriction patterns. Lane 4: DNA molecular weight standards (kb). (C) The cells entrapped inside the plaques have been extracted from the plates, revitalized in liquid medium and then streaked onto solid medium to obtain single colonies. The comparative analysis with extrachromosomal DNAs from non-transformed recipient cells (lanes 1 and 5) and from the cells transfected with both viral and satellite DNAs (lanes 3 and 7) revealed the absence of the pSSVx plasmid in one colony isolated on plate and analyzed (lanes 2 and 6). Lanes 1–3: uncut DNAs; lanes 5–7: EcoRI digests; lanes 4 and 8: DNA molecular weight standards (kb).

transcription termination region, and far upstream of the ORF c68 stop codon. Since the AflIII site is present in five copies on the pSSVx sequence (positions 677, 814, 982, 2812 and 4994), singly cut pSSVx genomes were generated by digestion with the restriction enzyme under conditions that allowed single cleavage of the DNA molecules presumably at every specific site (37). After suitable modification of termini, these DNAs were inserted into *E. coli* pUC19 plasmid vector and specific insertion at the position 2812 produced the fusion plasmid pSSVrt (Figure 2).

pSSVrt shuttle vector transfection and spreading

The plasmid DNA pSSVrt selected from *E. coli* was transferred by electroporation into *Sulfolobus*, using different strains, namely SSV2 lysogens of the strains P2, MT4 and G0W, which is a stable β -galactosidase mutant with an extended deletion in the *lacS* genetic locus (32). To test transformation efficiency, *S. solfataricus* was transformed with varying amounts of the plasmid pSSVrt (10–100 ng) and then checked for the presence and amount of the vector at different cell densities and after several generations. Three hours after electroporation no extrachromosomal DNA could be detected, confirming the low transformation efficiency already determined both by Schleper *et al.* (14) and Cannio *et al.* (23). Nevertheless the plasmid pSSVrt spread efficiently throughout a culture after transformation; in fact after the first scale-up from 3 to 50 ml of the culture, the amount of the plasmid and of the helper virus SSV2 increased and could be detected by ethidium bromide fluorescence on agarose gels (Figure 3). Identical results were obtained with all the different DNA concentrations used for electroporation but a lower amount (\sim 5-fold) of the hybrid plasmid could be detected in the P2 and MT4 (data not shown) strains when compared with the G0W strain (Figure 3).

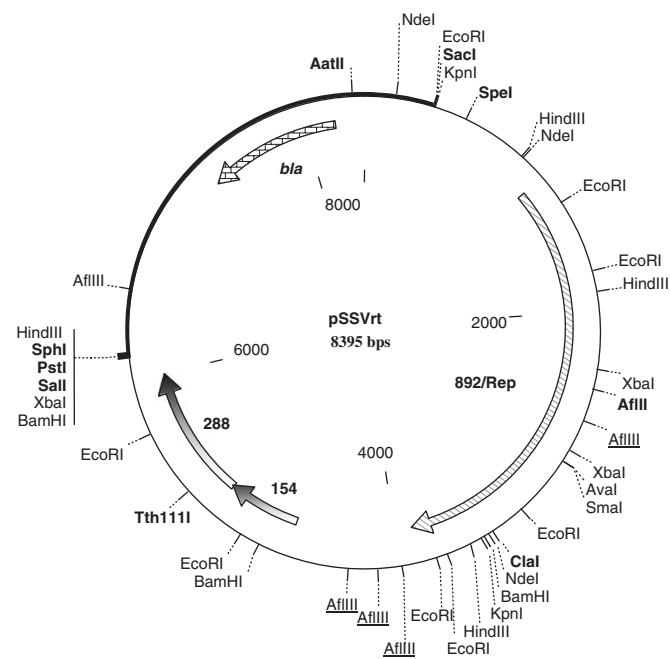


Figure 2. Plasmid map of the pSSVx/pUC19 shuttle vector. The pSSVrt shuttle vector has been obtained by cloning the pUC19 bacterial sequence (heavy bar) into an AflIII restriction site (all AflIII sites are underlined) downstream of the ORF288 of the pSSVx plasmid and by propagation in *E. coli*. Main ORFs allow easy location of archaeal (replicase ORF892/Rep as well as ORF154 and ORF288 putative DNA packaging components of pSSVx) and bacterial (*bla*, ampicillin resistance gene) sequences; bold face letters indicate unique restriction sites.

As expected, the same experiments performed on wild-type strains not infected with SSV2 produced no transformation by the shuttle vector, confirming the need of the helper virus for the propagation of the engineered plasmid.

A long-term growth experiment was also carried out with transformed *S.solfataricus* G0W. A culture containing pSSVrt was grown under standard conditions until it reached an optical density of 0.8, then diluted 1:10 in the same medium and grown and diluted again twice in the same manner. At this point samples were withdrawn at different cell densities and the DNA from the cells was analyzed; no change in the DNA replication and accumulation was observed (Figure 4) even after storing the propagated culture as a frozen -80°C glycerol stock and repeating the dilution/growth cycles. The relative fluorescence intensities of the pSSVrt and pSSVx were almost identical and the comparison with DNA fragments loaded at known concentrations on

the gels allowed the estimation of ~ 130 and 150 copies per cell (density of 1.0–1.2 OD_{600}) for the engineered and the unmodified viral DNAs.

pSSVx-derived shuttle vectors for the *lacS* gene transfer and expression in the β -galactosidase deficient mutant G0W

β -D-galactosidase activity of *S.solfataricus* is displayed by the *lacS* gene product (40,44) and is responsible for the typical blue stain of the cells when exposed to the chromogenic substrate, X-gal; the lack of gene function in defective mutant strains is hence responsible for colorless colonies (45). The *lacS* coding sequence with a 648 bp 3'-untranslated region (3'-UTR) was fused to a 448 bp fragment containing the promoter region of *tf55 α* and the first five codons of its ORF; this 2578 bp expression cassette was inserted into the poly-cloning site of the pSSVrt vector, increasing its size to ~ 11 kb. A similar gene fusion was already demonstrated to efficiently complement a β -galactosidase defect in *Sulfolobus* when carried by a viral SSV1-derived shuttle vector (35). The plasmid was used to transform the strain G0W ($\Delta lacS$) and after electroporation, cell culture was regenerated to allow spreading and subsequently seeded as spots onto plates as previously described (32). After 2–3 days of incubation, the colonized areas were overlaid with X-gal and reincubated at 75°C . No color was developed even after prolonged incubation, namely no expression of β -galactosidase activity could be detected, suggesting the failure of the recombinant plasmid to transport and/or to express the *lacS* gene. Extrachromosomal DNA preparation did not contain the recombinant pSSVx confirming that interference could have occurred either at the level of DNA propagation or of the recombinant particle proliferation/spreading. Southern analysis of extrachromosomal DNAs from this primary transformants demonstrated that the DNA transfer and maintenance were unaffected whereas spreading was impeded, as indicated by specific bands that could be detected at constant but very

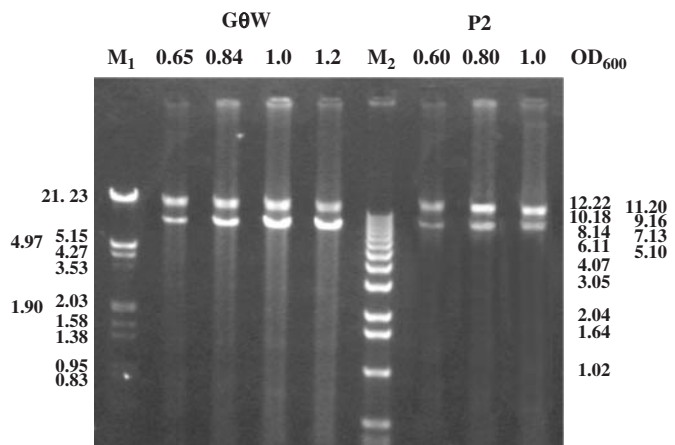


Figure 3. Transformation of *S.solfataricus* with the shuttle vector pSSVrt. Transfer into *S.solfataricus* G0W and P2 strains was performed by electroporation and after propagation of the cultures, the cells were harvested at different growth phases (indicated as optical density values at 600 nm on the top) for extrachromosomal DNA preparation. The upper and lower bands in each preparation correspond to SSV2 virus and recombinant satellite pSSVrt, respectively, both linearized with *Sal*I. M₁ and M₂: DNA molecular weight markers (kb).

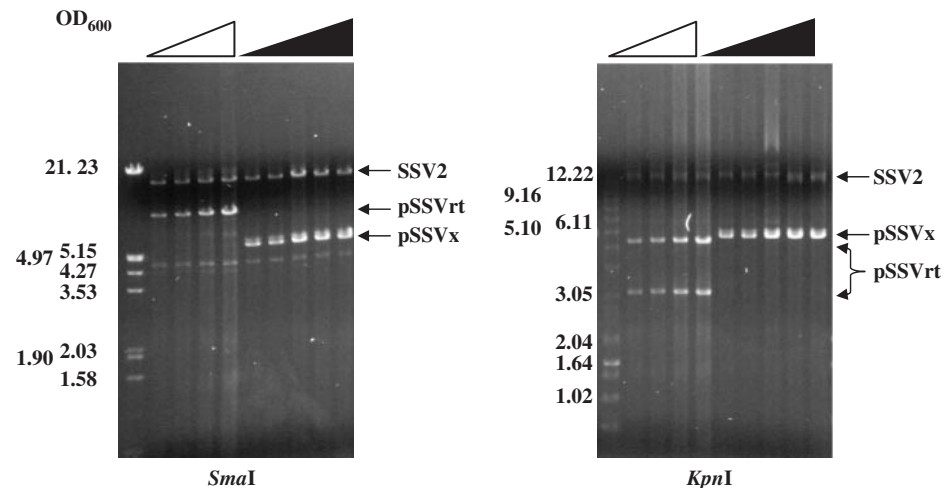


Figure 4. Maintenance and propagation of pSSVrt in *S.solfataricus*. After storage, SSV2 lysogens of *S.solfataricus* G0W transformed with pSSVrt and wild-type pSSVx were grown for several generations to check transformation stability. The propagated cultures were tested by restriction analysis of extrachromosomal DNAs (extracted at increasing cell densities from ~ 0.5 to 1.2OD_{600}); the *Sma*I enzyme produced single cuts in both satellite and SSV2 elements, whereas *Kpn*I had 1 and 2 recognition sites in pSSVx and pSSVrt, respectively, and no site on SSV2. Standards of DNA molecular weight are indicated (kb).

weak intensity for cells withdrawn at different generation stages (data not shown). To confirm this preliminary results a size reduction of the pSSVrt vector was obtained by eliminating a redundant sequence in the *E.coli* plasmid moiety not necessary for replication and ampicillin selection and producing the vector pMSSV. Moreover a smaller (2025 bp)

lacS expression cassette provided by Dr C. Schleper (35) and containing a shorter 3'-UTR replaced the one used in the first attempt. This *lacS* expression cassette was inserted into the polycloning site of pMSSV, generating the expression vector pMSSV*lacS* (Figure 5). Both pMSSV and pMSSV*lacS* were transferred into *S.solfataricus* G0W/SSV2

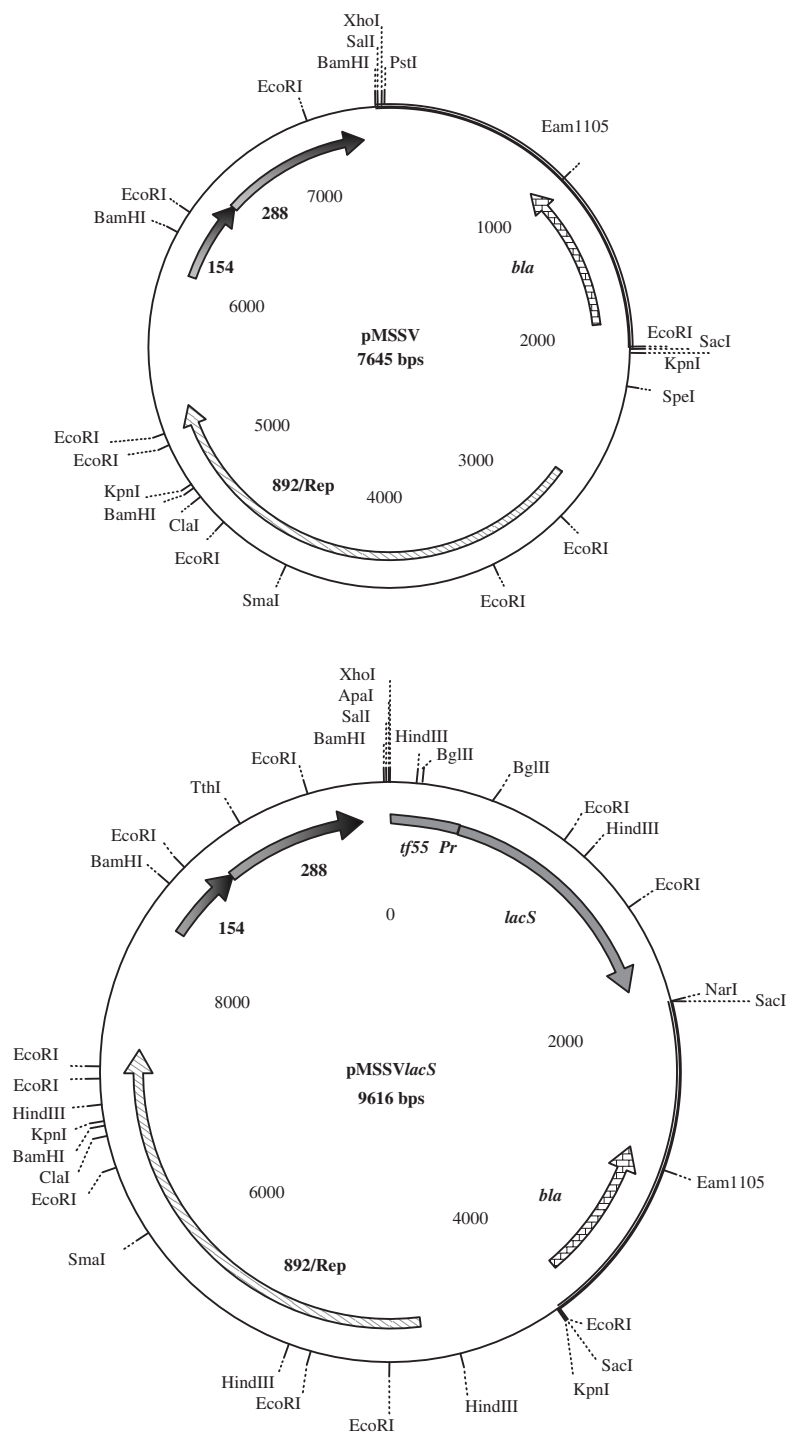


Figure 5. Plasmid maps of the minimal plasmid pMSSV and of the β -glucosidase expression vector pMSSV*lacS*. The bacterial moiety (indicated as a solid bar) of the plasmid pSSVrt was reduced in size, by eliminating every redundant sequence from the pUC19 vector and maintaining its ColE1 replicon and the ampicillin resistance marker (*bla*), to generate a new shuttle vector named pMSSV. An expression cassette containing the *lacS* gene fused to the thermosome *tf55 α* subunit promoter was inserted into the newly constructed vector producing the expression vector pMSSV*lacS*.

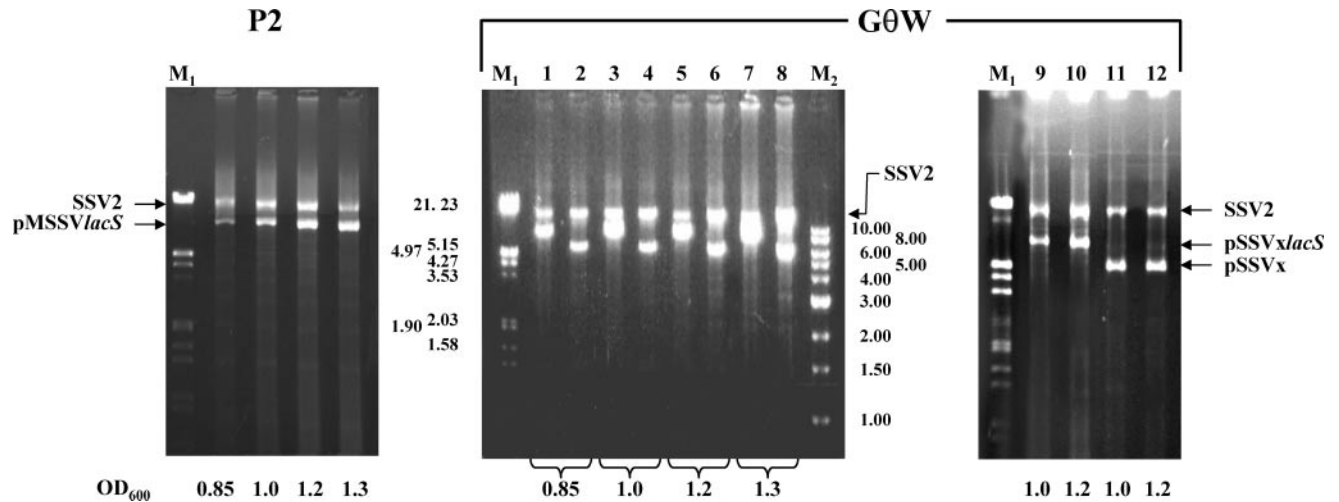


Figure 6. Transformation of *S.solfataricus* with the vectors pMSSV and pMSSVlacS. Competent cells of *S.solfataricus* P2 and G0W infected with the SSV2 virus were electroporated with the vector carrying the β -glycosidase gene pMSSVlacS. Strain G0W was also transformed with pSSVxlacS, a pMSSVlacS-derived plasmid obtained by excision of the *E.coli*-specific sequences and re-ligation. pMSSV and wild-type pSSVx were also transferred into the G0W/SSV2 lysogen for comparison of vector transfer and propagation efficiency in the presence and absence of the lacS gene. Extrachromosomal DNAs extracted from transformed cells after propagation of the cultures were checked by agarose electrophoresis for the presence of pMSSVlacS (all lanes in P2 and lanes 1, 3, 5 and 7 in G0W) and pMSSV (lanes 2, 4, 6 and 8 in G0W) at the different growth stages indicated as cell densities (OD_{600}). Similarly, recovery of the pSSVxlacS plasmid (SacI cut, lanes 9 and 10) was monitored in comparison to the parental pSSVx (SmaI cut, lanes 11 and 12). Mobility of the virus SSV2 is highlighted. M₁ and M₂: molecular weight markers (kb).

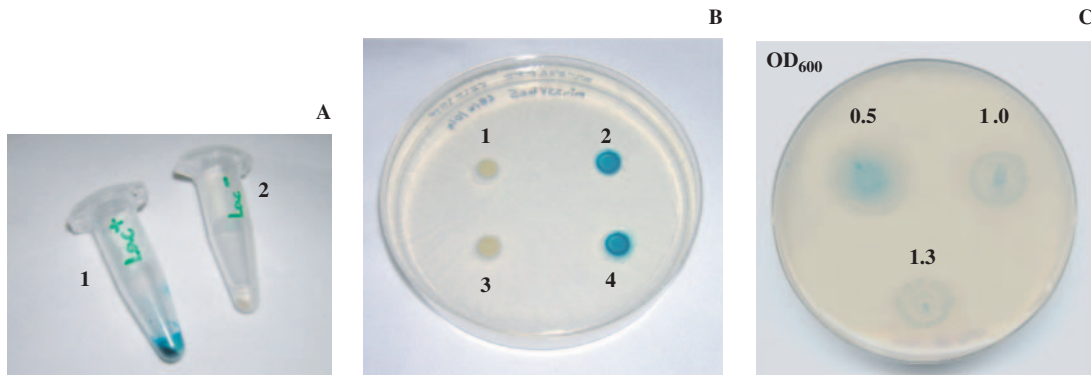


Figure 7. Complementation of the β -glycosidase mutation in *S.solfataricus* G0W and spreading of the pMSSVlacS vector. The expression vector pMSSVlacS was transferred into *S.solfataricus* G0W cells lysogens for SSV2. Successful transformation was checked after propagation for several generation by direct exposure of cell pellets from liquid cultures to X-Gal and development of the blue color [A (1) Lac⁺, pMSSVlacS transformed cells; (2) Lac⁻, cells transfected with pSSVx]. Maintenance of the plasmid was also confirmed for colonized areas on plates and X-Gal test [B (1) Strain G0W; (2) wild-type strain G0; (3) SSV2/pMSSV-infected G0W; (4) SSV2/pMSSVlacS -infected G0W]. C. 4- μ l aliquots of culture supernatant of SSV2/pMSSVlacS-infected G0W, withdrawn at the different cell densities indicated, were spotted onto a continuous lawn of uninfected G0W cells. The spreading of the recombinant satellite particles was revealed by the blue color developed on the plaques (primary infected cells) upon X-Gal exposure. Stain intensity depended on the extent of cell growth retardation in the plaques and was progressively less intense for increasingly higher virus titers.

cells by electroporation, whereas pMSSVlacS was also transferred into cells of the P2/SSV2 strain. After culture propagation, extrachromosomal DNAs were prepared and analyzed by agarose electrophoresis (Figure 6). The presence and growth-dependent accumulation of both vectors in G0W/SSV2 and of pMSSVlacS in P2/SSV2 revealed successful transformation and DNA replication, confirming that the plasmid size was critical for particle formation and spreading; moreover the lacS gene is harmless and does not induce recombination of the vector also in the P2 strain which already contains a wild-type chromosomal copy of the gene. Identical results were obtained when SSV2 lysogens of

Sulfolobus G0W were transformed with the plasmid pSSVxlacS lacking the *E.coli* sequences and obtained by cleavage with SacI and re-ligation (Figure 6). All pSSVx derived plasmid failed to transform *Sulfolobus* if linearized prior to transfer. In fact, X-Gal staining test was positive on *Sulfolobus* G0W/SSV2 transformed with pMSSVlacS (or pSSVxlacS, data not shown) on liquid-cultured (Figure 7A), plated (Figure 7B), and primary infected cells (Figure 7C). Plaques depicted in Figure 7C stained after incubation with the chromogenic substrate as a result of propagation of the engineered satellite virus. Since cell growth retardation in the plaques is directly proportional to the SSV2 virus titer

which is maximal in the stationary phase cultures (P. Contursi, unpublished data), turbidity and color faded progressively with increasing cell density.

Therefore, the β -galactosidase was expressed from the recombinant plasmid and the engineered satellite virus had spread efficiently throughout the culture.

pMSSVlacS DNA prepared from *S.solfataricus* could be re-transferred into *E.coli* without suffering recombinational rearrangements. Plasmid preparations and total DNA from *Sulfolobus* transformants were analysed by restriction analyses and Southern hybridization that confirmed the maintenance of the vector at the same levels of the parental pSSVrt. No vector integration into the host chromosome occurred since the recombinant vector exhibited the same restriction pattern as the DNA prepared from *E.coli* and no signal relative to the plasmid could be detected on genomic DNAs in the Southern blots (Figure 8). Bands corresponding to chromosomal *tf55 α* promoter (in all strains tested) and *lacS* gene (absent in G0W) could also be visualized as internal controls for detection of single copy chromosomal sequences. Signal assignment of the restriction fragments of *tf55 α* gene was performed on the basis of the sequence and localization on the P2 strain genome, whereas the mapping performed by Bartolucci *et al.* (32) allowed the identification of *lacS* gene specific signals. A similar Southern analysis confirmed that the pMSSVlacS copy number varied from 10–15 (mid-log phase) to \sim 130 molecules per cell (stationary phase).

Isolation of single pMSSVlacS transfectants

Single colonies formed by suitably diluted mid exponential pMSSVlacS transformant cells (0.3 OD₆₀₀) on rich solid medium (M182, glucose 0.1%) resulted positive to X-Gal staining only in the fraction of $1-3 \times 10^{-3}$; below this cell density value the presence of the plasmid was undetectable in plated cells. The fraction of positive clones could be increased to 40% when stationary phase cultures were plated, namely when the plasmid had reached its maximum copy number per cell. Unfortunately, blue stained clones loosed the recombinant plasmid when suspended and directly propagated in liquid cultures as indicated by the analysis on extrachromosomal DNA and negative staining with X-Gal. This result was nevertheless expected, since also wild-type pSSVx has been demonstrated to be lost in cells of single colonies on plates and/or of progressively diluted cultures (9); indeed we confirmed and took advantage of this feature for the SSV2 lysogen selection of G0W and P2.

In order to stabilize the pMSSVlacS transformants, selection on minimal media containing lactose as the only nutrient source was performed (cells not complemented for *lacS* function are unable to grow because of the lack of any β -galactosidase activity) (32). Streaking (and/or seeding of suitable dilutions) revealed that the culture had 100% colony forming efficiency on lactose. Moreover, all colonies resulted positive in the X-Gal test (Figure 9A), demonstrating that they were able to retain the plasmid under selective nutrient conditions. Interestingly, cells plated as spots after resuspension and immediate re-seeding onto rich medium, maintained the β -galactosidase activity (for the analysis of 10 independent clones see Figure 9B). This procedure

(resuspension and immediate re-seeding) overcame critical dilution and was successful also for the smaller fraction of positive transformants isolated from solid rich medium.

These experiments demonstrate that cultures of pMSSVlacS transformants before selection on plates were homogeneously infected (all cells contained the plasmid) and that in diluted cell suspensions (such as those necessary for single colony formation on plates and/or obtained by transfer of single colonies in liquid medium), actively dividing cells loosed the vector, unless a selective pressure was imposed.

β -galactosidase assays on pMSSVlacS transfectants

Detection of β -galactosidase activity on denaturing gels by enzyme staining revealed that the *lacS* gene product was indistinguishable when expressed as heterologous in *E.coli* and xenologous in *S.solfataricus*, namely the *Sulfolobus* expression system did not interfere with the correct polypeptide synthesis (data not shown).

β -galactosidase activity was measured using a spectrophotometric assay with the specific substrate ONPG in crude cell extracts of both transfected mutant G0W strain and wild type G0. The mutant recipient strain G0W has been shown to exhibit no detectable activity (32), whereas the specific activities of pMSSVlacS transformants in primary transformation mixtures rose from undetectable levels to 1.2 U/mg protein; this value was \sim 2-fold higher than that found in wild-type cells expressing *lacS* under the control of its own promoter. The activity remained stable in diluted and propagated cells cultured as described above, when assays were performed at the same growth phase. Under identical growth conditions, the value of endogenous β -galactosidase activity in the P2 wild-type strain (i.e. the natural source of both the *lacS* gene and *tf55 α* promoter sequences inserted in the pMSSVlacS), reached 0.1 U/mg as the highest value. Therefore, using this value as reference the expression level in G0W/pMSSVlacS is \sim 12-fold higher. For a single culture, activity increased up to late logarithmic growth phase and then maintained approximately the same value up to late stationary phase (Figure 10). This result confirmed that the recombinant satellite viral DNA was replicated inside the cells and that virus particle formed and spread throughout the culture; the increasing activity should therefore depend only on the copy number of the plasmid and hence on the number of the *lacS* gene copies per cell.

Heat shock of the stably transfected cells, shifting the culture temperature from 75° to 88°C, induced an increase of the specific activity up to 2.5-fold (3.0 U/mg) after 3 h and remained constant in cells exposed to thermal stress for 24 h.

DISCUSSION

In this paper, we have developed a relatively small-sized and high copy number shuttle vector for *S.solfataricus* based on the satellite virus pSSVx from *S.islandicus* REY15/4.

An extended intergenic region between the still uncharacterized ORFs c68 and 288 on the pSSVx genome sequence

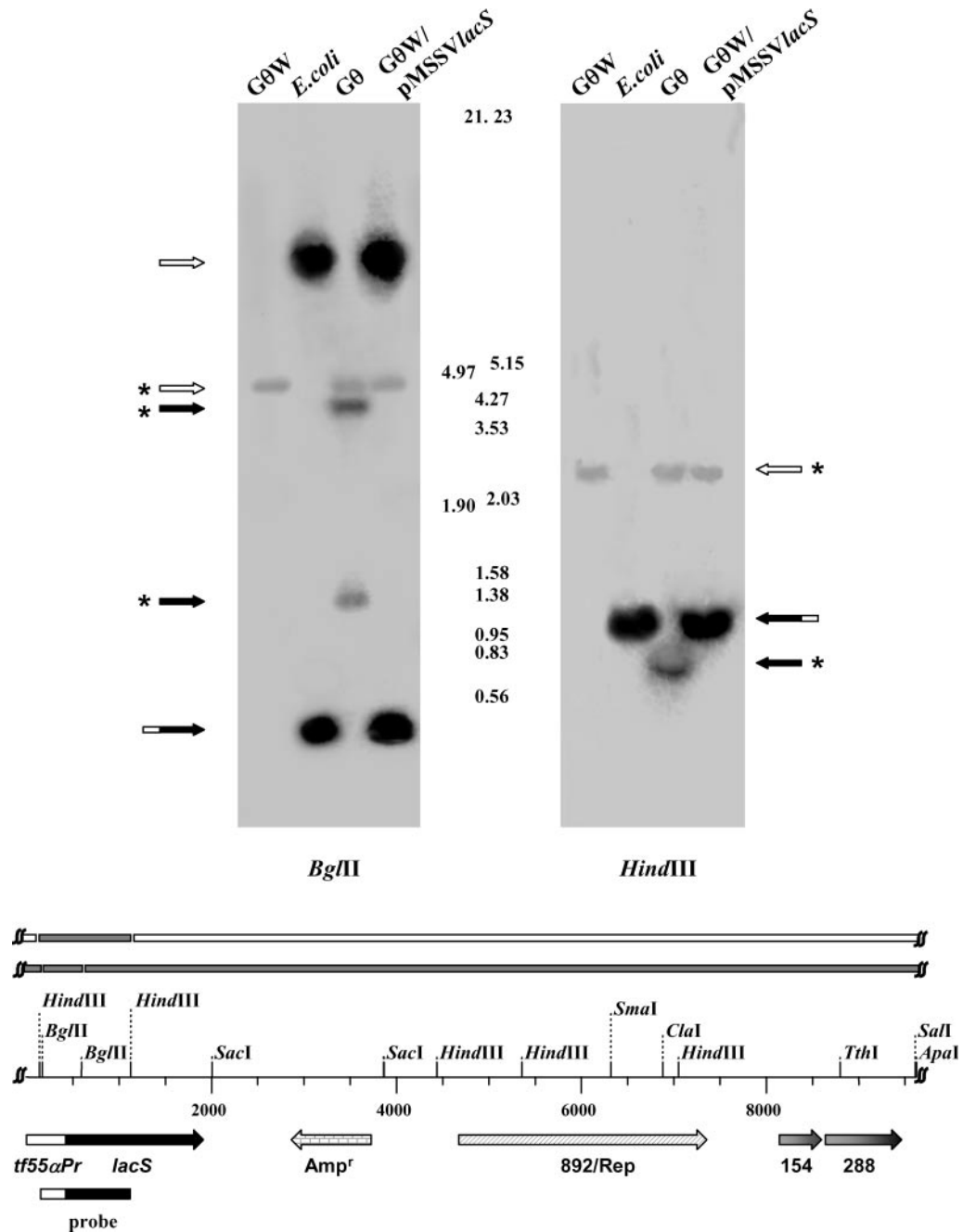


Figure 8. Southern analysis of pMSSVlacS transformants. For hybridization, total DNAs from transformant cells (G0W/pMSSVlacS) and from the recipient mutant strain G0W as well as from wild-type cells G0 were cut with BglII and HindIII as indicated. The recombinant pMSSVlacS vector prepared from *E.coli* before transformation was used as a reference for correct restriction patterns (molecular weight standards are indicated). White and black arrows indicate hybridization to *tf55α* and *lacS* gene sequences, respectively, the asterisks distinguishing signals of the chromosomal copies. Signals of the *tf55α-lacS* gene fusion on the vector are highlighted by white/black arrows. The scheme on the bottom represents the restriction patterns for the two enzymes on the map of the linearized pMSSVlacS and the fragments producing positive signals for hybridization (grey bars).

appeared potentially useful for inserting foreign DNA. In fact, the vector pSSVrt clearly showed efficient replication and maintenance in SSV2 lysogens of the strains G0W and P2.

The insertion of foreign sequences into the pSSVrt vector allowed also to determine the limiting upper size (~11 kb) of the DNA to be accommodated in the virus particles. The additional sequences severely affected packaging and spreading rather than the transfer and/or the replication/maintenance

of the viral DNA. These results also indicated that transport in viable virions and no other mechanism, such as conjugation (29,46–48), is responsible for cell-to-cell transfer of this genetic element.

A ‘minimal’ vector pMSSV was devised to accommodate inserts of a wider size range at least up to the 2.0 kb of a smaller *tf55α/lacS* expression cassette (35). In fact, the deletion of a region non-essential for replication and

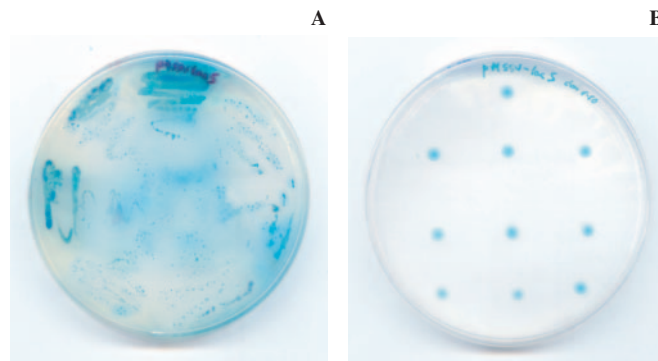


Figure 9. Selection of the pMSSVlacS transformants as independent clones. SSV2/G0W cells from a stationary phase culture (1.2 OD₆₀₀) grown in rich medium and containing pMSSVlacS at maximum copy number were streaked onto minimal 0.25% lactose medium to obtain single colonies. After blue color development with X-Gal at 75°C (A), 10 independent clones were picked, resuspended in liquid minimal medium and seeded drop wise onto rich solid medium for growth in circumscribed colonized areas. All clones maintained the plasmid as indicated by the positive X-Gal test (B).

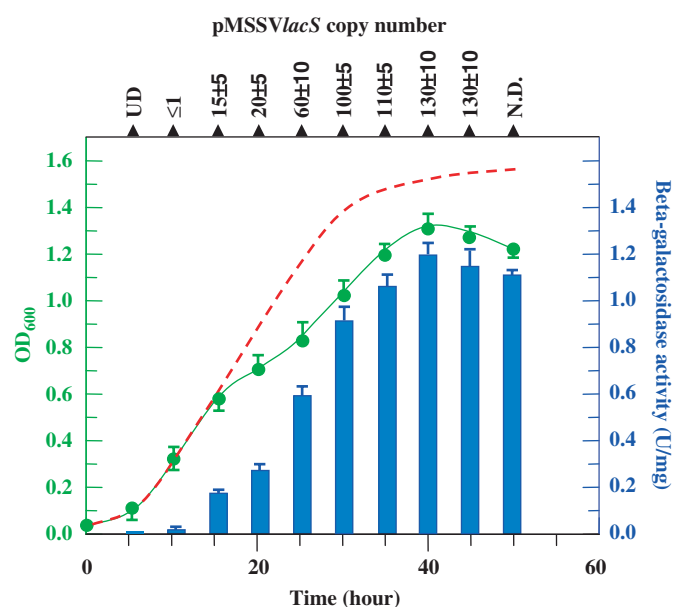


Figure 10. β -galactosidase activity and plasmid copy number in cultures of pMSSVlacS transformants. The β -galactosidase activity was measured in crude extracts of pMSSVlacS transformants harvested at different optical densities from a single culture. Specific activity (indicated by solid bars) is plotted together with the growth curve and the relative plasmid copy number. All values are the average of independent experiments performed on three different cultures and standard deviation is indicated for optical density and activity as well as for plasmid copy number. The dashed red line indicates a typical growth curve of the empty strain G0W. UD, undetectable; N.D., not determined.

selection in *E. coli* and a shortened *lacS* 3'-UTR resulted in an efficient shuttle vector able to transform *S. solfataricus* and to spread in primary cultures. The vector did not suffer either integration into the host chromosome or rearrangements and resided with undetectable to 130 copies per chromosome, the number depending on the cell growth phase. Interestingly, β -galactosidase activity in complemented mutant cells followed an increasing trend which correlated to the plasmid accumulation up to stationary phase and was not dependent on the number of the generations. *lacS* gene expression was

inducible by thermal stress, namely it was confirmed to be transcriptionally regulated by the chaperonin gene promoter. However, heat shock did not produce the same β -galactosidase activity increase (~ 10 -fold) described by Jonuscheit *et al* (35), although maximum gene expression resulted at comparable levels in the two host/vector systems (3 versus 5 U/mg).

Maximum size for DNA insertion into pMSSVlacS was determined to be ~ 1.3 kb (data not shown). In principle longer inserts up to 3.1 kb can be accommodated after cloning in *E. coli* and excision of the bacterial sequences prior to the transfer into *Sulfolobus*.

Maintenance of pMSSVlacS in early exponential grown and/or plated cells seemed to be the critical point for the efficacy of this system, because of both the low copy number (this work) and the reduced viral production in metabolically active cells (P. Contursi, unpublished data). In fact, segregation of the plasmid in an actively dividing cell (in which SSV2 is also maintained at low copy number and hence hardly sustains active replication and/or particle proliferation of pSSVx and derivatives) can be very asymmetrical until it is lost. We were able to overcome this bottleneck; as expected, efficient selection and stabilization of single pMSSVlacS transformants was indeed obtained by plating cells grown up to stationary phase (maximum copy number and highest viral production), by avoiding critical dilutions in the resuspension of single colonies and/or by imposing selective metabolic pressure in lactose minimal media.

This system presents many advantages compared with others already mentioned: (i) after DNA transfer, the vector propagates efficiently throughout the culture as a virus, overcoming the usual low transformation efficiency of *Sulfolobus* cells; (ii) the vector is stably maintained at high-copy number with no integration into the chromosome and hence no reduction in the number of episomal molecules and (iii) in stable transfectants, the β -galactosidase activity is dependent only on the copy number of the vector.

The availability of this new two-element transformation systems based on SSV2 and the engineered pSSVx will contribute to clarify the mechanisms responsible for the satellite/helper dependence as well as for replication, gene regulation and packaging of the episomal DNAs.

Further work will explore the use of this vector for the expression of both homologous and heterologous genes in *S.solfataricus* as well as for testing *Sulfolobus* regulatory sequences.

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