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A Genetic Study of SSV1, the Prototypical Fusellovirus

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Viruses of thermophilic Archaea are unique in both their structures and genomic sequences. The most widespread and arguably best studied are the lemon-shaped fuselloviruses. The spindle-shaped virus morphology is unique to Archaea but widespread therein. The best studied fusellovirus is SSV1 from Beppu, Japan, which infects Sulfolobus solfataricus. Very little is known about the function of the genes in the SSV1 genome. Recently we have developed genetic tools to analyze these genes. In this study, we have deleted three SSV1 open reading frames (ORFs) ranging from completely conserved to poorly conserved: VP2, d244, and b129. Deletion of the universally conserved ORF b129, which encodes a predicted transcriptional regulator, results in loss of infectivity. Deletion of the poorly conserved predicted DNA-binding protein gene VP2 yields viable virus that is indistinguishable from wild-type. Deletion of the well-conserved ORF d244 that encodes a predicted nuclease yields viable virus. However, infection of S. solfataricus with virus lacking ORF d244 dramatically retards host growth, compared to the wild-type virus.

Keywords: DNA binding, nuclease, transcription factor
are limited. Therefore, Long Inverse PCR (LIPCR) using high-fidelity highly processive DNA polymerases (e.g., Phusion®) was developed to specifically change the SSV1 genome at single nucleotide resolution. LIPCR was used to delete precisely the SSV1 viral integrase gene. Surprisingly, this “integrase-less” SSV1 was functional (Clore and Stedman, 2006). However, consistent with its conservation, the virus lacking the integrase gene is at a competitive disadvantage relative to integrase-containing viruses (Clore and Stedman, 2006). All of the SSV1 ORFs that can be deleted or tolerate insertion without abrogating virus function are in the “early” transcript, T5, that is induced soon after UV-irradiation of SSV-infected cultures (Reiter et al., 1987b; Fröls et al., 2007).

Three ORFs in the SSV1 genome were targeted for gene disruption in this study. The VP2 gene (NP_039802.1) was chosen for disruption because it is only present in SSV1 and the very distantly related SSV6 (Held and Whitaker, 2009; Redder et al., 2009), and is in the middle of the most highly conserved part of fusellovirus genomes (Figure 1). VP2 has DNA-binding activity (Reiter et al., 1987a; Iverson and Stedman, unpublished) that is presumably required for DNA packaging. ORF b129 (NP_039795.1) was chosen because it is intolerant of insertional mutagenesis (Stedman et al., 1999), a high resolution structure is known (Lawrence et al., 2009) and the gene is completely conserved in all SSVs (Figure 1). Finally, ORF d244 (NP_039781.1) was chosen for gene disruption because a high-resolution structure of its homolog from SSVRH...
is known (Menon et al., 2008) and it is conserved in most SSV genomes with the exception of SSVK1.

MATERIALS AND METHODS

CULTURE CONDITIONS

Sulfolobus solfataricus strains, Table 1, were grown aerobically at 76°C on plates or in liquid media containing yeast extract and sucrose as carbon and energy sources (YS Media), both as in (Bonasir and Stedman, 2003). Escherichia coli strains were grown in LB medium at 37°C as suggested by the manufacturer (Novagen).

PURIFICATION OF DNA

Plasmid DNA used for LIPCR was purified from E. coli using the alkaline lysis method of Birnboim and Doly (1979). Plasmid DNA used to transform Sulfolobus was purified using the GeneJet Plasmid Purification Kit (Fermentas) following the manufacturer’s protocols. Total genomic DNA was isolated from S. solfataricus in late log phase growth (OD600 ∼0.6) as in Stedman et al. (1999). Plasmid DNA was purified from a 50 mL culture of S. solfataricus transformed with SSV-Δ244 (late log, OD600 ∼0.6) using the GeneJet plasmid purification kit (Fermentas) following the manufacturer’s protocols. This DNA was retransformed into E. coli (Novagen), purified therefrom and analyzed by restriction endonuclease digestion with EcoRI (Fermentas).

CONSTRUCTION OF SSV1 DELETION MUTANTS

Deletion mutants were constructed from the pHJC97 shuttle vector using LIPCR (Clare and Stedman, 2006). Primers were designed to overlap with the start and stop codon of the ORF to be deleted to keep the deletion in frame. Initially primers were designed using the archaea genome browser. Primer melting temperatures were matched and then checked for potential primer dimer and secondary structure formation using online tools from IDT. Table 2 contains a list of oligonucleotide sequences used. LIPCR was performed using Phusion High-Fidelity DNA Polymerase (NEB/Finnzymes) at a final concentration of 0.005 U/µL. LIPCR cycling conditions as follows: initial denaturation at 98°C for 3 min; 35 cycles of 98°C for 6 min, and a final extension at 72°C for 5 min. DNA was precipitated directly from LIPCR reactions using sodium acetate at a final concentration of 0.3 M and 95% EtOH. This DNA was phosphorylated using T4 polynucleotide kinase according to the manufacturer’s protocols (Fermentas). DNA was ligated overnight (∼20 h) at 16°C using 3 Weiss units of T4 DNA ligase (Fermentas). Ligated DNA was transformed into NovaBlue Singles chemically competent E. coli following the manufacturer’s protocol (Novagen). Plasmids were purified from single colonies and deletion constructs were identified by restriction endonuclease digestions. The deletion borders were confirmed by sequencing of the plasmids.

ELECTROPORATION OF SULFOLOBUS

Purified plasmid DNA was electroporated into Sulfolobus strain G10 as in Schleper et al. (1992). Following electroporation (400V, 1.5 kV, 25 µF), cells were immediately resuspended in 1 mL of YS media at 75°C and incubated for 1 h at 75°C. The cells were then added to 50 mL of prewarmed YS media (75°C) and grown in liquid media as outlined below.

SCREEN FOR FUNCTIONAL INFECTIOUS VIRUS/HALO ASSAY

To confirm the presence of infectious virus, halo assays were performed in duplicate 48 and 72 h post-electroporation (Stedman et al., 2003). Uninfected Sulfolobus G10 cells were diluted to an OD600 nm of ∼0.3 and allowed to grow until the OD600 nm reached ∼0.35 (about 2.5 h). Half of a milliliter of this uninfected culture was added to 5 mL YS media containing 0.2% wt/vol Gelrite® as a soft layer and poured onto prewarmed YS plates. Two microliters of supernatant from electroporated cultures was spotted onto the lawns and plates were incubated at 75°C for up to 3 days. A halo of host growth inhibition, typically observed 48–72 h after incubation, indicated the presence of an infectious virus (Figure 2).

GROWTH CURVES

Portions of halos of growth inhibition from infected S. solfataricus G10 cells were removed from plates with a sterile pipette tip and inoculated into liquid YS media. The culture was grown to an OD600 nm of ∼0.6. One milliliter of this culture was diluted in 100 mL YS media to an OD600 ∼0.050. Cultures were placed in a shaking incubator at 75°C and the OD600 nm was measured
every 24 h. After 96 h, 1 mL of culture was diluted into 100 mL fresh YS media and returned to 75°C. One milliliter of culture was removed 72 h after each dilution, cells removed by centrifugation (14000 rpm for 5 min in a microcentrifuge) and the supernatant was screened for virus using the halo assay above.

**TRANSMISSION ELECTRON MICROSCOPY**

Supernatant from infected cultures was collected by centrifugation at 14,000 rpm for 5 min in a microcentrifuge. Five microliters of supernatant was absorbed onto a 400 mesh carbon/formvar grid (Ted Pella) for 2 min and negatively stained with 2% uranyl acetate for 20 sec. Grids were viewed on a JEOL 100CX TEM operated at 100 kV and images captured with a Gatan imager.

**RESULTS**

**SSV1 IS INFECTIOUS WITHOUT THE VP2 GENE**

The VP2 protein was purified from SSV1 virus particles and reported to be a DNA-binding protein (Reiter et al., 1987a). Surprisingly, a gene for VP2 was not found in SSV2 (Stedman et al., 2003) or SSVRH or SSVK1 (Wiedenheft et al., 2004). Moreover, a homolog is not present in the *S. solfataricus* or *S. islandicus* genomes (She et al., 2001). However, a very distant relative of SSV1, SSV6, which also contains an atypical putative tail fiber protein, has a VP2 gene (Redder et al., 2009). Thus, it is not clear whether SSV1 can function without a VP2 gene.

Therefore, we made an in-frame deletion of the majority of the VP2 gene by LIPCR in the context of the pAJC97 SSV1 shuttle vector (Clore and Stedman, 2006), leaving the first four codons and the last four codons (including the stop codon) of the ORF intact (Table 1). The putative promoter for the T9 “early” transcript was also left intact. The construct containing the deletion, pAJC97-ΔVP2, is hereafter referred to as SSV-ΔVP2.

To determine if the SSV-ΔVP2 was able to make infectious virus, the shuttle vector was electroporated into *S. solfataricus* strain G8h. Two days after electroporation, the supernatant from the transformed strains caused inhibition of growth of uninfected *S. solfataricus* strain G6h on plates (Figure 2) that was indistinguishable from growth inhibition caused by the virus containing the VP2 gene. Similar growth inhibition was also observed on lawns of uninfected *S. solfataricus* strain S443, a new *S. solfataricus* isolate from Lassen Volcanic National Park that is a host for all tested SSVs (Ceballos et al., in preparation). Moreover, the supernatant contained SSV-like particles when observed by transmission electron microscopy (Figure 3).

Infection by wild-type SSV1 and shuttle vectors does not drastically slow growth of cells in liquid culture for unknown reasons (Martin et al., 1984; Schleper et al., 1992; Stedman et al., 1999). The same is true of SSV-ΔVP2 (Figure 4). Infection with SSV-ΔVP2 was confirmed via PCR amplification (data not shown). **SSV1 CONSTRUCTS LACKING THE CONSERVED ORF b129 DO NOT APPEAR TO MAKE INFECTIVE VIRUSES**

The b129 ORF in SSV1 is universally conserved in all fuselloviruses (Redder et al., 2009). Moreover shuttle vectors with plasmid inserted into ORF b129 did not produce infective virus when electroporated into *Sulfolobus* (Stedman et al., 1999). However, a similar insertion mutant in the equally conserved SSV1 viral integrase appears to be non-functional (Stedman et al., 1999), but an in-frame deletion was functional (Clore and Stedman, 2006). A structure for the b129 ORF is also known (Lawrence et al., 2009) and it contains two Zn-finger putative DNA-binding motifs.

The b129 ORF was deleted with LIPCR. The deletion of the b129 ORF left the first four and last two codons of the ORF intact and maintained the predicted T3 promoter (Reiter et al., 1987b). This construct is referred to as SSV-Δb129. Unlike the SSV-ΔVP2 construct, supernatants from *Sulfolobus* cells electroporated with SSV1-Δb129 did not cause zones of growth inhibition when spotted on lawns of uninfected *S. solfataricus* strain G6h. A total of nine independent transformations were performed in which the wild-type virus consistently caused growth inhibition but SSV-Δb129 did not. Moreover, no halos of growth inhibition were formed on lawns of *S. solfataricus* strain S443. It is not currently known at which step of virus replication the SSV-Δb129 is deficient.

**SSV1 LACKING ORF d244 IS INFECTIOUS BUT HAS A NOVEL PHENOTYPE**

SSV1 ORF d244 is in the UV-inducible transcript T5, upstream of the viral integrase gene (Figure 1). The entire plasmid insert in the ORF directly upstream of ORF d244 without abrogating SSV1 function (Stedman et al., 1999). ORF d244 is well conserved in other Fusellovirus genomes with the exception of SSVK1 (Wiedenheft et al., 2004; Redder et al., 2009). The X-ray crystal structure of the homolog of SSV1 ORF d244, SSVRH ORF d212 has been solved and it is predicted to be a nuclease (Menon et al., 2010). Moreover, the product of ORF d244 has been reported to be in purified SSV1 particles (Menon et al., 2008).

The SSV1 d244 ORF was deleted with LIPCR. The deletion of the d244 ORF left the first two and last three codons of the ORF intact as well as maintained the ORF to avoid polar effects. This construct is referred to as SSV-Δd244.

To determine if SSV-Δd244 was able to make infectious virus, the shuttle vector was electroporated into *S. solfataricus* strain G6h. Two days after electroporation, the supernatant from the transformed strains caused inhibition of growth of uninfected *S. solfataricus* strain G6h on plates (Figure 2) and also inhibited growth of *S. solfataricus* strain S443 (data not shown).

| Strain/vector | Description | Reference |
|---------------|-------------|-----------|
| *S. solfataricus* G4h | MT4 Derivative | Cannio et al. (1998) |
| *S. solfataricus* S443 | Novel *Sulfolobus* isolate | Unpublished data |
| E. coli NovaBlue® | Expression strain | Novagen, Inc. |
| pAJC97 | SSV1 with TOPO PCR Blunt II | Clore and Stedman (2006) |
| pAJC97-ΔVP2 | pAJC97 lacking VP2 gene | This Work |
| pAJC97-Δb129 | pAJC97 lacking ORF d244 | This Work |
| pAJC97-Δb129 | pAJC97 lacking ORF b129 | This Work |
Table 2 | Oligonucleotides used in this work.

| Name      | Sequence                                                                 | Description                      |
|-----------|---------------------------------------------------------------------------|----------------------------------|
| VP2 LIPCR F | 5′-CAC CGC AAG TAG GCC-3′                                                  | Flanks VP2 gene for deletion     |
| VP2 LIPCR R | 5′-ATC CATT AAG TAG TCC ACC-3′                                             | Flanks VP2 gene for deletion     |
| d244 LIPCR R | 5′-GGAA AAA TGA TAT TCA ACT AGG-3′                                         | Flanks ORF d244 for deletion     |
| b129 LIPCR R | 5′-ATTAG GCT CTT TTT AAA GTC TAC C-3′                                     | Flanks ORF b129 for deletion     |
| VP2 Check F | 5′-ATTAG GCT CTT TTT AAA GTC TAC C-3′                                     | Amplifies VP2 gene and flanking sequences |
| VP2 Check R | 5′-TCC CCT AAC GCA CTC ACG TAC C-3′                                       | Amplifies VP2 gene and flanking sequences |
| d244 Check F | 5′-GAT CAT CAA CGA GTA TAT TGA C-3′                                       | Amplifies ORF d244 and flanking sequences |
| d244 Check R | 5′-GGA ACT CCT CTC ATT AAC C-3′                                            | Amplifies ORF d244 and flanking sequences |
| b129 Check F | 5′-ATG AAG GCT GAG GAA ACA ATC GTG-3′                                     | Amplifies ORF b129 and flanking sequences |
| b129 Check R | 5′-TTA ATA TAG CTG CGA TGC TGC TGC TGC-3′                                 | Amplifies ORF b129 and flanking sequences |

*Underlined sequence indicates ORF.

The supernatant contained SSV-like particles when observed by transmission electron microscopy (Figure 3). Infection by wild-type SSV1, shuttle vectors and SSV-ΔVP2 does not slow growth of cells in liquid culture (Martin et al., 1984; Schleper et al., 1992; Stedman et al., 1999; see above). However, infection by SSV-Δd244 drastically slows growth of S. solfataricus strains G/Theta1 and S443 in liquid culture (Figure 4). Infection with SSV-Δd244 was confirmed via PCR. Moreover, restriction endonuclease digestion of viral DNA recovered from transformed S. solfataricus cells and retransformed into E. coli revealed no obvious alterations of the SSV-Δd244 construct (data not shown).

**DISCUSSION**

**THE PUTATIVE DNA PACKAGING PROTEIN VP2 IS NOT REQUIRED FOR SSV1 FUNCTION**

The deletion of VP2 from SSV1 results in a functional virus that is indistinguishable from the wild-type virus (Figures 2–4). Based on the lack of conservation of VP2 this result is not completely unexpected. However, almost all viruses contain a genome packaging protein. There is no clear sequence homolog of VP2 in the host genome, but there are a number of small DNA-binding proteins, such as Sso7d or Cren7 that may be able to functionally substitute for VP2 in SSV1 genome packaging (Choli et al., 1988; Guo et al., 2008). This will be tested with mass spectrometry of SSV-ΔVP2 particles. Alternatively, the VP2 protein may be involved in maintenance of the positive supercoiling of the SSV1 viral genome (Nadal et al., 1986). It would be interesting to know if the topology of the viral DNA is affected by the absence of VP2. It is predicted that positive supercoiling should increase the thermal stability of the DNA, so SSV-ΔVP2 may be less thermally stable than the wild-type virus.

The VP2 gene may be more prevalent than previously thought. VP2-like sequences have been reported from metagenomic studies, one in an acid mine drainage metagenome (Andersson and Banfield, 2008) and the other from Boiling Spring Lake in California (Dünser and Stedman, unpublished). These VP2 genes may be in the context of a SSV6 or ASV-like genome (Redder et al., 2009).
and have genome replication induced by UV-irradiation or some subset of these activities. Experiments to test these hypotheses are underway.

**TRANSFECTION WITH SSV-Δd244 PRODUCES VIRUS AND RETARDS HOST CELL GROWTH**

The SSV1 d244 ORF is well-conserved in fuselloviruses with the exception of SSVK1 (Wiedenheft et al., 2004; Redder et al., 2009). However, SSV1 lacking ORF d244 clearly makes infectious virus particles (Figures 2 and 3). Moreover, the zones of clearing produced by supernatants of cells transfected with SSV-Δd244 are clearer than those produced by either the wild-type or SSV-ΔVP2 viruses (Figure 3; unpublished data). They are reminiscent of zones of clearing produced by SSVK1 (data not shown). Unlike wild-type virus and SSV-ΔVP2, transfection by SSV-Δd244 leads to drastically reduced host growth (Figure 4). The reasons for this growth inhibition are unclear. Similar growth phenotypes have been observed in SSVK1 infections (Stedman et al., in preparation). SSVK1 consistently produces more virus than similar cultures of the wild-type virus, so this may account for the growth defect (unpublished data). Whether SSV-Δd244 consistently produces more virus than the wild-type or SSV-ΔVP2 is currently unknown.

The structure of the product of SSV1 ORF d244 is a predicted nuclease (Menon et al., 2010), similar to Holiday junction resolvase enzymes. Why the lack of a resolvasse leads to slower host growth is unclear. Possibly SSV1 ORF d244 is involved in the specificity of SSV1 integration. SSV-K1 is known to integrate into multiple positions in the host genome (Wiedenheft et al., 2004), which may contribute to its higher copy number. Whether SSV-Δd244 integrates into multiple positions in the host genome is under investigation. On the other hand, there may be a defect in SSV-Δd244 replication or resolution of SSV replication intermediates that leads to accumulation of aberrant DNA, which, in turn, leads to slower host growth.

After multiple transfers of *Sulfolobus* cultures transfected with SSV-Δd244 into fresh media, growth rates recover to near wild-type rates (unpublished data). The virus is still present in these cultures by PCR and is able to inhibit *Sulfolobus* growth on plates (unpublished data) so the virus is not lost or apparently rearranged (see Results). Whether there are other genetic changes in the virus or host under these conditions remains to be determined. One attractive possibility is changes to the CRISPR repeat structures that are proposed to be important for acquired immunity in *Sulfolobus* (Held and Whithaker, 2009).

**SUMMARY AND OUTLOOK**

Comparative and structural genomics has identified a number of targets for gene disruption in the SSV1 genome. Here precise gene disruptions of the poorly conserved VP2 gene, and the well-conserved ORFs b129 and d244 are described. Deletions in VP2 may allow insights into DNA packaging in the SSV1 genome. Deletion of ORF b129 may allow the identification of the second viral transcriptional activator. Deletion of ORF d244 may allow insight into copy number regulation in SSVs, previously thought to be regulated by ORF d63 (Lawrence et al., 2009). Clearly, there are many more genes to be analyzed in the SSV1 genome, which seems unlikely since the SSV1 integrase is at the end of the T5 transcript, or that insufficient replicates for gene disruption were performed in the earlier study.

Nine replicate transformations of *S. solfataricus* with SSV-Δb129 did not generate functional virus. However, we cannot absolutely determine that SSV1 ORF b129 is essential for virus function without complementation experiments, which is underway. The reasons for the apparent necessity of SSV1 ORF b129 are unclear, but the structure of the b129 ORF product, a predicted transcriptional regulator (Lawrence et al., 2009) and induction of the T6 transcript containing ORF b129 after UV-irradiation (Reiter et al., 1987b; Fröls et al., 2007) provides clues to its function.

The assay used herein for virus infection, ability to cause a zone of growth inhibition on a lawn of uninfected cells, is for virus spread and infectivity. There are many other aspects of virus replication that could be affected by disruption of ORF b129. An attractive hypothesis is that the b129 protein activates transcription of virus structural genes encoded by the "late" transcripts T7/8/9, T1, and T2 (Reiter et al., 1987b; Fröls et al., 2007; see Figure 1). This would be one of very few archaeal transcriptional activators characterized to date and the only the second archaeal viral transcriptional activator (Kessler et al., 2006). Thus, the SSV-Δb129 construct may be able to replicate its genome, integrate into the host, and have genome replication induced by UV-irradiation or some subset of these activities. Experiments to test these hypotheses are underway.

**THE PRODUCT OF ORF b129 APPEARS TO BE ESSENTIAL FOR SSV1 INFECTIVITY**

Homologs of SSV1 ORF b129 are present in all known SSVs (Redder et al., 2009). The b129 ORF also does not tolerate insertion of the plasmid construct (Stedman et al., 1999). Thus, it is not surprising that deletion of ORF b129 leads to an incompletely replicating virus. However, the SSV1 integrase, a gene also conserved in all fuselloviruses, did not appear to tolerate insertion of the plasmid construct (Stedman et al., 1999), but could be deleted with LIPCR without abrogating virus function (Clue and Stedman, 2006). This indicates that either polar effects are important, which seems unlikely since the SSV1 integrase is at the end of the T5 transcript, or that insufficient replicates for gene disruption were performed in the earlier study.

Deletion of ORF b129 may allow the identification of the second transcriptional activator (Kessler et al., 2006). Thus, the SSV-Δb129 construct may be able to replicate its genome, integrate into the host, and have genome replication induced by UV-irradiation or some subset of these activities. Experiments to test these hypotheses are underway.

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