Archaeal Viruses from Yellowstone's High Temperature Environments

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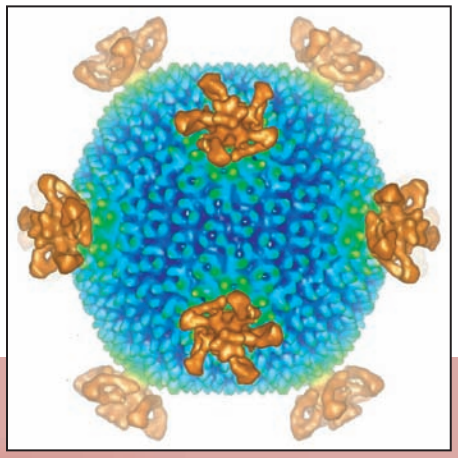

ABSTRACT

In general, our understanding of *Archaea* lags far behind our knowledge of the other two domains of life—*Bacteria* and *Eukarya*. Unlike the other domains of life, very few viruses of *Archaea* have been characterized. Of the approximately 4000 viruses described to date, only 36 are associated with archaeal hosts—many of these from thermophilic Crenarchaeota. In this work we describe the discovery, isolation, and preliminary characterization of viruses and novel virus-like particles isolated directly from diverse thermal environments in Yellowstone National Park. In addition, total environmental DNA extracted from three distinct locations is used to assess the diversity of the resident archaeal community and to evaluate the diversity of thermophilic viruses. The unprecedented diversity of thermal features (~10,000) in Yellowstone National Park has provided us the opportunity to assess host and virus diversity within a single site and among geographically separated sites with distinct geochemical signatures.

Key Words

Archaea
archaeal viruses
SSV, STIV, SIRV, and SIFV *Sulfolobus*
thermal viruses
viral diversity

1.0 INTRODUCTION

Historically, the isolation and characterization of viruses has led to new insights into virus relationships and to a more detailed understanding of the biochemical environment of their host cells. An examination of archaeal viruses will prove useful in helping understand the unique features of archaeal biochemistry.

Archaeal species are well represented in extreme environments, and recent progress in isolation and characterization of these organisms and their associated viruses has contributed to their use as model organisms. The more than 10,000 unique thermal features—in the form of hot springs, mud pots, fumaroles, and geysers—in Yellowstone National Park (YNP) provide a wide range of geochemical environments that harbor a diversity of archaeal thermophiles and their associated viruses. This thermal diversity offers an unprecedented opportunity for environmental examination of organisms inhabiting neighboring thermal features with distinct geochemical signatures.

The domain *Archaea* is divided into two major kingdoms—the Euryarchaeota and the Crenarchaeota. Two additional kingdoms, the Korarchaeota and the Nanoarchaeota, are under consideration. These groups are defined principally on 16S rDNA sequences (Garrity 2001; Woese et al. 1990). The Euryarchaeota comprise many phylogenetically distinct groups, but mainly consist of the methanogens (methane-producing organisms) and the extreme halophiles (organisms that inhabit high salt environments). Most euryarchaeotes do not live at high temperatures; however, there are some hyperthermophiles (orders Thermococcales, Archaeoglobales, Methanopyrus and Methanothermus) in this kingdom. The Korarchaeota branch, with its proximity to the root of the archaeal tree, may display novel biological properties relevant to our understanding of ancient organisms (Barns et al. 1996). Members of Nanoarchaeota are suspected to be small symbiotic organisms (Huber et al. 2002). Recent reports have identified nanoarchaeal species associated with high temperature environments in YNP (Hohn et al. 2002). The Crenarchaeota are distinct from the Euryarchaeota and contain organisms that live at both ends of the temperature spectrum (Hohn et al. 2002). Most cultured crenarchaeotes are hyperthermophiles that grow optimally above 80°C. Hyperthermophilic crenarchaeotes have been isolated from both terrestrial and marine environments. The three most studied orders are Thermoproteales, Sulfolobales, and Desulfurococcales. The recent completion of several archaeal genomes is raising many questions about archaeal gene structure, expression, and functions. In general, these genomes have revealed the composite nature of archaeal biochemistry, where the archaeal information processing systems (i.e. replication, transcription, and translation) resemble a simplified version of that in eukaryotic organisms; while regulation of these systems and metabolic processes are more similar to those associated with bacterial organisms. Archaeal biochemistry appears to be a mosaic of bacterial-like, eukaryal-like, and unique archaeal components that are harmoniously implemented in a single cell. Clearly, much of the archaeal biochemistry remains to be revealed.

Sulfolobus solfataricus is one of the best-characterized *Archaea* members. *Sulfolobus* spp. are aerobic acidophiles that grow at an optimum of 80°C (with a range of 70-87°C) and pH 3 (with a range of pH 1.5-5.5), and have been isolated from acidic hot springs in YNP (Wiedenheft et al. 2004), Japan (Yeats et al. 1982), Iceland (Zillig et al. 1994), New Zealand (Arnold et al. 2000a), Russia (Wiedenheft et al. 2004), and Italy (She et al. 2001). *Sulfolobus* is considered ubiquitous in thermal features around the world that maintain this temperature and pH range. Methods for culturing *S. solfataricus* and *Sulfolobus* spp. in general are well established; thus, these hyperthermophiles represent attractive model systems for examining life at high temperatures. The complete genome sequence of *S. solfataricus* has recently been made available (She et al. 2001), and two transformation systems have been developed both of which have greatly facilitated genetic characterization of this species (She et al. 2001; Stedman et al. 1999; Cannio et al. 1998; Schleper et al. 1992).

Unlike the other domains of life, very few archaeal viruses have been characterized; of the approximately 4000 viruses described to date, only 36 are associated with archaeal hosts

(**http://www.ncbi.nlm.nih.gov**). *Archaea's* known viruses are listed in **Table 1**. Most of these viruses have been isolated from extreme halophiles (especially *Halobacterium* spp.) or methanogens (*Methanobacterium* spp.). With two exceptions, these viruses have head and tail morphologies similar to T-phages and lambdoid phages belonging to the Myoviridae and Siphoviridae groups (Arnold et al. 1999a). In contrast, the seven viruses isolated from crenarchaeal hosts are morphologically and genetically novel. These unique futures have necessitated the formation of new taxonomic groups (described below).

Most of the viruses isolated thus far that replicate in hyperthermophilic hosts belong to the order Sulfolobales, within the kingdom Crenarchaeota of the domain *Archaea* (Snyder et al. 2003). So far, none of these viruses has been found to be lytic. To date, these viruses have been unique, both in their genome sequence and virion morphology. Viruses of *Sulfolobus* spp. are the best studied of the crenarchaeal viruses and represent five new viral families: Rudiviridae, Lipothrixviridae, Fuselloviridae, Guttaviridae, and the proposed Globulovirdae **(http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fr-fst-g.htm)**. Members of the family Fuselloviridae have been isolated from acidic hot springs in Japan (*Sulfolobus* spindle-shaped virus, SSV1; Palm et al. 1991), Iceland (SSV2; Stedman et al. 2003), Russia (SSV K1; Wiedenheft et al. 2004), and YNP (SSV-RH; Wiedenheft et al. 2004). All members of this virus family are characterized by their unique 60 x 90 nm spindle-shaped morphology. Each virion packages an approximately 15.5 kb circular dsDNA genome that codes for ~ 35 open reading frames (ORFs). SSV genomes

Table 1. Known viruses of Crenarchaeota.

Virus	Host	Dimensions (nm)	Genome Size	References
SSV1	*Sulfolobus shibatae*	spindle 60/90	ccc 15.5kb dsDNA	(Martin et al. 1984)
SSV2	*S. islandicus*	spindle 55/80	ccc 14.8kb dsDNA	(Stedman et al. 2003; Arnold et al. 1999b)
SSV3	*S. islandicus*	spindle 55/80	ccc 15kb dsDNA	(Zillig et al. 1998)
SSV-RH	*S. solfataricus*	spindle 60/90	ccc 16kb dsDNA	(Wiedenheft et al. 2004)
SSV-K1	*Sulfolobus* sp.	spindle 60/90	ccc 17.8 kb dsDNA	(Wiedenheft et al. 2004)
SIRV1	*S. islandicus*	stiff rod 780/23	linear 32kb dsDNA	(Prangishvili et al. 1999)
SIRV2	*S. islandicus*	stiff rod 900/23	linear 35kb dsDNA	(Prangishvili et al. 1999)
SIRV-Y1	*S. solfataricus*	stiff rod 900/25	linear 33kb dsDNA	(Rice et al. 2001)
SIFV	*S. islandicus*	filamentous 1950/24	linear 41kb dsDNA	(Arnold et al. 2000b)
SNDV	*S. neozealandicus*	droplet 110-185/95-75	ccc 20kb dsDNA	(Arnold et al. 2000a)
STIV	*Sulfolobus* sp.	icosahedral 70	ccc 19kb dsDNA	(Rice et al. 2004)
TTV1	*Thermoproteus tenax*	flexible rod 40/400	linear 15.9 dsDNA	(Janekovic et al. 1983)
TTV2	*T. tenax*	filamentous 20/1250	linear 16kb dsDNA	(Janekovic et al. 1983)
TTV3	*T. tenax*	filamentous 30/2500	linear 27kb dsDNA	(Janekovic et al. 1983)
TTV4	*T. tenax*	stiff rod 30/500	linear 17kb dsDNA	(Zillig et al. 1988)
PSV	*Pyrobaculum* sp. *Thermoproteus* sp.	spherical 100nm	linear 28kb dsDNA	(Haring et al. 2004)
DAFV	*Acidianus ambivalens*	filamentous 2200/27	linear 56kb dsDNA	(Zillig et al. 1994)
AFV1	*Acidianus* sp.	filamentous 900/24	linear 21kb dsDNA	(Bettstetter et al. 2003)

*Table modified from Snyder 2003

have been shown to site-specifically integrate into the host chromosome via a virally encoded tyrosine recombinase (Wiedenheft et al. 2004; Stedman et al. 2003).

Members of the family Rudiviridae have been isolated from Iceland as well as YNP (Prangishvili et al. 1999). Currently all members of this family belong to the *Rudivirus* genus and include two sequenced species of the *Sulfolobus islandicus* rod-shaped virus—SIRV1 and SIRV2—with *Thermoproteus tenax* virus 4 (TTV-4) currently under consideration. Confirmed members of the Rudiviridae species are all rod-shaped virions (23 x 800-900 nm) containing a single segment of linear dsDNA (33-36 kb), and infect archaeal host cells in the kingdom Crenarchaeota.

The Lipothrixviridae is currently the most diverse family of archaeal viruses, with three different genera (*Alphalipothrixvirus*, *Betalipothrixvirus* and *Gammalipothrixvirus*). All virions of this family have a complex morphology that consists of an envelope and a nucleocapsid. Virions assume a 38 x 410 nm rod–shaped morphology, with a tight fitting membrane. The genomes consist of a single molecule of linear dsDNA that varies in length (~13-16kb). Each genus in this family is currently represented by a single viral species. *T. tenax* virus 1 (TTV-1) is currently the only proposed representative of the *Alphalipothrixvirus* genus (D.-G. Ahn, unpublished). The *Betalipothrixvirus* genus is represented by *S. islandicus* filamentous virus (SIFV) with three other viral species currently under consideration—*Desulfurolobus ambivalens* filamentous virus (DAFV; Arnold et al. 2000b), *T. tenax virus* 2 (TTV-2; Zillig et al. 1994), and *T. tenax* virus 3 (TTV-3; Zillig et al. 1994). The recent discovery of *Acidianus* filamentous virus 1 (AFV-1) completes this family and is currently the only proposed representative of the *Gammalipothrixvirus* genus (Bettstetter et al. 2003). It is of interest to note that the host range of Lipothrixviridae viruses extends beyond Sulfolobales, and includes the order Thermoproteales, both members of the Crenarchaea kingdom.

The fourth family of *Sulfolobus* viruses is represented by *Sulfolobus neozealandicus* droplet-shaped virus (SNDV), which is characterized by its bearded droplet-like morphology and a circular, highly-modified dsDNA genome estimated to be 20 kb (Arnold et al. 2000a).

Surprisingly, with the exception of SIRV, very few ORFs from the viral genomes have any significant identity to sequences (outside their own genus) that are available in the public databases. This lack of homology to other genes may represent the unique biochemical requirements of these archaeal viruses. Understanding the function of these unique genes will be critical in understanding fundamental biological processes in *Archaea*.

In this work, we describe the discovery, isolation, and preliminary characterization of viruses and novel virus-like particles (VLPs) isolated directly from diverse thermal environments in YNP. In addition, total environmental DNA extracted from three distinct locations is used to assess the diversity of the resident archaeal community and to evaluate the diversity of thermophilic viruses. YNP's unprecedented diversity of thermal features (~10,000) has provided us the opportunity to assess host and virus diversity within a single site and among geographically separated sites with distinct geochemical signatures.

2.0 MATERIALS AND METHODS

2.1 Sampling Locations

Approximately 200 different thermal sites, from locations distributed across YNP, were selected according to temperature (70-92°C) and pH (1.0-4.5). Samples from these sites have primarily been used as inoculum to establish enrichment cultures. Hot springs, mud pots, and soils were included in this sampling population.

Three geographically distinct features were selected for more comprehensive chemical and microbial characterization. Water samples collected from Crater Hills (44°39.31'N, 110°28.951"W), Rabbit Creek (44°31.287'N, 110°48.647"W), and Ragged Hills (44°43.653'N, 110°42.862"W) were chemically analyzed using standard methods. In brief, inorganics were analyzed by ion chromatography (chloride and sulfate) or by pH titration ($CaCO_3$ and HCO_3); dissolved organic carbon was analyzed by infrared spectroscopy (IR) and persulfate

ultraviolet oxidation; ammonia (as N) and total phosphorus were analyzed by colorometric detection; and metal ions were detected either by atomic emission spectroscopy or by inductively coupled plasma mass spectroscopy (ICP-MS). All analyses were performed at Energy Laboratories.

2.2 Establishing Enrichment Cultures

Both aerobic and anaerobic techniques were used to establish enrichment cultures from environmental samples. Environmental samples were collected directly in sterile 50 mL centrifuge tubes. All environmental samples were transported at ambient temperature and used as inoculum to establish both aerobic and anaerobic enrichment cultures within eight hours after collection. Aerobic enrichment cultures were established by addition of 1 mL of environmental sample into long-necked Erlenmeyer flasks containing a revised version of a basal media previously developed for culturing *Sulfolobus* (Zillig et al. 1994). Enrichment cultures from soil samples were established by addition of 1 g of soil directly into *Sulfolobus* liquid media. Inoculated flasks were incubated while shaking at 80°C for up to 24 days and monitored for microbial growth. Virus-positive cultures (described below) were spread on solid media containing 0.6% Gelrite (Kelco) and incubated at 80°C in order to obtain single colony isolates.

Anaerobic enrichment cultures were established from a multitude of YNP sites. Briefly outlined, anaerobic sampling vials were prepared using the base medium described above for aerobic cultures with several modifications. Media was supplemented with 1 g of yeast extract (Difco), 0.5 g of NaS_2, and the addition of 1 mg of resazurin per liter. Once prepared, 5 mL of media was then placed into a 15 mL serum vial, flushed with 100% N_2, and sealed with a butyl rubber stopper. Once sealed, serum vials were autoclaved to remove any remaining oxygen. After autoclaving, vials were pressured to 14 psi with 100% N_2. Anaerobic vials were inoculated with 1 mL of environmental sample by direct injection into the base media and incubated at 78-90°C. Growth in the anaerobic vials was monitored by visual turbidity, examination by light microscopy, and by the production of H_2S gas. Single colony isolates were established from anaerobic enrichment cultures using the end-point dilution method.

2.3 16S rDNA Analysis

Total DNA was extracted directly from environmental samples, representative enrichment cultures, and selected single colony isolates using UltraClean™ Water DNA Kit (Mo-Bio). The 16S rDNA gene was amplified from DNA by standard polymerase chain reaction (PCR) protocols using the archaeal-specific primer 0023a forward (CTCCGGTTGATCCTGCC), and the universal 1492 reverse primer (GGTTACCTTGTTACGACTT). The resulting PCR products (approximately 1500 bp in length) were subsequently cloned into pCR2.1 using TOPO-TA cloning protocols (Invitrogen). The complete 16S rDNA inserts were sequenced by Big Dye Terminator protocols using an ABI 310 or 3700 automated capillary sequencer (Applied Biosystems). Multiple independent clones were sequenced, and the complete sequence for each clone was determined. The resulting environmental sequences were compared to publicly available 16S rRNA sequences (**http://www.ncbi.nlm.nih.gov/BLAST/**).

2.4 Virus Detection Methods

2.4.1 Direct screening of environmental samples for VLPs.

Environmental samples collected from YNP hot springs were directly visualized for the presence of VLPs in both unprocessed and concentrated samples. Briefly outlined, particulates and cells were removed from a 200 mL environmental sample by centrifugation (6000 x g for 10 min) and filtered through Acrodisc PF 0.8/0.2 mm filters (Pall Gelman Laboratory). The supernatants were directly visualized by transmission electron microscopy (TEM) or further concentrated by centrifugation at 54,000 x g for 2 hours or by polyethylene glycol precipitation (15% PEG 8000; Sigma). Pellets were re-suspended in 30 µL of ddH_2O, stained with 1% uranyl acetate, and examined with a Leo 912AB or Zeiss 100CA TEM. In addition, up to 200 L of environmental sample was processed through a tangential flow filtration system (Amersham Bioscience) to a final volume of 200 mL examined directly by TEM or further concentrated by centrifugation or by polyethylene glycol (PEG) precipitation followed by TEM examination.

2.4.2 Screening for VLPs in enrichment cultures.

Enrichment cultures were processed for visualization

by TEM. Briefly outlined, both aerobic and anaerobic enrichment cultures were grown to late stationary phase (8-10 days, 80°C) as determined by optical density at 650 nm and visualized directly. VLP concentrates were prepared (as described above) for cultures where VLPs could not be detected directly by TEM.

2.5 DNA Library Construction and Analysis from Virus and VLPs

Total DNA libraries were constructed from concentrated virus samples. Total DNA was extracted from isolated VLP preparations by proteinase K, sodium dodecyl sulfate (SDS), and phenol method (Sambrook et al. 1989) digested with either *Eco*RI, *Sac*II, or *BamH*I (Promega) restriction endonucleases, ligated into pBlueScript® II SK+, and transformed into XL2-Blue MRF' *Escherichia coli* (Stratagene). In addition, randomly sheared viral DNA was used to construct clone libraries. Recombinant plasmids were screened for insert size. Libraries were sequenced using M13 forward and reverse primers as outlined above. Contig assemblies were performed using Sequencher version 4.2.2 (Gene Codes Corp). Sequences were queried against the public database using the BLAST algorithm (Altschul et al. 1997). Multiple sequence alignments were assembled using CLUSTAL W.

2.6 Direct Detection of Viral DNA from Environmental Samples

Total DNA was extracted from environmental samples using the UltraClean™ Water DNA Kit (Mo-Bio). The yield of DNA extracted from the environment was estimated by ethidium bromide staining of agarose gels. Two primer sets were used to amplify SSV-like sequences from isolated environmental DNA. Primer set UnvSSV#7F (ATTCAGATTCTGWATWCAGAAC) and UnvSSV#8R (GATGAGTGCGTTAGGSGA) flanks the viral coat proteins and amplify 650 to 1100 nt, depending on the presence or absence of the VP2 gene in the isolate (Wiedenheft et al. 2004). Primer set UnvSSV#3F (CAATCGCCATAGGCTACGG) and UnvSSV#4R (CGTTTAYTACTATAACGGTAC) amplifies approximately 256 nt of the largest ORF in the SSV genome (B812 in SSV-RH). An additional primer set was designed to amplify the coat protein of SIRVs. Primer set DBPF (GATATTGACCAAAAATGGCAAAAGG) and DBPR (GCTTAAATATTTTAACTTACGTATCC) amplifies the entire 405 nt of the viral coat protein gene. Standard PCR reactions were performed with a Perkin-Elmer DNA Thermal Cycler 9700, using *Taq* DNA polymerase. PCR products were visualized using agarose gels to estimate concentration and size of amplicons.

The PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) as described above. The plasmid DNA was sequenced using BigDye Terminator Mix on an ABI 3700 automated sequencer (Applied Biosystems).

Sequences were edited and analyzed using Sequencher 4.2.2 (Gene Codes Corp). Sequence alignments were performed using CLUSTAL X (Higgins et al. 1996). The alignments were subsequently analyzed by PAUP* 4.0b10 (Swofford 2002), and a maximum parsimony analysis was conducted. Bootstrap values were obtained by re-sampling the data 10,000 times. A maximum likelihood analysis was conducted using MrBayes with Bayesian clade credibility values (Huelsenbeck and Ronquist 2001).

3.0 RESULTS AND DISCUSSION

3.1 Survey of Yellowstone's Acidic Thermal Environments

YNP is estimated to have more than 10,000 geothermal features. Many of these thermal features are acidic and are likely to be favorable environments for archaeal species and their associated viruses. In this survey we sampled a total of 183 acidic hot springs, mud pots, and soils within eight different regions of YNP. A total of 51 enrichment cultures, representing all of the geographically distributed regions, were successfully established. Enrichment cultures were examined by TEM for the presence of VLPs, and total DNA was used in phylogenetic analyses to assess host and virus diversity.

3.2 Survey of Archaeal Hosts in Yellowstone's Acidic Thermal Environments

Full-length 16S rDNA analysis was performed on total DNA isolated from enrichment cultures and on single colony isolates from these cultures. Further, culture-independent 16S rDNA analysis was performed on total

Figure 1. Map of Yellowstone National Park and the three monitor sites. Red stars indicate site locations: **A.** Crater Hills, **B.** Rabbit Creek, **C.** Ragged Hills

environmental DNA extracted from three locations (Crater Hills, Rabbit Creek, and Ragged Hills; **Figure 1**). This analysis reveals a diversity of archaeal species. Sequences sharing ≥98% identity to sequences in the rDNA database were considered the same species. At this cutoff value, ~70% of the YNP 16S rDNA clones represent new species and ~30% represent previously identified species or their close relatives. In spite of this observed diversity, we expect that this limited sampling effort significantly underestimates the true diversity of archaea present in high temperature acidic environments of YNP. Only a limited number of PCR primers were used in this analysis, which are unlikely to capture all archaeal species. Despite this limitation, it is evident from this 16S rDNA analysis and that done by others that archaea are ubiquitous in YNP thermal features (Whitaker et al. 2003).

A more detailed monitoring of three geographically separated thermal sites (Crater Hills, Rabbit Creek, and Ragged Hills) revealed that each hot spring harbored a unique archaeal community (**Table 3**). Distinctions in this observed population diversity are expected to be a consequence of the varying water chemistry (**Table 2**). In contrast to what had been previously suggested for YNP (Brock 1978), these thermal features are not dominated by *Sulfolobus*. Instead, anaerobic archaeal species tend to

Table 2. **Water chemistry of selected Yellowstone thermal features.**

	Rabbit Creek (90º, pH 2.4) June 3, 2004	Ragged Hills (69º, pH 3.2) June 3, 2004	Crater Hills (81º, pH 1.9) June 14, 2004
Chloride	3*	588	4
Sulfate	45	122	1320
DOC	8.1	0.5	6.2
Ammonia (N)	2.8	0.8	11.4
Nitrate + Nitrite	<0.05	<0.05	0.07
Phosphorus	0.81	0.2	0.33
Aluminum	0.2	1.9	26.2
Antimony	0.06	0.106	<0.05
Arsenic	0.051	0.464	0.034
Barium	<0.1	<0.1	0.1
Boron	<0.1	8.7	1.3
Calcium	1	6	<1.0
Copper	0.02	<0.1	<0.1
Iron	6.93	1.52	1.47
Lead	<0.01	<0.01	0.03
Manganese	0.05	0.04	0.03
Molybdenum	0.009	0.112	<0.005
Potassium	7	59	9
Silicon	23.2	149	104
Sodium	9	355	2
Zinc	0.28	0.01	0.02

*Units= ppm
Magnesium (1 mg/l), Mercury (0.001 mg/l), Nickel (0.01 mg/l), Cadmium (0.001 mg/l), and Selenium (0.005 mg/l) were not detected at their respective reporting limits.

be the predominant member of these environments. It is likely that the unique chemical environment plays a major role in determining the archaeal species present in thermal features. Overall, thermal features in YNP should not be treated the same merely because they have similar pH values and temperatures.

Table 3. **Organisms identified by rDNA sequence analysis.**

Closest match*	Percent matches	Number of Sequences	Location(s)
Caldococcus noboribetus D85038+	86-97%	136	Crater Hills; Rabbit Creek
Vulcanisaeta distributa AB063640	87-98%	115	Crater Hills; Rabbit Creek
Sulfolobus solfataricus X03235	87-98%	67	Crater Hills; Ragged Hills; Rabbit Creek
Acidianus species X89852, D26489, D85506, AF226987	93-99%	36	Crater Hills; Rabbit Creek
Metallosphaera sedula X90481	94-97%	12	Ragged Hills; Rabbit Creek
Pyrodictium occultum M21087	90-91%	2	Crater Hills; Rabbit Creek
Stygiolobus azoricus X90480	87-99%	280	Crater Hills; Ragged Hills
Thermocladium modestius AB005296	96-97%	2	Ragged Hills
Uncultured Archaeal clones	86-95%	11	Crater Hills; Rabbit Creek

*Closest match by using NCBI BLAST with 1000-1400 bp of the environmental rDNA gene.
+Genbank accession numbers are indicated for each species.

3.3 Effects of Culturing on Archaeal Host Diversity

The 16S rDNA analysis of selected primary enrichment cultures from all three monitor sites demonstrated an apparent drop in the diversity of archaeal species as

compared to the direct 16S rDNA analysis of environmental samples. Primary aerobic enrichment cultures were dominated by *Sulfolobus* species. The 16S rDNA sequence determined from most aerobic single colony isolates revealed a single sequence with >99% identity to that of *S. solfataricus* or *Sulfolobus acidocaldarius*. This is not unexpected, since the media used in these studies was originally developed for isolation of *Sulfolobus* species and is not likely to support the growth of the diversity of archaeal species found in the YNP thermal features. 16S rDNA analysis of anaerobic enrichment cultures detected *Acidianus*, *Desulfurococcus*, *Thermocladium*, *Stygiolobus*, *Vulcanisaeta,* and *Thermoproteales* species depending on the selective media used for culturing.

3.4 Detection of VLPs from Yellowstone's High Temperature Acidic Environments

Viruses and VLPs can be detected directly from environmental samples or from enrichment cultures established from environmental samples (**Figure 2**). Although VLPs can be observed directly from the environment, they are in low abundance and rarely observed prior to concentration. Occasionally, 23-25 nm spherical particles (spindle-shaped SSV-like) and VLPs with new morphologies have been observed directly from environmental samples. The potential hosts of these VLPs are not yet known. To our knowledge, this is the first report of the direct isolation of VLPs directly from hydrothermal environments. Although, VLPs can be isolated directly from YNP thermal features, they do not appear to be as abundant as those reported in marine environments (107-108 VLPs/mL; Wilhelm and Suttle 1999). The low abundance of virus particles residing outside of their hosts may be a consequence of the harsh chemical environment present in high temperature acidic environments that limit virus particle stability. This may favor selection of virus replication strategies that maximize the time the virus remains associated with their hosts and minimizes the time the virus spends as free particles in the environment. In support of this conclusion, no high temperature archaeal viruses isolated to date have been shown to lyse their host. At the same time, virus production of several of the YNP viruses can be induced by applying cell stress to enrichment cultures (UV or chemical), suggesting that

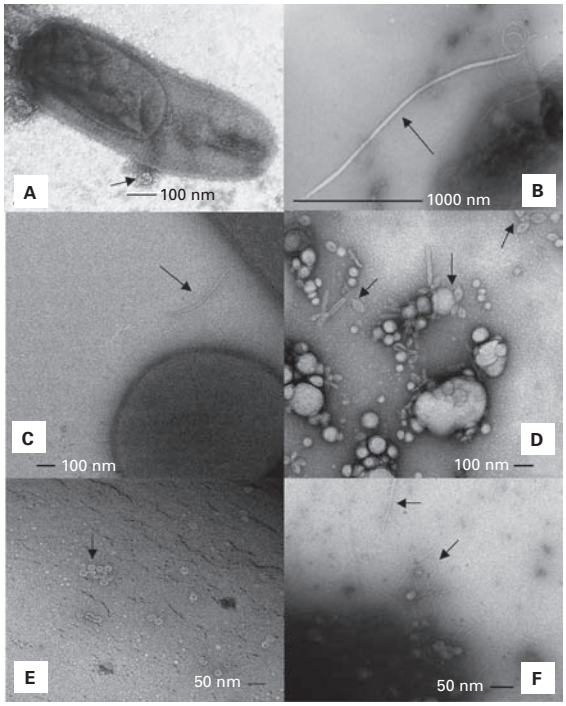

Figure 2. Transmission electron micrographs of VLPs. A-D and F. Virus-like particles (indicated by arrows) isolated from anaerobic enrichment cultures. **E.** Spherical virus-like particles isolated directly from the environment.

virus particles are released into the environment only when their host cell is compromised.

In contrast to environmental samples, viruses and VLPs are typically much more abundant in enrichment cultures and rarely require concentration. A diversity of virus and VLP morphologies can be detected in *Sulfolobus* and anaerobic enrichment cultures (**Figures 2 and 3**). To date, more than 8 VLP morphologies have been detected in various aerobic and anaerobic enrichment culture supernatants.

Four predominant morphologies are routinely detected. These are (i) spherical particles 18-100 nm in diameter; (ii) spindle-shaped particles 60x100 nm in diameter; (iii) stiff rod-shaped particles 300-1000 nm in length; and (iv) flexuous rod-shaped virus particles >2000 nm in length. The most common morphology observed in our high temperature enrichment cultures is the spindle-

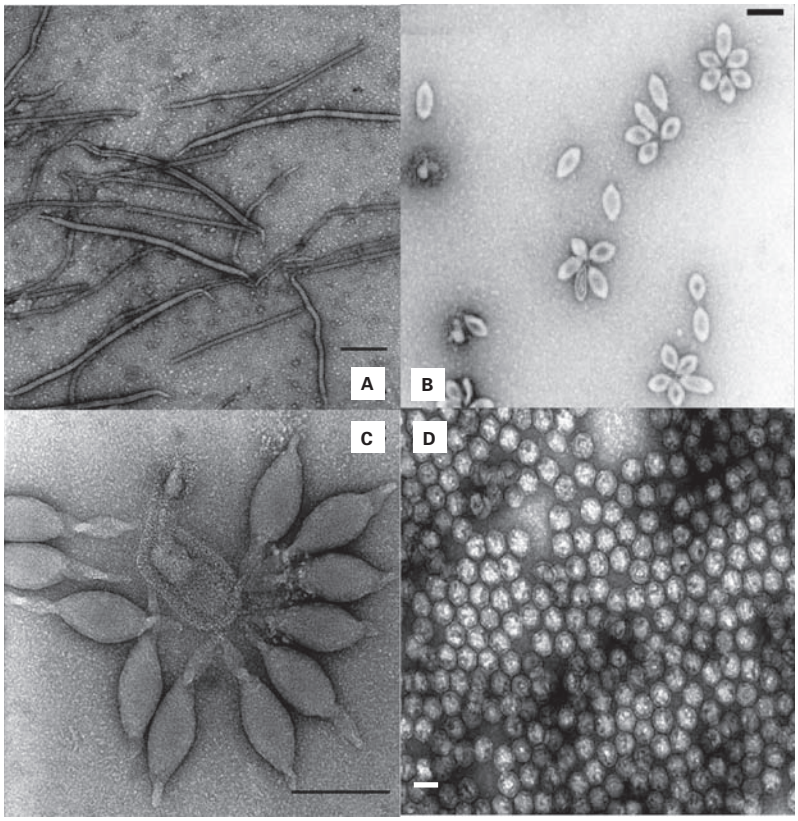

Figure 3. **Transmission electron micrographs of *Sulfolobus* viruses isolated from Yellowstone National Park. A.** SIFV-like, **B.** SSV-like, **C.** double-tailed particles, and **D.** STIV are illustrated. Scale bars = 100 nm.

shaped viruses that are characteristic of the Fuselloviridae. Other less well-defined morphologies are also commonly observed. These include, double-tailed morphologies, elongated cigar-shaped morphologies, bulbous head-like morphologies with elongated tails, and roughly spherical particles with asymmetrical appendages. An estimated 40% of enrichment cultures were positive for at least one VLP morphology type. These results indicate that viruses are likely a common feature of organisms growing in YNP high temperature acidic environments.

Five distinct VLP morphologies have been observed in enrichment cultures of *S. solfataricus* strains isolated from YNP thermal features. Several of the VLP morphologies appear similar to viruses isolated from *Sulfolobus* species from Japan and Iceland (Stedman et al. 2003; Zillig et al. 1994; Palm et al. 1991; Martin et al. 1984). The familiar spindle-shaped morphology characteristic of the Fuselloviridae is the most common virus morphology present in our enrichment cultures from YNP's high temperature environments. The virus particles were similar in size and shape to SSV-1 isolates from Japan (Palm et al. 1991; Martin et al. 1984) and SSV-2 isolates from Iceland (Stedman et al. 2003). The 90 x 60 nm spindle-shaped particles present tail fibers on one end. These tail fibers are presumed to be involved in viral attachment to the host cell membrane and are likely responsible for viral clustering into observed rosette-like structures (**Figure 3B**). These particles contain a circular covalently closed dsDNA genome of approximately 15 kb. Phylogenetic analysis of SSV ORFs reveals little or no sequence similarity to non-fusellovirus genes. However, the complete genome sequence of one YNP isolate (termed SSV-RH) demonstrates a clear relationship to SSV1, SSV2 and SSV-K1 (recently isolated from Kamchatka, Russia; Wiedenheft et al. 2004). Eighteen of the approximately 34 ORFs encoded by each SSV isolate shared significant sequence similarity among the four isolates. This set of 18 genes may represent a minimum gene complement that defines the Fuselloviridae. While this analysis demonstrates that YNP SSV isolates are clearly related to the Japanese, Icelandic, and Russian isolates, there are clearly differences in each viral genome that may reflect unique evolutionary histories or viral adaptations required for replication in a particular thermal environment. Comparative sequence analyses among the SSV isolates has also allowed us to design 'universal' PCR primers for detection of SSV-like sequences from thermal features worldwide (Wiedenheft et al. 2004). These primers

have afforded us the opportunity to address questions concerning viral population structures.

A second virus particle morphology that is commonly associated with *S. solfataricus* is characterized by rigid helical 25 x 900 nm rods with characteristics similar to the Rudiviridae (SIRV1 and SIRV2) from Iceland (Peng et al. 2001; Prangishvili et al. 1999). SIRV-like particles from YNP are observed in approximately 10% of the enrichment cultures and are sometimes found in association with YNP SSV-like particles. Similar to SIRV isolates from Iceland, SIRV-like particles from YNP possess dsDNA genomes 33-36 kb in length. Partial sequence analysis of the YNP isolates indicates some sequence similarity with SIRV-1. The YNP SIRV isolated showed a range of 50% to 88% nucleotide similarity to the Icelandic SIRVs. The highest degree of similarity was in the predicted coat protein gene, which revealed an 88% identity between the Icelandic and YNP isolates. Other regions of the genome had less than 50% sequence identity between the two isolates.

A third virus particle morphology replicating in *S. solfataricus* is characterized by a long flexible rod with properties similar to those of the *Lipothrixvirus*, SIFV, previously isolated from Iceland (Arnold et al. 2000b). SIFV-like particles were observed in approximately 5% of the YNP enrichment cultures. The SIFV-like particles from YNP are 50 x 900-1500 nm flexible rods with apparent attachment fibers at both ends. Partial sequence analysis (8.5 kb of non-contiguous sequence) showed a 45-65% range of identity with SIFV at the nucleotide level. Similar virus particle morphologies were also evident in anaerobic enrichment cultures that do not support *S. solfataricus* growth. The relationship of these viruses to SIFV is currently under investigation.

Three unique virus morphologies not previously associated with *Sulfolobus* have also been observed. Thirty-two-nanometer icosahedral VLPs are observed in approximately 15% of the YNP enrichment cultures. These VLPs do not appear to be enveloped by TEM and do not form growth inhibition zones on lawns of *Sulfolobus* spp. A second morphology was observed in 2 of the 51 enrichment cultures. These VLPs have a large central spindle-shape that is reminiscent of the Fuselloviridae

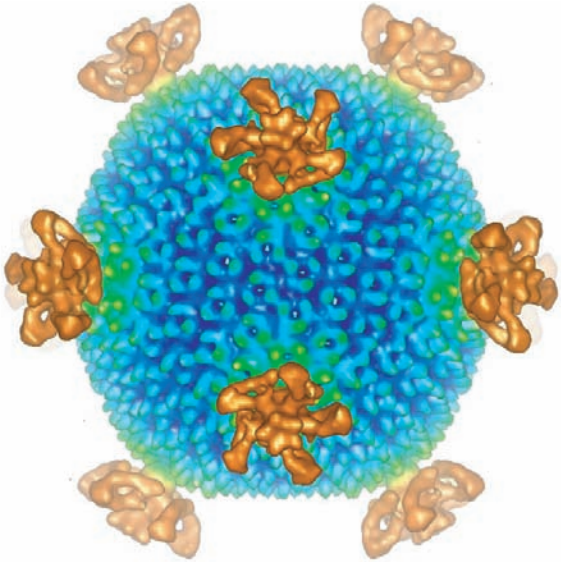

Figure 4. Cryo TEM reconstruction image of STIV.

in diameter and appear to be icosahedral in morphology. Cryo transmission electron microscopy combined with 3-D image reconstruction at ~27 Å resolution revealed unique features of this *Sulfolobus* turreted icosohedral virus (STIV) particle (Rice et al. 2004; **Figure 4**). The structural features of the capsid indicate that the virion is built upon a pseudo T=31 icosahedral lattice, which is the first of this type to be observed. The diameter of the capsid is ~74 nm with a ~6.4 nm shell thickness. The icosahedral asymmetric unit consists of 5 trimers (pseudo-hexamers) of the major capsid protein plus one additional minor capsid protein at the fivefold vertex. The virus structure also revealed appendages at each of the 12 fivefold vertices. These appendages are five-sided turret-like structures that have an average diameter of 24 nm, and that extend 13 nm above the particle surface. The center of each turret contains an approximately 3-nm-channel, which could provide access between the interior and exterior of the virion. There appears to be density at each end of the channel, indicating that the channel may be blocked and suggesting a possible regulatory role associated with this density.

An analysis of the STIV genome has been performed. The virus encapsidates a 17,663 bp circular dsDNA genome

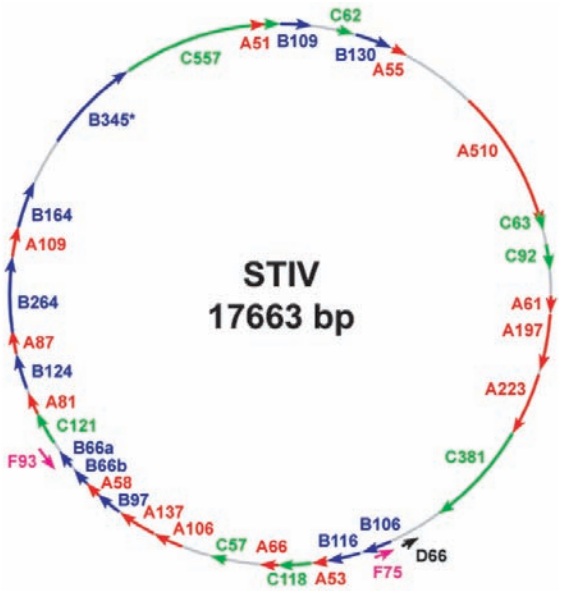

Figure 5. Genetic map of the STIV genome.

(**Figure 5**). Like its host genome, the viral genome has a low G+C content (Rice et al. 2004). Sequence analysis indicates that there are 50 ORFs potentially encoding for products greater than 50 amino acids. Thirty-six of these ORFs are associated with a TATA-like element. These 36 ORFs potentially encode for proteins ranging in size from 5.1-57 kDa. The inferred codon usage of these ORFs shows a codon usage bias that is similar to that of its host, *S. solfataricus* (She et al. 2001). Three TATA-like elements (TTTTTAAA) that are prevalent within the order Sulfolobales have been identified. Sixteen TATA-like elements similar to the Sulfolobales consensus sequence have also been identified in the STIV genome. If the position of TATA-like elements in the STIV genome are considered along with the degree of overlap between downstream ORFs, then 31 of the ORFs are expected to be transcribed into 6 polycistronic RNAs. The 5 other ORFs have individual TATA-like elements suggesting that they may be transcribed separately. Nearly all of the predicted amino acid sequences share no significant similarity to proteins in the public databases. The exceptions to this observation include ORF C557, which is similar to a hypothetical protein encoded by *Sulfolobus tokodaii* (Kawarabayasi et al. 2001), and ORFs B116 and C92 that show similarity to ORFs of the *Sulfolobus* rudiviruses, SIRV1 and SIRV2 (Peng et al. 2001). Interestingly, one of these ORFs, ORF B116, also is homologous to an ORF found in the *Lipothrixvirus* SIFV (Arnold et al. 2000b) and AFV (Bettstetter et al. 2003).

3.5 Effect of Culture Bias on Virus Diversity

We were interested in determining whether a culture bias exists for these hyperthermophilic viruses in YNP acidic hot springs. Most investigators use viruses replicating in culture to examine fundamental aspects of viral replication and virus-host interactions. However, viruses replicating in culture may not accurately represent the virus behavior and ecology found in the natural environment. Interest in the role of viruses in microbial ecology has been heightened by the completion of multiple microbial genomes, which has implicated the importance of viruses in horizontal gene transfer (Hughes and Friedman 2003; Sullivan et al. 2003; Peng et al. 2001; Nelson et al. 1999).

SSV diversity was monitored directly in the environment and in subsequent enrichment cultures by phylogenetic analysis of a conserved region within SSV viral genomes. We detected a strong culturing bias in SSVs between the environment and the primary enrichment culture. Two hundred fifty-six nucleotides within the largest conserved SSV ORF (SSV RH ORF B812) were amplified from total environmental DNA. Seventy-four PCR-generated SSV clones were phylogenetically analyzed. SSV diversity at the Rabbit Creek monitor site (RCMS), in the Midway Geyser Basin, is dominated by two major clusters and one minor cluster—the minor cluster consisting of only a single clone (**Figure 6, next page**). Surprisingly, phylogenetic analysis of 31 viral clones from the primary enrichment culture reveals an obvious culture bias (RCMS Cult1; blue in **Figure 6**). SSV diversity observed in the environmental sample from Rabbit Creek was reduced to a single cluster upon the first passage of this enrichment culture. The primary enrichment culture was associated with only one of the dominant environmental clusters. This trend was maintained when 20 additional clones were analyzed from the first passage of the primary enrichment culture (RCMS Cult2; green in **Figure 6**). The enrichment culture

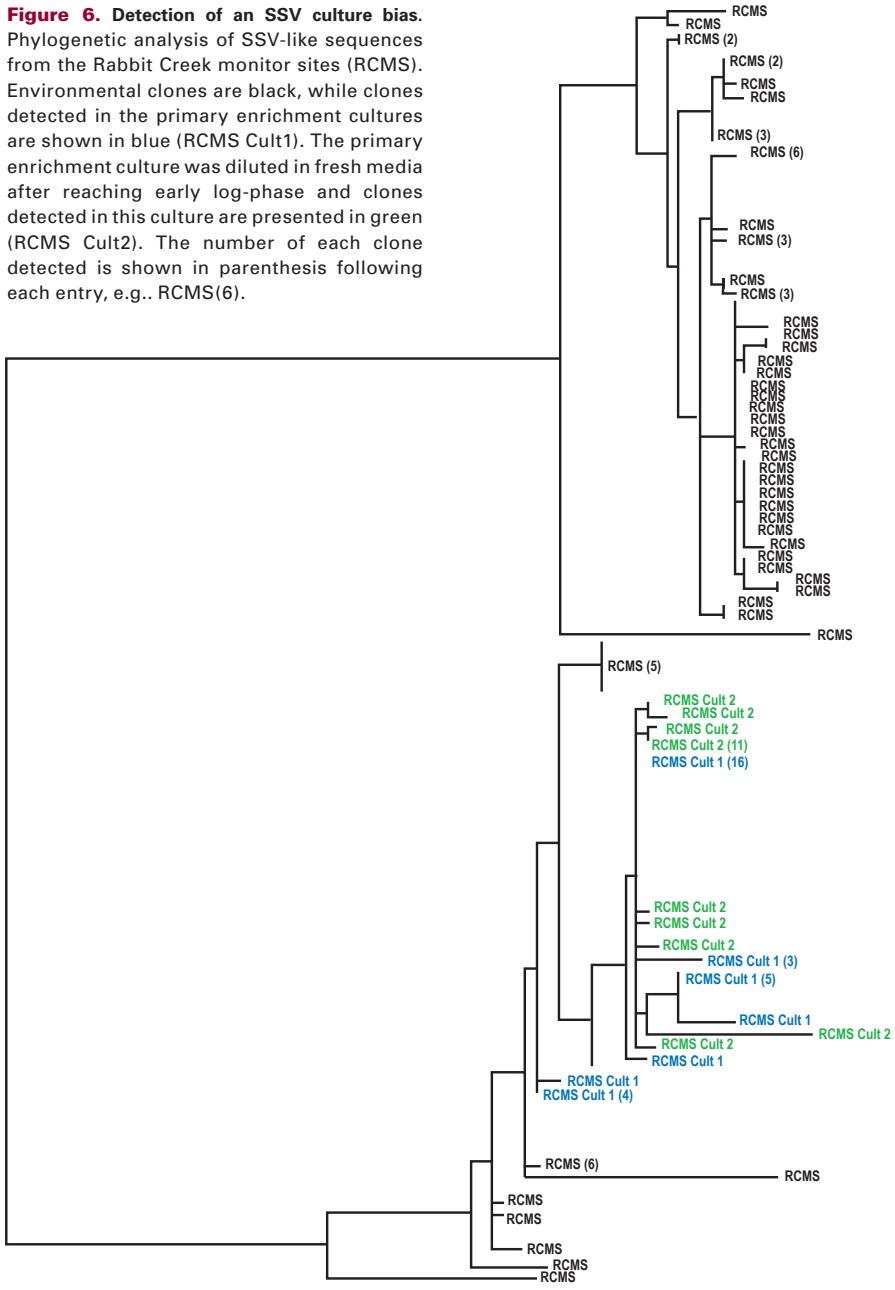

Figure 6. Detection of an SSV culture bias. Phylogenetic analysis of SSV-like sequences from the Rabbit Creek monitor sites (RCMS). Environmental clones are black, while clones detected in the primary enrichment cultures are shown in blue (RCMS Cult1). The primary enrichment culture was diluted in fresh media after reaching early log-phase and clones detected in this culture are presented in green (RCMS Cult2). The number of each clone detected is shown in parenthesis following each entry, e.g.. RCMS(6).

clones may form a separate cluster distinct from those observed in the environment, but this separation is not well supported by high bootstrap values, and more clones will have to be considered. These results identify a strong culture bias when comparing the diversity of SSVs in the environment to SSVs in culture. These results indicate that describing the diversity and population structure based on viruses isolated from cultures would severely underestimate the viral diversity actually present in a particular environment.

4.0 CONCLUSIONS

It is evident that viruses and VLPs can be isolated from archaeal hosts found in the diverse high temperature environments in YNP. A high proportion of enrichment cultures established from YNP contain viruses and VLPs, suggesting that viruses are commonly associated with host organisms residing in YNP thermal features. It is likely that this initial survey has underestimated host and viral diversity that are present in these environments. Our limited ability to directly isolate viruses from these environments and our inability to currently culture a majority of the

host organisms present in these environments limits our ability to achieve a more comprehensive set of viruses. In addition, thus far we have examined only a limited number of representative high temperature acidic sites. These sites represent a fraction of the more than 10,000 thermal features of varying pH and temperature.

Detection of the high number of virus morphologies suggests that there are many more viruses yet to be discovered in YNP thermal environments. The fact that the new viral morphologies mentioned here do not resemble other known viruses from either thermal or non-thermal environments suggests that their analysis will provide further insights into the evolution of archaeal virus structure and function. A detailed understanding of the viral replication cycle of these unusual viruses will likely provide insight into cellular processes present in *Archaea*. It is anticipated that a detailed understanding of these viruses will lead to new insights into archaeal biochemistry, genetics, and evolution. We are entering a new field of virology that will likely expand with more discoveries of archaeal viruses.

One of the major challenges facing archaeal virologists is to unravel the regulation of replication and gene expression of these viruses. At present, we have only an elementary understanding of how these viruses replicate and interact with their host's biochemical machinery. This task will require multiple approaches using the tools of cell biology, molecular biology, and biophysics. A thorough genetic analysis of viral genomes and the effects on host gene expression is underway. It is likely that this analysis will lead to major discoveries in archaeal biology. The field of archaeal virology is quickly proving to be unique and exciting.

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