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AFV1, a novel virus infecting hyperthermophilic archaea of the genus *Acidianus*

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Abstract

We describe a novel virus, AFV1, of the hyperthermophilic archaeal genus *Acidianus.* Filamentous virions are covered with a lipid envelope and contain at least five different proteins with molecular masses in the range of 23–130 kDa and a 20.8-kb-long linear double-stranded DNA. The virus has been assigned to the family *Lipothrixviridae* on the basis of morphotypic characteristics. Host range is confined to several strains of *Acidianus* and the virus persists in its hosts in a stable carrier state. The latent period of virus infection is about 4 h. Viral DNA was sequenced and sequence similarities were found to the lipothrixvirus SIFV, the rudiviruses SIRV1 and SIRV2, as well as to conjugative plasmids and chromosomes of the genus *Sulfolobus.* Exceptionally for the linear genomes of archaeal viruses, many short direct repeats, with the sequence TTGTT or close variants thereof, are closely clustered over 300 bp at each end of the genome. They are reminiscent of the telomeric ends of linear eukaryal chromosomes.

Keywords: Virus; Archaea; Hyperthermophile; Linear genome

Introduction

The observation that viruses are probably the most abundant biological entities on the planet (reviewed by Wommack and Colwell, 2000) has generated a major interest in the characterization of the extent and distribution of viral diversity in different ecosystems. Studies on viruses in hot aquatic ecosystems, at temperatures above 80°C, were pioneered by Wolfram Zillig. The unusual features of viruses characterized in his laboratory warranted the establishment of the following novel virus families: the *Fuselloviridae*, comprising spindle-shaped enveloped particles with circular double-stranded DNA genomes (SSV1, SSV2, and SSV3); the *Lipothrixviridae*, enveloped lipid-containing filamentous viruses with linear double-stranded DNA genomes (TTV1, TTV2, TTV3, DAFV, and SIFV), and unenveloped, stiff-rod-shaped *Rudiviridae* with linear double-stranded DNA genomes (SIRV1 and SIRV2) (reviewed by Zillig et al. 1996, 1998; Prangishvili et al., 2001; Prangishvili and Zillig, 2002). The fourth family, the *Guttaviridae*, has been suggested but not yet approved by the ICTV for the bearded-droplet-shaped particles with double-stranded DNA genome (SNDV) (Arnold et al., 2000a). Hosts of all these viruses are hyperthermophilic archaea of the orders Sulfolobales and Thermoproteales.

The uniqueness and diversity of hyperthermophilic viruses were surprising from the very beginning of their study. This was especially surprising because Zillig and co-workers were mainly aiming to isolate virus-host systems rather than to obtain a comprehensive picture of viral diversity in hot habitats. Our recent research specifically aimed at estimating viral diversity in hot habitats has revealed an even more remarkable picture. In enrichments of two samples from hot springs in Yellowstone National Park we have observed 12 different morphotypes of virus-like particles (Rachel et al., 2002; Prangishvili, 2003). This diversity included particles similar to fuselloviruses, rudiviruses, and lipothrixviruses, as well as typical head-and-tail phages and unusual particles not previously observed in nature. Here we report on the isolation from this collection of a novel fila-

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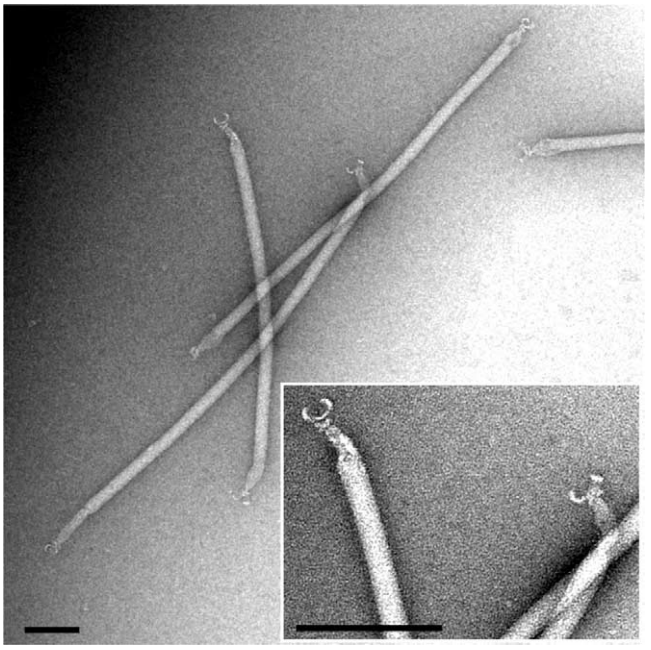


Fig. 1. Electron micrographs of particles of AFV1 with tail structures in their native conformation, negatively stained with 3% uranyl acetate. Bars, 100 nm.

mentous virus and its characteristics. The host range is confined to several strains of the hyperthermophilic archaeal genus *Acidianus*. The virus named AFV1, meaning *Acidianus* filamentous virus 1, is the first virus of *Acidianus* to be described in any detail.

Results

Virus hosts and virus purification

The virus AFV1 was observed in an enrichment culture of a sample from a hot spring in Crater Hills region in Yellowstone National Park (Rachel et al., 2002). Two rounds of dilution of the enrichment culture (1:100) did not result in any significant decrease in the number of virus particles, indicating active growth of the host strain. We attempted to isolate it from the enrichment culture by colony purification on Gelrite plates, as well as with a help of optical tweezers. Twenty-nine single isolates, named YS1–10, OV1–13, and W1–6, were screened for virus production by electron microscopy of cell-free growth cultures. Strains YS6, YS7, OV5, OV9-13 all yielded particles of the AFV1.

Cells of all 29 isolates were morphologically homogeneous irregular cocci of about 1.5 μm in diameter. DNA was extracted from cells of these isolates, and 16S rDNA genes were amplified by polymerase chain reaction (PCR) using the primers 8aF and 1512 uR (Eder et al., 1999). The PCR products were sequenced directly and sequences of all the strains were 100% identical. A comparative analysis of the sequence revealed that the isolates belong to the genus *Acidianus* of the Crenarchaeota. They were assigned to a novel species of this genus provisionally named "*A. hospitalis*" (M. Bettstetter and D. Prangishvili, in preparation).

Virus AFV1 was purified to homogeneity by precipitation with polyethylene glycol (PEG) 6000 and 1 M NaCl from the cell-free supernatant of late exponential culture of "*A. hospitalis*" YS6, followed by isopycnic gradient centrifugation in CsCl, yielding a sharp bluish-white opalescent band with a buoyant density of about 1.3 g/ml.

To study the viral host range, purified virus particles were added to growing cultures of potential hosts. Propagation of the virus was suggested by retardation of cell growth and was confirmed by electron microscopy. Several strains of *A. hospitalis* colony-purified from the same enrichment as *A. hospitalis* YS6, namely YS8, YS9, and W1-6, served as hosts for the virus. In addition, the AFV1 turned out to infect the type strain of the genus *Acidianus*, *A. infernus*. The two other known representative of this genus, *A. ambivalens* and *A. brierleyi*, were not hosts for the virus.

Virus structure

Particles of AFV1 are filaments measuring 900 × 24 nm (Fig. 1). Both ends carry claw-like structures with a diameter of 20 nm (Fig. 1). The claws are connected to the virus body by appendages, 60 nm long and 12 nm wide; linking the appendages and the claws is a collar-like structure 12 nm in diameter and 8 nm thick (Fig. 1).

The virus core is covered by an envelope that could be partially dissolved by treatment with 0.3% Triton X-100

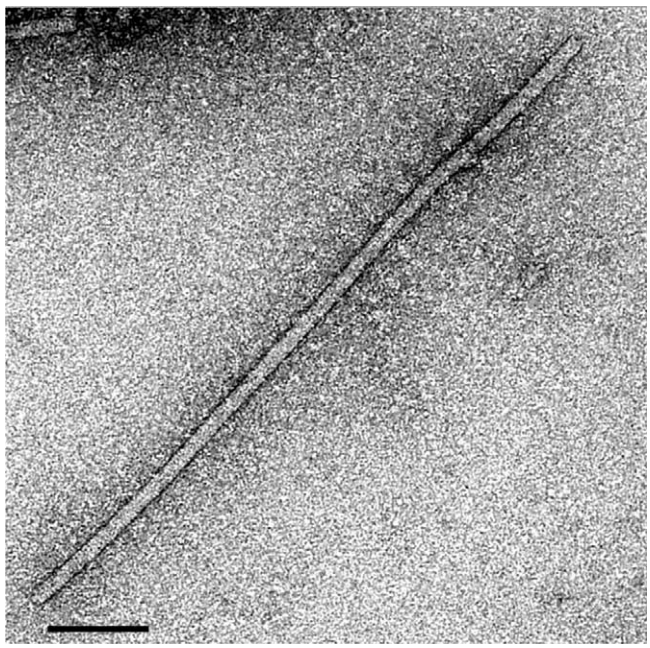


Fig. 2. Electron micrograph of a particle of AFV1 with an envelope removed by 0.1% Triton X-100, negatively stained with 3% uranyl acetate. Bar, 100 nm.

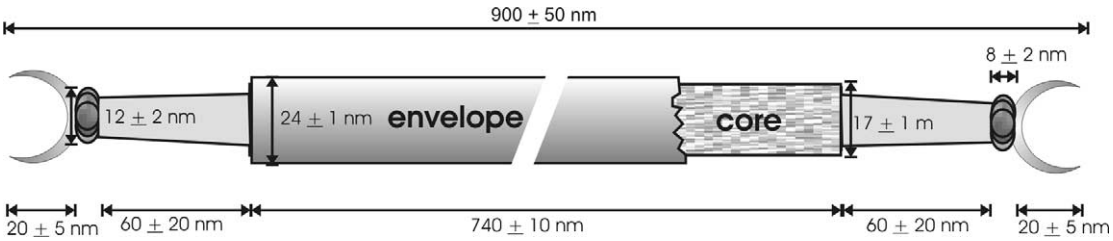


Fig. 3. A schematic model of a virion of AFV1.

(not shown). Treatment with 0.1% sodium dodecyl sulfate (SDS) resulted in complete removal of the envelope from virions. One such particle, completely devoid of an envelope, is shown in Fig. 2. The viral core has a diameter of 17 nm. The virion structure is represented schematically in Fig. 3.

Apparently, the unusual termini of the virions have a special function in a process of adsorption. We never observed direct attachment of virus particles to host cells but we often observed attachment of virus termini to the sides of thin filaments, most probably pili (Fig. 4). When particles of AFV1 were mixed separately with fractions of different cellular components, S-layer, cellular membrane, and pili, prepared as described under Materials and methods, preferential attachment was observed to the pili. The result of mixing virus particles with cellular membranes indicated by "M" is shown in Fig. 5. As can be seen, virions do not attach to the membranes; instead, they attach to the pili which were present in small numbers in the membrane preparation (Fig. 5). The two termini seem to be identical in their function, while sometimes we observed particles, both ends of which were specifically attached to the pili (Fig. 4). As a result of adsorption to the host receptor, viral claws apparently close and keep a particle attached to a pilus (Fig. 4). The contact seems rather strong, while on the pili we have often observed knob-like structures with 18 nm in diameter, apparently representing the viral termini which have been separated from the virus body (shown with white arrows in Fig. 4).

Virus-host relationships

During a cycle of productive infection with AFV1 there was no formation of any significant amounts of cell debris nor was a decrease in the cell density observed. This indicated that virus production was apparently not accompanied by lysis of host cells.

Growth rate of the host cells, *A. hospitalis* W1, in the initial stage of virus infection, was nearly totally blocked (Fig. 6). Two hours postinfection (p.i.) cell growth was slowly recovered, however, with a generation time of 20 h in contrast to a generation time of 11 h for uninfected cells. In later stages of infection, 8 h p.i., cells overcame this

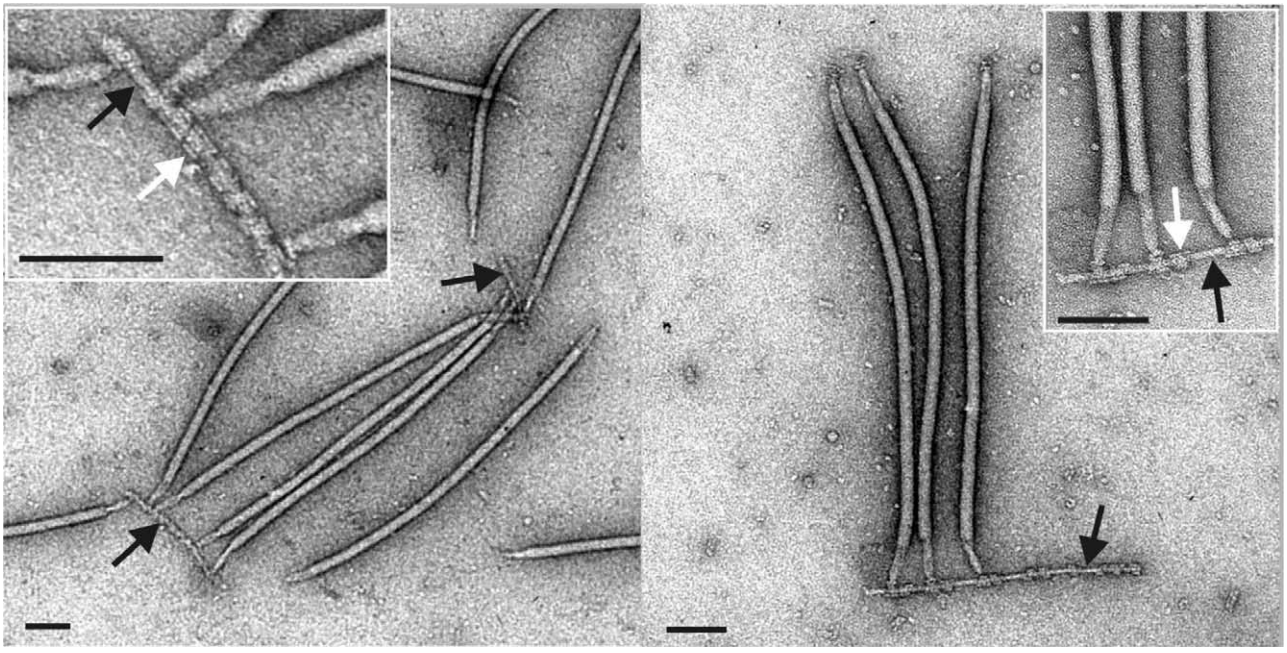


Fig. 4. Electron micrographs of particles of AFV1 adsorbed to pili of host *A. hospitalis* CH10/1, stained with 3% uranyl acetate. Black arrows indicate pili; white arrows show knots which are putative viral terminal structures separated from the virus body. Bars, 100 nm.

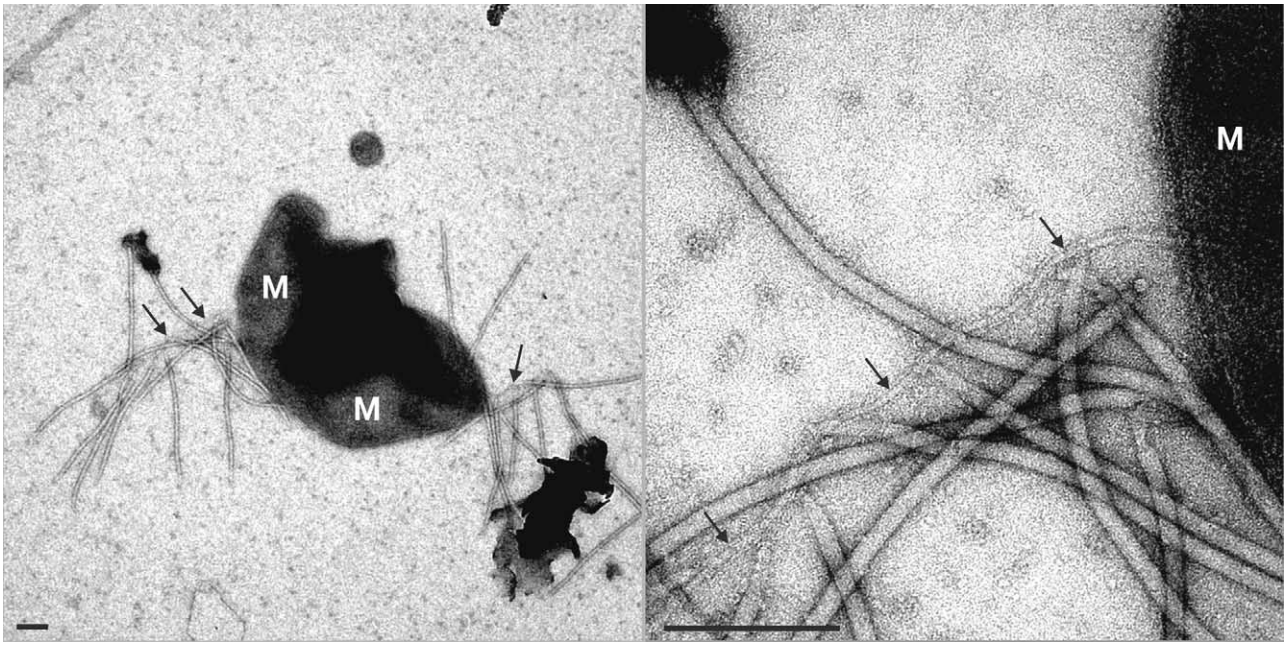


Fig. 5. Electron micrographs of particles of AFV1 incubated with membrane fragments (indicated by M) of cells of virus host, *A. hospitalis* CH10/1, stained with 3% uranyl acetate. Bars, 100 nm.

inhibition and finally reached the cell density identical to the uninfected control culture (Fig. 6). Retardation of growth at about 5 h p.i., observed in Fig. 6, is apparently caused by infection of cells which remained uninfected by newly produced virus particles. This implies that mature virus particles start to be released from infected cells 4–5 h after infection.

Infected host cells were not cured of the virus after several successive transfers into fresh medium (dilution 1:1000) and continuous growth for 2 months. The results suggested that AFV1 persists in its host in a stable carrier state.

Neither of the hosts of AFV1 was able to form lawns on Gelrite plates in conditions similar to those which we routinely use for lawn formation of *Sulfolobus* (Zillig et al., 1994). Variation in the density of the lawn inoculum, or in composition of the growth medium, did not cause any improvement. Thus plaque assays could not be established for AFV1 and, therefore, the virus titer could not be easily quantified. The only parameter of virus-host interactions which we were able to measure was the latent period, that is, the time interval between infection and release of first virus particles. Release of virus particles was detected by a decrease of intracellular viral DNA. This in its turn was quantified by measurements of signal intensities from viral DNA in Southern hybridization experiments. In these experiments, total DNA was isolated from virus-infected cells in 50-min intervals up to 7 h p.i., and later at 24 and 32 h p.i. Exactly equal amounts (5 μg) of all prepared DNAs were digested with *Eco*RI and the fragments generated were separated electrophoretically (Fig. 7). A blot of the gel was hybridized with an oligonucleotide OTR, 5′-GGTGA-GAGTGGATGAGTGAAATGAGTGTGGTTG-3′, the sequence of which was retrieved from an 8-kb *Eco*RI fragment of AFV1 comprising one terminus of the linear genome. Hybridization results are shown in Fig. 7b, and quantified signal intensities from the viral DNA are shown in Fig. 8. Apparently, a significant decrease in signal intensity is caused by a release of the mature virus particles, and a corresponding time interval, 4 h postinfection, is equal to a latent period. The result corresponds to the above described observation of a second wave of retardation of host growth apparently caused by a second round of virus infection at about 5 h p.i. (Fig. 6).

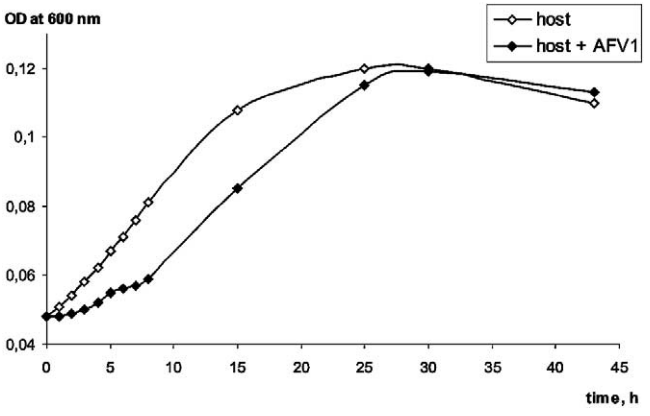


Fig. 6. Growth of uninfected (□) and AFV1-infected (■) cells of *A. hospitalis* W1.

It is noteworthy that at no stage of infection were frag-

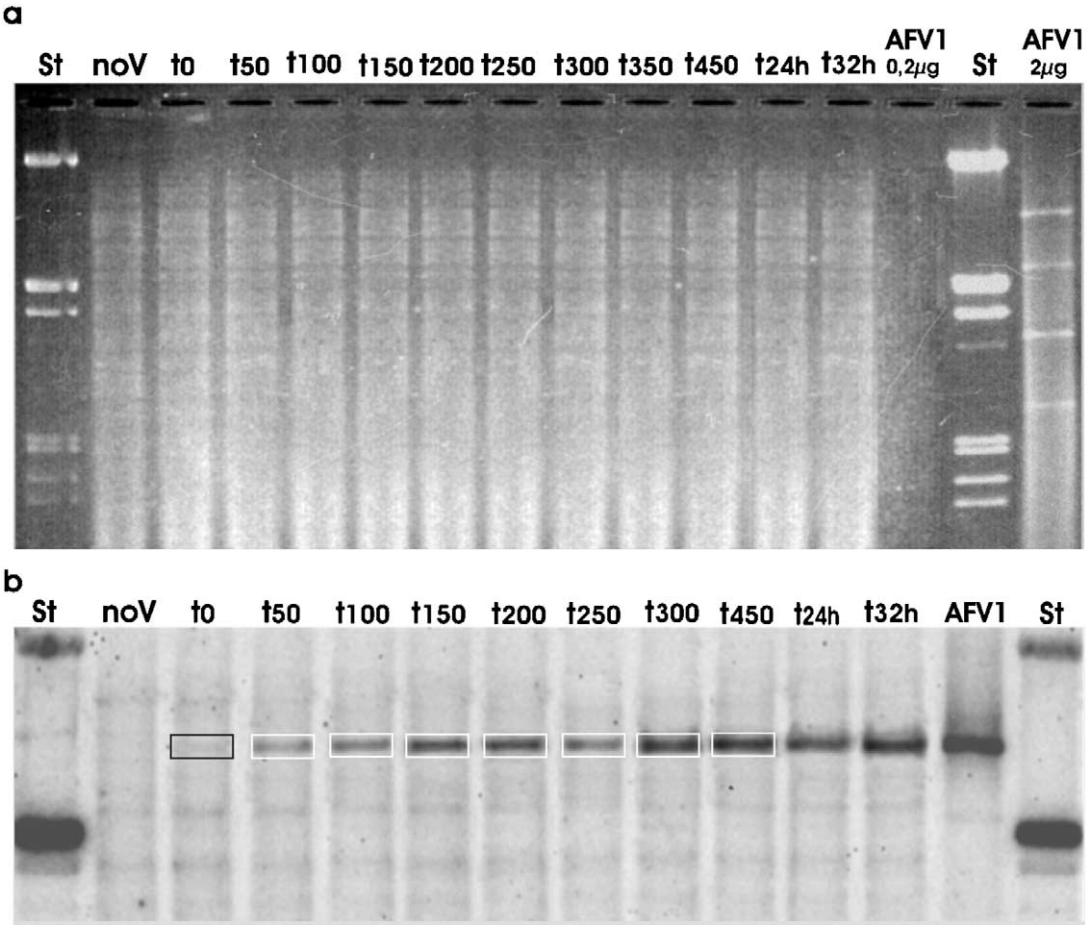


Fig. 7. Agarose gel electrophoresis of *Alu*I fragments of host and virus DNA. (a) Stained with ethidium bromide; (b) phosphoscreen image of hybridization signals of the blot of gel "a" with ^{32}P-labeled oligonucleotide AFV33N, representing a portion of the largest *Alu*I fragment of AFV1 DNA. Lanes t_x, 5 µg DNA from virus-infected host cells prepared at corresponding time intervals after infection, in minutes (where not indicated) or hours. Lane noV, DNA from uninfected host cells. Lane St, *Eco*RI-*Hin*dIII-digested λ DNA. Rectangles represent areas of signal quantification; black rectangle shows area with background signal which was substracted from signals quantified in white rectangles.

ments of viral DNA prominent in restriction digestion patterns of cellular DNA (Fig. 7a), nor were such bands prominent in a digest of cellular DNA of the original host of AFV1, *A. hospitalis* W1 (not shown). The results indicate a low intracellular copy number of virus DNA, probably less than 10 copies per chromosome.

Proteins

The protein pattern of AFV1 on SDS–PAGE showed five major bands of polypeptides with molecular masses of 130, 100, 80, 30, and 23 kDa (Fig. 9, lane AFV1). Viral origin of these polypeptides was confirmed by their absence among host proteins which were separated on the same gel (Fig. 9, lane Host). The origin of several minor proteins bands observed (Fig. 9, lane AFV1) is difficult to assess. A significant portion of viral proteins did not migrate in the separating gel even after intensive treatment with 1% SDS, 5 mM dithiothreitol, and 5% β-mercaptoethanol (Fig. 9, lane AFV1).

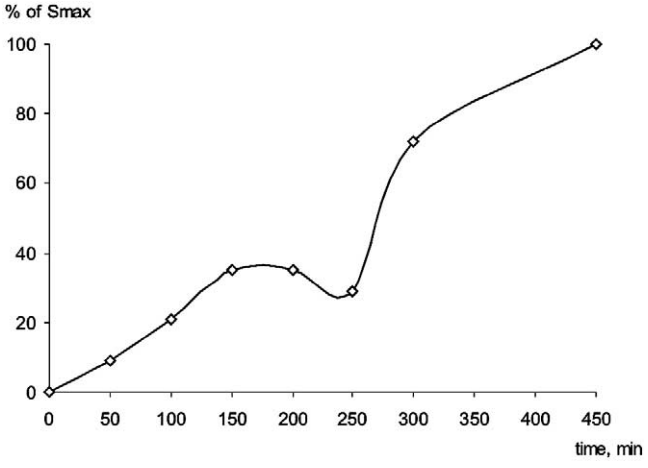


Fig. 8. Increase of intracellular AFV1 DNA in a course of an infection cycle. Relative amount of DNA is represented in relation to an amount at 450 min postinfection, S_{max}, and was measured by comparing corresponding quantified signal intensities shown in Fig. 7b.

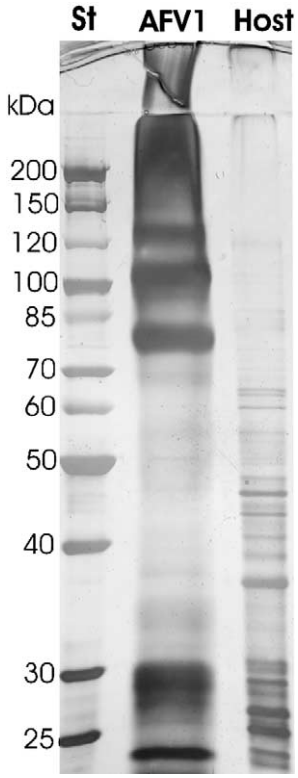


Fig. 9. SDS–PAGE of proteins of virus AFV1 and its host, *A. hospitalis* W1, silver stained. The size of markers is indicated (lane St).

Lipids

Thin-layer chromatography of lipids extracted from virus particles by chloroform/methanol treatment revealed a pattern of bands resembling that of host lipids prepared in the same way (Fig. 10). It was unlikely that these lipids were derived from cell debris present in virus preparation because the virus prepared for lipid analysis was purified by CsCI buoyant density and was free from any contamination by cell debris, e.g., membrane vesicles, as judged by electron microscopic studies. Moreover, with the exception of the largest lipid component, band mobilities were different, and one of the host lipids was apparently absent in lipid preparation from virus particles. The results suggested that the AFV1 lipids originate from a pool of lipids of host cells, although most of them are apparently modified (Fig. 10).

The genome and its organization

Nucleic acid extracted from purified virus particles could be digested by type II restriction endonucleases and was not sensitive to RNase, indicating that viral genome is double-stranded DNA. From fragment patterns obtained by digestion with restriction endonucleases, the size of the viral genome was estimated at about 21 kb. These patterns were independent of the host from which AFV1 was obtained.

Both DNA strands of the viral genome were sequenced (see Materials and methods) with approximately three-fold coverage and ambiguities were checked by sequencing PCR fragments amplified from the appropriate viral region. No sequence was detected on the sequence traces beyond the terminal inverted repeat GCCCCCC, at either end, and we infer that they constitute the termini. The terminal sequences were read on each strand except for the C-rich sequence at the left end of the virus (Fig. 11). The total sequence comprised 20,869 bp.

An ORF map was constructed by initially selecting for the largest possible ORFs employing all three start codons (AUG, GUG, and UUG) and then examining each individual gene for evidence of a promoter and/or Shine–Dalgarno motifs. When their presence supported the existence of a shorter coding region, then this was selected. Several ORFs end at UGA codons and some of these may extend further downstream. The map for the whole viral genome is presented in Fig. 11. Forty ORFs were identified when a size limit was lowered to 48 amino acids. Several other small ORFs were detected on opposite DNA strands overlapping ORFs shown in Fig. 11 and we cannot exclude that some of these are also expressed. About 75% of the ORFs shown in Fig. 11 are preceded by putative promoter sequences, and 60% are preceded by putative Shine–Dalgarno sequences. Moreover, 25% of the ORFs exhibit downstream T-rich sequences which are likely to be transcriptional terminators (Reiter et al., 1988). For one-third of the genes the promoters are located so close to the start codons that transcription must start just before the start codon. This occurs commonly in the genome of *Sulfolobus solfataricus* for single ORFs or for the first ORF in a operon (Tolstrup et al., 2000). Details of putative promoter and Shine–Dalgarno sequences, as well as possible operon structures, are presented in Table 1.

Two ORFs located at the left terminus, ORF150 and ORF190, and one located at the right terminus, ORF75,

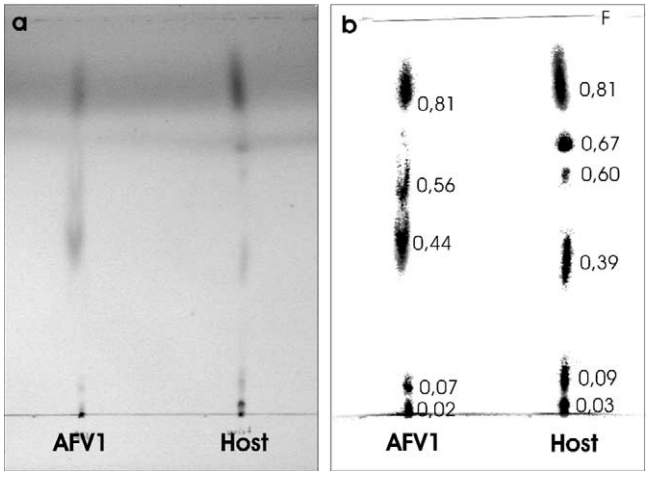
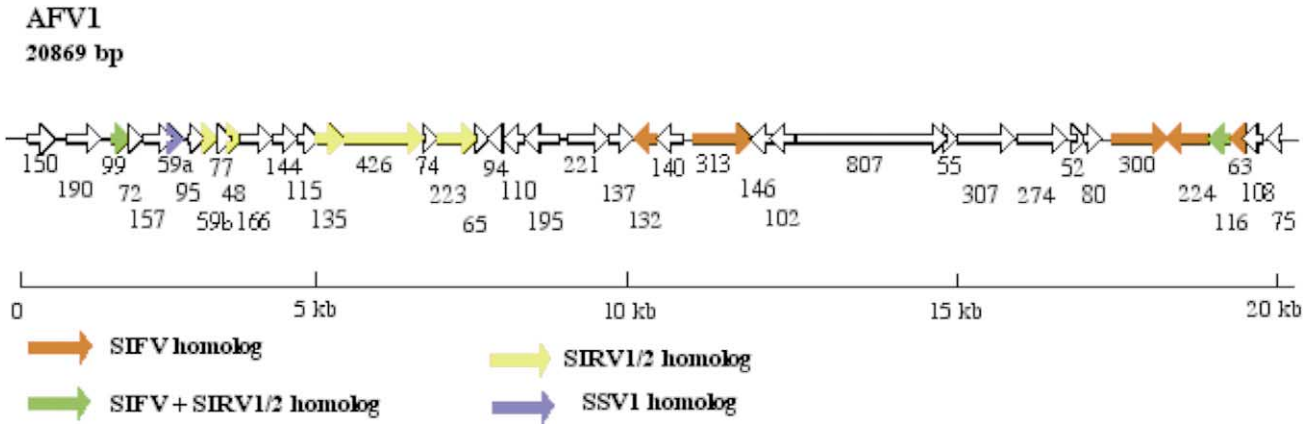


Fig. 10. Thin-layer chromatogram of lipids extracted from AFV1 particles and membrane vesicles of uninfected host, *A. hospitalis*. "b" is a digitally modified image of "a". RF (ratio to front) values of all spots are indicated. Position of the solvent front is indicated as a black line.



11

Left end

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   1 CGGGGGGGAG CATGTGGTTT GCGGTTTGAG GTTCGCACTT GTGATTTTTG
  51 TTCAGTTCAG TGTGGTAATT GTTTTTTGTT CTTGTGTGTG TTTTTATCTG
 101 TTTGTTTTTG TGTTTTTCTG TTTAGTTCTG TTCTTGTTCT GTTTTTGAAT
 151 TTATTTCTGG ACCTAAAGTG TTTTAATTGT TTTTTGTTCT TGTGTGTGTT
 201 TTTATCTGTT CTGTTCAGTT AAGTTCAGTT TTGTTTCTGT TCTGTTTTGT
 251 TTTTGAATTT TGGCTAACTG CAGCCGTGAT AAAAATTCAA GAAAATCCTT
 301 TTAAACCTAG AAAGCGGGTA TGAAAATGGG GAAAAAAATG AGTGAAGACC
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Right end

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20521 TATCTCTATT TTTGCATGCA TTTTTTTCCC CGTTTTTATA TCCGTAAAGC
20571 GGATATAAAA ACCTTTTCCT GAATTTTAAA CAGAACTGAA CTAAACAGAA
20621 CTGAACAGAA CTATATAACA ATAACATAAC ACAAAACTGA ACTTAACAGA
20671 ACTGAACAGA TACAAACACT ATAACAGAAC TAAACAGAAT AGAACAGAGC
20721 AGAACAGAAA CAAACAACAA AACAACACCA AACTGAACAG AACTATACAG
20771 AACAGATAAA AACACCAAAC AGATAATAAC ACAACACACC ACACCAACCA
20821 CCAACACCAC AACCATCACC AAAACCACCA CCAATATAGT TCCCCCCCG
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13

Fig. 11. Genome map of AFV1 showing the size and direction of transcripts of the putative genes present on the two DNA strands. Homologs that are shared with *Sulfolobus* viruses, SIFV, SIRV1/2, and SSV viruses are color coded.

Fig. 13. Terminal sequences of one strand of AFV1. The 350 nucleotide termini contain numerous short direct repeats, commonly TTGTT, at one end and the complementary sequence AACAA at the other. There is an 11-bp G-C inverted repeat at the extremities, while the other end of the region exhibits larger imperfect inverted repeats shown with arrows. Direct repeats of 37-mer sequence on the left end are underlined without arrowheads. Pentanucleotide repeats are shown in similar colors.

show identical sequences AAAA/TGGGGAAAAAA(ATG), immediately upstream from the start codon and high sequence similarity extends upstream to the promoter region (Fig. 12). The same sequence precedes a short, presumably truncated ORF12 located between ORFs 190 and 99 at the left end (Fig. 11). The sequence is presumably important for regulation of expression of the terminal genes. However, such sequences were not observed in genomes of the linear *Sulfolobus* viruses SIRV and SIFV (Arnold et al., 2000b; Peng et al., 2001).

BlastP searches against the NCBI database (Altschul et al., 1997) revealed 13 ORFs homologous to viral ORFs from the *Sulfolobus* genus. They include the lipothrixvirus SIFV, the rudiviruses SIRV1 and SIRV2, and the SSV fuselloviruses. Their sizes, positions, and orientation in AFV1 are indicated in Fig. 11. Three of these homologs, ORFs 135, 426, and 223, are ordered similarly to their respective homologs, ORFs 131b, 436, and 207, in both SIRV viral genomes.

Functions have been assigned to a few genes on the basis of sequence comparisons (Table 2). Thus ORFs 300 and 313 both show significant sequence similarity to the same three ORFs in the SIFV genome (Fig. 11) but show very low

Table 1
Analysis of the ORFs of virus AFV1

AFV1 ORFs[a]	Promoter sequence (location[b])	Shine–Dalgarno sequence (location[b])	Distance to upstream ORF (bp)	Possible terminator sequences and operons
150	TTTTAAA (−32)	GGGGA[c] (−5)	—	ttttttttcttctttctgtttgtcttctgtttct
190	TTATATA (−31)	GGGGA (−5)	129	tctttttttctctgttcttctttcttctgtcctc
99	TTTTTATA (−37)	GGTAG (−6)	172	operon
72	AAATATA (−28)	GGTGT (−6)	−16	
157	TTAAAGAA (−33)	GGTGA (−6)	−4	
59a	—	—	−8	atatctttttttctctctgtttctccttctggtct
95	TTTTTAA (−32)	GGTGA (−5)	87	operon
59b	—	TGTGA (−4)	4	
77	TATAATA (−38)	GGTGA (−5)	5	
48	—	GGTGG (−4)	1	
166	AATAATA (−31)	—	−8	
144	AATAAATTGAT (−33)	GGAGA (−6)	−14	
115	AGCTTTAAA (−35)	GGGGA (−5)	−20	
135	TTTAAGAA (−38)	GGTGT (−5)	−8	
426	AAGAAAAG (−27)	GATGA (−3)	−4	
74	TTTTTATAT (−32)	GGTGA (−6)	48	
223	—	GGTGG (−4)	−34	
65	TATATT (−28)	GGTGT (−7)	−22	gtctttttctctctctttc
94 (−)	—	GGTGA (−6)	−20	
110 (−)	TTTATAA (−26)	GTTGA (−6)	−4	
195 (−)	TAATATA (−22)	—	—	Operon
221	TTTTAAGAA (−40)–TATTACTG (−24)	—	—	Operon
137	—	GGTGA (−6)	−11	taaagtctgctatttttt
132 (−)	AATTTATAA (−36)	GGTGG (−6)	−1	aacttttttcttctttttctagttc
140 (−)	AATAAAATAT (−29)	—	—	Operon
313	TATATTA (−22)	—	—	ctgttt
146 (−)	—	GGTGA	−32	cttttcattatctct
102 (−)	TATTTTTAATT (−21)	—	—	Operon
807	ACTTATAT (−24)	—	—	Operon
55	ATTAA (−23)	GGAGA (−8)	−1	
307	AGAATT (−35)	GTGA (−7)	−4	
274	TTTAATAA (−22)	—	33	
52	—	GTGA (−6)	133	
80	—	GGTGG (−5)	−8	
300	TTTTTAAT (−23)	—	180	
224 (−)	AAAAATATATTAT (−20)	—	−4	gctttttgct
116 (−)	TTTTTATA (−24)	—	33	
63 (−)	TATATAAA (−36)	—	1	
108 (−)	TTTTTAATT (−21)	—	43	
75 (−)	TTTTTATAT (−31)	—	—	taagctttttttgttttt

Note. All ORFs considered significant are listed in column 1.

[a] ORFs followed by (−) are coded on the complementary strand. Putative promoters and Shine–Dalgarno motifs are given for putative ORFs.

[b] Numbers in parentheses indicate the number of nucleotides between the last nucleotide of the motif, in each column, and the first nucleotide of the putative start codon. Two ORFs at the left end, ORFs 150 and 190, and one at the right, ORF75, show the identical sequences immediately upstream of the start codon: AAAA/TGGGGAAAAAA(ATG), and partial sequence similarity extends further upstream to the promoter motif. Several promoter motifs are preceded, or proceeded, by a CAG sequence. Putative terminators are T-rich sequences similar to those described for many *Sulfolobus* genes (Reiter et al., 1988).

sequence similarity to one another. All exhibit group 1 motifs characteristic of glycosyl transferases which were considered to be involved in synthesis of the viral coat lipid in SIFV (Arnold et al., 2000b). ORFs 48 and 59b show high sequence similarity to hypothetical proteins of several archaeal chromosomes (She et al., 2001) and of the Sulfolo-

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ORF 150   CAAGAAAATCCTTTTAAACCTAGAAAGCGGGTATGAAAAT-GGGGAAAAAA
ORF 190   CA-GAAA-GCCTTATATACTGTTTCTTTGTATATAAAAATAGGGGAAAAAA
ORF 75    CAGGAAAAGGTTTTTATATCCGCTTTACGGATATAAAAAC-GGGGAAAAAA
ORF 12              CTAGTTTATCTGATTTATGGGCATAAAAAC-GGGGAAAAAT
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Fig. 12. Alignment of the sequences upstream of the start codon of the terminal ORFs 150, 190 (left end), and 75 of AFV1 (right end). The additional sequence precedes a short, presumably truncated, ORF immediately downstream from ORF190. Underlined are putative promoters.

Table 2
Homologous ORFs shared by virus AFV1 and *Sulfolobus* viruses SIFV, SIRV1, SIRV2, or SSV1

AFV1	SIFV	SIRV2	SSV1	Protein motif
807				oxidored q1/photosystem 1
426		440 (33/52)		
313	0041 (30/48)[a]			Glycosyl transferase group 1
300	0041 (23/44)			Glycosyl transferase group 1
224	0066 (44/60)			
223		207 (27/47)		
135	167 (28/51)			
132		131 (27/49)		
116	0026 (28/51)	114 (39/68)		
99	0014 (35/58)	102 (29/45)		
		95 (26/52)		
63	0074 (64/75)			
59a			A-45 (40/66)	Zinc finger-C2H2
59b		59 (50/74)		Ribbon-helix-helix, CopG family
48		59 (54/71)		Ribbon-helix-helix, CopG family

[a] Sequence identity/similarity percentages are listed in parentheses.

bus conjugative plasmid pNOB8 (She et al., 1998). Moreover, both exhibit a ribbon-helix-helix motif common to the CopG family. ORF59a, which is a homolog of the SSV1 A-45 protein (Fig. 11), contains a C2H2-type zinc finger motif and may be involved in transcriptional regulation. The largest protein, ORF807, exhibits five putative transmembrane helices and shows low sequence similarity to two related motifs found in the photosystem I reaction center subunit XI and in the oxidored_q1 of the NADH:ubiquinone oxidoreductase (complex I). The latter motifs are also associated with membrane-binding proteins and we infer, therefore, that ORF807 is a membrane-binding protein that may be involved in the viral transfection process.

The terminal 300 nucleotides of the AFV1 genome are rich in short direct repeats (Fig. 13). Many copies of the repeat TTGTT and variants thereof including CAGTT and NTGTT are present on opposite strands of the two termini. Moreover, two direct repeats of 37 bp occur at the left end of the genome (underlined without arrowheads in Fig. 13). At the genome termini there is an inverted repeat containing eight G-C pairs, and, moreover, between 270 and 340 bp from each end there are two almost perfect inverted repeats of 14 and 26 bp (Fig. 13).

The exact structure of the genomic termini is unclear at present. Analysis of restriction digests of the DNA of AFV1 revealed that the terminal fragments were underrepresented and, according to the staining intensity, their relative molarity was about half of that of the nonterminal fragments (Fig. 14); this is clear for both the *Eco*RI digest (lane 2) and the *Hind*III digest (lane 3). In the former, the 8271-bp left end band and the 5582-bp right end band are both much weaker than the smaller internal fragments bands of 3733 and 2448 bp. Similarly, in the *Hind*III digest, the 3823-bp left end band is a weak lower edge of the strong internal 4467 bp band and the right end band at 562 bp is almost nondetectable.

Discussion

Information on viruses infecting hyperthermophilic archaea of the genus *Acidianus* is very limited. Production of filamentous virus by a species presumably belonging to *Acidianus* has been reported previously (Zillig et al., 1994). However, the exact phylogenetic position of this species,

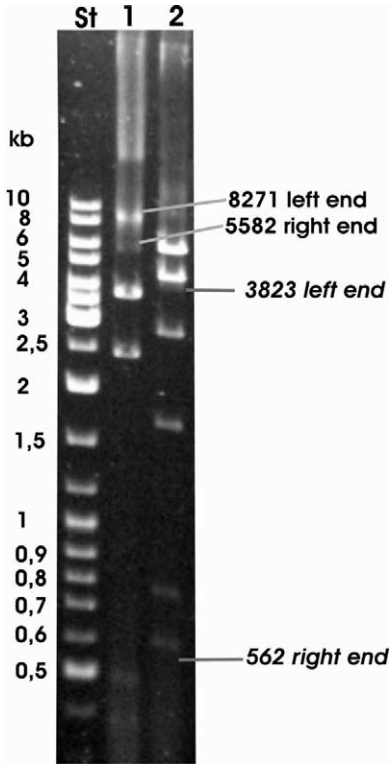

Fig. 14. Restriction digests of AFV1 DNA. St, size marker fragments; lane 2, 240 ng DNA digested with *Eco*RI; lane 3, 240 ng DNA digested with *Hind*III. DNA fragments corresponding to the left and right termini are indicated.

which was isolated from a sample from an Icelandic solfatara, has not been demonstrated. Moreover, the host range of the virus has not been probed. Available information on these filamentous particles, which are about 2 μm long and 27 nm in width, is restricted to a suggestion that they are enveloped and that the core contains equal amounts of two major proteins of about 30 and 20 kDa and a 56-kb double-stranded genome DNA (Zillig et al., 1994). These properties do not coincide with those of AFV1 but they resemble characteristics of the filamentous virus SIFV of *Sulfolobus*, also isolated from a sample from an Icelandic solfatara (Arnold et al., 2000b).

The original host of the AFV1 represents a new species of the genus *Acidianus*, provisionally named *A. hospitalis* (M. Bettstetter and D. Prangishvili, in preparation). Several novel *Acidianus* isolates apparently representing the same species could be infected by AFV1. Of the previously described members of the genus *Acidianus*, only *A. infernus* is a host for the virus.

Filamentous virions of AFV1 are covered with a lipid envelope and contain double-stranded linear DNA. These features are characteristic of representatives of the family *Lipothrixviridae* and, therefore, we assign AFV1 to this family. It is noteworthy that hosts for all known members of this virus family are hyperthermophilic archaea of the orders *Thermoproteales* and *Sulfolobales* (phylum Crenarchaeota).

As for other archaeal linear viruses, e.g., rod-shaped rudiviruses (Prangishvili et al., 1999), direct adsorption of AFV1 virions to host cells was not observed. Moreover, studies on the interaction of AFV1 virions with different fractions of host cells have shown their specific absorption to pili. AFV1, and probably other archaeal linear viruses, share this feature with filamentous, single-stranded DNA bacteriophages of the family *Inoviridae*, which use the tip of a pilus as a cell receptor. In contrast to the latter viruses, which bind to receptors at only one end of the virion, linear archaeal viruses have identical terminal structures at both ends, and both ends are able to bind to cell receptors. Archaeal viruses differ in their terminal structures. The claw-like structure of AFV1 has not been observed in any other known virus.

AFV1 shares certain characteristics of virus-host interactions with other viruses of Crenarchaeaota. Similar to most of them, it does not kill its host during productive infection (reviewed by Prangishvili et al., 2001). Under certain growth conditions there is a balance between production of virus and multiplication of host cells. After several successive dilutions of virus-carrying cultures and prolonged incubation, the virus was still present in host cells, indicating a stable carrier state of host-virus relationship. Cellular DNA bands originating from the viral genome could not be distinguished in the restriction digestion pattern at any stage of infection with AFV1. Therefore, we infer that the intracellular copy number of AFV1 DNA is less than 10 copies per cell.

Assignment of AFV1 to the *Lipothrixviridae* was corroborated by its genome organization. In particular, 15% of AFV1 ORFs have homologs in the genome of *Sulfolobus* lipothrixvirus SIFV. The same percentage of AFV1 ORFs also has homologs among ORFs of *Sulfolobus* rudiviruses SIRV1 and SIRV2. These data correlate with the previously observed close relationship between lipothrixviruses and rudiviruses, and a suggestion that the two viral families form a superfamily (Peng et al., 2001).

As for other viruses of Crenarchaeota, functions of most encoded proteins could not be predicted from similarity with sequences in databases. Among few predicted functions are two glycosyltransferases with homologs among SIFV proteins. In SIFV, these proteins were suggested to be involved in virus-specific modification of host lipids, due to their presumed ability to transfer activated sugars to a variety of substrates, including lipopolysaccharides (Arnold et al., 2000b). The putative glycosyl transferases of AFV1 may have the same function. The finding of modified host lipids in AFV1 virions points to the existence of a virus-encoded enzymatic apparatus responsible for such modification.

The nature of the ends of the linear genome of AFV1 is still unclear. The substoichiometric yields of the terminal fragments in the restriction endonuclease digests (Fig. 14) could indicate that a large proportion of the virus DNA is modified at their termini, possibly by a covalently bound protein which would affect the mobility of these fragments. Another possibility is that the termini can interact producing larger fragments that would tend to dissociate in the gel and produce a background smear.

The terminal sequence regions of the viral genome are very unusual. The linear dsDNA genomes of other crenarchaeal viruses exhibit very large inverted terminal repeats, e.g., about 1500 bp for the SIRV viruses. The latter viruses also contain regularly spaced (about 40 bp) direct repeat sequences of consensus sequence AAATTCC (Blum et al., 2001; Peng et al., 2001). AFV1 shows smaller, and less regular, inverted terminal repeats and many closely clustered direct repeats of the sequence TTGTT, and close variants thereof, spread over the terminal 300 bp, and on opposite strands (Fig. 13). They differ from the regularly spaced direct repeats of SIRV terminal sequences and are much more reminiscent of the telomeric ends of eukaryal chromosomes where the telomerase produces multiple short, imperfect, repeat sequences when generating a 3′-overhang on the lagging strand to prevent shortening of the linear genome during DNA replication (Wellinger and Sen, 1997; Kelleher et al., 2002). Possibly, a more primitive telomeric mechanism also operates in some archaeal viruses with linear DNA.

Materials and methods

Enrichment culture, isolation of virus hosts, and virus purification

A sample was taken from an acidic hot spring (pH 2, 85°C) in the Crater Hills region in Yellowstone National

Park and an enrichment culture was established as described by Rachel et al. (2002). Single strains were isolated from the culture either by plating on colloidal sulphur-containing Gelrite (Kelco, San Diego, CA) plates as described by Zillig et al. (1994), or by using optical tweezers as described by Barns et al. (1994) and Huber et al. (1995). They were grown in the same medium as the enrichment culture and were screened for virus production.

For production of AFV1, the strain of the original host *A. hospitalis* CH6 was grown until late exponential phase. Cells were removed by low-speed centrifugation (4500 rpm in a Beckman JA 10 Rotor). The virus particles were precipitated from the supernatant by the addition of NaCl to 1 M and PEG 6000 to 10% (w/v) and incubated overnight at 4°C. The sediment was collected by centrifugation in a Beckman JA14 rotor at 12,000 rpm for 30 min and suspended in buffer TA (20 mM Tris–acetate, pH 6). Remaining cell debris was partially removed from the suspension by centrifugation at 2500 rpm for 10 min in Laborfuge 400R (Heraeus). The supernatants containing virus particles and a reduced amount of cells debris were pooled. AFV1 particles were purified from the suspension by centrifugation in a CsCl buoyant density gradient (0.45 g/ml) in a Beckman SW50 rotor at 48,000 rpm for 40 h. The fractions were collected with a help of a syringe, dialyzed against buffer TA, and analyzed in an electron microscope for a presence of virus particles.

To obtain large amounts of AFV1, the virus was propagated in the laboratory host *A. hospitalis* CH10/1. Infected cells were harvested in late exponential growth phase. The virus was isolated and purified from cell-free growth medium as described above.

Preparation of DNA

For preparation of DNA, *A. hospitalis* CH10/1 was grown until the midexponential phase. Cells were collected and suspended in a buffer TE containing 10 mM Tris–HCl, pH 8, and 1 mM EDTA. Cells were lysed by addition of SDS and Triton X-100 to final concentrations of 0.8% (w/v) and 0.06% (v/w), respectively, and lysates were extracted twice with phenol and twice with phenol/chloroform. DNA was precipitated from the aqueous phase by adding of 0.1 vol of 3 M sodium acetate, pH 5.3, and 0.8 vol of isopropanol. The DNA pellet was washed with 70% ethanol, air dried, and resuspended in an appropriate volume of buffer TE.

Virus DNA was prepared in the same way as the cellular DNA. Virus particles for DNA preparation were disrupted by treatment with 1% SDS for 1 h at room temperature.

DNA hybridization

DNA fragments obtained by digestion with restriction endonucleases and separated on 1% agarose gel were transferred from the gel onto a Biodyne B nylon membrane (Pall) as described in Sambrook and Russel (2001). Oligonucleotides were with [α-^{32}P]ATP using T4 polynucleotide kinase, and Southern hybridization was performed using standard procedures (Sambrook and Russel, 2001). The filter was washed once in 5× SSC/0.1% SDS at 20°C, and twice in 1× SSC/0.1% SDS at 42°C.

Preparation of membrane vesicles and S-layer sacculi

Membrane vesicles and S-layer sacculi of *A. hospitalis* W1 were prepared according to Grogan (1996).

Preparation of pili

Pili were sheared from growing cells of *A. hospitalis* W1 using a standard Waring blender (2 min). Cells were removed from the culture by centrifugation at 4500 rpm for 10 min in Laborfuge 400R (Heraeus). The remaining cells and cell debris were removed by additional centrifugation at 20,000 rpm in the rotor SW41 (Beckman). Pili were collected from the supernatant by centrifugation at 45,000 rpm in the rotor SW60 (Beckman) and suspended in a corresponding volume of the buffer TA.

Protein analysis

Proteins were analyzed in SDS–polyacrylamide gel, as described by Schägger and Jagow (1987), and stained with Coomassie brilliant blue R-250 (Serva), or silver according to Blum et al. (1987).

Lipid extraction and analysis

Lipids were extracted from cells of *A. hospitalis* W1 as described by Arnold et al. (2000b) and analyzed according to Trincone et al. (2002).

Electron microscopy

Samples were deposited on a carbon-coated copper grid, negatively stained with 3% uranyl acetate, pH 4.5, and examined using a CM12 transmission electron microscope (FEI, Eindhoven, The Netherlands) operated at 120 kV. Magnification was calibrated using catalyzed crystals negatively stained with uranyl acetate (Reilein, 1998). All images were digitally recorded using a slow-scan CCD camera, connected to a PC running TVIPS software (TVIPS GmbH, Gauting, Germany).

DNA sequencing and sequence analysis

A shot-gun library was prepared for the AFV1 virus. DNA was sonicated to produce fragments of about 2 kb and these were cloned into the *Sma*I site site of the pUC18 vector. DNA was extracted from single colonies using a Model 8000 Robot (Qiagen, Westburg) and sequenced in a MegaBACE

1000 sequenator (Amersham Biotech). The viral sequence was assembled using the Sequencher program 3.1. Motifs were identified by searching the PFAM database (http://pfam.wustl.edu/cgi-bin/). Sequence searches were made in public sequence databases (Altschul et al., 1997; Tatiana and Thomas, 1999).

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