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SNDV, a Novel Virus of the Extremely Thermophilic and Acidophilic Archaeon *Sulfolobus*

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We describe a novel virus, SNDV (*Sulfolobus neozealandicus* droplet-shaped virus), of the crenarchaeotal archaeon *Sulfolobus,* which was found in a carrier state in a *Sulfolobus* strain isolated from a field sample from New Zealand. SNDV particles are droplet-shaped and densely covered by thin tail fibers at their pointed ends. The virion consists of a core and a coat. The latter has the appearance of a beehive and has a surface that is either helically ribbed or a stack of hoops. The genome is cccDNA of 20 kb, which is modified by *dam*-like methylation. It is cleaved by only a few type II restriction enzymes e.g., *Dpn*I but not *Mbo*I, demonstrating an N(6)-methylation of the adenine residue in GATC sequences. The DNA-modifying system differentiates between virus and host. We postulate a virus-encoded methylase that is active on hemimethylated DNA. The host range of SNDV is confined to few *Sulfolobus* strains from New Zealand. The virus persists in an unstable carrier state rather than as a prophage. Due to its uniqueness we propose to assign it to a novel virus family termed *Guttaviridae.*
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Key Words: Crenarchaeota; virus taxonomy; *Guttaviridae;* DNA modification; methylation.

INTRODUCTION

The archaeal domain consists of two kingdoms, the Euryarchaeota and the Crenarcharchaeota, the latter comprising the two orders Sulfolobales and Thermoproteales (Woese and Fox, 1977; Woese *et al.,* 1990). Almost all viruses of euryarchaeota resemble bacterial head-and-tail phages. Together with these they belong to either of the well known virus families *Siphoviridae* or *Myoviridae* (reviewed in Reiter *et al.,* 1988; Zillig *et al.,* 1988; Arnold *et al.,* 1999). In contrast, all crenarchaeotal viruses known to date are morphologically unique. To account for their peculiarity three novel virus families, the *Fuselloviridae,* the *Lipothrixviridae,* and the *Rudiviridae* had to be introduced (reviewed in Zillig *et al.,* 1998). Here we describe the novel crenarchaeotal virus Sulfolobus neozealandicus droplet-shaped virus (SNDV) from a *Sulfolobus* strain from New Zealand. It exhibits an unusual droplet-shape and harbors a genome of double-stranded cccDNA. These two features are not found jointly in any other virus. Therefore SNDV has been assigned to a floating genus termed Guttavirus (International Committee on Taxonomy of Viruses, Taxonomic Proposal No. 97.05) not belonging to an established virus family. Based on the following description of SNDV we propose the introduction of the novel virus family *Guttaviridae.*

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RESULTS

Virion structure

SNDV was discovered via a standard screening procedure to detect extrachromosomal genetic elements in *Sulfolobus* strains (see Materials and Methods). The culture supernatant of the *Sulfolobus* strain STH3/1 isolated from a solfataric field at Steaming Hill, south of Lake Taupo in New Zealand, contained droplet-shaped particles that were densely covered by beards of thin tail fibers at their pointed ends (Figs. 1A–1D). The particle ranged from 110 to 185 nm in length and from 95 to 70 nm in width. It is not clear whether the differences in shape and size were "snapshots" of a flexible virion or whether the SNDV population consisted of particles with different shapes. However, in SNDV specimens that were embedded in vitreous ice the droplet shape was most dominant (Fig. 1B). Virus particles that had been isolated via centrifugation in a CsCl density gradient were highly irregular, apparently due to osmotic disruption. Broken particles harbored or released an oblong virus core (Fig. 1C) from which in some cases a thin, looped filament emerged (Fig. 1D). The core was covered by a coat that had a beehive-like structure, the surface structure of which could be either helical or stacked (Fig. 1A).

Virus–host relationship

A concentrated virus suspension produced zones of growth inhibition on lawns of different *Sulfolobus* strains. Five of eight *Sulfolobus* strains from New Zealand including strain STH1/3 but none of six *Sulfolobus* strains from Iceland proved sensitive in this test. STH1/3 was used as

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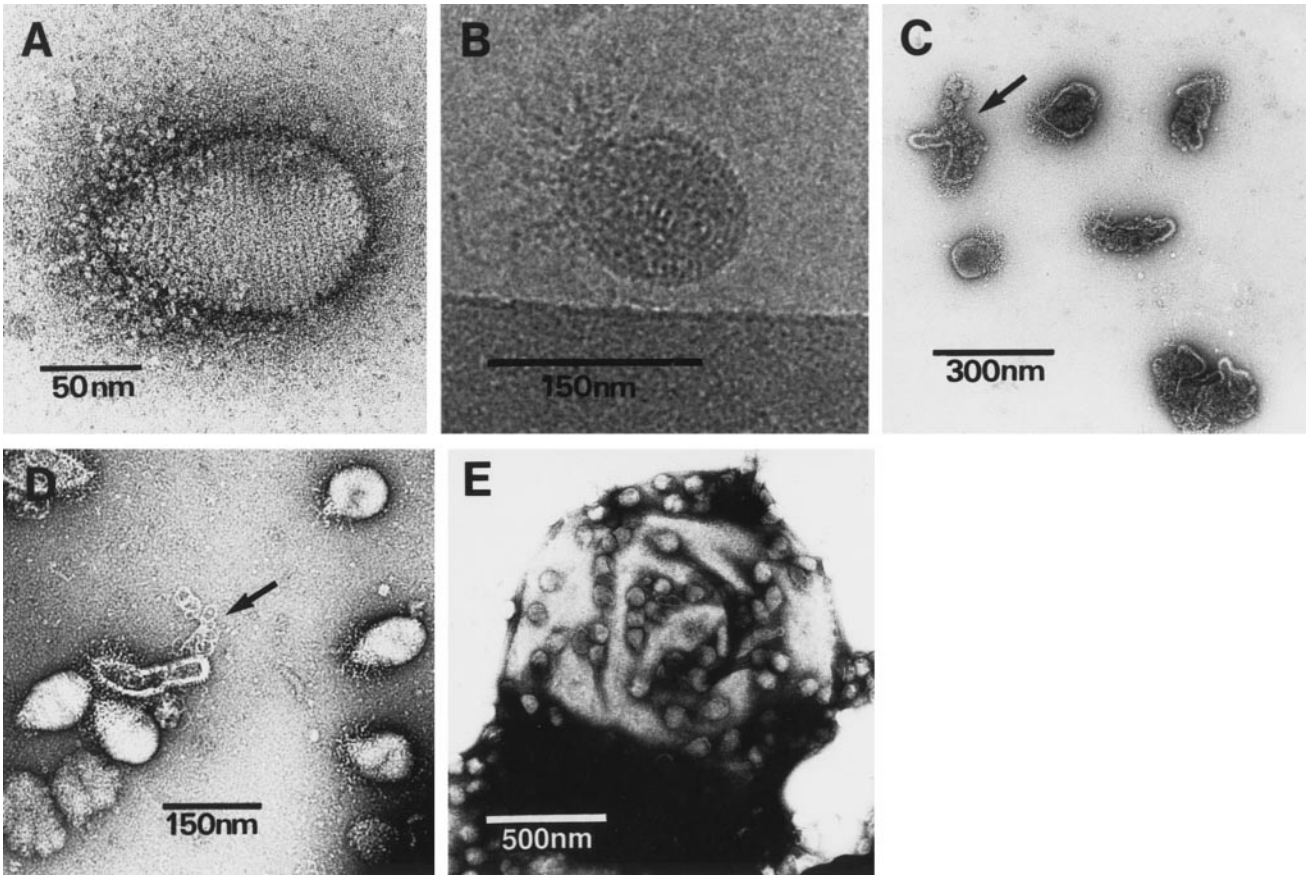

FIG. 1. Electron micrographs, all negatively stained, except (B). (A) SNDV particle showing the beard of tail fibers at its pointed end and the ribbed surface of the coat. (B) A vitreous ice-embedded specimen showing an SNDV particle without negative staining. (C) Disrupted SNDV particles obtained after centrifugation in a CsCl buoyant density gradient. The released core is indicated by an arrow. (D) SNDV particles. One broken particle releasing a filamentous matrix that might be the DNA molecule is indicated by an arrow. (E) SNDV particles adsorbed to the cell surface of STH1/3.

laboratory host for further physiological characterization of SNDV. Infection with SNDV produced neither significant amounts of cell debris nor a decrease in the cell density, showing that SNDV was not lytic. The original virus-carrying host STH3/1 produced 5.6 times more virus particles in a medium containing 0.1% tryptone as sole carbon source than in a medium containing a mixture of 0.1% tryptone, 0.05% yeast extract, and 0.2% sucrose (data not shown). Independent of the carbon source, virus production started in the early stationary growth phase.

SNDV inhibited the growth of the laboratory host STH1/3 (Fig. 2). Cultures of STH1/3 infected late in the exponential growth phase started to release virus particles in the early stationary growth phase, about 17 h after infection. Immediately before the liberation of virus, cells densely filled with virions were observed in the electron microscope (Zillig *et al.,* 1996). As in the laboratory host STH1/3, in the original host STH3/1 virus production started in the early stationary growth phase but the amount of virus increased more slowly and reached a peak that was five times lower. Lysis of STH1/3 cells was observed only after infection with a large amount of concentrated virus suspension, which led to an immediate reduction of the optical density of the culture, indicating partial lysis (data not shown).

Virus-free clones of the original host STH3/1 were obtained by picking colonies from plates, indicating curing and thus an unstable carrier state. In cultures of STH3/1 as well as the laboratory host STH1/3, the amount of virus in the supernatant decreased upon repeated transfer of culture aliquots into fresh medium and further growth, finally leading to virus-free cultures. SNDV DNA was not detectable via Southern hybridization in the total DNA extracted from such cultures. Infected cultures of the laboratory host STH1/3 had lost the virus already after the first transfer at a dilution of 1:25.

Electron microscopic inspection revealed that virus particles adsorbed to STH1/3 host cells particularly in folds of the S-layer (Fig. 1E). Even though the laboratory host STH1/3 produced five times more particles than the original host, a plaque assay could not be established with that strain as indicator. Neither a reduction of the carbon source in the medium nor the use of indicator cells harvested at different growth stages nor changed concentrations of the indicator cells led to the formation

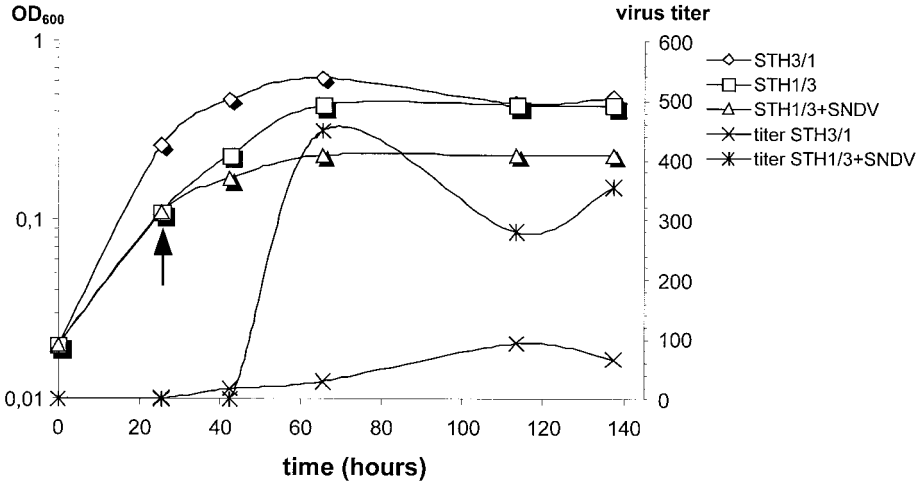

FIG. 2. Growth inhibition of the lab host STH1/3 after infection with SNDV (arrow) in comparison to an uninfected culture of STH1/3 and to a culture of the original virus-carrying host STH3/1. The virus amount was estimated as described under Materials and Methods.

of plaques derived from single virus particles in the indicator lawn. Only a concentrated SNDV suspension (see Materials and Methods) produced halos when spotted onto an STH1/3 indicator lawn. Under these conditions, the diameter of the halo corresponded to the area covered by the spot. Integration of viral DNA into the chromosome of STH3/1 could not be demonstrated by Southern hybridization.

Proteins and DNA

According to SDS–PAGE the SNDV virion contained at least three different proteins: one major component of approximately 17.5 kDa and two minor components of approximately 13.5 and 13 kDa (Fig. 3). The viral DNA formed a cccDNA band in CsCl ethidium bromide gradient equilibrium centrifugation and was sensitive to cleavage by DNase I and some type II restriction enzymes. It is therefore double-stranded and circular. The genome size was 20 kb. The type II restriction endonucleases *Alu*I, *Bam*HI, *Bst*EII, *Dra*I, *Sau*3AI, *Ssp*I, and *Taq*I cleaved the viral DNA, whereas the type II restriction enzymes *Apa*I, *Asp*700I, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Hpa*I, *Mlu*I, *Mro*I, *Pvu*I, *Pvu*II, *Sca*I, and *Xho*I did not. A comparative cleavage analysis with the three endonucleases *Sau*3AI, *Dpn*I, and *Mbo*I was performed to check whether the virus DNA was specifically methylated. The three enzymes share the recognition site GATC but differ in their dependence on N(6)-methylation of the adenine residue (i.e., *dam*-like methylation; see Table 1). *Sau*3AI cleaves independent of *dam*-like methylation, *Dpn*I cleaves only at methylated sites, and *Mbo*I cleaves only at unmethylated sites (for a review see McClelland *et al.,* 1994). DNA isolated from SNDV particles was not cleaved by *Mbo*I but showed the same cleavage pattern after incubation with *Sau*3AI and *Dpn*I. This indicated that the viral DNA was *dam*-like methylated. Viral DNA isolated as episomal DNA from the host cells was insensitive to cleavage by *Mbo*I. However, in contrast to DNA from virus particles it yielded mainly small DNA fragments when digested with *Sau*3AI but additionally larger fragments when split by *Dpn*I (Fig. 4). The comparative cleavage analysis of the total DNAs extracted from the virus-carrying host

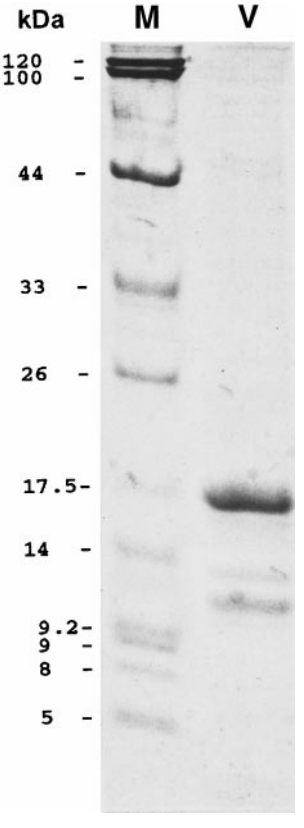

FIG. 3. Coomassie-stained SDS–PAGE of the viral proteins (V). RNA polymerase of *S. acidocaldarius* was used as size marker (M); the subunit sizes are listed in kDa at the left.

TABLE 1

Restriction Endonucleases That Were Used to Detect *dam*-Like Methylation of the SNDV DNA

Enzyme	Recognition site	Cleaves	Does not cleave	Cleavage of SNDV DNA
*Bam*HI	GGATCC	GGATC^{m5}C GG^{m6}ATCC GG^{m6}ATC^{m5}C GGATC^{m4}C	GGAT^{m4}CC GGAT^{m5}CC GGAT^{hm5}C^{hm5}C	Yes
*Sau*3AI	GATC	G^{m6}ATC	GAT^{m4}C GAT^{m5}C GAT^{hm5}C	Yes
*Dpn*I	GATC	G^{m6}ATC G^{m6}AT^{m5}C G^{m6}AT^{m4}C	GATC GAT^{m4}C GAT^{m5}C	Partial
*Mbo*I	GATC	GAT^{m4}C GAT^{m5}C	G^{m6}ATC	No
*Alu*I	AGCT	?	^{m6}AGCT AG^{m4}CT AG^{m5}CT AG^{hm5}CT	Yes
*Taq*I	TCGA	T^{m5}CGA T^{hm5}CGA	TCG^{m6}A	Yes

Note. The recognition sites of the endonucleases, the effect of site-specific methylation on the enzymatic activities (reviewed in McClelland *et al.,* 1994), and the sensitivity of the viral DNA to cleavage by the endonucleases are listed.

STH3/1 and from a virus-free clone of this strain revealed that only the virus DNA was sensitive to *Dpn*I and therefore *dam*-like modified, whereas the host DNA was not (Fig. 5).

DISCUSSION

Like other crenarchaeotal viruses, SNDV is morphologically unique (see Zillig *et al.,* 1994, 1996, 1998). Although SSV1, which belongs to the *Fuselloviridae,* resembles SNDV in its 15.5-kb cccDNA genome size, the differences in lifestyle and shape are sufficient to introduce a new virus family for SNDV. In contrast to SSV1, which is a temperate spindle-shaped virus with an electron microscopically smooth surface and very short tail fibers (Martin *et al.,* 1984), SNDV, which persists in a carrier state, is droplet-shaped with a beehive-like ribbed surface pattern and is densely covered by a beard of long tail fibers at its pointed end. SNDV has already been proposed to be a member of a floating genus, Guttavirus. We suggest the introduction of a corresponding virus family and to name it *Guttaviridae*.

The pointed end of the virion is densely covered by a beard of thin fibers that are not resolved at lower magnification but appear like strings of beads at high magnification (Figs. 1A–1C). Because SNDV particles adsorb to the host with their bearded ends (Fig. 1E), the physiological function of the tail fibers might be adsorption to a receptor. According to electron micrographs showing virions adsorbed to host cells (Fig. 1E), the particles seem to attach preferentially to folds of the S-layer. Since the negative staining procedure, however, notoriously produces dehydration-induced distortions in biological specimens, it is more likely that the particles attached to breaks in the hosts' S-layer, which are characteristic for *Sulfolobus.*

Osmotically disrupted particles reveal details of the virion structure (Fig. 1C). The core of SNDV is oblong in shape and appears to be a densely wound spiral that sometimes, however, is relaxed, yielding a tightly looped filament (Fig. 1D). This filament, however, is too thick to be naked DNA. The core is covered by a beehive-like ribbed coat (Figs. 1C and 1D). Some views of broken particles revealed cross sections of the coat, which was about 7 nm thick (Fig. 1B). The ribs extended only through its outer third (Fig. 1D). Only three proteins were clearly detected by SDS–PAGE. The virions contain at least three structural components: coat, core, and tail fibers.

A plaque assay for SNDV could not be established, probably because virus particles are released only in the stationary phase where visible plaques cannot form in the lawn of the host. Only in concentrated suspensions was the virus titer high enough to cause growth inhibition and thus a halo when spotted on a soft layer seeded with host cells for lawn formation.

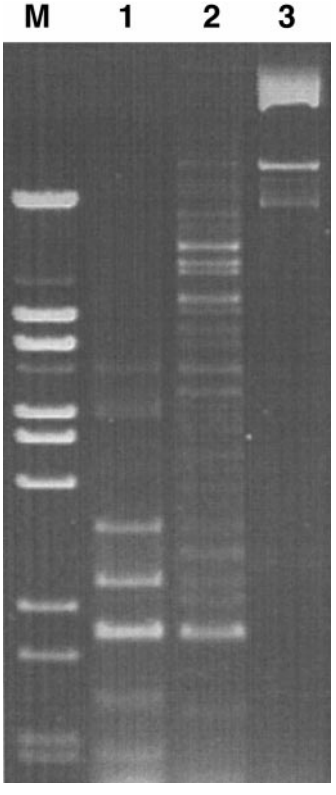

FIG. 4. Cleavage of SNDV DNA with *Sau*3AI (lane 1), *Dpn*I (lane 2), and *Mbo*I (lane 3). Lane M shows *Eco*91I-digested bacteriophage λ DNA (MBI Fermentas) as a size marker.

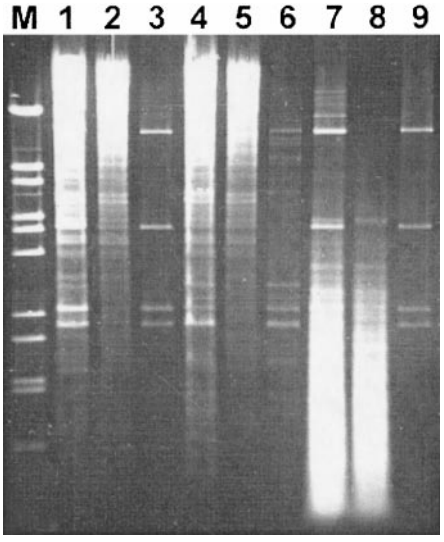

FIG. 5. Comparative restriction of host DNA and SNDV DNA with *Dpn*I and *Mbo*I: *Bam*HI cleavage pattern of the DNA of the original virus-carrying (lane 1) and the virus-free (lane 2) host STH3/1 and of purified SNDV DNA (lane 3); *Bam*HI/*Dpn*I cleavage pattern of the original virus-carrying (lane 4) and the virus-free (lane 5) host STH3/1 and of purified SNDV DNA (lane 6); *Bam*HI/*Mbo*I cleavage pattern of the original virus-carrying (lane 7) and the virus-free (lane 8) host STH3/1 and of purified SNDV DNA (lane 9); (lane M) *Eco*91I-digested λ DNA size marker. The restriction endonuclease *Bam*HI was added to all samples to obtain better resolution (the *Bam*HI recognition site GGATCC contains the cleavage site GATC of *Dpn*I and *Mbo*I and is unaffected by methylation).

Considering that the virus genome is not integrated into the host chromosome and in particular that the virus is lost upon prolonged incubation of a virus-carrying culture, we conclude that SNDV does not persist as a stable prophage in its host as, for example, the haloarchaeophage ΦH (Schnabel and Zillig, 1984). In view of the rapid curing of SNDV-carrying host cells, it appears astonishing that the virus was isolated from a clone obtained from a single *Sulfolobus* colony. The synchronized multiplication of virus and host is essential for the stable maintenance of a virus that, like SNDV, persists in a carrier state. When this synchronization becomes unbalanced the virus can become lost. Circumstances that influence the growth rate of the host can thus lead to the loss of virus. The establishment of a stable carrier state of the archaeal halophage Hs1, for example, depends on the sodium chloride concentration of the medium (Torsvik and Dundas, 1974). SNDV is produced only in the stationary phase in which the growth of its host ceases. In the exponential growth phase both STH1/3 and STH3/1 multiply faster than the virus. The rate of virus production does not surmount the cell growth rate until the cells enter the stationary phase. The ratio of infected to uninfected host cells in the stationary phase thus appears crucial for the successful persistence of SNDV. The conditions under which the multiplication of SNDV and that of its host remain synchronized have yet to be elucidated. The only lytic effect of SNDV observed after infection with a large amount of virus was most likely caused by lysis from without, which is also known from bacteriophage T4 at an excessive multiplicity of infection (Fraenkel-Conrat, 1982).

SNDV is thus far the only crenarchaeotal virus with a modified genome. Modifications of viral genomes are known for euryarchaeotal head-and-tail phages, e.g., the phage ΦCh1 of the halophilic archaeon *Natronobacterium magadii* with a genome methylated in a *dam*-like manner (Witte *et al.,* 1997) or the phage Hs1 of *Halobacterium salinarum* with a fully cytosine-methylated genome (Vogelsang-Wenke and Oesterhelt, 1988). In principle genomes can be modified in two ways: (a) modified nucleotide residues are introduced by incorporation of unusual desoxyribonucleoside triphosphates (e.g., Mathews and Allen, 1983) or (b) nucleotide residues are modified in DNA after replication, e.g., by methylation (e.g., Magrini *et al.,* 1997). The results obtained by comparative restriction analysis with the three endonucleases *Sau*3AI, *Dpn*I, and *Mbo*I indicate that the SNDV genome is modified in a sequence-dependent manner as reported for many methylases (reviewed in McClelland *et al.,* 1994). This modifying system differentiates between the virus and the host DNA, most likely by a virus-encoded methylase that recognizes only hemimethylated GATC sites. Such methylases that are preferentially active on hemimethylated DNA have already been reported (e.g., Yusuf *et al.,* 1991). The intermediary formation of hemimethylated SNDV DNA during replication by the host enzymatic machinery could explain the incomplete cleavage of viral DNA by *Dpn*I compared to *Sau*3AI because *Dpn*I cleaves only fully methylated DNA (Lacks and Greenberg 1977; Vovis and Lacks, 1977). Therefore it is most likely that the intracellular SNDV DNA is a mixture of DNA at various stages of methylation, whereas the host DNA is not methylated at all as documented by its sensitivity to *Mbo*I.

MATERIALS AND METHODS

Detection of SNDV

Cultures of *Sulfolobus* strains isolated from solfataric field samples were screened for the presence of extrachromosomal genetic elements as described by Zillig and co-workers (1994). In a first step such elements were detected as prominent DNA bands after agarose gel electrophoresis of cleaved DNA extracted from *Sulfolobus* cultures. In addition, the supernatants of suspicious cultures were checked for the presence of virus particles in the electron microscope. By this standard screening routine SNDV was detected in the *Sulfolobus* strain STH3/1, which had been isolated as a single colony clone from an enrichment culture of a field sample taken at Steaming Hills in New Zealand (Zillig *et al.,* 1998).

Purification of virus and virus DNA

To obtain large amounts of virus, the strain STH3/1 was cultivated at 80°C and pH 3.0 in the medium described by Zillig *et al.* (1994) containing 0.1% tryptone as the sole carbon source. The cultures were harvested in the stationary growth phase and the cells were removed by low-speed centrifugation (6000 rpm in a Beckman JA10 rotor). Because of the lack of a plaque assay, the best time for harvesting was estimated by the examination of the unconcentrated supernatant in the electron microscope in various growth stages. The virus particles were precipitated from the supernatant by the addition of NaCl to 1 M and polyethylene glycol 6000 to 10% (w/v) and incubation overnight at 4°C. The sediment was collected by centrifugation in a Beckman JA14 rotor at 10,000 rpm for 10 min, drained well, and resuspended in TE buffer (10 mM Tris–hydroxyaminomethane/HCl, 1 mM EDTA, pH 7.4). Cell debris was removed by centrifugation at 6000 rpm for 10 min in a Junior Christ centrifuge (Heraeus). The supernatant was kept and the pellet extracted two more times in this manner with reduced volumes of TE. The supernatants containing virus particles and small amounts of cell debris were pooled. SNDV particles were not further purified from this suspension via centrifugation in a CsCl buoyant density gradient as reported for the isolation of other *Sulfolobus* viruses (Zillig *et al.,* 1994) because the structural integrity of the particles was lost. A virus suspension prepared by this method from a 500-ml culture of the original strain STH3/1 is termed "concentrated virus suspension" hereafter. Due to the lack of a quantitative plaque assay it was not possible to determine the amount of viable virus in this suspension. Virus DNA was isolated from 100 μl of the concentrated particle suspension in the presence of 1% sodium lauryl sulfate (SDS) by extraction with 1 vol of phenol. The two phases were mixed and centrifuged for 10 min at 13,000*g*. The aqueous phase was removed and extracted, first with 1 vol of a mixture of phenol and chloroform (1:1) and second with 1 vol of chloroform. The DNA was precipitated from the aqueous phase by adding $\frac{1}{10}$ vol of 3 M sodium acetate (pH 5.3) and 0.8 vol isopropanol followed by centrifugation for 30 min at 13,000 rpm in an Eppendorf centrifuge. The DNA pellet was washed in 100 μl ethanol (70%) and was again collected by centrifugation. The air-dried pellet was resuspended in 30 μl TE buffer.

Medium and plates

The salt base of the medium was basically that used by Brierley and Brierley (1973). The medium contained 5 ml Wolin's vitamin mixture (Wolin *et al.,* 1963) per liter and 0.1 or 0.2% tryptone (w/v) as carbon source. It was buffered with 0.7 g glycine per liter adjusted to pH 3.0 with diluted sulfuric acid (see also Zillig *et al.,* 1994). The best colony plating efficiency was obtained using the soft-layer technique and employing medium with 0.2% tryptone (see below). Gellan gum K9A40 (Gelrite, Kelco, San Diego, CA) was used as gelling agent (8 g per liter). The gels were stabilized by the addition of Mg^{2+} (10 mM) and Ca^{2+} ions (3 mM) after melting and before pouring (Zillig *et al.,* 1994).

Attempts to establish an SNDV plaque assay

Plaque assays were attempted as described by Zillig and co-workers (1994). The final soft layer was 1.8 ml containing 0.2% gellan gum without additional Mg^{2+} and Ca^{2+} ions. The plates were incubated for up to 6 days at 80°C. *Sulfolobus* strains were screened for their virus sensitivity by spotting the concentrated SNDV suspension onto lawns. On the basis of adsorption studies (see below), we chose strain STH1/3 to establish a quantitative plaque assay but failed, although different concentrations of indicator cells and reduced concentrations of the carbon source were tested.

Electron microscopy

For negative staining, a droplet of the virus specimen was placed onto a carbon-coated copper grid that had been pretreated in a Plasma Cleaner (Hattrick, medium setting, 20 s). The specimen was allowed to adsorb to the carbon layer for 1 min and excess liquid was removed with filter paper. A solution of 2% uranyl acetate was added to the carbon grid for 20 s and excess liquid was removed again. After the specimen had been air-dried it was examined in a Philips CM 12 microscope equipped with a LaB6 cathode at 120 kV. Frozen hydrated samples of SNDV particles were prepared for cryo-electron microscopy by fast-freezing thin films of sample solution on grids covered with a holey carbon film in liquid ethane with a gravity-driven plunger. Specimens were observed using a Gatan cryoholder in a Philips CM200FEG electron microscope. Images were recorded using a slow-scan CCD camera attached to the microscope. The microscope was operated at 120 kV accelerating voltage and with the CCD camera at 20,000× total magnification. The calibrated CCD pixel size referring to the specimen level was 0.48 nm.

Adsorption studies

Small volumes of host cell cultures of the strain STH1/3 (1×10^7 cells/ml) were incubated with aliquots of the concentrated SNDV suspension for 45 min under normal growth conditions. The cultures were concentrated 10 times by low-speed centrifugation (6000 rpm, Heraeus Christ zentrifuge) and prepared for examination in the electron microscope.

One-step growth experiments

Host cultures of STH1/3 (1×10^7 cells/ml) were infected with aliquots of the concentrated SNDV suspen-

sion. The inhibition of the growth rate of the host was determined by following optical densities of infected and uninfected cultures at 600 nm. The relative amounts of virus particles were determined in the electron microscope by counting the particles in the visual field at 10,000-fold magnification.

Protein analysis

Proteins were analyzed by SDS–PAGE (Schägger and Jagow, 1987) and stained with Coomassie brillant blue R-250 (Serva). The acrylamide concentration in the separating gel was 16%.

Enzymatic digestion

SNDV DNA was subjected to cleavage by type II restriction enzymes according to the producers' instructions (MBI-Fermentas, Boehringer Mannheim, NEB). The virus DNA was checked for the presence of methylation by the use of the endonucleases *Sau*3AI, *Dpn*I, and *Mbo*I. DNA isolated from virions, episomal virus DNA, and total DNA extracted from the infected host were subjected to cleavage by *Dpn*I and *Mbo*I in order to determine whether the modifying apparatus could discriminate between these DNAs.

Labeling of DNA and hybridization reactions

DNA was transferred onto a Biodyne B nylon membrane (PaII) as described in Sambrook *et al.* (1989). DNA was labeled using the DIG labeling and detection kit from Boehringer Mannheim. Labeling, hybridization, and detection reactions were performed according to the manufacturer's instructions using the standard hybridization buffer containing 50% formamide. Labeled DNA probe and target DNA were hybridized overnight at 42°C. Stringent washing was performed at 62°C in a solution of 0.5× SSC (20× SSC: 175.3 g sodium chloride and 88.2 g sodium citrate per liter, pH 7.0) and 0.1% SDS for 20 min.

DNA isolation from host cells

Cells were isolated from 25 ml of infected cultures by low-speed centrifugation (6000 rpm, Heraeus Christ centrifuge). The cell pellet was completely resuspended in 450 μl TE buffer (pH 7.4) and mixed with 50 μl of a 10% SDS solution and 10 μl of a DNase-free RNase solution (20 mg/ml). After incubation for 30 min at 56°C the DNA was isolated by phenol extraction and isopropanol precipitation as described above. Instead of centrifugation the precipitated DNA was isolated from the isopropanol mix after gentle mixing with a heat-sealed pasteur pipette, dipped in 70% ethanol for washing, and transferred into a fresh Eppendorf tube. The DNA was briefly air-dried and redissolved overnight in a fresh Eppendorf tube. The DNA was briefly air-dired and redissolved overnight in 100 μl TE buffer at 56°C before subjection to cleavage analysis by agarose gel electrophoresis. The DNA was transferred and further analyzed as described above using a SNDV DNA probe to check for the presence of viral DNA.

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