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Viral Diversity in Hot Springs of Pozzuoli, Italy, and Characterization of a Unique Archaeal Virus, *Acidianus* Bottle-Shaped Virus, from a New Family, the *Ampullaviridae*

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Virus-like particles with five different morphotypes were observed in an enriched environmental sample from a hot, acidic spring (87 to 93°C, pH 1.5) in Pozzuoli, Italy. The morphotypes included rigid rods, flexible filaments, and novel, exceptional forms. Particles of each type were isolated, and they were shown to represent viable virions of five novel viruses which infect members of the hyperthermophilic archaeal genus *Acidianus*. One of these, named the *Acidianus* bottle-shaped virus, ABV, exhibits a previously unreported morphotype. The bottle-shaped virion carries an envelope which encases a funnel-shaped core. The pointed end of the virion is likely to be involved in adsorption and channeling of viral DNA into host cells. The broad end exhibits 20 (± 2) thin filaments which appear to be inserted into a disk, or ring, and are interconnected at their bases. These filaments are apparently not involved in adsorption. ABV virions contain six proteins in the size range 15 to 80 kDa and a 23.9-kb linear, double-stranded DNA genome. Virus replication does not cause lysis of host cells. On the basis of its unique morphotype and structure, we propose to assign ABV to a new viral family, the *Ampullaviridae*.

Evidence supporting an extremely high abundance of viruses in the biosphere has stimulated a strong interest in determining the degree of viral diversity in different ecosystems (19). One group of ecosystems which has yielded exciting new results is geothermally heated hot aquatic environments. Screening for viruses in terrestrial hot springs with temperatures above 80°C in Iceland (20) and in Yellowstone National Park (13, 15) has revealed numerous, different virus morphotypes, many of which have not been previously observed in nature. Several of the isolated viruses infect hyperthermophilic archaea from the *Crenarchaeota* kingdom, and they all have double-stranded DNA (dsDNA) genomes. Moreover, on the basis of their exceptional morphological and genomic properties, five novel virus families have been established: the spindle-shaped *Fuselloviridae*, the filamentous *Lipothrixviridae*, the rod-shaped *Rudiviridae*, the droplet-shaped *Guttaviridae* (reviewed in references 12 and 21), and the spherical *Globuloviridae* (5).

In order to confirm that hot aquatic environments constitute favorable habitats for viruses with unusual morphotypes, we examined viral diversity in a volcanic area near Naples, Italy, and the results reinforced previous observations. Virus-like particles with five different morphotypes were isolated from the environmental samples, and they were shown to be infectious virions. One of these novel viruses, the *Acidianus* bottle-shaped virus (ABV), is described in detail.

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MATERIALS AND METHODS

Enrichment cultures. A sample was taken from a water reservoir in the crater of the Solfatara volcano at Pozzuoli, Italy. Although the temperature of the lake was on average about 60°C, undercurrents close to the surface were hotter and generated steam. These undercurrents were caused by submerged hot springs, and a sample was taken directly from one such spring with a temperature range of 87 to 93°C and a pH of 1.5 to 2.

Two enrichment cultures were established, one aerobic and the other anaerobic, under conditions favorable for growth of members of the order *Sulfolobale*s, which are known to dominate in hot acidic terrestrial springs (8). Growth conditions were similar to those described by Zillig et al. (20). Each 50-ml culture was inoculated with 1 ml of the sample. Cultures were grown at 75°C, pH 3, with a gas phase containing N_2 and CO_2 (80:20, vol/vol) for the anaerobic culture.

Preparation of DNA. Cells were collected and suspended in a Tris-EDTA buffer containing 10 mM Tris-HCl, pH 8, and 1 mM EDTA. They were lysed by adding sodium dodecyl sulfate (SDS) and Triton X-100 to final concentrations of 0.8% (wt/vol) and 0.06% (vol/vol), respectively, to the suspension, and DNA was extracted once with phenol, once with phenol-chloroform, and once with chloroform. DNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M sodium acetate, pH 5.3, and 0.8 volume of isopropanol. The DNA pellet was washed with 70% ethanol, air dried, and resuspended in an appropriate volume of Tris-EDTA buffer.

Viral DNA was prepared by disruption of virus particles with 1% (wt/vol) SDS for 1 h at room temperature, and extraction and precipitation were done according to the protocol used for cellular DNA.

16S rDNA analysis. The 16S rRNA gene was amplified from cellular DNA by PCR, using *Taq* polymerase and a forward primer, either 8aF, which is specific for archaeal 16S rDNA, or 9bF, which is specific for bacterial 16S rDNA, and the reverse primer 1119uR, which is universal for any prokaryotic 16S rDNA (4). The PCR product was cloned, using the pDrive-TA cloning kit (QIAGEN, Hilden, Germany). The presence of inserts of the predicted sizes was analyzed by direct PCR screening of 50 transformants without plasmid extraction. A small part of each colony was used for each PCR with the plasmid-specific primers M13F(−40) and M13R. Insert sizes were checked by electrophoresis on a 1% (wt/vol) agarose gel. Restriction fragment length polymorphism analysis was performed as previously described (16). Representative transformants were selected on the basis of the 16S rDNA fingerprint patterns, the corresponding plasmid DNA was extracted using the Qiaprep Spin Miniprep kit (QIAGEN

GmbH, Hilden, Germany), and the 16S rDNA inserts were sequenced. After alignment against a database including more than 11,000 bacterial and archaeal 16S rDNA sequences, phylogenetic distances were determined using the maximum parsimony method and, finally, a phylogenetic tree was constructed (an ARB project; see reference 10).

Protein analysis. Proteins were analyzed in 10% (wt/vol) SDS polyacrylamide gels (9) and stained with silver (2).

Purification of strains and screening for virus production. Single isolates were obtained by plating dilutions of the enrichment culture onto Gelrite plates containing colloidal sulfur (20). Brownish colonies were picked up with a sterile needle, transferred into 20 ml of fresh growth medium, which was used for enrichment cultures, and incubated at 75°C. At an optical density at 600 nm (OD_{600}) of about 0.2, the supernatant of each cell culture was screened for virus production. Cells were removed by centrifugation (at 4,500 rpm for 10 min in a Laborfuge 400R; Heraeus). The cell-free supernatant was filtered through a 0.8/0.2-μm filter (VWR, Darmstadt, Germany), and particles were concentrated by ultracentrifugation (48,000 rpm, 1 h, Beckman Coulter Optima LE-80 K ultracentrifuge, SW 60 rotor) and examined by transmission electron microscopy (TEM).

Isolation of viruses. After removal of cells (4,500 rpm, 20 min, Sorvall GS3 rotor) from the enrichment culture, a mixture of viruses was isolated by adding NaCl to 1 M and polyethylene glycol 6000 to 10% (wt/vol) to the supernatant of the cell-free culture. After being incubated at 4°C overnight, the particles were pelleted by centrifugation (12,000 rpm, 30 min, Sorvall RC5C Plus, GSA rotor) and suspended in buffer containing 20 mM Tris-acetate, pH 6. The remaining cell debris was partially removed from the suspension by low-speed centrifugation (2,500 rpm, 10 min, Heraeus Laborfuge 400R). Virions were purified by centrifugation in a CsCl buoyant density gradient (48,000 rpm, 24 h, Beckman Coulter Optima LE-80 K ultracentrifuge, SW 60 rotor). The fractions were collected with a syringe and analyzed by TEM for the presence of virus particles.

ABV virions were purified from the culture of infected "*Acidianus convivator*" (proposed name) by following the same protocol.

Viral infection. The susceptibility of strains to viral infection was tested by adding 1 μl of the CsCl-purified virion suspension to 1 ml of fivefold-concentrated cell culture grown to an OD_{600} of about 0.02. After being incubated at 75°C for 1 h, the infected cell culture was used to inoculate 50 ml of medium. Infected cells were grown to late logarithmic growth phase and examined for the presence of virions by TEM.

Electron microscopy. Samples were applied to carbon-coated copper grids, negatively stained with 3% uranyl acetate, pH 4.5, and examined with a CM12 transmission electron microscope (FEI, Eindhoven, The Netherlands) operated at 120 keV. The magnification was calibrated using catalase crystals negatively stained with uranyl acetate (14). The images were digitally recorded, using a slow-scan CCD camera connected to a personal computer running TVIPS software (TVIPS GmbH, Gauting, Germany).

For electron tomography, negatively stained samples were mounted in a high-tilt grid holder (3). Tilt series were recorded at a magnification of ×34,000 or ×44,000 at about −1-μm defocus at room temperature under low-dose conditions with a tilt range from −70° to +70° and at 2° increments. After the tilted projections were aligned, the three-dimensional reconstruction was performed by weighted back projection, using the EM software package (7). Visualization of the three-dimensional volume was performed using the Amira software package (Mercury Computer Systems, Düsseldorf, Germany).

RESULTS

VLPs in enrichment cultures. Enrichment cultures were established from a sample taken from a hot acidic spring of the Solfataric field in Pozzuoli, Italy, under aerobic and anaerobic conditions which favor growth of hyperthermophilic members of the genera *Sulfolobus* and *Acidianus* of the crenarchaeal order *Sulfolobales* (20). After concentrating cell-free supernatants from each of the two enrichment cultures, we observed a variety of virus-like particles (VLPs) by TEM (Fig. 1A through E). The diversity of VLP morphotypes was similar in both cultures, and they could be classified into five distinct types. One particle type was morphologically novel. It was bottle-shaped, with an overall length of 230 ± 20 nm and a maximal width of 75 ± 5 nm at the broad end, which was densely covered with thin filaments (Fig. 1A). Another particle type had an ellipsoid body, 145 ± 30 nm long and 85 ± 5 nm wide, with appendages of various lengths at each pointed end, yielding a maximum particle length of about 1,000 nm (Fig. 1 B). The latter resembles particles we observed earlier in an enrichment culture from a hot acidic spring in Yellowstone National Park (13). Three other particle types were similar to known hyperthermophilic viruses. Rigid helical rods of about 610 ± 50 nm × 22 ± 3 nm (Fig. 1C) resemble rudiviruses SIRV1 and SIRV2, while flexible filaments in the size ranges 1,100 ± 50 nm × 24 ± 1 nm (Fig. 1D) and 2,000 ± 100 nm × 25 ± 1.5 nm (Fig. 1E) resemble members of the *Lipothrixviridae*.

Cells in enrichment cultures. Several serial dilutions of the enrichment cultures to 1:1,000 affected neither the quantity nor the diversity of observed VLPs. This suggested that strains harboring each particle type were growing in both aerobic and anaerobic enrichment cultures. Light microscopy confirmed the presence in these cultures of irregular coccoid cells (0.5 to 1.5 μm), which probably belong to the hyperthermophilic genus *Acidianus* (8). All subsequent work was performed with the aerobic enrichment culture.

In order to characterize the host strains further, cellular DNA was isolated from the enrichment culture and subjected to a 16S rDNA analysis. Whereas PCR primers specific for bacterial 16S rDNA yielded no amplification products, primers specific for archaeal 16S rDNA yielded products of ~1.1 kb. The amplified archaeal 16S rDNA was cloned, and 30 randomly selected clones were screened by restriction fragment length polymorphism analysis. Ten different restriction fragment patterns were distinguished. Their 16S rDNA sequences were determined, and comparative gene sequence analysis revealed that they all represented organisms which cluster in two groups within the genus *Acidianus*, one closely related to *A. infernus* and *A. ambivalens* and another closely related to *A. brierleyi*. A phylogenetic tree showing their positions was constructed by maximum-parsimony analysis of 16S rDNA sequences (Fig. 2).

VLP-producing strains. In order to isolate VLP-producing strains, dilutions of the enrichment culture were plated onto colloidal sulfur-containing Gelrite plates. After ten days of growth, brown colonies of about 2 to 5 mm in diameter were observed. Since virus-infected cells may be retarded in growth, the smallest colonies were picked and examined further. A total of 200 colonies were picked and grown in liquid cultures. After reaching an OD_{600} of about 0.2, each of the 200 cultures was screened for virus production by TEM. In this way, five strains which produced VLPs were identified. Their phylogenetic position is shown in Fig. 2, and the morphotypes of the produced particles are also indicated. *Acidianus* sp. strain Acii26 produced rod-shaped particles, and *Acidianus* sp. strain AciF28 yielded filamentous particles 1.1 μm in length, while *Acidianus* sp. strains Acii25, Acii18, and Acii19 were each associated with filamentous particles 2 μm in length.

Infectivity of VLPs. In order to identify hosts for the other types of particles observed in the enrichment culture, we chose a different approach: after the mixture of all the VLPs from the enrichment culture was isolated and purified, we attempted to replicate the VLPs in each of the 195 virus-free novel *Acidianus* spp. isolates.

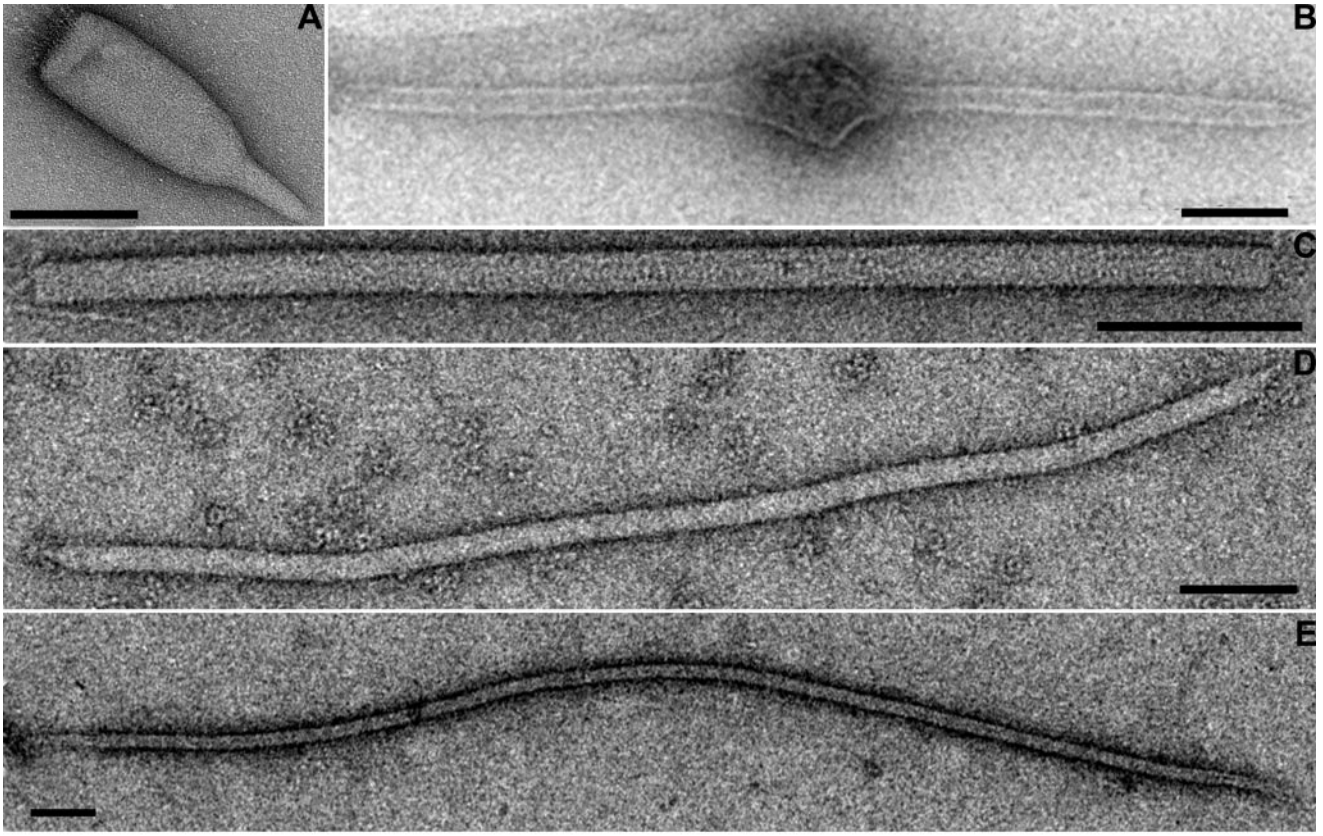

FIG. 1. (A through E) Transmission electron micrographs of viruses observed in an enrichment culture of a water sample from Pozzuoli, Italy. Samples were negatively stained with 3% uranyl acetate. Bars, 100 nm.

All the VLPs present in the cell-free supernatant of the enrichment culture were coprecipitated by adding PEG 6000, and the VLP mixture was centrifuged in a CsCl density gradient. Two distinct opalescent bands, with densities of 1.3 mg ml^{-1} (upper band) and 1.4 mg ml^{-1} (lower band), were collected and examined by TEM. The upper band mainly contained particles with the morphotypes illustrated in Fig. 1B, C, D, and E, while the lower band exhibited the morphotypes shown in Fig. 1A and E. For further studies, the bands were combined and the VLP mixture was incubated with freshly grown cells from each of the 195 novel *Acidianus* spp. isolates at 75°C. "Infected" cells were grown to stationary phase, and then the cell-free supernatant of each culture was examined by TEM for the presence of VLPs indicative of virus replication. In two species, *Acidianus* sp. strain AD1 and "*Acidianus pozzuoliensis*" (proposed name), the rod-shaped particles could replicate, which indicated that they represented viable virions. The virus was named *Acidianus* rod-shaped virus 1 (ARV1). Particles of three different morphotypes were replicated in another species, "*A. convivator*": the bottle-shaped particles, pleomorphic particles with two long tails, and 2-μm-long filamentous particles. The three novel viruses were named, respectively, *Acidianus* bottle-shaped virus (ABV), *Acidianus* two-tailed virus (ATV), and *Acidianus* filamentous virus 3 (AFV3). AFV3 is identical in morphotype and size to the viruses produced by *Acidianus* sp. strain Acii25 and *Acidianus* sp. strains Acii18 and Acii19. The virus produced by *Acidianus* sp. strain AciF28 was named *Acidianus* filamentous virus 2 (AFV2).

In this paper we focus on the characterization of ABV. In separate papers we describe the other four novel viruses: ATV (M. Häring, G. Vestergaard, L. Chen, R. Rachel, R. A. Garrett, and D. Prangishvili, submitted for publication), AFV2 (6), ARV1 (18), and AFV3 (G. Vestergaard, M. Häring, R. Rachel, R.A. Garrett, and D. Prangishvili, unpublished data).

Purification of ABV. In order to isolate ABV, we started with the mixture of VLPs present in the CsCl gradient band of density, 1.4 g ml^{-1} (see above), which contained viruses ABV and AFV3. When cells of "*A. convivator*" were coinfected with this mixture, both viruses were replicated. Separation of the two viruses was finally achieved by a second CsCl density gradient centrifugation step, yielding two closely migrating bands, each of which was enriched by virions of one of the viruses.

Host range. The host range of ABV was tested by adding purified virus particles to growing cultures of different species and strains of the genera *Acidianus* and *Sulfolobus*, including *A. ambivalens*, *A. brierleyi*, *A. infernus*, "*A. hospitalis*" (proposed name) (1), *Sulfolobus solfataricus* strains P1 and P2, and "*S. islandicus*" (proposed name) strains LAL14/1, REN2H1, and HVE10/4. No virus replication was observed in any of these cultures. Thus, "*A. convivator*" was the only host which could be infected by ABV. As is true for many species and strains of *Acidianus*, "*A. convivator*" did not grow as a lawn, and therefore, a plaque assay could not be established for ABV.

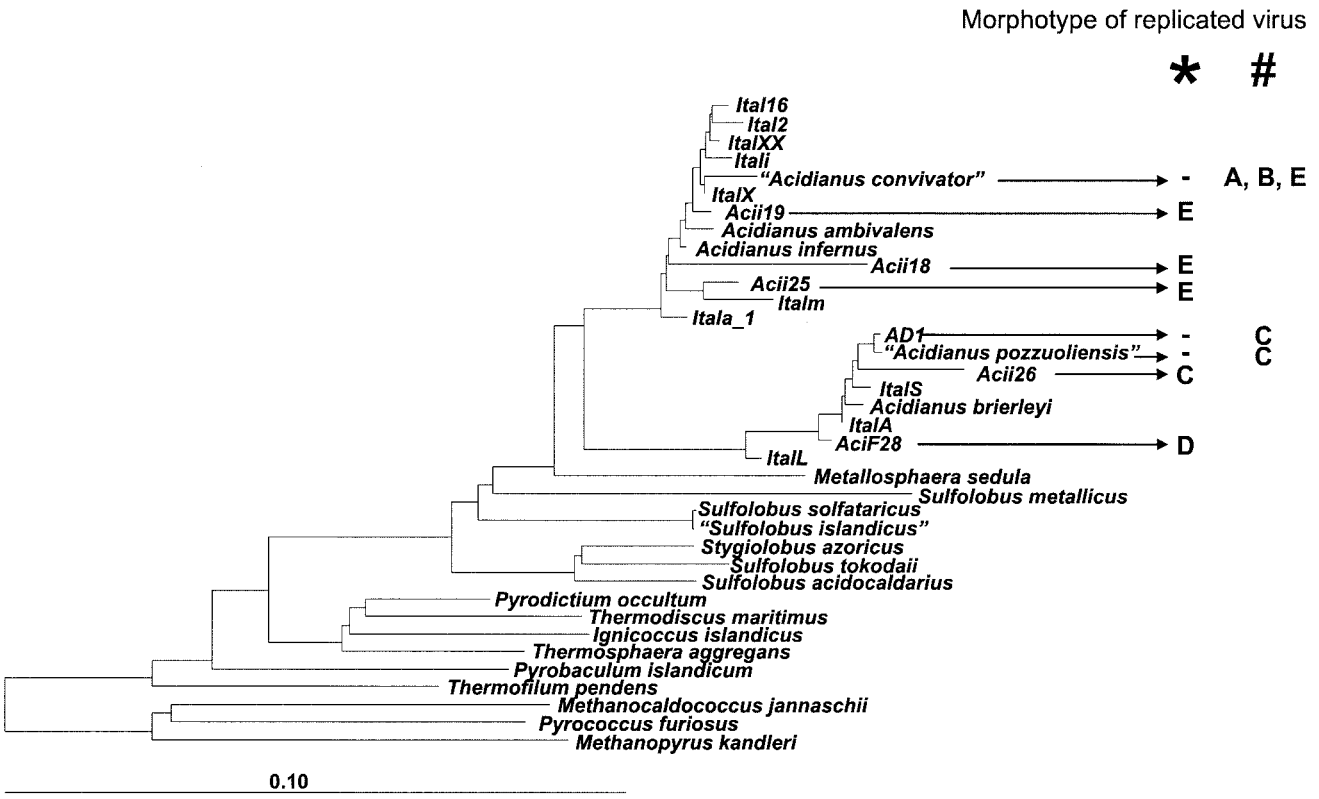

FIG. 2. Phylogenetic tree for *Crenarchaeota*, determined by a maximum-parsimony analysis of 16S rDNA sequences, showing the positions of the hosts described in the text. The scale bar represents 0.10 fixed mutations per nucleotide position. Viral morphotypes naturally replicated by corresponding hosts are indicated in the column marked with an asterisk (*). Viral morphotypes replicated by corresponding hosts as a result of infection by virions are indicated in the column marked with the pound sign (#). −, no virus replication. The morphotypes of the viruses are indicated by letters corresponding to the panels in Fig. 1.

Virus-host interactions. The generation time of "*A. convivator*" at 75°C was about 24 h, and the maximum OD_{600} reached was 0.2. Virus infection resulted in significant growth retardation: the doubling time increased by nearly a factor of two. However, after prolonged growth the infected cell culture reached a density similar to that of the uninfected cell culture. Virus replication caused neither a detectable decrease of OD_{600} nor formation of cell debris. TEM studies of the infected cell cultures revealed that ABV particles were first detectable 10 days postinfection. Since the detection limit using TEM was shown to be about 10^5 particles per ml (5), particles in cell-free culture supernatants were concentrated 100-fold by ultracentrifugation prior to TEM analysis.

Protein and nucleic acid composition. Electrophoretic analysis of the ABV proteins by SDS-PAGE revealed a complex protein pattern, showing six major bands corresponding to proteins with apparent molecular masses of 15, 22, 65, 66, 70, and 80 kDa (Fig. 3A). We do not exclude the possibility that the protein composition of the virion is even more complex, and minor protein components could not be visualized.

All proteins observed in Fig. 3A were apparently of viral origin, since the virus preparation used for this analysis was highly purified and contained no cell debris detectable by TEM.

Nucleic acid isolated from purified ABV particles was insensitive to RNase A but was digestible by type II restriction endonucleases, consistent with its being dsDNA. Analysis of digestion by the restriction endonuclease NcoI yielded a size estimate of 23.9 kb for the ABV genome (Fig. 3B).

Virion structure. Virions of ABV isolated from a culture of infected "*A. convivator*" cells exhibited the same exceptional bottle shape as the VLPs observed in the enrichment culture. They had an overall length of 230 ± 20 nm, and their width varied from 75 ± 5 nm at the broad end to 4 ± 1 nm at the pointed end. The broad end was densely covered with short thin filaments (Fig. 4A).

Electron tomography of the negatively stained virions demonstrated that the thin filaments had a length of about 20 nm and a width of 3 nm. They were arranged in a circle (Fig. 4E) which, owing to the effects of negative staining and air drying, is collapsed onto the carbon support (see the vertical slice through the three-dimensional data set in Fig. 4D). The filaments appeared to be inserted into a disk or a ring at the broad end of the virion. Most likely they are also interconnected at the base. Moreover, in partially disrupted particles the filaments were observed to detach from the virion as a separate structural unit (Fig. 5E). Analysis of the three-dimensional volume (Fig. 4E) yielded an estimate for the number of filaments, which was 20 ± 2 per virion.

A horizontal section through the three-dimensional data set

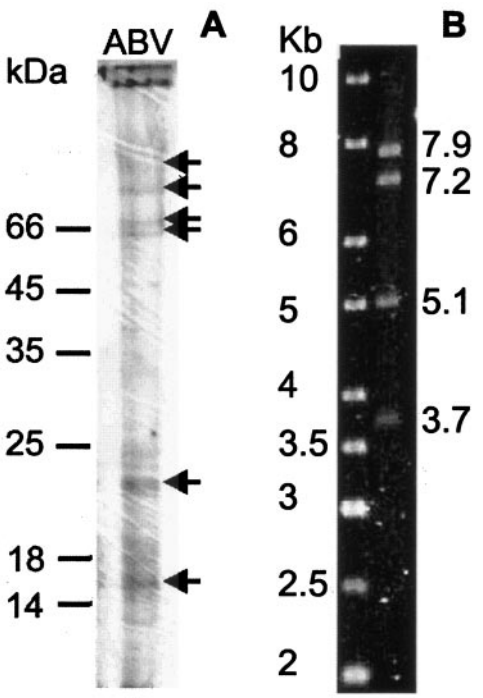

FIG. 3. Protein and nucleic acid composition of ABV. (A) SDS-PAGE of proteins of the virus ABV, silver stained. The size of markers is indicated. (B) Fragments of ABV DNA digested with the restriction endonuclease NcoI (right lane). The left lane shows size markers. Fragment sizes are given in kilobases for both the markers (left) and the digested ABV DNA (right).

(Fig. 4B) gave insight into the architecture of the body of the virion. It appears to be built of two layers encasing a complex core filled with densely packed material which lacks any visible symmetry (Fig. 4B and E). The two presumed layers had a thickness of about 7 nm (inner layer) and 9 nm (outer layer) where the latter showed longitudinal striations (Fig. 4C).

After being ultracentrifuged (38,000 rpm, 1 h, Beckman SW 60 rotor), ABV virions were often seen to be partially disrupted. Analysis of such particles by TEM gave further insight into the virion structure (Fig. 5). The results confirmed the presence of the 9-nm-thick outer envelope. Moreover, this envelope was seen to encase a cone-shaped structure, about 125 nm in length, with a width of 30 nm at the small end increasing to 78 nm at the wide end (Fig. 5A). The structure showed striations running perpendicularly to the long axis, with periodicities of 13 nm^{-1} and 4.3 nm^{-1}, indicative of a helical arrangement of subunits.

The cone shape of the core appeared not to be determined by the outer envelope because its structure was still maintained even after the outer envelope of the virion had been partially destroyed and had lost its shape (Fig. 5A). However, the cone-shaped structure was destroyed in many particles, probably because of the harsh treatment of the samples during ultracentrifugation followed by negative staining with uranyl acetate and air-drying. Disappearance of the structure coincided with release of a nucleoprotein filament (Fig. 5 C, F, and G), and it is noteworthy that the width of this filament is about 7 nm and equal to the width of the presumed inner layer observed by electron tomography. The results suggest that this inner layer is formed by a toroidally supercoiled nucleoprotein filament (Fig. 5F and G). The cone-like packaging of the nucleoprotein might be the result of a precisely controlled self-assembly process from protein subunits and nucleic acid.

The inner core of ABV particles, as visualized by electron tomography, contains granular material with no obvious symmetry (Fig. 4B and E). Since it constitutes a separate compartment with a distinct volume, it remains to be determined what

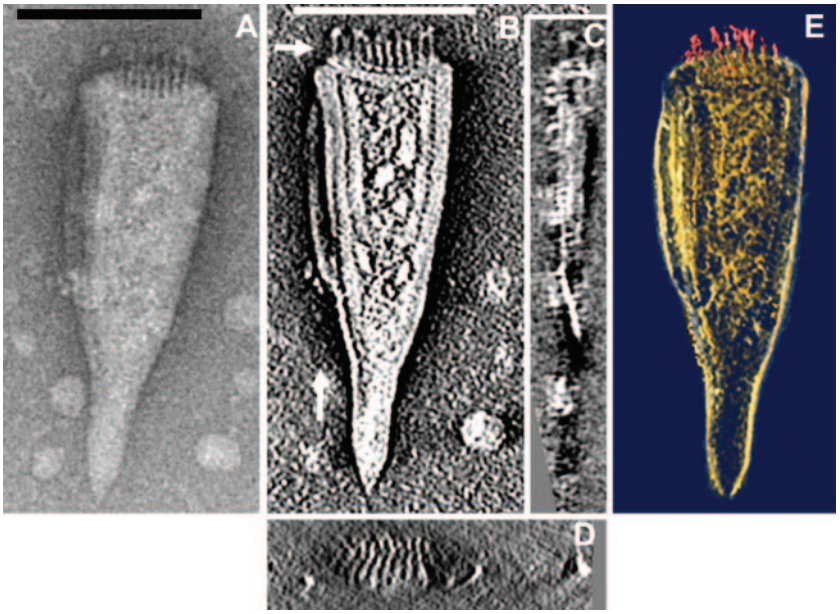

FIG. 4. Electron micrograph and three-dimensional reconstruction of ABV negatively stained with 3% uranyl acetate. (A) Original micrograph at 0 degree tilt. (B) Horizontal slice (0.7 nm) through the three-dimensional data set of the three-dimensional reconstruction of ABV. (C) Vertical slice (YZ). (D) Vertical slice (XZ) through the same data set. The positions of the slices in panels C and D are indicated by the white arrows in panel B. (E) Color-coded representation of the virion's three-dimensional structure. Bars, 100 nm.

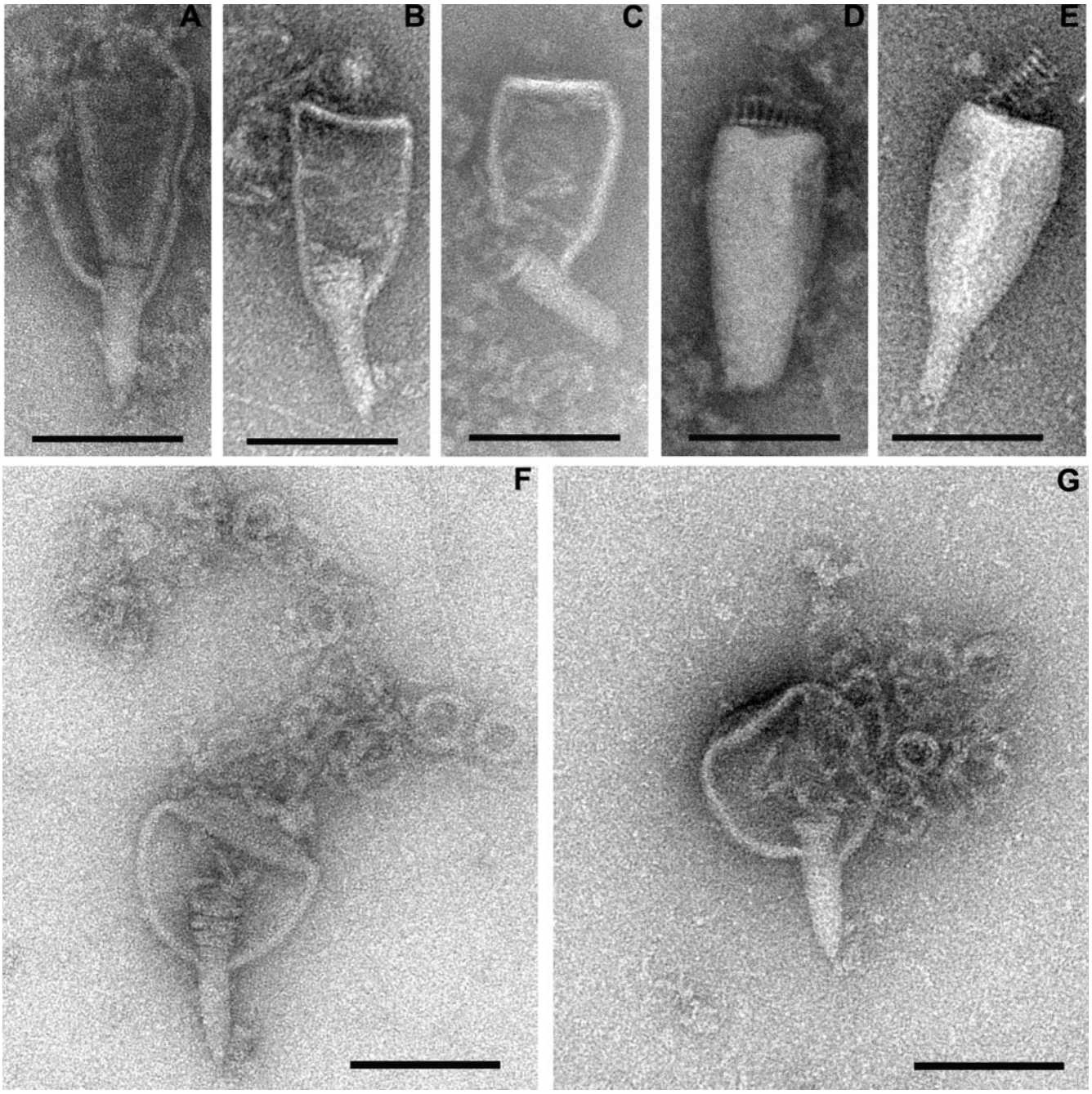

FIG. 5. Electron micrographs of partially disrupted particles of ABV negatively stained with 3% uranyl acetate. Bar, 100 nm.

material it contains, i.e., which molecules, proteins, or even enzymatic activities are enclosed.

Analysis of the partly disrupted virions also provided evidence for the structure of the tip. Occasionally, it was observed to be partially separated from the rest of the virion, suggesting that it constitutes a separate structural unit (Fig. 5D). Maintaining the bottle analogy, it looks like a stopper inserted into a neck-less bottle, rather than the neck of a bottle. The "stopper" measures 90 ± 5 nm in length and 30 ± 2 nm in maximal width (Fig. 5A through C, F, and G). While the results of the three-dimensional reconstruction did not reveal whether it is tightly enclosed by the outer envelope (Fig. 4B), observations of partially disrupted virions indicate that the outer envelope connects to the sides of the "stopper," close to its base, rather than encasing it (Fig. 5A through C, F, and G). Electron tomography demonstrated that the "stopper" consists of densely packed material and suggested that a specific structure, or "opening," occurs at the tip (Fig. 4E). The base of the "stopper" was seen to be connected to the nucleoprotein filament (Fig. 5A, F, and G), and this is the only structure in the virion to which the DNA appears to be directly attached (Fig. 5F and G). Therefore, it is likely to be involved in cellular

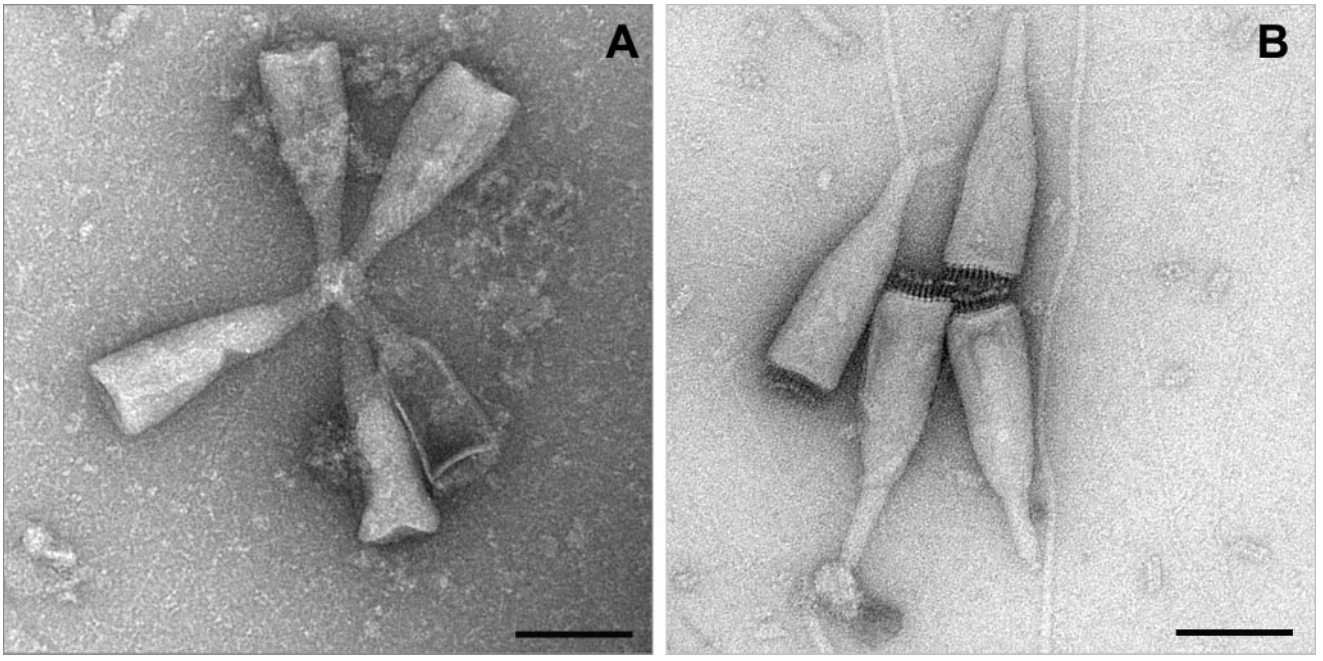

FIG. 6. Electron micrographs of particles of ABV after negative staining with 3% uranyl acetate. (A) ABV particles adsorbed with their pointed end toward a membrane vesicle of the host "*A. convivator*." (B) ABV particles attached to each other with their thin filaments at the broad end. Bars, 100 nm.

adsorption and injection of viral DNA. This inference is supported by other electron microscopic observations showing virions attached to membrane vesicles of the host cells via their tips, sometimes forming rosette-like structures (Fig. 6A). In contrast, the 20 thin filaments were often observed connecting virions but were not attached to the membrane vesicles (Fig. 6B). A schematic structure of ABV, based on the results from electron microscopy and electron tomography, is presented in Fig. 7.

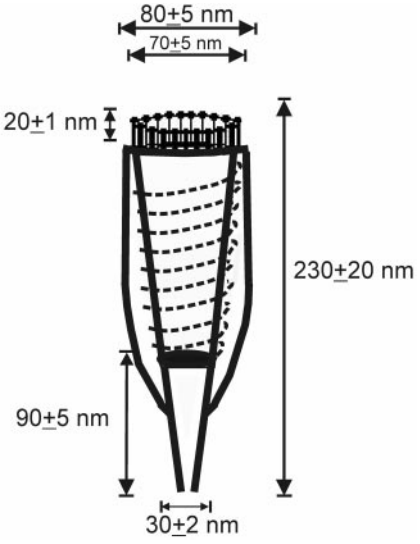

FIG. 7. A scheme of the structure of an ABV virion.

DISCUSSION

Analysis of the viral diversity present in a sample taken from a hot, acidic spring (87 to 93°C, pH 1.5 to 2) in the crater of the Solfatara volcano at Pozzuoli, Italy, demonstrated the presence of a complex mixture of virus-like particles, including both known and previously unreported morphotypes. This strongly supports earlier findings that viruses in geothermally heated hot terrestrial habitats are diverse and exhibit exceptional morphotypes which differ from those of viruses observed in other ecosystems (11). In order to identify hosts for these viruses, 200 isolates from the enrichment culture, all belonging to the hyperthermophilic archaeal genus *Acidianus*, were screened for production of VLPs and for their ability to propagate them. VLPs representative of each of the observed morphotypes were shown to be infectious and therefore to constitute novel viruses.

The structure of one of the novel viruses, ABV, which carries six different structural proteins and a dsDNA genome of 23.9 kb, was studied in detail. We propose assigning it to a new viral family, the *Ampullaviridae* (from the Latin "ampulla," for bottle) because of the unique bottle-shaped morphology of the virions, which has not previously been observed in the viral world (17). Moreover, the complex morphotype of asymmetric virions, lacking elements with icosahedral or regular helical symmetry, with two completely different structures at each end and an envelope encasing a funnel-shaped core, represents a novel type of virus particle. Based on our present understanding of virus biology, we infer that the rationale for this complex morphotype is that it facilitates infection.

The funnel-shaped core of the enveloped ABV virions appears to be well designed for transferring DNA into a host cell.

Apparently it consists of three distinct structural units: the "stopper," the nucleoprotein cone, and the inner core (Fig. 7). Several observations yield insight into possible functions of these components. Evidence from electron microscopy suggests that the "stopper" is a structural element involved in recognition of cellular receptors and adsorption, and it is the only component to which DNA is directly attached. Moreover, it is the only structure which can fulfil the role of an "injection needle" which can generate a transmembrane pathway for channeling viral DNA into the host cell. Such a function would require the presence of a channel inside the "stopper" and an opening at its tip, and such a special tip structure is clearly demonstrated by the three-dimensional reconstructions (Fig. 4E).

Observations through electron microscopes also suggest that the inner core is the most labile part of the virion, since it appears to be partially destroyed under conditions where the other structural elements remain intact (Fig. 4). Structural changes in the protein core could facilitate release of the toroidally supercoiled nucleoprotein filament (while remaining attached to the "stopper"; Fig. 5F and G), and it is possible that such a mechanism for triggering DNA release is employed during the initial steps of infection. Whether the energy accumulated in the toroidally supercoiled structure is sufficient to transport the genetic material into the cell is unclear. However, unwinding of the nucleoprotein filament, wound up as an inverse cone, concomitant with its "funneling" into the cell, appears to be an efficient way of utilizing the energy of DNA packaging for its injection.

The broad end of the particle also exhibits an unusual structure, with the 20 (± 2) thin filaments regularly distributed around, and inserted into, a disk or ring. TEM observations indicate that these filaments are not involved in adsorption, and their function remains unclear. The design of the virion suggests that the filaments could be involved in unidirectional motility of the virus either intracellularly or extracellularly. Extracellularly, this could reflect that the cell density in hyperthermophilic aquatic environments is significantly lower than that which occurs in similar mesophilic environments.

Further progress in our understanding of the biology of ABV will require genomic analysis and correlation with the functions of the viral structural elements, focusing on the mechanism and dynamics of the virus-host interaction during adsorption and virus production.

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