The virophage Sputnik is a satellite virus of the giant mimivirus and is the only satellite virus reported to date whose propagation adversely affects its host virus’ production. Genome sequence analysis showed that Sputnik has genes related to viruses infecting all three domains of life. Here, we report structural studies of Sputnik, which show that it is about 740 Å in diameter, has a T=27 icosahedral capsid, and has a lipid membrane inside the protein shell. Structural analyses suggest that the major capsid protein of Sputnik is likely to have a double jelly-roll fold, although sequence alignments do not show any detectable similarity with other viral double jelly-roll capsid proteins. Hence, the origin of Sputnik’s capsid might have been derived from other viruses prior to its association with mimivirus.
was passed through a 0.8-µm filter to remove amoeba debris and then through a 0.22-µm filter to remove mamavirus. Sputnik was pelleted by polyethylene glycol (PEG) precipitation and further purified on a CsCl gradient. The virus band was collected, and buffer was exchanged to phosphate-buffered saline (PBS) buffer and concentrated to about 1 mg/ml.

**CryoEM.** Sputnik particles were flash-frozen on holey grids in liquid ethane. Images were recorded at a 39k magnification with a CM200 FEG microscope with electron dose levels of approximately 20 e⁻/Å². All micrographs were digitized at 6.35 Å/pixel⁻¹ by using a Nikon scanner. Individual particle images were boxed, floated, and deprocessed to normalize mean intensities and variances and to remove linear background gradients by using the program ROBEM (http://cryoem.ucsd.edu/programs.shtml). Contrast transfer function (CTF) parameters were determined and phases were flipped by using the program EMAN assuming isosceles symmetry. The number of particles incorporated into the final reconstruction was 6,780, giving a final resolution of 10.7 Å.

**Mass spectroscopy.** A 50-µl aqueous sample of Sputnik was mixed with 300 µl methanol and 100 µl chloroform. Subsequently, 200 µl water was added and mixed, followed by 5 min of centrifugation at 10,000 x g to separate phases. The lower phase was analyzed by size-exclusion chromatography (SW2000 XL; Tosoh Biosciences) with a buffer composed of chloroform, methanol, and 1% formic acid in water (4:4:1, vol/vol/vol) at 40°C. The major peak was then analyzed by electron spray ionization mass spectroscopy (19). The magnitudes of individual masses were compared to a standard lipid sample (dipalmitoyl phosphatidyl choline [DPPC]) in order to determine the amount of lipid in the sample. A chloroform blank was used as a negative control.

The percentage of the putative lipid membrane would represent as a fraction of the weight of the whole virus was calculated as follows. The mean diameter of the virus was taken as the weighted average of the 5-, 3-, and 2-fold diameters and found to be 741 Å. Thus, the volume of the whole virus (\(V_0\)) is 4\(\pi(741^2)\)/3. The thickness of the outer protein coat is 75 Å, and the thickness of the lipid membrane is 40 Å. Thus, the mean radius of the lipid is 370 – 75 = 275 Å. Hence, the volume of the lipid membrane (\(V_L\)) would be about 4\(\pi(275^2)\)/3 x 40. The lipid density is approximately 0.9 g/ml, whereas protein and DNA densities are approximately 1.2 g/ml. The ratio of the mass of lipid/mass of the whole virus would then be 0.9\(\pi(V_0 - V_L)\) + 0.9\(\pi(V_L)\), which gives about 14%.

According to the mass spectroscopy results, the amount of the ionized lipid was between 5 and 10 µg in a starting Sputnik sample of about 40 µg, corresponding to between 12.5% and 25% lipid.

**Fitting of PBCV-1 Vp54 into the Sputnik cryoEM map.** A trimer of PBCV-1 Vp54 was reconstituted from the deposited Protein Data Bank (PDB) coordinates (accession number 1M3Y) by applying crystallographic symmetry. When using the Colores program, the five independent capsomers (four in general positions and one associated with a 3-fold axis in the icosahedral asymmetric unit) were fitted separately as trimers. In EMfit, the starting position of the center of mass for each capsomer was taken from the best fit obtained by the Colores program. The best fits from the four generally positioned capsomers and one-third of one pseudohexameric capsomer were then combined into one PDB file representing the fitted icosahedral asymmetric unit. Icosahedral 2-, 3-, and 5-fold matrices were applied to the fitted asymmetric unit to generate the model for the whole virus. The fitting results are summarized in Table 1.

### Table 1. Fit of the crystal structure of PBCV-1 Vp54 to the Sputnik cryoEM density

<table>
<thead>
<tr>
<th>Capsomer</th>
<th>Colores best fit</th>
<th>EMfit</th>
<th>Colores second-best fit</th>
<th>EMfit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colores highest-density peak (Å)</td>
<td>EMfit</td>
<td>Colores highest-density peak (Å)</td>
<td>EMfit</td>
</tr>
<tr>
<td>1</td>
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<td>34.2</td>
<td>1.3</td>
<td>7.31</td>
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<tr>
<td>2</td>
<td>8.96</td>
<td>32.8</td>
<td>1.4</td>
<td>6.4</td>
</tr>
<tr>
<td>3</td>
<td>10.19</td>
<td>32.2</td>
<td>1.9</td>
<td>6.87</td>
</tr>
<tr>
<td>4</td>
<td>9.08</td>
<td>32.6</td>
<td>1.9</td>
<td>6.77</td>
</tr>
<tr>
<td>5</td>
<td>12.18</td>
<td>32.7</td>
<td>0.7</td>
<td>8.45</td>
</tr>
</tbody>
</table>

* The program EMfit measures the fit (Sumf) in terms of the average density at all fitted Cα atoms normalized by the maximum density fixed at 100 (18).

**RESULTS AND DISCUSSION**

**Icosahedral reconstruction of Sputnik.** Sputnik particles (~1 ng/ml) were purified as described previously (10) and frozen in vitrified ice, and data were recorded on film and processed (see Materials and Methods) (Fig. 1). The resolution of the icosahedrally averaged 3D map was estimated to be ~10.7 Å, based on a 0.5 Fourier shell correlation threshold. The dimensions of Sputnik measured 840 Å, 730 Å, and 710 Å along the 5-, 3-, and 2-fold axes, respectively (Fig. 2A). The MCP is organized into a hexagonal lattice with a triangulation number of \(T=27\) (h=3, k=3). Each icosahedral asymmetric unit contains four generally positioned, pseudoicosahedral capsomers, one-third of one pseudoicosahedral capsomer on the icosahedral 3-fold axis, and one-fifth of one pentameric capsomer at the 5-fold axis. The distance of the capsomers from the center of the virus decreases successively from the pentameric capsomers, the capsomers surrounding the pentameric capsomers...
as there are 18,343 bp in the genome, the density of the packed DNA in the genome is about 3.6 A^3/bp.

The inside and outside lipid leaflets of the membrane are presumably layers of packaged DNA that are distinct from the protein capsid. These layers become progressively more fused toward the center of the virus. These layers are "cavities" in the middle of the pentameric capsomers (Fig. 3). The density of the fibers is lower than that of the capsid, suggesting either that the fibers are not fully occupied in each capsomer or that the fibers are flexible. There are "cavities" in the middle of the pentameric capsomers (Fig. 3), one of which could serve as a gate for DNA entry or exit. Indeed, the pentameric vertices of many viruses, especially in those with double jelly-roll capsid structures, are "cavities" in the middle of the pentameric capsomers (Fig. 3).

Inside the capsid shell, there are two layers of densities that together are about 40 Å thick, consistent with capsomer dimensions in viruses of the PRD1-adenovirus lineage. There is a 55-Å-long "mushroom"-like fiber with a triangular head protruding from the center of each pseudohexameric capsomer but not from the pentameric capsomers (Fig. 3). The density of the fibers is lower than that of the capsid, suggesting either that the fibers are not fully occupied in each capsomer or that the fibers are flexible. There are "cavities" in the middle of the pentameric capsomers (Fig. 3), one of which could serve as a gate for DNA entry or exit. Indeed, the pentameric vertices of many viruses, especially in bacteriophages, act as genome entry or exit gates (5, 11, 21, 22).

There are weak interactions between the protein capsid shell and the likely lipid bilayer, represented by "foggy" densities (Fig. 3). The putative membrane surrounds a series of layers separated by about 25 Å that become progressively more fused toward the center of the virus. These layers are presumably layers of packaged DNA that are distinct from the membrane in being less resolved from each other than are the inside and outside lipid leaflets of the membrane. The volume enclosed by the lipid layer is about 3.6 × 10^7 Å^3, and as there are 18,343 bp in the genome, the density of the packaged DNA is about 1.966 Å^3/bp. This is comparable to other viruses such as T4, phi29, PRD1, and adenovirus, which have densities of 2.907, 2.100, 2.148, and 2.057 Å^3/bp, respectively.

Underneath the icosahedral 2-fold axes, there is some weak density that spans across the putative membrane, representing a transmembrane protein (Fig. 3).

**Lipid content of Sputnik.** Lipid was extracted with chloroform from a Sputnik sample of known mass. Mass spectroscopy showed that the sample contained between 12% and 24% lipid, by weight, and that there were a number of types of lipids in the extract with the dominant species (764.7 mass units) having a molecular mass corresponding to phosphatidylserine. This can be compared with the anticipated volume of the membrane relative to the volume of the virus assuming appropriate densities for lipid, protein, and DNA showing that the putative lipid membrane would represent about 14% of the mass of the virus. Judging from the cryoEM images of the sample, the contamination from host amoeba debris was not significant. Thus, the measured amount of lipid corresponds well with the structural information, confirming the presence of a lipid membrane within Sputnik.

**Surface fibers.** Quite a few viruses have fiber-like structures on their surface. Among viruses with double jelly-roll capsid structures, PBCV-1 has fibered capsomers in special positions (5); adenovirus has long fibers emanating from the pentameric vertices, which function to recognize the host (6); Chilo iridescent virus (CIV) has short fibers emanating from every capsomer whose function is unknown; and mimivirus has a forest of fibers whose function may be to act as a decoy for amoeba phagocytosis (21). Other viruses, especially bacteriophages such as T4 and phi29, have hexameric capsomers with protruding fibers whose function is uncertain. Equally, it is unclear what the function is of the fibers protruding from the Sputnik surface. Some possibilities are that the fibers of Sputnik, as perhaps also the fibers of other viruses, help to stabilize each individual capsomer or that the fibers are associated with host cell recognition or entry.

**Fitting of the PBCV-1 Vp54 structure into the Sputnik cryoEM map.** The thickness of the Sputnik capsomer and the distance between adjacent capsomers suggest that the MCP of Sputnik is similar to the MCPs from the PRD1-adenovirus lineage. Therefore, the crystal structure of PBCV-1 MCP Vp54 was fitted into the density map of Sputnik by using both the Colores (20) and EMfit (18) programs (Fig. 4A). The results obtained from both programs were essentially the same and produced reasonably good fits for each of the five independent capsomers (Table 1).

The protein encoded by open reading frame 20 (ORF20), gene product 20 (gp20), is the most abundant protein in Sputnik and, therefore, is probably the MCP. Its length (595 amino acids) is comparable to that of the mimivirus MCP (591 amino acids) and is 158 amino acids longer than PBCV-1 Vp54 (437 amino acids). Therefore, there should be unoccupied capsomer densities in the Vp54-fitted Sputnik map. Indeed, this is what was observed, and the unoccupied densities are mostly on the external side of the capsomers. Upon further examination, the unoccupied densities are close to Ala 291 in the Vp54 structure, which is in the DE loop of the second jelly-roll fold (Fig. 4B). It was shown by sequence alignment that mimivirus is likely to have a large insertion around this position. This would account for most of the length difference in MCPs between PBCV-1 on the one hand and mimivirus or Sputnik on the other (21).
Sputnik gp20 does not have any detectable sequence identity to the mimivirus MCP, nor does gp20 have any homologues in current sequence databases. Hence, Sputnik is probably not a result of divergent evolution from the *Mimiviridae* but, rather, diverged from the NCLDVs at an earlier time. Thus, possibly, this virophage had been associated with another viral host before being associated with mimivirus. Genome sequence analysis showed Sputnik to represent an unknown family of viruses (10). The structural work reported here supports this conclusion and shows that Sputnik would be a new branch in the PRD1-adenovirus lineage.

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We declare that we have no conflict of interest.

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