Inside the Lifestyle of the Virophage

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Key Words

Amoeba viruses · Giant viruses · Mamavirus · Microscopy · Mimivirus · Satellite virus · Sputnik virophage · Viral factory · Viral RNAs

Abstract

Objective(s): We sought to better characterize Sputnik, the first isolated virophage, and to analyze its parasitic lifestyle during co-infection with Marseillevirus (a new giant virus) in *Acanthamoeba castellanii*. **Methods:** A combination of electron microscopy, immunofluorescence microscopy, and real-time PCR was used to characterize the kinetics of the viral replication cycle. RT-PCR was performed to detect RNAs inside the Sputnik virions. **Results:** Sputnik is a new viral entity carrying an almost complete ready-to-use set of viral RNAs (20 out of 21). Sputnik does not replicate with Marseillevirus but delays its replication cycle. While Marseillevirus is successfully internalized by *A. castellanii* following co-infections with Mamavirus and Sputnik, it does not initiate a replication cycle. In contrast, both Marseillevirus and Mamavirus can replicate in the amoeba in case of co-infection, but the development of one is exclusive from the other inside a single amoeba cell. **Conclusions:** This work provides new insight into the Sputnik replication cycle with another giant virus and confirms that Sputnik is a virophage. It shows new dimensions of the interactions existing among giant viruses.

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Introduction

In 2003, a new giant virus named *Acanthamoeba* polyphaga Mimivirus (Mimivirus) was discovered in water collected from a cooling tower in Bradford, England [1]. By its size (~650 nm diameter) and its gene repertoire (>900 ORFs), Mimivirus outcompeted all other giant viruses described thus far [2]. For five years, Mimivirus was the only member of the Mimiviridae family, but in 2008, a new strain of Mimivirus was isolated from another cooling tower in Les Halles, Paris, France [3]. This virus was named Mamavirus. While the appearance (size and presence of fibrils) of Mamavirus largely resembled that of Mimivirus, its genome was slightly bigger [3]. However, the major distinctive feature of this new giant virus was its close association with a tiny ‘companion’ that we named Sputnik.

Sputnik is a small icosahedral virus (50 nm diameter). It harbors an 18-kb genome composed of 21 ORFs. More than a third of these ORFs do not have homologs in current databases, but some of them can be retrieved in environmental metagenomic datasets. Further, Sputnik’s genome contains a mosaic of genes related to bacteriophages, archaea viruses and its Mamavirus host [3]. Mamavirus infection by Sputnik leads to the formation of some abnormal Mamavirus particles and a significant decrease in amoebal lysis. Sputnik is also able to infect Mimivirus, but the number of co-infected amoebae is lower [3]. Through its ability to generate defective Mamavirus virions and to attenuate Mamavirus infectivity for the amoeba, Sputnik is a legitimate parasite rather than a simple satellite. This unprecedented discovery led us to

coin the generic term of virophage to describe a virus infecting another virus. Whether Sputnik should be classified as a virophage or a satellite has been a matter of debate ever since, and through this work (and others to come), we want to address this issue.

Here, we provide insight on new elements of the cell cycle of Mamavirus and its virophage. After a brief description of the Mamavirus and Sputnik replication cycle, we characterize the effect of Sputnik infection on another giant virus named Marseillevirus (described in [4]). We further present results concerning the co-infection of the amoebal host with Marseillevirus, Mamavirus and the Sputnik virophage. In addition, we demonstrate the presence of viral RNAs packaged inside the Sputnik virions. Finally, in light of these results, we explain the reasons why Sputnik can undoubtedly be called a virophage.

Material and Methods

Virus Isolation

Mamavirus and the Sputnik virophage were isolated as previously described [3]. Marseillevirus is a new giant virus isolated from biofilms formed in the waters of an industrial cooling tower. Details about Marseillevirus isolation are given elsewhere [4].

Viral Infection

Acanthamoeba castellanii cultures were seeded in peptone-yeast extract glucose medium at 10^5 cells/ml and infected with a mixture of Mamavirus and Sputnik, as previously described [3]. For co-infection, *A. castellanii* cultures were simultaneously infected with a mixture of Mamavirus, Sputnik and Marseillevirus, or Mamavirus and Marseillevirus. To survey the effect of Sputnik on the replication cycle of Marseillevirus, *A. castellanii* cells were first infected by Sputnik for 24 h, and then Marseillevirus particles were added. At different times post-infection (p.i.), cultures were processed for electron and fluorescence microscopy.

Electron Microscopy

Infected *A. castellanii* cells were washed three times in PBS and fixed overnight with 2% glutaraldehyde in 0.1 M cacodylate buffer. Cells were washed again with 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide in 0.1 M potassium ferricyanide for 1 h, and dehydrated in increasing ethanol concentrations (from 30 to 100%). Samples were embedded in Epon 812 resin. Sections (70 nm) were stained with 3.5% uranyl acetate and lead citrate before examination with a transmission electron microscope (Philips Morgagni 268D).

Fluorescence Microscopy

For immunofluorescence (IFF), 100 μ l of cell suspension was placed into a cytospin chamber, centrifuged at 800 rpm for 10 min in a Shandon Cytospin 4 (Thermo Electron Corp.), and fixed for 10 min with a drop of methanol. DAPI (4',6'-diamidino-2-phenylindole) was used to stain nucleic acids contained both in the cell nuclei and in the viral factories. Indirect IFF labeling was

performed using rabbit anti-Mimivirus serum and mouse anti-Sputnik serum, as previously described [3].

Viral Nucleic Acid Extraction, Real-Time and Reverse Transcriptase PCR

At different times p.i., DNAs were extracted from 200 μ l of culture following the fluid/blood protocol of the QIAamp DNA Mini Kit (Qiagen, Valencia, Calif., USA).

Real-time PCR was performed with a LightCycler FastStart DNA Master SYBR Green I™ kit in a standard PCR reaction, as described by the manufacturer (Roche Diagnostics, Mannheim Germany). Each reaction contained 3 mM MgCl₂, 1 μ M of each primer (primers for Mamavirus polymerase, Marseillevirus capsid, or Sputnik ORF20; table 1), and 5 μ l of template DNA in a 20- μ l PCR mixture. The amplification started with an initial denaturation step at 95° for 10 min, followed by 40 cycles of 95° for 15 s, 57° for 5 s, and 72° for 8 s, with a temperature transition rate of 2°/s. Fluorescence signals were measured once in each cycle at the end of the extension step. After PCR amplification, T_m curve analysis was performed. For each sample, the C_T (Cycle Threshold) was given. The C_T corresponds to the PCR cycle number at which the fluorescence reaches a threshold value of 10 times the standard deviation of the baseline emission. The C_T values are inversely proportional to the starting amount of target DNA.

Detection of Viral RNA Packaged in the Sputnik Particle

Culture supernatants containing Sputnik particles were filtered through a 0.22- μ m membrane to remove contaminant cells and Mamavirus particles, and the filtrate was concentrated by ultracentrifugation (2 h at 22,000 rpm). To remove nucleic acids bound externally to the virions, 50 μ l of the concentrate was treated with RNase (Roche) and DNase I (Qiagen) for 1 h at 37°. Nucleases were inactivated at 70° for 10 min in a mixture of 0.5 M EDTA and 10% SDS, and then virions were digested with proteinase K (20 mg/ml) for 1 h at 56°. RNAs were extracted using the RNeasy Mini kit (Qiagen) and resuspended in 60 μ l of ultrapure water. Residual DNAs were removed via Turbo DNase treatment (Ambion).

Reverse transcription of the mRNA was performed with M-MLV reverse-transcriptase (Invitrogen) using random hexamers and following the manufacturer's recommendations. PCR of the cDNAs (2 μ l), using the HotStarTaq DNA Polymerase kit (Qiagen) and Sputnik specific primers (Sputnik ORF1 to Sputnik ORF21; table 1), were performed with one cycle of denaturation at 94° for 15 min, followed by 30 cycles of 30 s at 94°, 30 s at 59°, 45 s at 72°, and a final extension of 7 min at 72°. PCRs were also performed on mRNAs (2 μ l) to check for DNA contamination. RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel and sequenced in both directions with an ABI Prism 3100 sequencer.

Results

Morphological Characteristics of Mamavirus and Sputnik Particles

Mamavirus shared numerous morphological features with its Mimivirus counterpart. For instance, Mamavirus particles appeared as purple dots under Gram staining (fig. 1a). Mamavirus has an icosahedral capsid, and

Table 1. Primers used in this study

Target	Primer name	Sense	Sequence (5'–3')
Mamavirus polymerase	322F	Forward	TTGGAAAGATGTTAAACCGTCA
Mamavirus polymerase	322R	Reverse	TCTGCTTGAGGGAAATTTTGA
Marseillevirus capsid	T19-capsid-F	Forward	GCGGTAACCCCTCCCATTAT
Marseillevirus capsid	T19-capsid-R	Reverse	CTTTTGCACCTGCTTGATGA
Sputnik ORF1	Sput-ORF1-F	Forward	GCCAATGAGAGGTGGTTGTT
Sputnik ORF1	Sput-ORF1-R	Reverse	TGGCTTTAGGAGATTGCATTG
Sputnik ORF2	Sput-ORF2-F	Forward	GATGATCCTGGAGTCCCATT
Sputnik ORF2	Sput-ORF2-R	Reverse	GGTCAATTATGACCCCTCA
Sputnik ORF3	Sput-ORF3-F-ATPase	Forward	GCTGGAGGGAAATTCAAAGG
Sputnik ORF3	Sput-ORF3-R-ATPase	Reverse	CCACTACCACTTTGTCCAGCA
Sputnik ORF4	Sput-ORF4-F	Forward	TGTTGGGAGGAGATACACCTT
Sputnik ORF4	Sput-ORF4-R	Reverse	TCGACCATTCCATGAATTAGC
Sputnik ORF5	Sput-ORF5-F	Forward	CAAAGACCTTTTGATAAAGAAGCA
Sputnik ORF5	Sput-ORF5-R	Reverse	TCAGTAAGCATTTTCATTGTTG
Sputnik ORF6	Sput-ORF6-F-collagen	Forward	CAGGCATCACTCCATCAACA
Sputnik ORF6	Sput-ORF6-R-collagen	Reverse	ATTCTGCGGTACTTGCTGT
Sputnik ORF7	Sput-ORF7-F-collagen	Forward	CCCTTTCAAACACACCATCA
Sputnik ORF7	Sput-ORF7-R-collagen	Reverse	CCTTTTGAACCAGGATCACC
Sputnik ORF8	Sput-ORF8-F	Forward	GCTCAAGCAACAACCTTTAGCAA
Sputnik ORF8	Sput-ORF8-R	Reverse	CCAGATCCACCTGAACCTACA
Sputnik ORF9	Sput-ORF9-F	Forward	TGGTCATTGGTGTGTTTATTCA
Sputnik ORF9	Sput-ORF9-R	Reverse	CAGAACACACCATCTTCCACA
Sputnik ORF10	Sput-ORF10-F-integrase	Forward	TGGAGCTCCTCAAGTAGCTAAAG
Sputnik ORF10	Sput-ORF10-R-integrase	Reverse	TCTTGAGCCAGCAAATCTTTT
Sputnik ORF11	Sput-ORF11-F	Forward	CCATTGCTATGCCAATTGAA
Sputnik ORF11	Sput-ORF11-R	Reverse	TTCATCTGTATTTCATTCAATTTTG
Sputnik ORF12	Sput-ORF12-F	Forward	TTGGGAAAGTTCCAAAAGGA
Sputnik ORF12	Sput-ORF12-R	Reverse	TTGCTTCTTTTACCATTCTTTTACC
Sputnik ORF13	Sput-ORF13-F-helicase	Forward	GCTTGTGCATTAGCTGGTGA
Sputnik ORF13	Sput-ORF13-R-helicase	Reverse	TGCCCATCTGTATTTTGCAG
Sputnik ORF14	Sput-ORF14-F-znribbon	Forward	GAATGTCCTATTTGTGGTGCTC
Sputnik ORF14	Sput-ORF14-R-znribbon	Reverse	TTTTTGTTCAATATCCACAAGCA
Sputnik ORF15	Sput-ORF15-F	Forward	TCGACAATTATGACTTATTTTCTCA
Sputnik ORF15	Sput-ORF15-R	Reverse	GCCAAAATTTGATGGATAATCTT
Sputnik ORF16	Sput-ORF16-F	Forward	TGAACTTCTCATGGTTGCAGA
Sputnik ORF16	Sput-ORF16-R	Reverse	TGTAAAGAGAACCTTTAGGAGCAAA
Sputnik ORF17	Sput-ORF17-F-transposase	Forward	TGGATTTAAGATTACAACAAAA
Sputnik ORF17	Sput-ORF17-R-transposase	Reverse	TTATTTTCTTTTAGTAGTTTCCTGT
Sputnik ORF18	Sput-ORF18-F	Forward	GCTCCAAATGCACCAACAGT
Sputnik ORF18	Sput-ORF18-R	Reverse	GCACCAACTGGAGCAGAAAT
Sputnik ORF19	Sput-ORF19-F	Forward	GCACCTTCTTATCCACCTGAA
Sputnik ORF19	Sput-ORF19-R	Reverse	TCCAAGGTCCAGTAGCTACAAA
Sputnik ORF20	Sput-ORF20-F-capsid	Forward	GAGATGCTGATGGAGCCAAT
Sputnik ORF20	Sput-ORF20-R-capsid	Reverse	CATCCCAAGAAAGGAGGA
Sputnik ORF21	Sput-ORF21-F	Forward	GAGAAATGTGGGCATATTCAAGA
Sputnik ORF21	Sput-ORF21-R	Reverse	TTTTCAGGTGTCGATTTACCG

including fibrils, the size of the particle reached ~650 nm in diameter (fig. 1b). Sputnik also displayed an icosahedral capsid (fig. 1c), which was ~50 nm in diameter. Sputnik particles were frequently observed at the periphery of or trapped inside Mamavirus fibrils (fig. 1b, d).

Sputnik Particles Enclose Ready-to-Use Viral RNAs

RT-PCR on purified viral particles showed that Sputnik virions enclosed all viral RNAs except one, ORF17 which encodes for a transposase [3] (fig. 2). ORF17 mRNA was not detected inside the sputnik particle but was ex-

Fig. 1. **a** Direct observation of Gram-stained Mamavirus particles inside *A. castellanii*. **b** Transmission electron microscopy image of Mamavirus particles. **c**, **d** Transmission electron microscopy image of negatively stained Sputnik particles (**c**) and Mamavirus surrounded by Sputnik (**d**).

pressed during infection, because all Sputnik mRNAs, including ORF17, were detected at 4 h p.i. (data not shown). Control for DNA contamination and sequencing of the PCR products confirmed that these RT-PCR products were representative of Sputnik mRNAs.

Ultrastructure of Mamavirus and Sputnik Infection

Mamavirus and Sputnik co-infections were followed by IFF and electron microscopy at different time points p.i. (T_0 , H_4 , H_8 and H_{16}). The results are presented in figure 3.

At T_0 (30 min after infection), a large number of Mamavirus particles were seen inside the amoeba cytoplasm as bright and individualized red dots. In contrast, Sputnik particles were observed as a diffuse green signal inside the amoeba cytoplasm with some distinct spots of viral concentration. Electron microscopy showed the presence of numerous Mamavirus-containing vacuoles. Some of them contained several viruses and likely resulted from the fusion of individual vacuoles.

At 4 h p.i. (H_4) the Mamavirus viral factories (VFs) were seen as distinct, strongly stained patches in some cells. The appearance of VFs was concomitant with a decrease in the intensity of the cell nuclei. In these cells, no

or only a few Mamaviruses were observed, indicating an eclipse phase. In most cases, production of Sputnik virions was initiated at the end of the Mamavirus eclipse phase (around 6 h p.i.).

At 8 h p.i., the VFs exhibited a diffuse fluorescent DAPI signal, and Mamavirus production was observed around them. Progeny Sputnik virions were also observed as a bright green fluorescent signal and were localized at one pole of the VF.

At 16 h p.i., almost all of the cell cytoplasm was filled with newly synthesized Mamavirus and Sputnik viral particles. Some vacuoles containing Sputnik particles could also be discriminated. At later times, amoeba lysis resulted in the release of viral particles in the supernatant (not shown).

Sputnik Hijacks the Mamavirus Factory

Both IFF (fig. 4a) and electron microscopy (fig. 4b) revealed the presence of Sputnik particles inside the Mamavirus VF. Detection of sputnik particles within the VF suggested that Sputnik assembly and genome encapsidation occurs at this site. In general, Sputnik particles were produced at one side of the VF, but in some cases, the VF harbored two distinct parts: one filled

Fig. 2. Detection of Sputnik viral transcripts inside the virion.

Fig. 3. a Immunofluorescence of Sputnik- and Mamavirus-infected amoeba at 0, 4, 8 and 16 h p.i. Labeling was obtained with rabbit anti-Mimivirus serum (red) and mouse anti-Sputnik serum (green). Nucleic acids were stained with DAPI (blue). The composite image was created by merging each of the pictures presented above. **b** Electron microscopy of Sputnik- and Mamavirus-infected amoeba at 0, 4, 8, and 16 h p.i.

with Sputnik and the other with Mamavirus (fig. 4b). We frequently observed Sputnik production before that of Mamavirus. In the cases of simultaneous production, the spatial separation of the VF may result from competition between Sputnik and Mamavirus for the available space.

Co-Infection of A. castellanii with Marseillevirus and Sputnik

Marseillevirus is a new giant virus isolated from biofilms formed in the waters of an industrial cooling tower [4]. This virus has a particularly fast cell cycle, as the formation of new virions is observed after only 5 h of infec-

Fig. 4. **a** Fluorescence microscopy image showing Sputnik particles (labeled with mouse anti-Sputnik serum, green) inside and at the periphery of the VF (stained with DAPI, blue). **b** Electron micrograph of a VF divided in two parts: one filled with Mamavirus and the other with Sputnik.

Fig. 5. *A. castellanii* infection. **a** Infection by Sputnik (filled square) and Marseillevirus (empty square). **b** Infection by Sputnik (filled square), Mamavirus (triangle), and Marseillevirus (empty square) measured by real-time PCR at different times p.i.

tion in *A. castellanii* [4]. To assess the effect of Sputnik on the replication cycle of Marseillevirus, *A. castellanii* cells were infected with Sputnik for 24 h before addition of Marseillevirus particles (T₀). The infection cycle was then monitored by electron microscopy and by real-time PCR at H₃, H₆, H₁₂, and H₁₆ (table 1 and fig. 5a).

The results showed that, from T₀ to H₁₂, Sputnik did not replicate with Marseillevirus and started to be eliminated at H₁₆. Similarly, no Sputnik particles were detected inside the amoeba under electron microscopy. However, the presence of Sputnik had a noticeable effect on the Marseillevirus replication cycle, which was delayed for several hours.

Co-Infection of A. castellanii with Mamavirus, Marseillevirus, and with or without Sputnik

To evaluate the effect of Marseillevirus on the multiplication cycle of Sputnik and Mamavirus, *A. castellanii* cells were infected with a mixture of Mamavirus, Sputnik, and Marseillevirus. Infection was followed by real-time PCR targeting specific genes of Mamavirus, Marseillevirus and Sputnik (table 1 and fig. 5b) and by electron microscopy (fig. 6).

At T₀ p.i., Mamavirus and Marseillevirus particles were observed inside distinct vacuoles (fig. 6, T₀, the Marseillevirus particle is indicated with a black arrow) or inside the same vacuole (fig. 6, T₀, black arrow in the inset)

Fig. 6. Electron microscopy and schematic interpretation of Mamavirus, Sputnik, and Marseillevirus replication cycles over a 24-h period.

in the amoeba cytoplasm. The presence of both Mamavirus and Marseillevirus in the same vacuole suggests that either they have been internalized at the same time, or that distinct vacuoles have fused together. At 3 h p.i., it was difficult to detect Mamavirus particles (due to the eclipse phase), but a few Marseillevirus particles were present in the amoeba. The eclipse phase of Mimivirus corresponds to the release of its genetic material into the amoeba cytoplasm. To do this, the Mimivirus particle opens through a unique portal, the stargate, which is located at a single vertex position [5]. This allows the fusion of the viral and host membranes and the formation of a conduit by which the Mimivirus genome is delivered into the host cytoplasm [5] (fig. 6, H₃). Once in the cytoplasm, Mamavirus genome replication may be similar to that of *Asfarviridae*, with a transient nuclear replication phase followed by a cytoplasmic replication phase inside the VF [6, 7] (fig. 6a, H₃). The increase in the AT-content of the nucleus observed between 0 and 3 h p.i. favors this hypothesis [7]. On the other hand, the Mamavirus replication cycle (genome replication and transcription) might exclusively occur in

the cytoplasm, as is the case for *Poxviridae* (fig. 6b, H₃). Indeed, we observed circular inclusions inside the early Mamavirus factories (fig. 7a), which have previously been hypothesized to represent a ready-to-use core particle holding a functional replication complex [8]. This takeout machinery would be in charge of the transcription of the early Mamavirus genes, initiating the development of the VF [8]. During co-infection with Marseillevirus and Sputnik, multiple Mamavirus seeds were observed, giving a multi-lobular aspect to the early VFs (fig. 7b). This multi-lobular morphology has previously been observed with Mimivirus [7] and might also indicate the presence of multiple replication centers inside the VF. Finally, after 6 h p.i., no Marseillevirus could be detected in the cells, and the VFs started to produce Sputnik at one pole. From this time point forward, the course and kinetics of the cycle were similar to that observed in the case of co-infection with Mamavirus and Sputnik only.

IFF and electron microscopy of amoeba infected with Mamavirus alone and Marseillevirus showed at T₀ p.i., and as described above, that both viruses were able to en-

Fig. 7. a Electron microscopy showing inclusions with an electron-dense center inside the early VF. **b** Multilobular viral factory stained with DAPI.

ter the amoeba cells (data not shown). However, at 16 h p.i. individual cells containing either *Marseillevirus* or *Mamavirus* VF were observed. No single cell evidenced the simultaneous presence of both VF, suggesting a competitive exclusion of the viruses. Unlike co-infection in presence of *Sputnik* and *Mamavirus*, *Marseillevirus* replication was not inhibited by the presence of *Mamavirus* alone (data not shown).

Discussion

Effect of Sputnik on Another Giant Virus

In our previous study, we showed that *Mamavirus* was negatively affected by *Sputnik*. The adverse effect of *Sputnik* was also confirmed with *Mimivirus*, another member of the *Mimiviridae* family. Thus far, none of the other nucleocytoplasmic large DNA viruses (NCLDV) [9, 10], a group including *Poxviruses*, *Iridoviruses*, *Phycodnaviruses*, and the *Asfarvirus*, has been associated with a *Sputnik*-like virus. Here we wanted to go beyond this observation and test the effect of *Sputnik* on another giant virus. We focused on *Marseillevirus*, a recently isolated virus that represents a new family of the NCLDV group [4]. *Marseillevirus* was selected because of its ability to generate VFs in *A. castellanii* (unlike the other NCLDVs) and its unusually rapid replication cycle (~5 h). The results showed that *Sputnik* did not enter the amoeba and did not replicate with *Marseillevirus* but delayed its replication cycle. The first VFs were detected 6 h p.i. in *A. castellanii* cells instead of only 3–4 h p.i. with *Marseillevirus* alone (data not shown). However, once the VFs were produced in the cells, viral replication of *Marseillevirus* followed the same kinetics whether *Sputnik* was added or not (data not shown).

When added together, both *Mamavirus* and *Marseillevirus* achieved their replication cycle in independent *A. castellanii* cells. In the presence of *Sputnik*, *Marseillevirus* was still internalized but did not replicate anymore. The mechanism by which *Sputnik* interferes with *Marseillevirus* and/or *A. castellanii* is unclear, but it might target the early stages of the replication cycle (i.e. adsorption and/or penetration) of the virus through for instance the production of soluble inhibitors.

Mechanism of Sputnik Entry

Sputnik particles were frequently observed at the periphery of or trapped inside the *Mamavirus* fibrils (fig. 1b, d). The chemical nature of the fibrils surrounding the outer layer of the *Mamavirus* particle is not clearly defined. However, it has previously been hypothesized that, according to the large number of ORFs with collagen triple helix repeats in the *Mimivirus* genome, these fibrils might consist of cross-linked glycosylated collagen [2, 11, 12]. The *Sputnik* genome also contains two predicted coding sequences containing collagen triple helix motifs (ORF6 and 7). These sequences share homologies with the *Mimivirus* R196 and R238, respectively. In addition, *Sputnik* displays mushroom-like fibers at the surface of its capsid [13]. While the function of these fibrils is still uncertain, we can speculate about their role in the adhesion observed between *Sputnik* and *Mamavirus*. By interacting with the *Mamavirus* fibrils, *Sputnik* would then have an ideal gateway to enter the amoeba. In light of these results, the absence of fibrils in *Marseillevirus* would, at least in part, explain the inability of *Sputnik* to replicate with this virus.

Sputnik Virophage

We first identified *Sputnik* as a satellite, according to the International Committee on Taxonomy of Viruses,

Table 2. Traditional satellites (viruses and nucleic acids)

	Satellite type	Example ¹	Genome size	Helper	Host	Ref.
ssDNA	Satellite virus	<i>Adeno-associated virus (Dependovirus, Parvoviridae)</i> ²	4,700 nt	Adenoviruses or Herpesviruses	Vertebrate	[26]
Positive ssRNA	Satellite virus	Tobacco necrosis satellite virus	1,000 nt	Chronic bee-paralysis virus	Animal	[27]
		Chronic bee-paralysis satellite virus	1,239 nt	Diverse groups	Plant	[28]
dsDNA	Satellite nucleic acids	Enterobacteria phage P4 satellite DNA	11,627 bp	Enterobacteria phage P2 (<i>Myoviridae</i>)	Bacteria	[29]
ssDNA	Satellite nucleic acids	Tomato leaf curl virus satellite DNA	682 nt	Geminiviruses (Begomovirus)	Plant	[30]
dsRNA	Satellite nucleic acids	Saccharomyces cerevisiae L-A virus satellite	500–1,800 bp	<i>Totiviridae</i>	Fungi	[31]
Positive large ssRNA	Satellite nucleic acids	Arabidopsis mosaic virus large satellite RNA	800–1,500 nt	Arabidopsis mosaic virus	Plant	[32]
Positive small ssRNA	Satellite nucleic acids	Cucumber mosaic virus satellite RNA	<700 nt	Cucumber mosaic virus	Plant	[33]
Positive circular ssRNA	Satellite nucleic acids	Velvet tobacco mottle virus satellite RNA (virusoid)	220–350 nt	Mostly Sobemoviruses	Plant	[34]
Negative ssRNA	Satellite nucleic acids	Hepatitis delta virus (<i>Deltavirus</i>)	1,700 nt	Hepatitis B virus	Vertebrate (human)	[35]

Modified from [25].

¹ Satellite viruses encode their own coat protein, while satellite nucleic acids use the coat protein of their helper virus.

² Can replicate independently of the helper in some cell cultures.

Table 3. Similarities and differences among satellite viruses, bacteriophages and the Sputnik virophage

	Satellite viruses	Bacteriophages	Sputnik virophage
Nucleic acid	ssDNA or ssRNA	ssDNA, dsDNA, dsRNA, ssRNA	dsDNA (presence of mRNAs)
Negative effect on host replication cycle ¹	Possible	Yes	Yes
Independent viral family	Possible	Yes	Yes
Genes from host	No	Yes	Yes
Genes from other organisms	No	Yes	Yes
Independent replication cycle	Possible	No	No

¹ Host is the helper virus (satellite viruses), bacteria (bacteriophages) or Mamavirus (Sputnik virophage).

which defines satellites as ‘sub-viral agents composed of nucleic acid molecules that depend for their productive multiplication on co-infection of a host cell with a helper virus’ [14]. In contrast to satellite nucleic acids (e.g. the enterobacteria phage P4 satellite DNA; table 2), satellite viruses (e.g. the human dependovirus adeno-associated virus, AAV) encode their own coat protein, which would make Sputnik the first dsDNA satellite virus isolated so far (table 2).

However, several lines of evidence show that Sputnik is more than a satellite and represents a new biological entity (table 3). First, from a biological point of view, Sputnik

infection results in a 70% decrease of the Mamavirus cytopathic effect on the amoeba [3]. AAV interference in the replication of its adenovirus helper has previously been observed [15]. However, the formation of some ‘diseased’ Mamavirus forms in case of co-infection with Sputnik [3] is a phenomenon that has never been described for traditional satellites. In addition, in the absence of a helper virus, AAV particles can penetrate into human cells and viral sequences can integrate into a specific region of the genome [16]. Sputnik alone did not penetrate and replicate inside *A. castellanii* and no integration sites have been identified thus far in the amoeba genome [3].

Second, the genetic definition of the satellites states that ‘satellite nucleic acids have substantially distinct nucleotide sequences from those of the genomes of either their helper virus or host’ [14]. However, Sputnik shares three ORFs with Mamavirus [3]. More surprisingly, Sputnik’s genome also contains homologs of genes from viruses infecting archaea and bacteriophages. Sputnik is likely a ‘gene robber’ that acquired these genes through horizontal gene transfer. In addition, a previous study reported the presence of 16 Mimivirus-like hairpin structures (a polyadenylation signal unique to Mimivirus [17]) in the Sputnik genome, supporting the fact that Sputnik uses the Mamavirus apparatus to carry out its own replication cycle [8].

In our previous study, proteomic analysis of Sputnik particles identified three proteins, i.e. the major capsid protein and two minor virion proteins (proteins encoded by ORF20, ORF08, and ORF19, respectively) [3]. Here we further show that all viral RNAs, except one, were packaged inside the Sputnik virions. This phenomenon has also been observed among Herpesviruses [18, 19], Adenoviruses [20–22] and Mimivirus [2]. As for Adenoviruses [21], the packaging of RNAs into the Sputnik particle was not selective since almost all of the RNAs were detected in the virions. The functions of these ready-to-use RNAs in the virus life cycle are currently unknown but likely reflect the adaptation of Sputnik to its parasitic and competitor behavior toward Mamavirus and other giant viruses.

In this light, Sputnik is then a complete viral entity containing genomic DNA and mRNAs. Sputnik infects Mamavirus and for all of the reasons presented above, a different term is necessary to characterize this new virus. In 1917, Francis d’Herelle described bacteriophages as

‘living, filterable, ultramicroscopic microorganisms parasitizing and rapidly multiplying at the expense of actively growing bacteria’ [23, 24]. Genetically, bacteriophages are professional gene pickpockets and are engaged in extensive horizontal gene exchange. Both biological and genetic definitions of bacteriophages, which rely on the notion of parasitism and horizontal gene transfer, are the closest to what we observed with Sputnik. While the original name of Sputnik was kept, the ‘virophage’ neologism was suggested by analogy to the bacteriophages of d’Herelle. We believe that Sputnik is not the only virophage and that other giant viruses may be parasitized by such virophages. This is confirmed by our recent discovery of a new *Mimiviridae* member infected by a second strain of virophage (data not shown).

Acknowledgments

Marie Suzan-Monti, Bernard Campana, and Lina Barassi are acknowledged for their expert assistance. We also thank Benoit Desnues for critical reading of the manuscript. This work was funded by the Centre National de la Recherche Scientifique (crédits récurrents).

Addendum

During the edition process of this article, a study has shown the entirely cytoplasmic viral replication of Mimivirus (Mutsafi Y, Zauberman N, Sabanay I, Minsky A: Vaccinia-like cytoplasmic replication of the giant Mimivirus. *Proc Natl Acad Sci USA* 2010;107:5978–5982). According to these results, panel b of figure 6 (H₃ p.i.) reflects the accurate replication pathway of Mimivirus.

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