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Amoebae: Hiding in Plain Sight: Unappreciated Hosts for the Very Large Viruses

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Keywords

amoebae, giant virus, NCLDV, *Nucleocytoviricota*, viral diversity, genome complexity

Abstract

For decades, viruses have been isolated primarily from humans and other organisms. Interestingly, one of the most complex sides of the virosphere was discovered using free-living amoebae as hosts. The discovery of giant viruses in the early twenty-first century opened a new chapter in the field of virology. Giant viruses are included in the phylum *Nucleocytoviricota* and harbor large and complex DNA genomes (up to 2.7 Mb) encoding genes never before seen in the virosphere and presenting gigantic particles (up to 1.5 μm). Different amoebae have been used to isolate and characterize a plethora of new viruses with exciting details about novel viral biology. Through distinct isolation techniques and metagenomics, the diversity and complexity of giant viruses have astonished the scientific community. Here, we discuss the latest findings on amoeba viruses and how using these single-celled organisms as hosts has revealed entities that have remained hidden in plain sight for ages.

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INTRODUCTION

Importance

Viruses are the most abundant biological entities on Earth (1). As intracellular parasites of organisms from the three domains of life, they exert important top-down selective pressures. They are agents of evolution and play fundamental roles in ecosystems (1–3). Viral studies are important for medicine, economics, and biotechnology (4). Therefore, it is not surprising that the known virosphere is extremely anthropocentric (5). Nonetheless, most viruses are not associated with humans, and their study can unravel an unprecedented part of the world. The discovery of Acanthamoeba polyphaga mimivirus (APMV) is a notable example (6).

The description of a virus with genome and particles of such complexity opened the door to an innovative area of study. Virologists revisited the concepts that defined viruses and even led to the discovery of satellite-like associated viruses, i.e., virophages (7). Therefore, it is not surprising that the search for other viruses that infect amoebae has recently intensified. With advances in molecular techniques, studies of metagenomics have detected these ubiquitous and abundant viruses in different parts of the world (8, 9). These prospective studies and viral isolation have made it possible to make an in-depth characterization of some of these viruses to help shed light on viral diversity, evolution, structure, genomics, replication cycle, interactions with hosts, and what role these complex organisms play in nature.

Discovery of Giant Viruses of Amoebae

A pneumonia outbreak in 1992 in Bradford, England, led researchers to seek to identify the causative agent of the disease. Water samples were collected from cooling towers, and different microorganisms were isolated in association with amoebae including an apparent Gram-positive bacteria described at the time as the Bradford coccus (6). Several molecular attempts to identify the new microorganism failed. The mystery began to be solved when transmission electron microscopy analysis was performed at Aix-Marseille University, France. This revealed gigantic icosahedral virus-like particles surrounded by extensive fibril structures (6). The new species was then named *Acanthamoeba polyphaga* mimivirus in reference to its host organism and for mimicking another microorganism. The newly discovered mimivirus had a particle size of approximately 700 nm in diameter that could not be filtered in standard 0.2-μm membranes. The viral particle was nonenveloped and had an ∼1.2-Mb genome that was larger than some cellular organisms (6, 10). The mimivirus was associated with a group of nucleocytoplasmic large DNA viruses (NCLDVs), thus establishing the new family *Mimiviridae*.

The search for new giant viruses intensified after the discovery of APMV. New mimivirus isolates, as well as metagenomic analyses, revealed the ubiquity and diversity of these viruses on the planet (11–14). In 2009, six years after the discovery of mimiviruses, a new *Acanthamoeba*-infecting virus was discovered from water samples collected from cooling towers in Paris and was named marseillevirus (15). The novel virus was an icosahedral particle with an approximate size of 250 nm and a circular genome of 370 kb characterized by genomic mosaicism (15). This was the first representative of the new family *Marseilleviridae*, which expanded over the years to include different marseillevirus lineages (16–19). Since then, several groups of giant amoeba viruses have been discovered, pushing the limits once imagined for viral particle size and genomic complexity as evidenced by pandoraviruses, pithoviruses, and tupanviruses, thus expanding the diversity of putative members of the phylum *Nucleocytoviricota* (20–23).

Advances in Virus Isolation Techniques

Rowbotham (24, 25) pioneered the use of *Acanthamoeba* coculture techniques. At that time, Pontiac fever was a recently solved mystery, and he proposed an enrichment step for the isolation of *Legionella* spp. directly from patient samples. This technique involved culturing *Acanthamoeba* spp. in xenic culture with UV-irradiated *Klebsiella aerogenes*. This technique allowed the characterization of many intracellular bacteria (26, 27).

During the first decade after the description of APMV, the coculture strategy for amoebae was mainly based on the use of *Acanthamoeba polyphaga* (Linc-AP1) and *Acanthamoeba castellanii*. A total of 7,000 samples were inoculated to discover the A, B, and C lineages of *Mimiviridae* and *Marseilleviridae* using different cocktails of antimicrobials and using Page's amoebal saline medium to avoid microbial proliferation (28). Some viruses remained unexplored because they were considered endosymbionts (29). This high-throughput procedure paved the way for new viral isolates even though many modifications and adaptations were subsequently made (30). The ingenuity and creativity deployed allowed one to explore and revealed new viruses notably by adapting amoebae as for the isolation of pandoravirus (20) or by a pre-enrichment step using rice grains and incubation of samples in the dark for almost a month, thus resulting in the isolation of megavirus chilensis and samba virus with its virophage (11, 31). Finally, a high-throughput strategy was developed for amoebae by coupling coculture with flow cytometry and electron microscopy (32). In parallel, new cell types such as *Vermamoeba vermiformis* have been explored in an appropriate medium called TS (starvation buffer) (33, 34).

This past decade has shown a rapid expansion of viral isolates on different platforms including nonamoebal hosts (35–38). Ultimately, a high-throughput system was developed to detect nonlytic viruses. This system could be coupled with different image techniques including optic and electron microscopy as well as fluorescence staining for the rapid detection of giant viruses directly from culture (39). Moreover, the use of a new support is always a challenge, such as the use of the xenic amoeba *Stenamoeba* sp. to isolate virus with a virophage named "Sissivirus" (under characterization) (35). Another strategy is based on the separation of amoebae by capturing the infected amoeba via double viral infections as for faustovirus and clandestinovirus via a single microaspiration technique (40). More recently, myriad new viruses have been identified, causing our perception of the virosphere to change regularly.

Unexpected Diversity of Amoebae Viruses

The family *Mimiviridae* is the most diverse among the groups of giant viruses known to date. Mimiviruses ushered in a new era of virology and expanded the limits of the virosphere in terms of both structure and genomic complexity (41). These viruses have pseudoicosahedral particles approximately 700 nm in diameter with long fibrils immersed in a layer of peptidoglycan-like component, thus justifying the erroneous identification as Gram-positive cocci in the early 1990s (42) (**Figure 1**). This family contains one of the most complex amoebae viruses isolated to date: the tupanviruses (22).

Tupanviruses have a capsid similar to mimiviruses but have a long cylindrical tail attached, thus leading to an average size of 1.2 μm of viral particles (**Figure 1**). In 2017, metagenomic analysis from samples collected at a wastewater treatment plant in Klosterneuburg, Austria, revealed the existence of klosneuviruses (43). These viruses have a unique genomic complexity with several genes related to protein synthesis in an amount and diversity never seen before (second only to tupanviruses) (22, 43). Recently, other members of the possible subfamily "Klosneuvirinae" were identified by metagenomics of soil samples, thus increasing the diversity of this group (44).

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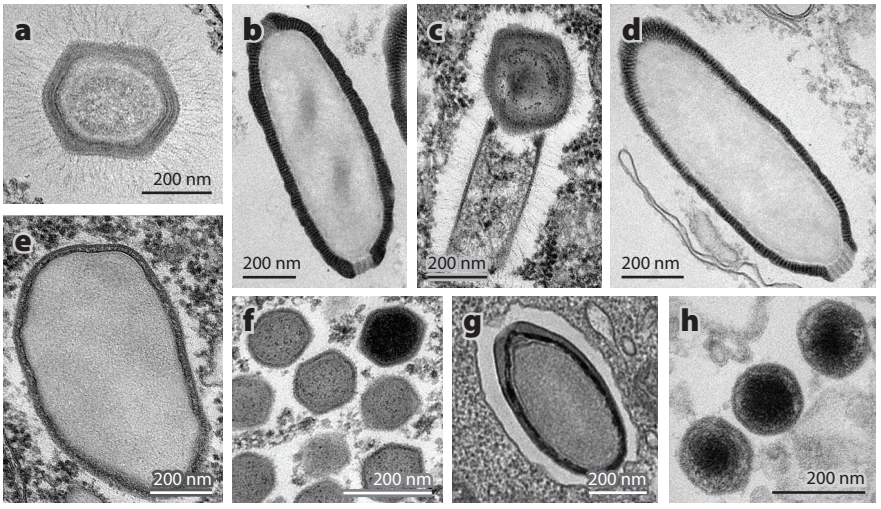


Figure 1

Diversity of amoebae-infecting viruses. Electron microscopy images evidence the structural diversity and complexity of viruses isolated from different amoebae hosts: (*a*) mimivirus, (*b*) cedratvirus, (*c*) tupanvirus, (*d*) pithovirus, (*e*) orpheovirus, (*f*) marseillevirus, (*g*) pandoravirus, and (*h*) kaumoebavirus.

Over the past 15 years, diverse groups of amoeba viruses have been isolated and characterized. Viruses with widely varied structures and genomes were uncovered using *Acanthamoeba* as an isolation platform. Marseilleviruses have icosahedral particles of approximately 250 nm (**Figure 1**) and circular genomes, thus constituting a diverse family of viruses with at least five recognized lineages (A–E) (15–19). Pithoviruses and cedratviruses have ovoid particles that can reach 1.5 μm in length and present one and two cork structures, respectively, in apical positions of the viral particles, i.e., regions where their circular genomes are released during the replication cycle (21, 45) (**Figure 1**). There are currently only a few isolated pithoviruses, but several others have been identified by metagenomics, thus forming the putative family "Pithoviridae" (46, 47). Pandoraviruses are 1.0-μm ovoid particles (**Figure 1**), and linear genomes reach up to 2.7 Mb. These are the largest viral genomes known to date (20, 48). These viruses have already been isolated on different continents, thus revealing distinct lineages and an enormous genetic diversity (48–50). Pandoraviruses are phylogenetically close to molliviruses—another group of *Acanthamoeba*-infecting viruses with only two known isolates (51, 52). Pacmanvirus and medusavirus are other viruses isolated in *A. castellanii*, with two and one known isolates to date, respectively (53–55).

Other groups also infect *Vermamoeba* sp.: Faustoviruses were the first to be isolated in this system with circular genomes and 250-nm icosahedral particles (34). These viruses are phylogenetically close to the kaumoebaviruses—a newly discovered group of viruses with only two isolates to date (56–58) (**Figure 1**). Clandestinovirus was identified in a mixed coculture with a faustovirus and has similar characteristics to medusavirus, possibly constituting a new family called "Medusaviridae" (59). Finally, viruses with very large genomes and particles were also identified and infect *Vermamoeba*. Yasminevirus was the first amoeba virus of "Klosneuvirinae" to be isolated. It has a large set of translation-related genes (60). Orpheovirus is currently the largest virus isolated using *Vermamoeba* and is part of the putative family "Pithoviridae" (61). This virus has ovoid particles nearly 1.1 μm in diameter (**Figure 1**) and a circular genome of 1.4 Mb, in contrast to pithoviruses and cedratviruses with genomes up to 610 kb.

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Finally, a recent analysis by global metagenomics highlighted the enormous diversity of giant viruses. There are more than 2,000 new viral genomes, thus increasing the phylogenetic diversity of NCLDVs by eleven-fold in relation to what was previously known (8). This reveals the importance of this type of analysis and expands our knowledge about viral diversity. It is also an important limitation because it highlights fundamental questions about the biology of these viruses including the difficulty of identifying rare viruses in the environment. This was evidenced with the discovery of yaravirus—a small amoeba virus with 90% of the gene content unknown and undetectable in a set of more than 8,500 available metagenomes (62).

BASIC VIROLOGY

The Complex Genomes of Amoebae Viruses

One of the most striking features of viruses is the presence of short genomes encoding only a few genes due to their intracellular parasitic nature. However, some viruses break this pattern and encode hundreds of genes such as large DNA viruses such as poxviruses, chloroviruses, and jumbophages (63–65). The discovery of mimiviruses crossed this limit—these viruses have genomes over 1.0 Mb that encode more than 1,000 genes, thus exhibiting a genomic complexity never conceived of before in the virosphere (66) (**Figure 2*a***). Mimiviruses have dozens of genes related to metabolic processes with an emphasis on elements related to protein translation—something hitherto exclusive to the cellular world (10, 66). Proteins such as IF4A, eF-TU, and eRF1 are involved in initiation, elongation, and release of the polypeptide chain, respectively. These are found in mimivirus genomes and a diverse set of aminoacyl-transfer RNA (tRNA) synthetases (aaRSs)—this is unprecedented in the virosphere (10). The discovery of klosneuviruses and later tupanviruses dramatically increased the genomic complexity of *Mimiviridae*. These viruses have 20 aaRSs covering all amino acids and up to 70 tRNAs covering all cognate amino acids found in the tupanvirus genome (22, 43) (**Figure 2*a*,*b***). Other giant viruses such as pandoraviruses and orpheovirus also have genes related to protein synthesis, but in much less abundance and diversity than *Mimiviridae* (20, 61) (**Figure 2*b***).

In addition to the translational machinery, other features stand out in the genome of several groups of giant viruses. Marseilleviruses, medusavirus, and clandestinovirus have genes that encode histones. These proteins are homologous to those found in eukaryotes and are involved in the formation of nucleosomes and DNA organization (54, 59, 67, 68). Mimiviruses have genes related to post-translational modification processes and are involved in different sugar pathways such as UDP-D-viosamine and UDP-L-rhamnose, thus having elements that form their own glycosylation machinery (69, 70). Pandoraviruses have a set of enzymes involved in the tricarboxylic acid cycle for energy production. These genes are expressed during the viral replication cycle, and at least one of the encoded proteins was verified to have biological activity based on isocitrate dehydrogenase—a key enzyme in the process (71). Furthermore, some giant viruses have their own set of mobile genetic elements (named as mobiloma) such as mimiviruses, marseilleviruses, and faustoviruses that have genes containing introns [e.g., RNA polymerase and major capsid protein (MCP)] and the presence of linear transposable elements, or transpovirons, detected in all three mimivirus lineages (72–75). Furthermore, mimiviruses have satellite-like viruses that depend on their enzymatic machinery for replication, called virophages, which can also integrate into the genomes of their hosts, thus forming provirophages (7, 75).

Finally, a surprising amount of ORFans in all known amoeba viruses reached approximately 90% in pandoraviruses (20) (**Figure 2*a***). This reflects the enormous diversity and genetic novelty in these viruses and highlights the importance of isolating and characterizing new amoeba viruses. Furthermore, pan-genome studies performed with different groups of amoeba viruses such as

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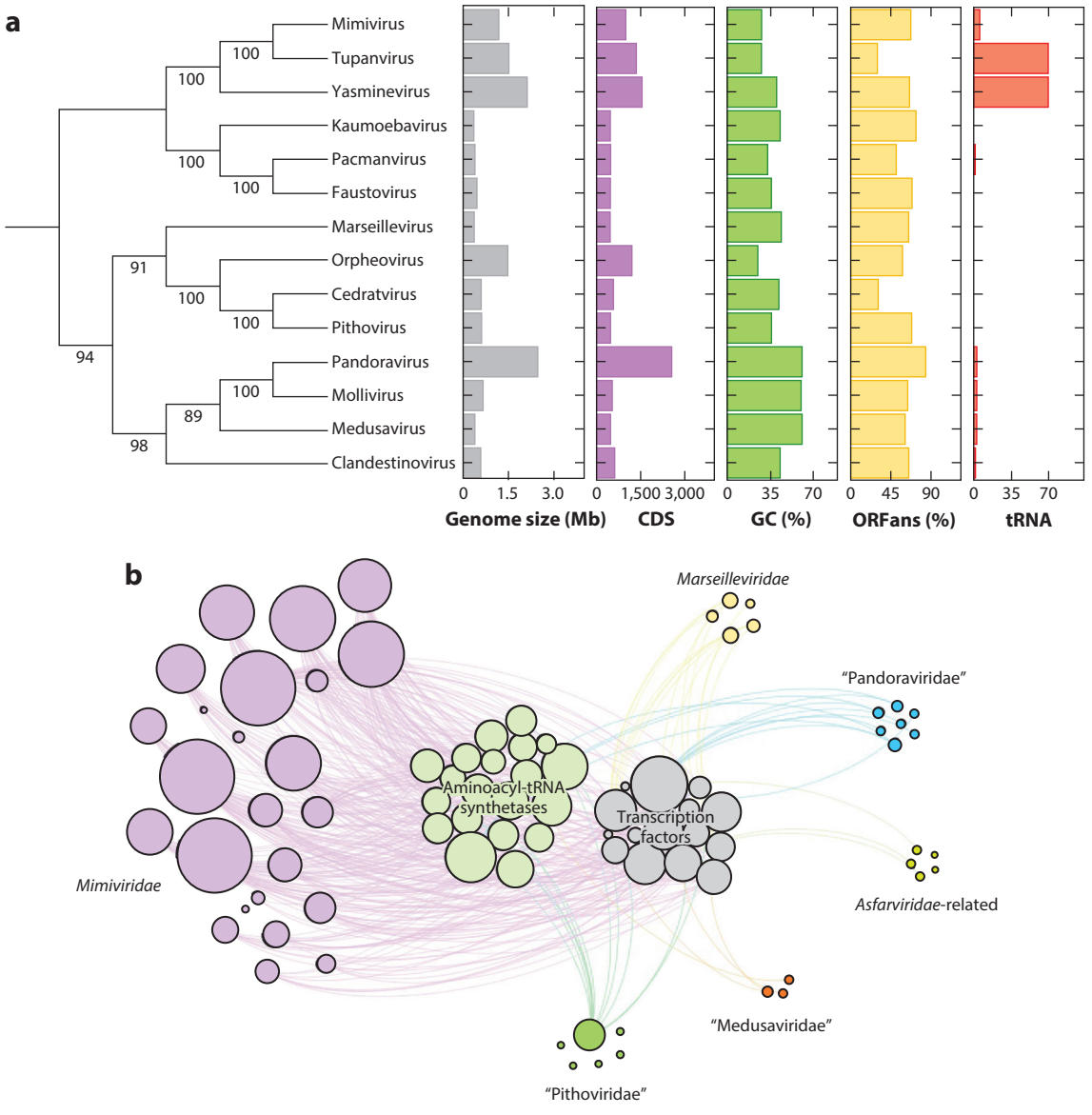


Figure 2

The genomic complexity of giant viruses. (*a*) Phylogenetic tree based on concatenate alignment of the three most conserved genes among *Nucleocytoviricota* (DNA polymerase B family, D5-primase-helicase, and viral late transcription factor 3) including representatives of different groups of giant viruses of amoebae. Simple bar charts indicate general genomic features of these viruses. (*b*) Network graph connecting representatives of distinct groups of giant viruses of amoebae to translation-related elements. Aminoacyl-tRNA synthetases and translation factors (initiation, elongation, and release) are represented with light green and gray colors, respectively. Different groups of viruses are indicated with distinct colors. The node sizes are proportional to the degree of connections. Abbreviations: CDS, coding DNA sequence; GC (%), percentage of guanine and cytosine content in the nucleic acid; ORFans (%), percentage of protein-coding regions that have no recognizable homolog; tRNA, transfer RNA.

mimivirus, marseillevirus, faustovirus, and pandoravirus revealed an open pan-genome, showing that the genomic diversity and complexity of amoeba viruses is far for reach stability (18, 50, 56, 76).

Evolution and Taxonomy of Giant Viruses

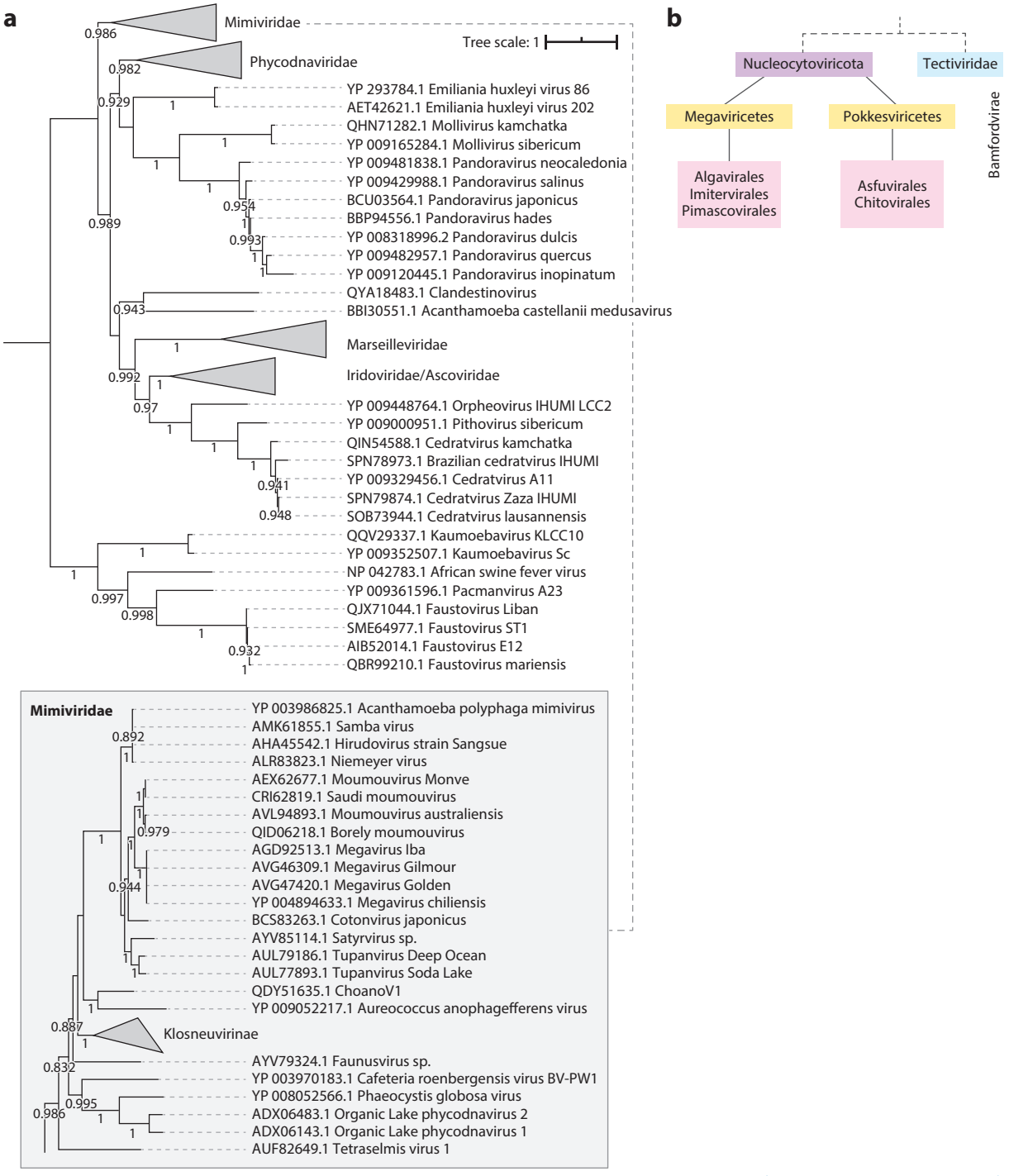
After the discovery of mimiviruses and other amoeba viruses, they were grouped together with NCLDVs [now officially the phylum *Nucleocytoviricota*, kingdom *Bamfordvirae* (23)]. Some genes are conserved in most known viruses in this group, e.g., DNA polymerase B family, which allows for reconstruction of the evolutionary history of the group, thus evidencing the phylogenetic relationship between the different amoeba viruses (**Figure 3*a***). Mimiviruses are phylogenetically close to phycodnaviruses including some viruses formerly included in the family *Phycodnaviridae* that should be reclassified and will be part of the *Mimiviridae* family. It is now clear that both families group together viruses that infect microalgae found either in freshwater or in the marine environment (77, 78). These families are currently included in the orders *Algavirales* and *Imitervirales*, respectively. Both are included in the class *Megaviricetes* (**Figure 3*b***). Pandoraviruses and molliviruses are not yet officially classified into any group, but they have a close relationship to viruses of the *Phycodnaviridae* family—especially the coccolithoviruses (**Figure 2*a***), thus suggesting their inclusion in the order *Algavirales*. Marseilleviruses and pithoviruses, as well as related viruses (cedratviruses and orpheovirus), are phylogenetically close to iridoviruses and ascoviruses; they are classified in the order *Pimascovirales* (**Figure 3**). Other large amoeba viruses such as faustoviruses, pacmanviruses, and kaumoebaviruses are phylogenetically related to the African swine fever virus, family *Asfarviridae*, thus suggesting that they belong to the order *Asfuvirales*, class *Pokkesviricetes* (**Figure 3*b***). Other evidence points to a common origin among these viruses as regulatory elements of gene transcription (79), although new and more robust analyses must be conducted to better elucidate the relationship between these amoeba viruses and the asfarviruses.

Other evidence suggests a common origin of these giant viruses where they would have shared a common ancestor with smaller viruses—possibly belonging to the group of bacteriophages of the family *Tectiviridae* (80, 81) (**Figure 3*b***). Molecular and morphological aspects support this hypothesis (82). Phylogenetic analyses based on different genes conserved within *Nucleocytoviricota* point to a common origin of these viruses that would have occurred before the emergence of modern eukaryotes from smaller viruses, thus emphasizing how ancient giant viruses are (83). Furthermore, analyses based on genes associated with the protein translation process point to a similar pathway in which the giant amoeba viruses would have acquired several genes through horizontal gene transfer events, thus substantially increasing the size and genomic complexity of these viruses (43, 81). However, analyses based on different proteins of these viruses point to an opposite pathway in which giant viruses coexist with cellular ancestors as they were a separate group of complex organisms that evolved mostly due to a loss of genes and adaptation to a parasitic lifestyle (84, 85). Given this scenario, further studies must be carried out to settle this important question about giant amoeba viruses leading to important information about the origin and evolution of one of the most complex groups in the virosphere.

Structure of Giant Viruses

Giant viruses of amoeba have complex particles that come in a wide variety of shapes and sizes (15, 21, 22, 51, 59–62, 86–91). Isolates with smaller dimensions have particles of icosahedral symmetry (15, 34, 53, 54, 57, 59, 60, 62, 87). Some (faustoviruses, kaumoebaviruses, and yaraviruses) seem to present two-capsid protein shells (62, 86). Different shapes start to appear in particles with 400 nm or more. Molliviruses have spherical particles ranging from 500 to 600 nm (51, 52).

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Figure 3 (*Figure appears on preceding page*)

Evolution and classification of giant amoeba viruses. (*a*) Maximum likelihood phylogenetic tree of *Nucleocytoviricota* based on the DNA polymerase B family gene. Sequences in amino acid were aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) software. The tree was built using FastTree with the Jones-Taylor-Thornton (JTT) model and 1,000 bootstrap replicates and then visualized in Interactive Tree Of Life (iToL). The *Mimiviridae* clade is shown in the light gray box. (*b*) Schematic representation of the classification and evolution of giant viruses based on the International Committee on Taxonomy of Viruses–approved megataxonomy. A putative evolutionary relationship between *Nucleocytoviricota* and *Tectiviridae* is represented by dotted lines.

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Pandoraviruses, orpheovirus, pithoviruses, and cedratviruses have ovoidal shaped particles that frequently present over 1,000 nm in length (20, 21, 45, 61). Different formats of particles can be observed among representatives of the family *Mimiviridae*. Their capsids have a pseudo/quasi-icosahedral symmetry and are often covered by fibrils, ranging from 450 to 527 nm in representatives of the genus *Mimivirus*, making the total particle size range between 700 and 834 nm in diameter (88). Although tupanviruses' capsids are similar to mimiviruses', these viruses' particles have a complex symmetry in which the capsid is followed by a cylindrical tail that varies in size in the viral progeny (22).

Furthermore, it is possible to observe the delivery portal in those larger particles—a differentiated area of the capsid through which the internal content of the particle is released. The delivery portal in molliviruses is a circular depression 160–200 nm in diameter (51). For pandoraviruses and orpheovirus, the internal content is released through an ostiole-like structure located on the apex of the particle (49, 92). In pithoviruses and cedratviruses, the delivery portal is sealed by a structure named cork. While pithoviruses present a single cork on the apex of the particle (21), cedratviruses present two corks—one at each end of the particle—but the internal content is delivered just from one (91). Capsids within family *Mimiviridae* members present a fivefold vertex called stargate that is sealed by a proteinaceous complex, the starfish, which collectively prevents the capsid from opening (93). The processes that trigger the opening of the capsids are described in the section titled Replication Cycle. The delivery portal is usually not covered by fibrils, whether they are short (molliviruses and orpheovirus) or long (family *Mimiviridae*) (51, 92, 94).

Proteomic analysis of the purified viral particles showed that the majority of amoebal viruses' particles are composed of a large number of proteins ranging from 26 to over 200 proteins, thus forming a mature particle of different groups (62, 95). Studies have shown that a smaller number of proteins are detected in the mature viral particle when compared to the diversity of proteins present in the viral factory. Moreover, the number of proteins is not associated with the particle or genome size (21, 22, 95). In contrast to icosahedral viruses, the MCP of marseilleviruses is not the most abundant protein in the mature viral particle (95). Even so, proteomic analyses reveal that most of the proteins that form amoebal viruses' particles still have no known function or orthologs (54, 62, 95, 96). Nevertheless, other methodologies have helped to elucidate some components of viral particles. A recent study demonstrated that the *Pandoravirus massiliensis* capsid is composed of a peripheral layer of polysaccharide nature that resembles cellulose (97). Interestingly, *P. massiliensis* particles also present membrane potential (proton gradient). This result, in association with bioinformatics data, suggests that the *P. massiliensis* genome has distant homolog genes predicted to encode tricarboxylic acid elements. This raises questions about energy generation associated with pandoravirus particles during replication (71).

Replication Cycle

Many viruses within the family *Mimiviridae* present fibrils on the surface of the capsid (11, 22, 94, 98). In APMV, fibrils play a role in particle attachment on the amoeba's surfaces (99). But the fibrils do not seem to be essential for viral replication, since the virus loses many fibrils when passaged

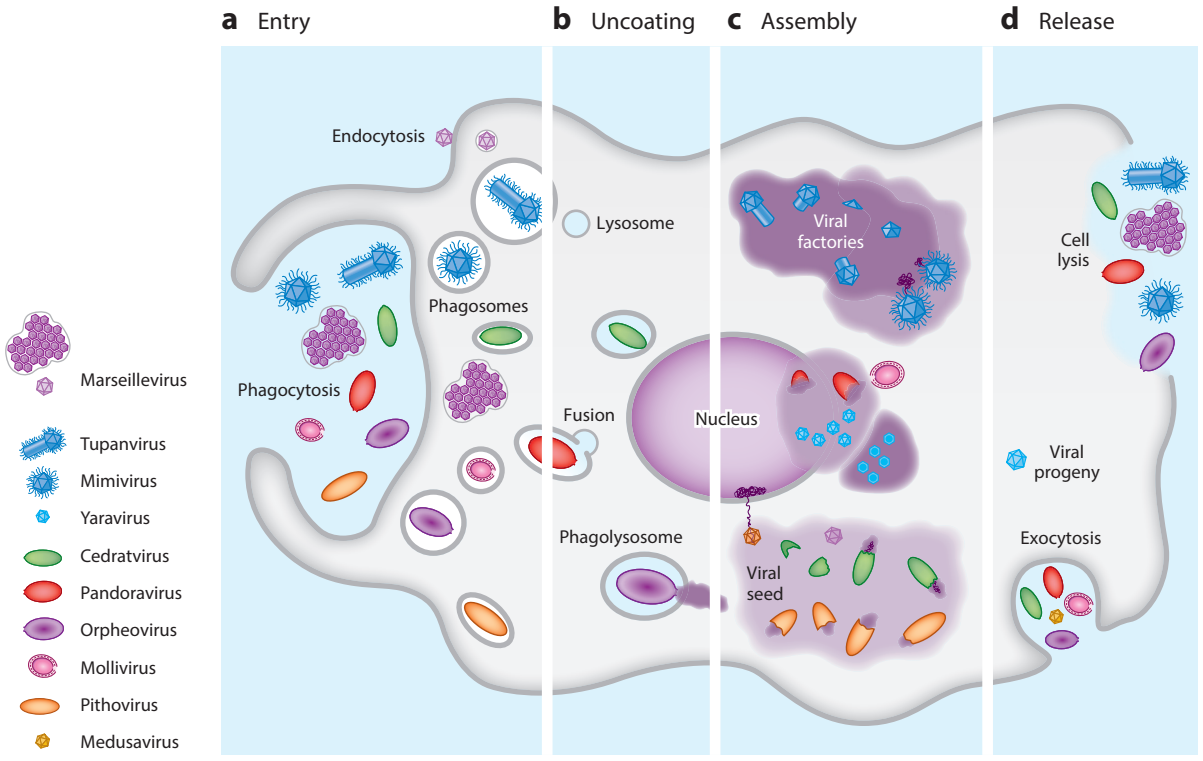


Figure 4

Representative scheme of the amoeba viruses' replication cycle. (*a*) Giant virus entry can occur through the phagocytic pathway or endocytic pathway. (*b*) The drop in pH due to fusion with the lysosome triggers the release of the viral seed. (*c*) Establishment of the viral factories where the assembly of the viral progeny takes place. (*d*) Viral release can occur via exocytosis, cell lysis, or both.

multiple times in cell culture (100). Other giant viruses also have fibrils and, although shorter, they can also play a similar role in viral particle adhesion: the ~12-nm fibrils of marseilleviruses, the 50- to 100-nm fibrils of molliviruses, the ~50-nm fibrils of orpheovirus, and amoeba klosneuvirus fibrils (15, 51, 60, 87, 92). Aside from fibrils, medusavirus presents flexible spherical-headed spikes of ~14 nm that also might be involved with viral attachment and entry (54).

The size of the particle is a determinant element for the initial steps of the replication cycle. It was experimentally demonstrated that latex beads over 500 nm trigger phagocytosis in *Acanthamoeba* cells (101). Many giant viruses have dimensions greater than 500 nm, and it has been frequently hypothesized that they are consequently phagocyted (61, 92, 102) (**Figure 4*a***). In fact, being able to explore the phagocytic pathway as an entry route seems so advantageous that strategies to bypass the minimum size requirement were selected in the smaller giant viruses. This is the case of 250-nm viruses within the *Marseilleviridae* family. By the end of their replication cycle, the viral progeny can be grouped together, thus forming viral aggregates inside or outside vesicles that can reach more than 1,000 nm and are successfully phagocyted. In addition to the phagocytic pathways, individual particles can enter the host cell through endocytosis (103) (**Figure 4*a***). The endocytic pathway might be a route for other amoebal viruses that do not fulfill the size requirement as suggested for yaravirus (62). Another entry route for these viruses can be macropinocytosis (103).

Once inside the host cell, the particle uncoating generally occurs during the endosomal or phagosomal escape. Some redox proteins have been described as components of amoebal virus

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virions and may interact with oxidative elements inside amoebal endosomes and phagosomes. An inner membrane has been described inside most giant virus capsid/tegument shells. This membrane is essential during virus uncoating because it fuses with the cell compartment membrane (endosome/phagosome). Such fusion occurs through the delivery portal whose opening is triggered by a drop in pH. This is likely due to fusion with lysosomes during the natural course of infection (94). Low pH alone was insufficient to fully open samba virus and tupanvirus capsids in in vitro studies, thus indicating that other interactions are required for the complete release of the internal contents (93) (**Figure 4*b***).

After the internal content/viral seed is released inside the host cell, an intense rearrangement occurs until the formation of a mature viral factory. In most giant viruses, this formation occurs near to the amoeba's nucleus, but the nucleus is no longer observed after the formation of the mature viral factory of pandoraviruses, molliviruses, yaraviruses, and clandestinovirus (51, 59, 62, 89). Some viral factories have specialized areas, which are important for particle morphogenesis. The periphery of mimiviruses' electron-dense viral factory is electron lucent and has an apparent fibrillar nature. When the newly formed capsids pass through this region, they acquire long polysaccharidal fibrils. Therefore, this part of the viral factory was named the fibril acquisition area (94). Furthermore, the electron-lucent viral factory of yaravirus presents an electron-dense periphery. When the newly formed empty capsids migrate to this part of the viral factory, they are filled with their internal content (62) (**Figure 4*b*,*c***).

For most amoebal viruses, DNA replication occurs inside the viral factory, except for medusaviruses, whose DNA replicates in the periphery of the host nucleus, and the particle morphogenesis occurs in the cytoplasmatic viral factory (54). Along with medusavirus and the viruses that form their viral factories in the cell nucleus, other viruses, such as marseilleviruses, also depend on host nuclear functions for their replication. Marseilleviruses replicate in the cytoplasm, but they transiently recruit the nuclear transcription machinery to their cytoplasmic viral factory to generate their earliest transcripts (95) (**Figure 4*c***).

The particle morphogenesis and the internal content of marseilleviruses, molliviruses, pandoraviruses, and pithoviruses were described to occur simultaneously (15, 21, 51, 89), but these can also occur separately. Most giant viruses begin particle morphogenesis with the formation of semicircular structures, named crescents. These are usually filled by the internal content as they expand (62, 91, 92, 94, 104). For mimiviruses, the capsid assembly starts with the formation of the stargate, and genome acquisition then occurs through the opposite vertex concomitantly with fibril acquisition (94) (**Figure 4*c***).

Almost every infection culminates in cellular lysis at the end of the replication cycle, but yaravirus, pandoraviruses, orpheovirus, and cedratviruses start releasing their particles before the cell lysis by exocytosis (62, 89, 91, 92). Only clandestinovirus and molliviruses seem to not cause significant cell lysis (51, 59) (**Figure 4*d***).

VIRUS-HOST INTERACTION

Virus-Amoeba Interaction

To provide a good understanding of the relationships surrounding a virus and its host, studies generally focus on key elements regarding the contagium of that cell. During infection by giant viruses, a series of structural and morphological changes occur in the amoebal cells as part of the natural outcome of most viral infections (**Figure 5**). These changes, also called cytopathic effects (CPEs), are generally the consequence of an efficient viral replication and have been extremely important to help identify novel isolates of giant viruses (105). Each cell may develop different CPEs depending on which virus is currently replicating. For most infections involving amoebae and giant

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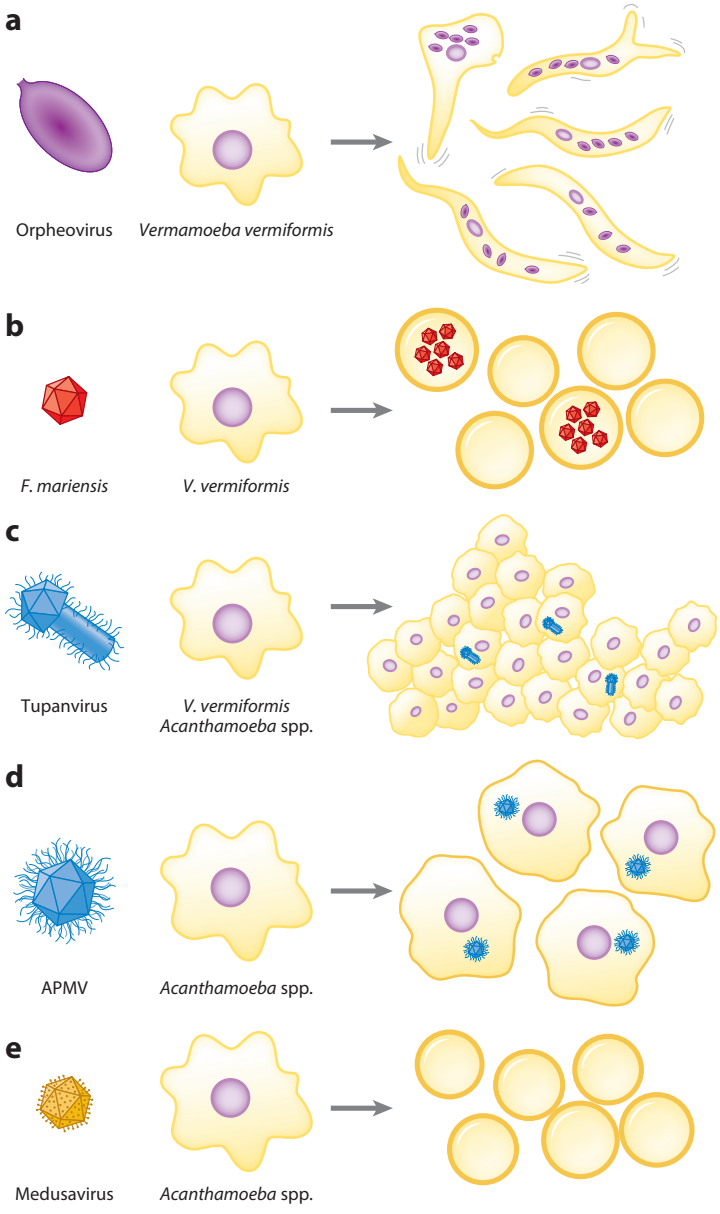


Figure 5

Representative scheme of the CPEs. (*a*) Orpheovirus causes cells to shift to a stretched fusiform shape during the early stages of infection. (*b*) *Faustovirus mariensis*–infected trophozoites release a soluble factor that promotes the encystment of infected and uninfected cells. (*c*) Tupanvirus's infected cells form bunches with uninfected cells. (*d*) APMV decreases the levels of serine-proteinase transcription and translation of infected cells, thus preventing encystation. (*e*) Medusavirus stimulates an encystment-like process. Abbreviations: APMV, Acanthamoeba polyphaga mimivirus; CPE, cytopathic effect.

viruses, the standard observed CPE involves progressive cell rounding and loss of the trophozoite motility and lysis with subsequent release of the viral progeny from inside the cell (106). Amoebal viruses found in the above-described CPEs are mimiviruses, marseilleviruses, pandoraviruses, cedratviruses, faustoviruses, yasminevirus, and yaravirus (19, 34, 49, 60, 62, 91, 106, 107).

Different sets of CPEs may be observed in a different context of infection by other groups of amoebal viruses (**Figure 5**). For example, in addition to promoting rounding of *A. castellanii* cells, infection by medusaviruses was described as stimulating an encystment-like process of a subpopulation of amoebal cells as early as 2 days post infection (54) (**Figure 5*e***). A similar effect was also observed for *Vermamoeba vermiformis* cells when infected by the giant *Faustovirus mariensis* (104). A detailed description of the replication cycle of orpheovirus showed the occurrence of different steps of CPEs before amoebal cells lyse (92). During the Souza et al. study (92), the authors found that *V. vermiformis* amoebae shift to a stretched fusiform shape as early as 3 hpi. The effect becomes more obvious as the time of infection progresses. At 12 hpi, the morphology evolves to a branched fusiform shape. Later, around 24 hpi, amoebae start to emerge as rounded cells until they are lysed by the virus (92) (**Figure 5*a***).

In another study, an interesting biological feature that links the successful replicative cycle of tupanvirus to the CPEs that are established during infection of *A. castellanii* cells was described (108). When amoebae are infected by tupanviruses, they progressively develop some of the standard effects described above for most of the infections with giant viruses. First, the cell becomes round. Then the massive viral production forces lead to amoebal lysis. However, when these amoebal cells are just beginning to become round, several neighboring uninfected cells are induced to aggregate to them and form bunches with other cells that were already infected (108). This behavior was very important during infection, thus establishing an advantage for viral dissemination. Such behavior has also been linked to relevant changes in the expression of mannose-binding protein genes by tupanvirus (108) (**Figure 5*c***). Interestingly, a similar CPE has also been described for viruses belonging to a marseillevirus lineage (109).

As known hosts of giant viruses, amoebae of the *Acanthamoeba* and *Vermamoeba* genera may also play important roles in many of the biological and evolutionary aspects of the giant viruses mentioned above. Both amoebae are considered to be free-living organisms and are encountered in a diversity of environments with a worldwide distribution (110, 111). Amoebae's life cycle proceeds through two stages: The first is called the trophozoite and is a form that is generally observed under favorable growth conditions (abundant food supply, appropriate temperature, neutral pH) (110, 111). During this stage, amoebae are motile and can perform most of their life tasks including feeding and reproducing (110, 111). The second stage, best known as cysts, is a natural form of resistance against adverse/stressful conditions. In contrast to trophozoites, the cysts are static (110–112).

Since the discovery of the first amoebal giant viruses, only a few studies have investigated aspects of viral infection in a context that considers both cellular stages. One of the first studies regarding this subject explored some specific kinds of interactions among the APMV and amoebae of the *Acanthamoeba* spp. in the framework of the encystment stimulation commonly observed in the natural environment. *Acanthamoeba* cysts are resistant to APMV infection (113). Interestingly, this resistance was maintained as long as the stimulus for cell encystment was triggered before the viral infection, even if the amoebae had not yet suffered massive turnover of the cellular components necessary to become a cyst (113). However, the protection is severely nullified when the mimivirus gets to the cell before the stimulus of encystation. The virus can then infect the amoeba (113). Strategies for viral evasion of this antiviral mechanism were also uncovered. Prior work found a serine proteinase that is a crucial component in mediating *A. castellanii* cells to encyst (114). Mimivirus decreases the levels of amoebal serine-proteinase transcription and translation,

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thus preventing *Acanthamoeba* encystations (113) (**Figure 5*d***). Finally, a novel antiviral mechanism involving *F. mariensis* and its amoebal host, *Vermamoeba vermiformis*, was described (104).

Giant viruses invade amoebal trophozoites and use their metabolic machinery to produce the viral progeny and induce cell lysis to disseminate the progeny. However, upon *F. mariensis* infection, *V. vermiformis* trophozoites release a soluble factor (likely containing Mg^{2+}). This soluble factor induces encystment of neighboring cells to enclose faustovirus progeny inside the newly formed cysts. These cysts are no long viable and therefore reduce the viral progeny in the supernatant (104) (**Figure 5*b***).

Virophages

The discovery in 2008 of satellite-like viruses, named Sputnik virophages, was exciting in the biology of giant amoeba virus. Virophages are large-viruses dependent or associated viruses classified in the family *Lavidaviridae*. They have 50- to 70-nm icosahedral capsids and double-stranded DNA genomes ranging from 17 to 30 kb, and they are dependent on giant viruses to complete their replication in host cells. The fundamental basis of the virophage/giant virus relationship still requires further study, but virophages might exploit the giant viruses' transcriptional machinery especially during the late phase of giant virus replication. Consequently, virophages might impact, to different degrees, the production of giant viruses' progeny. Virophages have a structural module (MCP with a double–jelly roll structure), thus suggesting a common origin among those viral entities (7, 115–118). Although virophages were first described in association with amoebal mimiviruses, a few years later, a novel virophage named mavirus was described in association with Cafeteria roenbergensis virus (CrOV) infecting the flagellate *Cafeteria roenbergensis*. Later metagenomics studies revealed that virophages are widespread especially in aquatic environments (116–119).

Comprehensive studies have demonstrated that the virophage-giant virus-protist tripartite relationship is complex and ancient. Remarkably, virophages seem to integrate into either giant viruses or protist genomes (provirophages). In one notable example, the virophage mavirus could integrate into the chromosome of the flagellate *C. roenbergensis* (120). Virophage genes are expressed upon CrOV infection, thus resulting in production of mavirus virions. Such newly formed virophage progeny promote the overall protection of the flagellate *C. roenbergensis* population. In contrast, giant viruses also developed mechanisms to mitigate virophage impacts on their replication. A defense system against a specific group of virophages (group named Zamilon) has been described in mimiviruses lineage A. The mechanisms of the so-called mimivirus virophage resistance element system need further investigation, but it was hypothesized that it acts analogously to the CRISPR-Cas system and is guided by the *R349* gene containing 15- or 28-nucleotide Zamilon-like repeats (121). Therefore, the discovery of virophages has fueled debates regarding the nature of nested parasitism and the definition of satellite viruses.

FINAL CONSIDERATIONS

Throughout history, the study of viruses has provided unique insights to understand complex events of nature. This has helped to develop biology and anthropology (4). The discovery of giant viruses broke the mold of classical virology and demonstrated that even the most accepted definitions within basic science are not immutable. These new findings open the field to even more exciting discoveries in the future (6). New advances in viral prospection and isolation are needed to explore this new field of study. In addition to changes in isolation techniques, the use of other amoebae as prospecting platforms has contributed to the discovery of new viruses. This has led to a broader view of the virosphere (34). Even with the diversity of amoeba viruses already isolated,

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some viral groups still have only one representative isolated (59, 61, 62). Metagenomic studies have indicated that giant viruses are diverse and ubiquitous on Earth (8). Indeed, different representatives of giant viruses have been isolated among the most diverse samples worldwide, but some of these viruses have such singular genomes that isolation and characterization studies are critical to identify these organisms. It could not be done solely by metagenomics (62). Therefore, further prospecting and characterization studies are essential to understand the virosphere.

Although APMV was found in 1992, giant viruses that infect amoebae remained hidden in plain sight for more than 10 years without being identified as viruses (6). One of the main reasons why this organism was not considered to be a virus was its large size, which contradicted the definition of viruses at the time (6). In fact, giant viruses' particles are so unique and diverse that some reported finding a noncultivable endocytobiont even after the description of APMV as a virus (29, 122, 123).

A universe of viral diversity was revealed through prospective and characterization studies of giant viruses. This work significantly contributed to our biological understanding of these unique viruses and the virosphere. Nevertheless, much remains to be elucidated about giant viruses, and studies regarding these viruses remain an important field of research to understand possible ecological roles, the host spectrum and evolutionary aspects of this group, and the biological diversity of viruses.

DISCLOSURE STATEMENT

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