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Giant Viruses of Amoebae: A Journey Through Innovative Research and Paradigm Changes

Philippe Colson, Bernard La Scola, and Didier Raoult

Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), Aix
Marseille Université, UM63, CNRS 7278, IRD 198, INSERM 1095, Institut
Hospitalo-Universitaire (IHU) Méditerranée Infection, Assistance Publique-Hôpitaux de
Marseille (AP-HM), 13005 Marseille, France; email: didier.raoult@gmail.com

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giant virus, mimivirus, Megavirales, virophage, amoeba

Abstract

Giant viruses of amoebae were discovered serendipitously in 2003; they are visible via optical microscopy, making them bona fide microbes. Their lifestyle, structure, and genomes break the mold of classical viruses. Giant viruses of amoebae are complex microorganisms. Their genomes harbor between 444 and 2,544 genes, including many that are unique to viruses, and encode translation components; their virions contain >100 proteins as well as mRNAs. Mimiviruses have a specific mobilome, including virophages, provirophages, and transpovirons, and can resist virophages through a system known as MIMIVIRE (mimivirus virophage resistance element). Giant viruses of amoebae bring upheaval to the definition of viruses and tend to separate the current virosphere into two categories: very simple viruses and viruses with complexity similar to that of other microbes. This new paradigm is propitious for enhanced detection and characterization of giant viruses of amoebae, and a particular focus on their role in humans is warranted.

INTRODUCTION

It has been 60 years since Andre Lwoff (1) launched the concept of virus and about twice that time since Ivanovsky (2), Beijerinck (3), and Loeffler & Frosch (4) pointed out that infectious agents other than microbes may exist. These infectious entities were primarily deemed to be of a new kind, as they appeared to be anomalies with respect to existing knowledge about microbes. Indeed, they escaped direct examination by light microscope; were not retained by Chamberland filters, which are impervious to microbes; and were not cultivable on inert nutrient media (1, 5). Since then and until the early twenty-first century, any observation of viruses by light microscopy was considered to be an anomaly, and this paradigm delayed the discovery of the first giant viruses of amoebae (6, 7). The identification of *Acanthamoeba polyphaga mimivirus* (commonly referred to simply as Mimivirus) and other subsequently discovered giant viruses of amoebae brought upheaval to previous concepts regarding the definition and evolution of viruses (8–13). It also led to the description of three new virus families and the delineation of several additional putative virus families. We review here how the giant virus journey has been marked by innovative research with new technologies and tools as well as by changes in existing paradigms (**Figure 1**).

THE DISCOVERY OF MIMIVIRUS: USING *ACANTHAMOEBA* SPP. TO ISOLATE NEW PATHOGENS

From the outset, *Acanthamoeba* spp. have been the platform for isolation of giant viruses of amoebae (6). This culture strategy was implemented and described as the amoebal enrichment method by T. Rowbotham (14), who used it during the investigation of a pneumonia outbreak in an English hospital with the aim of isolating new amoeba-resistant microorganisms, primarily *Legionella* spp. and *Legionella*-like bacteria (14). This approach was more fruitful than expected, as it allowed the isolation of Mimivirus. For more than a decade after it was first isolated, Mimivirus was considered to be a bacterium because it was visible by light microscopy. It had a coccus morphology, revealed by a positive Gram stain (6). However, it grew only on *Acanthamoeba* spp. and could not be identified. Subsequent attempts to identify this microbe used molecular biology to amplify 16S ribosomal DNA, a tool that has revolutionized bacteriology since the 1990s and had been implemented precociously in our laboratory. However, polymerase chain reaction (PCR) amplification could not be obtained with any of various protocols. This was unexpected, and the next step in the search for clues about the identity of this microbe was to use electron microscopy, which showed giant icosahedral virions (**Figure 2a**). A similar story happened with pandoraviruses, other giant viruses of amoebae, which were considered to be amoebal eukaryotic parasites before genome sequencing revealed their viral nature (7, 15). This finding demonstrated that viruses as large as microbes but devoid of ribosomal DNA exist. Thus, the isolation of Mimivirus relied on a new tool and gave a serendipitous result (16).

Since then, giant viruses of amoebae have been hunted by multiple teams (**Table 1, Figure 3**). Tools and strategies used to isolate new giant viruses of amoebae have been improved during the past 14 years by miniaturization, sensitization, and adaptation to highly motile and nonadherent protozoa (17). Initially fastidious, particularly because of bacterial contamination, the coculture strategy was enhanced through the use of various mixtures of antibiotics and antifungals, including amphotericin B or thiabendazole and then voriconazole; pre-enrichment procedures with nutrients; and dark incubation of amoebae or sample resuspension in Prescott and James medium (15, 18–23). Thereafter, high-throughput isolation was implemented by observing lysis of amoebae first deposited on agar plates (24), then in liquid media with automated detection based on assessment of the size and DNA content of giant viral particles stained with SYBR Green (25).

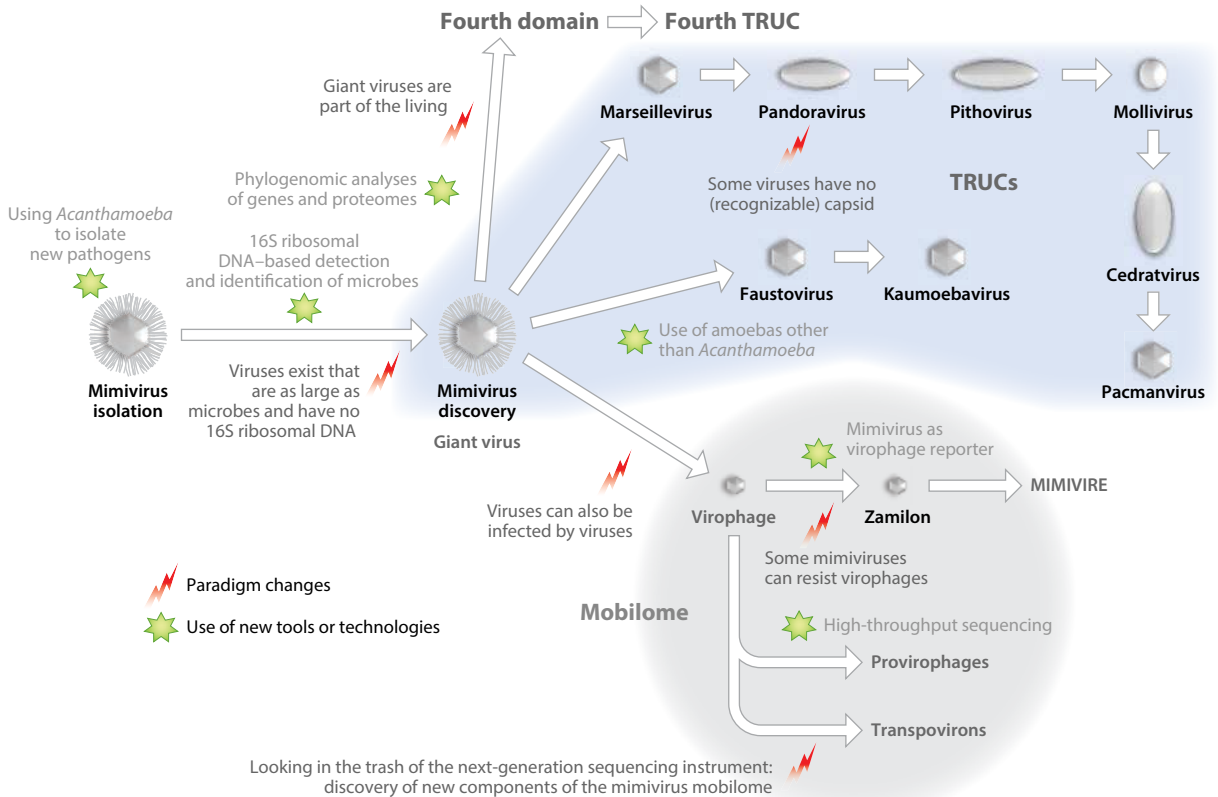


Figure 1

The long journey of research on giant viruses of amoebae through the perspective of innovation and paradigm changes. The path has been marked by discoveries related to innovative research, the study of previously unexplored fields, and changes in existing paradigms. Work on the giant virus Mimivirus led to the discovery of new biological entities (blue labels), including the first known virus that infects viruses, and to the description of three new virus families and representatives of several additional putative virus families. In addition, the study of Mimivirus led investigators to coin several concepts and terms, such as virophage, provirophage, transpoviron, fourth domain, TRUC (things resisting uncompleted classification), and MIMIVIRE (mimivirus virophage resistance element). The steps in the discovery of Mimivirus, other giant viruses of amoebae, and the mimivirus mobilome illustrate the preponderant role of new technologies and paradigm changes in research advances. The blue shaded background area encompasses giant viruses of amoebae that have been named TRUCs, and the gray shaded background area encompasses virophages and transposons that are associated with mimiviruses and are described to be major components of the giant viruses' mobilome.

PHYLOGENOMIC ANALYSES OF GENES AND PROTEOMES

One year after the discovery of Mimivirus, its genome was described (9). The genome is unexpectedly large (1.18 Mbp) and contains a surprisingly large number of predicted genes (more than 900). This gene repertoire contains several genes unique among viruses, including nine that encode proteins homologous to proteins of the translation apparatus (9). Moreover, four main groups of genes can be delineated: core genes, genes transferred horizontally, duplicated genes, and orphan open reading frames (ORFans). Core genes are those shared with nucleocytoplasmic large DNA viruses (NCLDVs), smaller double-stranded DNA viruses that had previously been the largest known viruses and include asfarviruses, poxviruses, iridoviruses, ascoviruses, and phycodnaviruses (26, 27) (Figure 4). A total of 42 core genes were described, 9 of which are concurrently present

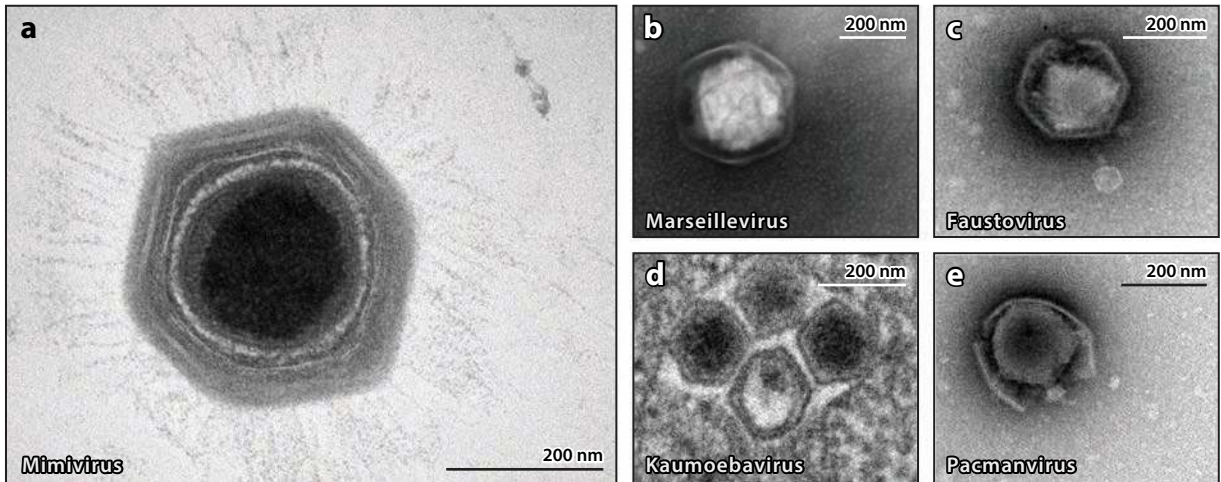


Figure 2

Electron micrographs of giant virions with icosahedral capsids: (a) Mimivirus, (b) Marseillevirus, (c) Faustovirus, (d) Kaumoebavirus, (e) and Pacmanvirus.

in the genomes of Mimivirus and all the NCLDVs. Duplicated genes represent one-quarter to one-third of the Mimivirus genome, according to the threshold used to consider genes as paralogs (28), indicating their considerable involvement in the genome-shaping process. They include several genes encoding proteins that might be involved in virus-host interactions and may interfere with host processes such as transcription, protein degradation, and cellular regulatory mechanisms. The largest paralogous gene family corresponds to ankyrin repeat-containing proteins. Gene sequences transferred horizontally also represent a substantial proportion of Mimivirus genes, around 10% (9, 29), and include sequences found in bacteria, eukaryotes, archaea, and other viruses; estimates of their proportions vary according to differing analyses and interpretations. Aside from their number, the sense of these transfers (i.e., whether Mimivirus is the donor or the acceptor) has been much debated for some genes (9, 29–32). The last major group of genes is the ORFans, which are genes without homologs in sequence databases. These genes, which represent new putative functions, accounted for almost one-half of the gene content of Mimivirus (48%). The proportion of these ORFans among the 114 proteins identified in the virion was in the same order of magnitude (40%) (33), which highlights the fact that many of the structural and functional components of Mimivirus remain unknown.

MARSEILLEVIRUSES

Six years after the Mimivirus report, a giant virus of amoebae was described that differs from Mimivirus strains; it was named Marseillevirus (34) (**Figure 2b**). Like Mimivirus, Marseillevirus was isolated from water collected in a cooling tower, this time in Paris. It is smaller than Mimivirus but is also visible by electron microscopy. Its genome is a double-stranded circular DNA. Its gene content (457 genes) largely differs from that of Mimivirus, with 20 genes encoding proteins containing membrane occupation and recognition nexus (MORN) repeat domains and 2 encoding histone-like proteins. The Marseillevirus genome also harbors numerous paralogous genes, ORFans, genes laterally transferred, and core genes shared by Mimivirus and the NCLDVs. The preponderance of genes laterally transferred suggests many exchanges of sequences with

Table 1 Main characteristics of isolates of viruses of amoebae whose annotated genomes are available

Family/ taxon	Sublineage	Name	Virion		Genome			
			Size (nm)	Morphology	Genome size (kbp) ^a	Gene number	GC%	NCBI GenBank, EMBL, or ENA accession number
<i>Mimiviridae</i>	A	<i>Acanthamoeba polyphaga mimivirus</i>	~500 (~750 ^b)	Icosahedral	1,182	979	28.0	NC_014649
		Acanthamoeba polyphaga mamavirus	~500	Icosahedral	1,192	1,023	—	JF801956
		Hirudovirus	~410 (~630 ^b)	Icosahedral	1,155	998	28.0	KF493731
		Samba virus	~527 (~834 ^b)	Icosahedral	1,181	971	28.0	KF959826
		Niemeyer virus	~463 (~616 ^b)	Icosahedral	1,299	1,033	28.0	KT599914
		Fauteuil virus	—	Icosahedral	1,181	—	—	LN871163
		Amazonia virus	—	Icosahedral	1,180	979	27.9	LN867402
		Oyster virus	—	Icosahedral	1,200	948	27.9	KM982401
		Kroon virus	—	Icosahedral	1,222	944	27.5	KM982402
		Lentille virus	—	Icosahedral	—	807	—	AFYC01000001–10
		Mimivirus Bombay	435	Icosahedral	1,182	898	28.0	KU761889
		Mimivirus shirakomae	—	Icosahedral	1,183	986	—	AP017645
		Mimivirus kasaii	—	Icosahedral	1,183	988	—	AP017644
	B	<i>Acanthamoeba polyphaga moumouvirus</i>	~420	Icosahedral	1,021	930	24.6	NC_020104
		Moumouvirus Monve	—	Icosahedral	—	—	—	JN885994-JN886001
		Saudi moumouvirus	~500	Icosahedral	1,030	953	25.8	KY110734
		Moumouvirus goulette	—	Icosahedral	1,017	970	—	KC008572
	C	<i>Megavirus chilensis</i>	~590	Icosahedral	1,259	1,123	25.2	NC_016072
		LBA111 virus	~554	Icosahedral	1,231	1,178	—	NC_020232
		Courdo 11 virus	~450	Icosahedral	1,246	1,166	—	JX975216
		Courdo 7 virus	—	Icosahedral	—	—	—	JN885990-JN885993
Courdo 5 virus		—	Icosahedral	—	—	—	LN868540	
Powai lake megavirus isolate 1 virus		425	Icosahedral	1,209	996	25.0	KU877344	
<i>Marseilleviridae</i>	A	<i>Marseillevirus marseillevirus T19</i>	~250	Icosahedral	368	457	44.7	NC_013756
		Cannes 8 virus	~250	Icosahedral	374	483	44.6	KF261120
		<i>Senegalvirus marseillevirus</i>	~250	Icosahedral	373	479	—	JF909596–602
		Melbournevirus	~250	Icosahedral	369	403	44.7	KM275475
	B	<i>Lausannevirus</i>	~250	Icosahedral	347	444	42.9	NC_015326
		Port-Miou virus	—	Icosahedral	349	410	—	KT428292
	C	<i>Tunisvirus</i>	~250	Icosahedral	380	484	43.0	KF483846
		Insectomime virus	225	Icosahedral	386	477	42.7	KF527888
		Tokyovirus A1	~200	Icosahedral	373	387	—	NC_030230
	D	Brazilian marseillevirus	~250	Icosahedral	362	491	43.3	NC_029692
	E	Golden marseillevirus	~200	Icosahedral	361	483	43.1	NC_031465
	Unclassified	Noumeavirus	200	Icosahedral	376	452	—	NC_033775
	Unclassified	Kurlavirus BKC-1	—	Icosahedral	361	386	—	KY073338
	Pandora-viruses	—	<i>Pandoravirus salinus</i>	~1,000 × 500	Ovoid	2,474	2,544	61.7
Pandoravirus dulcis			~1,000 × 500	Ovoid	1,909	1,488	63.7	NC_021858
Pandoravirus inopinatum			—	Ovoid	2,243	1,902	60.7	NC_026440

(Continued)

Table 1 (Continued)

Family/ taxon	Sublineage	Name	Virion		Genome			NCBI GenBank, EMBL, or ENA accession number
			Size (nm)	Morphology	Genome size (kbp) ^a	Gene number	GC%	
Pithoviruses	—	Pithovirus sibericum	~1,500 × 500	Ovoid	610	467	35.8	NC_023423
		Pithovirus massiliensis	1,200–1,600 × 500	Ovoid	683	520	35.4	LT161893
Faustoviruses	M	Faustovirus E12	~200	Icosahedral	466	457	36.2	KJ614390
		Faustovirus E23	~200	Icosahedral	466	519	36.2	KU702952
		Faustovirus E24	~200	Icosahedral	466	518	36.2	KU702948
		Faustovirus D5a	~200	Icosahedral	466	517	36.2	KU702950
	D	Faustovirus D3	~200	Icosahedral	456	495	37.8	KU556803
		Faustovirus D5b	~200	Icosahedral	465	507	37.7	KU702949
		Faustovirus D6	~200	Icosahedral	462	509	37.7	KU702951
	L	Faustovirus Liban	~200	Icosahedral	471	518	36.7	—
	E9	Faustovirus E9	~200	Icosahedral	491	511	39.6	—
	Molliviruses	—	Mollivirus sibericum	~500–600	Spherical	652	523	60.1
Kaumoebaviruses	—	Kaumoebavirus Sc	~250	Icosahedral	351	465	43.7	KX552040
Cedratviruses	—	Cedratvirus A11	~1,200 × 500	Ovoid	589	574	42.6	NC_032108
	—	Cedratvirus lausannensis CRIB-75	750–1,000 × 400–600	Ovoid	575	643	42.8	PRJEB18669
Pacmanviruses	—	Pacmanvirus A23	175	Icosahedral	395	465	33.6	NC_034383
Virophages	—	Sputnik 1 virophage	~50	Icosahedral	18	21	27.0	NC_011132
		Sputnik 2 virophage	~50	Icosahedral	18	21	27.0	JN603369
		Sputnik 3 virophage	~50	Icosahedral	18	21	27.0	JN603370
		Rio Negro virophage	~35	Icosahedral	—	—	—	KJ183141
		Zamilon virophage	50–60	Icosahedral	17	20	29.7	NC_022990

The prototype virus of each family or putative family is indicated by bold type. A dash indicates information is not available. Abbreviations: EMBL, European Molecular Biology Laboratory; ENA, European Nucleotide Archive; NCBI, National Center for Biotechnology Information.

^aAll genomes are double-stranded DNA.

^bIncluding fibers.

eukaryotes, including *Acanthamoeba* spp., bacteria, archaea, and viruses, including giant ones. Furthermore, it highlights that coinfection of amoebae by sympatric microorganisms that survive and replicate in these phagocytic protists was likely involved in the generation of such genomes with high levels of mosaicism (34, 36). In 2012, it was proposed that mimiviruses, marseilleviruses, and NCLDV be classified in a new viral order, Megavirales, as these viruses have a common origin and virion architecture and share major biological characteristics, such as replication within viral factories (37, 38).

PANDORAVIRUSES

Four years after the Marseillevirus report, two additional giant viruses of amoebae were described; they were named Pandoravirus salinus and Pandoravirus dulcis (15). Meanwhile, new mimiviruses and marseilleviruses were isolated from environmental samples collected worldwide (20). As was the case for mimiviruses, pandoraviruses were considered for years to be nonviral parasites of *Acanthamoeba* spp. because their large size was not compatible with the existing concept of a virus

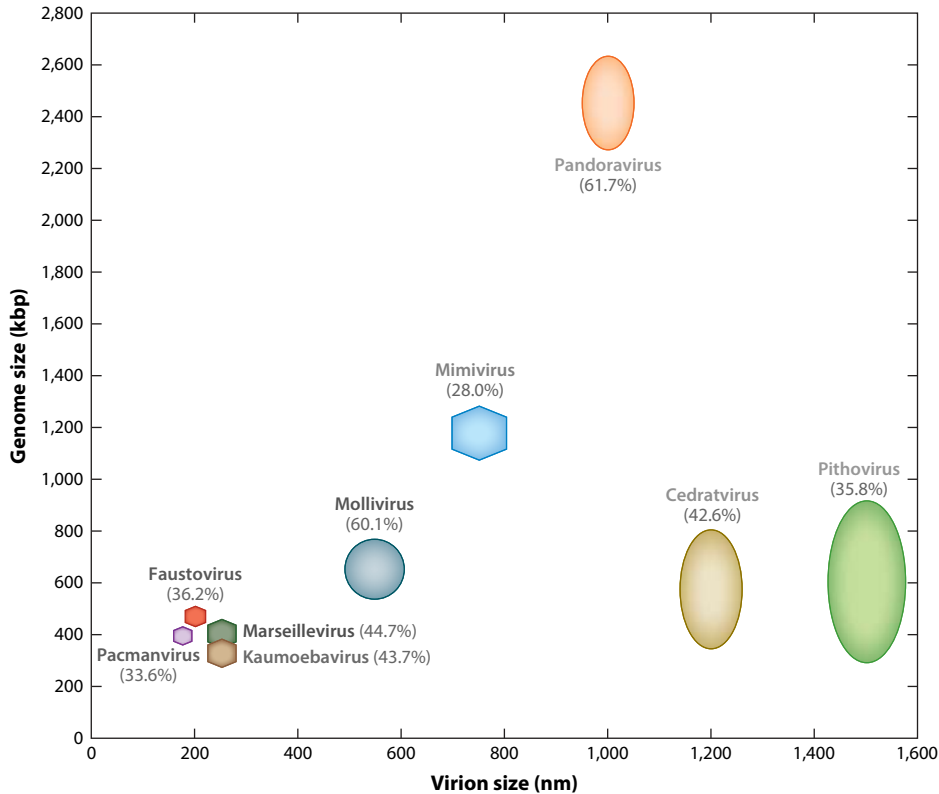
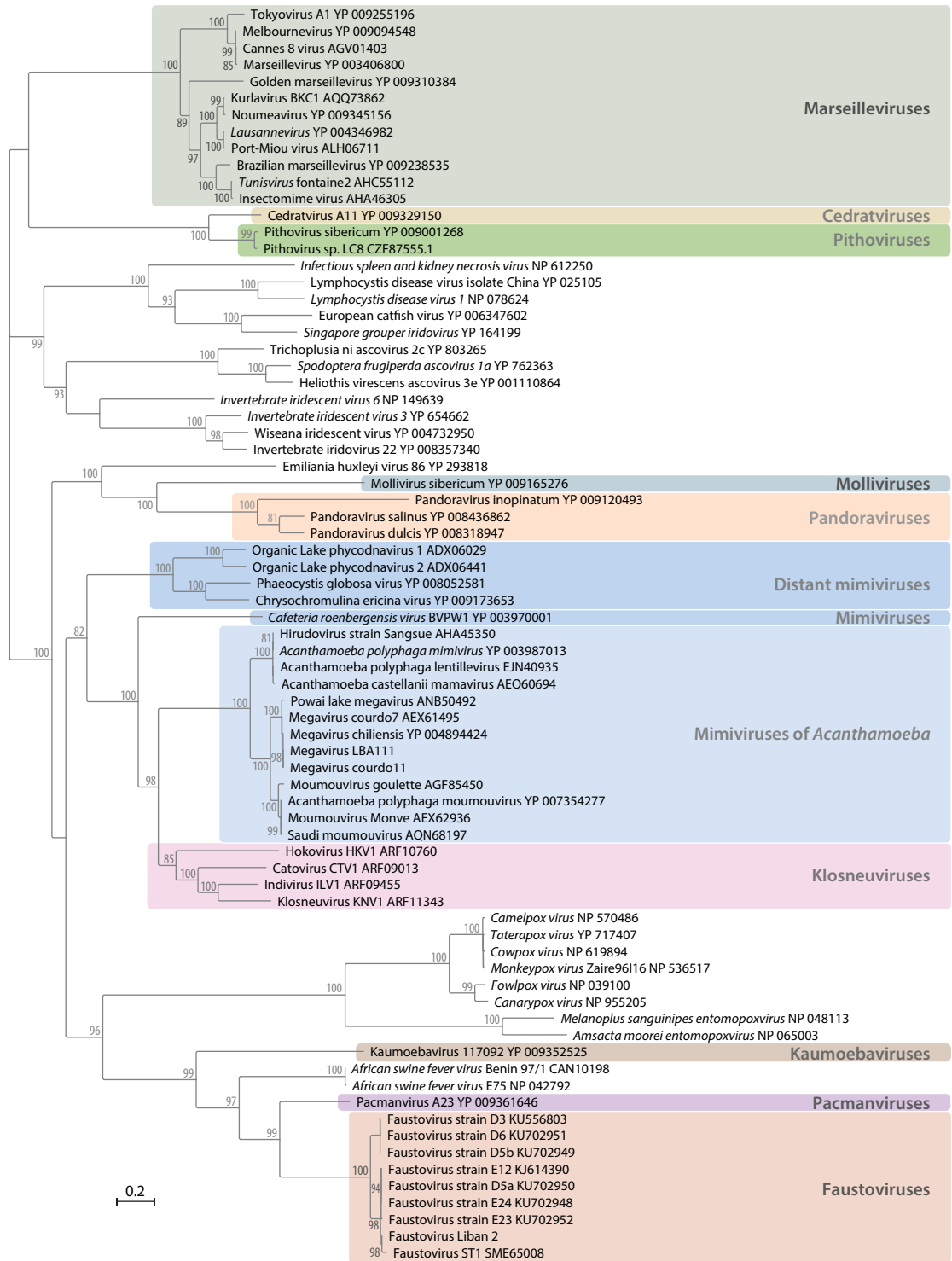


Figure 3

Size distributions of genomes and virions for giant viruses of amoebae. Values are for the prototype virus of each family or putative lineage. The G+C content is indicated as a percentage after the name of each giant viral family or putative lineage.

(7, 39, 40). Pandoraviruses have still larger virions than mimiviruses (~1 μm in length and 0.5 μm in diameter) (**Figure 5a**), and their genomes range in size between 1.9 and 2.5 Mbp (15). These genomes also contain a tremendous proportion of ORFans, 84% for *Pandoravirus salinus*. Among genes with homologs in the National Center for Biotechnology Information sequence database, more than half encode proteins with ankyrin, MORN, and F-box motifs, which comprise large families of paralogs. The *Pandoravirus salinus* genome harbors peculiar transposons named MITEs for miniature inverted repeat transposable elements (41). However, the most significant features of pandoraviruses are the absence of genes homologous to any known capsid gene (15, 42) and the concurrent absence of a structure resembling a capsid, even a complex one. This challenges the definition of viruses (12, 43) and even one of the criteria of the proposed order Megavirales (38). Other giant viruses have unusual capsids. These include ascoviruses with allantoid capsids and poxviruses with brick-shaped virions (38). But the case of pandoraviruses stands apart due to the complete absence of a genetically or morphologically recognizable capsid (15, 42). Nevertheless, a candidate gene was recently proposed to encode a pandoravirus capsid on the basis of both amino acid and nucleotide sequence similarity (44). Besides, pandoraviruses have other viral features, including replication with an eclipse phase, and they lack major hallmarks of cellular organisms such as ribosomal components and enzymes involved in cell division (15). Pandoravirus virions



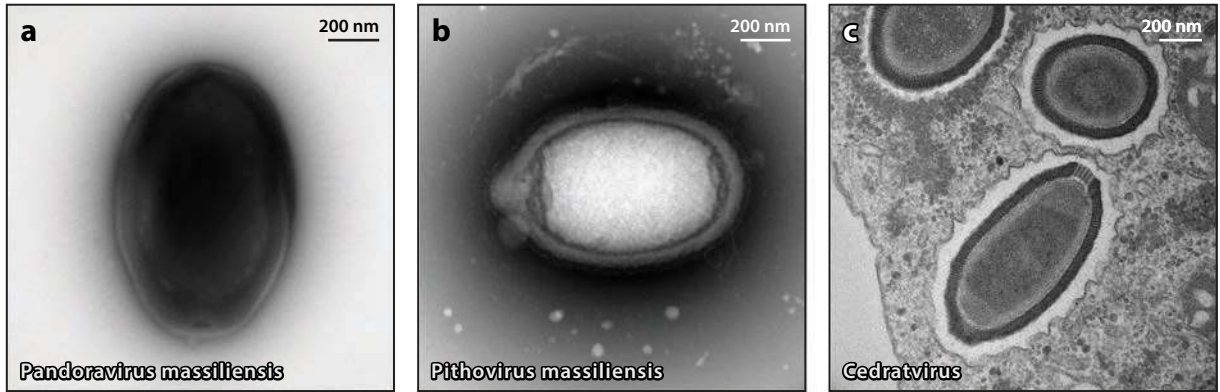


Figure 5

Electron micrographs of giant virions with an ovoid shape and a tegument-like envelope: (a) *Pandoravirus massiliensis*, (b) *Pithovirus massiliensis*, and (c) *Cedratvirus*.

are surrounded by a tegument-like structure that is ~ 70 nm thick. A pore at the virion apex allows the delivery of internal components to the amoebal cytoplasm.

PITHOVIRUSES

Expansion of the diversity of giant viruses of amoebae has accelerated since 2013. Shortly after the discovery of pandoraviruses, *Pithovirus sibericum* was isolated from a $>30,000$ -year-old Siberian permafrost sample inoculated on *Acanthamoeba castellanii* (23). Its morphology resembles that of pandoraviruses, and it has a still longer virion (~ 1.5 μm). As is the case for pandoraviruses, its virions are surrounded by a tegument that is ~ 60 nm thick; the apical pore has the aspect of a honeycomb grid. No capsid-like structure is recognizable, but unlike in the case of pandoraviruses, one gene is related, although very distantly, to an iridovirus capsid gene. Its gene content is most closely related to those of marseilleviruses and iridoviruses. More than one-fifth of the genome is composed of multiple regularly interspersed copies of a noncoding repeat with lower G+C content than coding regions and consisting of 2-kbp-long tandem arrays of conserved 150-bp-long palindromic motifs. The isolation of *Pithovirus sibericum* was claimed to be the revival of an old virus that may no longer exist. However, another isolate, *Pithovirus massiliensis* (**Figure 5b**), was obtained in 2016 from sewage in Southeastern France, and it was genetically highly similar to its Siberian counterpart (45). The most striking feature in pithoviruses is the large discrepancy between the virion size and the genome size (610 kbp for *Pithovirus sibericum*), which is about half that of *Mimivirus* (23).

Figure 4

Phylogenetic tree based on amino acid sequences of the DNA-dependent RNA polymerase subunit 1 for giant viruses of amoebae and other members of the proposed order Megavirales. Phylogeny reconstruction was performed using the maximum-likelihood method with the FastTree program (34) and was visualized with the MEGA6 software (<http://www.megasoftware.net/>). The scale bar represents the number of estimated changes per position. Bootstrap values are shown when $>80\%$.

MOLLIVIRUSES

Mollivirus sibericum is another giant virus of amoebae that replicates in *A. castellanii*; it was described in 2014 (46). This virus was also retrieved from Siberian permafrost, from the same sample as Pithovirus sibericum. The spherical virion is 500–600 nm in diameter and shelters a 652-kbp genome. Phylogenomics indicates that this virus is most closely related to pandoraviruses, albeit distantly. Viral progeny seem to emerge at the periphery of the amoebal nucleus during the replication cycle, which is not followed by lysis of infected amoebae. Numerous amoebal proteins are packaged in the virions, including ribosomal proteins.

FAUSTOVIRUSES

Adding a new amoeba, *Vermamoeba vermiformis*—a predominant amoeba in human stools (47) and in hospital water samples (48)—into the culture support panel allowed isolation of a previously unknown icosahedral giant virus, named Faustovirus (**Figure 2c**), from sewage samples (19). Other isolates were thereafter obtained from *V. vermiformis*, in all cases from sewage samples collected in Southern France, Senegal, or Lebanon (49). This exclusive association with sewage suggests that faustoviruses may be indicators of fecal contamination. Among the giant viruses of amoebae and previously described NCLDV, faustoviruses are the most closely related to asfarviruses, which are pig pathogens (19, 49). However, their genomes are approximately three times larger than those of asfarviruses. Faustoviruses have 456–491-kbp genomes that contain 457–519 predicted genes (49). While annotating the Faustovirus genome, Reteno et al. (19) were surprised by the fact that capsid-encoding sequences were scattered along a 17,000-kbp region. This led them to suspect that substantial splicing of the gene encoding the major capsid protein occurs. Although splicing was first observed in adenoviruses (50) and was previously demonstrated for the Mimivirus capsid gene (51), it is mostly restricted to eukaryotic genes; however, it might have an astonishing extent in the case of Faustovirus capsids. In addition, Faustovirus was found to use two protein shells to encapsidate (52).

KAUMOEBAVIRUSES

The use of *V. vermiformis* as culture support subsequently allowed isolation of another new giant virus, only distantly related to those previously characterized, named Kaumobavirus (53) (**Figure 2d**). As was the case for faustoviruses, Kaumobavirus was obtained from a sewage sample, and comparative genomics and phylogenetic analyses showed that it is distantly related to faustoviruses and asfarviruses. The capsid-encoding region appears to be intermediate between that in faustoviruses and that in asfarviruses, as it spans 5 kbp. Besides, Kaumobavirus has an icosahedral capsid and a genome size most similar to those described for marseilleviruses.

CEDRATVIRUSES

Cedratvirus strain A11 (**Figure 5c**), described in 2016, is a distant relative of pithoviruses, although only one-fifth of its genes are involved in best reciprocal hits with pithovirus genes (54). It was isolated from environmental water collected in Algeria, by culturing on *A. castellanii*. One of the most remarkable features of Cedratvirus is its double-cork structure. An ~40-nm-thick tegument can be seen during early steps of infection; in mature virions, the tegument is ~55 nm thick. Apical pores with a grid shape appear smaller than those in pithoviruses. The genome size is comparable to those of pithoviruses. A close relative to Cedratvirus A11 isolated from a drinking water plant

was described in 2017 (55). The genomes of cedratviruses do not harbor the noncoding repeats that abound in the Pithovirus sibericum genome (46).

PACMANVIRUSES

Pacmanvirus was described in 2017 (56) (**Figure 2e**). It was isolated from an environmental sample of Algeria inoculated on *Acanthamoeba castellanii*. Pacmanvirus owes its name to a broken aspect of its capsid that can be seen by electron microscopy with negative staining. Viral replication is very fast, as amoebal burst and complete amoebal lysis occur at 6 and 8 h postinfection, respectively. The sizes of the virion and of the DNA genome are in the same order of magnitude as those of Kaumoebavirus and faustoviruses. According to phylogenomic analyses, Pacmanvirus is most closely related to faustoviruses, asfarviruses, and Kaumoebavirus.

THE EXPANDING DIVERSITY OF GIANT VIRUSES OF AMOEBAE

Multiplying protozoal supports adapted to liquid medium for high-throughput isolation was confirmed as a fruitful approach to isolate new kinds of giant viruses of amoebae that would have remained unknown if a more conservative strategy was used. It has become clear that giant viruses of amoebae are common in environmental water and soil worldwide. They have been isolated from samples collected on five continents by several teams (15, 20, 53, 57). In addition, sequences highly similar to those of giant viruses of amoebae have been detected in metagenomes generated from environmental samples (58–62). Recently, a 1.6-Mbp genome from a putative giant virus, named Klosneuvirus and most closely related to mimiviruses, was discovered in metagenomes from a wastewater treatment plant in Austria (63). This allowed the subsequent discovery, by screening 7,000 environmental metagenomes, of three additional 0.9- to 1.5-Mbp genomes from putative mimivirus relatives, named Indivirus, Hokovirus, and Catovirus.

However, giant viruses of amoebae were likely overlooked until the discovery of Mimivirus and for years thereafter. This oversight was due to the paradigm existing at that time that viruses were necessarily small and capable of passing through filters whose pore size prevents the passage of bacteria (64, 65). Such procedures led investigators to neglect giant viruses of amoebae. Indeed, they are absent from the filtered fraction comprising classical viruses due to their virion size. Although they are present in the unfiltered fraction comprising cellular organisms, notably bacteria, they cannot be detected by PCR amplification or ultradeep sequencing of 16S ribosomal DNA because they lack ribosomal components. Thus, the virus paradigm that existed at the time when Mimivirus was isolated slowed its discovery, and then continued to impede giant virus detection for some time despite the advent of dramatically powerful technologies such as next-generation sequencing. It is likely that the diversity of giant viruses of amoebae is still largely untapped. A recently implemented new tool consists of flow cytometry that allows sorting of viable giant viruses from supernatants, including from viral mixtures (25, 66). This will allow isolation of slow-growing viruses even when they are associated with fast-growing ones, as was demonstrated with a mixture of Cedratvirus and Mimivirus (66).

VIROPHAGES

From the very onset of virology, viruses were identified as pathogens, or potential pathogens: first for plants, nonhuman animals, and humans, since the 1890s (2–4, 67); then for bacteria, since 1915; and eventually for archaea, since 1974 (68–70). Hence, viruses were known to infect cellular organisms of the three domains of life described by Woese & Fox (71). One of the upheavals

brought by giant viruses of amoebae was the realization that viruses can also be infected by other, smaller viruses (72). This was revealed at the time of the isolation of a new mimivirus strain, named Mamavirus. Abnormal morphologies of Mamavirus were observed, including accumulation of external shells at one side of or around the virions, as well as opened virions. Concurrently, a small icosahedral virus, approximately 50 nm in diameter, was observed in the giant virus factory and the culture supernatant. Further analyses showed that this virus was unable to multiply in the absence of the mimivirus and that its replication in the giant virus factory was deleterious to that of the mimivirus and was associated with the production of defective and abnormal giant virions. By analogy with bacteriophages, this virus infecting a mimivirus was named a virophage. Virophages have 35–74-nm icosahedral capsids and 17–29-kbp double-stranded DNA genomes that harbor 16–34 genes, a few of them homologous to giant virus genes (73). Like giant viruses of amoebae, virophages have been isolated or detected by metagenomics in various locations and habitats worldwide, mostly in environmental water.

Next-generation sequencing, which developed concurrently with the discoveries of giant viruses of amoebae, allowed the timely characterization of giant viral genomes and gene repertoires. This technology produces a wealth of sequences, but some of them are automatically discarded by bioinformatics software because they are redundant or, in the case of genome assembly by a mapping strategy, because they are unmapped on a reference genome. Looking in the trash of genome assembly software while analyzing the genomes of new mimiviruses allowed investigators to detect sequences with a large number of copies that corresponded to short regions of mimivirus genomes and increased by up to 10 times the excess of sequence coverage (74). These observations led to the realization that virophages may integrate into mimivirus genomes as provirophages and to the discovery of a new type of transposon, named the transpoviron. Further analyses detected different transpovirons associated with amoebal mimiviruses of different lineages. They are linear DNA elements of approximately 7 kbp that harbor six to eight genes, including two genes shared with virophages. They replicate inside mimiviral factories and accumulate in the amoebal cytoplasm and in giant virus and virophage particles. Both virophage DNA and transpovirons can integrate at various locations in the genomes of mimiviruses, and they therefore represent a specific mobilome, alongside self-splicing introns previously detected in giant virus genes.

The first three isolated virophages associated with mimiviruses of *Acanthamoeba* spp. were Sputnik strains and were found to replicate with mimiviruses of the three described lineages—A, B, and C—indicating a broad host range (75). This led to the implementation of a new tool to detect and isolate new virophages. This tool is based on a reporter system that allows the isolation and discovery of new virophages by a coculture procedure using *Acanthamoeba polyphaga* and mimiviruses, without the isolation of their giant mimiviral host (75). However, when applied to the Zamilon virophage (76), which is closely related to but divergent from Sputnik virophages, this system produced an unexpected result. Lineage A mimiviruses, but not lineage B or C mimiviruses, were resistant to Zamilon. To try to elucidate the mechanisms of this resistance, investigators searched for Zamilon short sequences in mimivirus A genomes. This was done by analogy with the defense strategy that consists of the integration of sequences from genetic or viral invaders (77). Such a strategy is widespread among cellular organisms and is used in the CRISPR-Cas systems of bacteria and archaea (78). Insertions of four 15-nucleotide-long repeated sequences of the Zamilon virophage were found in the genome of resistant mimivirus A isolates, within an operon that was named MIMIVIRE, for mimivirus virophage resistance element (77). In contrast, such short sequences were absent from the genomes of susceptible isolates of lineages B and C. The exact molecular mechanism of MIMIVIRE resistance is currently unclear, but it is suspected to rely on sequence-specific recognition of a nucleic acid. The MIMIVIRE-associated genes include

genes that encode a helicase and a nuclease whose functions were validated experimentally, as well as a gene that contains the repeated insert. Restoration of susceptibility to the Zamilon virophage in lineage A mimiviruses was observed by independently silencing these three genes with RNA interference. An alternative mechanism has been hypothesized to explain mimivirus A resistance to Zamilon that would involve protein-protein interactions and a proteic restriction factor of the virophage replication machinery (79).

PECULIAR GENETIC AND STRUCTURAL FEATURES OF GIANT VIRUSES OF AMOEBAE

Giant viruses of amoebae have numerous peculiar genetic and structural characteristics. With regard to their genetic features, the double-stranded DNA genomes of these viruses notably harbor considerable proportions of ORFans, which ranged between 31% of genes for Cedratvirus (54) and 84% for Pandoravirus salinus (15) at the time of their descriptions. This indicates numerous new putative functions and structures encoded by these genes. The genomes of giant viruses also contain introns and inteins (80), as well as peculiar transposons, including transpovirons in mimivirus genomes (74) and MITEs in the Pandoravirus salinus genome (15, 41). Multiple regularly interspersed copies of a noncoding repeat are present in Pithovirus sibericum. Early and late promoters and polyadenylated transcripts with hairpin-like structures were predicted in Mimivirus (81, 82). The most emblematic of the giant viral genes that are absent from other viruses are those encoding translation components (80, 83, 84). They include aminoacyl-tRNA synthetases, various translation-associated factors, and tRNAs. Such genes are absent only from Pithovirus sibericum (23). tRNA-encoding genes are absent from marseilleviruses (85), pithoviruses (23), faustoviruses (49), Kaumoebavirus (53), and cedratviruses (54, 55). Giant viruses of amoebae also display several unique structural features. Mimivirus virions are surrounded by a layer of fibers, the structure of which has been only partially deciphered (84, 86). These fibers have different lengths and were not observed in all mimiviruses (86). Short fibers were also observed on Marseillevirus virions (35). Giant viruses of amoebae can also harbor pores at the capsid vertices, or at the particle apex for viruses with an ovoid or spherical shape and a tegument-resembling envelope (80). These pores allow the release of the internal contents of the virions, including the genomic DNA, into the amoebal cytoplasm. In mimiviruses, this pore is called a stargate, because of the five-branch starfish-shaped structure that covers it until its aperture (87). Mimivirus DNA is packaged in neosynthesized virions by another portal. In giant viruses of amoebae with icosahedral capsids, the major capsid protein has a double jelly-roll fold (87). Faustovirus virions harbor two shells: The outer shell consists of a double jelly-roll protein, whereas the inner shell is composed of a structure that differs from all other known capsid proteins (52). In pandoraviruses (7, 15), pithoviruses (23, 45), and cedratviruses (54, 55), the virion is surrounded by a thick tegument whose nature is currently unknown. For these viruses, the content of the internal viral core is released through a portal with a cork aspect. Cedratviruses can have a double cork (54, 55).

INFECTION OF AMOEBAE BY GIANT VIRUSES AND HOST-VIRUS INTERACTIONS

Giant viruses of amoebae have been shown to infect amoebae of the genus *Acanthamoeba* (Figure 6). However, it is currently unknown whether there are other hosts for these viruses. *Acanthamoeba* spp. are phagocytic protists that are among the predominant organisms in soil and water (88). These free-living amoebae can ingest any particle with a size greater than 0.5 μm (36). They are known to graze on multiple organisms and microorganisms, including bacteria, yeasts, fungi,

Acanthamoeba castellanii

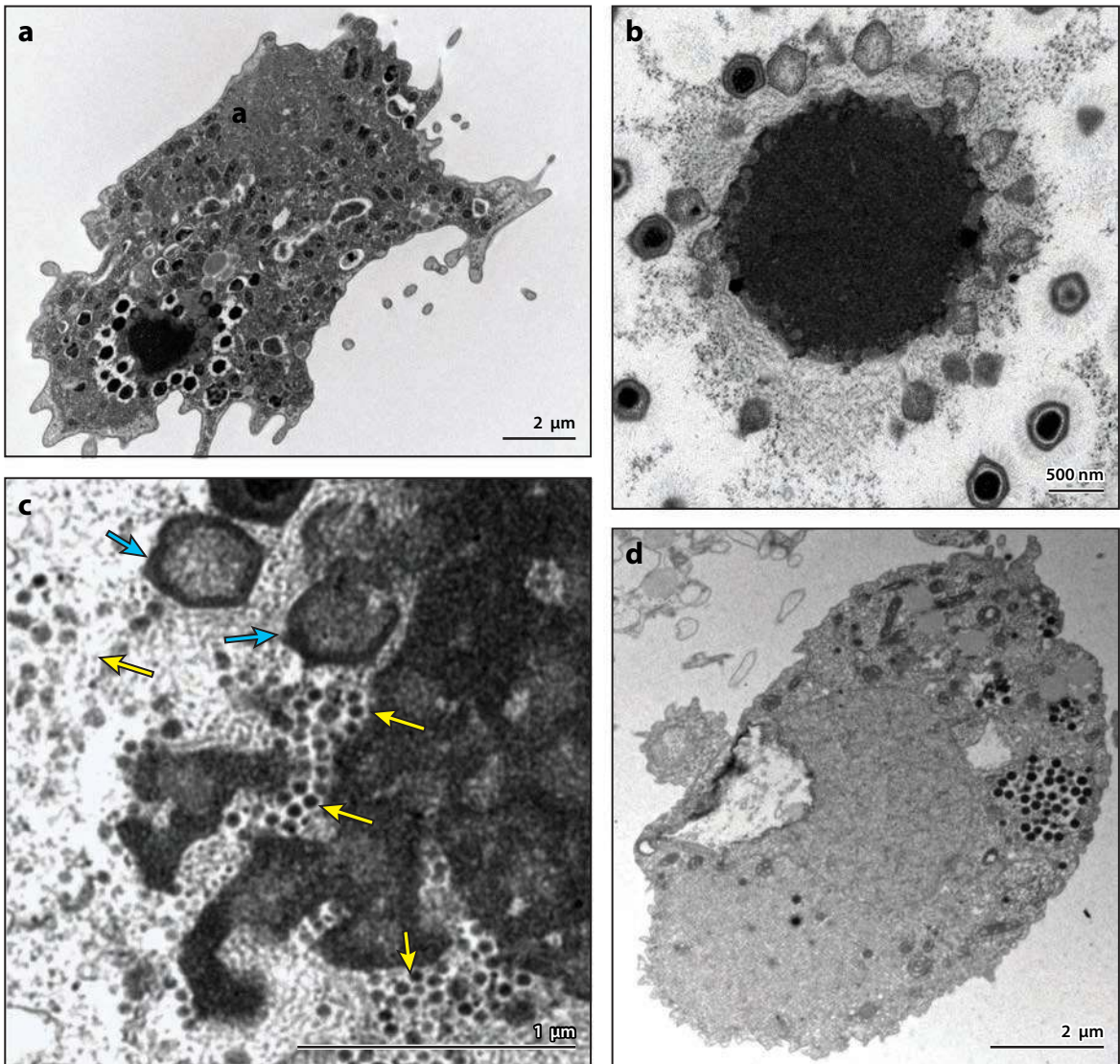


Figure 6

Electron micrographs of *Acanthamoeba castellanii* infected with giant viruses and of giant viral factories. (a) *A. castellanii* infected with a mimivirus. (b) A mimivirus factory in the cytoplasm of *A. castellanii*. (c) The external border of a mimivirus factory in the cytoplasm of *A. castellanii*, which shows the presence of mimivirus virions (blue arrows) as well as virophages (yellow arrows). (d) *A. castellanii* infected with Marseillevirus.

viruses, and algae, and they therefore engulf large amounts of foreign DNA (88, 89). Some of these microorganisms, among which are giant viruses, are capable of surviving and multiplying in *Acanthamoeba* spp., which in such cases serve as replicative niches as well as reservoirs, armor, and vectors for so-called amoeba-resisting microorganisms (88–90). The association of giant viruses with amoebae is likely one of the reasons for their common detection in environmental samples.

Using amoebae other than *Acanthamoeba* spp. as culture support enabled the isolation of new, divergent giant viruses. *Acanthamoeba* spp. have been and remain to date the main culture support to isolate giant viruses of amoebae. *A. polyphaga* exclusively enabled isolation of mimiviruses and marseilleviruses. In contrast, *A. castellanii* enabled isolation of pandoraviruses, Pithovirus sibericum, and Mollivirus sibericum (15, 23, 46). Adding *A. castellanii* to *A. polyphaga* as culture support quickly broadened the diversity of giant viral isolates at the laboratory scale, yielding for the first time a pandoravirus and a pithovirus (45, 57). This indicated that *A. castellanii* was more permissive than *A. polyphaga* to these giant viruses of amoebae. It was further demonstrated that a given sample can or cannot induce amoebal lysis according to the *Acanthamoeba* species it is inoculated into (57). A still more obvious example of the interest in using new amoebae to isolate new viruses is the introduction of *V. vermiformis* into the culture support panel, which allowed rapid isolation of faustoviruses and Kaumoebavirus. Moreover, distantly related mimiviruses, including *Cafeteria roenbergensis virus*, Phaeocystis globosa virus, and Chrysochromulina ericina virus, infect marine flagellates and microalgae that are widespread in water worldwide (91). Attempts to infect a wide range of cells other than amoebae with mimiviruses and other giant viruses of amoebae have generally failed (16, 55). However, there are hints, and some evidence, that the cellular tropism of these viruses is broader than currently known. Mimivirus was experimentally shown to enter professional phagocytes, including myeloid cells from humans (monocytes, monocyte-derived macrophages, and myelomonocytic cells) and mice, and productive infection of mouse macrophages with Mimivirus was reported (92). Mimivirus was also demonstrated to replicate in human peripheral blood mononuclear cells and to induce type I interferon production and inhibit the expression of interferon-stimulated genes in these cells (93). In addition, a marseillevirus was shown to enter immortalized human T lymphocyte cells (94), and marseilleviruses were detected in lymph node macrophages by immunohistochemistry, immunofluorescence, and fluorescence in situ hybridization (FISH) (94, 95).

The replicative cycle of giant viruses in their amoebal hosts lasts between 6 and 24 h (**Figure 7**). Entry occurs through phagocytosis and possibly, for marseilleviruses, through giant vesicles and endosome-stimulated pathways (96). Then, the DNA genome is released in the amoebal cytoplasm from the internal viral core, which precedes the eclipse phase (80, 97). Thereafter, DNA replication begins in nascent virus factories. The nucleus most often exhibits morphological changes during the course of giant virus replication, except in the case of infection with Pithovirus sibericum. In addition, nuclear membrane invaginations can be seen with pandoraviruses and Mollivirus sibericum. During Mollivirus sibericum replication, the virus factory incorporates the altered nucleus. The virion assembly step also differs among giant viruses. In the case of mimiviruses, virion internal membrane biogenesis and assembly, capsid assembly, DNA packaging, and fiber layer assembly occur successively from the inside to the outside of the virus factory. For pandoraviruses, pithoviruses, and Mollivirus sibericum, the envelope and interior of the virion are assembled simultaneously (97). The release of neosynthesized giant viruses occurs through amoebal lysis in all cases except for Mollivirus sibericum, for which this event seems to involve exocytosis without cell lysis. It is worth noting that the virus factory, which is a replication organelle where viral and cellular components are recruited, was assimilated to the nucleus of a cell infected by viruses, itself called the virocell (29).

The large gene armamentarium of giant viruses of amoebae, and particularly the presence of genes involved in the transcription and translation apparatus, suggests relative independence of these viruses from their hosts with regard to replication. However, different patterns exist for different giant viruses. Notably, pandoraviruses, Mollivirus sibericum, and the marseillevirus Noumeavirus were described to be devoid of transcription-associated proteins, and it has been suggested that this conditions the implication of the amoebal nucleus in viral replication (97, 98).

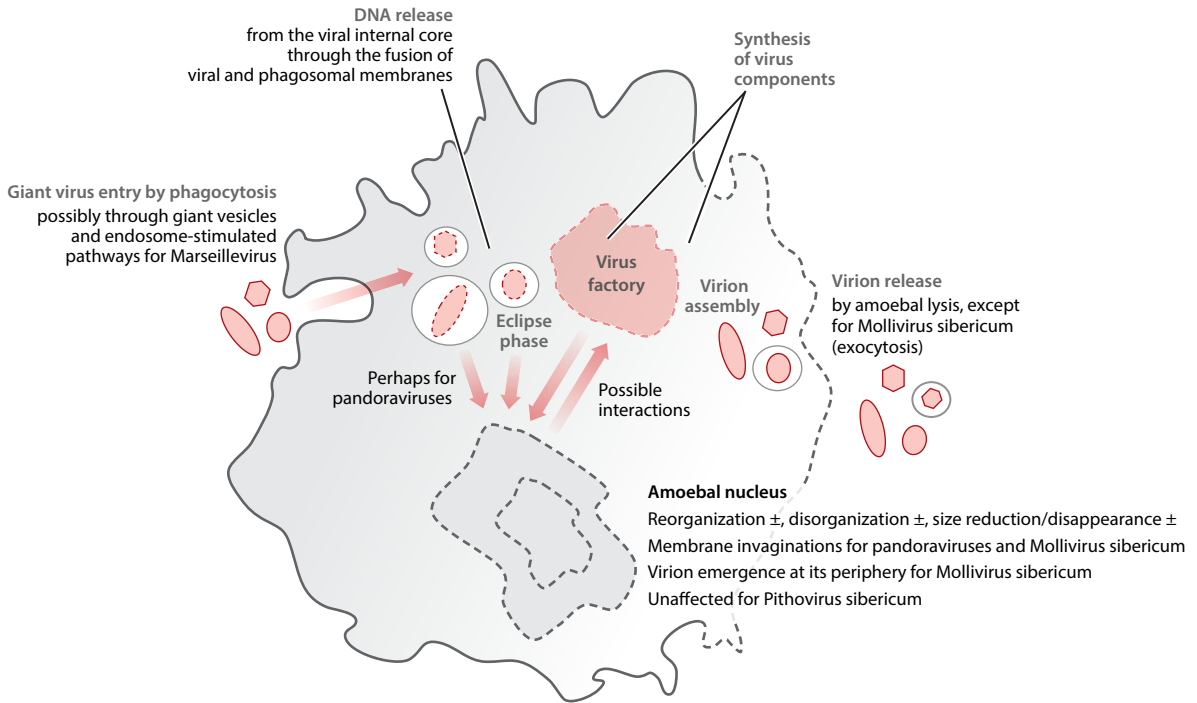


Figure 7

Schematic depiction of the main steps of the replicative cycle of giant viruses in amoebae.

In the case of Noumeavirus, transcription is initiated in the virus factory, which may occur through transient recruitment of the nuclear transcription machinery (98).

THE NEED FOR NEW APPROACHES TO DEFINE AND CLASSIFY GIANT VIRUSES

The names and definitions of the main groups of infectious agents have been greatly influenced over time by the tools and technologies available for their characterization. The term microbes was coined in 1878 by C.E. Sédillot (99) to designate living entities that could be seen only by using a light microscope (100). In 1925, E. Chatton proposed that the absence or presence of a nucleus was a morphological feature that could be used to distinguish between microbes that were named prokaryotes and eukaryotes, respectively (101). During the 1970s, Woese & Fox (71) used ribosomal RNA sequences to propose three major domains of cellular organisms: eukaryotes, archaea, and bacteria. Virus research began in the late nineteenth century, a few decades after it had been established that living microbes were the cause of infectious diseases, and originated from observations that were anomalous with respect to existing knowledge on microbes. Unlike microbes, viruses were invisible, ultrafilterable, and uncultivable on inert standard culture media and were initially characterized only by their pathogenicity (102). The modern concept of the virus was unraveled during the 1950s by A. Lwoff and colleagues (1, 103, 104), who described viruses as simple entities composed of a single type of nucleic acid enclosed in a symmetric proteic shell, the capsid, that strictly rely on cellular hosts to replicate and are devoid of components of the translation apparatus and of enzymes for energy production.

According to many criteria, giant viruses of amoebae more closely resemble intracellular bacteria than they do classical viruses. They represent anomalies with respect to the virus paradigm established at the onset of virology, which includes invisibility by light microscopy and ultrafilterability. Giant virions are visible by light microscopy because their diameter exceeds 200 nm (6, 105). At the time of the discovery of Mimivirus, this had been the case only for rare viruses, such as the poxviruses that Buist (106) observed in 1887 as minute bodies in lymph from vaccinia and smallpox vesicles and deemed to be spores. In addition, giant virions are not ultrafilterable; they are retained by filters with pore sizes of 0.20–0.45 μm (64). The size of their DNA genomes is larger than 300 kbp and can exceed 2 Mbp (80). Moreover, giant viruses of amoebae are complex microorganisms in terms of their genomic and protein contents. Their genomes harbor between 444 and 2,544 predicted genes, including many that are absent from any other viral genomes, and encode translation components. The recent description of four new genomes of giant viruses related to mimiviruses that were assembled from environmental metagenomes expands the set of translation system components present in giant viruses (63). The Klosneuvirus genome encodes 25 tRNAs and 40 proteins related to translation, including 19 aminoacyl-tRNA synthetases. Giant virions produced in amoebae contain mRNA and more than 100 proteins (80). Based on current knowledge, other features that characterize only mimiviruses are the presence of a specific mobilome (74), susceptibility to virophages, and the MIMIVIRE defense mechanism against those virophages (77). Furthermore, giant viruses of amoebae, together with NCLDV (27), comprise a monophyletic group with an ancient origin (107, 108). In addition, comparison of the genomes of a fossil pithovirus and a modern pithovirus suggested that the mechanisms of evolution and the mutation rate in giant viruses are similar to those in bacteria (45). Indeed, a high level of conservation and selective pressure on conserved genes, over a period of thousands of years, were observed. This comparative analysis also showed that giant viral genes inferred as horizontally transferred had been selected and then conserved and adapted to viral codon usage. High selective pressure was also observed for ORFans, suggesting they have biological relevance. Overall, giant viruses of amoebae are highly complex viruses that mostly lack ribosomal components and energy production machinery.

Since its discovery, Mimivirus has been connected to the tree of life based on phylogenetic analyses of a small set of genes shared with cellular organisms. Indeed, despite the absence of ribosomal genes, a feature that characterizes all known viruses to date, Mimivirus harbors core genes that are conserved among NCLDVs and are homologous to bacterial, archaeal, and eukaryotic genes (9). The phylogeny based on these genes was congruent with that based on ribosomal genes and showed that Mimivirus is rooted deeply in the eukaryotic clade. In 2010, the term fourth domain was coined with the description of new phylogenies along with a hierarchical clustering analysis (109). These phylogenies were based on a few genes involved in the biosynthesis of DNA precursors, in DNA replication and repair, and in transcription, whereas hierarchical clustering analyzed the presence/absence patterns among NCLDVs and cellular organisms of homologs of such informational genes (109). These analyses were redone with other Megavirales representatives with the same results (110, 111), and because the topology of the four branches did not rely on ribosomal genes, the proposed additional branch was named a fourth TRUC of microbes, TRUC being an acronym for things resisting uncompleted classification (8). The existence of a fourth branch in the tree of life is still hotly debated. It has been contested with arguments that the phylogenies were biased by the occurrence of lateral gene transfer from cellular organisms (mostly eukaryotes) to giant viruses, and by inappropriate methodologies (29, 32, 112–115). In contrast, phylogenetic studies based on conserved genes and sequences from marine metagenomes showed additional branches (116). Moreover, phylogenies of entire repertoires of protein structural domains favored the existence of a fourth branch in the tree of life (117, 118), but this has

also been contested based on methodological arguments (119). Phylogenomic reconstructions based on highly conserved genes present in Megavirales members as well as analyses of protein fold superfamilies further suggested that giant viruses have an archaic origin, contemporary to protoeukaryotes (108, 118). The origin and evolution of giant viruses of amoebae still continue to be debated (32, 63).

Finally, the best way to classify giant viruses of amoebae and the most appropriate taxonomic approach are issues that need to be addressed (120), especially because the number and diversity of these viruses have considerably expanded. To date, three families of virus that replicate in *Acanthamoeba* spp. have been recognized by the International Committee on Taxonomy of Viruses. They correspond to the giant viral families *Mimiviridae* (121) and *Marseilleviridae* (122) and to a family of virophages named *Lavidaviridae* (123). Giant virus classification and taxonomy may require consideration of new criteria and tools that are implemented for bacteria in the current era of expansion of phylogenomic data (124).

GIANT VIRUSES OF AMOEBAE IN HUMANS

The pathogenicity of giant viruses of amoebae is an emerging field in medicine that should not be neglected. Due to the ubiquity of giant viruses of amoebae in environmental water and soil samples worldwide, and their presence in food items such as oysters and mussels (125, 126), humans are probably exposed to them. A growing body of data shows that giant viruses of amoebae are present in humans and that their presence may not be neutral. This has been assessed by a polyphasic approach. The large majority of the data involves mimiviruses and marseilleviruses, because they were discovered first. Mimivirus was suspected to be linked to pneumonia from the very outset (16). Serological studies showed several cases of seropositivity and seroconversion to Mimivirus in patients with unexplained pneumonia (127–130). In particular, this was demonstrated in a laboratory technician who handled Mimivirus in large quantities, in circumstances reminiscent of those that led investigators to suspect a link between Epstein–Barr virus and infectious mononucleosis (131, 132). Antibodies to the mimivirus Sputnik virophage were also detected in two Laotian patients presenting with fever (133). Isolation of mimiviruses from two Tunisian patients was described ten years after the discovery of Mimivirus (18, 134). These two patients presented with unexplained pneumonia, and mimiviruses were isolated from bronchoalveolar fluid in the first patient and from feces in the second. Positive PCR findings on human samples have been reported in only three cases, for respiratory specimens (18, 127, 135). This may suggest that mimiviruses are not common in the human body, including in patients with pneumonia. However, mimiviruses, like other giant viruses of amoebae, display considerable genetic diversity. Indeed, the genome of each newly isolated mimivirus differs by numerous mutations from those previously sequenced, and this hampers the efficiency of PCR systems (18, 136, 137). In addition, mimivirus and marseillevirus virions were found to be very resistant in various physicochemical conditions, and it is possible that DNA extraction protocols for these giant viruses have low efficiency (138, 139). Finally, the development of histopathologically proven lesions of pneumonia was demonstrated in mice inoculated intracardially with Mimivirus (140). Another finding is that, compared with sera from healthy people, a significantly greater proportion of sera from patients with rheumatoid arthritis are positive for Mimivirus L71 protein, which is a viral collagen present on the surface of Mimivirus (141).

In contrast with mimiviruses, marseilleviruses have mostly been detected in humans in blood and lymphoid tissues. However, in 2012, a marseillevirus was the first giant virus of amoebae to be isolated from a human—a young healthy Senegalese man—and this was from feces (65, 142). Thereafter, marseillevirus-matching sequences were found to represent 2.5% of the metagenomic

reads generated from the blood of asymptomatic blood donors, and two contigs of 13.6 and 10.2 kbp were assembled that belong to a marseillevirus named giant blood marseillevirus (143). Anti-Marseillevirus antibodies were detected in the blood of one of the Marseillevirus-positive blood donors, and the blood was also positive for Marseillevirus DNA by PCR and FISH. Antibodies to Marseillevirus and/or Marseillevirus DNA were subsequently detected in healthy young people, blood donors, and polytransfused thalassemic patients in France and Switzerland (144, 145). PCR failed to detect Marseillevirus DNA in blood from other blood donors and from polytransfused patients in other studies (146–148). However, a large metagenomic study of the blood DNA virome from 8,000 humans detected four sequences in one individual and two in another matching marseilleviruses of lineage A, which includes the prototype Marseillevirus isolate (149). Furthermore, an 11-month-old child was then found to have a high titer of antibodies to Marseillevirus (94). He presented with lymphadenitis, and Marseillevirus was detected by FISH and immunohistochemistry in his lymph node. Immunoglobulin G to Marseillevirus was also detected in a 30-year-old woman with Hodgkin’s lymphoma, and the giant virus was concomitantly detected by PCR, FISH, direct immunofluorescence, and immunohistochemistry in her lymph node (95). The most recent observation was the detection via PCR—twice during a one-year interval—of Marseillevirus DNA in the pharynx of a patient presenting with neurological disorders (150). These findings raise the possibility that giant viruses of amoebae can enter and replicate in human cells. Mimivirus was found to enter into human monocytes and macrophages (92) and to replicate in peripheral blood mononuclear cells (93); nevertheless, no virus propagation was demonstrated on these human blood cells. Also, Marseillevirus virions were detected in human T lymphocytes after inoculation with a serum positive by PCR and serology for giant blood marseillevirus (143). Finally, sequences similar to those of giant viruses of amoebae have been identified in metagenomes generated from human samples (61, 65, 143, 151–153). This involved sequences related to mimiviruses, virophages, and marseilleviruses (65, 149, 151, 152, 154, 155) but also to pithoviruses, pandoraviruses, molliviruses, and faustoviruses (61, 149), which are the most recently described giant viruses of amoebae.

CONCLUSION

The lifestyle, structure, and genomes of giant viruses of amoebae break the mold of what was traditionally considered a virus. Giant viruses of amoebae bring upheaval to the definition of the virus and tend to separate the currently known virosphere into two categories: very simple viruses and viruses with a complexity similar to that of other microbes. This new paradigm offers a more suitable context to continue to improve the detection and characterization of giant viruses of amoebae, and a particular focus on their role in humans is warranted.

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