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Alohomora! What the entry mechanisms tell us about the evolution and diversification of giant viruses and their hosts

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The virosphere is fascinatingly vast and diverse, but as mandatory intracellular parasites, viral particles must reach the intracellular space to guarantee their species' permanence on the planet. While most known viruses that infect animals explore the endocytic pathway to enter the host cell, a diverse group of ancient viruses that make up the phylum *Nucleocytoviricota* appear to have evolved to explore new access' routes to the cell's cytoplasm. Giant viruses of amoeba take advantage of the phagocytosis process that these organisms exploit a lot, while phycodnavirus must actively break through a algal cellulose cell wall. The mechanisms of entry into the cell and the viruses themselves are diverse, varying in the steps of adhesion, entry, and uncoating. These are clues left by evolution about how these organisms shaped and were shaped by convoluting with eukaryotes.

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Introduction

If there is anything fascinating in the virosphere, it would be its diversity besides its surprising vastness [1]. The diverse morphologies, structures, and various genome types of viruses present us with intriguing pieces of a puzzle that tells how viruses shaped and were shaped by interaction with their hosts and their role in the evolution of life on Earth [2–4]. These organisms have inhabited a unique and invisible universe to humans [5]. Although optical microscope revealed many other microorganisms, it kept viruses hidden on an even smaller scale [6]. Two centuries had passed since discovering the first virus [5], when the discovery of Acanthamoeba polyphaga mimivirus, a giant virus of amoeba, revealed that these entities were not restricted to this smaller scale and were even more diverse [7,8].

Giant viruses brought new enigmas in searching for the evolution path. Despite that they mainly infect free-living amoebae, their genomes and particles' colossal size were never seen before in the virosphere. Genomes including translational apparatus, containing diverse translation factors, such as tRNAs and aminoacyl-tRNA synthetases [9–11], also surprising genes linked to metabolic routes, such as glycolysis [12] and Tricarboxylic acid [13], or even practically whole genomes that were unknown to humankind, as seen in the non-giant, Yaravirus [14]. Their genomes also revealed a new taxonomic arrangement, in which giant viruses of amoeba clustered with viruses from various other hosts, such as algae and even animals, forming a hypothetically monophyletic group, although highly diverse, the phylum *Nucleocytovirricota* [15,16,17•].

However, even with their wealth of genes, proteins, and the diversity observed in this group [11], all viruses share a mandatory intracellular and parasitic lifestyle. Therefore, to guarantee their permanence on the planet, they need to find, adhere and penetrate a host cell. These steps are essential for virus entry and uncoating, the process by which the genome of a virus particle is delivered to the replication site [18].

Adhesion and entry

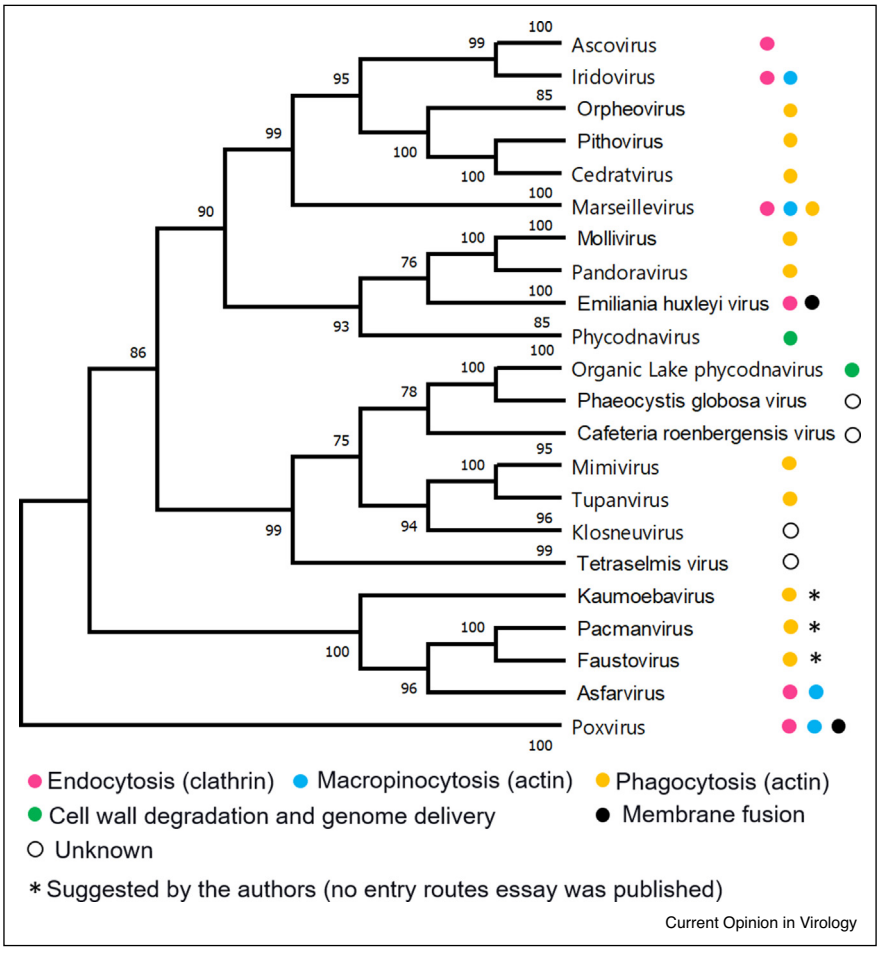
Virus-receptor interaction is the key to cell invasion. Viruses use elegant strategies to orchestrate adhesion and cross the plasmatic membrane to control cellular machinery [19]. Before penetration, adhesion is a crucial step [20]. Different factors influence it, including the external form of virions (such as surface protrusions and their topology), the type of target cell, cell receptors, and the viral protein content [19]. Mechanisms that guarantee effective adhesion of viruses to the cell surface are undoubtedly essential to start the penetration step. Structures like the fibrils present in mimiviruses are

strong suggestions of how important the development of structures that favor adhesion is for viruses [21••].

Viral entry can occur in different ways, but the most used endocytic pathways taken by viruses are, in general, the clathrin-mediated routes, which are continuous, fast, and effective processes that transports incoming viruses together with their receptor into endosomes [22]. However, many other routes have already been identified: (1) micropinocytosis, an actin-driven process in which the plasma membrane ruffles' extension forms a cup-like structure that seals at its distal tips and can also bring viruses (e.g. adenovirus) [23]; (2) clathrin-independent pathway (e.g. influenza virus and arenavirus); (3) the caveolar path (e.g. coxsackievirus); (4) the cholesterol-dependent endocytic pathway devoid of clathrin and caveolin- (e.g. polyomavirus) [20,21••,22]. In addition to these pathways, viruses can also fuse their membrane to the host membrane (e.g. measles virus) [20,21••,24].

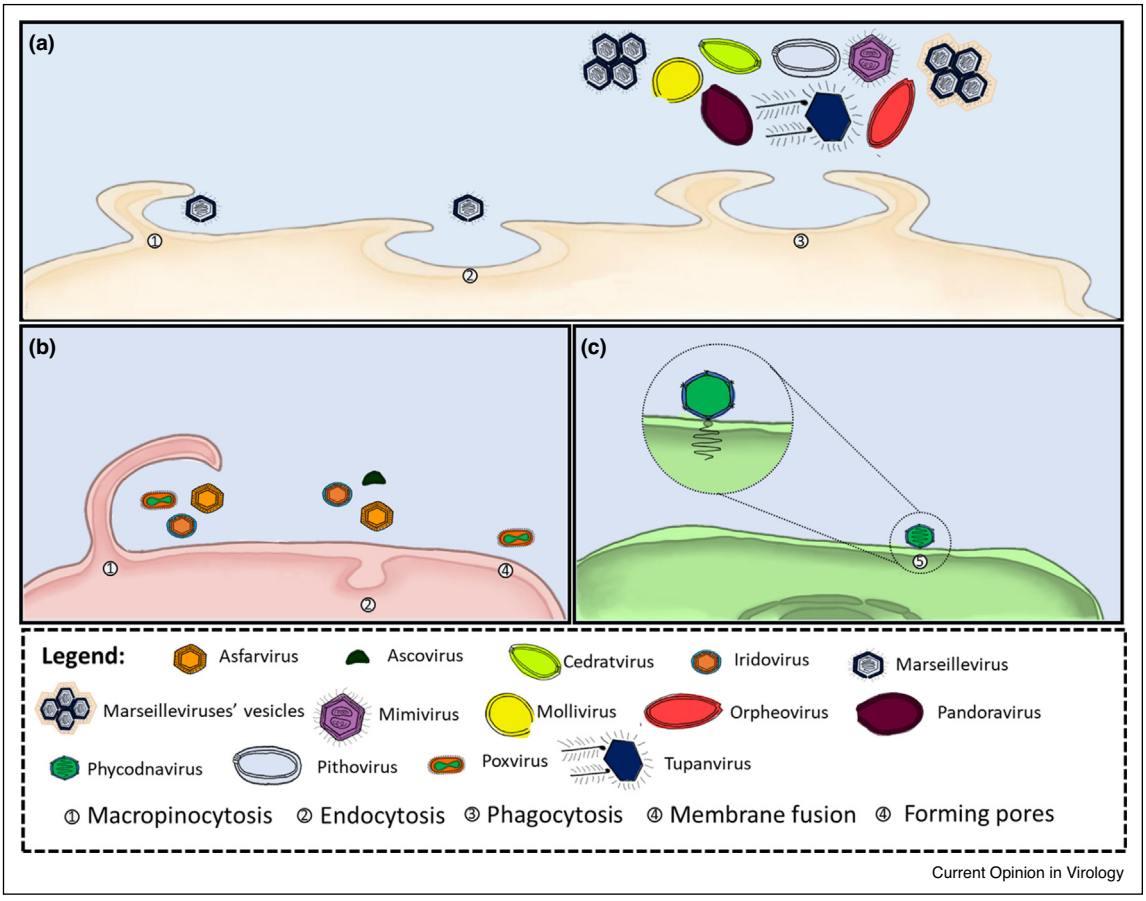
Cell-to-cell transmission is also a possibility. Viruses can induce the fusion of membranes of an infected cell with an uninfected one (e.g. herpes virus varicella-zoster) [22,25]. Viruses can also be transmitted as information (viral genome) via viral synapses, through an adhesive and stable connection between these cells, without fusion of membranes (e.g. human T cell leukemia virus type 1 and human immunodeficiency virus type 1) [26,27]. When integrated into the genome, they will replicate and spread as proviruses [28]. What is intriguing about the phylum *Nucleocytoviricota* is that these viruses, which apparently form a monophyletic group, can explore a fantastic diversity of entry routes (Figure 1), unlike other viruses' groups, such as RNA viruses. Although more closely

Figure 1



The viruses of the phylum *Nucleocytoviricota* present a great diversity of entry routes in host cells.
The phylogenetic analysis of the DNA polymerase B gene shows the evolutionary relationships among *Nucleocytoviricota* members. The right dots highlight that in such hypothetically monophyletic group different routes of virus entry into host cells are explored: clathrin-mediated endocytosis, macropinocytosis (actin-mediated), phagocytosis (actin-mediated), membrane fusion, and cell wall degradation followed by genome delivery. Maximum likelihood tree, with a bootstrap analysis based on the value of 1000 replicates. Image edited for better understanding.

Figure 2



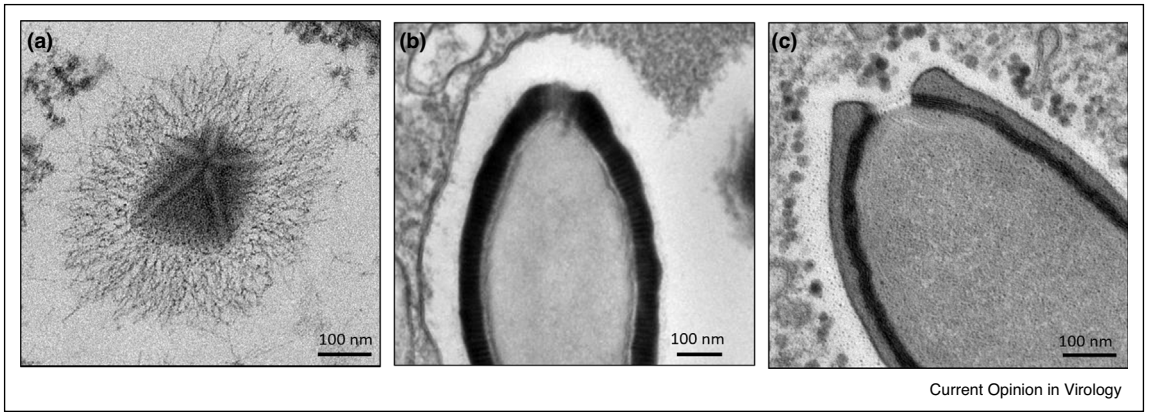
The viruses of the phylum *Nucleocytoviricota* explore different forms of entry according to their hosts.
(a) Amoeba viruses can penetrate the host cell by (1) Macropinocytosis, like the individual particles of Marseillevirus; (2) Receptor-mediated endocytosis, also by Marseillevirus particles; (3) phagocytosis is widely exploited by giant viruses, such as cedratvirus, mimivirus, orpheovirus, pandoravirus, pithovirus, and tupanvirus. Moreover, Marseilleviruses use this pathway when they group or are released in vesicles. **(b)** In animal cells, whether vertebrates or invertebrates, the explored routes are basically: (1) macropinocytosis, by asfarvirus, iridovirus, and poxvirus, and (2) receptor mediated. Poxvirus can also penetrate by (4) fusion of the viral membrane to the cell membrane. **(c)** In algae cells, the phycodnavirus needs to attach to the cell wall, degrade it, and use its cell membrane as a tunnel through which its genome has entered the cell.

related viruses appear to have inherited similar entry mechanisms (Figure 1).

Mimiviruses, such as APMV, were one of the responsibles for including phagocytosis as an entry route for viruses [25,29•]. It is undoubtedly related to the fact that they were the first giants to be discovered, and their size favors phagocytosis. After all, it is already well established that particles larger than 500 nm can trigger phagocytosis in *Acanthamoeba* sp. [30], which is a tremendous advantage for viruses that infect amoebae, considering that phagocytosis is the leading way for these organisms to feed themselves [31]. Therefore, this strategy seems to be widely exploited by these viruses [21••], which infect *Acanthamoeba* sp., such as those belonging to the *Mimivirus* genus [29•,32,33], pandoravirus [34,35], pithovirus [36], mollivirus [37], and cedratvirus [38,39]. Moreover, those that infect *Vermamoeba vermiformis* as orpheovirus [40], or both, as tupanvirus [41,42]. All these viruses are particularly large and appear to have been selected to exploit this entry route (Figure 1 and 2a).

Phagocytosis is an advantageous route for giant viruses of amoeba since smaller viruses, which do not fulfill the requirements to trigger it, have other strategies that favor their entry using this route. That is the case of Marseilleviruses, in which the newly formed particles can be grouped to induce phagocytosis or be released within vesicles, which will later be phagocytized by another amoeba [43••]. However, this is not the only entry route explored by Marseilleviruses since individual particles' infection is also observed and can be initiated by receptor-mediated and clathrin-dependent endocytosis or even by macropinocytosis (Figure 2a). Finally, phagocytosis can

Figure 3



Examples of uncoating features in amoebal giant viruses.
(a) The stargate/starfish in a mimivirus; **(b)** The cork in a cedratvirus; **(c)** The ostiole in a pandoravirus. The images were obtained from the Transmission Electron Microscope (Spirit Biotwin FEI-120 kV) at the Microscopy Center of UFMG.

also be triggered by grouped Marseillevirus particles, even without being enveloped by a vesicle [43••].

Clathrin-mediated endocytosis and actin-mediated macropinocytosis are pathways widely exploited by smaller viruses of the phylum *Nucleocytoviricota* (Figure 2b) [21••]. Many of them are responsible for infections in both vertebrate animals, such as asfarvirus [44,45], invertebrates like iridovirus [46] and ascovirus [47], or both, as does poxvirus [48,49]. The poxvirus particles can also penetrate, promoting the membrane's fusion to the host membrane [49]. Nevertheless, the entry is not defined only by the virus. As it is known, the cell must be permissive for the infection to occur successfully [50]. That includes being able to carry out these mechanisms of particle interiorization. Grouping the virus by their penetration strategies and their hosts seems an appropriate way to understand how they were selected to interact with each other (Figure 2).

An interesting example in this discussion is the phycodnavirus's entry route, a virus of about 200 nm that also belongs to the *Nucleocytoviricota* phylum. These viruses infect *Chlorella variabilis* algae and, for this reason, have a rigid barrier to overcome: the cellulose cell wall. This wall does not allow endocytosis or phagocytosis of these particles. The viruses must then actively penetrate the cell. It binds quickly and specifically to the cell walls' outer surface from its algae host [51] (Figure 2c). The spike structure of the virus makes the first contact with the host cell wall, which will be degraded by an enzyme associated with the virus, the internal membrane of the virus will merge with the host membrane, forming a membrane-lined tunnel between the virus and the host, leaving an empty capsid attached to the surface [51].

Uncoating

Once the cell barriers were overcome, these viruses seem to converge to the same strategy in the uncoating step. In a very simplified way, in this stage, the virus membrane's fusion to the host cell membrane (e.g. poxvirus and phycodnavirus) or the phagosome/endosome membranes occurs. Although membranes' fusion is recurrent for all known viruses' uncoating in this phylum, the mechanisms that trigger this fusion are not fully understood. The phagosome or endosome's acidic pH is often cited as a factor that generates the changes that lead to capsids disassembly and membrane's fusion for viruses that use these routes [18,45,46,52•,53]. However, unique structures were identified and suggested to be fundamental for uncoating, especially in giant viruses [54] (Figure 3).

These structures were selected to allow the opening of the resistant capsids' structure only after penetration [18,52•,53]. For example, Mimiviruses have a pseudo icosahedral capsid and on its apex a protein structure in the shape of a starfish that acts as a seal for the stargate (Figure 3a), the apex of the capsid [53]. These structures ensure that the stargate remains closed until the phagosome's interior environment promotes a new protein arrangement in the starfish, promoting the stargate's opening and releasing the capsid content after the membranes' fusion. This is a fascinating mechanism; although *in vitro* testing that combined low pH and great temperatures promoted the stargate opening, little is known about what induces the opening inside the host cell [53].

The stargate is also present in tupanviruses and seems to work in the same way since the molecular forces that stabilize the stargate vertex are conserved among mimiviruses [53]. However, tupanviruses brought another

intriguing structure: a tail. Tupanvirus tail is about ~550 nm extension, ~450 nm diameter, including fibrils. It is responsible for giving tupanvirus the longest isolated virus's status so far [41,42]. During uncoating, the tail also seems to release its contents in the cytoplasm after the invagination of the phagosome membrane in the tail's interior [42].

Non-icosahedral giant viruses of amoeba, such as pandoravirus, pithovirus, cedratvirus, mollivirus, and orpheovirus, do not have such stargate structure. However, they still need to overcome the capsid's resistance to guarantee its uncoating. Sealing complexes similar to corks are present in pithovirus [36,55] and cedratvirus (Figure 3b) [38,39] and are differentiated from the ostioles of orpheovirus [40,55] and pandoravirus [35] (Figure 3c). The ostile represents an aperture in the viral tegument [56]. Beneath the tegument, a lipid membrane protects the core of the particle [56]. The genome uncoating occurs similarly for viruses that present ostioles or corks; inside the phagosome, the virus membrane fuses with the phagosome membrane and promotes the viral genome's release in the cytoplasm.

During evolution, these structures were selected to release the genome after penetration in giant non-icosahedral viruses. It is not difficult to imagine that the phagosome's environment would have to be much more aggressive to stop the inertia in these resistant capsids without these structures. Metastability is an attribute that allows a stable virion to change its conformation when disturbed. In particular, the physical properties of virions facilitate infectious uncoating triggered by the host [43••]. Nature and evolution found a balance between a resistant capsid to keep particles viable in hostile environments, such as the soda lake in which one of the tupanviruses was found, but malleable enough to allow the uncoating inside the host cells when triggered by the right stimulus.

Pandoraviruses are good examples of this balance. Apparently, this virus lost the gene encoding double-jelly roll major capsid protein, the main building block of icosahedral capsids in this virus assemblage, acquiring an ovoid shape [57••]. This change in the viral particle's topology is essential, as it can influence the virus's entry into the cell [21••]. The tegument of its capsid is divided into three layers. Recent studies suggest that part of it is composed of cellulose, probably from the host amebic cellulose, which naturally uses this substance in its encistment [56]. Reinforcing that interaction of pandoravirus and its possible host, by this example of gene transfer process [56]. In another layer of pandoravirus, it is suggested that there is a proton-motive force across the virion membrane, promoted by enzymes linked to the tricarboxylic acid cycle, which could create an electrochemical gradient necessary for proper fusion of virus membranes to the host phagosome membrane [13].

Conclusions

As mandatory intracellular parasites, viral entry may represent one of the most crucial steps for species' perpetuation. In contrast to other groups of viruses that often present similar topologies, proteins, sequences, and structures that lead them to explore similar entry routes, the *Nucleocytoviricota* phylum has an immense diversity of characteristics, including a great variety of entry strategies. While RNA viruses basically exploit the routes of endocytosis or the fusion of the membrane with the host cell, the *Nucleocytoviricota* phylum can explore almost all examples of possible viral entry pathways (endocytosis — caveolin clathrin receptors, phagocytosis, macropinocytosis, membrane puncture, phagocytosis of vesicles) that were selected throughout the evolution and diversification of eukaryotes. With teguments almost like armors, giant viruses needed to co-develop structures that compensate for their capsids to guarantee the success of the second step of viral entry; the uncoating. The acquisition of these different structures and entry strategies results from the selective pressures that act in this ancient group and the differences it underwent during the divergence and coevolution with its remarkable broad range of hosts.

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Conflict of interest statement

Nothing declared.

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