

MICROSPORIDIA: Biology and Evolution of Highly Reduced Intracellular Parasites

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■ **Abstract** Microsporidia are a large group of microbial eukaryotes composed exclusively of obligate intracellular parasites of other eukaryotes. Almost 150 years of microsporidian research has led to a basic understanding of many aspects of microsporidian biology, especially their unique and highly specialized mode of infection, where the parasite enters its host through a projectile tube that is expelled at high velocity. Molecular biology and genomic studies on microsporidia have also drawn attention to many other unusual features, including a unique core carbon metabolism and genomes in the size range of bacteria. These seemingly simple parasites were once thought to be the most primitive eukaryotes; however, we now know from molecular phylogeny that they are highly specialized fungi. The fungal nature of microsporidia indicates that microsporidia have undergone severe selective reduction permeating every level of their biology: From cell structures to metabolism, and from genomics to gene structure, microsporidia are reduced.

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INTRODUCTION

The microsporidia (or Microspora) are an unusual group of eukaryotic, obligate intracellular parasites that have attracted the curiosity of biologists for more than 100 years. Like many other intracellular parasites, microsporidia are highly specialized and have evolved an extremely sophisticated and unique infection mechanism along with other adaptations to life inside other cells. These adaptations are primarily characterized by reduction. Compared with other eukaryotes, microsporidia

are highly reduced at every level: from morphology and ultrastructure, to biochemistry and metabolism, and even at the level of their molecular biology, genes and genomes.

The first microsporidian was described in the middle of the nineteenth century when pébrine, or “pepper disease,” was ravaging silkworms in southern Europe and threatening to destroy the European silk industry. The pébrine agent was observed to be a microscopic parasite that was named *Nosema bombycis* by Nägeli in 1857 (66). Nägeli considered *Nosema* to be a member of the schizomycete fungi, although classification at that time did not reflect the true diversity of microbial life, and schizomycetes were a grab bag of yeasts and bacteria. After further study, Balbiani accordingly created a new group for *Nosema* in 1882, calling it Microsporidia (2), the name still in use.

Today, the microsporidia are known to be an extremely diverse group of parasites. There are currently approximately 150 described genera of microsporidia with over 1200 individual species (78, 79). By far, most microsporidia infect animals, where they have been characterized in all vertebrate orders as well as most invertebrates, including the parasitic myxosporidia (95). While these animal parasites account for the vast majority of microsporidia, a few species have been shown to infect certain protists, such as ciliates and gregarine apicomplexa (95). It is interesting that these gregarines and some of the ciliates are themselves animal parasites, which suggests that the microsporidian probably once infected the same animal hosts as these protists and later adapted to parasitize its neighbor. Given the diversity and abundance of microsporidia known in animals today, it seems likely that the actual number of microsporidia far exceeds those which have been described and that the number of microsporidian species could perhaps approach the number of species of animals.

Although widespread among animals, microsporidia are apparently most prevalent in arthropods and fish. They are used as biological control agents against insect pests and, under natural circumstances, are found to be destructive to apiculture, fish, and some crustacea important to aquaculture (4, 75). The first microsporidian infection described in mammals was *Encephalitozoon cuniculi*, originally found in rabbits in 1922 (106). This species is now known to frequently infect a broad range of mammalian hosts. In 1959 the first clear case of a human microsporidian infection was recorded (60), but such cases were relatively rare until the mid-1970s, when a dramatic increase in recorded infections accompanied the increased prevalence of immunosuppressed individuals, either resulting from infection with HIV or due to the use of immunosuppressing drugs (98). The most common of these opportunistic human microsporidia is *Enterocytozoon bienersi*, which was first described as a gastrointestinal parasite causing “wasting syndrome” (a potentially lethal diarrhea) in 1985 (20). *E. bienersi* is now known to infect a wide range of human tissues in AIDS patients and occasionally infects healthy immunocompetent humans where it results in an acute but self-limiting intestinal disorder (74). Presently, 13 species of microsporidia have been found to infect humans (30, 92, 99) leading to a long list of human diseases, including chronic diarrhea and wasting

syndrome, keratoconjunctivitis, pneumonia, bronchitis, nephritis, urethritis, pro-
statitis, hepatitis, encephalitis, myostitis, and peritonitis (30, 98, 99, 105).

THE MICROSPORIDIAN SPORE

The focal point of the microsporidian infection strategy, life history, and diagnosis is the spore, a single, highly organized cell (Figure 1). Spores are the only easily recognizable stage of microsporidia, they are the stage where species can be differentiated, and they are the only stage of microsporidia that is viable outside of a host cell. Spores range in size from as little as 1 μm in *E. bienewisi* to 40 μm in *Bacillidium filiferum* (92) and can be spherical, ovoid, rod-shaped, or crescent-shaped, although most are ovoid. Within a species, spore morphology tends to be fairly regular, although some species do possess different spore types in different stages of their life cycles (92). The spore is bound by a normal unit membrane and two rigid extracellular walls. The exospore wall is composed of a dense, granulofibrous, proteinaceous matrix (7, 93) and is generally uniform at the surface, although it can be highly ornamented in aquatic microsporidia (92). The endospore wall is composed of alpha-chitin (92) and proteins and is of uniform thickness, except at the apex of the spore where the endospore wall is considerably thinner than elsewhere. Within the spore membrane is the sporoplasm, or the cytoplasm of the spore, which is the infectious material of microsporidia. The sporoplasm contains a single nucleus or two nuclei arranged as a diplokaryon (two closely appressed nuclei), cytoplasm enriched with ribosomes, and is otherwise dominated by structures relating to infection. There are three principal structures related to infection: the polaroplast, the polar filament or polar tube, and the posterior vacuole. The polaroplast is a large organization of membranes occupying the anterior

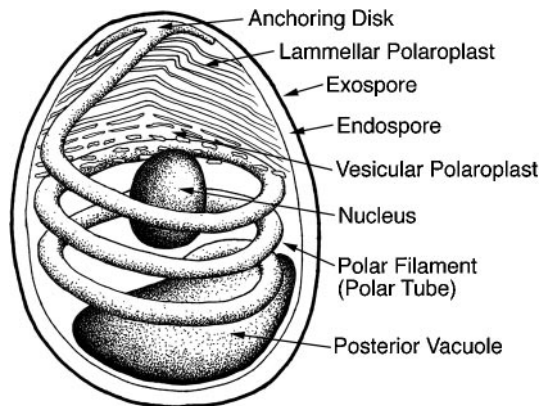


Figure 1 Diagram of a microsporidian spore showing the major structures discussed in the text.

part of the spore. The anterior portion of the polaroplast exists as highly organized, closely stacked membranes called the lammellar polaroplast, whereas the posterior portion is more loosely organized and is called the vesicular polaroplast. The most obvious organelle associated with infection is the polar filament or polar tube. In the sporoplasm this filament is composed of membrane and glycoprotein layers and ranges from 0.1 to 0.2 μm in diameter and 50 to 500 μm in length (51, 92). The polar filament is attached at the apex of the spore via an umbrella-shaped structure called the anchoring disk, from which it extends to the posterior of the spore. For approximately one third to one half the length of the spore the polar filament is straight, and the remainder is helically coiled about the contents of the sporoplasm. The number of coils, their arrangement relative to one another, and even the angle of helical tilt are conserved and diagnostic for a particular species (79, 92). The polar filament terminates at the third major organelle associated with infection, the posterior vacuole. There is apparently some physical association between the end of the polar filament and the posterior vacuole (59, 100), but whether the polar filament actually enters the vacuole or simply contacts it is not known, as the point of contact has never been observed (51, 92, 94).

MICROSPORIDIAN INFECTION AND LIFE CYCLE

Germination of the microsporidian spore is one of the most interesting and dramatic series of subcellular events in biology, involving a build-up and controlled release of tremendous force, a cascade of extremely rapid events in close succession, and a complete alteration of the sporoplasm, which includes some unique restructuring of membrane topology.

Spore germination begins with an environmental trigger that varies for different species depending on their habitat (85) but is largely poorly understood (51). *In vitro*, spores may be germinated by a number of physical and chemical stimuli including, but not limited to, alterations in pH, dehydration followed by rehydration, hyperosmotic conditions, the presence of anions or cations, or exposure to ultraviolet light or peroxides [for an extensive review of these conditions for various species see (51)]. When a spore is induced to germinate, the first sign is a general swelling of the spore and a specific swelling of the polaroplast and posterior vacuole (59). This is the result of an increase in osmotic pressure in the spore (52, 67, 86), but how this pressure builds up is the subject of some debate. Spores are equipped with aquaporins that specifically transport water across the sporoplasm membrane (31), which explains how the influx of water can take place, but not necessarily why. One intriguing possibility was raised after it was observed that levels of trehalose drop significantly during the germination of *Nosema algerae* (84, 88). Trehalose is a glucose-glucose disaccharide found widely in nature and is the major carbohydrate storage material of microsporidian spores (84, 90) and fungal spores (1, 29). It was suggested that during spore activation in microsporidia, trehalose is degraded to constituent glucose monomers, effectively increasing the number of soluble molecules within the spore and leading to the import of large quantities of

water and the concomitant increase in osmotic pressure (84, 86, 88). This model is a fascinating possibility and would represent an ingenious system to generate force in the cell. However, the germination of the spore involves a great number of changing conditions, so the levels of trehalose cannot be tied unequivocally to the rise in internal osmotic pressure. In fungal spores trehalose does not appear to primarily act as an energy reserve but rather as an anti-stress metabolite (1). This also may be the role of trehalose in microsporidia, and trehalose degradation may only be a step in the germination process. Moreover, the trehalose levels in a related species of *Nosema* do not change during germination (19), suggesting some, or potentially many, other causes for water influx. Indeed, alternative models point to the intracellular concentration of calcium ions as a cause for the influx of water (50) and a possible role for calmodulin in the process (102). Here it is suggested that membrane breakdown during spore activation could release calcium ions from the endomembrane system into the sporoplasm. These ions could induce the influx of water and could also induce the activation of enzymes such as trehalase whose activity could further enhance the hypertonic shift in the sporoplasm (50).

Whatever the proximal cause, the osmotic pressure of the spore builds up and this pressure seems to be the driving force of the subsequent events of germination. The internal pressure of the spore and the breakdown in sporoplasmic membranes culminate in the rupturing of the anchoring disk and the discharge of the polar filament by eversion (Figure 2). Eversion begins at the spore apex, where the discharging polar filament actually breaks through the thinnest region of the spore wall, and is frequently likened to turning the finger of a glove inside-out. As

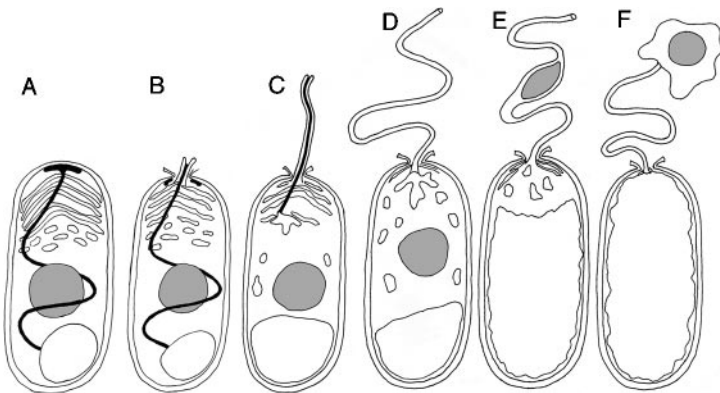


Figure 2 Polar tube eversion during spore germination. (A) Dormant spore, showing polar filament (*black*), nucleus (*gray*), polaroplast and posterior vacuole. (B) Polaroplast and posterior vacuole swelling, anchoring disk ruptures, and polar filament begins to emerge, everting as it does so. (C) Polar filament continues to evert. (D) Once the polar tube is fully everted, the sporoplasm is forced into and (E) through the polar tube. (F) Sporoplasm emerges from the polar tube bound by new membrane.

the filament everts, it becomes a tube and the dense granular, glycoproteinaceous material that filled the filament when inside the sporoplasm is deposited on the outside of the tube (51). The remarkable nature and violent speed of this event cannot be overstated. The discharged polar tube can range in length from 50–500 μm in length (potentially 100 times the length of the spore) and is generated by turning inside out a filament that is coiled around the sporoplasm. Yet, the entire event of germination takes place in fewer than two seconds and the tip of the discharging tube can move through the medium at velocities exceeding 100 $\mu\text{m/s}$ (32).

Clearly, the polar tube is a significant projectile, and if a potential host cell lies nearby, the discharging tube can strike this cell and pierce its membrane. Once the polar tube is fully discharged, the continued pressure within the spore (most likely from the swelling of the posterior vacuole) forces the sporoplasm through the polar tube. Although somewhat elastic, the polar tube is a narrow conduit ranging from just 0.1 to 0.25 μm in diameter, but the sporoplasm is nevertheless forced through the polar tube rapidly, emerging at the tip of the tube in only 15–500 ms (32). If the polar tube has penetrated a host cell, the sporoplasm emerges from the tube directly into the host cytoplasm, thus infecting without the host recognizing the parasite as a foreign invader. This invasion is a remarkable event, since the sporoplasm membrane has been left behind in the now empty spore (87, 101), raising some intriguing questions about membrane topology surrounding spore germination. First, an intracellular membrane-based organelle has broken through the sporoplasm membrane and spore wall while being turned inside out. Yet, the sporoplasm passes out of its bounding membrane and through the polar tube to emerge at the other side with a brand-new membrane. This is thought to be possible because polaroplast membrane is forced into the polar tube at the onset of germination, and it is this membrane that forms the bounding plasma membrane of the intracellular stage of the parasite (101), a remarkable feat of membrane manipulation.

Once inside the host cell, the parasite is referred to as a meront and it begins a stage of growth and division characterized by a high degree of interspecies variation. In general, however, the parasite is found directly within the host cytoplasm and not within a host phagocytotic vacuole, as is the case for many other intracellular parasites. Occasionally the parasite induces the formation of a surrounding membrane known as a parasitophorous vacuole at an early stage of infection (55), but most often they do not (although they commonly do during sporogenesis). At this stage, the parasite is intimately associated with its host and often induces significant changes to the host that are not obviously deleterious. Often the host cell reorganizes around the parasite, so that the parasite can be found surrounded by host organelles such as endoplasmic reticulum (ER), nuclei, or mitochondria (92). In some instances the microsporidian also physically interacts with the host nucleus, resulting in the enlargement of host nuclear pores (57) or even the invasion and infection of the nucleus itself (35). Host cells may also transform in shape and size, often enlarging (103). The archetype of microsporidian-induced host cell transformation is the xenoma, a host cell harnessed and transformed by the parasite

to promote its own growth and development. In such cases, the host cell is induced to enlarge and undergo many rounds of nuclear division resulting in an enormous plasmodium filled with parasites in highly ordered strata depending on their stage of development: mature spores at the center with the earlier stages radiating toward the periphery of the xenoma (12, 54, 103).

The onset of sporogony is marked in some species by the separation of diplokaryotic nuclei (11) and in other specific cases by meiosis (34), although synaptonemal complexes have been observed in all life stages of other microsporidia. Morphological features are more consistent indicators of sporogony and include an apparent thickening of the plasma membrane (due to the accumulation of electron-dense material on the membrane) and the increased presence of ER and ribosomes. Both the ER and ribosomes change organization throughout sporogony, with the ER becoming highly ordered, and the ribosomes increasingly forming arrays attached to the ER, known as polyribosomes (92). Although sporogony can occur in direct contact with the host cytoplasm, some species produce a sporophorous vesicle in which the sporonts develop. In most species, this stage of the life cycle is also accompanied by some degree of division, although the number of sporoblasts (presporal cells) produced varies among species from two (bisporous) to many (polysporous). Following division, the extrusion apparatus (including the polar filament, polaroplast, and posterior vacuole) begins to develop. Early observations implicated the Golgi as giving rise to the polar filament (91), and this has been confirmed by histochemical labeling for activity of the Golgi marker, thiamine pyrophosphatase (80). Further, histochemical studies assaying activity of nucleoside diphosphatase, a marker of the ER and sometimes of the outer *cis*-Golgi, also implicate the ER in the formation of the polar filament and associated polaroplast membranes (81). These results suggest that the Golgi and ER (which themselves are homologous membrane systems) give rise to the sophisticated extrusion apparatus of microsporidia. As the extrusion apparatus nears complete formation and the sporoblasts approach maturity, the cells decrease in size and the chitinous endospore layer develops. Once complete, the mature spores are released. Some species produce autoinfective spores that germinate immediately in an attempt to infect the same host, thus spreading the infection quickly though an individual (76). Alternatively, spores are released into the environment (i.e., via host urine, feces, or decomposition) where they can infect other individuals, most often by way of the digestive system.

MICROSPORIDIAN GENOMICS

The genomes of microsporidia have aroused interest since they were first karyotyped and found to be much smaller than expected for eukaryotes. Presently, the sizes of 13 microsporidian genomes are known and they fall between 19.5 Mbp and only 2.3 Mbp [(6, 70); for a summary table see (63)]. Apart from their small size, microsporidian genomes are in all characteristics eukaryotic: They have multiple

linear chromosomes, telomeres, and segregate by closed mitosis. However, the reduced size of microsporidian genomes has led to two genome sequence surveys (25, 36), the sequencing of an entire chromosome (69) and now the completely sequenced genome of *E. cuniculi* (44). The general characteristics of the 2.9-Mbp *Encephalitozoon* genome mirror what has been observed in other highly reduced eukaryotic genomes (23): Genes are typically flanked by short intergenic regions (although there are no overlapping genes), there are few repeat sequences, little evidence of selfish elements, and few introns. Introns were predicted to be present in microsporidia based on studies that characterized elements of the spliceosome (21, 27) and one putative intron in a ribosomal protein-coding gene (5). In the 1997 predicted open reading frames in the complete *E. cuniculi* genome, only 11 introns were identified, giving *Encephalitozoon* one of the lowest intron densities among eukaryotes. One of the surprising characteristics of the *Encephalitozoon* genome that demonstrates the extreme degree of reduction is the finding that *Encephalitozoon* genes themselves are actually shorter on average than their homologs from other organisms (44). This suggests that the selection for reduction is very strong indeed, perhaps even overwhelming selection against marginally disadvantageous deletions in protein- and RNA-encoding gene sequences.

ORIGIN AND EVOLUTION OF MICROSPORIDIA

Our conception of the evolutionary history of microsporidia has been radically rewritten on a number of occasions since their original description in the middle of the nineteenth century. When Nägeli considered *Nosema* to be a yeast-like fungus, the concept of a “protist” or a “protozoan” was in its infancy, so it was common to pigeonhole microbial organisms into animals, plants, or sometimes fungi depending on their characteristics. The unique mode of infection seen in *Nosema* eventually led to their separation from fungi, but with no obvious similarity to any other group of eukaryotes, the evolutionary origins of microsporidia were not much clearer. As the real diversity of microbial eukaryotes began to dawn on biologists, more complex hierarchical classification schemes were developed based on certain common features of morphology. One of the cell types to be identified and classified together were spore-forming parasites, collectively called Sporozoa. This group contains what are now known as apicomplexa, myxosporidia, actinomyxidia, haplosporidia, microsporidia, and a handful of individual genera. Within Sporozoa, microsporidia were considered to be most closely related to a variety of other parasites at different times but were most often believed to be akin to myxosporidia and actinomyxidia, which, with microsporidia, were collectively called the Cnidosporidia (53).

For some time, microsporidia were considered to be either Cnidosporidia [although the vast differences between microsporidia on one hand and myxosporidia and actinomyxidia on the other were pointed out (58)] or an independent protist

lineage of uncertain affinity (56). However, in 1983, attention was drawn to the possible evolutionary significance of microsporidia in a new way. Cavalier-Smith (13) proposed that the origin of eukaryotes might have preceded the endosymbiotic origin of the mitochondrion by some considerable span of time, implying that there may be protists that evolved before the mitochondrial origin. In other words, there may be primitively amitochondriate eukaryotes, and focusing attention on these protists could unlock some of the secrets surrounding the origin of eukaryotes. Four lineages of amitochondriate protists that could hold this pivotal position were identified, and these were collectively named Archezoa: Archamoebae (e.g., *Entamoeba*), Metamonada (e.g., *Giardia*), Parabasalia (e.g., *Trichomonas*), and Microsporidia (13). Archezoa were also known to have other characteristics that could be considered primitive, for instance the microsporidia and some other Archezoa contain 70S ribosomes with bacterial-sized rRNAs rather than the 80S ribosomes typical of eukaryotes (18, 39). In some articulations of the Archezoa hypothesis, microsporidia were actually singled out as perhaps the most primitive and ancient lineage of all eukaryotes, since in addition to lacking mitochondria they also lack flagella and other 9 + 2 structures and were sometimes thought to lack Golgi as well (68). Shortly after the Archezoa hypothesis was formulated, the tools of molecular phylogenetics began to be applied vigorously to microbial eukaryotes, and the first molecular data from microsporidia lent extraordinary support to the Archezoa hypothesis. The small subunit ribosomal RNA (SSU rRNA) from the microsporidian *Vairimorpha* was shown to be the earliest branch on the eukaryotic tree (96), and of greater interest, the microsporidia were found to be the only eukaryotes to retain the prokaryotic trait of having their 5.8S rRNA fused to the large subunit (LSU) rRNA (97). These two pieces of evidence bolstered the notion that microsporidia were indeed an ancient and primitive lineage, and further evidence seemed to accumulate with the sequencing of every new microsporidian gene: Phylogenies based on elongation factor 1 α (43), elongation factor 2 (42), as well as isoleucyl tRNA synthetase (9), all showed the microsporidia branching deeply.

The same apparent early phylogenetic position was also seen with other Archezoa (with the possible exception of *Entamoeba*), and altogether the case of an ancient origin and primitive lack of mitochondria for microsporidia and other Archezoa seemed neatly sewn up. Despite the accumulated evidence in favor of the Archezoa, the highly adapted parasitic lifestyle of the microsporidia was always a source of doubt: Such specialized parasites may appear primitive when in fact they reflect a process of reduction from a more complex ancestor. It was also noted that microsporidian gene sequences are highly divergent and possibly misleading due to an artifact in phylogenetic reconstruction known as "long branch attraction." It was even noted that the fused 5.8S-LSU rRNA in microsporidia could easily be a secondarily derived state since microsporidian rRNAs are extremely strange compared with other eukaryotes, or even when compared with prokaryotes. Microsporidian rRNAs are exceptionally small because they have sustained a number

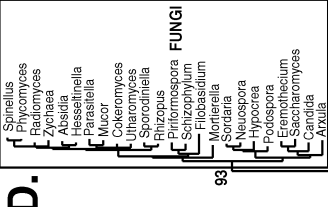
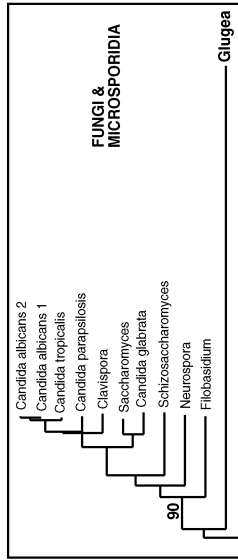
of deletions, sometimes in regions of the sequence that are highly conserved even between prokaryotes and eukaryotes. It was reasoned that a deletion that affected one of the rRNA operon processing sites could generate the fused microsporidian rRNA from an ancestral eukaryotic rRNA operon (14).

These concerns eventually proved to be well founded, as further sampling of microsporidian genes soon revealed that not all evidence supported an ancient origin for the group. The first phylogenies to contradict the evidence for an early origin of microsporidia were those of alpha-tubulin and beta-tubulin (24, 46). Here the microsporidia formed a surprising but extremely well-supported group with fungi. This alternative, fungal origin for microsporidia is in complete disagreement with earlier evidence that microsporidia are ancient or primitive organisms because fungi are not considered ancient or primitive and are now known to be close relatives of animals. How could different genes from the same organisms provide such different phylogenetic trees? One possibility is that the accelerated rates of substitution common to many microsporidian genes led to artifacts in the phylogenetic reconstruction, and it was proposed that the ancient origin of microsporidia was erroneously suggested by the highly derived genes of rRNA and elongation factors. However, tubulin phylogenies are not immune to this problem, and it was noted that fungal and microsporidian tubulins are both highly divergent [perhaps because neither group contains 9 + 2 structures, releasing evolutionary constraints on their tubulin gene sequences (46)], suggesting that these phylogenies may be in error. It has now been demonstrated, however, that both alpha- and beta-tubulins from the flagellated chytrid fungi are highly conserved, and in phylogenies where chytrids are the only fungal representatives, microsporidia still branch with fungi with high statistical support. This shows that the relationship between microsporidia and fungi in tubulin phylogenies is not a long branch artifact (48).

The evidence from tubulin phylogenies led to an immediate re-evaluation of certain characteristics of microsporidia such as an insertion in the EF-1 α protein, (43) as well as unusual features of their meiosis that had previously been recognized as resembling homologous processes in fungi (28). In addition, genes encoding TATA-box-binding protein (26), mitochondrial HSP70 (33, 37, 71), glutamyl-tRNA synthetase (10), and the largest subunit of RNA polymerase II (RPB1) (38) were also sequenced from microsporidia, and the phylogenies of each of these genes further supported the fungal origin of the group—strongly in the case of RPB1. The genome of *E. cuniculi* also yielded a number of proteins with strong fungal affinities (44), and a recent analysis of combined molecular data from four genes further supports the fungal origin of microsporidia (3). Also of critical importance is that much of the evidence for the ancient origin of microsporidia has recently been undermined by the re-analysis of genes that had previously supported the early-branching position. Re-analyzing EF-2 and LSU rRNA data using methods that take into account variations in substitution rates at different sites in a sequence showed that these genes not only fail to support the ancient position of microsporidia but actually weakly support the fungal relationship (38, 89). Some examples of these phylogenies are shown in Figure 3, and a summary of molecular



Figure 3 (Continued on next page)



GARDIA	GINMIESDKMFWYE-----GFLCDLADQLGAK
Spironucleus	GUSINESFNPMWYS-----GFLCDLADQLGAK
Trichomonas	GNNTEKSTNFWYN-----GFLLEALDQLQFP
Trichonympha	GNNTEKSKFMWKK-----GFLLEALDQLQFP
Trichomyxa	GNNTEKSKFMWKK-----GFLLEALDQLQFP
Leishmania	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Euglena	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Clatispora	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Zychnochia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Marasmiella	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Colearomyces	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Ultraromyces	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Sporodielia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Hirizopus	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Periomospora	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Spinizopylium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Filobasidium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Zea	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Mordania	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Sordaria	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Neurospora	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Hypocrea	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Podospira	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Eremothelium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Entamoeba	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Trichomyces	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Candida	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Aradia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Psychotomophra	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Heliothis	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Bombyx	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Drosophila	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Tentonia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Peromyscus	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Xenopus	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Danio	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Caenorhabditis	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Onchocerca	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Ephydratia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Hydra	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Planorostellium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Spinellium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Planorostellium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Arabidopsis	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Zea	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Oryza	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Glycine	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Arabidopsis	GNNMDESSEMAWYK-----GFLLEALDQLQFP
99	GNNMDESSEMAWYK-----GFLLEALDQLQFP
73	GNNMDESSEMAWYK-----GFLLEALDQLQFP
T. cruzi	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Leishmania	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Podipha	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Stylonichia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Entamoeba	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Plasmodium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Tetrahymena	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Paramecium	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Oryzias	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Blastocystis	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Trichomonas	GNNMDESSEMAWYK-----GFLLEALDQLQFP
S. barkhanus	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Giardia	GNNMDESSEMAWYK-----GFLLEALDQLQFP
Hexamita	GNNMDESSEMAWYK-----GFLLEALDQLQFP
S. muris	GNNMDESSEMAWYK-----GFLLEALDQLQFP

Glugea
MICROSPORIDIA

0.1

0.1

phylogenies including microsporidia is given in Table 1. Furthermore, the notion that microsporidia are primitively amitochondriate has been disproved by two means. First, if they are fungi then we know that microsporidia must be derived from mitochondrial ancestors since fungi and all their close relatives contain mitochondria. Second, genes for proteins derived from mitochondria have been found in microsporidia. Most mitochondria contain a small genome, but the majority of genes for mitochondrial proteins are encoded in the nucleus and their products are post-translationally targeted to the organelle. Therefore, it has been reasoned that an organism that has drastically altered its mitochondrion, or even lost it altogether, could retain relic mitochondrial genes in the nuclear genome (17). One such gene, encoding mitochondrial HSP70, has now been characterized in three species of microsporidia: *N. locustae*, *Vairimorpha necatrix*, and *E. cuniculi*. The presence of this gene in these organisms gives solid confirmation that microsporidia had a mitochondriate ancestry, and in some analyses even supports a fungal origin (33, 37, 71). Now, several more mitochondrion-derived genes have been found in microsporidia (25, 44), the evolutionary implications of which will be considered when the current presence or absence of mitochondria is discussed below.

Altogether, there are now a number of gene phylogenies that provide robust support for some relationship between microsporidia and other fungi, but what exactly is this relationship? Most genes that have been used to test this have only included ascomycetes and occasionally basidiomycetes. With such poor sampling of fungi, it is not clear from these studies if microsporidia actually are fungi, or if they are merely a closely related sister group of fungi. Unfortunately, only two genes have currently been sampled from diverse fungi to better define this relationship, and these are alpha- and beta-tubulins. In the case of beta-tubulin there is strong support for microsporidia actually evolving from within the fungi, but phylogenies fail to distinguish whether microsporidia are specifically related to ascomycetes or zygomycetes (48). Alpha-tubulin also strongly supports microsporidia evolving from within the fungi, but in this case, and in analyses combining both genes, the microsporidia show a specific relationship to zygomycetes (P.J. Keeling, unpublished

Figure 3 Phylogenies of four microsporidian proteins showing several types of microsporidian phylogeny. (A) Beta-tubulin and (B) RPB1 phylogenies show strong support for microsporidia being related to, or derived from, fungi. (C) Elongation factor-2 was originally reported to show microsporidia branching early among eukaryotes, but re-analysis taking into account site-to-site rate variation actually shows the microsporidia branching weakly with fungi. (D) Elongation factor-1 α is an example of a phylogeny that consistently places microsporidia early among eukaryotes; however, it is an extremely divergent gene, calling into question its use as a phylogenetic marker for microsporidia. This is particularly interesting in the case of EF-1 α since the microsporidian gene actually contains an insertion otherwise found only in animals and fungi (*inset*). All trees were inferred using gamma-corrected distances and weighted neighbor joining as described in (25).

TABLE 1 Summary of published phylogenetic trees showing position of microsporidia

Gene	“Deep” branching	Unresolved/ intermediate	Fungal
Alpha-tubulin			(24, 46, 48)
Beta-tubulin			(24, 46, 48)
RPB1			(38)
TBP			(26)
Glu-tRNA synthetase			(10)
Ser-tRNA synthetase			(44)
V-ATPase-A			(44)
TF IIB			(44)
GTPase			(44)
mt HSP70	(37, 71)	(71)	(33, 37)
LSU rRNA	(70)	(70)	(89)
EF-2	(42)		(38)
Ile-tRNA synthetase		(9)	
mt PDH-alpha		(25)	
mt PDH-beta		(25)	
EF-1 α	(42, 43)	(38)	
SSU rRNA	(96)	(89)	
eIF-2 γ	(47)		
Gln-tRNA synthetase	(10)		
Proteosome alpha	(8)		

data). Interestingly, a zygomycete origin for microsporidia was proposed based on the superficial similarities between the polar tube and the apical spore body of harpellalean zygomycetes (15). However, molecular evidence to date does not suggest a specific relationship between microsporidia and harpellalean zygomycetes or any other group of zygomycetes, except in certain analyses where microsporidia are related to entomophthorales (48).

While the exact relationship between microsporidia and fungi remains to be clarified, nearly all current evidence does support one major conclusion: Microsporidia are not ancient eukaryotes, but are instead highly evolved fungi. This conclusion colors nearly all other aspects of microsporidia in a new light: No longer are they primitive in lacking mitochondria, flagella, or peroxisomes—these features result from reductive evolution, probably in response to their growing adaptation to intracellular parasitism (the mitochondrion is a special case discussed below). Similarly, microsporidian biochemistry is not primitive, it is reduced. Even at the molecular level, the tiny genomes of microsporidia evolved from larger genomes

by gene loss and compaction, and the unusual genes and gene sequences we find today are highly derived, not ancient (49).

CORE METABOLISM

Much of the metabolism of eukaryotes centers around the mitochondrion, the so-called powerhouse of the cell. However, ultrastructural studies on microsporidia in the 1960s revealed no mitochondrion (91), and no study since then has actually visualized an organelle answering to the description of a typical mitochondrion in any microsporidian (63, 92). We now know that microsporidia evolved from mitochondriate fungi, but two interesting questions remain: What was the fate of the microsporidian mitochondrion, and how has their core metabolism adapted?

Metabolic pathways for energy generation from carbohydrates have more or less been worked out from several amitochondriate protists, in particular the parabasalian *Trichomonas vaginalis*, the diplomonad *Giardia lamblia*, and the entamoebid *Entamoeba histolytica* (data are also beginning to accumulate from the apicomplexan, *Cryptosporidium*) (65, 73). In addition, a number of genes encoding enzymes involved in core metabolic pathways have also been characterized from these organisms. The metabolic picture that has emerged from these studies shows some commonality between these disparate organisms, but also a great deal of variation. In general all these organisms lack electron transfer chains, oxidative phosphorylation, and the tricarboxylic acid (TCA) cycle. All break down glucose using the glycolytic pathway, which is like that of other eukaryotes except that phosphofructokinase is pyrophosphate-dependent rather than ATP-dependent (65). From phosphoenol pyruvate, pyruvate is formed directly or can be formed using a malate bypass not found in typical eukaryotes. Pyruvate metabolism is perhaps the defining difference between these amitochondriates and other eukaryotes. Typically, pyruvate enters the mitochondrion and is oxidatively decarboxylated by the pyruvate dehydrogenase complex (PDHC), but in the amitochondriate parabasalia, diplomonads, and entamoebids, it is decarboxylated by a single enzyme, pyruvate:ferredoxin oxidoreductase (PFOR), an iron-sulphur protein also used by anaerobic bacteria. Electrons are transferred from pyruvate to ferredoxin by PFOR, then to NADH, and ultimately to an organic terminal electron acceptor. In the parabasalia, the terminal electron acceptor is ionic hydrogen, resulting in the production of hydrogen gas, and the reactions following pyruvate production take place within a membrane-bound organelle called the hydrogenosome (64). Conversely, in *Giardia* and *Entamoeba* all the reactions of core carbon metabolism are cytosolic. Altogether, sugar metabolism in these organisms is substantially less efficient than that of mitochondriate eukaryotes, relying as it does on what has been termed "extended glycolysis" and substrate-level phosphorylation (65).

Until recently, microsporidia could not be compared to these other "amitochondriates" since next to nothing was known about their metabolic capacities, largely because their obligate intracellular growth and division presents serious challenges to biochemical assays (83). Nevertheless, some biochemical assays

were carried out using purified spores and *in vitro*-germinated spores maintained for a short time in a cell culture medium (102). Altogether, these studies confirmed the suspected lack of TCA cycle, showed a requirement for ATP in the sustaining media (buttressing suspicions that these parasites probably import ATP from their hosts), determined that the parasites produce lactic acid and pyruvic acid, and demonstrated the presence of several enzymes involved in glycolysis, the pentose-phosphate pathway, as well as trehalose synthesis and degradation (22, 84, 102).

These studies indicate that microsporidia have retained the glycolytic pathway and suggest that they probably use extended glycolysis. However, the first microsporidian gene encoding an enzyme for core carbon metabolism proved that they are very different indeed. The *N. locustae* genome was found to encode both the alpha and beta subunits of pyruvate dehydrogenase (PDH, or PDHC E1), the first enzyme of the pyruvate dehydrogenase complex (25). In mitochondriate eukaryotes, PDH is responsible for the decarboxylation of pyruvate and gives rise to the “active aldehyde” intermediate, 2-alpha-hydroxyethyl-thiamine pyrophosphate (HETPP), which is then converted by PDHC E2 (dihydrolipoamide acetyl transferase) and E3 (dihydrolipoamide dehydrogenase) into acetyl-CoA. Genes encoding PDH subunits have also been found in the genome of *E. cuniculi*, but PDHC E2 and E3 are absent, which suggests that the role of PDH in microsporidia is unique. One possible explanation stems from the observation that PDH and PFOR actually share certain similarities in both structure (16) and biochemical activity; in particular, both use HETPP as an active intermediate (62). Accordingly, microsporidian PDH might be synthesizing HETPP to reduce the iron-sulphur center of a second protein, which then transfers electrons to ferredoxin, in effect mimicking the activity of PFOR but without actually using the enzyme (25). The catalog of genes present in the *Encephalitozoon* genome does indeed confirm that microsporidia use a completely novel form of core metabolism. Most importantly, the *Encephalitozoon* genome encodes genes for PDHC E1 (but not E2 or E3) as well as ferredoxin, ferredoxin:NADPH oxidoreductase, and a number of proteins involved in synthesizing iron-sulphur centers (44). Microsporidia also differ from other amitochondriates in that phosphofructokinase is related to the ATP-dependent enzyme of other fungi rather than being pyrophosphate dependent. A schematic representation of certain pathways inferred to be important in core energy metabolism in microsporidia is shown in Figure 4.

The presence of PDH-based pyruvate metabolism in *Nosema* and *Encephalitozoon* breaks one of the main “rules” of amitochondriate metabolism, namely the use of PFOR or some derivative of PFOR rather than PDH (65). Yet, this difference also epitomizes “amitochondriate” metabolism in that the core carbon metabolic machinery of these protists appears to have been cobbled together piece by piece, probably directed more by what enzymes happened to be available than by which ones might be brought together to make the most efficient system (65). This metabolism provides a fine example of evolution working as a “tinkerer” as proposed by Jacob (40, 41, 45).

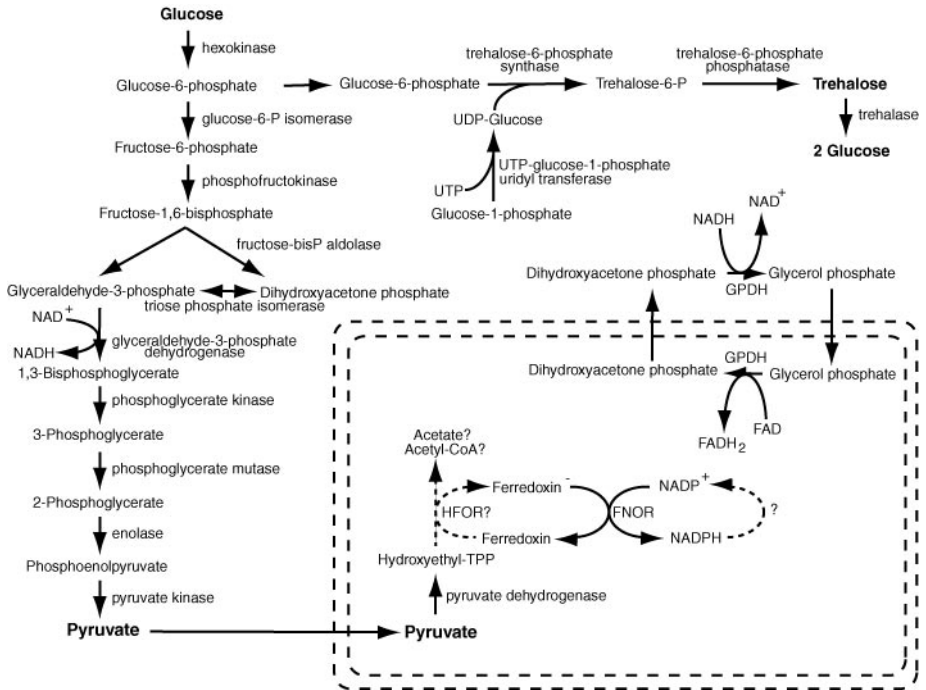


Figure 4 Schematic of microsporidian core carbon metabolism inferred from presence and absence of genes in microsporidia. Only certain pathways focusing on glucose metabolism are shown; others have been left out for clarity and can be seen in (44).

It is abundantly clear from the discovery that microsporidia are related to fungi, their possession of mitochondrion-derived HSP70 and PDH, and from a wealth of data from the *Encephalitozoon* genome that microsporidia evolved from a mitochondrion-containing ancestor. But none of these data actually tells us whether they still contain mitochondria today. The lack of mitochondria was originally proposed based on the absence of identifiable structures in ultrastructural investigations (91), but it has been shown repeatedly that organelles can easily go unnoticed. The apicomplexan plastid was only identified by its tell-tale genome (61, 104), and the mitochondrion of *Entamoeba* was only just identified by localizing one of the handful of proteins predicted to be derived from the mitochondrial endosymbiont; since it lacks a genome its identification is much more difficult (82). Conversely, the mitochondrion-derived Cpn60 protein from the diplomonad *Giardia* has also been localized, and it is apparently not contained in a membrane-bound organelle; so it appears to be possible that mitochondria can be completely lost while the host genome retains genes derived from the endosymbiont (72, 77). In the case of microsporidia, there are conflicting views on whether the mitochondrion may have been lost outright or persists today in a reduced and derived form.

The best evidence to date focuses on the potential amino-terminal leaders of mitochondrion-derived proteins to see if they are potentially mitochondrion-targeting transit peptides. The three initial reports of mitochondrial HSP70 genes all came to different conclusions regarding the amino termini of these genes: It was proposed that they were targeted to a mitochondrion, that they likely were not, or that they could be targeted to peroxisomes instead. A re-analysis of the HSP70 leaders, as well as those of PDH alpha and beta, came to the conclusion that they did not appear to have amino-terminal mitochondrion-targeting sequences (25). However, there is now credible evidence from a variety of proteins encoded in the *Encephalitozoon* genome that several proteins may contain mitochondrial-targeting transit peptides, leading to the proposition that a cryptic mitochondrion, a mitosome, would likely be found in microsporidia (44). If the metabolic profile of microsporidia inferred from the presence and absence of genes in core metabolic pathways is examined, one can also see that some reactions may necessitate the presence of a membrane-bound organelle. One example from the *Encephalitozoon* genome is the glycerol-3-phosphate shuttle, where dihydroxyacetone phosphate (DHAP) is reduced to glycerol-3-phosphate (oxidizing NADH in the process), which is transported to the mitochondrion where it is oxidized to re-form DHAP (reducing FAD in the process). *Encephalitozoon* contains both cytosolic and mitochondrial homologs of the key enzyme in this shuttle, glycerol phosphate dehydrogenase (GPDH) (44). The significance of this pathway in microsporidia is that the shuttle is present in order to move electrons across the mitochondrial envelope, so the presence of this pathway implies that microsporidia do indeed contain a membrane-bound relic mitochondrion and not just a few leftover enzymes. Conclusive evidence for this hypothesis will only come from localizing mitochondrion-derived enzymes to an organelle in microsporidia.

MICROSPORIDIA IN THE NEXT 150 YEARS

In 2001, microsporidian research was transformed by the completion of the *E. cuniculi* genome sequence (44). These data immediately provided insight and compelling answers to a number of questions about microsporidian biology that had previously been matters of hypothesis. Yet, we are really only beginning to scratch the surface of understanding just how these parasites have become so well adapted to their parasitic lifestyle. In terms of microsporidian genome size, *Encephalitozoon* is more of an exception, rather than the rule, having one of the most reduced genomes. Different lineages of microsporidia have reduced their genome sizes at different rates, doubtless losing and retaining pathways differentially. One question that arises from the *Encephalitozoon* genome is, "What makes up the difference between small and large microsporidian genomes?" Are larger genomes less compact, have they undergone less-severe reduction, or have their environments simply allowed for the loss of fewer genes? In addition to questions of biology, our current interpretations of microsporidian origins are still clouded by our lack of specific information about their ancestors. Although we are confident

that microsporidia and fungi are related, the specifics of their relationship await further analysis. Once the origin of microsporidia has been unambiguously resolved, it may be possible to reconstruct how they might have evolved to be the highly specialized parasites we see today. This reconstruction process could provide important insights into microsporidian biology, and may also give us a glimpse of the process of adapting to parasitism.

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