Charles University in Prague, Faculty of Science
Department of Parasitology

Evolution of hydrogenosomes: adaptation of free living protists *Mastigamoeba balamuthi* and *Naegleria gruberi* to oxygen-poor environment

PhD. Thesis

Mgr. Eva Nývltová

Thesis supervisor: prof. RNDr. Jan Tachezy, PhD.

Prague, 2015

"Most people say that it is the intellect which makes a great scientist.
They are wrong: it is character."

(Albert Einstein)

Many thanks to my very patient supervisor Jan Tachezy, great advisor Ivan Hrdý, computer and antibody champion Honza Mach and always helpful laboratory assistant Michaela Marcinčíková, as well as and to all fellows for support and help during my studies.

I declare that I substantially contributed to the results presented in this thesis.

Eva Nývltová

Data presented in this thesis resulted from team collaboration at the Laboratory of biochemical and molecular parasitology and during projects with our partners. I declare that the involvement of Mgr. Eva Nývltová in this work was substantial and that she contributed significantly to obtain the results.

.....

prof. RNDr. Jan Tachezy, PhD.
Thesis supervisor

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 26. 2. 2015

.....

Eva Nývltová

Contents

Abstract

Various protists from different eukaryotic groups are able to live in the oxygen-poor niches. Their metabolic adaptation to anaerobiosis is usually associated with loss of the typical mitochondrial functions, including the tricarboxylic acid cycle and oxidative phosphorylation. The anaerobic forms of mitochondria generate ATP exclusively by the substrate level phosphorylation as observed in the hydrogen-producing hydrogenosomes, or the ATP synthesis is completely lost in mitosomes. Consequently, the proteomes of such organelles are considerably reduced. It is a question of debate whether the anaerobic forms of mitochondria evolved directly from premitochondrial organelles that might be present in ancient anaerobic eukaryotes or during the secondary adaptation of aerobic eukaryotes to anaerobic niches.

The protist from the super group Amoebozoa, *Mastigamoeba balamuthi*, is very attractive for study of mitochondria evolution, because it is closely related with two very different organisms: the aerobic, free-living slime molds such as *Dictyostelium* that possesses classical aerobic mitochondria, as well as the anaerobic parasite *Entamoeba histolytica* that contains mitosomes, the most reduced form of mitochondria. The mitochondria derived organelles in anaerobic, free-living *Mastigamoeba balamuthi* can represent the intermediate stage between mitochondria and mitosomes. The functional analysis of *M. balamuthi* organelles revealed that these organelles are metabolically active and possess the hydrogen and ATP-generating pathway, which is typical for anaerobic energy metabolism in the hydrogenosomes. This pathway (extended glycolysis) includes the enzymes pyruvate:ferredoxin oxidoreductase (PFO), [FeFe]-hydrogenase, and ADP-forming acetyl-CoA synthetase (ACS). Interestingly, extended glycolysis is duplicated in the cytosol. The most conserved function of all mitochondria is the FeS cluster assembly, which is present also in Mastigamoeba hydrogenosomes. However, the typical mitochondrial ISC machinery is replaced by the bacterial NIF-like system and this system is also duplicated in the cytosol. Based on the functional and phylogenetic analysis we proposed that the transition of mitochondria-to-hydrogenosomes in *Mastigamoeba* included acquisition of genes encoded the enzymes of anaerobic metabolism by lateral gene transfer. The products of these genes initially operated in the cytosol. After the gene duplication and acquisition of mitochondrial targeting signal, the pathways were parallelized in the mitochondria, whereas most of the mitochondrial pathways were lost.

The mitochondria of *Naegleria gruberi*, a member of Excavata supergroup, might represent another example of the aerobic-to-anaerobic transition of mitochondrial metabolism. Although these organelles possess the typical pathways of aerobic mitochondria including oxidative phosphorylation, it has been recently predicted that they also possess [FeFe]-hydrogenase, a typical enzyme of anaerobic metabolism. Using gas chromatography, we confirmed that aerobically grown *N. gruberi* indeed produces molecular hydrogen. However, the active hydrogenase was detected only in the cytosolic fraction, while no hydrogenase activity was associated with the mitochondria. This result supports our hypothesis that the cytosol is the primary site of hydrogenase upon the acquisition of corresponding gene by LGT and only in some organism; the hydrogenase was duplicated and targeted to the mitochondria that were transformed to the hydrogenosomes.

1 Introduction

Eukaryotic organisms developed from prokaryotes during the evolutionary process taking almost 2 billion years (Kurland and Andersson, 2000). One of the major differences between prokaryotes and eukaryotes is compartmentalization of various metabolic function into specialized compartments and formation of organelles (Williams and Keeling, 2003). Although prokaryotes ruled the Earth for 1,5 billion years before eukaryotic expansion (Knoll, 2003), they failed to evolve in more elaborated morphological complexity. Even though some bacteria are able to form multicellular organisms (Kroos, 2005; Velicer and Yu, 2003), it seems that the recent bacteria are not much different than those in the earliest known fossils (Knoll, 2003).

One of the notable compartments of the eukaryotic cell is the mitochondrion. The presence of mitochondrial in all extant lineages eukaryotes implies that mitochondria occurred at an early stage of eukaryotic evolution, long before acquisition of plastids (Embley and Martin, 2006; Hjort et al., 2010; Williams and Keeling, 2003). Mitochondria as ATP-generating organelles usually function as the center of energy metabolism in the cell. In addition, mitochondria are involved in many other processes such as synthesis of heme (Scheffler, 2001), steroids (Miller, 1995), amino acid metabolism, iron-sulfur (FeS) cluster biogenesis and programmed cell death (Kroemer et al., 1995; Lill et al., 1999; Lill and Mühlenhoff, 2005; Williams and Keeling, 2003). Mitochondria are always surrounded by a double membrane; however their internal morphology is diverse and depends on the physiological state of the cell. Mitochondria can be present as individual organelles as well as a highly dynamic network, with membrane envaginations (cristae) as well as cristae-less morphology (Lloyd, 1974; Priest and Hajduk, 1994; van der Giezen and Tovar, 2005).

The prevailing hypothesis for the origin of mitochondria is the serial endosymbiotic theory (Sagan, 1967). This hypothesis suggests that mitochondria evolved during a unique endosymbiotic event between the α-proteobacterium-like mitochondrial ancestor and the host cell some 1,5 billion years ago (Dyall et al., 2004; Makiuchi and Nozaki, 2014; Martin and Müller, 1998; Williams and Keeling, 2003). Their monophyletic origin is supported by comparative genomic studies of mitochondrial DNA (Gray et al., 1999; Kurland and Andersson, 2000; Martin, 2005; van der Giezen and Tovar, 2005). During eukaryogenesis, the endosymbiont and the host cell became mutually dependent. The endosymbiont-to-organelle transition was associated with a large-scale reduction of the endosymbiont, especially its genome (Gray et al., 1999; Kurland and Anderson 2000; Williams and Keeling 2003). Most of the endosymbiont genes were lost or transferred to the host-cell nucleus (Adams and

Palmer, 2003; van der Giezen and Tovar, 2005). Although mitochondria usually retain a complete system for gene expression, less than 1% of mitochondrial proteins are encoded by the organellar genome, therefore the majority of the mitochondrial proteins is encoded by the nucleus and post-translationally delivered to the organelles. This process required evolution of a specific eukaryotic system for targeting and translocation of proteins across outer and inner mitochondrial membranes (Pfanner and Geissler, 2001; Williams and Keeling, 2003).

Several hypotheses were proposed to describe the character of the endosymbiosis between an ancestral α-proteobacterium and the original host cell. Before discovery of reduced forms of mitochondria, the Archaezoa hypothesis attracted high attention and become very popular. This hypothesis proposed the existence of a primitive amitochondrial eukaryote that gained mitochondria in later step of eukaryogenesis. Based on this hypothesis the taxon Archaezoa was proposed uniting "amitochondrial" eukaryotes such as Metamonada, Microsporidia, Parabasalia, and Archamoebae that were believed to have developed from the direct descendants of the ancient eukaryotes (Cavalier-Smith, 1986; Cavalier-Smith, 1989). However, phylogenetic trees reconstruction the evolutionary history of the ribosomal DNA sequences demonstrated that many of these "amitochondriates" branched with other eukarytic lineagues in the eukaryotic tree (Cavalier-Smith, 1993; Gray et al., 1999; Leipe et al., 1993; Sogin et al., 1993). As well as identification of genes of mitochondrial origin in "amitochodriates" as well as the discovery of reduced forms of mitochondria in these organisms weakened this attractive hypothesis (Bui et al., 1996; Hirt et al., 1997; Keeling and Doolittle, 1995).

The syntrophy hypothesis proposed a bacterial symbiotic relationship between methanogenic archaea and facultative fermentative-sulfate reducing δ-proteobacteria (ancestral myxobacteria). The syntrophic symbiosis was based on possible transfer of molecular hydrogen between two partners in anaerobic environments: proteobacteria that could synthesize hydrogen as the metabolic end-product, and archaea that could utilize hydrogen for synthesis of methane with concomitant ATP production. This relationship possibly led to close cell-cell interactions and to highly evolve symbiotic structures, such as a complex membrane system. The symbiont genome extinction was accomplished by gradual transfer to the archaeal host, where genes adapted to a new genetic environment. The driving force of the evolution of the eukaryotic nucleus might not have been to isolate the genetic material from the cytoplasm, as is generally believed, but to allow the coexistence of two inter-dependent metabolic pathways in the protoeukaryotic cell. However the newly-emerged chimeric proto-eukaryote was amitochondriate until a second symbiosis with an α-

protobacterial metanotroph. The methanogenic proto-eukaryote might provide methane for the anaerobic or microaerophilic methanotrophic endosymbiont, which released carbon dioxide for the host cell (Moreira and Lopez-Garcia, 1998;Moreira and Lopez-Garcia, 2002).

The hydrogen hypothesis is another hypothesis based on a symbiotic metabolic association (syntrophy). This hypothesis assumed a symbiosis between a strictly anaerobic hydrogen-dependent methanogenic archaeon and a facultatively anaerobic α-proteobacterium which produced hydrogen but was able to respire and generate ATP in aerobic environments as well. The archaeon became dependent on hydrogen production by the α-proteobacterium, which was gradually engulfed and became an endosymbiont, which gave rise to mitochondria. According to this hypothesis, the first mitochondrial organelle was more similar to the recent anaerobic hydrogen-producing mitochondria or hydrogenosomes (organelles producing hydrogen) than to classical contemporary mitochondria (Embley and Martin, 2006; Martin and Müller, 1998). The phylogenetic analysis of most enzymes of aerobic energy metabolism (pyruvate dehydrogenase complex, *cytochrome b*, and subunits of the respiratory chain) support an α-proteobacterial ancestry of the mitochondrial endosymbiont (Gabaldon and Huynen, 2003; Karlberg et al., 2000; Kurland and Andersson, 2000). However, the evolutionary history of anaerobic enzymes does not support α-proteobacterial origin and are not uniquely associated with the mitochondrial organelles. Moreover, eukaryotic genes of the typical anaerobic enzyme [FeFe]-hydrogenase are not monophyletic in origin suggesting they have not originated from a common endosymbiont, though it is interesting that all eukaryotes contain non-hydrogen producing NARF proteins with homology to [FeFe]-hydrogenase (Barton and Worman, 1999; Embley et al., 2003a; Hug et al., 2010). Interestingly, the genes of another key anaerobic enzyme pyruvate:ferredoxin oxidoreductase are possibly monophyletic; however, their α-proteobacterial origin can be excluded (Embley et al., 2003b; Horner et al., 2000; Hug et al., 2010; Rotte et al., 2001).

An aerobic origin of mitochondria is proposed by the Ox-Tox model, according to which the first eukaryotic cell arose in an early-oxygen habitat. This hypothesis is supported by phylogenetic analysis of ATP transporters, which seem to have evolved after eukaryotic radiation (Andersson and Kurland, 1999; Andersson et al., 1998; Karlberg et al., 2000). It has been proposed that mitochondria protected the host cell from oxidative stress derived from oxidative phosphorylation at the later stages eukaryogenesis (Kurland and Andersson, 2000). It is very probable that initially endosymbiont-host cell system had very different functions than modern mitochondria. The integration of the ATP/ADP translocase into the workings of

the endosymbiont may been a driving force in the transformation of the endosymbiont into an organelle (Andersson and Kurland, 1999).

More recent isotope studies indicate that although oxygen was present in the atmosphere since approximately 2 billion years ago, the oceans still existed in an intermediate oxidation stage, where the lower layers were anoxic until about 600 Myr ago (Poulton et al., 2004; Shen et al., 2003). It has been proposed that the development of the first eukaryotic lineages with mitochondria (1,5 billion years ago) is connected with water habitat of anoxic waters called the Canfield ocean (Canfield, 1998; Gray et al., 1999; Javaux et al., 2001). In this case the selection pressure was probably not the benefit of oxygen detoxification provided by the endosymbiont and the first eukaryotes possibly evolved in anoxic waters (Canfield et al., 2007).

Hydrogenosomes are organelles with anaerobic energy metabolism producing molecular hydrogen, carbon dioxide and ATP. Presently, there are no doubts that hydrogenosomes and mitochondria are organelles of a common origin (Embley et al., 2003a). However, it is still debated whether hydrogenosomes evolved directly from premitochondrial organelles that might be present in ancient anaerobic eukaryotes or during secondary adaptation of aerobic eukaryotes to anaerobic niches. To contribute to this discussion we investigated double membrane bound organelles that were identified in the free-living protist *Mastigamoeba balamuthi*. This organism belongs to the Amoebozoa group, which is very attractive for study of mitochondria evolution as it includes closely related aerobic organisms with conventional mitochondria such as *Distyostelium* (Ogawa et al., 2000) as well as anaerobes such as *Entamoeba* with highly reduced forms of mitochondria called mitosomes (Tovar et al., 1999). The distribution of the enzyme hydrogenase, marker of anaerobic metabolism, is not monophyletic and can be found in mitochondrial organelles of several non-related anaerobically living eukaryotes. We investigated the cellular localization of the anaerobic enzyme [FeFe]-hydrogenase that was identified in the genome of the aerobic protist *Naegleria gruberi*, a member of the Excavata group and predicted to localize to the aerobic mitochondria (Fritz-Laylin et al., 2010).

2 Classification of organelles of mitochondrial type

Mitochondrial type organelles have been found so far in all studied eukaryotes, living in aerobic, microaerophilic as well as anaerobic habitats. Based on the character of energy metabolism, and the contribution of bacterial anaerobic enzymes, particularly hydrogenase, five types of mitochondrial organelles were distinguished: aerobic mitochondria, anaerobic mitochondria, hydrogen-producing mitochondria, hydrogenosomes and mitosomes (Müller et al., 2012). Importantly, neither energy metabolism nor other mitochondrial functions are common to all mitochondrial type organelles. Moreover, with increasing knowledge on functional diversity of mitochondria in various organisms, more exceptions and lineage-specific adaptations are observed, which complicate mitochondria categorization (Maguire and Richards, 2014).

2.1 Aerobic mitochondria

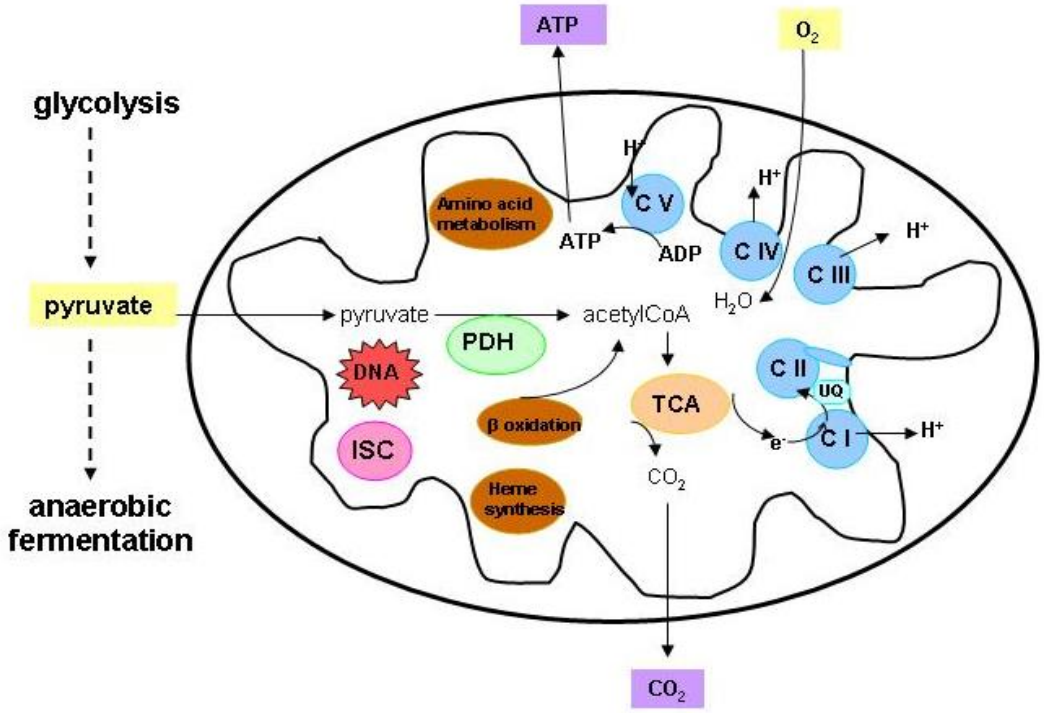

Figure 1. Schematic metabolic pathways in aerobic mitochondria. PDH – pyruvate dehydrogenase complex, TCA – tricarboxylic acid cycle, ISC – FeS cluster assembly machinery, C I, C II, C III, CIV– Complex I – IV of respiratory chain, C V – Complex V (ATP synthase), UQ - ubiquinone

Classical aerobic mitochondria generate ATP by the complete oxidation of substrates to carbon dioxide and water via the combined action of TCA (tricarboxylic acid) cycle

mitochondrial respiratory chain (Complex I – IV) and ATP synthase (Complex V). Pyruvate, as the main mitochondrial substrate, is produced by glycolysis in the cytosol and imported into the organelles. Here, pyruvate is metabolized by the pyruvate dehydrogenase complex (PDH) to acetyl-CoA, carbon dioxide and NADH. Acetyl-CoA condenses with oxaloacetate by activity of citrate synthase and enters the TCA cycle. Electrons that are generated during the TCA cycle are transferred via NADH and $FADH_2$ by respiratory chain Complexes I and II, respectively, to ubiquinone and subsequently by activity of Complex III to *cytochrome c*. Finally, Complex IV donates electrons to oxygen forming water (van Hellemond and Tielens, 1994; Voet D. and Voet J.G., 2004). Transfer of electrons from NADH and $FADH_2$ to oxygen is associated with the pumping of protons from the mitochondrial matrix, generating a pH gradient and membrane potential that is employed by ATP synthase (Fig.1). This process is also called oxidative phosphorylation and generates 23 moles of ATP per mole of glucose.

In addition, mitochondria play important roles in amino acid metabolism, biosynthesis of folate, biotin, phospholipids, and heme (Lange et al., 1999) and in formation of FeS clusters. The latter function is of particular importance. FeS cluster assembly is considered to be the only essential function of all mitochondria (Lill and Mühlenhoff, 2005) and is usually mediated by FeS cluster assembly (ISC) machinery that consists of over 10 proteins. The ISC machinery is required for formation of FeS cofactors that are essential for function of a large number of FeS proteins that are present in mitochondria as well as in other cellular compartments. In mitochondria, FeS clusters are pre-assembled on scaffold proteins and subsequently transferred to mitochondrial apoproteins. In the case of apoproteins that are present outside of mitochondria, the ISC system generates a so far poorly understood compound that is exported to the cytosol and utilized by the cytosolic FeS cluster assembly (CIA) system for maturation of FeS proteins outside of mitochondria (Lill and Mühlenhoff, 2008).

The majority of well-studied mitochondria possess a circular or linear genome with size ranging from 6 kbp in *Plasmodium* to 2000 kbp in some plants (Burger et al., 2003). However, mitochondria genome encodes only a small subset of genes relative to the observed proteome of mitochondria. Most of the mitochondrial proteins are encoded in the nucleus and translated in the cytosol. Proteins that are targeted to the mitochondrial matrix usually possess cleavable N-terminal mitochondrial targeting presequences (MTS), while membrane proteins possess inner targeting signals. Complex import machinery is present in mitochondrial outer and inner membranes as well as in the intermembrane space. The main components include translocase of the outer membrane (TOM), sorting and assembly machinery (SAM),

intermembrane space assembly machinery (MIA), and translocases of the inner membrane (TIM22 and TIM23), along with presequence translocase-associated motor (PAM) (Bauer et al., 1996; Dolezal et al., 2006; Neupert and Herrmann, 2007). The MTS is cleaved upon import of protein precursors into the mitochondria by mitochondrial processing peptidase (MPP). MPP comprises two subunits: a catalytic β-subunit, which binds a zinc cation, and a regulatory α-subunit with a flexible glycine-rich loop that serves for substrate recognition. Finally, processed proteins are folded into their mature form with the participation of the matrix chaperones Hsp70, Cpn60 and Cpn10 (Emanuelsson et al., 2000; Gakh et al., 2002). Evolution of protein import machinery was a key event for transformation of the bacterial endosymbiont to a eukaryotic organelle, a notion which is supported by presence of complex protein import machinery in all extant eukaryotic lineages (Embley and Martin, 2006).

2.2 Anaerobic mitochondria

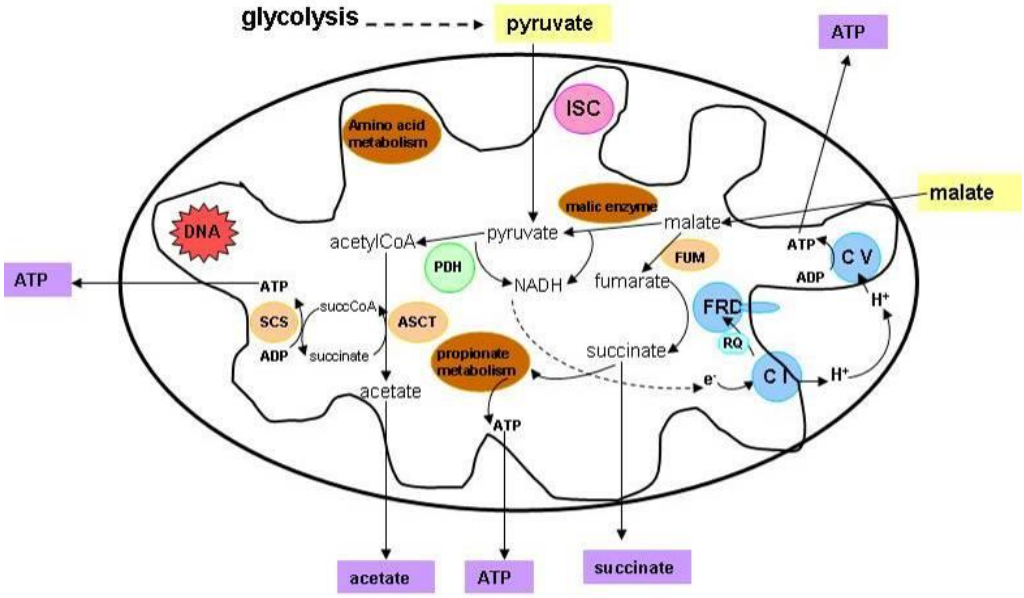

Figure 2. Schematic metabolic pathways in anaerobic mitochondria of helminths. PDH - pyruvate dehydrogenase complex, ASCT - acetate:succinate CoA transferase, SCS - succinyl-CoA synthetase, FUM - fumarase, FRD - fumarate reductase, ISC – FeS cluster assembly machinery, C I – Complex I of respiratory chain, C V - Complex V, RQ - rhodoquinone

Anaerobic mitochondria have been described in several eukaryotic species such as parasitic helminths, which occupy anaerobic conditions in part of their life cycle. In addition, some aerobically living eukaryotes are able to switch to anaerobic metabolism when they are

temporarily exposed to environmental hypoxic conditions such as marine worms, *Euglena* or the alga *Chlamydomonas reinhardtii*. Under oxygen-restricted conditions, their mitochondria have undergone various alterations that allow ATP synthesis without oxygen (Benichou et al., 1988; Dubini et al., 2009; Mus et al., 2007; Tucci et al., 2010).

The main differences from classical aerobic mitochondria are: (i) utilization of an alternative final electron acceptor: an endogenously produced electron acceptor, such as fumarate and succinate, or external acceptors such as nitrate, (ii) replacement of rhodoquinone instead of ubiquinone (if it is present), and (iii) production of different metabolic end products such as acetate, succinate, propionate or formate (Müller et al., 2012; Tielens et al., 2002; van Hellemond and Tielens, 1994).

In the anaerobic mitochondria of helminthes, malate is the main substrate of anaerobic mitochondria where it is metabolized to pyruvate by malic enzyme or fumarate by fumarase to fumarate. Pyruvate can be catabolized by PDH to acetyl-CoA can serve as a (i) CoA donor for acetate:succinate CoA transferase (ASCT) and succinyl-CoA synthetase (SCS) functioning in ATP generation or (ii) substrate for fatty acid biosynthesis. Electrons generated by malic enzyme are transferred via NADH to the membrane associated rhodoquinone by NADH-rhodoquinone oxidoreductase activity of respiratory Complex I and finally to fumarate through Complex II (rhodoquinol-fumarate reductase). The transfer of electrons by Complex I is coupled with pumping of protons across the inner mitochondrial membrane, this proton gradient fuels ATP synthesis via ATP synthase in the absence of oxygen (Fig. 2) (Tielens et al., 2002). Other important functions of anaerobic mitochondria are usually fatty acid synthesis, propionyl-CoA synthesis (Inui et al., 1984; Komuniecki et al., 1989), amino acid metabolism and FeS cluster biosynthesis by the mitochondrial ISC system.

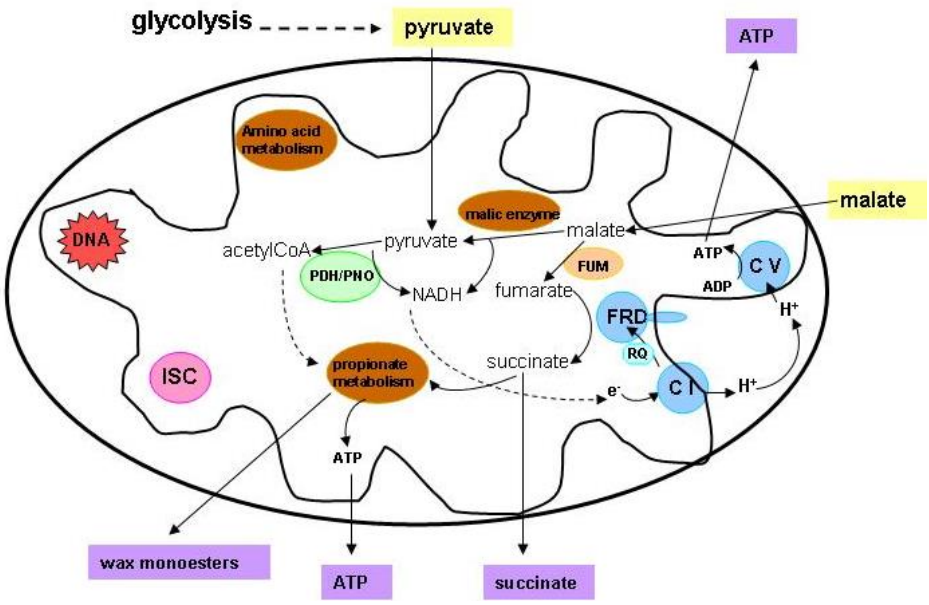

Figure 3. Schematic metabolic pathways in mitochondria of *Euglena gracilis* under anaerobiosis. PDH - pyruvate dehydrogenase complex, PNO - pyruvate:NADP oxidoreductase, FUM - fumarase, FRD - fumarate reductase, ISC – FeS cluster assembly machinery, C I – Complex I of respiratory chain, C V - Complex V, RQ - rhodoquinone

Euglena gracilis is a model euglenid, which is able to grow under aerobic as well as anaerobic conditions. Aerobically, *Euglena* uses oxygen as final electron acceptor and possesses regular aerobic mitochondria with slightly modified TCA and respiratory chain complexes. However, in anaerobic habitats, acetyl-CoA is used as the terminal electron acceptor, which is coupled with production of wax ester. Similarly to helminthes, malate is converted to either pyruvate or fumarate by malic enzyme or fumarase, respectively (Fig. 3). Fumarate is converted by rhodoquinol-fumarate reductase activity to succinate and subsequently a CoA moiety is added by SCS. Succinyl-CoA is then metabolized in three steps to propionyl-CoA and serves in lipid synthesis (Hoffmeister et al., 2004; Müller et al., 2012; Tucci et al., 2010).

In *Euglena*, pyruvate is oxidatively decarboxylated by activity of the PDH complex or alternatively by the typical anaerobic enzyme, pyruvate:NADP oxidoreductase (PNO) (Inui et al., 1987). Propionyl-CoA and acetyl-CoA together with NAD(P)H are used for production of acyl-CoA, which is exported to the cytosol where wax esters are formed (Hoffmeister et al., 2005). *Euglena* organelles lack substrate level phosphorylation and ATP is generated only by ATP synthase (Fig. 3) (Müller et al., 2012; Perez et al., 2014; Tucci et al., 2010). In addition,

the mitochondria of *Euglena* possess an ATP-producing glycolate metabolic pathway, which could markedly enhance photoheterotrophic growth (Collins et al., 1975).

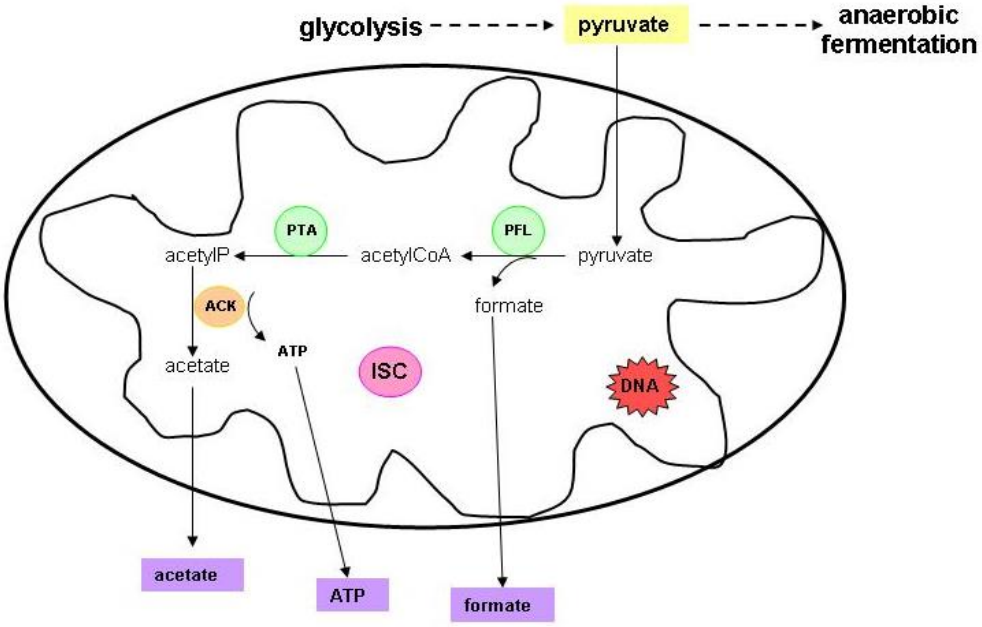

Figure 4. Schematic metabolic pathways in mitochondria of *Chlamydomonas reinhardtii* under anaerobiosis. PFL - pyruvate formate lyase, PTA - phosphotransacetylase, ACK - acetate kinase, ISC - FeS cluster assembly machinery

Chlamydomonas reinhardtii is a unicellular green microalga with oxygen-dependent respiration in mitochondria and active photosynthesis in green plastids (Atteia et al., 2009; Cardol et al., 2005; Eriksson et al., 1995). Under aerobic conditions the mitochondria work as aerobic mitochondria (Godman and Balk, 2008). However, under anaerobic conditions, *Chlamydomonas* induces pyruvate-dependent fermentative metabolism in mitochondria as well as in chloroplasts with the main end products including acetate, formate, ethanol and hydrogen. Interestingly, mitochondrial functions are very reduced and most of the metabolic function is present in the chloroplast including the enzymes pyruvate:ferredoxin oxidoreductase (PFO) and [FeFe]-hydrogenase, typically found in mitochondria-related organelles called hydrogenosomes (see also page 20). In the chloroplast, pyruvate is oxidatively decarboxylated to CO_2 and acetyl-CoA by PFO. Electrons that are released during this reaction are transferred by ferredoxin to hydrogenase, which synthesizes molecular hydrogen. Alternatively pyruvate can be converted to acetyl-CoA by the strictly anaerobic enzyme pyruvate formate lyase (PFL), which is probably localized in the chloroplast as well as in mitochondria (Atteia et al., 2006; Terashima et al., 2011). In chloroplasts acetyl-CoA is

metabolized by phosphotransacetylase (PTA) to acetyl-phosphate and then by acetate kinase (ACK) to acetate with concomitant synthesis of ATP. It has been suggested that the same anaerobic pathway including PFL, PTA and ACK is induced by anaerobiosis also in mitochondria (Fig. 4) (Atteia et al., 2006). However, PFO and [FeFe]-hydrogenase seem to be present only in plastids. In addition a complete set of six SUF (the sulfur mobilization) proteins was found in the *Chlamydomonas* genome (Godman and Balk, 2008). The SUF system appears to function under oxidative stress conditions (Nachin et al., 2003) and is probably localized in the chloroplasts, similarly to plants (Godman and Balk, 2008; Ye et al., 2006).

2.3 Hydrogen-producing mitochondria

These organelles combine characteristics of classical mitochondria with hydrogenosomes. As anaerobic mitochondria, hydrogen-producing mitochondria possess a complete or partial membrane-associated, proton-pumping electron transport chain. In addition, these organelles have an [FeFe]-hydrogenase that allows them to use protons as terminal electron acceptors, resulting in hydrogen production (Müller et al., 2012). The organisms with hydrogen-producing mitochondria include free-living protists that inhabit mainly aerobic niches such as *Naegleria gruberi* with fully active aerobic mitochondria or aero-tolerant protists such as *Acanthamoeba castellanii* and a recently described *Pygsuia biforma* with the most reduced form of hydrogen producing mitochondria (Fritz-Laylin et al., 2010; Leger et al., 2013; Stairs et al., 2014). Other representatives with hydrogen-producing mitochondria inhabit oxygen-poor niches of intestinal tracts of insects and vertebrates, such as *Nyctotherus ovalis* or *Blastocystis hominis* respectively (Akhmanova et al., 1998; Stechmann et al., 2008). With the exception of *P. biforma,* the hydrogen-producing organelles contain their own genomes.

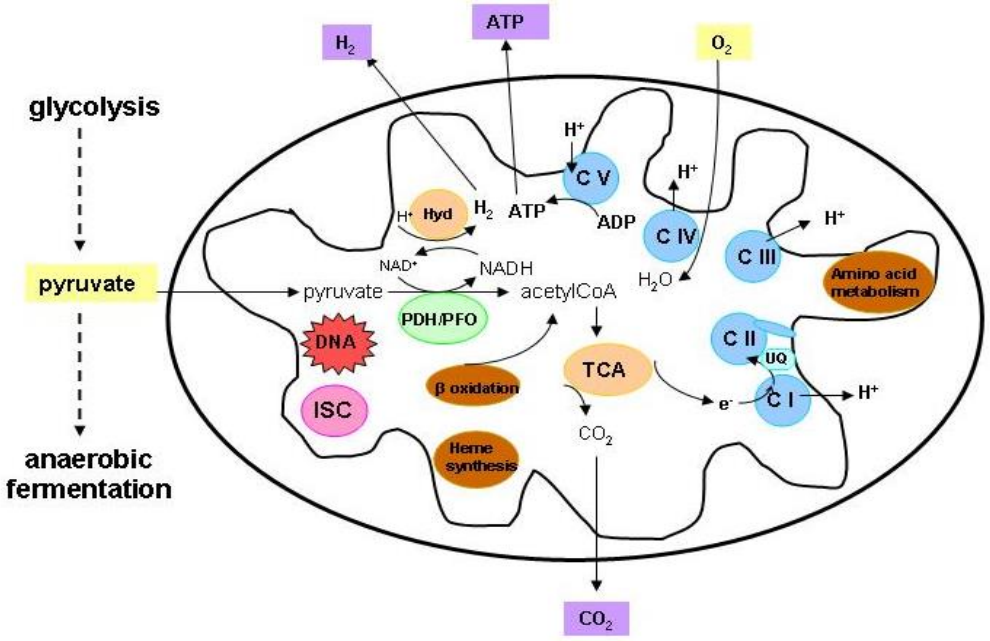

Figure 5. Schematic metabolic pathways in hydrogen-producing mitochondria of *Naegleria* and *Acanthamoeba*. PDH - pyruvate dehydrogenase complex (in *Naegleria* and *Acanthamoeba*), PFO - pyruvate: ferredoxin oxidoreductase (in *Acanthamoeba*), TCA – tricarboxylic acid cycle, Hyd - hydrogenase, ISC – FeS cluster assembly machinery, C I, C II, C III, C IV – Complex I – IV of respiratory chain, C V – Complex V (ATP synthase), UQ - ubiquinone

The free-living amoeboflagellate *Naegleria gruberi* lives predominantly in aerobic environments and possesses mitochondria with a complete set of pathways for cytochrome-dependent respiration and oxidative phosphorylation. Surprisingly, genome sequence analysis revealed the presence of genes for several components of anaerobic metabolism. Localization of the corresponding gene products has been predicted into the mitochondria (Fritz-Laylin et al., 2010). These components include [FeFe]-hydrogenase as well as three maturase proteins (hydE, hydF and hydG) for assembling of H-clusters in the hydrogenase. In the mitochondria pyruvate is catabolised by a conventional PDH complex and acetyl-CoA enters the TCA cycle (Fig.5) (Fritz-Laylin et al., 2010). Under oxygen-limited conditions, acetyl-CoA could be converted by ASCT to acetate and succinyl-CoA. Succinyl-CoA is further utilized by SCS to produce ATP and succinate, which is recycled (Fig.6). The genome also contains a gene for acetyl-CoA synthetase (ACS) that may catalyze a direct conversion of acetyl-CoA to ATP and acetate. It was suggested that *Naegleria* is capable of mitochondrial hydrogen production under anaerobic conditions (Opperdoes et al., 2011). However, the source of electrons for hydrogen synthesis is unclear, because PFO, a typical enzyme generating electrons during oxidative decarboxylation of pyruvate under anaerobic conditions, is not present.

Unfortunately, no biochemical data about hydrogenase activity or hydrogen production by *Naegleria gruberi* are available.

Acanthamoeba castellani is a free-living amoeba that also causes opportunistic infections in vertebrates. Based on *in vitro* experiments, *A. castellani* was believed to be strictly dependent on the presence of oxygen (Neff et al., 1958). However in nature, *Acanthamoeba* also encounters microaerobic or anaerobic habitats and is able to grow even faster in oxygen-low conditions (Cometa et al., 2011; Turner et al., 1997). Not surprisingly, mitochondria of *Acanthamoeba* are equipped with the classical mitochondrial pathways including complete TCA cycle and a cytochrome-dependent respiratory chain including Complex V (Fig.5) (Gawryluk et al., 2012; Gawryluk et al., 2014). However, the results from an EST and genome survey suggested that, in addition to aerobic pathways, mitochondria of *A. castellani* possess components of anaerobic energy metabolism as observed in hydrogenosomes, including PFO, [FeFe]-hydrogenase and ASCT. Mitochondrial localization of PFO, together with hydF and ASCT, was confirmed by a recent proteomic study (Gawryluk et al., 2014; Leger et al., 2013). However, as in the case of *Naegleria*, no biochemical data confirming the activity of PFO and hydrogenase have been provided.

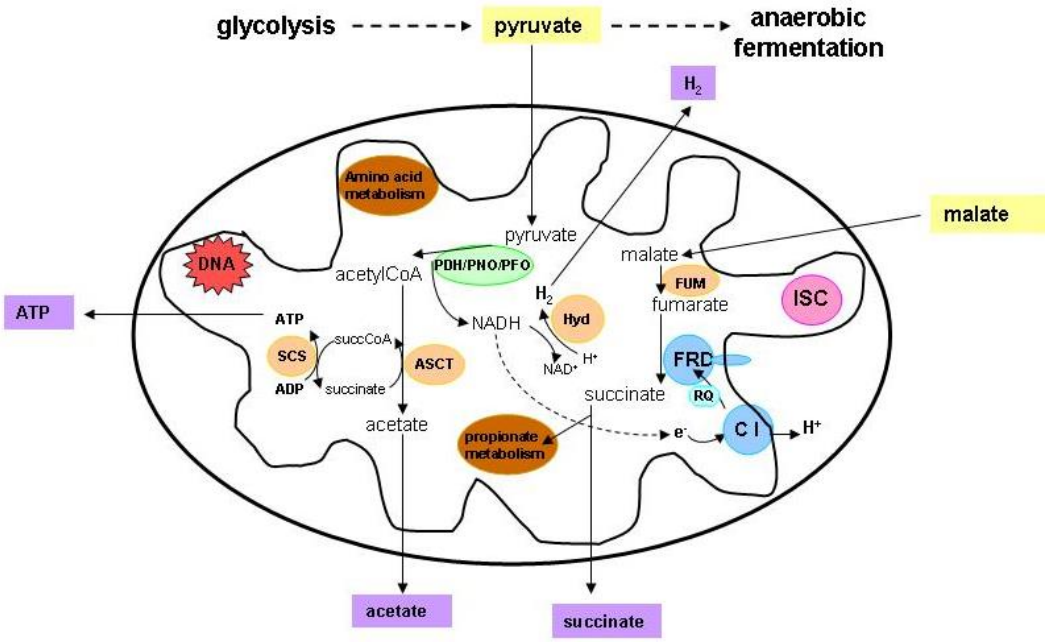

Figure 6. Schematic metabolic pathways in hydrogen-producing mitochondria of *Neocalimastix*, *Blastocystis* and *Pygsuia*. PDH - pyruvate dehydrogenase complex, PNO - pyruvate:NADP oxidoreductase, PFO - pyruvate: ferredoxin oxidoreductase, Hyd - hydrogenase, ASCT - acetate:succinate CoA transferase, SCS - succinyl-CoA synthetase, FUM - fumarase, FRD - fumarate reductase, ISC – FeS cluster assembly machinery, C I – Complex I of respiratory chain, RQ - rhodoquinone

The hydrogen-producing mitochondria of the anaerobic ciliate *Nyctotherus ovalis* from the hindgut of cockroaches were originally described as hydrogenosomes with a genome (Akhmanova et al., 1998; Boxma et al., 2005). However, based on the presence of functional Complex I, II and PDH complex, several components of TCA cycle and the genome, it has been proposed that these organelles represent an intermediate stage between mitochondria and hydrogenosomes (Martin, 2005). Recently, these organelles were included in a group of hydrogen-producing mitochondria (Müller et al., 2012). The main substrate for energy metabolism is pyruvate that is catabolised by mitochondrial PDH complex to acetyl-CoA. Acetyl-CoA is subsequently utilized by the typical hydrogenosomal enzyme ASCT, followed by SCS for production of ATP and acetate. The partial TCA cycle of *Nyctotherus* includes fumarase, succinate dehydrogenase (Complex II) and α-ketoglutarate dehydrogenase (KGD). However, the pathway proceeds in a reductive direction where the Complex II operates as fumarate reductase (FRD) (de Graaf et al., 2011; Müller et al., 2012). The electrons generated by activity of PDH and KGD are transferred to rhodoquinone via Complex I and ultimately to fumarate via Complex II (Fig.6). The electron transfer reactions performed by Complex I and II collectively contribute to the proton gradient via Complex I (Boxma et al., 2005). However this proton gradient is probably not used for ATP synthesis, because ATP synthase was not found (de Graaf et al., 2011). *Nyctotherus* lives in an endosymbiosis with methane-producing archaea, which use the hydrogen produced by the ciliate as a substrate (Gijzen et al., 1991).

It is likely that hydrogen-producing mitochondria/hydrogenosomes are present in several other ciliates living usually as endosymbionts. Occurrence of hydrogenosomes was reported for the rumen ciliates *Dasytricha ruminantium* (Yarlett et al., 1981), *Isotricha* species (Yarlett et al., 1983), Ophryoscolecidae (Snyers et al., 1982), and the entodiniomorphid *Polyplastron multivesiculatum* (Paul et al., 1990). *Metopus contortus* is a free-living ciliate with putative hydrogenosomes that inhabits anoxic marine sands (Biagini et al., 1997). However, limited experimental data are available to categorize these organelles.

Hydrogen-producing mitochondria with similar metabolism were described in stramenophile *Blastocystis hominis*. In *Blastocystis* organelles, pyruvate is converted to acetyl-CoA mostly by the anaerobic enzyme PNO (Lantsman et al., 2008), although the genes for two other pyruvate metabolizing enzymes were found including the PDH complex and PFO (Denoeud et al., 2011; Stechmann et al., 2008). However, only weak activity of PFO could be detected (our unpublished data), while contribution of the PDH complex to pyruvate metabolism remains unclear. Similar to *Nyctotherus*, acetyl-CoA enters the hydrogenosomal pathway of substrate level phosphorylation that leads to production of acetate and ATP

(Fig. 6) (Lantsman et al., 2008). A gene coding [FeFe]-hydrogenase was identified in the *Blastocystis* genome and presence of corresponding protein was detected in their mitochondria (Stechmann et al., 2008). However, neither hydrogenase activity nor production of hydrogen by the growing cells has been detected thus far (Lantsman et al., 2008, our unpublished data). Whether the TCA cycle is present is not clear. Genes for TCA cycle enzymes that may catalyze conversion of oxaloacetate to succinate were identified (Denoeud et al., 2011; Stechmann et al., 2008). However, Lantsman et al. (2008) detected activities of four other enzymes for which corresponding genes have not been identified so far. Genes coding for components of Complex I (20 subunits) and II (4 subunits) were identified, while other respiratory complexes are apparently absent (Denoeud et al., 2011; Stechmann et al., 2008). The other mitochondrial pathways including fatty acid biosynthesis, amino acid metabolism and FeS cluster assembly were predicted (Denoeud et al., 2011). Protein import machinery seems to contain standard composition including TOM, SAM, MIA, TIM and PAM complexes. Interestingly, a gene for the protein Tom40 has not been identified so far, probably due to its divergent nature; however, a gene for a receptor of inner targeting signals, Tom70, is present (Tsaousis et al., 2011).

Most recently, hydrogen-producing mitochondria were suggested to be present in the free-living breviate *Pygsuia biforma*. *Pygsuia* can be found in aerobic as well as anaerobic environments (Brown et al., 2013). Unlike in the others, the mitochondria of *Pygsuia* do not possess a genome and are more similar to the hydrogenosome. Reconstruction of metabolic pathways suggested that pyruvate is oxidatively decarboxylated by PFO to acetyl-CoA, which enters the pathway of substrate level phosphorylation with acetate and ATP as end products via the ASCT/SCS system. From typical aerobic mitochondrial function, partial TCA cycle, two components of Complex I (51 and 24 kDa subunits also found in *Trichomonas vaginalis*, see page 22) and a complete Complex II are present in *Pygsuia* (Fig.6). The partial TCA cycle possibly works in reverse direction from malate to succinate with a connection to fumarate reductase (Complex II). Complex I is incomplete but probably able to generate electrons and an electrochemical proton gradient, since the organelles can be visualized using the membrane potential dependent MitoTracker dye. Presence of carriers that mediate electron transport between Complex I and II is unclear (Stairs et al., 2014). Interestingly, in *Pygsuia* no evidence for mitochondrial ISC system was found except presence of Nfu1 protein, however, transcriptomic data revealed presence of fusion protein of SUFC and SUFB that is closely related to archael SUFC and SUFB proteins. The mechanism of FeS cluster assembly is thus unclear (Stairs et al., 2014).

2.4 Hydrogenosomes

Hydrogenosomes represent the next step in the reductive evolution of mitochondria. In contrast to hydrogen-producing anaerobic mitochondria, hydrogenosomes have completely lost the membrane-associated electron transport chain and genome. Since 1973 when they were first described in *Tritrichomonas foetus* (Parabasalids) (Lindmark and Müller, 1973), hydrogenosomes have been identified in various distant anaerobic lineages including diplomonads (*Spironucleus salmonicida*), chytridiomycetes (such as *Neocallimastix* and *Piromyces*), heteroloboseans (*Psalteriomonas lanterna* and *Sawyeria*) and some ciliates (Barbera et al., 2010; Boxma et al., 2004; de Graaf et al., 2009; Jerlstrom-Hultqvist et al., 2013; van Bruggen et al., 1984; Yarlett et al., 1981; Yarlett et al., 1986). Like mitochondria, hydrogenosomes are double membrane bound organelles; however, the inner membrane does not usually have any cristae. DAB (3,3'-diaminobenzidine) positive vesicles rich in calcium and N-acetyl-glucosamine-containing glyco-conjugates as well as phosphatase activity have been observed between the outer and inner membranes (Benchimol et al., 1996; Benchimol and Desouza, 1983). The hydrogenosomes are typically spherical, but can also be beam-like or dumb bell-shaped with a homogeneous electron-dense matrix (Fig.7). The size of hydrogenosomes ranges between 200 to 500 nm in diameter, but may reach up to 2 μm in *Monocercomonas sp.* (Benchimol, 2009; Diniz and Benchimol, 1998).

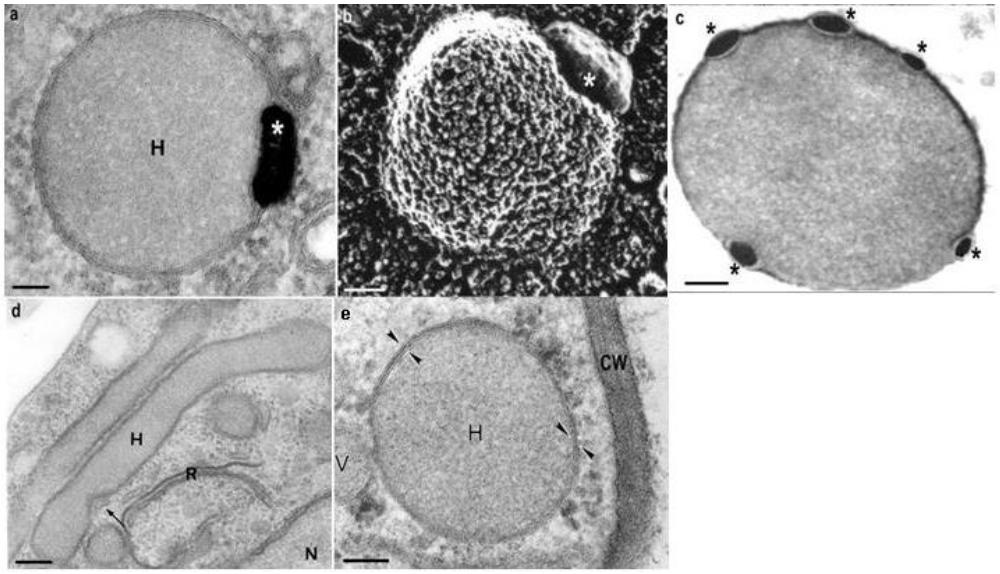

Figure 7. Different shapes and sizes of hydrogenosomes. (a, b) *Tritrichomonas foetus*, (c) *Trichomonas vaginalis*, (d) *Monocercomonas sp.*, (e) the anaerobic fungus *Neocallimastix frontalis*; H - hydrogenosomes; CW - cell wall; R - endoplasmic reticulum; N - nucleus, V - vesicle; * - peripheral vesicle, Bar = 50 nm (Benchimol, 2009).

The origin of hydrogenosomes is not monophyletic. The patchy distribution of hydrogenosome-bearing protists in the eukaryotic tree indicates that hydrogenosomes evolved multiple times during protist evolution. The organelles were named hydrogenosomes according to their ability to produce hydrogen via [FeFe]-hydrogenase (Müller, 1993). Hydrogenases typically depend on a specialized FeS cluster (H-cluster) for proper activity and folding. Proper aseembly of the H-cluster requires three hydrogenase maturases (hydE, hydF and hydG) are present in the organellar matrix (Putz et al., 2006).

Hydrogenosomes utilized pyruvate and/or malate as substrates that are converted to H_2, CO_2 and acetate. Acetyl-CoA, an intermediate product of pyruvate catabolism, provides a CoA moiety for formation of succinyl-CoA by ASCT and serves as a substrate for SCS that catalyzes ATP synthesis (Müller, 1993). Pathways of mitochondrial origin that are retained in hydrogenosomes include amino acid metabolism and FeS cluster assembly (Morada et al., 2011; Mukherjee et al., 2006a; Sutak et al., 2004). However, besides cytochrome-dependent electron transport, hydrogenosomes lack the TCA cycle, heme synthesis, and beta oxidation as well as an organellar genome (Müller, 1993). All hydrogenosomal proteins are encoded in the nucleus, translated in the cytosol and imported to the hydrogenosomes by translocase machinery, which is usually reduced in comparison to the classical mitochondrial machinery. The translocases Tom40 and Sam50 in the outer membrane and Tim17 in the inner membrane are the most conserved (Rada et al., 2011). The proteins targeted to the hydrogenosomal matrix usually carry N-terminal MTS, which deliver the proteins into the organelle. Upon translocation MTS is cleaved by hydrogenosomal processing peptidase (HPP) (Bradley et al., 1997; Brown et al., 2007; Smid et al., 2008). However, targeting of matrix proteins to the organelles without N-terminal MTS has been also reported (Burstein et al., 2012; P. Rada unpublished data).

2.4.1 Parabasalids

The presence of hydrogenosomes is a common feature of all studied parabasalids (Cepicka et al., 2010). The most detailed biochemical study was performed on the hydrogenosomes of *Trichomonas vaginalis*, a parasitic protist of the human urogenital tract (Petrin et al., 1998). Pyruvate and malate, the main substrates of energy metabolism, can be imported from the cytosol into the organelle. Subsequently, malate is converted via malic enzyme to pyruvate. Pyruvate is decarboxylated by PFO to acetyl-CoA, which enters the substrate phosphorylation pathway with acetate and ATP as end products. The electrons from

PFO are transferred via ferredoxin to the enzyme [FeFe]-hydrogenase to produce molecular hydrogen (Lindmark and Müller, 1973). The electrons generated by malic enzyme are first accepted by NAD^+ and subsequently transferred by NADH dehydrogenase to ferredoxin. NADH dehydrogenase consists of two soluble subunits (Tvh47 and Tvh22) that represent remnants of mitochondrial Complex I (Hrdy et al., 2004). In addition to ACST, *T. vaginalis* also possesses a gene coding for a putative hydrogenosomal ACS, which could metabolise acetyl-CoA directly to acetate. However, its function has not been studied yet (I. Hrdý, personal communication). Amino acid metabolism includes the activity of arginine deiminase (ADI), the first enzyme of the arginine dehydrolase pathway, which is typically present in the cytosol of eukaryotic cells (Yarlett et al., 1996). In *T. vaginalis*, ADI is in hydrogenosomes while other components of the arginine dehydrolase pathways are cytosolic as in other eukaryotes (Morada et al., 2011). In addition, two proteins (GSC-L and GSC-H) of the glycine cleavage system (GCS) have been found in hydrogenosomes (Mukherjee et al., 2006a). The genes coding other GCS components (P and T proteins) have not been found in the *T. vaginalis* genome (Mukherjee et al., 2006a; Mukherjee et al., 2006b). Phylogenetic analysis suggests that L and H proteins are not of mitochondrial origin and most likely were acquired from bacteria by LGT. Because of the lack of P and T proteins, the role of GCS-L and GSC-H in hydrogenosomes is unclear.

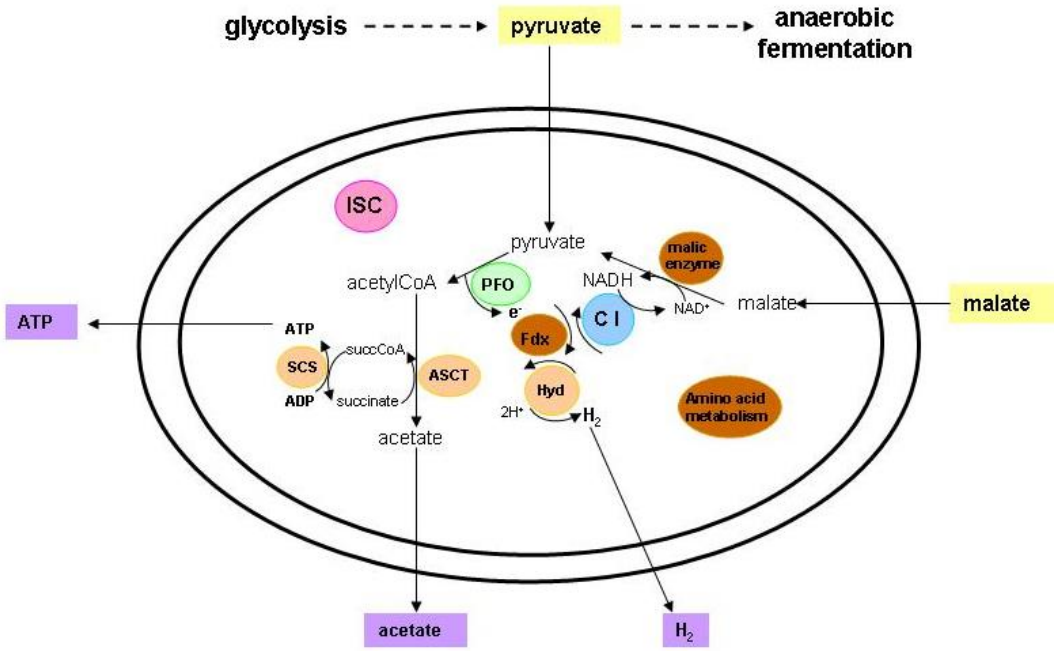

Figure 8. Schematic metabolic pathways in the hydrogenosome of parabasalids and heteroloboseans. PFO - pyruvate: ferredoxin oxidoreductase, Hyd - hydrogenase, ASCT - acetate:succinate CoA transferase, SCS - succinyl-CoA synthetase, ISC – FeS cluster assembly machinery, C I – Complex I of the respiratory chain, Fdx - ferredoxin

Hydrogenosomes of *T. vaginalis* possibly possess enzyme cysteine synthase (CS). CS usually synthesizes cysteine from substrate O-acetylserine. Surprisingly, the *T. vaginalis* CS has been shown to utilize the less common substrate O-phosphoserine (Schneider et al., 2011), which was previously observed exclusively in mycobacteria and Archaea (Agren et al., 2008; Westrop et al., 2006). Produced cysteine serves as a substrate for cysteine desulfurase (IscS), which generates alanine and molecular sulfur used for FeS cluster formation (Sutak et al., 2004). In the next step alanine can be converted to glutamate by the enzyme alanine aminotransferase and glutamate by glutamate dehydrogenase to 2-oxoglutarate. In addition, aspartate aminotransferase was identified in the hydrogenosomal proteome. This enzyme catalyses the interconversion of oxaloacetate to aspartate and in parallel glutamate to 2-oxoglutarate (Schneider et al., 2011).

2.4.2 Chytridiomycetes

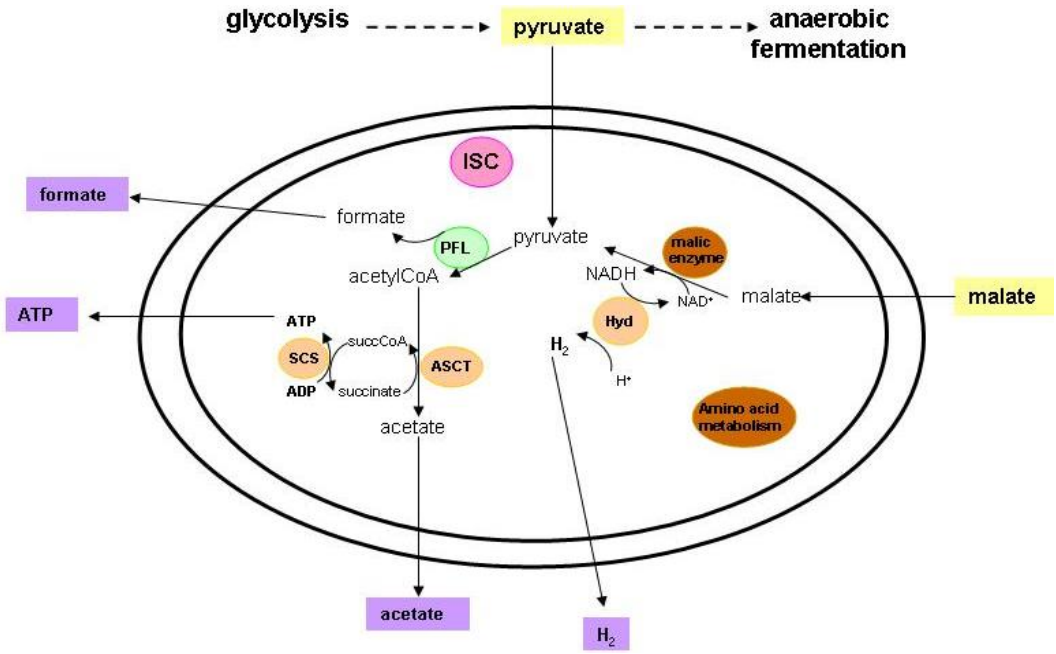

Figure 9. Schematic metabolic pathways in the hydrogenosome of Chytridiomycetes. PFL - pyruvate formate lyase, Hyd - hydrogenase, ASCT - acetate:succinate CoA transferase, SCS - succinyl-CoA synthetase, ISC – FeS cluster assembly machinery

Rumen anaerobic fungi of genera *Neocallimastix* and *Piromyces* (Chytridiomycetes) represent another group of hydrogenosome-bearing protists. A specific feature of chytrid hydrogenosomes is the presence of the strictly anaerobic enzyme pyruvate formate lyase (PFL). This enzyme catalyzes reversible conversion of pyruvate to acetyl-CoA (Fig.9). The

presence of hydrogenosomal PFO in the chytrid *Neocallimastix* is still unclear. Low enzymatic activity was detected in *N. sp.* L2 and *N. patriciarum* (Marvinsikkema et al., 1994;Marvinsikkema et al., 1993;Yarlett et al., 1986) whereas no PFO activity was confirmed in *N. frontalis* (Ofallon et al., 1991) as well as in *Piromyces sp.* E2. (Akhmanova et al., 1999; Boxma et al., 2004). Interestingly in *Piromyces*, the pyruvate conversion by PFL is duplicated in the organelles and the cytosol (Akhmanova et al., 1999).

Unlike PFO, conversion of pyruvate by activity of PFL does not generate the reductive equivalents that are required for hydrogen production. It was suggested that an alternative source of electrons provides NAD(P)H-dependent malic enzyme which catalyzes oxidative decarboxylation of malate (Boxma et al., 2004). However, the electron flow from NAD(P)H to the low redox potential carrier ferredoxin is energetically rather unfavourable. In *T. vaginalis*, this reaction is catalyzed by a unique NADH dehydrogenase. However, it is not known whether this enzyme is present in chytrids.

2.4.3 Heterolobоseans

In addition to parasitic and symbiotic protists, hydrogenosomes have been found in free-living anaerobic protists of the group Heterolobosea. These organisms, such as *Sawyeria marylandensis* (O'Kelly et al., 2003) and *Psalteriomonas laterna* (Broers et al., 1990) can be found in anaerobic sediments. Unfortunately, our knowledge on heterolobosean biochemistry is rather limited, as currently there is no system for axenic cultivation of these organisms thus far and the majority of information is based on EST surveys and transcriptomic data.

The free-living amoeba *Sawyeria marylandenis* inhabits the microaerophilic environment of iron-rich freshwater streams. Light and electron microscopy have revealed the presence of organelles with morphology similar to hydrogenosomes, with an electron-dense matrix surrounded by a double membrane (O'Kelly et al., 2003). Interestingly, some of these organelles appeared as round bodies with central holes (Barbera et al., 2010). Unlike in other heterolobosean, an endoplosmic reticulum was not observed around *Sawyeria* hydrogenosomes. Analysis of EST survey data suggested that the putative hydrogenosomes of *Sawyeria* contain surprisingly similar anaerobic pathways to those in trichomonad hydrogenosomes. The predicted anaerobic pathway comprises PFO, [FeFe]-hydrogenase, ASCT and SCS that can catalyze conversion of pyruvate to acetate, CO_2 and ATP (Fig.8). In addition to [FeFe]-hydrogenase, two hydrogenase maturases hydG and hydF were identified. Similar to *T. vaginalis* hydrogenosomes, the *Sawyeria* organelles most likely possess the

catalytic part of complex I (NuoF, NuoE). The EST survey data also revealed the presence of several components of FeS cluster assembly, fatty acid metabolism, amino acid metabolism and mitochondrial import pathways, several of them with predicted MTS. However, no biochemical data are available (Barbera et al., 2010).

The amoeboflagellate *Psalteriomonas lanterna* possesses an unusual type of hydrogenosome that was originally described based on electron microscopy by Broerst et al., (1990). In flagellates, these organelles could be observed as a large globule that is formed by stack of over 20 double membrane bound hydrogenosomes and interleaved with endosymbiotic methanogenic archaea. In the amoeboid form, the globular structure is less prominent. Individual hydrogenosomes are surrounded by rough endoplasmic reticulum (Hackstein et al., 2001). The presence of [FeFe]-hydrogenase within the organelles was demonstrated using a specific antibody as well as histochemistry (de Graaf et al., 2009) (Broers CAM,. PhD Thesis 1992). Based on EST survey data, partial sequences for PFO, [FeFe]-hydrogenase and one subunit of Complex I (NuoF) were identified (de Graaf et al., 2009). The other putative hydrogenosomal protein includes an ADP/ATP carrier, propionyl-CoA carboxylase and glutamate dehydrogenase (de Graaf et al., 2009).

2.4.4 Diplomonads

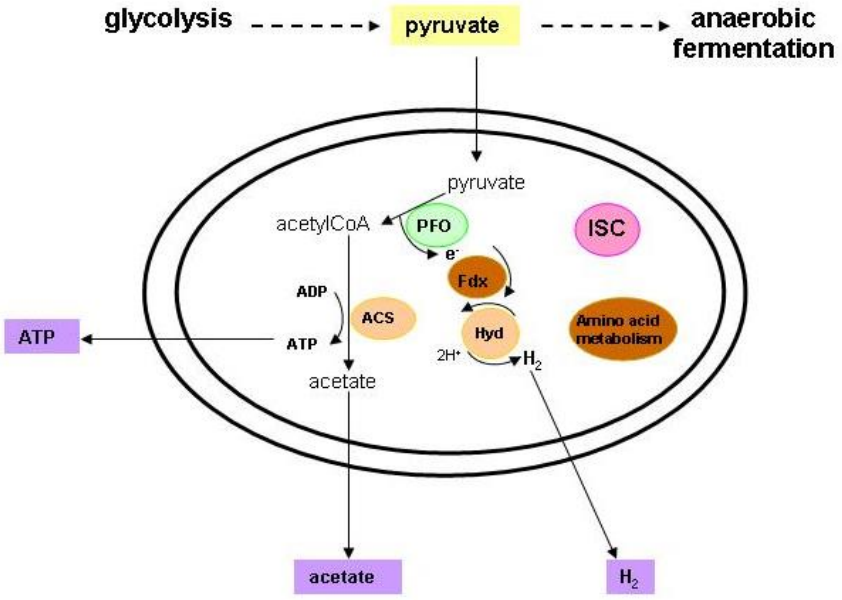

Figure 10. Schematic metabolic pathways in the hydrogenosome of *Spironucleus salmonicida*. PFO - pyruvate: ferredoxin oxidoreductase, Hyd - hydrogenase, ACS - acetyl-CoA synthetase, ISC – FeS cluster assembly machinery, Fdx - ferredoxin

The fish parasite *Spironucleus salmonicida* is the only representative of diplomonads that possesses hydrogenosomes thus far. Based on genomic and mitochondrial proteomic data, hydrogenosomal metabolism pathways were identified. It has been proposed that pyruvate is converted via PFO to acetyl-CoA, which is metabolized directly by ADP-forming ACS to acetate and ATP (Fig.10). The organelles also possess the enzyme [FeFe]-hydrogenase and all three maturases (hydE, hydG and hydF). Interestingly 5 genes for PFOs and 7 genes for hydrogenases were found in the genome. However, only PFO-5 and 2 hydrogenases (5 and 6) were localized in the hydrogenosomes. Thus, it is not clear what the contribution of hydrogenosomal metabolism is to overall hydrogen production by *S. salmonicida*. The other identified pathways include FeS cluster assembly (ISC) and possibly glycine metabolism, although only the H protein of four components of the GCS has been found as well as serine hydroxylmethyltransferase (SHMT) (Jerlstrom-Hultqvist et al., 2013).

The presence of mitochondria derived organelles was proposed for other *Spironucleus* species, the fish parasites *S. vortens* and *S. barkhanus.* Imunofluorescence and electron microscopy of *S. vortens* suggested that this organism possesses two types of mitochondrial organelles including large hydrogenosomes (200 nm – 1 μm in diametr) and smaller mitosomes (100 – 150 nm in diametr) (Millet et al., 2013). However, this suggestion needs to be verified because the activity of hydrogenase is present only in the cytosol (our unpublished data). In *S. barkhanus*, partial genes for the enzymes PFO and [FeFe]-hydrogenase were identified (Horner et al., 2000; Horner et al., 1999). However their cellular localization has not been studied.

2.5 Mitosomes

The most reduced form of the mitochondrion is named the mitosome (Tovar et al., 1999) and/or the crypton (Mai et al., 1999). Mitosomes have been described exclusively in parasitic protists from distant eukaryotic groups that inhabit the oxygen-poor environment of the intestinal tract such as *Entamoeba histolytica* (Archamoeba), *Giardia intestinalis* (Diplomonadida), and in intracellular parasites including microsporidians (Fungi), *Cryptosporidium parvum* (Apicomplexa) and *Mikrocytos mackini* (Rhizaria). They share only a few characteristics with mitochondria: a double membrane bound electron-dense matrix, the presence of the mitochondrial chaperones Hsp70, Cpn60, and Cpn10, and with the exception of Archamoebae, the presence of mitochondrial FeS cluster assembly machinery (ISC)

(Maguire and Richards, 2014). Mitosomes lack energy metabolism and ATP synthesis is localized exclusively in the cytosol and including extended glycolysis. Similar to hydrogenosomes, mitosomes do not contain an organellar genome and all mitosomal proteins are imported to the organelles by mitochondrial translocases. However, only the most conserved components of the outer membrane translocation machinery have been identified, including Tom40 and Sam50 (Makiuchi and Nozaki, 2014), while inner membrane translocases have not been found so far.

2.5.1 Parasites of intestinal tract

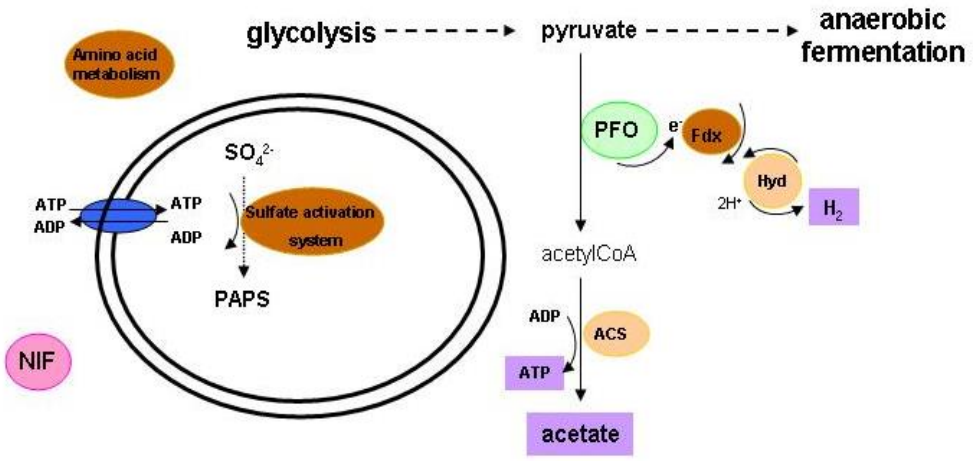

Figure 11. Schematic metabolic pathways in mitosome and cytosol of *Entamoeba histolytica*. PAPS - 3'-phosphoadenosine 5'-phosphosulfate; in the cytosol: NIF - FeS cluster assembly machinery, PFO - pyruvate: ferredoxin oxidoreductase, Hyd - hydrogenase, ACS - acetyl-CoA synthetase, Fdx - ferredoxin

Entamoeba histolytica, a causative agent of amoebic dysentery, was the first protist in which mitosomes were described based on cellular localization of the mitochondrial marker protein Cpn60 (Mai et al., 1999; Tovar et al., 1999). Later other mitochondrial proteins including mtHsp70, Cpn10 and an unusual ATP/ADP transporter were co-localized with Cpn60 in the same organelles (Chan et al., 2005;Tovar et al., 2007;van der Giezen et al., 2005). As these organelles have completely lost energy metabolism, an intriguing question is what is the source of ATP is that is required for protein import and function of Cpn60 or Hsp70. The explanation may be provided by the presence of an unusual mitochondrial-type ADP/ATP carrier (AAC) that possibly functions in the opposite direction than conventional AAC and thus allows the import of ATP from the cytosol to the organelle (Chan et al., 2005).

Unlike mitosomes described in other protists, *Entamoeba* mitosomes do not possess complex ISC machinery for formation of FeS clusters. This machinery was replaced in Archamoebae by simple bacterial NIF (nitrogen fixation) machinery. However, its cellular localization is unclear. Although Maralikova et al. (2010) suggested that NIF components are present in the *Entamoeba* cytosol as well as in mitosomes; the mitosomal localization of NIFs has not been confirmed by others (Dolezal et al., 2010; Mi-ichi et al., 2009). The only known function of *Entamoeba* mitosomes is activation of sulfate (Fig.11) (Mi-ichi et al., 2009). A sulfate activation pathway is typically present in eukaryotic cytosol where it mediates conversion of inert inorganic sulfate to its biologically active form. The active sulfate is then transferred into proteins, lipids or small organic molecules (Patron et al., 2008). Surprisingly, proteomic analysis of *Entamoeba* mitosomes revealed presence of three enzymes of the sulfate activation pathway: ATP sulfurylase (AS), inorganic pyrophosphatase (IPP) and APS kinase (APSK) within the organelle. Moreover, this proteomic study identified 95 putative mitosomal proteins (Mi-ichi et al., 2009). Import of proteins into the *Entamoeba* mitosome is mostly independent of cleavable MTS. An N-terminal presequence was identified and experimentally tested only in a single mitosomal protein - Cpn60 (Tovar et al., 1999). Moreover, *Entamoeba* lacks both subunits of MPP. Known components of the outer membrane translocase machinery are Tom40 and Sam50. More recently a novel component of the TOM complex named Tom60 has been identified (Makiuchi et al., 2013).

Giardia intestinalis, a parasite of human gut, possesses two types of mitosomes that can be distinguished according to their cellular distribution: (i) "central" mitosomes that are invariably present between the two nuclei of diplomonad cell, and (ii) "peripheral" mitosomes that are scattered within the cytosol, typically in caudal part of the cells. The central mitosomes seems to be associated with the basal bodies of caudal flagella, which may facilitate their regular segregation to the daughter cell during cytokinesis. Segregation of peripheral mitosomes seems to be stochastic (P. Dolezal, personal communication). The main function of the Giardial mitosome is biogenesis of FeS clusters by mitochondrial ISC type machinery (Fig. 12). Proteomic analysis of *Giardia* mitosomes revealed the presence of all essential ISC components (such as cysteine desulfurase IscS and scaffold proteins IscU, Nfu, and IscA), electron carrier [2Fe2S]-ferredoxin, mtHsp70, co-chaperonin Jac1 and nucleotide exchange factor Mge, as well as monothiol glutaredoxin (Jedelsky et al., 2011;Rada et al., 2009). Over 638 other proteins of unknown function have been proposed to be localized in these organelles, of which about 139 possess a predicted MTS. Mitosomal localization was experimentally confirmed for 20 proteins (Jedelsky et al., 2011). Interestingly, the MTS is

cleaved by a unique processing peptidase that consists of only a single catalytic β-subunit, while the α-subunit is absent (Smid et al., 2008). The only known component of the *Giardia* TOM complex is Tom40, while Sam50 has not been identified. Similarly to *Entamoeba*, no component of the TIM channel has been identified so far. However, the *Giardia* inner mitosomal membrane possesses Tim44, Pam18 and Pam16 (Dagley et al., 2009; Jedelsky et al., 2011).

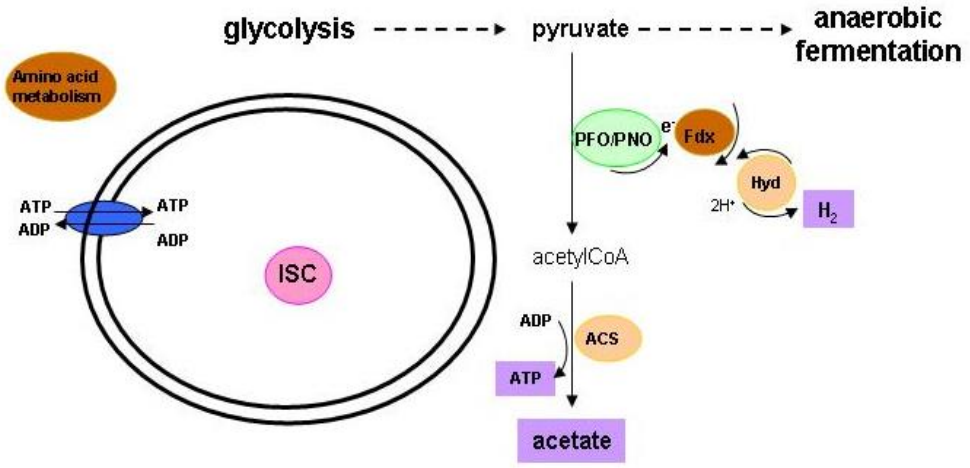

Figure 12. Schematic metabolic pathways in mitosome and cytosol of Microsporidia, *Cryptosporidium parvum* and *Giardia intestinalis*. ISC - FeS cluster assembly machinery; in the cytosol: PFO - pyruvate: ferredoxin oxidoreductase (in *Giardia*), PNO - pyruvate:NADP oxidoreductase (in *Cryptosporidium*), Hyd - hydrogenase, ACS - acetyl-CoA synthetase, Fdx - ferredoxin

2.5.2 Intracellular parasites

The microsporidia *Mikrocytos mackini* and *Cryptosporidium parvum* are obligate intracellular parasites. Their simplified metabolic organization is most likely a result of reductive evolution due to their adaptation to an intracellular environment. Their energy metabolic pathways are usually much reduced, including glycolysis and/or extended glycolysis.

The microsporidian *Encephalitozoon cuniculi* belongs among the most reduced eukaryotes with genome size of only 2.9 Mbp (Biderre et al., 1995). The presence of mitosomes in this organism was first predicted based on genome sequence analysis (Katinka et al., 2001). The N-terminal MTS of mitosomal proteins seems to be absent in most of the proteins (an MTS was identified only in IscU) and also none of the MPP subunits have been

identified (Burri et al., 2006; Williams et al., 2002). In the mitosomal membrane, only some translocase components were identified: Tom 40 and Tom 70 in the outer membrane and Tim 22 in the inner membrane (Katinka et al., 2001). The only clear function of the mitosomes is FeS cluster biosynthesis via mitochondrial ISC machinery (Fig. 12) (Burri et al., 2006; Katinka et al., 2001). The most important components of the ISC machinery were co-localized with the organellar marker mtHsp70 (Goldberg et al., 2008). Interestingly, *E. cuniculi* possesses four unusual bacterial ADP/ATP transporters. Three of them are present in the cell membrane and probably import ATP from the host cell into microsporidians. The signal of the fourth transporter was co-localized with mitochondrial marker mtHsp70 (Tsaousis et al., 2008). It has been proposed that this transporter imports ATP into the mitosome, where it is required for ISC machinery and protein import.

Based on imunufluorescence and electron microscopy, it has been suggested that mitosomes of other microsporidian *Trachipleistophora hominis* possess the ICS component IscS. Surprisingly, the component IscU as well as protein frataxin were found exclusively in the cytosol (Goldberg et al., 2008). In addition, mitosomal protein import is not dependent on a MTS (Williams et al., 2002).

Based on MTS prediction into mitosomes of the microsporidian *Antonospora locustae* several proteins were suggested. As putative mitosomal matrix proteins were predicted components of ISC machinery and the enzymes PDH and superoxide dismutase. However MTS signal was also found in clearly cytosolic proteins such as glyceraldehyde 3- phosphate dehydrogenase, which means that the predicted MTS signal is not always essential for protein targeting (Burri et al., 2006).

In comparison with other intracellular mitosome-containing parasites, energy metabolic pathways of the apicomplexan *Cryptosporidium parvum* seem to be more complex. Unlike in microsporidians, *C. parvum* possesses complete glycolysis as well as pathway of extended glycolysis in the cytosol, similar to *G. intestinalis* and *E. histolytica*. Here, pyruvate is converted by activity of PNO to acetyl-CoA, which is metabolized by ACS to acetate and ATP. Interestingly, *C. parvum* mitosomes contain an ADP/ATP transporter similar to *E. cuniculi* (Mogi and Kita, 2010). The organelles also possess the mitochondrial chaperones Cpn60 and mtHsp70 (Riordan et al., 2003) as well as ISC machinery for FeS cluster biogenesis (Fig. 12) (Abrahamsen et al., 2004; Lagier et al., 2003).

Recently, the mitosomes were described in *M. mackini*, the intracellular parasite of Pacific oysters that represents the first mitosome-bearing organisms of the Rhizaria group. Similarly to other mitosomes, the only known function is probably biosynthesis of FeS clusters by ISC

machinery. Based on a transcriptomic survey the presence of genes coding IscS, IscU, Hsp70 and ferredoxin reductase was revealed (Burki et al., 2013). However, no more information about this microcell protist is currently available.

3 *Mastigamoeba balamuthi*

Mastigamoeba balamuthi is an Archamoebae within the eukaryotic supergroup Amoebozoa. Group Archamoebae includes four lineages: Mastigamoebidae, Pelomyxidae, Entamoebidae and Rhizomastixidae (Ptackova et al., 2013). These organisms are adapted to inhabit anaerobic or oxygen-restricted environments. The representants include both free-living (e.g. *Mastigamoeba balamuthi*) and parasitic species (e.g. *Entamoeba histolytica*). It has been proposed that a common ancestor of Archamoebae and other amoebozoan lineages possessed aerobic mitochondria that were retained in groups such as the closely related Eumycetozoa, while Archamoebae converted their aerobic mitochondria to their anaerobic forms (Gill et al., 2007). However, although various morphologically unusual forms of mitochondria in amoebozoans have been observed using electron microscopy, the functional adaptations have been studied only in *E. histolytica* which possesses the most reduced mitochondrial form, the mitosome.

M. balamuthi was originally described by Chavez et al. (1986) as *Phreatamoeba balamuthi*. The relation to *E. histolytica* and *Dictyostelium* was proposed based on a phylogenetic analysis of 100 genes (Bapteste et al., 2002). *Mastigamoeba* can form three morphological stages: an amoeboid form, an uniflagellate form and a cyst. The amoeboid form is the dominant stage in the life cycle. They move by monopodia and are predominantly multinucleate. The nucleus contains a single central nucleolus and the nuclear membrane persists at least through metaphase (Chavez et al., 1986). The size of the amoeboid forms varies from 11 to 160 μm in length. Flagellates possess a single flagellum associated with a cone of microtubules around a single nucleus. The size of flagellates ranges from 6 to 60 μm in length (Chavez et al., 1986). When *Mastigamoeba* grows in axenic culture, they can be transformed to the flagellate forms by addition of rice powder to the growth medium. However, the flagellates do not reproduce. The cyst is surrounded by a resistant wall that lacks pores and ranges from 9 to 18 μm in diameter (Chavez et al., 1986).

In *M. balamuthi*, double membrane bound organelles with an electron-dense matrix were identified by electron microscopy and considered to represent mitosomes. Based on an

EST survey, 69 candidates of organellar proteins have been identified, 7 of them with a putative MTS, although neither translocase complexes nor a MPP has been found (Gill et al., 2007). A function-oriented ortholog gene analysis suggested that the organelles possibly harbour mitochondrial chaperones (Cpn60, Cpn10), some components of the TCA cycle (SDH, malate dehydrogenase, aconitase, isocitrate dehydrogenase) and amino acid metabolism (GCS, SHMT, threonine dehydrogenase). The EST survey also revealed presence of genes for enzymes of extended glycolysis including PFO, [FeFe]-hydrogenase and ACS. However, their cellular localization was not clarified. Similarly to *Entamoeba*, *Mastigamoeba* possesses genes for NIF components for FeS cluster biogenesis, while no genes for mitochondrial ISC system were found (Gill et al., 2007). This analysis suggested that function of *Mastigamoeba* organelles is much more complex than the extremely reduced function of *Entamoeba* mitosomes. However, no biochemical data are available to support the presence of predicted metabolic pathways in *Mastigamoeba* mitosomes. Moreover, previous an EST survey allows prediction of only a partial organellar proteome. Thus, analysis of more genomic and transcriptomic data are required to obtain more complete information.

4 Aims

1) To characterize mitochondria organelles of *Mastigamoeba balamuthi* based on biochemical analysis of the organelles and new informations obtained from analysis of ongoing *Mastigamoeba* genome project:
   (i) to develop a protocol for isolation of these organelles,
   (ii) to investigate their energy metabolism and
   (iii) to characterize machinery that is required for biogenesis of FeS clusters

2) To investigate proposed hydrogen-production in the mitochondria of *Naegleria gruberii*.

5 References

Abrahamsen, M.S., et al. (2004). Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science.* 304, 441-445.

Adams, K.L. and Palmer,J.D. (2003). Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. *Mol. Phylogenet. Evol.* 29, 380-395.

Agren, D., Schnell, R., Oehlmann,W., Singh, M., and Schneider, G. (2008). Cysteine synthase (CysM) of *Mycobacterium tuberculosis* is an O-phosphoserine sulfhydrylase evidence for an alternative cysteine biosynthesis pathway in *Mycobacteria*. *J. Biol. Chem.* 283, 31567-31574.

Akhmanova, A., Voncken, F., van Alen, T., van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M., and Hackstein, J.H. (1998). A hydrogenosome with a genome. *Nature.* 396, 527-528.

Akhmanova, A., Voncken, F.G.J., Hosea, K.M., Harhangi, H., Keltjens, J.T., den Camp, H.J.M.O., Vogels, G.D., and Hackstein, J.H.P. (1999). A hydrogenosome with pyruvate formate-lyase: anaerobic chytrid fungi use an alternative route for pyruvate catabolism. *Mol. Microbiol.* 32, 1103-1114.

Andersson, S.G. and Kurland, C.G. (1999). Origins of mitochondria and hydrogenosomes. *Curr. Opin. Microbiol.* 2, 535-541.

Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., Naslund, A.K., Eriksson, A.S., Winkler, H.H. and Kurland, C.G. (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* 396, 133-140.

Atteia, A. et al. (2009). A proteomic survey of *Chlamydomonas reinhardtii* mitochondria sheds new light on the metabolic plasticity of the organelle and on the nature of the alpha-proteobacterial mitochondrial ancestor. *Mol. Biol. Evol.* 26, 1533-1548.

Atteia, A., van Lis,R., Gelius-Dietrich, G., Adrait, A., Garin, J., Joyard, J., Rolland, N. and Martin,W. (2006). Pyruvate formate-lyase and a novel route of eukaryotic ATP synthesis in *Chlamydomonas* mitochondria. *J. Biol. Chem.* 281, 9909-9918.

Bapteste, E. et al. (2002). The analysis of 100 genes supports the grouping of three highly divergent amoebae: *Dictyostelium*, *Entamoeba*, and *Mastigamoeba*. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1414-1419.

Barbera, M.J., Ruiz-Trillo, I., Tufts, J.Y., Bery, A., Silberman, J.D. and Roger,A.J. (2010). *Sawyeria marylandensis* (Heterolobosea) has a hydrogenosome with novel metabolic properties. *Eukaryot. Cell* 9, 1913-1924.

Barton, R.M. and Worman, H.J. (1999). Prenylated prelamin a interacts with Narf, a novel nuclear protein. *J. Biol. Chem.* 274, 30008-30018.

Bauer, M.F., Sirrenberg, C., Neupert,W. and Brunner, M. (1996). Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* 87, 33-41.

Benchimol, M. (2009). Hydrogenosomes under microscopy. *Tissue & Cell* 41, 151-168.

Benchimol, M., Almeida, J.C. and de Souza, W. (1996). Further studies on the organization of the hydrogenosome in *Tritrichomonas foetus*. *Tissue & Cell* 28, 287-299.

Benchimol, M. and de Souza,W. (1983). Fine-structure and cyto-chemistry of the hydrogenosome of *Tritrichomonas foetus*. *J. Protozool.* 30, 422-425.

Benichou, P., Calvayrac, R. and Claisse, M. (1988). Induction by antimycin a of cyanide-resistant respiration in heterotrophic *Euglena gracilis*: Effects of growth, respiration and protein biosynthesis. *Planta* 175, 23-32.

Biagini, G.A., Finlay, B.J. and Lloyd, D. (1997). Evolution of the hydrogenosome. *FEMS Microbiol. Lett.* 155, 133-140.

Biderre, C., Pages, M., Metenier, G., Canning, E.U. and Vivares,C.P. (1995). Evidence for the smallest nuclear genome (2.9 Mb) in the microsporidium *Encephalitozoon cuniculi*. *Mol. Biochem. Parasitol.* 74, 229-231.

Boxma, B. et al. (2005). An anaerobic mitochondrion that produces hydrogen. *Nature* 434, 74-79.

Boxma, B. et al. (2004). The anaerobic chytridiomycete fungus *Piromyces sp.* E2 produces ethanol via pyruvate:formate lyase and an alcohol dehydrogenase E. *Mol. Microbiol.* 51, 1389-1399.

Bradley, P.J., Lahti, C.J., Plumper, E. and Johnson, P.J. (1997). Targeting and translocation of proteins into the hydrogenosome of the protist *Trichomonas*: Similarities with mitochondrial protein import. *EMBO Journal* 16, 3484-3493.

Broers, C.A.M., Stumm, C.K., Vogels, G.D. and Brugerolle,G. (1990). *Psalteriomonas lanterna gen. nov. sp. nov.*, a free-living ameboflagellate isolated from freshwater anaerobic sediments. *Eur. J. Protistol.* 25, 369-380.

Broers, CAM. (1992) Anaerobic psalteriomonad amoeboflagellates. PhD Thesis, Catholic University Nijmegen, Nijmegen

Brown, M.T., Goldstone, H.M.H., Bastida-Corcuera, F., Delgadillo-Correa, M.G., McArthur, A.G. and Johnson, P.J. (2007). A functionally divergent hydrogenosomal peptidase with protomitochondrial ancestry. *Mol. Microbiol.* 64, 1154-1163.

Brown, M.W., Sharpe, S.C., Silberman, J.D., Heiss, A.A., Lang, B.F., Simpson, A.G. and Roger, A.J. (2013). Phylogenomics demonstrates that breviate flagellates are related to opisthokonts and apusomonads. *Proc. Biol. Sci.* 280, 20131755.

Bui, E.T., Bradley, P.J. and Johnson, P.J. (1996). A common evolutionary origin for mitochondria and hydrogenosomes. *Proc. Natl. Acad. Sci. U. S. A* 93, 9651-9656.

Burger, G., Gray, M.W. and Lang, B.F. (2003). Mitochondrial genomes: anything goes. *Trends Genet.* 19, 709-716.

Burki, F., Corradi, N., Sierra, R., Pawlowski, J., Meyer, G.R., Abbott, C.L. and Keeling, P.J. (2013). Phylogenomics of the intracellular parasite *Mikrocytos mackini* reveals evidence for a mitosome in Rhizaria. *Curr. Biol.* 23, 1541-1547.

Burri, L., Williams, B.A., Bursac, D., Lithgow, T. and Keeling, P.J. (2006). Microsporidian mitosomes retain elements of the general mitochondrial targeting system. *Proc. Natl. Acad. Sci. U. S. A* 103, 15916-15920.

Burstein, D., Gould, S.B., Zimorski, V., Kloesges, T., Kiosse, F., Major, P., Martin,W.F., Pupko, T., and Dagan, T. (2012). A machine learning approach to identify hydrogenosomal proteins in *Trichomonas vaginalis*. *Eukar. Cell* 11, 217-228.

Canfield, D.E., Poulton, S.W. and Narbonne, G.M. (2007). Late-neoproterozoic deep-ocean oxygenation and the rise of animal life. *Science* 315, 92-95.

Canfield, D. E. (1998). A new model for Proterozoic ocean chemistry. *Nature* 396, 450-453.

Cardol, P., Gonzalez-Halphen, D., Reyes-Prieto, A., Baurain, D., Matagne, R.F. and Remacle, C. (2005). The mitochondrial oxidative phosphorylation proteome of *Chlamydomonas reinhardtii* deduced from the genome sequencing project. *Plant Physiol* 137, 447-459.

Cavalier-Smith,T. (1986). The kingdoms of organisms. *Nature* 324, 416-417.

Cavalier-Smith,T. (1989). Molecular phylogeny. Archaebacteria and Archezoa. *Nature* 339, l00-l01.

Cavalier-Smith,T. (1993). Kingdom protozoa and its 18 phyla. *Microbiol. Rev.* 57, 953-994.

Cepicka, I., Hampl, V., and Kulda, J. (2010). Critical taxonomic revision of parabasalids with description of one new genus and three new species. *Protist* 161, 400-433.

Chan, K.W. et al. (2005). A novel ADP/ATP transporter in the mitosome of the microaerophilic human parasite *Entamoeba histolytica*. *Curr. Biol.* 15, 737-742.

Chavez, L.A., Balamuth, W. and Gong, T. (1986). AlLight and electronmicroscopic study of a new polymorphic free-living amoeba, *Phreatamoeba balamuthi ng. nsp. J. Protozool.* 33, 397-404.

Collins, N., Brown, R.H. and Merrett, M.J. (1975). Oxidative phosphorylation during glycollate metabolism in mitochondria from phototrophic *Euglena gracilis*. *Biochem. J.* 150, 373-377.

Cometa, I., Schatz, S., Trzyna,W. and Rogerson, A. (2011). Tolerance of naked amoebae to low oxygen levels with an emphasis on the genus *Acanthamoeba*. *Acta Protozool.* 50, 33-40.

Dagley, M.J., Dolezal, P., Likic,V.A., Smid, O., Purcell, A.W., Buchanan, S.K., Tachezy, J. and Lithgow, T. (2009). The protein import channel in the outer mitosomal membrane of *Giardia intestinalis*. *Mol. Biol. Evol.* 26, 1941-1947.

de Graaf, R.M., Duarte,I., van Alen,T.A., Kuiper, J.W., Schotanus, K., Rosenberg, J., Huynen, M.A. and Hackstein, J.H. (2009). The hydrogenosomes of *Psalteriomonas lanterna*. *BMC. Evol. Biol.* 9, 287.

de Graaf, R.M. et al. (2011). The organellar genome and metabolic potential of the hydrogen-producing mitochondrion of *Nyctotherus ovalis*. *Mol. Biol. Evol.* 28, 2379-2391.

Denoeud, F. et al. (2011). Genome sequence of the stramenopile *Blastocystis*, a human anaerobic parasite. *Genome Biol.* 12, R29.

Diniz, J.A.P. and Benchimol, M. (1998). *Monocercomonas sp.*: Cytochemistry and fine structure of freeze-fractured membranes. *J. Eukar. Microbiol.* 45, 314-322.

Dolezal, P. et al. (2010). The essentials of protein import in the degenerate mitochondrion of *Entamoeba histolytica*. *PLoS Pathog.* 6, e1000812.

Dolezal, P., Likic,V., Tachezy, J. and Lithgow, T. (2006). Evolution of the molecular machines for protein import into mitochondria. *Science* 313, 314-318.

Dubini, A., Mus, F., Seibert, M., Grossman, A.R. and Posewitz, M.C. (2009). Flexibility in anaerobic metabolism as revealed in a mutant of *Chlamydomonas reinhardtii* lacking hydrogenase activity. *J. Biol. Chem.* 284, 7201-7213.

Dyall, S.D., Brown, M.T. and Johnson, P.J. (2004). Ancient invasions: from endosymbionts to organelles. *Science* 304, 253-257.

Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300, 1005-1016.

Embley, T.M. and Martin, W. (2006). Eukaryotic evolution, changes and challenges. *Nature* 440, 623-630.

Embley, T.M., van der Giezen, M., Horner, D.S., Dyal, P.L., Bell, S. and Foster,P.G. (2003a). Hydrogenosomes, mitochondria and early eukaryotic evolution. *IUBMB. Life* 55, 387-395.

Embley, T.M., van der Giezen, M., Horner, D.S., Dyal, P.L., and Foster, P. (2003b). Mitochondria and hydrogenosomes are two forms of the same fundamental organelle. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 358, 191-201.

Eriksson, M., Gardestrom, P., and Samuelsson, G. (1995). Isolation, purification, and characterization of mitochondria from *Chlamydomonas reinhardtii*. *Plant Physiol* 107, 479-483.

Fritz-Laylin, L.K. et al. (2010). The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* 140, 631-642.

Gabaldon, T. and Huynen, M.A. (2003). Reconstruction of the proto-mitochondrial metabolism. *Science* 301, 609.

Gakh, O., Cavadini, P., and Isaya, G. (2002). Mitochondrial processing peptidases. *Biochem. Biophys. Acta* 1592, 63-77.

Gawryluk, R.M., Chisholm, K.A., Pinto, D.M., and Gray, M.W. (2012). Composition of the mitochondrial electron transport chain in *Acanthamoeba castellanii*: structural and evolutionary insights. *Biochem. Biophys. Acta* 1817, 2027-2037.

Gawryluk, R.M., Chisholm, K.A., Pinto, D.M., and Gray, M.W. (2014). Compositional complexity of the mitochondrial proteome of a unicellular eukaryote (*Acanthamoeba*

castellanii, supergroup Amoebozoa) rivals that of animals, fungi, and plants. *J. Proteomics.* 109, 400-416.

Gijzen, H.J., Broers, C.A.M., Barughare, M., and Stumm, C.K. (1991). Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. *Appl. Environ. Microbiol.* 57, 1630-1634.

Gill, E.E., Diaz-Trivino, S., Barbera, M.J., Silberman, J.D., Stechmann, A., Gaston, D., Tamas, I. and Roger, A.J. (2007). Novel mitochondrion-related organelles in the anaerobic amoeba *Mastigamoeba balamuthi*. *Mol. Microbiol.* 66, 1306-1320.

Godman, J. and Balk, J. (2008). Genome analysis of *Chlamydomonas reinhardtii* reveals the existence of multiple, compartmentalized iron-sulfur protein assembly machineries of different evolutionary origins. *Genetics* 179, 59-68.

Goldberg, A.V., Molik, S., Tsaousis, A.D., Neumann, K., Kuhnke, G., Delbac, F., Vivares, C.P., Hirt, R.P., Lill, R. and Embley, T.M. (2008). Localization and functionality of microsporidian iron-sulphur cluster assembly proteins. *Nature* 452, 624-628.

Gray, M.W., Burger, G. and Lang, B.F. (1999). Mitochondrial evolution. *Science* 283, 1476-1481.

Hackstein, J.H.P. et al. (2001). Hydrogenosomes: convergent adaptations of mitochondria to anaerobic environments. *Zoology* 104, 290-302.

Hirt, R.P., Healy, B., Vossbrinck, C.R., Canning, E.U., and Embley, T.M. (1997). A mitochondrial Hsp70 orthologue in *Vairimorpha necatrix*: molecular evidence that microsporidia once contained mitochondria. *Curr. Biol.* 7, 995-998.

Hjort, K., Goldberg, A.V., Tsaousis, A.D., Hirt, R.P., and Embley, T.M. (2010). Diversity and reductive evolution of mitochondria among microbial eukaryotes. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 365, 713-727.

Hoffmeister, M., Piotrowski, M., Nowitzki, U., and Martin, W. (2005). Mitochondrial trans-2-enoyl-CoA reductase of wax ester fermentation from *Euglena gracilis* defines a new family of enzymes involved in lipid synthesis. *J. Biol. Chem.* 280, 4329-4338.

Hoffmeister, M., van der Klei, A., Rotte, C., van Grinsven, K.W., van Hellemond, J.J., Henze, K., Tielens, A.G. and Martin,W. (2004). *Euglena gracilis* rhodoquinone:ubiquinone ratio and mitochondrial proteome differ under aerobic and anaerobic conditions. *J. Biol. Chem.* 279, 22422-22429.

Horner, D.S., Foster, P.G. and Embley, T.M. (2000). Iron hydrogenases and the evolution of anaerobic eukaryotes. *Mol. Biol. Evol.* 17, 1695-1709.

Horner, D.S., Hirt, R.P., and Embley, T.M. (1999). A single eubacterial origin of eukaryotic pyruvate: ferredoxin oxidoreductase genes: implications for the evolution of anaerobic eukaryotes. *Mol. Biol. Evol.* 16, 1280-1291.

Hrdy, I., Hirt, R.P., Dolezal, P., Bardonova, L., Foster, P.G., Tachezy, J. and Embley, T.M. (2004). *Trichomonas* hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. *Nature* 432, 618-622.

Hug, L.A., Stechmann, A. and Roger, A.J. (2010). Phylogenetic distributions and histories of proteins involved in anaerobic pyruvate metabolism in eukaryotes. *Mol. Biol. Evol.* 27, 311-324.

Inui, H., Miyatake, K., Nakano, Y., and Kitaoka, S. (1984). Fatty acid synthesis in mitochondria of *Euglena gracilis*. *Eur. J. Biochem.* 142, 121-126.

Inui, H., Ono, K., Miyatake, K., Nakano, Y. and Kitaoka, S. (1987). Purification and characterization of pyruvate:NADP+ oxidoreductase in *Euglena gracilis*. *J. Biol. Chem.* 262, 9130-9135.

Javaux, E.J., Knoll, A.H. and Walter, M.R. (2001). Morphological and ecological complexity in early eukaryotic ecosystems. *Nature* 412, 66-69.

Jedelsky, P.L. et al. (2011). The minimal proteome in the reduced mitochondrion of the parasitic protist *Giardia intestinalis*. *Plos One* 6, e17285.

Jerlstrom-Hultqvist, J., Einarsson, E., Xu, F., Hjort, K., Ek, B., Steinhauf, D., Hultenby, K., Bergquist, J., Andersson, J.O., and Svard, S.G. (2013). Hydrogenosomes in the diplomonad *Spironucleus salmonicida. Nat. Commun.* 4, 2493.

Karlberg, O., Canback, B., Kurland, C.G., and Andersson, S.G. (2000). The dual origin of the yeast mitochondrial proteome. *Yeast* 17, 170-187.

Katinka, M.D. et al. (2001). Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature* 414, 450-453.

Keeling,P.J. and Doolittle,W.F. (1995). Archaea: narrowing the gap between prokaryotes and eukaryotes. Proc. Natl. Acad. Sci. U. S. A 92, 5761-5764.

Knoll, A.H. (2003). The geological consequences of evolution. *Geobiology* 1, 3-14.

Komuniecki, R., McCrury, J., Thissen ,J. and Rubin, N. (1989). Electron-transfer flavoprotein from anaerobic *Ascaris suum* mitochondria and its role in NADH-dependent 2-methyl branched-chain enoyl-CoA reduction. *Biochem. Biophys. Acta* 975, 127-131.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J.L., and Mignotte, B. (1995). The biochemistry of programmed cell death. *FASEB J.* 9, 1277-1287.

Kroos, L. (2005). Eukaryotic-like signaling and gene regulation in a prokaryote that undergoes multicellular development. *Proc. Natl. Acad. Sci. U. S. A* 102, 2681-2682.

Kurland, C.G. and Andersson, S.G. (2000). Origin and evolution of the mitochondrial proteome. *Microbiol. Mol. Biol.* Rev. 64, 786-820.

Lagier, M.J., Tachezy, J., Stejskal, F., Kutisova, K. and Keithly, J.S. (2003). Mitochondrial-type iron-sulfur cluster biosynthesis genes (IscS and IscU) in the apicomplexan *Cryptosporidium parvum*. *Microbiology* 149, 3519-3530.

Lange, H., Kispal, G., and Lill, R. (1999). Mechanism of iron transport to the site of heme synthesis inside yeast mitochondria. *J. Biol. Chem.* 274, 18989-18996.

Lantsman, Y., Tan, K.S., Morada, M., and Yarlett, N. (2008). Biochemical characterization of a mitochondrial-like organelle from *Blastocystis sp.* subtype 7. *Microbiology* 154, 2757-2766.

Leger, M.M., Gawryluk, R.M., Gray, M.W. and Roger, A.J. (2013). Evidence for a hydrogenosomal-type anaerobic ATP generation pathway in *Acanthamoeba castellanii. PLoS. One.* 8, e69532.

Leipe, D.D., Gunderson, J.H., Nerad, T.A., and Sogin, M.L. (1993). Small subunit ribosomal RNA of *Hexamita inflata* and the quest for the first branch in the eukaryotic tree. *Mol. Biochem. Parasitol.* 59, 41-48.

Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C., and Kispal, G. (1999). The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins. *Biol. Chem.* 380, 1157-1166.

Lill, R. and Mühlenhoff, U. (2005). Iron-sulfur-protein biogenesis in eukaryotes. *Trends Biochem. Sci.* 30, 133-141.

Lill, R. and Mühlenhoff, U. (2008). Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. *Annu. Rev. Biochem.* 77, 669-700.

Lindmark, D.G. and Müller, M. (1973). Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. *J. Biol. Chem.* 248, 7724-7728.

Lloyd, D. (1974) The Mitochondria of Microorganisms. 553 S., 125 Abb., 63 Tab. London—New York—San Francisco, Academic Press.

Maguire, F. and Richards, T.A. (2014). Organelle evolution: a mosaic of 'mitochondrial' functions. *Curr. Biol.* 24, R518-R520.

Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R. and Samuelson, J. (1999). Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("crypton") in the microaerophilic protozoan parasite *Entamoeba histolytica. Mol. Cell Biol.* 19, 2198-2205.

Makiuchi, T., Mi-ichi, F., Nakada-Tsukui, K. and Nozaki, T. (2013). Novel TPR-containing subunit of TOM complex functions as cytosolic receptor for *Entamoeba* mitosomal transport. *Sci. Rep.* 3, 1129.

Makiuchi, T. and Nozaki, T. (2014). Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa. *Biochemie* 100, 3-17.

Maralikova, B., Ali, V., Nakada-Tsukui, K., Nozaki, T., van der Giezen,M., Henze, K., and Tovar, J. (2010). Bacterial-type oxygen detoxification and iron-sulfur cluster assembly in amoebal relict mitochondria. *Cell Microbiol.* 12, 331-342.

Martin,W. (2005). The missing link between hydrogenosomes and mitochondria. *Trends Microbiol.* 13, 457-459.

Martin,W. and Müller,M. (1998). The hydrogen hypothesis for the first eukaryote. *Nature* 392, 37-41.

Marvinsikkema, F.D., Driessen, A.J.M., Gottschal, J.C., and Prins, R.A. (1994). Metabolic energy generation in hydrogenosomes of the anaerobic fungus *Neocallimastix* - evidence for a functional relationship with mitochondria. *Mycol. Res.* 98, 205-212.

Marvinsikkema, F.D., Kraak, M.N., Veenhuis, M., Gottschal, J.C. and Prins, R.A. (1993). The hydrogenosomal enzyme hydrogenase from the anaerobic fungus *Neocallimastix sp* L2 is recognized by antibodies,directed against the C-terminal microbody protein targeting signal. *Eur. J Cell Biol.* 61, 86-91.

Mi-ichi, F., Abu, Y.M., Nakada-Tsukui, K. and Nozaki, T. (2009). Mitosomes in *Entamoeba histolytica* contain a sulfate activation pathway. *Proc. Natl. Acad. Sci. U. S. A* 106, 21731-21736.

Miller,W.L. (1995). Mitochondrial specificity of the early steps in steroidogenesis. *J. Steroid Biochem. Mol. Biol.* 55, 607-616.

Millet, C.O.M., Williams, C.F., Hayes, A.J., Hann, A.C., Cable, J., and Lloyd, D. (2013). Mitochondria-derived organelles in the diplomonad fish parasite *Spironucleus vortens. Exp. Parasitol.* 135, 262-273.

Mogi, T. and Kita, K. (2010). Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. *Parasitol. Int.* 59, 305-312.

Morada, M., Smid, O., Hampl, V., Sutak, R., Lam, B., Rappelli, P., Diessi, D., Fiori, P.L., Tachezy, J. and Yarlett, N. (2011). Hydrogenosome-localization of arginine deiminase in *Trichomonas vaginalis. Mol. Biochem. Parasitol.* 176, 51-54.

Moreira, D. and Lopez-Garcia, P. (1998). Symbiosis between methanogenic archaea and delta-proteobacteria as the origin of eukaryotes: the syntrophic hypothesis. *J. Mol. Evol.* 47, 517-530.

Moreira, D. and Lopez-Garcia, P. (2002). The molecular ecology of microbial eukaryotes unveils a hidden world. *Trends Microbiol.* 10, 31-38.

Mukherjee, M., Brown, M.T., McArthur, A.G., and Johnson, P.J. (2006a). Proteins of the glycine decarboxylase complex in the hydrogenosome of *Trichomonas vaginalis. Eukar. Cell* 5, 2062-2071.

Mukherjee, M., Sievers, S.A., Brown, M.T., and Johnson, P.J. (2006b). Identification and biochemical characterization of serine hydroxymethyl transferase in the hydrogenosome of *Trichomonas vaginalis. Eukar. Cell* 5, 2072-2078.

Müller,M. (1993). The Hydrogenosome. *J.Gen. Microbiol.* 139, 2879-2889.

Müller, M., Mentel, M., van Hellemond, J.J., Henze, K., Woehle, C., Gould, S.B., Yu, R.Y., van der Giezen, M., Tielens, A.G., and Martin,W.F. (2012). Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol. Mol. Biol. Rev.* 76, 444-495.

Mus, F., Dubini, A., Seibert, M., Posewitz, M.C., and Grossman, A.R. (2007). Anaerobic acclimation in *Chlamydomonas reinhardtii*: anoxic gene expression, hydrogenase induction, and metabolic pathways. *J. Biol. Chem.* 282, 25475-25486.

Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003). SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *EMBO J.* 22, 427-437.

Neff, R.J., Neff, R.H., and Taylor, R.E. (1958). The nutrition and metabolism of a soil amoeba, *Acanthamoeba sp. Physiol. Zoology* 31, 73-91.

Neupert, W. and Herrmann, J.M. (2007). Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76, 723-749.

O'Kelly, C.J., Silberman, J.D., Zettler, L.A.A., Nerad, T.A. and Sogin, M.L. (2003). *Monopylocystis visvesvarai n. gen., n. sp* and *Sawyeria marylandensis n. gen., n. sp.*: Two new amitochondrial heterolobosean amoebae from anoxic environments. *Protist* 154, 281-290.

O'Fallon, J.V., Wright, R.W., and Calza, R.E. (1991). Glucose metabolic pathways in the anaerobic rumen fungus *Neocallimastix frontalis*. *Biochem. J.* 274, 595-599.

Ogawa, S. et al. (2000). The mitochondrial DNA of *Dictyostelium discoideum*: complete sequence, gene content and genome organization. *Mol. Gen. Genet.* 263, 514-519.

Opperdoes, F.R., de Jonckheere, J.F., and Tielens, A.G. (2011). *Naegleria gruberi* metabolism. *Int. J. Parasitol.* 41, 915-924.

Patron, N.J., Durnford, D.G., and Kopriva, S. (2008). Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. *BMC. Evol. Biol.* 8, 39.

Paul, R.G., Williams, A.G., and Butler, R.D. (1990). Hydrogenosomes in the rumen entodiniomorphid ciliate *Polyplastron multivesiculatum. J. Gen. Microbiol.* 136, 1981-1989.

Perez, E. et al. (2014). The mitochondrial respiratory chain of the secondary green alga *Euglena gracilis* shares many additional subunits with parasitic Trypanosomatidae. *Mitochondrion*. 19, 338-349.

Petrin, D., Delgaty, K., Bhatt, R., and Garber, G. (1998). Clinical and microbiological aspects of *Trichomonas vaginalis. Clin. Microbiol. Rev.* 11, 300.

Pfanner, N. and Geissler, A. (2001). Versatility of the mitochondrial protein import machinery. *Nat. Rev. Mol. Cell Biol.* 2, 339-349.

Poulton, S.W., Fralick, P.W., and Canfield, D.E. (2004). The transition to a sulphidic ocean approximately 1.84 billion years ago. *Nature* 431, 173-177.

Priest, J.W. and Hajduk, S.L. (1994). Developmental regulation of mitochondrial biogenesis in *Trypanosoma brucei. J. Bioenerg. Biomem.* 26, 179-191.

Ptackova, E., Kostygov, A.Y., Chistyakova, L.V., Falteisek, L., Frolov, A.O., Patterson, D.J., Walker, G., and Cepicka, I. (2013). Evolution of Archamoebae: morphological and molecular evidence for pelobionts including *Rhizomastix, Entamoeba, Iodamoeba*, and *Endolimax*. *Protist* 164, 380-410.

Putz, S., Dolezal, P., Gelius-Dietrich, G., Bohacova, L., Tachezy, J., and Henze, K. (2006). Fe-hydrogenase maturases in the hydrogenosomes of *Trichomonas vaginalis*. *Eukaryot. Cell* 5, 579-586.

Rada, P. et al. (2011). The core components of organelle biogenesis and membrane transport in the hydrogenosomes of *Trichomonas vaginalis*. *Plos One* 6.

Rada, P., Smid, O., Sutak, R., Dolezal, P., Pyrih, J., Zarsky, V., Montagne, J.J., Hrdy,I., Camadro, J.M. and Tachezy, J. (2009). The monothiol single-domain glutaredoxin is conserved in the highly reduced mitochondria of *Giardia intestinalis*. *Eukaryot. Cell* 8, 1584-1591.

Riordan, C.E., Ault, J.G., Langreth, S.G., and Keithly, J.S. (2003). *Cryptosporidium parvum* Cpn60 targets a relict organelle. *Curr. Gen.* 44, 138-147.

Rotte, C., Stejskal, F., Zhu, G., Keithly, J.S., and Martin,W. (2001). Pyruvate: NADP+ oxidoreductase from the mitochondrion of *Euglena gracilis* and from the apicomplexan *Cryptosporidium parvum*: a biochemical relic linking pyruvate metabolism in mitochondriate and amitochondriate protists. *Mol. Biol. Evol.* 18, 710-720.

Sagan, L. (1967). On the origin of mitosing cells. *J. Theor. Biol.* 14 (3), 225–274.

Scheffler, I.E. (2001). Mitochondria make a come back. *Adv. Drug Deliv. Rev.* 49, 3-26.

Schneider, R.E., Brown, M.T., Shiflett, A.M., Dyall, S.D., Hayes, R.D., Xie, Y.M., Loo, J.A., and Johnson, P.J. (2011). The *Trichomonas vaginalis* hydrogenosome proteome is highly reduced relative to mitochondria, yet complex compared with mitosomes. *Int. J. Parasitol.* 41, 1421-1434.

Shen, Y., Knoll, A.H., and Walter, M.R. (2003). Evidence for low sulphate and anoxia in a mid-Proterozoic marine basin. *Nature* 423, 632-635.

Smid, O. et al. (2008). Reductive evolution of the mitochondrial processing peptidases of the unicellular parasites *Trichomonas vaginalis* and *Giardia intestinalis*. *Plos Pathog.* 4.

Snyers, L., Hellings, P., Bovykesler, C., and Thinessempoux, D. (1982). Occurrence of hydrogenosomes in the rumen ciliates Ophryoscolecidae. *Febs Letters* 137, 35-39.

Sogin, M.L., Hinkle, G., and Leipe, D.D. (1993). Universal tree of life. *Nature* 362, 795.

Stairs, C.W., Eme, L., Brown, M.W., Mutsaers, C., Susko, E., Dellaire, G., Soanes, D.M., van der Giezen,M., and Roger, A.J. (2014). A SUF Fe-S cluster biogenesis system in the mitochondrion-related organelles of the anaerobic protist *Pygsuia*. *Curr. Biol.* 24, 1176-1186.

Stechmann, A., Hamblin, K., Perez-Brocal, V., Gaston, D., Richmond, G.S., van der Giezen, M., Clark, C.G., and Roger, A.J. (2008). Organelles in *Blastocystis* that blur the distinction between mitochondria and hydrogenosomes. *Curr. Biol.* 18, 580-585.

Sutak, R., Dolezal, P., Fiumera, H.L., Hrdy, I., Dancis, A., Delgadillo-Correa, M., Johnson, P.J., Müller, M., and Tachezy, J. (2004). Mitochondrial-type assembly of FeS centers in the hydrogenosomes of the amitochondriate eukaryote *Trichomonas vaginalis*. *Proc. Natl. Acad. Sci. U. S. A* 101, 10368-10373.

Terashima, M., Specht, M., and Hippler, M. (2011). The chloroplast proteome: a survey from the *Chlamydomonas reinhardtii* perspective with a focus on distinctive features. *Curr. Genet.* 57, 151-168.

Tielens, A.G., Rotte, C., van Hellemond, J.J., and Martin, W. (2002). Mitochondria as we don't know them. *Trends Biochem. Sci.* 27, 564-572.

Tovar, J., Cox, S.S., and van der Giezen, M. (2007). A mitosome purification protocol based on percoll density gradients and its use in validating the mitosomal nature of *Entamoeba histolytica* mitochondrial Hsp70. *Methods Mol. Biol.* 390, 167-177.

Tovar, J., Fischer, A., and Clark, C.G. (1999). The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica. Mol. Microbiol.* 32, 1013-1021.

Tsaousis, A.D., Gaston, D., Stechmann, A., Walker, P.B., Lithgow, T. and Roger,A.J. (2011). A functional Tom70 in the human parasite *Blastocystis sp.*: Implications for the evolution of the mitochondrial import apparatus. *Mol. Biol. Evol.* 28, 781-791.

Tsaousis, A.D., Kunji, E.R., Goldberg, A.V., Lucocq, J.M., Hirt, R.P. and Embley, T.M. (2008). A novel route for ATP acquisition by the remnant mitochondria of *Encephalitozoon cuniculi. Nature* 453, 553-556.

Tucci, S., Vacula, R., Krajcovic, J., Proksch, P., and Martin,W. (2010). Variability of wax ester fermentation in natural and bleached *Euglena gracilis* strains in response to oxygen and the elongase inhibitor flufenacet. *J. Eukaryot. Microbiol.* 57, 63-69.

Turner, N.A., Biagini, G.A., and Lloyd, D. (1997). Anaerobiosis-induced differentiation of *Acanthamoeba castellanii. Fems Microbiol. Lett.* 157, 149-153.

van der Giezen, M., Leon-Avila, G., and Tovar, J. (2005). Characterization of chaperonin 10 (Cpn10) from the intestinal human pathogen *Entamoeba histolytica. Microbiology* 151, 3107-3115.

van der Giezen, M. and Tovar, J. (2005). Degenerate mitochondria. *EMBO Rep.* 6, 525-530.

van Hellemond, J.J. and Tielens, A.G. (1994). Expression and functional properties of fumarate reductase. *Biochem. J.* 304 (Pt 2), 321-331.

van Bruggen, J.J.A., Zwart, K.B., Vanassema, R.M., Stumm, C.K., and Vogels, G.D. (1984). *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus mcmurrich. Archiv. Microbiol.* 139, 1-7.

Velicer, G.J. and Yu, Y.T. (2003). Evolution of novel cooperative swarming in the bacterium *Myxococcus xanthus. Nature* 425, 75-78.

Voet D. and Voet J.G. Biochemistry. 3rd. 2004. John Wiley and Sons, Int. NJ

Westrop, G.D., Goodall, G., Mottram, J.C., and Coombs, G.H. (2006). Cysteine biosynthesis in *Trichomonas vaginalis* involves cysteine synthase utilizing O-phosphoserine. *J. Biol. Chem.* 281, 25062-25075.

Williams, B.A., Hirt, R.P., Lucocq, J.M., and Embley, T.M. (2002). A mitochondrial remnant in the microsporidian *Trachipleistophora hominis. Nature* 418, 865-869.

Williams, B.A. and Keeling, P.J. (2003). Cryptic organelles in parasitic protists and fungi. *Adv. Parasitol.* 54, 9-68.

Yarlett, N., Hann, A.C., Lloyd, D., and Williams, A. (1981). Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium Schuberg. Biochem. J.* 200, 365-372.

Yarlett, N., Hann, A.C., Lloyd, D. and Williams, A.G. (1983). Hydrogenosomes in a mixed isolate of *Isotricha prostoma* and *Isotricha intestinalis* from ovine rumen contents. *Comp Biochem. Physiol B* 74, 357-364.

Yarlett, N., Martinez, M.P., Moharrami, M.A., and Tachezy, J. (1996). The contribution of the arginine dihydrolase pathway to energy metabolism by *Trichomonas vaginalis. Mol. Biochem. Parasitol.* 78, 117-125.

Yarlett, N., Orpin, C.G., Munn, E.A., Yarlett, N.C., and Greenwood, C.A. (1986). Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum. Biochem. J.* 236, 729-739.

Ye, H., Pilon, M., and Pilon-Smits, E.A.H. (2006). *CpNifS*-dependent iron-sulfur cluster biogenesis in chloroplasts. *New Phytologist* 171, 285-292.

6 Publications

Nývltová E, Šuták R, Harant K, Šedinová M, Hrdy I, Paces J, Vlček Č, Tachezy J. (2013). NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*. *Proc Natl Acad Sci U S A.* 110(18): 7371-6.

Tsaousis AD, Nývltová E, Sutak R, Hrdy I, Tachezy J. (2014). A nonmitochondrial hydrogen production in *Naegleria gruberi*. *Genome Biol Evol.* 6(4):792-9.

Nývltová E, Stairs CW, Hrdý I, Rídl J, Mach J, Pačes J, Roger AJ, Tachezy J. (2015). Lateral gene transfer and gene duplication played a key role in the evolution of *Mastigamoeba balamuthi* hydrogenosomes. *Mol Biol Evol.*, in press

6.1 Publication: Nývltová et al., 2013

Nývltová E, Šuták R, Harant K, Šedinová M, Hrdy I, Paces J, Vlček Č, Tachezy J. (2013). NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*. *Proc Natl Acad Sci U S A.* 110(18): 7371-6.

NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*

Eva Nývltová[a], Robert Šuták[a], Karel Harant[b], Miroslava Šedinová[a], Ivan Hrdý[a], Jan Pačes[c], Čestmír Vlček[c], and Jan Tachezy[a,1]

[a]Department of Parasitology and [b]Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, 128 44 Prague 2, Czech Republic; and [c]Laboratory of Genomics and Bioinformatics, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic

Edited by Jeffrey D. Palmer, Indiana University, Bloomington, IN, and approved March 21, 2013 (received for review November 16, 2012)

In most eukaryotes, the mitochondrion is the main organelle for the formation of iron-sulfur (FeS) clusters. This function is mediated through the iron-sulfur cluster assembly machinery, which was inherited from the α-proteobacterial ancestor of mitochondria. In Archamoebae, including pathogenic *Entamoeba histolytica* and free-living *Mastigamoeba balamuthi*, the complex iron-sulfur cluster machinery has been replaced by an ε-proteobacterial nitrogen fixation (NIF) system consisting of two components: NifS (cysteine desulfurase) and NifU (scaffold protein). However, the cellular localization of the NIF system and the involvement of mitochondria in archamoebal FeS assembly are controversial. Here, we show that the genes for both NIF components are duplicated within the *M. balamuthi* genome. One paralog of each protein contains an amino-terminal extension that targets proteins to mitochondria (NifS-M and NifU-M), and the second paralog lacks a targeting signal, thereby reflecting the cytosolic form of the NIF machinery (NifS-C and NifU-C). The dual localization of the NIF system corresponds to the presence of FeS proteins in both cellular compartments, including detectable hydrogenase activity in *Mastigamoeba* cytosol and mitochondria. In contrast, *E. histolytica* possesses only single genes encoding NifS and NifU, respectively, and there is no evidence for the presence of the NIF machinery in its reduced mitochondria. Thus, *M. balamuthi* is unique among eukaryotes in that its FeS cluster formation is mediated through two most likely independent NIF machineries present in two cellular compartments.

hydrogenosome | mitosome | free-living protist

Iron-sulfur (FeS) clusters are among the most ancient protein cofactors and are essential for the function of a wide variety of FeS proteins in the Bacteria, Archaea, and Eukarya domains (1). Although FeS clusters spontaneously assemble into apoproteins in vitro, the formation of FeS clusters in living cells is a catalyzed process (1, 2). There are three distinct systems of bacterial origin that are responsible for the formation of iron-sulfur clusters: the ISC (iron-sulfur cluster), SUF (sulfur mobilization), and NIF (nitrogen fixation) systems (2). ISC and SUF are highly complex, multicomponent machineries that include two core proteins: cysteine desulfurase (IscS or SufS), which releases sulfur from cysteine to produce alanine, and a scaffold protein (IscU or SufB) that provides a platform for the assembly of transient FeS clusters (2). Both machineries are involved in the formation of FeS clusters in housekeeping proteins, although the SUF machinery is more important for FeS cluster formation and repair under oxygen stress (3). The NIF system is less complex, comprising the cysteine desulfurase NifS and scaffold protein NifU (4). The NIF system is generally thought to be specialized for the nitrogenase FeS cluster assembly of nitrogen-fixing bacteria (5).

Eukaryotes gained both the ISC and SUF machineries during eukaryogenesis from bacterial endosymbionts (6, 7). The ISC machinery present in mitochondria is related to the α-proteobacterial ancestor of these organelles (8). Similarly, the SUF machinery in plastids is related to a homologous system in cyanobacteria, which is the bacterial group from which these organelles originated (6, 9). The formation of FeS clusters through the activity of the ISC machinery is the only essential function of mitochondria identified thus far and is required not only for the maturation of mitochondrial FeS proteins but also for the formation of FeS clusters in other cell compartments (2, 10). It has been proposed that the maturation of cytosolic FeS proteins is dependent on the export of an unknown compound from the mitochondria, which is then used by the cytosolic FeS assembly (CIA) machinery (2).

Several lineages of unicellular eukaryotes possess highly modified forms of mitochondria that are adapted to function under anaerobic conditions (11). Hydrogenosomes, which are present in *Trichomonas vaginalis* and other anaerobic protists, generate hydrogen with the concomitant synthesis of ATP through substrate-level phosphorylation (11). This pathway of extended glycolysis depends on the activities of several FeS proteins, such as pyruvate: ferredoxin oxidoreductase (PFO), and hydrogenase (11). Similar to mitochondria, hydrogenosomes contain ISC machinery that catalyzes FeS cluster assembly (12). *Giardia intestinalis*, microsporidia, and *Cryptosporidium parvum* possess mitochondrion-derived organelles known as mitosomes, which completely lack energy metabolism and other metabolic pathways (13–15). The FeS cluster assembly mediated through the ISC machinery is the only known mitochondrial function retained in these organelles (13–15).

The free-living protist *Mastigamoeba balamuthi* and its parasitic relative *Entamoeba histolytica* are members of the Archamoebae. Unique among eukaryotes studied so far, these anaerobic organisms possess genes encoding the two components of bacterial NIF machinery: NifS and NifU (16, 17). Phylogenetic analyses have suggested that the amoebic NIF machinery was inherited through a common ancestor of *Mastigamoeba* and *Entamoeba* from an ε-proteobacterium via lateral gene transfer (LGT). This transfer is hypothesized to have occurred after the split of the anaerobic Archamoebae and aerobic Mycetozoa (e.g., *Dictyostelium*), which possess conventional ISC machinery (16).

Author contributions: J.T. designed research; E.N., R.S., K.H., M.S., I.H., J.P., and C.V. performed research; E.N., J.P., C.V., and J.T. analyzed data; and E.N. and J.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) (accession nos. JX970968–JX970972, JQ746594, JQ746595, JQ771319–JQ771322, JX982147, JX982148, KC522615, KC543490, KC543491, KC555230, and KC555231) and in the European Molecular Biology Laboratory (EMBL) database (www.ebi.ac.uk/ena) (accession no. PRJEB1507).

[1]To whom correspondence should be addressed. E-mail: tachezy@natur.cuni.cz.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1219590110/-/DCSupplemental.

E. histolytica possess mitosomes that do not generate ATP as do those in other mitosome-harboring organisms (11). However, these organelles do not possess the ISC machinery, and no component of either the ISC or SUF machinery has been identified in the *Entamoeba* genome. NIF components have been predominantly identified in the cytosol of *Entamoeba* (18, 19). However, it remains controversial whether these components are present exclusively in the cytosol or also operate in the mitosomes (18–20).

M. balamuthi is a more basal member of the Archamoebae group (16). The mitochondrion-related organelles of *M. balamuthi* have been suggested to represent an intermediate stage between "classic" mitochondria and the reduced mitosomes of *E. histolytica* (16). Although aerobic respiration is absent in the modified mitochondria of *M. balamuthi*, they may have retained some components of the tricarboxylic acid cycle (16). Interestingly, the expressed sequence tag survey revealed partial sequences encoding the NifS and NifU components, and these proteins have been predicted to be cytosolic in *M. balamuthi* (16). However, it remains unknown how FeS clusters are formed in the mitochondria of *M. balamuthi* or if the ISC machinery is present in these organelles. Therefore, we analyzed the *M. balamuthi* genome to identify and characterize the FeS cluster assembly machineries of this species and their corresponding substrates.

Results

Bacterial NIF Machinery Is Duplicated in *M. balamuthi*. Searches for components of the bacterial NIF machinery in the *M. balamuthi* genome sequence revealed two paralogs for each component, i.e., the cysteine desulfurase NifS (MbNifS) and the scaffold protein NifU (MbNifU) (Table S1). The protein sequences for the two MbNifS and MbNifU paralogs displayed high sequence identity (76.5% and 61.8%, respectively), except at their N-termini. Although the first copy of the cysteine desulfurase (MbNifS-C) gene was almost collinear with its bacterial and *E. histolytica* orthologs, the second copy (MbNifS-M) possessed a 16-amino acid extension at the amino terminus (Fig. 1). Similarly, a 55-amino acid N-terminal extension was identified in the MbNifU-M scaffold protein paralog, but this extension was absent in MbNifU-C. These extensions show features similar to mitochondrial targeting sequences, including predictable cleavage sites for the mitochondrial processing peptidase, with an arginine residue typically located at the −2 position (Fig. 1).

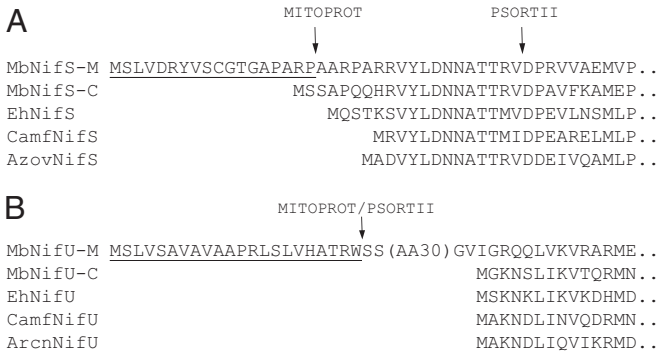

Fig. 1. Comparison of the amino-terminal sequences of *M. balamuthi* NifS/NifU paralogs. MTSs were predicted using PSORTII and MITOPROT servers. The predicted cleavage sites are labeled with arrows. The MITOPROT-predicted targeting sequences are underlined. (*A*) Paralogs of *M. balamuthi* NifS (MbNifS-M and MbNifS-C) and NifS orthologs of *E. histolytica* (EhNifS), *Campylobacter fetus* (CamfNifS), and *Azotobacter vinelandii* (AzovNifS). (*B*) NifU paralogs of *M. balamuthi* (MbNifU-M and MbNifU-C), and NifU orthologs of *E. histolytica* (EhNifU), *C. fetus* (CamfNifU), and *Arcobacter nitrofigilis* (ArcnNifU).

Phylogenetic analysis of the *Mastigamoeba* and *Entamoeba* NifS and NifU sequences suggested that a common ancestor of these two lineages possessed cytosolic NifS and NifU proteins, which were obtained via LGT from an ε-proteobacterium. The branching order within the Archamoebal group revealed that both Nif components were ancestrally duplicated in the common ancestor. The analysis further suggested that *Entamoeba* had retained the mitochondrial version of the Nif machinery, but the cytosolic Nif components were lost. However, the statistical support for the relative branching order was too weak to exclude other scenarios (Fig. S1). Searches for the components of the mitochondrial ISC assembly machinery in the *M. balamuthi* genome were negative; however, a single gene encoding [2Fe2S] ferredoxin (MbFdx), an electron carrier that provides reducing equivalents for FeS cluster assembly in the mitochondria, was identified. Similar to the NIF components, MbFdx possesses a putative N-terminal targeting sequence (Fig. S2). Components of the ISC export machinery [Atm1 (a mitochondrial ATP-binding cassette transporter) and Erv1 (a sulfhydryl oxidase)] were also not present. Interestingly, we identified all components of the CIA machinery, except electron transport proteins Tah18 and Dre2 (Table S1).

N-Terminal Mitochondrial Targeting Sequences Deliver MbNifS-M and MbNifU-M to the Mitochondria in *Saccharomyces cerevisiae*. Because there is no tractable system for protein expression in *M. balamuthi*, we used *S. cerevisiae*, which is a well-studied model organism belonging together with Amoebozoa to Unikonta (21). We first expressed the MbNifU-M fused to GFP at carboxyl terminus with and without the N-terminal mitonchondrial targeting sequences (MTS). The experiments showed that the targeting of MbNifU-M-GFP to the yeast mitochondria was entirely MTS-dependent. We obtained the same results for MbNifS-M-GFP. Both NIF paralogs that lacked the amino-terminal extensions (MbNifU-C and MbNifS-C) were localized to the yeast cytosol. Next, we examined whether MbNifU-M and MbNifS-M were translocated into the yeast mitochondria or remained attached to the surface of the membrane. The immunoblot signals for the two proteins were not significantly affected when isolated mitochondria were treated with trypsin, but the signals for both proteins disappeared after trypsin treatment when the organellar membranes were disintegrated with detergent (Fig. 2). These experiments confirmed that MbNifS-M and MbNifU-M were targeted and translocated into the yeast mitochondria through their MTSs.

Dual Localization of the NIF Machinery in *M. balamuthi*. The localization of MbNifS and MbNifU in the subcellular fractions of *M. balamuthi* revealed that distinct NIF components were present in the cytosol and mitochondrion-enriched fractions. MbNifS was visualized as a double band in the whole-cell lysate, but only single bands of different sizes corresponding to MbNifS-C and MbNifS-M were observed in the cytosol and mitochondria, respectively. A similar pattern was obtained using an antibody that recognizes MbNifU-C and MbNifU-M. The localization of MbNifS-M and MbNifU-M to the mitochondria was confirmed through a protease protection assay performed on the mitochondria-enriched fractions (Fig. 3).

Next, we measured the activity of cysteine desulfurase based on the detection of sulfur formation. The specific activity of cysteine desulfurase in the cytosol was 1.130 ± 0.177 nmol·min^{-1}·mg^{-1} (n = 8). Considerably lower specific activity was detected in the mitochondrial fraction (0.146 ± 0.062 nmol·min^{-1}·mg^{-1}; n = 8). However, a 67% increase in activity was observed after disintegration of the organellar membranes using detergent (0.443 ± 0.116 nmol·min^{-1}·mg^{-1}; n = 8) (Table S2). The latency of cysteine desulfurase activity indicated an intraorganellar localization for this protein. The organellar activity of NifS was further confirmed based on alanine production that was comparable with the sulfur

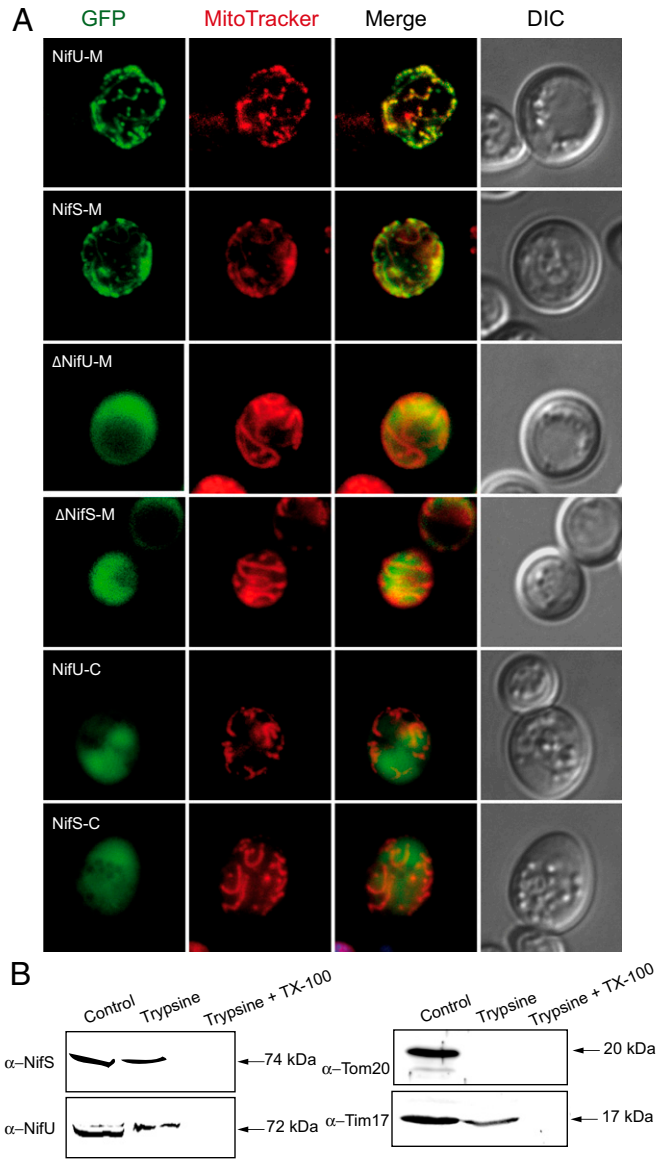

Fig. 2. N-terminal presequence-dependent targeting of *M. balamuthi* NifS-M and NifU-M into *S. cerevisiae* mitochondria. (*A*) The proteins were expressed with GFP tags in *S. cerevisiae* (green). Mitochondria were costained with Mitotracker (red). MTSs were deleted in ΔMbNifS-M and ΔMbNifU-M. MbNifS-C, and MbNifS-C paralogs did not contain the N-terminal MTSs. DIC, differential interference contrast. (Magnification: *A*, 1,000×.) (*B*) Protease protection assays. Isolated mitochondria (control) were treated with trypsin alone or together with Triton X-100. Antibodies against entamoebal NifS and NifU as well as yeast Tom20 (outer-membrane marker) and Tim17 (inner-membrane marker) were used for the immunoblot analysis.

formation (0.365 nmol·min^{-1}·mg^{-1}; n = 8) (Fig. S3). Finally, we determined whether the cytosolic and mitochondrial fractions catalyze the formation of FeS clusters in recombinant apo-MbFdx using S^{35}-cysteine. Reconstitution of FeS centers was observed with time-dependent incorporation of S^{35} into holo-MbFdx when either the cytosolic or mitochondrial fraction was added to the reaction mixture (Fig. 4). Taken together, these results indicate that the NIF machinery catalyzes independent FeS cluster assembly in two cellular compartments, the cytosol and the mitochondria.

Mitochondria Contain NIF Machinery Together with Substrate FeS Proteins. Searches of the *M. balamuthi* genome database revealed two complete gene sequences encoding candidate FeS proteins: succinate dehydrogenase subunit B (SdhB) and hydrogenase maturase E (HydE) (Table S1). Immunofluorescence microscopy revealed colocalization of SdhB with the signal for HydE in numerous round organelles corresponding to mitochondria (Fig. 5). Importantly, NifU and NifS staining was observed in the cytosol and in vesicles colabeled with the anti-SdhB antibody. The presence of mitochondrial NIF components together with HydE and SdhB in the same compartment was further confirmed through immunoblot analysis of the subcellular fractions (Fig. 3).

Next, we tested the enzymatic activities of two FeS enzymes for which the corresponding genes were identified in the *Mastigamoeba* genome: a hydrogenase, and PFO (Table S1). High activity of both PFO and hydrogenase was detected in the cytosol (Table S2). However, ∼2% of the activity of both the enzymes was associated with the mitochondrial fraction (Table S2). As in the case of cysteine desulfurase, PFO displayed 77% latency in the mitochondrial fraction, indicating an intraorganellar localization (Table S2). Accordingly, two hydrogenases and two PFOs possessed predictable MTSs that were absent in the other paralogs (Fig. S2 and Table S1). The activities of malate dehydrogenase and NADH oxidase were used as mitochondrial and cytosolic enzyme markers, respectively (Table S2). These data indicate that the NIF machinery is present in mitochondria, together with proteins whose maturation is dependent on FeS cluster assembly.

Components of *E. histolytica* NIF Machinery Do Not Possess MTSs and Are Found in the Cytosol. In contrast to *M. balamuthi*, single-copy genes encode *E. histolytica* NifS and NifU that do not possess a recognizable amino-terminal MTS (Fig. 1). The expression of both *E. histolytica* NIF components in *S. cerevisiae* revealed that these proteins are not targeted to the mitochondria but instead remain in the cytosol (Fig. 6). Subcellular fractionation of *E. histolytica* (Eh) revealed that the signals for EhNifS and EhNifU appear predominantly in the soluble fraction. A faint band for EhNifS was observed in the organellar fraction when large amounts of protein were loaded. However, this band disappeared upon trypsin treatment of the organelles (Fig. S4). These results do not

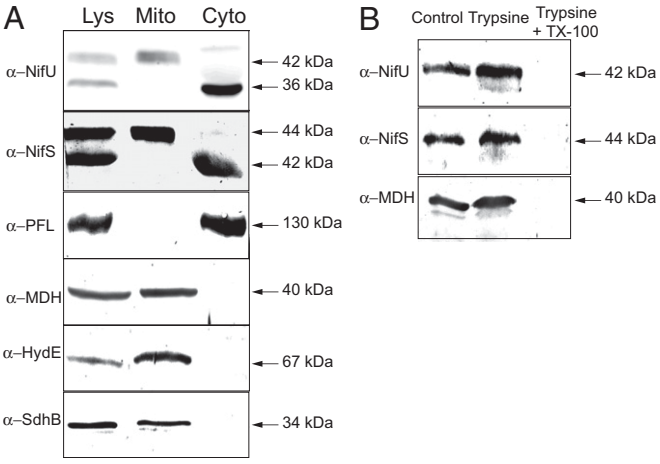

Fig. 3. Localization of the NIF components in *M. balamuthi* cellular fractions. (*A*) Immunoblot analysis of whole cell lysates (Lys), mitochondrial fractions (Mito), and cytosol (Cyto). NifS-M, NifS-C, NifU-M, and NifU-C were visualized using *E. histolytica* αNifS and αNifU antibodies that recognize both forms. Pyruvate formate lyase (PFL), malate dehydrogenase (MDH), HydE, and SdhB were recognized using homologous polyclonal antibodies. (*B*) Protease protection assay. Control, mitochondrial fractions without treatment; Trypsin+TX-100, treatment with trypsin and Triton X-100. The mitochondria were probed using antibodies against *E. histolytica* NifS, NifU, and *M. balamuthi* MDH.

EVOLUTION

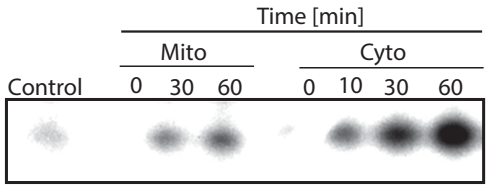

Fig. 4. Reconstitution of [FeS] clusters in *M. balamuthi* apoferredoxin catalyzed in mitochondrial (Mito) and cytosolic fractions. Recombinant apoferredoxin was incubated with [^{35}S]cysteine, Fe-ascorbate, and cellular fractions for the indicated time period and analyzed using native gel electrophoresis followed by autoradiography.

support previous report suggesting that NIF components function in the mitosomes in addition to the cytosol of *E. histolytica* (18).

Discussion

There are three unique features of the FeS cluster assembly machinery in *M. balamuthi*: (*i*) the archetypal mitochondrial ISC machinery that is present in virtually all eukaryotes has been replaced with ε-bacterial NIF components; (*ii*) the genes encoding the NIF machinery proteins have been duplicated, with one system being present in the cytosol and the second system targeted to the mitochondria; and (*iii*) both the cytosolic and mitochondrial cellular fractions independently catalyze the formation of FeS clusters.

In the majority of eukaryotes, the mitochondrial ISC machinery is essential for the maturation of cytosolic FeS proteins (10). This phenomenon has been functionally demonstrated in evolutionarily distant organisms, such as *S. cerevisiae* (10) and *Trypanosoma brucei* (22). The typical mitochondrial metabolic pathways have been lost during the course of reductive evolution in the mitosomes of *Giardia* and microsporidia (11). However, the mitosomes of these species have retained the functional mitochondrial ISC machinery, underscoring the essential function of this pathway in eukaryotic cells (13, 23). Maturation of extramitochondrial FeS proteins requires the export of a still unknown compound from the mitochondria to the cytosol, where it is used by the CIA machinery (2). It has been speculated that this compound contains a sulfur moiety or a preassembled FeS cluster that is transferred to apoproteins via the components of the CIA machinery (2). However, *M. balamuthi* does not follow this general scheme. The present results show that *Mastigamoeba* contains two distinct NIF-type FeS cluster assembly machineries that most likely function independently in the cytosol and mitochondria. The components of the cytosolic and mitochondrial machineries, including NifS and NifU, are encoded by distinct paralogs, and the presence of the corresponding gene products in both cellular compartments of *M. balamuthi* was confirmed through subcellular fractionation and immunofluorescence microscopy. Importantly, biochemical studies have demonstrated that both the cytosol and the mitochondria possess cysteine desulfurase activity and can catalyze the formation of FeS clusters on apoferredoxin. The dual localization of the NIF machinery observed in *M. balamuthi* is reminiscent of how some components of the ISC machinery are localized in human cells, where IscS, IscU, and Nfu are found predominantly in the mitochondria, although small amounts of these proteins are also detected in the cytosol and the nucleus. Unlike *Mastigamoeba*, human mitochondrial and cytosolic/nuclear proteins are synthesized from a single transcript through alternative translation or splicing (24). Although the involvement of cytosolic/nuclear ISC components in the de novo formation of FeS clusters is currently a matter of discussion (24, 25), it has been shown that depletion of cytosolic/nuclear IscU causes defects in cytosolic FeS cluster assembly (26). However, there is no information regarding the possible relationship between the cytosolic ISC components and the human CIA machinery. Thus, future studies should clarify whether the cytosolic NIF found in *Mastigamoeba* and the human ISC machineries function independently or whether the cytosolic NIF/ISC provide any substrate for the CIA machinery that is conserved in all eukaryotes, including *Mastigamoeba*.

The substitution of a multicomponent mitochondrial ISC system with simple NIF machinery was a unique evolutionary event in eukaryotes, but it is not unprecedented elsewhere in nature. In bacteria, the NIF system has been identified in nonnitrogen-fixing anaerobic ε-proteobacteria, such as *Helicobacter*, in which the NifS and NifU components function in FeS cluster assembly, whereas the ISC and SUF machineries are absent (27). Interestingly, the

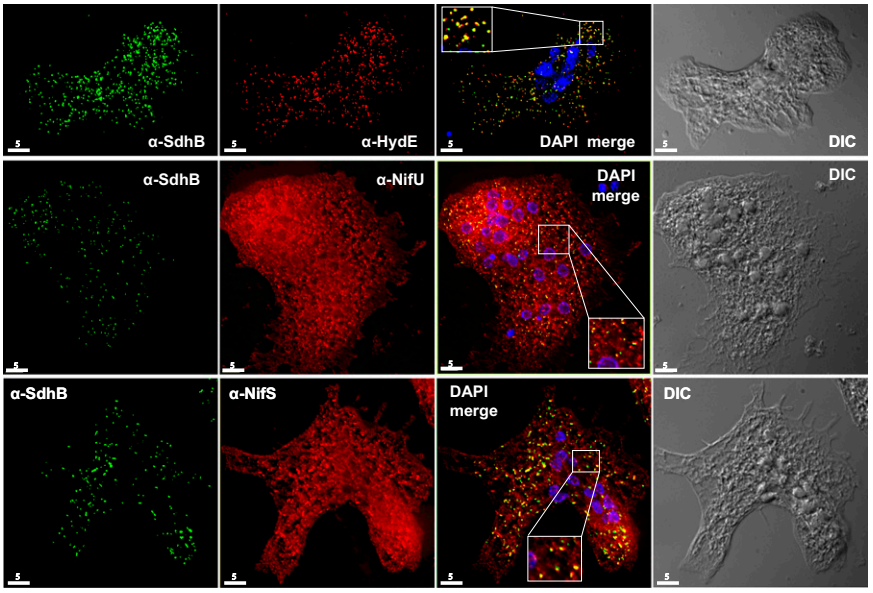

Fig. 5. Localization of FeS assembly machinery (NifS and NifU) and FeS proteins (SdhB and HydE) in *M. balamuthi*. The MbSdhB mitochondrial FeS protein (green) colocalized with the hydrogenase maturase HydE (red). The signal for NifS and NifU (red) showed the double localization of proteins in the organelles and cytosol. The SdhB colocalized with organellar NifS and NifU. DAPI (blue); DIC, differential interference contrast. (Scale bars: 5 μm.)

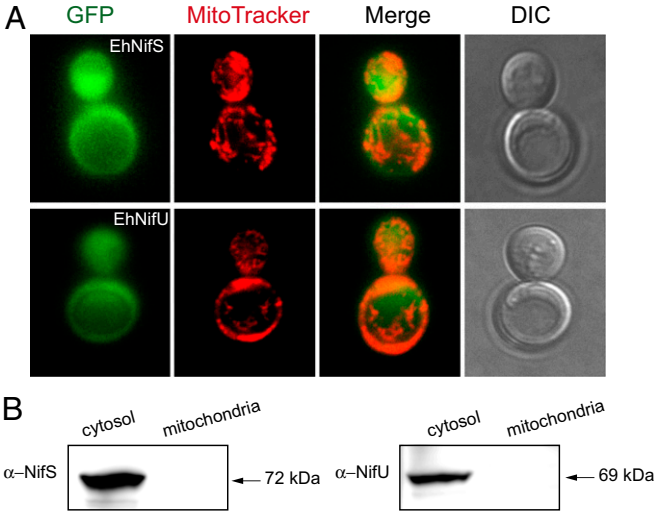

Fig. 6. Expression of *E. histolytica* NifS and NifU in *S. cerevisiae*. (*A*) Proteins were expressed with a GFP-tag at the carboxyl terminus in *S. cerevisiae* (green). (Magnification: *A*, 1,000×.) (*B*) Immunoblot analysis of mitochondrial and cytosolic fractions. The recombinant proteins were detected using anti-NifS and -NifU antibodies.

expression of *Helicobacter* NifS/NifU and *E. histolytica* EhNifS/EhNifU in *Escherichia coli*, in which both the *isc* and *suf* operons have been deleted, supports the growth of mutated bacteria. However, full complementation of the ISC and SUF systems is observed only under anaerobic conditions (17, 28). Thus, it seems plausible that the substitution of the ISC machinery with the oxygen-sensitive NIF machinery that occurred in the *Mastigamoeba* ancestor would have been possible only in anaerobic niches, where this species exists together with NIF-possessing ε-proteobacteria (16). The oxygen sensitivity of the NIF system might also explain why the NIF machinery has not been identified in any aerobic organism.

The replacement of the ISC assembly machinery in mitochondria with the NIF system requires the acquisition of mitochondrial signals to mediate the targeting and translocation of NifS and NifU to these organelles. There are two types of MTSs: (*i*) amino-terminal cleavable presequences, and (*ii*) inner signals embedded within the structure of the mature protein (29). In the present study, we showed that both NifS-M and NifU-M possess amino-terminal presequences that are strictly required for protein delivery to the yeast mitochondria, and deletion of these extensions results in the mislocalization of the NIF proteins to the cytosol. The strict presequence-dependent targeting of NifS-M and NifU-M to the mitochondria indicates that inner signals are absent from these NIF components. Hence, it is likely that gene duplication and acquisition of the N-terminal signals were essential for the targeting of the NIF components to these organelles upon LGT from ε-proteobacteria.

The dual localization of the NIF machinery observed in *M. balamuthi* is consistent with the dual localization of the proteins that require the assembly of FeS clusters for maturation. We demonstrated that the *Mastigamoeba* mitochondria contain several FeS proteins, including the SdhB subunit of the succinyl dehydrogenase complex, the hydrogenase maturase HydE and, most likely, [2Fe2S] ferredoxin. Interestingly, the activity of two typical FeS enzymes involved in extended anaerobic glycolysis, PFO and hydrogenase, was identified in both compartments, although this activity was predominantly localized to the cytosol. Hydrogenase is also encoded in the *E. histolytica* genome, and episomally expressed entamoebic hydrogenase has been detected in the cytosol of transformed cells (30). However, hydrogenase activity has not been observed in nontransfected entamoebas, and rather low activity has been identified in transfected cells (0.0035 μmol·min·mg) (31). In this context, the activity of hydrogenase in the cytosol of *M. balamuthi* (1.351 ± 0.176 μmol·min·mg) is unusually high.

The presence of aerobic mitochondria in Mycetozoa, which is a sister taxon of the anaerobic Archamoebae (32), indicates that the mitochondria of *M. balamuthi* represent secondarily adapted and reduced forms of these organelles and that reductive evolution has progressed further in entamoebids, which retain mitochondria in the form of mitosomes. In contrast to the mitochondria of *M. balamuthi*, there is no evidence for the presence of any protein with classic multiple iron FeS clusters in *E. histolytica* mitosomes. Therefore, the predominant cytosolic distribution of the NIF machinery should be sufficient to accommodate the cellular requirements for FeS cluster biogenesis in this organism. Surprisingly, dual localization of the NIF machinery in the cytosol and the mitosomes of *E. histolytica* has also been suggested, and FeS cluster biogenesis has been proposed as one of the fundamental functions of entamoebal mitosomes (18). However, following proteomic analysis of *E. histolytica* mitosomes has not confirmed the presence of NIF machinery (20). In our cell localization analyses, we detected components of the *E. histolytica* NIF machinery, particularly in the cytosolic fraction, which had been suggested previously (19). Moreover, the *E. histolytica* NIF components were found to be encoded by single copy genes, which lack the mitochondrial targeting signals. Neither the cytosolic NIF components of *M. balamuthi* nor the NIF components of *E. histolytica* were imported into the mitochondria in yeast, thereby suggesting an absence of inner mitochondrial targeting signals in these proteins. However, we cannot exclude the potential recognition of a small amount of NifS and NifU through the translocation machinery of the mitosomes, which are considerably modified compared with yeast mitochondria (19), although the present experiments do not provide support for this possibility.

In conclusion, based on the present and previous findings (16, 17), we propose the following scenario for the evolution of FeS cluster assembly in Archamoebae: (*i*) a common ancestor of *Mastigamoeba* and *Entamoeba* that inhabited anaerobic niches gained the NIF machinery from ε-proteobacteria through LGT; (*ii*) the genes encoding the NIF machinery were ancestrally duplicated and the components that acquired MTSs replaced the ISC machinery, as observed in *Mastigamoeba*; and (*iii*) in *Entamoeba*, the mitochondrial NIF machinery may have lost its organellar targeting sequences and replaced the cytosolic NIF version, as suggested by our phylogenetic analysis. However, the low resolution of the Archamoeba tree cannot exclude a more parsimonious scenario, in which the mitochondrial NIF machinery was lost and the cytosolic version was retained. Although the characteristics of the NIF system that were advantageous over the ISC system under anaerobic conditions remain enigmatic, the presence of the NIF system in Archamoebae highlights the diversity involved in the adaptation of eukaryotes to anaerobic niches, which has repeatedly occurred in distinct eukaryotic lineages (11).

Materials and Methods

Cell Cultivation and Fractionation. *M. balamuthi* (ATCC 30984), *E. histolytica* (strain HM-1:IMSS), and YPH499 *S. cerevisiae* strain were maintained axenically, as previously described (33–35). The preparation of subcellular fractions of *M. balamuthi* and *E. histolytica* through differential centrifugation of the cell homogenate is described in *SI Materials and Methods*. The yeast mitochondria were obtained according to previously described methods (36).

Genome Sequencing. The partial genome sequence of *M. balamuthi* was assembled from sequences generated using Illumina and 454 pyrosequencing. Based on the estimated genome size of 50 Mb, the genome coverage is 15× for long 454 reads and 20× for short paired-end Illumina reads (*SI Material and Methods*). The preliminary assembly of *M. balamuthi* genome and raw sequencing data have been submitted to EMBL (www.ebi.ac.uk/ena/data/view/PRJEB1507).

Phylogenetic Analysis. Maximum-likelihood and Bayesian phylogenetic analyses of selective NifS and NifU proteins from epsilon proteobacteria along with the homologs from the archamoebae species were performed as decribed in *SI Materials and Methods*.

Gene Searches and Cloning. The local *M. balamuthi* genome database was searched using the TBLASTN algorithm with protein sequences of known orthologs in *E. histolytica* and *T. vaginalis* (Table S3). The PCR primers were designed based on identified genomic sequences (Table S4) for the amplification of coding sequences using *M. balamuthi* cDNA as a template. The genes encoding MbNifS-M, MbNifU-M, MbNifS-C and MbNifU-C (the accession numbers are provided in Table S1) were cloned and expressed as described in *SI Materials and Methods*.

Protease Protection Assay. The topology of the mitochondrial proteins was tested using trypsin treatment as previously described (37). Details of the procedure are given in *SI Materials and Methods*. Rabbit polyclonal antibodies against *E. histolytica* NifS, NifU, and Cpn60 (a kind gift from T. Nozaki, National Institute of Infectious Diseases, Tokyo, Japan), and *S. cerevisiae* Tom20 and Tim17 (a kind gift from T. Lithgow and K. Gabriel, Monash University, Melbourne, Australia) were used for immonoblot analyses.

Immunofluorescent Microscopy. Image acquisition was performed using standard techniques (38) (*SI Materials and Methods*).

Enzyme Assays. The PFO, hydrogenase, NADH oxidase, and malate dehydrogenase activities were assayed as previously described (39). The cysteine desulfurase activity was monitored through the production of sulfide (40) or alanine (*SI Materials and Methods*).

Reconstitution of FeS Clusters. Recombinant MbFdx was expressed in BL21 *E. coli* (DE3) using the pET42b$^+$ vector (Novagen) and purified under native conditions (Qiagen). The reconstitution of FeS clusters in apoMbFdx was performed as previously described (12).

ACKNOWLEDGMENTS. We thank Alexandra Sequin for assistance in the reconstitution of iron-sulfur clusters; Anastasios Tsaousis and Vojtěch Žárský for assistance in phylogeny studies; Boris Striepen for expertise in immunofluorescent microscopy; and appreciate the access to computing and storage facilities owned by parties contributing to the National Grid Infrastructure MetaCentrum and European Life Sciences Infrastructure for Biological Information node CZ (LM2010005). This work was supported through funding from the Czech Science Foundation (P305/11/1061), and Czech Ministry of Education Grants MSM 0021620858 (to J.T.) and GAUK101710 (to E.N.).

1. Meyer J (2008) Iron-sulfur protein folds, iron-sulfur chemistry, and evolution. *J Biol Inorg Chem* 13(2):157–170.
2. Lill R (2009) Function and biogenesis of iron-sulphur proteins. *Nature* 460(7257): 831–838.
3. Djaman O, Outten FW, Imlay JA (2004) Repair of oxidized iron-sulfur clusters in *Escherichia coli*. *J Biol Chem* 279(43):44590–44599.
4. Bandyopadhyay S, Chandramouli K, Johnson MK (2008) Iron-sulfur cluster biosynthesis. *Biochem Soc Trans* 36(Pt 6):1112–1119.
5. Johnson DC, Dos Santos PC, Dean DR (2005) NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of isc-gene products in *Azotobacter vinelandii*. *Biochem Soc Trans* 33(Pt 1):90–93.
6. Tachezy J, Sánchez LB, Müller M (2001) Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as indicated by the phylogeny of IscS. *Mol Biol Evol* 18(10):1919–1928.
7. Embley TM, Martin W (2006) Eukaryotic evolution, changes and challenges. *Nature* 440(7084):623–630.
8. Tachezy J, Dolezal P (2007) *Origin of Mitochondria and Hydrogenosomes*, eds Martin WF, Müller M (Springer, Berlin), pp 105–133.
9. Ye H, Pilon M, Pilon-Smits EA (2006) CpNifS-dependent iron-sulfur cluster biogenesis in chloroplasts. *New Phytol* 171(2):285–292.
10. Lill R, et al. (2012) The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. *Biochim Biophys Acta* 1823(9):1491–1508.
11. Müller M, et al. (2012) Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol Mol Biol Rev* 76(2):444–495.
12. Šut'ák R, et al. (2004) Mitochondrial-type assembly of FeS centers in the hydrogenosomes of the amitochondriate eukaryote *Trichomonas vaginalis*. *Proc Natl Acad Sci USA* 101(28):10368–10373.
13. Tovar J, et al. (2003) Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. *Nature* 426(6963):172–176.
14. Goldberg AV, et al. (2008) Localization and functionality of microsporidian iron-sulphur cluster assembly proteins. *Nature* 452(7187):624–628.
15. Putignani L (2005) The unusual architecture and predicted function of the mitochondrion organelle in *Cryptosporidium parvum* and *hominis* species: The strong paradigm of the structure-function relationship. *Parassitologia* 47(2):217–225.
16. Gill EE, et al. (2007) Novel mitochondrion-related organelles in the anaerobic amoeba *Mastigamoeba balamuthi*. *Mol Microbiol* 66(6):1306–1320.
17. Ali V, Shigeta Y, Tokumoto U, Takahashi Y, Nozaki T (2004) An intestinal parasitic protist, *Entamoeba histolytica*, possesses a non-redundant nitrogen fixation-like system for iron-sulfur cluster assembly under anaerobic conditions. *J Biol Chem* 279(16): 16863–16874.
18. Maralikova B, et al. (2010) Bacterial-type oxygen detoxification and iron-sulfur cluster assembly in amoebal relict mitochondria. *Cell Microbiol* 12(3):331–342.
19. Dolezal P, et al. (2010) The essentials of protein import in the degenerate mitochondrion of *Entamoeba histolytica*. *PLoS Pathog* 6(3):e1000812.
20. Mi-ichi F, Abu Yousuf M, Nakada-Tsukui K, Nozaki T (2009) Mitosomes in *Entamoeba histolytica* contain a sulfate activation pathway. *Proc Natl Acad Sci USA* 106(51): 21731–21736.
21. Cavalier-Smith T (2003) Protist phylogeny and the high-level classification of Protozoa. *Int J Parasitol* 39(4):338–348.
22. Smíd O, et al. (2006) Knock-downs of iron-sulfur cluster assembly proteins IscS and IscU down-regulate the active mitochondrion of procyclic *Trypanosoma brucei*. *J Biol Chem* 281(39):28679–28686.
23. Williams BAP, Hirt RP, Lucocq JM, Embley TM (2002) A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. *Nature* 418(6900):865–869.
24. Rouault TA, Tong WH (2005) Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat Rev Mol Cell Biol* 6(4):345–351.
25. Biederbick A, et al. (2006) Role of human mitochondrial Nfs1 in cytosolic iron-sulfur protein biogenesis and iron regulation. *Mol Cell Biol* 26(15):5675–5687.
26. Li K, Tong WH, Hughes RM, Rouault TA (2006) Roles of the mammalian cytosolic cysteine desulfurase, ISCS, and scaffold protein, ISCU, in iron-sulfur cluster assembly. *J Biol Chem* 281(18):12344–12351.
27. Olson JW, Agar JN, Johnson MK, Maier RJ (2000) Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. *Biochemistry* 39(51):16213–16219.
28. Tokumoto U, Kitamura S, Fukuyama K, Takahashi Y (2004) Interchangeability and distinct properties of bacterial Fe-S cluster assembly systems: Functional replacement of the isc and suf operons in *Escherichia coli* with the nifSU-like operon from *Helicobacter pylori*. *J Biochem* 136(2):199–209.
29. Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: Machineries and mechanisms. *Cell* 138(4):628–644.
30. Ghosh S, Field J, Rogers R, Hickman M, Samuelson J (2000) The *Entamoeba histolytica* mitochondrion-derived organelle (crypton) contains double-stranded DNA and appears to be bound by a double membrane. *Infect Immun* 68(7):4319–4322.
31. Nixon JEJ, et al. (2003) Iron-dependent hydrogenases of *Entamoeba histolytica* and *Giardia lamblia*: Activity of the recombinant entamoebic enzyme and evidence for lateral gene transfer. *Biol Bull* 204(1):1–9.
32. Adl SM, et al. (2012) The revised classification of eukaryotes. *J Eukaryot Microbiol* 59(5):429–493.
33. Chaves LA, Balamuth W, Gong T (1986) A light and electron-microscopic study of a new, polymorphic free-living ameba *Phreatamoeba balamuthi* n. g., n. sp. *J Protozool* 33(3):397–404.
34. Diamond LS, Clark CG, Cunnick CC (1995) YI-S, a casein-free medium for axenic cultivation of *Entamoeba histolytica*, related Entamoeba, *Giardia intestinalis* and *Trichomonas vaginalis*. *J Eukaryot Microbiol* 42(3):277–278.
35. Lithgow T, Junne T, Suda K, Gratzer S, Schatz G (1994) The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. *Proc Natl Acad Sci USA* 91(25):11973–11977.
36. Murakami H, Pain D, Blobel G (1988) 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol* 107(6 Pt 1):2051–2057.
37. Rada P, et al. (2011) The core components of organelle biogenesis and membrane transport in the hydrogenosomes of *Trichomonas vaginalis*. *PLoS ONE* 6(9):e24428.
38. Dolezal P, et al. (2005) Giardia mitosomes and trichomonad hydrogenosomes share a common mode of protein targeting. *Proc Natl Acad Sci USA* 102(31):10924–10929.
39. Vanacova S, et al. (2001) Iron-induced changes in pyruvate metabolism of *Tritrichomonas foetus* and involvement of iron in expression of hydrogenosomal proteins. *Microbiology* 147(Pt 1):53–62.
40. Siegel LM (1965) A direct microdetermination for sulfide. *Anal Biochem* 11:126–132.

6.2 Publication: Tsaousis et al., 2014

Tsaousis AD, Nývltová E, Sutak R, Hrdy I, Tachezy J. (2014). A nonmitochondrial hydrogen production in *Naegleria gruberi*. *Genome Biol Evol*. 6(4):792-9.

A Nonmitochondrial Hydrogen Production in *Naegleria gruberi*

Anastasios D. Tsaousis[1,2,*], Eva Nývltová[1], Robert Šuták[1], Ivan Hrdý[1], and Jan Tachezy[1,*]

[1]Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic

[2]Laboratory of Molecular and Evolutionary Parasitology, School of Biosciences, University of Kent, Canterbury, United Kingdom

*Corresponding author: E-mail: tsaousis.anastasios@gmail.com; tachezy@natur.cuni.cz.

Accepted: March 12, 2014

Abstract

Naegleria gruberi is a free-living heterotrophic aerobic amoeba well known for its ability to transform from an amoeba to a flagellate form. The genome of *N. gruberi* has been recently published, and in silico predictions demonstrated that *Naegleria* has the capacity for both aerobic respiration and anaerobic biochemistry to produce molecular hydrogen in its mitochondria. This finding was considered to have fundamental implications on the evolution of mitochondrial metabolism and of the last eukaryotic common ancestor. However, no actual experimental data have been shown to support this hypothesis. For this reason, we have decided to investigate the anaerobic metabolism of the mitochondrion of *N. gruberi*. Using in vivo biochemical assays, we have demonstrated that *N. gruberi* has indeed a functional [FeFe]-hydrogenase, an enzyme that is attributed to anaerobic organisms. Surprisingly, in contrast to the published predictions, we have demonstrated that hydrogenase is localized exclusively in the cytosol, while no hydrogenase activity was associated with mitochondria of the organism. In addition, cytosolic localization displayed for HydE, a marker component of hydrogenase maturases. *Naegleria gruberi*, an obligate aerobic organism and one of the earliest eukaryotes, is producing hydrogen, a function that raises questions on the purpose of this pathway for the lifestyle of the organism and potentially on the evolution of eukaryotes.

Key words: *Naegleria*, hydrogenase, maturases, mitochondrial evolution, hydrogen hypothesis.

Introduction

Naegleria gruberi is a noteworthy microbial eukaryote for evolutionary, biochemical, and biomedical reasons. *Naegleria gruberi* is a nonpathogenic relative to *Naegleria fowleri*, the "brain-eating amoeba," and the causative agent of the primary amoebic meningoencephalitis (PAM), a disease currently with no efficient treatment. Both organisms have the ability to transform from an amoeba to a biflagellate form or a cyst depending on its habitat, alternating its biochemical functions in each stage (Cable and John 1986; Fritz-Laylin et al. 2011). Evolutionarily, *N. gruberi* is considered to be one of the earliest eukaryotes and consequently close to the last eukaryotic common ancestor (Koonin 2010). Recent analysis of its genome has backed up this hypothesis with the discovery of a metabolically flexible mitochondrion that possesses both classical aerobic pathways including branched respiratory chain and oxidative phosphorylation, and enzymes that are known to mediate a substrate-level phosphorylation in the hydrogenosome, an anaerobic form of mitochondrion (Embley et al. 2003; Embley 2006). Most importantly, in silico predictions strongly suggested that *Naegleria*'s mitochondrion possesses an [FeFe]-hydrogenase, a marker of energy metabolism in anaerobic or microaerophilic organisms that produced molecular hydrogen (Vignais and Billoud 2007; Fritz-Laylin et al. 2010). This discovery provided new support for the hydrogen hypothesis of the origin of mitochondria, in which it has been postulated that the endosymbiotic ancestor of mitochondrion possessed both anaerobic and aerobic pathways (Martin and Muller 1998). During adaptation of eukaryotes to aerobic or anaerobic niches, these pathways were selectively lost, resulting in the formation of mitochondrion or hydrogenosome. However, a mitochondrion with both types of metabolisms operating in any contemporary eukaryote has not been found thus far.

For this reason, we have decided to investigate the localization and functional characterization of the [FeFe]-hydrogenase of *N. gruberi* to provide experimental data in addition to previous in silico predictions. The [FeFe]-hydrogenase is an enzyme that acts as a sink to remove reducing equivalents from oxidative decarboxylation of pyruvate or malate.

Electrons generated during these reactions are accepted by low-redox potential electron carriers (usually ferredoxins) and transferred to the hydrogenase that synthesizes molecular hydrogen. In eukaryotes, these enzymes are found in the hydrogenosomes of several anaerobic protists (for further reading, see Embley and Martin [2006], Hug et al. [2010], and Muller et al. [2012]) including chytridiomycetes, anaerobic ciliates, trichomonads, *Blastocystis*, as well as in the cytosol of others such as *Giardia* and *Entamoeba*. In addition, an [FeFe]-hydrogenase-dependent hydrogen production has been found in the chloroplasts of green algae (Kamp et al. 2008).

The [FeFe]-hydrogenase is generally associated with three maturase proteins: the radical *S*-adenosylmethionine enzymes HydE and HydG, and the small GTPase HydF (Putz et al. 2006). These maturases are required for the assembly of H cluster at the catalytic site of the enzyme that is essential for hydrogen synthesis. The H cluster includes two subclusters; a diiron subcluster with several nonprotein ligands and a [4Fe-4S] subcluster (Peters and Broderick 2012). Interestingly, the maturases have been identified in protists with hydrogenosomes (*Trichomonas vaginalis*, *Mastigamoeba balamuthi*; Putz et al. 2006; Nyvltova et al. 2013), but they are absent in organisms with exclusively cytosolic [FeFe]-hydrogenase (*Giardia intestinalis* and *Entamoeba histolytica*; Lloyd et al. 2002; Nixon et al. 2003). Unsurprisingly, the activities of cytosolic hydrogenases in these organisms are very low (*Giardia*) or could be detected only upon overexpression of the hydrogenase gene in transformed cells (*Entamoeba*). Thus, hydrogenases in *Giardia* and *Entamoeba* might not be involved in the production of molecular hydrogen as have been proposed (Meyer 2007; Nicolet and Fontecilla-Camps 2012).

In the current article, we have combined immunolocalization techniques along with cell biology and biochemistry to clarify the cellular localization of [FeFe]-hydrogenase in the aerobic excavate *N. gruberi*. We demonstrated that *N. gruberi* is able to generate molecular hydrogen when grown under aerobic conditions. Unexpectedly, [FeFe]-hydrogenase as well as HydE were detected exclusively in the cytosol of the organism.

Materials and Methods

Cell Cultivation

Naegleria gruberi strain NEG-M (kindly provided by Lillian Fritz-Laylin) was grown axenically at 27 °C in M7 medium (Fulton 1974). Cells were subcultured every 3–5 days depending on their density. The YPH499 *Saccharomyces cerevisiae* strain was grown in a rich or selective medium as described (Lithgow et al. 1994).

DNA, RNA Extraction, and RACE

Genomic DNA was extracted using the phenol:chloroform protocol (Sambrook et al. 2001). Total RNA extraction was performed using TRIzol protocol (Stechmann et al. 2008). The total RNA was used as a template for cDNA synthesis with the GeneRacer Kit (Invitrogen). cDNA was amplified according to the manufacturer's guidelines and by using the GeneRacer RNA oligo and the SuperScript III RT Reaction provided with the kit. Rapid amplification of the 5′-cDNA ends was used according to the manufacturer's protocol to amplify the 5′ end of each gene, and multiple clones were sequenced to verify the initial start codon of the gene. The list of primers used for this technique can be found in supplementary table S1, Supplementary Material online.

Cell Fractionation of *Naegleria*

Naegleria gruberi cellular fractions were obtained by differential centrifugation of the cell homogenate. All steps were carried out at 4 °C and in the presence of the protease inhibitors (Complete Mini EDTA-free cocktail tablets, Roche). To separate cellular fractions, the cells were centrifuged at 1,200 × g for 15 min, and washed and resuspended in the buffer (250 mM sucrose and 10 mM MOPS-KOH, pH 7.4). The washed cells were disrupted using sonication on ice. The homogenate was centrifuged twice at 1,200 × g for 15 min to remove unbroken cells, membrane fragments, and nuclei, and the supernatant was carefully collected. The final mitochondrial fraction was obtained by centrifugation of supernatant at 13,000 × g for 20 min and washed twice in the buffer. The cytosolic fraction was centrifuged at 20,000 × g for 25 min. The separated fractions were analyzed by enzymatic assays and western blot analysis.

Genes Cloning, and Expression in *S. cerevisiae*

The genes encoding *N. gruberi* [FeFe]-hydrogenase (XP_002674266), HydE (XP_002671091.1), and succinate dehydrogenase subunit B (SdhB; YP_007890028) were amplified from *Naegleria* cDNA and cloned into the pUG35 plasmid using XbaI and HindIII restriction sites. The plasmid encodes the respective protein by expression of the recombinant proteins fused with green fluorescence protein (GFP) in *S. cerevisiae* (Niedenthal et al. 1996). The pUG35 vector has -URA (uracyl) promoter, so the cells are growing on the uracyl-depleted medium (for selection); the protein expression is unregulated because it is immediate expressed upon transfection. For transformation, the WT 499 yeast culture was grown in a yeast extract peptone dextrose (YPD) medium overnight at 30 °C. The yeast cells were centrifuged at 1,000 × g for 5 min at 10 °C and washed in sterile phosphate buffered saline (PBS). The pellet was resuspend in 1 ml of 100 mM lithium acetate (LiAC) buffer and incubated for 10 min at 30 °C. After incubation, the suspension was centrifuged at 1,000 × g for 1 min at 10 °C. The pellet was carefully resuspended in the transformation mixture (300 µl of 40% polyethylene glycol (PEG), 42 µl of 1 M LiAC, 10 µl of salmon sperm DNA (SSD), and 150 ng of plasmid DNA) and incubated

first for 30 min at 30 °C and then for 30 min at 42 °C. After incubation, the suspension was immediately centrifuged at 1,000 × g for 1 min at 10 °C and spread on selective –URA plate. After 4 days in 30 °C, the colonies were investigated by immunofluoresence microscopy and western blot analysis.

Cell Fractionation of *S. cerevisiae*

Saccharomyces cerevisiae expressing GFP-tagged *N. gruberi* [FeFe]-hydrogenase, HydE, and *N. gruberi* SdhB (NgSdhB) were grown in selective medium at 30 °C. Yeast mitochondria were obtained according to Niedenthal et al. (1996). To examine the topology of the mitochondrial proteins, the isolated mitochondria were incubated for 30 min at 37 °C in ST buffer (0.8 M sorbitol and 30 mM Tris buffer; pH 7.5) supplemented with 5 μg/ml trypsin (Sigma) or trypsin with 0.1% Triton X-100. The incubation was terminated upon the addition of 5 mg/ml soybean trypsin inhibitor followed by incubation on ice for 5 min. The proteins were precipitated with cold acetone for 1 h at −20 °C. The fractions were analyzed on western blot with α-GFP (Abcam) and *S. cerevisiae* α-Tom20 and α-Tim17 antibodies (kindly provided by T. Lithgow and K. Gabriel).

Immunofluorescent Microscopy

Naegleria gruberi cells were incubated for 20 min with the MitoTracker Red CMXRos mitochondrial marker (Molecular Probes), fixed by 1% formaldehyde and attached to coverslips. The cells were probed with rabbit polyclonal antibody (Ab) raised against *T. vaginalis* hydrogenase (kindly provided by P. Johnson, UCLA), rat polyclonal Ab against *M. balamuthi* SdhB (Nyvltova et al. 2013), *M. balamuthi* HydE (Nyvltova et al. 2013), and *S. cerevisiae* Nbp35 (kindly provided by Prof. Roland Lill). The proteins were visualized using secondary Alexa Fluor-588 donkey α-rabbit and Alexa-Fluor-488 goat α-rat IgG (Molecular Probes). *Saccharomyces cerevisiae* expressing GFP-tagged hydrogenase and NgSdhB were incubated for 30 min with the MitoTracker Red CMXRos mitochondrial marker (Molecular Probes), washed twice in PBS and stabilized in 1% agarose.

Enzymatic Assays

Hydrogenase (EC 1.18.3.1) activity was assayed spectrophotometrically at 600 nm in anaerobic cuvettes as the rate of methyl viologen reduction (Rasoloson et al. 2002). Mitochondrial enzyme $NADP^+$-glutamate dehydrogenase (GDH; EC 1.4.1.4) was determined spectrophotometrically at 340 nm. The reaction mixture (2 ml) contained 50 mM Tris-KCl buffer, pH 7.4, 1 mM $NADP^+$, 20 mM L-glutamate, and cell fractions (Helianti et al. 2001).

Cytosolic ATP-dependent hexokinase (EC 2.7.1.1) was measured spectrophotometrically at 340 nm as previously described (Fromm and Zewe 1962). The reaction mixture (2 ml) contained 50 mM Tris-KCl buffer (pH 7.4), 1 mM $NADP^+$, 1 mM ATP, 20 mM D-glucose, 1.0 U/ml NADP-glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and cell fraction. Protein concentration was assayed by Lowry method.

Hydrogen Production

In 30-ml tightly closed vials, 1×10^7 cells were placed in 10 ml of M7 medium. The vials were left in atmospheric air and incubated at 25 °C for 4 h. Hydrogen concentrations were measured in the gas phase by a GC-2014 gas chromatograph (Shimadzu) equipped with a thermal conductivity detector. Gases were separated using 3 mm × 2.1 m glass column filled with molecular sieve 5 Å, 60/80 mesh (Supelco). Argon was used as the carrier gas. The oven temperature was 60 °C.

Results and Discussion

Characteristics of the *N. gruberi* [FeFe]-Hydrogenase Sequence

In the recent genomic and transcriptomic study of *N. gruberi* (Fritz-Laylin et al. 2010), the authors have identified the full sequence of [FeFe]-hydrogenase gene, along with three predicted genes associated with the maturases of this enzyme. Bioinformatic analyses of the hydrogenase sequence have demonstrated that the encoded protein could have an N-terminal mitochondrial-targeting sequence (MTS; Fritz-Laylin et al. 2010) (supplementary figs. S1 and S2, Supplementary Material online). Interestingly, the *Naegleria* hydrogenase has an N-terminal region that is considerably longer than typical eukaryotic [FeFe]-hydrogenases (supplementary fig. S1, Supplementary Material online). This suspiciously long N-terminal expansion raised the question whether the predicted length of the sequence was of the correct size. To tackle this question, we used the rapid amplification of cDNA ends on *N. gruberi* cDNA. Using internal reverse primers to the coding region of the gene, we amplified the complete 5′-end, and after sequencing multiple clones, we demonstrated a 100% identity to the predicted coding region of the gene (supplementary fig. S2, Supplementary Material online). Similar approach has been used for verification of N-terminal regions of the hydrogenase maturases, with the same results (supplementary fig. S3, Supplementary Material online).

By examining the protein sequence, the *N. gruberi* [FeFe]-hydrogenase possesses conserved cysteine residues in the H-domain of the enzyme that are required for coordination of the H-cluster (supplementary fig. S1, Supplementary Material online). In addition, the N-terminal part of the protein possesses typical cysteine motives for coordination of two accessory [4Fe-4 S] clusters and an [2Fe2S] cluster that are involved in electron transfer from the electron donor to the active site (Vignais and Billoud 2007; Tsaousis et al. 2012).

All these predictions suggest that the *N. gruberi* hydrogenase is a functional enzyme and could be localized in the mitochondria of the cell as previously predicted (Fritz-Laylin et al. 2010).

Immunolocalization of *N. gruberi* [FeFe]-Hydrogenase

Using an Ab raised against *T. vaginalis* [FeFe]-hydrogenase (Bui and Johnson 1996), we demonstrated its high specificity for *N. gruberi* [FeFe]-hydrogenase that was recognized as a protein with an expected size of 83.8 kDa in *Naegleria*'s cell lysate (fig. 1*B*) as well as in a lysate of *Escherichia coli* cells heterologously expressing a fragment of *N. gruberi*'s hydrogenase (fig. 1*C*). Surprisingly, immunofluorescent microscopy of *N. gruberi* cells using α-hydrogenase Ab showed a cytosolic distribution of this enzyme, and the hydrogenase labeling do not colocalize with the MitoTracker (fig. 1*A*). The same cytosolic localization we also observed for hydrogenase maturase HydE (fig. 1*A* and supplementary fig. S4, Supplementary Material online). As controls, we used Abs against SdhB, the mitochondrial protein involved in TCA (tricarboxylic acid) cycle and against Nbp35, the cytosolic Fe-S cluster assembly factor (Netz et al. 2012). Immunofluorescent visualization of SdhB revealed its colocalization with the signal for MitoTracker in organelles corresponding to mitochondria (fig. 1*A*). Signal of the α-Nbp35 antibody had shown the same distribution as the α-hydrogenase and α-HydE Abs: it did not colocalize with MitoTracker and consequently showed a pattern corresponding to the cytoplasm of *Naegleria* (fig. 1*A*).

The localization of [FeFe]-hydrogenase and HydE observed by immunofluorecent microscopy was verified by cell fractionation experiments. Mitochondrial and cytosolic fractions were isolated from cell homogenate using differential centrifugation, followed by a immunoblot analysis. Using the α-hydrogenase Ab and α-HydE Ab, we have detected the signal for hydrogenase and HydE, respectively exclusively in the cytosolic fraction (fig. 1*B*). In contrast, SdhB was detected in the mitochondrial fraction.

Furthermore, we have decided to employ an alternative approach to evaluate the localization of the [FeFe]-hydrogenase and examine the function of predicted MTS. We expressed the 5′-end region of *N. gruberi*'s [FeFe]-hydrogenase (1,251 bp) and HydE (690 bp) in *S. cerevisiae* and investigated whether these proteins have the ability to deliver into mitochondria. As a positive control, we expressed the 5′-end region of SdhB (690 bp). Immunofluorescent microscopy revealed that the GFP-tagged *N. gruberi* SdhB localizes in the mitochondria (colocalization with MitoTracker; fig. 2*A*), whereas the *N. gruberi* [FeFe]-hydrogenase-GFP and HydE-GFP were found to be present exclusively in the cytosol of the cell (fig. 2*A*), which was consistent with the previous results. In addition, we examined the localization of hydrogenase-GFP, HydE-GFP, and NgSdhB-GFP in yeast subcellular fractions. The immunoblot analysis showed the signal for hydrogenase-GFP and HydE-GFP only in cytosolic fractions (fig. 2*B*). As expected, the signal for SdhB-GFP was found in isolated mitochondria. The NgSdhB topology within mitochondria was further tested by protease protection assay. The NgSdhB-GFP signal was not affected when the mitochondria were treated with trypsin; however, the signal disappeared after trypsin treatment when the organellar membranes were disintegrated with a detergent, which is consistent with the presence of NgSdhB-GFP in the mitochondrial matrix (fig. 2*B*). As a control for mitochondrial integrity, α-Tom20 was used as the outer membrane marker (digested with trypsin without the addition of detergent), and α-Tim17 was used as an inner membrane marker (not digested with trypsin without the addition of detergent; fig. 2*B*). These data demonstrated that even though *N. gruberi's* [FeFe]-hydrogenase and HydE proteins possess N-terminal extensions, these putative signals are not recognized by yeast mitochondria, and both proteins remain in yeast cytosol, which is consistent with their cytosolic localization in *N. gruberi*. In contrast, NgSdhB is recognized by yeast mitochondrial translocases and is delivered into the mitochondrial matrix.

Enzymatic Activity of [FeFe]-Hydrogenase

Both in vivo and in vitro experiments have revealed that the [FeFe]-hydrogenase localizes in the cytosol of *N. gruberi*. However, the possibility that there is a minor pool of the hydrogenase in the *N. gruberi's* mitochondria cannot be excluded. Therefore, to obtain independent line of evidence, we next tested the enzymatic activity of [FeFe]-hydrogenase in *N. gruberi* cellular fractions. Consistently, activity of [FeFe]-hydrogenase was found exclusively in the cytosolic fraction, and no activity was detected in the mitochondrial fraction of *N. gruberi* (table 1). For control reasons, the abundance and purity of mitochondrial and cytosolic fractions was tested using marker enzymes. GDH was used as a mitochondrial marker enzyme, which has been predicted to be present in the mitochondria of *N. gruberi* (Fritz-Laylin et al. 2010, 2011). Indeed, the GDH activity was exclusively associated with mitochondria (table 1). As a cytosolic marker, we used a glycolytic enzyme hexokinase that displayed about 9.36 nmol/min/mg activity in the cytosol and only 0.46 nmol/min/mg of activity was associated with mitochondria. The minor mitochondrial hexokinase activity is most likely due to its association with the outer mitochondrial membrane (table 1) (Majewski et al. 2004). These biochemical data not only support the cytosolic localization of *N. gruberi*'s [FeFe]-hydrogenase but also demonstrated that the protein is enzymatically active. Noteworthy, the activity of hydrogenase, which is an oxygen-sensitive enzyme, was measured in *N. gruberi* that was grown under standard aerobic conditions in axenic culture. Our attempts to maintain *Naegleria* under anaerobic atmosphere have been unsuccessful.

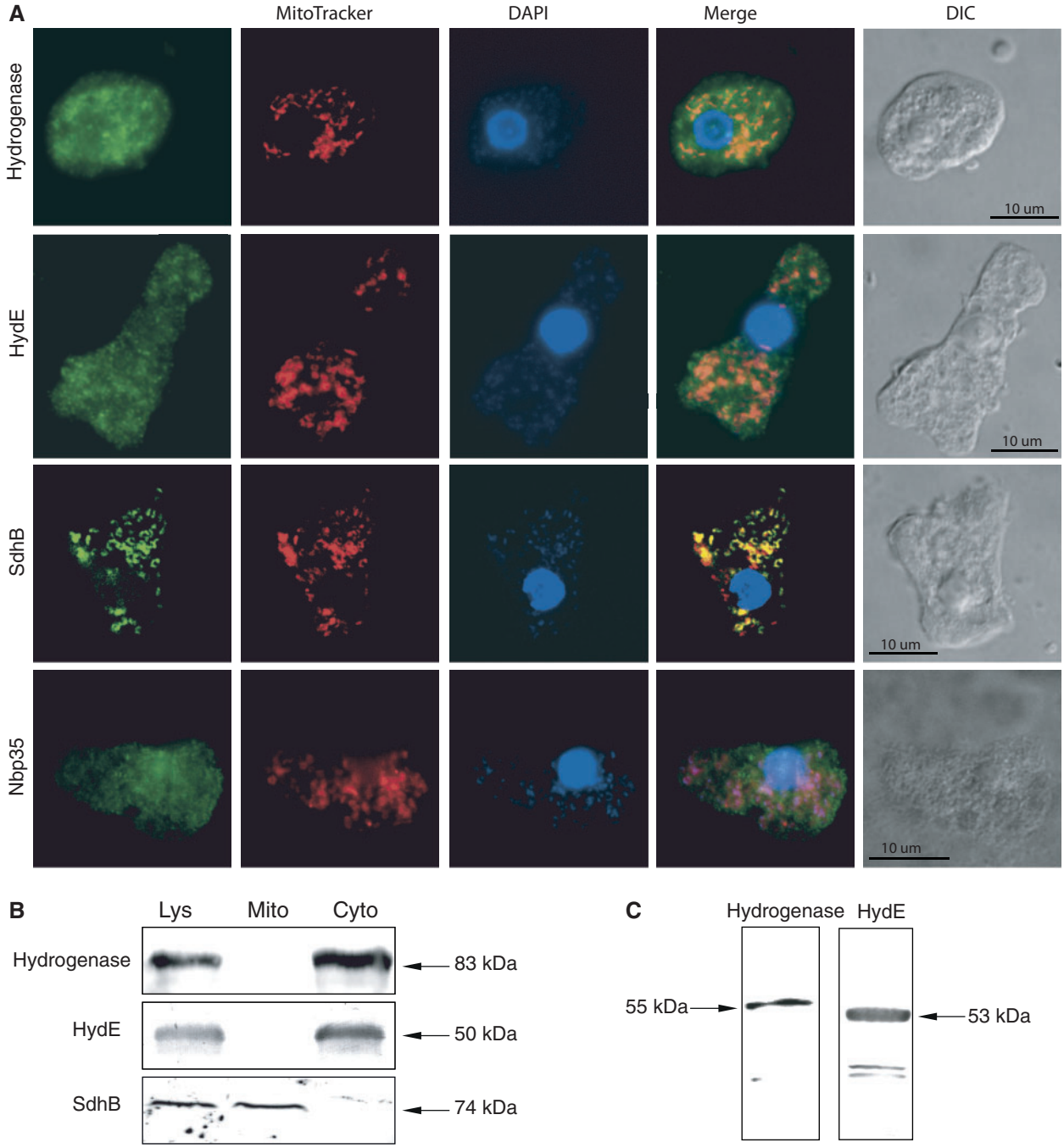

Fig. 1.—Cellular localization of hydrogenase and HydE in *N. gruberi* cells. (*A*) Immunofluorescent microscopy. [FeFe]-Hydrogenase, HydE, and Nbp35 (cytosolic marker) were detected in the cytosol of the cell. The signals for these proteins do not colocalize with the MitoTracker red. The Ab against SdhB (mitochondrial marker) labeled discrete structures corresponding to the *N. gruberi* mitochondria and colocalized with the MitoTracker red. 4′,6-Diamidino-2-phenylindole (DAPI) staining shows the presence of nucleus and mitochondrial DNA. DIC, differential interference contrast. Scale bar: 10 μm. (*B*) Localization of [FeFe]-hydrogenase, HydE, and SdhB in *N. gruberi* cellular fractions. Lys, whole cell lysates; Mito, mitochondrial fraction, Cyto, cytosolic fraction. SdhB, HydE and [FeFe]-hydrogenase were visualized using *M. balamuthi* α-SdhB, α-HydE and *T.vaginalis* α-hydrogenase, antibodies, respectively. (*C*) Specificity of α-[FeFe]-hydrogenase and α-HydE Abs were tested using partial recombinant *N. gruberi* [FeFe]-hydrogenase (55 kDa), and complete *N. gruberi* HydE (53 kDa) heterologously produced in *E. coli* and subsequently were probed using corresponding heterologous Ab.

Hydrogen Production

Even though we have demonstrated the functional activity of *N. gruberi* [FeFe]-hydrogenase, we have also questioned whether the cells can actually produce molecular hydrogen under standard cultivation conditions. For this reason, *N. gruberi* cells were incubated in the M7 growth medium under atmospheric oxygen, and after 4 h, the hydrogen concentrations were determined in the gas phase. As a result, we found that *N. gruberi* cells are able to produce ~6 nmol of hydrogen/min/10^7 cells (table 2). This level is about three times higher than hydrogen production observed in microaerophilic organisms *G. intestinalis* (2 nmol/min/10^7 cells; Lloyd et al. 2002) and about five times lower than in *T. vaginalis* (Sutak et al. 2012).

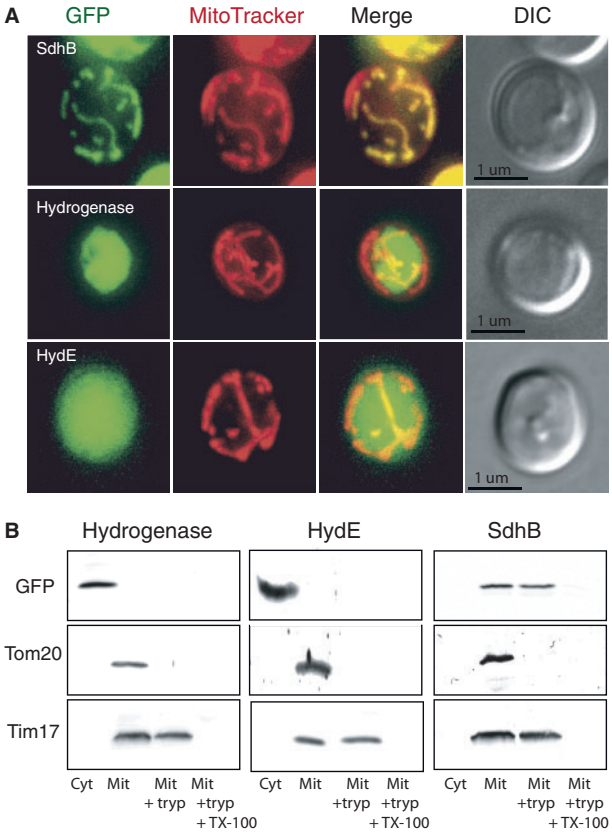

Fig. 2.—N-Terminal presequence-dependent targeting of *N. gruberi* hydrogenase, HydE, and SdhB into *S. cerevisiae* mitochondria. (*A*) The N-terminal region of the *N. gruberi* proteins (SdhB, Hydrogenase and HydE) were expressed with GFP tags in *S. cerevisiae* (green). Mitochondria were labeled with Mitotracker (red). DIC, differential interference contrast. (*B*) Western blot analysis of cellular fractions. Cyt, cytosol; Mit, mitochondrial fraction. Protease protection assays was performed for mitochondrial fraction: Mit+tryp, mitochondria were treated with trypsin; Mit+tryp+TX-100; mitochondria were treated with trypsin together with Triton X-100. Hydrogenase, NgSdhB, and HydE were detected using anti GFP tag antibody, and specific antibody was used to detect Tom20 (outer-membrane marker), and Tim17 (inner-membrane marker).

Concluding Remarks on the Anaerobic Metabolism of *Naegleria*

The recent completion of the genome project of *N. gruberi* has indicated that this amoeboflagellate is an organism with unique biology of its genome, cell biology, and biochemistry, leading to the capacity to alternate between aerobic and anaerobic metabolism (Fritz-Laylin et al. 2011). The genome encodes a [FeFe]-hydrogenase along with three enzymes required for its maturation; all four proteins were predicted to harbor N-terminal mitochondrial targeting signals suggestive of potential localization of this pathway in the organelles of *N. gruberi* (Fritz-Laylin et al. 2010; Fritz-Laylin et al. 2011; Opperdoes et al. 2011). As a result, having the capacity of aerobic respiration along with the anaerobic metabolism, the mitochondria of *N. gruberi* were considered to resemble the evolutionary intermediate proposed to have arisen within the ancestor of all extant eukaryotes (Mentel and Martin 2008; Fritz-Laylin et al. 2011). Even though this was a concrete hypothesis, there were still questions on the functionality of this pathway within mitochondria. For example, the pathway requires the presence of an enzyme producing reducing equivalents and a protein, which accepts these electrons and

Table 2

Hydrogen Production in Microbial Eukaryotes

Organism	Hydrogen Production (nmoles/min per 10^7 cells)	
Naegleria gruberi	5.814 ± 0.57	This study
Trichomonas vaginalis	29.098 ± 1.549	This study
		Ellis et al. (1992)
Giardia intestinalis	2	Lloyd et al. (2002)

Table 1

Biochemical Localization and Characterization of *Naegleria gruberi* [FeFe]-Hydrogenase

	Whole Cells (nmol/min/mg)	Mitochondrion (nmol/min/mg)	Cytosol (nmol/min/mg)	Localization
Hydrogenase	3.839 ± 0.233	0.0000	0.893 ± 0.241	Cytosol
GDH	5.132 ± 0.149	5.469 ± 0.152	0.000	Mitochondrion
Hexokinase	9.367 ± 1.714	0.465 ± 0.188	15.552 ± 3.24	Cytosol

transfers them to the [FeFe]-hydrogenase that subsequently produces molecular hydrogen. In anaerobic organisms, the proteins responsible for such function are the pyruvate:ferredoxin oxidoreductase (PFO) and the [2Fe-2S] ferredoxin. Even though two genes encoding [2Fe-2S] ferredoxin proteins have been discovered in the *N. gruberi* genome, a PFO gene has not been identified (Fritz-Laylin et al. 2010; Fritz-Laylin et al. 2011), raising questions on the functionality of the anaerobic metabolism within mitochondria. Moreover, the data presented on this study clearly demonstrate that functional [FeFe]-hydrogenase is not present in mitochondria; instead, the active enzyme is localized in the cytosol of the organism. Because a marker component of the maturases HydE is localized in the cytosol as well, we can confidently predict that maturation of hydrogenase takes place in the cytosol of *N. gruberi*. This is the first time that the [FeFe]-hydrogenase along with its maturases have been found in the cytosol of an organism, a result that raises more questions on the evolution and/or acquisition of this pathway within eukaryotes. Along with *Naegleria,* other organisms have a cytosolic version of [FeFe]-hydrogenase, including *M. balamuthi* (Nyvltova et al. 2013), *E. histolytica*, and *G. intestinalis.* Even in *T. vaginalis,* some of the [FeFe]-hydrogenases might be present outside of hydrogenosomes (Sutak et al. 2012); however, hydrogenase maturases were found exclusively within organelles, thus far.

The recent phylogenetic analysis of [FeFe]-hydrogenases proteins suggested multiple origins of [FeFe]-hydrogenases in eukaryotic cell evolution with lateral gene transfer (LGT), placing a huge part in it (Hug et al. 2010). If so, an interesting question is: What was the original cellular localization of hydrogenase upon its acquisition by LGT? The most plausible scenario seems to be that hydrogenase operated first in the cytosol and later, upon acquisition of mitochondrial targeting signals, it was imported together with hydrogenase maturases to the mitochondrion. If so, the situation in *Naegleria* may represent a stage that was conserved just after LGT of hydrogenase gene. Alternatively, acquisition of [FeFe]-hydrogenase could arise with the endosymbiotic ancestor of mitochondrion (Martin and Muller 1998), later in evolution, the ancient gene was replaced by LGT, and in the case of *N. gruberi* and other extant protists with cytosolic hydrogenases, the organellar hydrogenases gradually lost functional MTSs and localized to the cytosol. Indeed, [FeFe]-hydrogenase and HydE of *N. gruberi* possess unusual N-terminal extensions that reminds MTSs; however, the presence of these extensions did not facilitate protein delivery into the tested mitochondria. The situation will become clearer as we assemble more genomic data from diverse eukaryotes (especially those living in anaerobic or microaerophilic niches) within a range of lineages to provide a lucid picture on the evolution of this anaerobic pathway within eukaryotes. In addition, further investigations on the localization of proteins involved in anaerobic pathways of *N. gruberi* should come about, because we have clearly demonstrated that the current predictions are not consistent with experimental data. Last but not least, we should continue expanding our knowledge on the cell biology of *N. gruberi*, because it could subsequently provide us with new insights on how to combat its close relative *N. fowleri*, a deadly human pathogen without treatment.

Supplementary Material

Supplementary figures S1–S4 and table S1 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

This work was supported by an International Outgoing Fellowship (IOF) Marie Curie Fellowship to A.D.T., Charles University in Prague (UNCE 204017, GAUK101710) to E.N., the Czech Ministry of Education (MSM 0021620858) to J.T., and the project BIOCEV—Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0109) from the European Regional Development Fund. The authors thank Michaella Marcincikova for assisting with the culturing of *N. gruberi*, Patricia Johnson for providing us with the anti-*T. vaginalis* [FeFe]-hydrogenase antibody, and Roland Lill for providing us with the anti-yeast Nbp35 antibody.

Literature Cited

Bui ET, Johnson PJ. 1996. Identification and characterization of [Fe]-hydrogenases in the hydrogenosome of *Trichomonas vaginalis*. Mol Biochem Parasitol. 76:305–310.

Cable BL, John DT. 1986. Conditions for maximum enflagellation in *Naegleria fowleri*. J Protozool. 33:467–472.

Ellis JE, Cole D, Lloyd D. 1992. Influence of oxygen on the fermentative metabolism of metronidazole-sensitive and resistant strains of *Trichomonas vaginalis*. Mol Biochem Parasitol. 56:79–88.

Embley TM. 2006. Multiple secondary origins of the anaerobic lifestyle in eukaryotes. Philos Trans R Soc Lond B Biol Sci. 361:1055–1067.

Embley TM, Martin W. 2006. Eukaryotic evolution, changes and challenges. Nature 440:623–630.

Embley TM, van der Giezen M, Horner DS, Dyal PL, Foster P. 2003. Mitochondria and hydrogenosomes are two forms of the same fundamental organelle. Philos Trans R Soc Lond B Biol Sci. 358:191–201; discussion 201-192.

Fritz-Laylin LK, Ginger ML, Walsh C, Dawson SC, Fulton C. 2011. The *Naegleria* genome: a free-living microbial eukaryote lends unique insights into core eukaryotic cell biology. Res Microbiol. 162:607–618.

Fritz-Laylin LK, et al. 2010. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. Cell 140:631–642.

Fromm HJ, Zewe V. 1962. Kinetic studies of yeast hexokinase. J Biol Chem. 237:3027–3032.

Fulton C. 1974. Axenic cultivation of *Naegleria gruberi*. Requirement for methionine. Exp Cell Res. 88:365–370.

Helianti I, et al. 2001. Characterization of native glutamate dehydrogenase from an aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1. Appl Microbiol Biotechnol. 56:388–394.

Hug LA, Stechmann A, Roger AJ. 2010. Phylogenetic distributions and histories of proteins involved in anaerobic pyruvate metabolism in eukaryotes. Mol Biol Evol. 27:311–324.
Kamp C, et al. 2008. Isolation and first EPR characterization of the [FeFe]-hydrogenases from green algae. Biochim Biophys Acta. 1777: 410–416.
Koonin EV. 2010. Preview. The incredible expanding ancestor of eukaryotes. Cell 140:606–608.
Lithgow T, Junne T, Suda K, Gratzer S, Schatz G. 1994. The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. Proc Natl Acad Sci U S A. 91:11973–11977.
Lloyd D, Ralphs JR, Harris JC. 2002. *Giardia intestinalis*, a eukaryote without hydrogenosomes, produces hydrogen. Microbiology 148: 727–733.
Majewski N, et al. 2004. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol Cell. 16:819–830.
Martin W, Muller M. 1998. The hydrogen hypothesis for the first eukaryote. Nature 392:37–41.
Mentel M, Martin W. 2008. Energy metabolism among eukaryotic anaerobes in light of Proterozoic ocean chemistry. Philos Trans R Soc Lond B Biol Sci. 363:2717–2729.
Meyer J. 2007. [FeFe] hydrogenases and their evolution: a genomic perspective. Cell Mol Life Sci. 64:1063–1084.
Muller M, et al. 2012. Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. Microbiol Mol Biol Rev. 76: 444–495.
Netz DJ, et al. 2012. A bridging [4Fe-4S] cluster and nucleotide binding are essential for function of the Cfd1-Nbp35 complex as a scaffold in iron-sulfur protein maturation. J Biol Chem. 287:12365–12378.
Nicolet Y, Fontecilla-Camps JC. 2012. Structure–function relationships in [FeFe]-hydrogenase active site maturation. J Biol Chem. 287: 13532–13540.
Niedenthal RK, Riles L, Johnston M, Hegemann JH. 1996. Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12:773–786.
Nixon JE, et al. 2003. Iron-dependent hydrogenases of *Entamoeba histolytica* and *Giardia lamblia*: activity of the recombinant entamoebic enzyme and evidence for lateral gene transfer. Biol Bull. 204:1–9.
Nyvltova E, et al. 2013. NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*. Proc Natl Acad Sci U S A. 110:7371–7376.
Opperdoes FR, De Jonckheere JF, Tielens AG. 2011. *Naegleria gruberi* metabolism. Int J Parasitol. 41:915–924.
Peters JW, Broderick JB. 2012. Emerging paradigms for complex iron–sulfur cofactor assembly and insertion. Annu Rev Biochem. 81: 429–450.
Putz S, et al. 2006. Fe-hydrogenase maturases in the hydrogenosomes of *Trichomonas vaginalis*. Eukaryot Cell. 5:579–586.
Rasoloson D, et al. 2002. Mechanisms of in vitro development of resistance to metronidazole in *Trichomonas vaginalis*. Microbiology 148: 2467–2477.
Sambrook J, Russell DW, Maniatis T. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
Stechmann A, et al. 2008. Organelles in *Blastocystis* that blur the distinction between mitochondria and hydrogenosomes. Curr Biol. 18: 580–585.
Sutak R, et al. 2012. Secondary alcohol dehydrogenase catalyzes the reduction of exogenous acetone to 2-propanol in *Trichomonas vaginalis*. FEBS J. 279:2768–2780.
Tsaousis AD, Leger MM, Stairs CW, Roger AJ. 2012. The biochemical adaptations of mitochondrion-related organelles of parasitic and free-living microbial eukaryotes to low oxygen environments. In: Altenbach AV, Bernhard JM, Seckbach J, editors. Anoxia: cellular origin, life in extreme habitats and astrobiology. The Netherlands: Springer. p. 51–81.
Vignais PM, Billoud B. 2007. Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev. 107:4206–4272.

Associate editor: Bill Martin

6.3 Publication: Nývltová et al., 2015

Nývltová E, Stairs CW, Hrdý I, Rídl J, Mach J, Pačes J, Roger AJ, Tachezy J. (2015). Lateral gene transfer and gene duplication played a key role in the evolution of *Mastigamoeba balamuthi* hydrogenosomes. *Mol Biol Evol*., in press

Lateral Gene Transfer and Gene Duplication Played a Key Role in the Evolution of *Mastigamoeba balamuthi* Hydrogenosomes

Eva Nývltová,[1] Courtney W. Stairs,[2] Ivan Hrdý,[1] Jakub Rídl,[3] Jan Mach,[1] Jan Pačes,[3] Andrew J. Roger,[2] and Jan Tachezy*[1]

[1]Department of Parasitology, Faculty of Science, Charles University in Prague, Viničná, Prague, Czech Republic

[2]Centre for Comparative Genomics and Evolutionary Bioinformatics, Dalhousie University, Halifax, NS, Canada

[3]Laboratory of Genomics and Bioinformatics, Institute of Molecular Genetics AV CR, Vídeňská, Prague, Czech Republic

***Corresponding author:** E-mail: tachezy@natur.cuni.cz.

Associate editor: John Logsdon

Abstract

Lateral gene transfer (LGT) is an important mechanism of evolution for protists adapting to oxygen-poor environments. Specifically, modifications of energy metabolism in anaerobic forms of mitochondria (e.g., hydrogenosomes) are likely to have been associated with gene transfer from prokaryotes. An interesting question is whether the products of transferred genes were directly targeted into the ancestral organelle or initially operated in the cytosol and subsequently acquired organelle-targeting sequences. Here, we identified key enzymes of hydrogenosomal metabolism in the free-living anaerobic amoebozoan *Mastigamoeba balamuthi* and analyzed their cellular localizations, enzymatic activities, and evolutionary histories. Additionally, we characterized 1) several canonical mitochondrial components including respiratory complex II and the glycine cleavage system, 2) enzymes associated with anaerobic energy metabolism, including an unusual D-lactate dehydrogenase and acetyl CoA synthase, and 3) a sulfate activation pathway. Intriguingly, components of anaerobic energy metabolism are present in at least two gene copies. For each component, one copy possesses an mitochondrial targeting sequence (MTS), whereas the other lacks an MTS, yielding parallel cytosolic and hydrogenosomal extended glycolysis pathways. Experimentally, we confirmed that the organelle targeting of several proteins is fully dependent on the MTS. Phylogenetic analysis of all extended glycolysis components suggested that these components were acquired by LGT. We propose that the transformation from an ancestral organelle to a hydrogenosome in the *M. balamuthi* lineage involved the lateral acquisition of genes encoding extended glycolysis enzymes that initially operated in the cytosol and that established a parallel hydrogenosomal pathway after gene duplication and MTS acquisition.

Key words: **acetylCoA synthetase, sulfate activation pathway, hydrogenase, PFO, glycine cleavage system, succinate dehydrogenase.**

Introduction

In heterotrophic eukaryotes, the cytosol and mitochondrion are the main cellular compartments where energy metabolism takes place. Glycolysis is typically a cytosolic pathway that converts 1 mole of glucose to 2 moles of pyruvate with a net yield of 2–5 moles of ATP. In most aerobic eukaryotes, pyruvate is imported into mitochondria and then metabolized to CO_2 and H_2O. This pathway synthesizes 25 additional moles of ATP per mol of glucose, 23 of which are generated by oxidative phosphorylation. In contrast, the mitochondria of organisms that inhabit anaerobic niches have dramatically reduced or completely lost ATP synthesis (Müller et al. 2012). Anaerobic mitochondria have been characterized in some invertebrates (Müller et al. 2012); however, the most "reduced" mitochondria, such as hydrogenosomes and mitosomes, have been identified exclusively in unicellular eukaryotes (Embley and Martin 2006). Hydrogenosomal energy metabolism involves at its core the conversion of pyruvate to CO_2, acetate, and H_2 with the concomitant synthesis of a single mole of ATP per mole of pyruvate by substrate level phosphorylation. In mitosomes, ATP is not synthesized (Müller et al. 2012). Both hydrogenosomes and mitosomes lack a pyruvate dehydrogenase complex (PDH), the tricarboxylic acid cycle (TCA), a cytochrome *c*-dependent respiratory chain, and, consequently, ATP synthesis by chemiosmotic coupling (Müller et al. 2012). However, most of these organelles studied to date have retained the main components of the mitochondrial protein import machinery and the iron–sulfur cluster (ISC) assembly (Dolezal et al. 2006; Tachezy and Dolezal 2007), with the notable exceptions of the nitrogen fixation (NIF)- and putative sulfur mobilization (SUF)-type Fe-S cluster assembly systems in the Archamoebae and the breviate *Pygsuia biforma*, respectively (Nyvltova et al. 2013; Stairs et al. 2014). Anaerobic protists compensate for the lack of "typical" aerobic mitochondrial pyruvate metabolism (i.e., metabolism that is coupled to the TCA cycle) using the anaerobic pathway of extended glycolysis. Rather than undergoing oxidative decarboxylation via the PDH complex, pyruvate is converted to acetyl-CoA and CO_2 by pyruvate:ferredoxin oxidoreductase (PFO) or by pyruvate:$NADP^+$ oxidoreductase (PNO). Electrons that are generated during these reactions are transferred to an [FeFe] hydrogenase via ferredoxin to form molecular hydrogen or directly to $NADP^+$ to

Article

form NADPH. Acetyl-CoA is either directly converted to acetate, CoA, and ATP by acetyl-CoA synthetase (ACS; ADP-forming) or the CoA moiety of acetyl-CoA is transferred to succinate by acetate:succinate CoA-transferase (ASCT), whereupon succinyl-CoA serves as a substrate for ATP synthesis by succinyl-CoA synthetase (SCS). Alternatively, acetyl-CoA is produced by pyruvate formate lyase (PFL) that catalyzes the nonoxidative conversion of pyruvate. In organisms that contain hydrogenosomes, extended glycolysis is compartmentalized within these organelles, and ATP is generated via ASCT and SCS; however, in organisms that contain mitosomes, pyruvate-metabolizing enzymes and hydrogenase operate in the cytosol, and the cytosolic activity of ACS generates ATP (Müller et al. 2012).

Hydrogenosomes have been found in parasitic protists such as the human pathogen *Trichomonas vaginalis* (Lindmark et al. 1975) and the fish parasite *Spironucleus salmonicida* (Jerlstrom-Hultqvist et al. 2013), commensals such as rumen ciliates and chytrid fungi (de Graaf et al. 2011), and the free-living heteroloboseids (de Graaf et al. 2009; Barbera et al. 2010). Mitosomes have been described only in parasitic protists, including *Entamoeba histolytica* (Tovar et al. 1999), *Giardia intestinalis* (Tovar et al. 2003), *Cryptosporidium parvum* (Riordan et al. 1999), *Mikrocytos mackini* (Burki et al. 2013), and microsporidia (Williams et al. 2002). The punctuate distribution of hydrogenosomes and mitosomes across diverse eukaryotic lineages suggests that these organelles evolved repeatedly from mitochondria (Yarlett and Hackstein 2005). Phylogenetic analysis of components required for extended glycolysis (PFO and hydrogenase) coupled with their patchy distribution in the eukaryotic tree suggests that the corresponding genes were acquired most likely by lateral gene transfer (LGT) initially from bacteria and then subsequently transferred amongst eukaryotes (Hug et al. 2010). Alternatively, it is also possible that anaerobic energy-producing pathways in anaerobic forms of mitochondria might also reflect the anaerobic history of eukaryotes (Martin 2011).

Archamoebae are an interesting group of protists for understanding the evolutionary transitions between different types of anaerobic mitochondria as it includes closely related members with mitosomes, such as the parasitic *E. histolytica*, as well as the free-living amoeba *Mastigamoeba balamuthi* that contains putative hydrogenosomes (Nyvltova et al. 2013). Unlike most other eukaryotes, these archamoebae have entirely lost the mitochondrial ISC type of Fe-S cluster assembly machinery. Instead, these organisms possess a simpler NIF system that was most likely acquired by LGT from ε-proteobacteria (van der Giezen et al. 2004; Gill et al. 2007; Nyvltova et al. 2013). In *M. balamuthi*, duplicate NIF systems function within the hydrogenosomes and the cytosol, whereas in *E. histolytica*, the NIF system is present only in the cytosol, although mitosomal localization has also been suggested (Maralikova et al. 2009; Nyvltova et al. 2013).Although the mitosomes of *E. histolytica* are unable to synthesize ATP, they possess a unique sulfate activation pathway that is dependent on ATP supply (Mi-ichi et al. 2009). This pathway is not present in any known mitochondria, and phylogenetic analysis of its two key components, ATP sulfurylase (AS) and adenosine-5′-phosphosulfate kinase (APSK), suggests that the corresponding genes were acquired laterally from a bacterial source. Interestingly, in *Entamoeba*'s relative *M. balamuthi*, PFO and hydrogenase are localized both in the cytosol and in the putative hydrogenosomes (Nyvltova et al. 2013). A previous survey of *M. balamuthi* expressed sequence tags suggested that these organelles might also possess some components of the TCA cycle, such as the succinate dehydrogenase (SDH) complex, and components of the glycine cleavage system (GCS; Gill et al. 2007). However, to date, the presence of the sulfate activation pathway, ATP synthesis and the fate of acetyl-CoA in the *M. balamuthi* organelles have not been investigated.

Here, we analyze the *M. balamuthi* genome sequence to identify the components of extended glycolysis and energy metabolism in this organism and to investigate the role of LGT in shaping the metabolism of its putative hydrogenosomes. We found that *M. balamuthi* possesses two complete sets of enzymes for extended glycolysis, including ACS, PFO, and a hydrogenase that allows the acetyl CoA-dependent synthesis of ATP in both the cytosol and the hydrogenosomes. We also found that hydrogenosomes possess D-lactate dehydrogenase (D-LDH), components of the sulfate activation pathway, SDH, and the GCS. Phylogenetic analyses suggest that all components of extended glycolysis and two components of the sulfate activation pathway were acquired by LGT. Importantly, each component of extended glycolysis is present in at least two copies that differ in the presence/absence of mitochondrial targeting sequences (MTS), consistent with their dual localization. Based on these phylogenetic and biochemical data, we propose that LGT followed by gene duplication was an important evolutionary mechanism during the transition from an ancestral archamoebal organelle to the hydrogenosome of *M. balamuthi*.

Results

Purification of *M. balamuthi* Hydrogenosomes and the Cellular Localization of Malate Dehydrogenase

A hydrogenosome-rich fraction was separated from *M. balamuthi* homogenate using discontinuous OptiPrep gradient centrifugation. Homogenate centrifugation resulted in the formation of five distinct protein fractions (fig. 1*A*), which were separated and analyzed. Two proteins, mitochondrial type Hsp70 and Cpn60, were selected as hydrogenosomal markers. In addition, we used malate dehydrogenase (MDH) as a putative enzymatic marker based on the prediction of a mitochondrial targeting sequence in its N-terminal domain (Gill et al. 2007). Using immunoblot analysis, homologous antibodies raised against these three *Mastigamoeba* proteins recognized proteins of the predicted molecular weight in fractions 4 and 5 (fig. 1*B* and supplementary fig. S1*B*, Supplementary Material online). The observed specific activities for MDH across fractions correlated with these immunoblot analyses; fractions 4 (0.96 ± 0.024 μmol/min/mg; $n = 12$) and 5 (1.12 ± 0.084 μmol/min/mg; $n = 12$) contained 33 and 67% of the total MDH activity, respectively (fig. 1*C*).

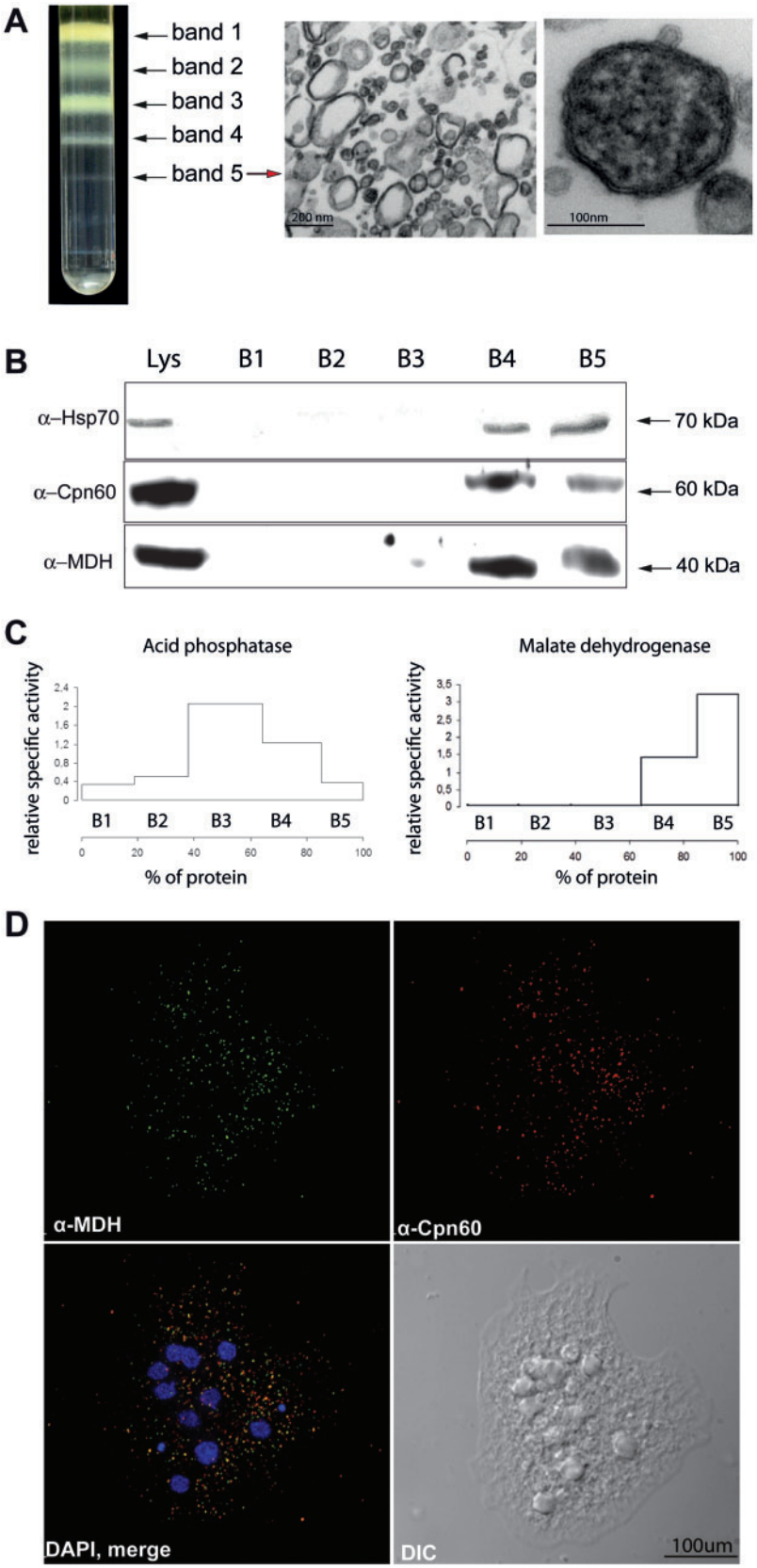

FIG. 1. Isolation of the hydrogenosome-enriched fraction from *Mastigamoeba balamuthi* cell lysate. (*A*) Separation of the cell lysate (Lys) on a discontinuous OptiPrep gradient (15–40%) resulted in five distinct fractions (B1-5). The presence of double membrane-bound organelles in fraction B5 was confirmed using electron microscopy. (*B*) Immunoblot analysis of Lys and fractions B1-5 using polyclonal antibodies against the marker proteins mitHsp70, Cpn60, and MDH. (*C*) Distribution of marker enzymes (acid phosphatase for lysosomes and MDH for *M. balamuthi* hydrogenosomes) in fractions B1-5 after OptiPrep gradient centrifugation. (*D*) Immunofluorescence microscopy. Signal for mitochondrial marker protein Cpn60 (red) colocalized with hydrogenosomal MDH (green; PCC = 0.878 ± 0.036, $n = 45$).

The majority of phosphatase activity, a lysosomal marker, was found in fraction 3 (51%); only 33% and 10% of total phosphatase activity was detected in fractions 4 and 5, respectively (fig. 1C). Thus, fraction 5 was used for further analysis. The presence of hydrogenosomes in fraction 5 was confirmed using electron microscopy, which showed numerous electron-dense organelles surrounded by a double membrane; however, this fraction also contained contaminating vesicles of similar sizes (fig. 1A). The integrity of the hydrogenosomes in fraction 5 was confirmed by a determination of MDH latency. Over 76% of MDH activity was liberated when the organellar membranes were disintegrated by addition of 0.1% Triton X-100, which indicates that most organelles remain intact through all steps of isolation.

The cellular distribution of MDH was investigated by immunofluorescence microscopy (fig. 1D). Specific anti-MDH antibodies identified MDH in approximately 100–200 organelles in cells that were colabeled with antibodies against Cpn60. To determine whether MDH activity was present exclusively in the organelles or was also present in the cytosol, we isolated the soluble cellular fraction of *M. balamuthi* using high-speed centrifugation (the supernatant obtained after centrifugation at 100,000 × g). We detected high NADH oxidase (a cytosolic marker enzyme) activity (table 1) but no MDH activity in this fraction. Collectively, these results suggest that MDH is exclusively localized in organelles. Therefore, we employed MDH as an enzymatic marker for *M. balamuthi* hydrogenosomes in the following experiments.

Remnants of Mitochondrial Pathways

Much like other anaerobic protists, *Mastigamoeba* has retained only a limited set of genes encoding enzymes that are involved in energy metabolism of aerobic mitochondria. In addition to MDH, we found all four subunits of Complex II (CII, the SDH complex [SDHA-D]). No other electron-transporting complexes were detected except the electron-transferring flavoprotein dehydrogenase (ETFDH). In aerobes, CII catalyzes the oxidation of succinate to fumarate with the concomitant reduction of ubiquinone (UQ) to ubiquinol (UQH_2), which is then reoxidized by Complex III. However, in some facultative anaerobes (e.g., *Ascaris suum*), CII catalyzes the reverse reaction, reducing fumarate to succinate and oxidizing rhodoquinol (RQH_2) to rhodoquinone (RQ). We therefore attempted to assay SDH or fumarate reductase (FRD) activity extensively in whole-cell extracts and hydrogenosome-enriched fractions using UQ and RQ substrates, without success (data not shown). We suspect that our inability to detect CII activity might be because it was 1) irreversibly inactivated during the preparation of cellular fractions and/or 2) it catalyzes a different reaction. Although we were not able to demonstrate this activity, all critical residues for substrate and cofactor binding are conserved in all four *Mastigamoeba* CIIs. Furthermore, homology-modeling analyses suggest that the *Mastigamoeba* sequence folds similarly to other, previously published structures (supplementary fig. S2, Supplementary Material online). The two largest SDH subunits (SDHA and SDHB) were subjected to phylogenetic analysis. The *Mastigamoeba* SDHA sequence emerged as a long branching basal taxon within eukaryotes and its SHDB sequence branched in an unresolved position in a eukaryote and α-proteobacteria (SDHB) clade (supplementary fig. S3A and B, Supplementary Material online). Both analyses are consistent with these proteins being ancestral "mitochondrial" homologs that were vertically inherited within the eukaryote lineage. Failure of the *Mastigamoeba* SDH subunits to branch within the closest amoebozoan relatives of *Mastigamoeba* (i.e., *Dictyostelium*) is likely due to their extreme sequence divergence, leading to long-branch attraction to the long internal branches in the tree. The marked sequence divergence of these proteins is most likely related to changing constraints due to altered structure or function relative to their aerobic counterparts. We were unable to detect a homolog of the assembly factor SDH5/SDHAF that acts in flavin attachment to SDHA (Hao et al. 2009), suggesting that the CII of *Mastigamoeba* is unusual.

The only other genes identified encoding quinone-utilizing enzymes were ETFa/b (table 3 and supplementary table S1B and fig. S4B and C, Supplementary Material online) and ETFDH (table 3 and supplementary table S1B and fig. S3C, Supplementary Material online). These proteins are typically involved in fatty acid oxidation reactions in which electrons are shuttled from ETFa/b to UQ via ETFDH. In some anaerobic bacteria, ETF and ETFDH are involved in butyryl-CoA metabolism (Herrmann et al. 2008). We identified partial sequences for ETFa/b and a full-length sequence of ETFDH encoding a putative MTS; however, no obvious homologs of butyryl-CoA metabolism enzymes were found. Furthermore, in phylogenetic analysis, the *Mastigamoeba* ETFDH emerges from within a typical mitochondrial ETFDH clade (supplementary fig. S3C, Supplementary Material online).

Table 1. Specific Activities of Selected Enzymes in Cellular Fractions of *Mastigamoeba balamuthi*.

Enzyme	Activity [μmol/min mg]	Localization
D-LDH (EC 1.1.1.28)	0.721 ± 0.019 (15)	Hydrogenosomes
MDH (EC 1.1.1.37)	1.009 ± 0.021 (18)	Hydrogenosomes
NAD malic enzyme (EC 1.1.1.37)	0.039 ± 0.008 (15)	Cytosol
NADH oxidase (EC 1.6.99.3)	0.213 ± 0.007 (18)	Cytosol
NADP malic enzyme (EC 1.1.1.40)	0.041 ± 0.008 (15)	Cytosol
PNO (EC 1.2.1.51)	0.447 ± 0.019 (13)	Cytosol
TDH (EC 1.1.1.103)	0.004 ± 0.0003 (4)	Cytosol

NOTE.—Mean ± standard deviation (*n*, number of experiments).

The GCS pathway is conserved in diverse aerobic mitochondria (Kikuchi 1973). This multienzyme system is critical for the methylenation of the one carbon-transferring cofactor, N5, N^{10}-methylenetetrahydrofolate (CH_2-THF), using glycine as a substrate. Glycine could be formed in mitochondria from threonine in two steps by the activities of mitochondrial threonine dehydrogenase (TDH) and α-amino-β-ketobutyrate CoA ligase (AKL; Bird et al. 1984). The CH_2-THF that is generated by mitochondrial GCS is used in the methylenation and subsequent hydroxylation of glycine to form serine (this reaction is catalyzed by serine hydroxymethyltransferase [SHMT]). The GCS comprises four enzymes: glycine decarboxylase (GCSP, P-protein), aminomethyltransferase (GCST, T-protein), lipoamide carrier protein (GCSH, H-protein), and dihydrolipoamide dehydrogenase (GCSL, L-protein). In *Mastigamoeba*, we identified homologs of all GCS components with MTS, except one of two L-proteins (GSCL2) in which MTS was not predicted (table 3). Using immunofluorescent microscopy, antibodies raised against human GCSH colocalized with antibodies directed against the hydrogenosomal marker MDH (fig. 4*A*) suggesting the GCS likely functions in these organelles. By immunoblotting analysis, the antihuman GCSH antibodies also recognized a protein of the predicted molecular weight in the hydrogenosome-enriched fraction (fig. 4*B* and supplementary fig. S1*B*, Supplementary Material online).

To assess the evolutionary history of the Mastigamoaeba GCS, phylogenetic analysis was performed on the larger proteins GCSP, GCST, and GCSL. In both the GCSP and GCST analyses, the *Mastigamoeba* GCS homologs branched with other mitochondrial sequences suggesting that these enzymes were likely inherited vertically from the common ancestor of mitochondria (supplementary fig. S3*D* and *E*, Supplementary Material online). GCSL1 branches with other eukaryotic homologs with weak support, whereas GCSL2 is a very long branch that clusters with bacteria and *Trichomonas* (supplementary fig. S3*F*, Supplementary Material online). This obvious sequence divergence might be explained by the different evolutionary pressures on GCSL in hydrogenosome-bearing taxa relative to mitochondriate model organisms, such as yeast and *Arabidopsis*. Moreover, GCSL is known to function in a variety of other protein complexes (Spalding and Prigge 2010). It is possible therefore, that GCSL2 is involved in a pathways unrelated to glycine cleavage. Finally phylogenetic analysis of SHMT revealed eukaryotes branching as a monophyletic clade to the exclusion of bacteria (of mixed taxonomic groupings). The Mastigamoeba SHMT sequence branches toward the base of the eukaryotic clade (supplementary fig. S3*G*, Supplementary Material online). The lack of resolution in the phylogeny makes it difficult to determine if the *Mastigamoeba* sequence is more similar to cytosolic or mitochondrial homologs. However, the presence of a MTS combined with the hydrogenosomal localization of proteins known to function in cooperation with SHMT (i.e., GCS) suggests that this protein functions in the hydrogenosome. In addition to the GCS and SHMT proteins, we identified a putatively organellar lipoamide protein ligase (LPLA) protein (responsible for attaching lipoamide to GCSH and related proteins) with an MTS (table 3 and supplementary table S1*B*, Supplementary Material online).

However, we did not confirm presence of predicted threonine degradation pathway (Gill et al. 2007). Although, we identified genes coding for TDH and AKL, none of them possessed putative MTS (table 3). In addition, we tested TDH activity in subcellular fractions, which revealed presence of the active enzyme in the cytosol (0.004 ± 0.0003 µmol/min/mg; $n = 4$) while no activity was associated with the hydrogenosomal fraction (table 1). Interestingly, using phylogenetic analysis, the *Mastigamoeba* TDH sequence branches sister to mitochondrial TDH sequences (supplementary fig. S3*H*, Supplementary Material online) suggesting this protein could have been ancestrally localized to the mitochondria but has since been relocalized exclusively to the cytoplasm.

Collectively, remnants of electron transport chain (CII, ETFa/b and ETFDH) and amino acid metabolism (GCS, SHMT, LPLA) likely represent common ancestral traits that are shared between hydrogenosomes of *Mastigamoeba* and mitochondria of aerobic amoebozoans.

Pyruvate Metabolism

Pyruvate is a glycolytic product that is imported to mitochondria where it is converted to acetyl-CoA by PDH. However, in the hydrogenosomes of *M. balamuthi*, pyruvate is converted to acetyl-CoA and CO_2 by PFO (Nyvltova et al. 2013), and we did not find any evidence for genes encoding PDH components. Pyruvate can also be oxidatively decarboxylated by PNO, a homolog of which we identified in the *M. balamuthi* genome (Nyvltova et al. 2013). Thus, we measured the PNO activity of cytosolic and hydrogenosomal fractions using a spectrophotometric assay as well as histochemical in-gel staining. Both assays revealed presence of PNO exclusively in the cytosolic fraction (table 1 and fig. 2). Malate is an alternative hydrogenosomal substrate that is oxidatively decarboxylated to pyruvate by an $NAD(P)^+$-dependent malic enzyme in *T. vaginalis* hydrogenosomes (Müller et al. 2012). Surprisingly, we did not detect either NAD^+- or $NADP^+$-dependent activities of malic enzymes in *M. balamuthi* hydrogenosomes; however, both activities were found in the cytosolic fraction. D- or L-lactate is converted to pyruvate

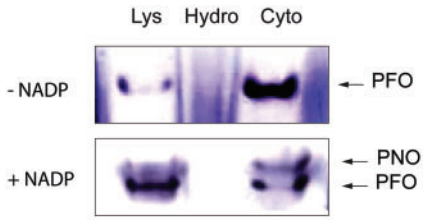

Fig. 2. Histochemical detection of PFO and PNO in *Mastigamoeba balamuthi* cell fractions. Cell lysate (Lys) was separated into cytosolic (Cyto) and hydrogenosome-enriched (Hydro) fractions using differential centrifugation. Samples were separated under native conditions in a 6% polyacrylamide gel and stained for the oxidative decarboxylation of pyruvate in the presence and absence of $NADP^+$ as an electron acceptor to distinguish PFO and PNO activity, respectively.

by cytochrome-dependent inner membrane-bound D-LDH or soluble NAD^+-dependent L-LDH; thus, lactate is another possible source of pyruvate. Surprisingly, in *M. balamuthi* hydrogenosomes, we exclusively found a soluble NAD^+-dependent D-LDH activity that has not previously been described in mitochondrion-related organelles (table 1). The activity displayed a latency (75%) similar to that of MDH, indicating that D-LDH is localized inside the organelles. Searches for the corresponding gene in the *M. balamuthi* genome revealed the presence of two D-LDH paralogs: D-LDH-M, which possesses a 75-amino acid N-terminal MTS with a predicted cleavage site between I75 and A76, and D-LDH-C, which lacks a presequence (supplementary fig. S4*A*, Supplementary Material online). Both paralogs possess conserved residues that are required for D-LDH function, including a coenzyme-binding domain GXGXXG17D sequence and Phe394, which is important for substrate specificity. To test whether D-LDH can be targeted to mitochondria, we expressed both D-LDH paralogs fused with green fluorescent protein (GFP)-tags at the carboxy-terminus in *Saccharomyces cerevisiae*, a model system that we have previously used successfully for testing the cellular localization of *M. balamuthi* proteins (Nyvltova et al. 2013). As expected, D-LDH-M was delivered into yeast mitochondria, a finding that is consistent with our measurements of organellar D-LDH activity in *M. balamuthi*; in contrast, D-LDH-C was localized in the cytosol (fig. 3).

The D-LDH protein belongs to a larger protein family of dehydrogenases, including hydroxyacid dehydrogenases (HDHs). Phylogenetic analyses of D-LDHs and HDHs indicate that the *M. balamuthi* homologs D-LDH-M1 and D-LDH-C2 branch together in a paraphyletic clade of bacteria and eukaryotes that is distinct from the HDHs. Although support across the phylogeny is poor, the putative *M. balamuthi* D-LDH homolog does not branch with the *Entamoeba* and *Trichomonas* HDH proteins (supplementary fig. S3*I*, Supplementary Material online). Subsequent analyses involving removal of the HDH sequences did not increase support within the D-LDH clade (data not shown). Bacterial sequences from a variety of phyla were interspersed within the D-LDH clade, suggesting the existence of multiple LGTs between bacteria and eukaryotes (even though the interrelationships between these sequences were largely unresolved).

Collectively, these data indicate that pyruvate but not malate serves as a substrate in *M. balamuthi* hydrogenosomes. In addition, these organelles possess NAD^+-dependent D-LDH of bacterial origin that converts lactate to pyruvate.

ATP Synthesis

Next, we investigated ATP synthesis in the organelles. Incubation of hydrogenosomal extract with ADP resulted in the formation of ATP, which was slightly increased when acetyl-CoA was added (table 2). This observation suggested the presence of adenylate kinase (AK), which catalyzes the interconversion of adenine nucleotides, and ADP-dependent ACS, which generates ATP and acetate. To distinguish these two ADP-dependent activities, we added the specific AK inhibitor, Ap5A [P1,P5-di(adenosine-5′)pentaphosphate] to the reaction. This inhibitor completely abolished AK activity, which allowed us to determine formation of ADP-dependent activity of ACS (table 2). We also tested for ASCT and SCS; however, these enzymatic activities were not detected, consistent with no evidence to date for corresponding genes in any genomic surveys.

Two ACS Paralogs with Dual Cellular Localization

Searches of the *M. balamuthi* genome for AK and ACS revealed the presence of the corresponding genes, corroborating our biochemical data. Both AK and ACS possessed an MTS, as predicted by the PSORT II software (table 3 and supplementary table S1*B*, Supplementary Material online). Of the four homologs of ACS we identified in the genome of *Mastigamoeba*, only one (ACS-M1) has a predicted MTS. Expression of ACS fused with a GFP-tag at its carboxy-terminus in yeast revealed that ACS-M1 was targeted to mitochondria, whereas the product of ACS-C2 was found in yeast cytosol (fig. 3). Next, we compared ACS activity in the cytosolic and hydrogenosomal fractions. We found that the specific activity of ACS in the cytosol was approximately eight times higher than that in hydrogenosomes (table 2). This dual localization was confirmed using immunoblot analyses: the signal for ACS was considerably stronger in the cytosolic fraction (fig. 4*B* and supplementary fig. S1*B*, Supplementary Material online). Using immunofluorescence microscopy, antibodies raised against ACS colocalized with antibodies raised against the hydrogenosomal marker MDH. However, ACS staining was also observed in the cytosol (fig. 4*A*).

Phylogenetic analysis of these ACS homologs revealed that *Mastigamoeba* encodes two distinct types of ACS-like proteins (fig. 5 and supplementary fig. S3*J*, Supplementary Material online). *Mastigamoeba balamuthi* ACS proteins, including *M. balamuthi* ACS-M1, ACS-C2, and ACS-C3, cluster together in a group encoded by organisms consisting mostly of Archaea (crenarcheotes and euarchaeotes; bootstrap value [BV] = 100, posterior probability [PP] = 1.0). The close relationship between the three *M. balamuthi* ACSs suggests that these homologs result from two sequential gene duplications, after which one copy (ACS-M1) likely acquired an MTS. The remaining eukaryotic taxa branched in similar positions, as reported previously (Jerlstrom-Hultqvist et al. 2013). The second type of ACS-like proteins includes the putatively cytosolic Acyl-CoA synthetase (AcCS-C4), which groups within proteins encoded by a mixed clade of eukaryotes and bacteria (BV = 94, PP = 0.99); this group does not include *Entamoeba* orthologs.

In general, the topology of ACS indicates that eukaryotic ACS proteins were separately acquired by eukaryotes though at least four independent LGT events including: 1) from Archaea to *Mastigamoeba* giving rise to ACS-M1, ACS-C2, and ACS-C3, 2) from firmicutes to the diplomonads, *Giardia* and *Spironucleus* (BP = 97, PP = 1.0), 3) from an unclear donor to the *Entamoeba* lineage (BV = 91, PP = 1.0) and 4) from an unclear donor to other eukaryotes, including AcCS-C3 (BV = 95, PP = 1.0).

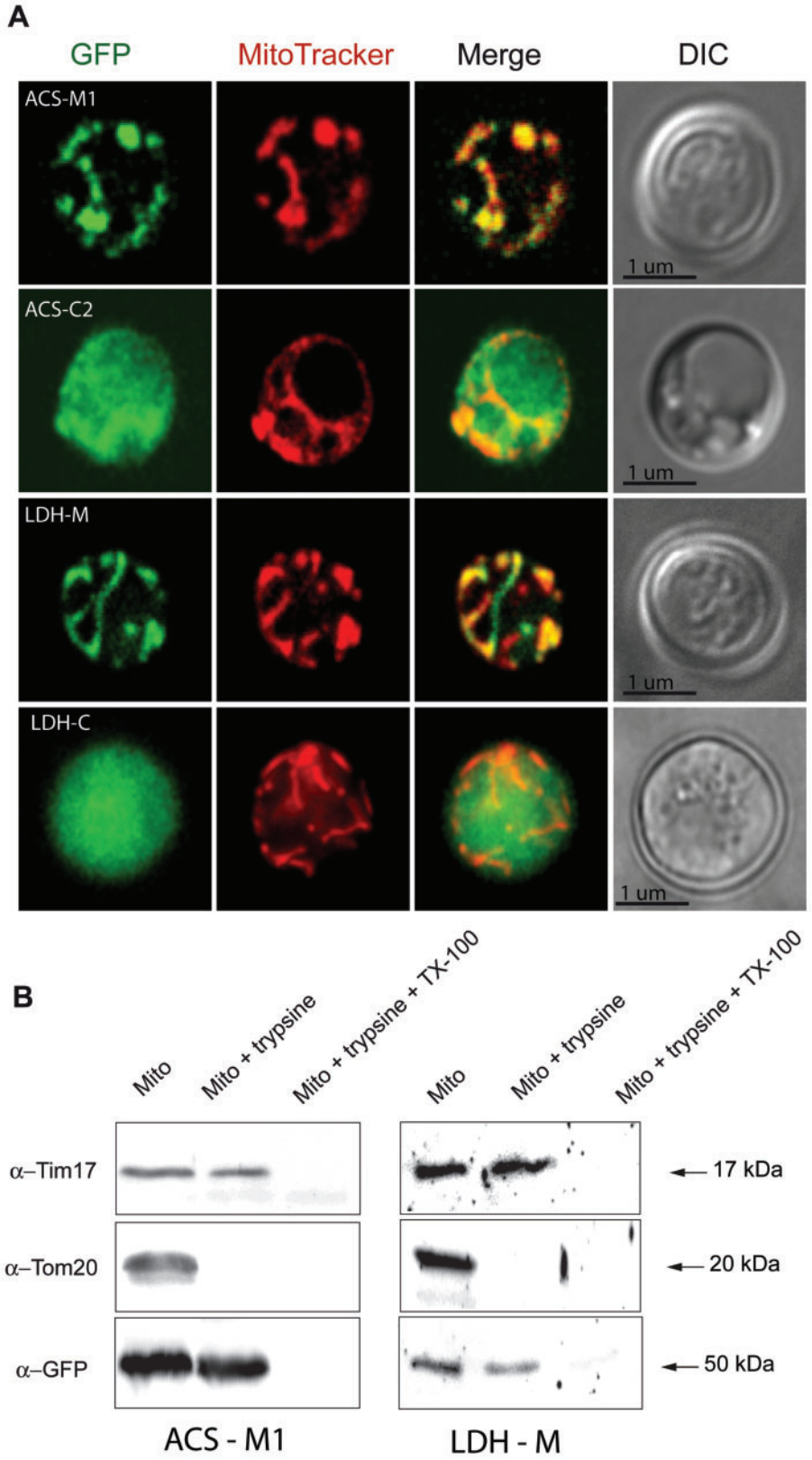

Fig. 3. Expression and cell localization of *Mastigamoeba balamuthi* ACS and LDH in *Saccharomyces cerevisiae*. (*A*) Immunofluorescence microscopy. Mitochondrial (-M) and cytosolic (-C) versions of ACS and LDH were fused at the C-terminus with GFP and expressed in yeast. Mitochondria were visualized using MitoTracker. DIC, differential interference contrast. (*B*) Protease protection assay. Mitochondria were isolated from yeast expressing *M. balamuthi* ACS-M and LDH-M and treated with trypsin to remove proteins that are associated with the outer membrane; the mitochondria were treated with a combination of trypsin and the detergent Triton-X100 to disintegrate both membranes. Transblotted samples were probed using antibodies against Tom20 (an outer membrane marker), Tim17 (an inner membrane marker), and GFP, which is present at the C-terminus of ACS and LDH.

Table 2. Acetyl-CoA-Dependent ATP Production in Cellular Fractions of *Mastigamoeba balamuthi*.

Substrates	ATP Production [pmol/min mg]	
	Hydrogenosomes	Cytosol
ADP	2.11 ± 0.34 (11)	16.67 ± 0.73 (6)
ADP + pyr + CoA	2.21 ± 0.18 (9)	19.58 ± 0.48 (6)
ADP + acetylCoA	2.29 ± 0.28 (9)	31.25 ± 0.51 (6)
ADP + inhibitor	0.05 ± 0.01 (9)	0.85 ± 0.09 (6)
ADP + inhibitor + acetylCoA	1.27 ± 0.24 (9)	20.86 ± 0.36 (4)
ADP + inhibitor + pyr + CoA	0.79 ± 0.26 (9)	18.91 ± 0.47 (4)

NOTE.—pyr, pyruvate; inhibitor, P1,P5-di(adenosine-5′) pentaphosphate. Mean ± standard deviation (*n* = number of experiments).

Sulfate Activation Pathway

Searches of the *M. balamuthi* genome revealed the presence of genes coding for all main components of the sulfate activation pathway, including AS, APSK, and inorganic pyrophosphatase (IPP; table 3). Interestingly, *M. balamuthi* possesses three paralogs of IPP, only one of which contains a predicted MTS (IPP-1) (supplementary table S1*B*, Supplementary Material online). To investigate the cellular localization of the sulfate activation pathway, we generated polyclonal antibodies against recombinant AS and IPP-1. Western blot analysis of *M. balamuthi* fractions revealed corresponding signals exclusively in the hydrogenosomal fractions (fig. 4*B* and supplementary fig. S1*B*, Supplementary Material online). Accordingly, we observed colocalization of AS and IPP1 with the hydrogenosomal marker MDH in numerous organelles using immunofluorescence microscopy (fig. 4*A*).

Phylogenetic analysis of IPP sequences from diverse eukaryotes and prokaryotes reveals two distinct clades. One clade is exclusively eukaryotic, whereas the other is predominantly bacterial (supplementary fig. S3*K*, Supplementary Material online). *Mastigamoeba* IPP-1 is grouped with the previously reported *Entamoeba* mitosomal IPP (Mi-ichi et al. 2009; BP = 90 PP = 1.0) and homologs from Amoebozoa (BP = 35 PP = 0.79) and eukaryotes (BP = 100 PP = 1.0), suggesting that this is the ancestral IPP common to all eukaryotes. The other two *Mastigamoeba* proteins (IPP2 and IPP3) cluster together and emerge from within a strongly supported clade comprising sequences from the Bacteroidetes and miscellaneous other bacterial groups (BP = 99 PP = 1.0). The absence of other eukaryotic sequences in this clade, combined with the strong support values found, suggests that *Mastigamoeba* acquired an ancestor of the genes encoding the IPP2 and IPP3 proteins via LGT from an unknown prokaryotic donor. After the transfer, a gene duplication event clearly occurred that gave rise to both versions. Analysis of the predominantly bacterial IPP clade alone did not resolve the exact position of the *Mastigamoeba* IPP2/3 group (data not shown).

Phylogenetic analyses of AS and APSK (supplementary fig. S3*L* and *M*, Supplementary Material online) were broadly congruent with previous studies (Mi-ichi et al 2009). As expected, the *Mastigamoeba* AS sequence clustered with the *Entamoeba* homolog, branching as a sister to a

Table 3. Accession Numbers of Selected Genes with Predicted Mitochondrial N-Terminal Targeting Presequences by PSORT II.

Accesion Numbers	Annotation	N-Terminal Sequence
KF927023	ACS-M1	Yes
KF927022	ACS-C2	No
KF941318	AK-M	Yes
KP030851	AK-C	No
0KF927026	APS kinase	Yes
KF927024	AS	Yes
KF941316	D-LDH-M	Yes
AAP76314	D-LDH-C	No
KP006414	ETF α	Yes
KP006415	ETF β	Yes
KJ993879	ETFDH	Yes
ABV54211	GCS-H	Yes
KJ993878	GCS-L1	Yes
KJ993877	GCS-L2	No
KJ993875	GCS-P	Yes
KJ993876	GCS-T	Yes
KJ558425	Hydrogenase 1	No
AGA37393	Hydrogenase 2	No
AGA37394	Hydrogenase 3	No
KJ558426	Hydrogenase 4	No
KJ558427	Hydrogenase 5	No
KJ920286	Hydrogenase 8	Yes
KJ920287	Hydrogenase 9	No
AGA37391	Hydrogenase 10	Yes
KF927025	IPP-1	Yes
KJ558423	IPP-2	No
KJ558424	IPP-3	No
KP030853	LPLA	Yes
KJ616392	PFO 1	No
AAM53401	PFO 2	No
AGA37395	PFO 3	Yes
KJ616393	PFO 4	No
KJ616394	PFO 5	No
AGA37396	PFO 6	Yes
AGA37390	PNO	No
KJ993872	SdhA	Yes
AGA37361	SdhB	Yes
KJ993873	SdhC	Yes
KJ993874	SdhD	Yes
KP057244	SHMT	Yes
KM979287	TDH	No

NOTE.—ETF α/β, electron transfer flavoprotein; Sdh, succinate dehydrogenase.

α-proteobacterial clade with high support (BV = 100, PP = 1.0). However, the overall resolution of the APSK phylogeny was poor. A collection of sequences from bacteria, fungi, and amoebozoans (*Entamoeba*, *Mastigamoeba*, *Acanthamoeba*, and dictyostelids) form a well-supported clade (BP = 83, PP = 1.0) excluding α-proteobacteria. Interestingly, all four lineages of Amoebozoa did not group together in maximum likelihood (ML) or Bayesian analyses, although resolution is extremely poor throughout the phylogeny (i.e., very low bootstrap values occur). The presence of APSK in these four amoebozoans suggests that this gene

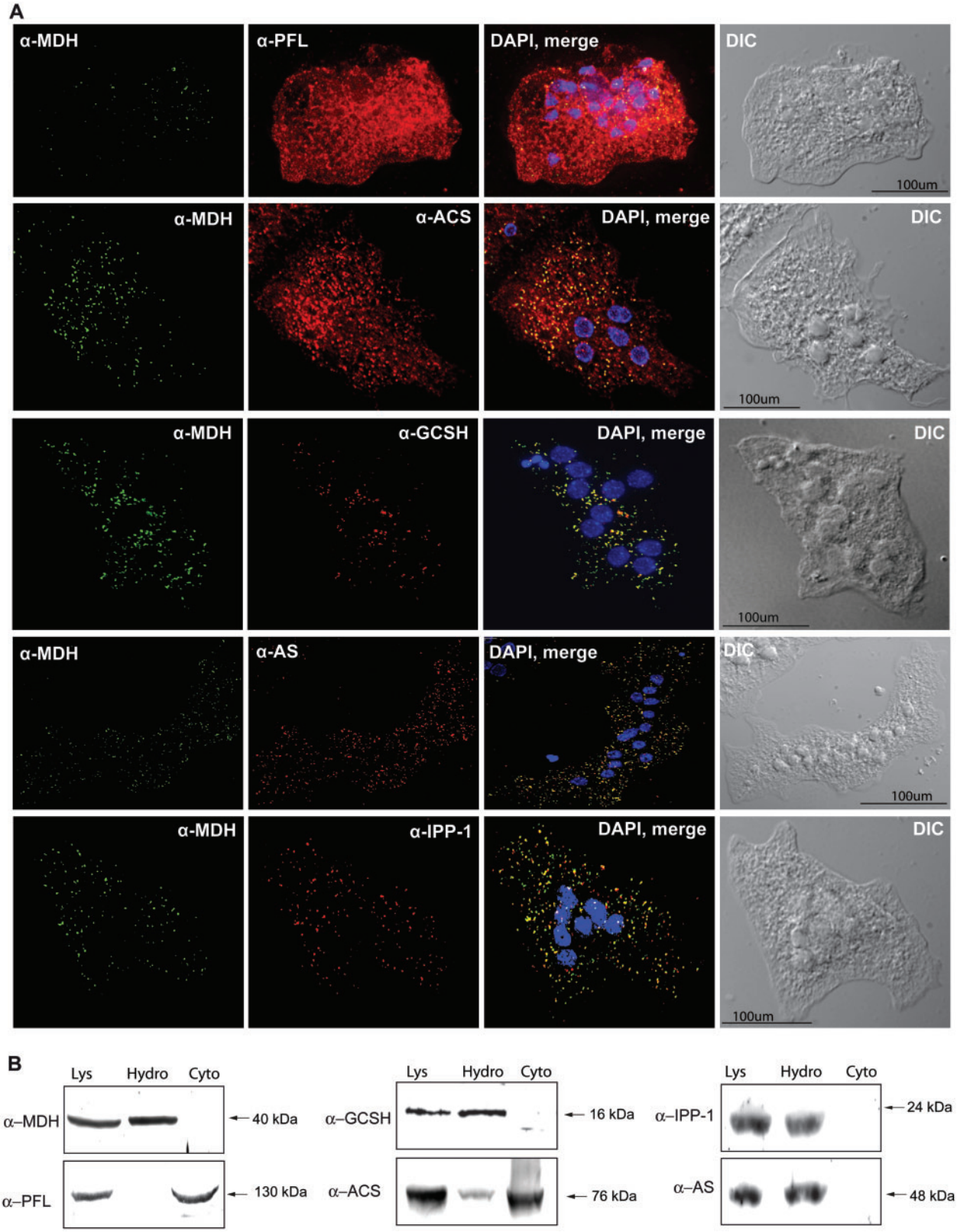

Fig. 4. Cellular localization of PFL, ACS, AS, and IPP in *Mastigamoeba balamuthi*. (*A*) Immunofluorescence microscopy. Signals for GCSH, AS, and IPP clearly colocalized with hydrogenosomal MDH (PCC = 0.907 ± 0.023, $n = 42$; 0.902 ± 0.025, $n = 41$; and 0.876 ± 0.023, $n = 38$, respectively). ACS colocalized with MDH. However, some signal was observed in the cytosol (PCC = 0.659 ± 0.047, $n = 43$). Signal for PFL was consistent with its cytosolic localization (PCC = 0.411 ± 0.063, $n = 39$). Proteins were visualized using antibodies against human GCSH and *M. balamuthi* AS, ACS, IPP, PFL, and MDH. Alexa Fluor 488 donkey α-rabbit and 594 donkey α-mouse and α-rat were used as secondary antibodies. (*B*) Immunoblot analysis of cellular fractions. Cell lysate (Lys) was separated into cytosolic (Cyto) and hydrogenosome-enriched (Hydro) fractions using differential centrifugation and probed with the appropriate antibodies.

could have been present in the common ancestor of the lineage. However, due to poor resolution, the exact evolutionary history of this gene remains unclear.

Phylogenetic Analysis of PFO and Hydrogenase

Phylogenetic analyses of ACS and D-LDH (fig. 5 and supplementary fig. S3*J* and *I*, Supplementary Material online), as well as NIF components (Nyvltova et al. 2013), revealed that for each of these components, recent gene duplications preceded the acquisition of MTS by one of the duplicate genes. Thus, we investigated whether similar evolutionary patterns are evident in hydrogenase or PFO phylogenies. Our searches in the *M. balamuthi* genome revealed at least ten predicted splice variants of [FeFe]-hydrogenase that are encoded by eight different genes. Of these hydrogenases, only one (hydrogenase-10 and isoform hydrogenase-7) has an MTS (table 3 and supplementary table S1*B*, Supplementary Material online). The predicted organellar localization of hydrogenase-10 is further supported by heterologous GFP-fusion protein expression in yeast, where hydrogenase-10 was shown to colocalize with the mitoreactive stain, MitoTracker (supplementary fig. S1*A*, Supplementary Material online).

Phylogenetic analysis of [FeFe] hydrogenase reveals two distinct clades of paralogs. *Mastigamoeba* hydrogenase-1 and -2 form a clade within a larger, well-supported clade comprising mainly bacterial sequences but also sequences of *Entamoeba* and the anaerobic protist *Trimastix pyriformis* (BV = 100, PP = 1.0) (supplementary fig. S3*N*, Supplementary

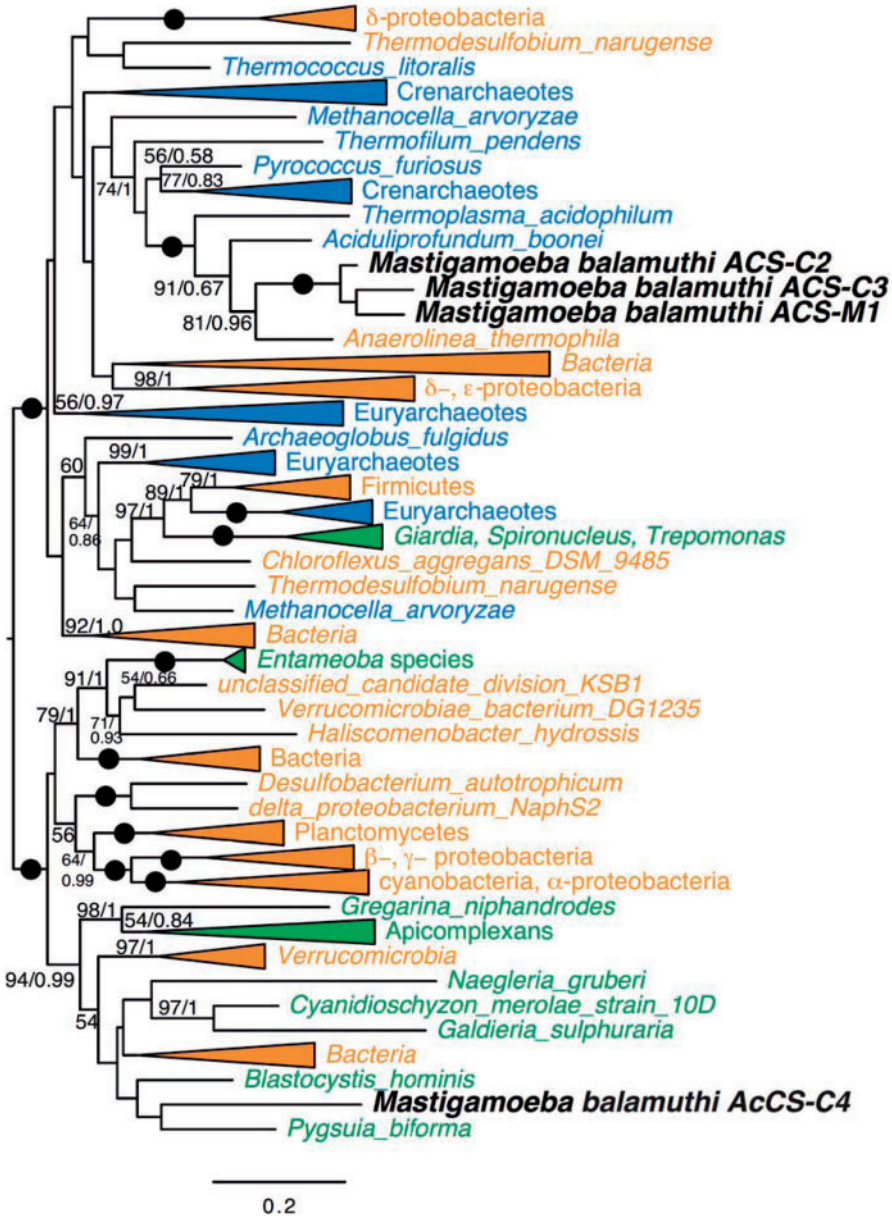

Fig. 5. Phylogeny of ACS. ML tree of ACS (187 taxa and 497 sites). Bootstrap support (BP) and PP values were calculated for each branch using RAxML and PhyloBayes, respectively. Only BV and PP values greater than 50% and 0.5, respectively, are shown. Branches with maximum support (BV = 100%; PP = 1.0) are depicted using black circles. Bacterial and archaeal clades are shown in blue and orange, respectively.

Material online). The remaining *Mastigamoeba* sequences, including the mitochondrial-targeting homolog, branch together with strong Bayesian support (PP = 0.76). These sequences are nested within a larger clade comprising Thermotogae and eukaryotic taxa. Previous reports have noted the presence of additional C-terminal domains on some eukaryotic hydrogenase sequences (Stechmann et al. 2008; Stairs et al. 2014). Similar to the hydrogenases of *Trichomonas*, *Blastocystis* species, and *P. biforma*, at least four of the *Mastigamoeba* sequences encode C-terminal FAD-binding and/or flavodoxin domains (see the mapped domain architecture, supplementary fig. S3*N*, Supplementary Material online).

We identified six PFO homologs in *Mastigamoeba*, but only one including an MTS (PFO-M3). In phylogenetic analysis, the PFO sequences are dispersed in a monophyletic clade of eukaryotes (BP = 60, PP = 0.94) (supplementary fig. S3*O*, Supplementary Material online). Although most internal branches are poorly supported, it appears that PFO-M3, PFO-C4, and PFO-C5 are the result of a sequence of two gene duplication events. Curiously, a PFO paralog from the diplomonad protist branch within this *Mastigamoeba* cluster is most closely related to PFO-M3. It is unclear if this finding is due to the "long branches" of the diplomonad sequences branching with the most divergent of the *Mastigamoeba* sequences or if it instead reflects a eukaryote-to-eukaryote LGT event. Together, these results suggest that, similarly to the ACS, D-LDH, and NIF proteins, hydrogenosomal forms of hydrogenase and PFO evolved by gene duplications of cytosolic enzymes and the subsequent acquisition of MTSs.

Discussion

Our investigations unequivocally confirmed that mitochondrion-related organelles in *M. balamuthi* match the current definition of "hydrogenosomes," which are class 4 mitochondrial family organelles (Müller et al. 2012); these organelles generate ATP by substrate-level phosphorylation and use protons as electron acceptors to form molecular hydrogen. Moreover, the hydrogenosomes of *M. balamuthi* display four unique features that are not found in the hydrogenosomes of other eukaryotes: 1) ATP is generated by the activity of an ACS (ADP-forming), 2) the organelles possess a sulfate activation pathway, 3) the organelles harbor homologs of CII (SDH) subunits (members of the electron transport chain), and 4) Fe-S clusters are formed by a bacterial-like NIF system (Nyvltova et al. 2013; fig. 6).

In hydrogenosomes, pyruvate and malate are the typical substrates for energy metabolism and are decarboxylated by PFO/PNO/PFL and malic enzyme, respectively. However, the absence of a malic enzyme suggests that pyruvate, not malate, is the main substrate metabolized by *M. balamuthi* hydrogenosomes. In mitochondria, pyruvate can be generated from

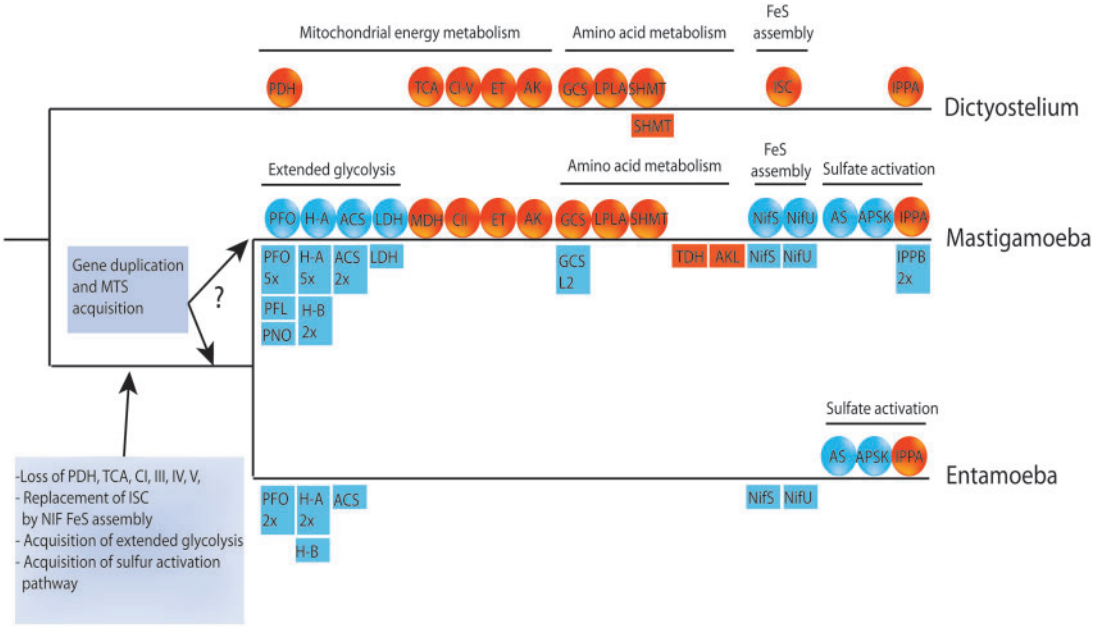

Fig. 6. Evolution of selected biochemical pathways in amoebae. A common ancestor of mastigamoeba and entamoeba possibly lost majority of canonical mitochondrial enzymes/pathways such as PDH complex, majority of enzymes of TCA cycle, and respiration chain complexes with exception of CII. Mitochondrial ISC assembly was replaced by bacterial NIF machinery. Oxidative phosporylation was replaced by anaerobic substrate level phosporylation catalyzed by enzymes of extended glycolysis that were acquired by LGT. A common ancestor also acquired two components of sulfur activation pathway (AS and APSK). Gene duplication and acquisition of MTS resulted in dual localization of NIF system and enzymes of extended glycolysis in mastigamoeba. However, it is not clear whether these events happened in a common ancestor or only in mastigamoeba lineage. CI-V, respiratory complexes; ET, electron-transferring flavoprotein dehydrogenase (ETFDH), ETFa, and ETFb; GCS, glycin cleavage system; H-A/B, hydrogenase clade A/B; IPPA/B, inorganic pyrophosphatase clade A/B PFL, pyruvate formate lyase. Genes acquired by LGT are given in blue, genes of mitochondrial origin that were vertically inherited are highlighted by orange; squares and circles indicate cytosolic and mitochondrial localization, respectively; the numbers represent the number of cytosolic genes.

D- or L-lactate via a cytochrome dependent, inner membrane-bound D-LDH or a soluble NAD^+-dependent L-D-LDH (de Bari et al. 2002). Interestingly, we did not detect activities of these enzymes in *M. balamuthi* hydrogenosomes; instead, we found soluble NAD^+-dependent D-LDH activity. In mammalian cells, D-lactate that forms in the cytosol via the methylglyoxal pathway is transported into mitochondria by three translocators, including a D-lactate/oxoacid antiporter, which mediates both the D-lactate/pyruvate and D-lactate/oxaloacetate exchanges (de Bari et al. 2002). *Mastigamoeba balamuthi* hydrogenosomes exhibit MDH activity that converts malate to oxaloacetate. However, neither a gene nor an activity corresponding to an oxaloacetate-metabolizing citrate synthase was identified. Thus, oxaloacetate might be available for D-lactate import. Future studies will endeavor to define which translocator is responsible for D-lactate import into hydrogenosomes and how D-lactate is formed in *M. balamuthi*. It has been proposed that *M. balamuthi* possesses a threonine degradation pathway that provides an alternative, intraorganellar means of forming pyruvate (Gill et al. 2007). The central reaction in this pathway is catalyzed by SHMT, which converts glycine and methylene-THF to serine and THF. The regeneration of THF to methyl-THF is mediated by the GCS. The presence of putative MTSs in SHMT and GCS components (and in the cofactor component, LPLA) strongly suggests that GCS operates in *M. balamuthi* hydrogenosomes. Furthermore, the phylogenetic analysis of GCS proteins supports a mitochondrial, and therefore ancestral, origin of these pathways. However, we found no evidence for the organellar localization of other components of threonine degradation pathways, including TDH, α-amino-β-ketobutyrate CoA ligase, and serine dehydrogenase. Nevertheless, the interconversion of glycine and serine, as catalyzed by the canonical mitochondrial complex of SHMT/GCS in hydrogenosomes, represents a putative source of active one-carbon units, which are required for various biosynthetic pathways (e.g., glutathione synthesis).

In *M. balamuthi*, we demonstrated that ACS synthesizes acetyl-CoA dependent ATP formation in the cytosol as well as in hydrogenosomes. We identified four genes in *Mastigamoeba* that are evolutionarily related to the ACS family. However, only one of these sequences (ACS-M1) codes for a putative MTS, suggesting that at least one ACS is present in the organelle and that multiple isoforms function in the cytoplasm. In other anaerobic eukaryotes, including chytrid fungi, parabasalids such as *T. vaginalis*, the ciliate *Nyctotherus ovalis*, and *Blastocystis* sp., hydrogenosomal ATP synthesis is catalyzed in two steps by the activities of ASCT and SCS (Müller et al. 2012). In contrast, ACS catalyzes the cytosolic, acetyl-CoA-dependent synthesis of ATP in organisms containing mitosomes, such as *G. intestinalis* and *E. histolytica* (Müller et al. 2012). Genes for *ACS* were also found in other eukaryotes with divergent lineages, such as the apicomplexan *Plasmodium falciparum*, the diatom *Thalassiosira pseudonana* (Armbrust et al. 2004), the excavate *Naegleria gruberi* (Fritz-Laylin et al. 2010), and the breviate *P. biforma* (Stairs et al. 2014). Interestingly, it has been predicted that ACS homologs of *N. gruberi* (Fritz-Laylin et al. 2010) and *Th. pseudonana* (Atteia et al. 2013) may function in mitochondria and in secondary plastids, respectively, whereas the homolog in *Pygsuia* appears to be targeted to its mitochondrion-related organelle (Stairs et al. 2014). However, no experimental data are available for the enzymatic activities of any of these enzymes from these organisms; thus, the hydrogenosome of *M. balamuthi* is the first eukaryotic organelle in which ACS activity has been experimentally demonstrated.

The dual localization of ACS activity in *M. balamuthi* is consistent with the dual localization of two other components used in extended glycolysis: PFO and hydrogenase (Nyvltova et al. 2013). Interestingly, in addition to PFO, *M. balamuthi* also possesses genes encoding two further pyruvate-metabolizing enzymes, PNO and PFL (Stairs et al. 2011; Nyvltova et al. 2013). Our enzymatic assays revealed that PNO participates in pyruvate metabolism exclusively in the cytosol. Cytosolic localization was also observed for PFL using immunodetection methods; however, our attempts to detect PFL activity were unsuccessful, possibly due to the high oxygen sensitivity of this enzyme (Lindmark et al. 1969). Three enzymes, PFO, PNO, and PFL, most likely participate in cytosolic pyruvate breakdown. These and previous results demonstrate that, uniquely among eukaryotes, *M. balamuthi* possesses dual extended glycolysis pathways together with dual machineries for Fe-S cluster assembly in the cytosol and in hydrogenosomes. We note that the specific enzymatic activities of PFO, hydrogenase, and ACS in the cytosol are considerably higher (by at least two orders of magnitude) than those of enzymes in the hydrogenosomal pathway. It is puzzling that such a weak hydrogenosomal pathway is retained in *M. balamuthi* and yet has been lost in *E. histolytica*.

It has been hypothesized that a common ancestor of the two amoebozoan lineages, Eumycetozoa and Archamoebae, possessed aerobic mitochondria and that these were retained in Eumycetozoa; meanwhile, Archamoebae adapted to anaerobic niches, converting their aerobic mitochondria to their anaerobic counterparts (hydrogenosomes, mitosomes; Gill et al. 2007). This organellar transition included the loss of most canonical mitochondrial pathways, such as the TCA cycle, cytochrome *c*-dependent respiration and oxidative phosphorylation, and the acquisition of anaerobic pathways by LGT (Hug et al. 2010). Our current and previous phylogenetic analyses of *M. balamuthi* support an LGT origin for the components of 1) anaerobic energetic metabolism including PFO, hydrogenase, ACS, and D-LDH, 2) ISC assembly machinery (NifS, NifU), and 3) sulfate activation (AS, APSK). Moreover, the dual cellular localization of pathways (1) and (2) raises interesting questions: What was the initial cellular localization of the components immediately after their acquisition by LGT, and what preceded their organellar localization? In general, there are two possible scenarios regarding the history of LGT-acquired components: 1) The acquired bacterial proteins were predisposed to be delivered to the mitochondrial matrix at low efficiency but later gained targeting presequences that improved the efficiency and specificity of protein delivery to the organelles. In this case, there is no need for gene duplication if the newly acquired function is not required in the cytosol. 2) Initially, the newly acquired

proteins operated in the cytosol. After gene duplications and the accidental acquisition of MTSs, one copy was targeted and translocated into the organelles. The first scenario is supported by bioinformatic analysis of proteobacterial proteins showing that at least 5% of the proteins are predisposed for targeting to mitochondria (Lucattini et al. 2004). In addition, it has been shown that key proteins of extended glycolysis, including PFO, α-SCS, and malic enzyme, can be delivered into the hydrogenosomes of *T. vaginalis* without a targeting presequence (Zimorski et al. 2013). Moreover, no MTS has been identified in proteins targeted to hydrogenosomes in *Sp. salmonicida* (Jerlstrom-Hultqvist et al. 2013). The second scenario is supported by the dual localization of extended glycolysis and the ISC assembly machinery, which we observed in *M. balamuthi*. We found that each gene coding for ACS, D-LDH, PFO, hydrogenase, NifS, and NifU is present in at least two copies that differ in presence or absence of MTS (fig. 6). Using the *S. cerevisiae* model, we showed that only forms possessing an MTS could be targeted and translocated to yeast mitochondria, whereas paralogs not possessing an MTS remain in the cytosol. Accordingly, we found acetyl-CoA-dependent ATP synthesis, PFO, hydrogenase, and NifS activities both in the hydrogenosomes and in the cytosol of *M. balamuthi*. Dual localization of the components of extended glycolysis was also observed in *Sp. salmonicida*. This organism possesses genes for five PFOs and seven hydrogenases; however, only a single PFO and two hydrogenase paralogs were found to be targeted to hydrogenosomes; the other paralogs were localized to the cytosol (Jerlstrom-Hultqvist et al. 2013). In the anaerobic breviate *P. biforma*, multiple copies of PFO and hydrogenases also exist; however, only some appear to be localized to the mitochondrion-related organelle (Stairs et al. 2014). Therefore, dual localization of extended glycolysis is not restricted to Archamoebae, strengthening the argument for the second scenario.

Another intriguing question concerns the evolutionary transition of ATP-generating hydrogenosomes to energetically "silent" mitosomes. Considering the dual localization of extended glycolysis and NIF system enzymes in *M. balamuthi* and the cytosolic localization of these pathways in *E. histolytica* (Nyvltova et al. 2013), it is tempting to speculate that these organisms arose from an ancestor possessing a common set of LGT genes and that copies with hydrogenosomal targeting signals were lost in *E. histolytica* during the hydrogenosome-to-mitosome transition. Alternatively, it is possible that the gene duplication events occurred only in the *M. balamuthi* lineage, whereas the mitosomes of *E. histolytica* evolved directly through the reduction of mitochondrial pathways; that is, without their common ancestor going through a hydrogenosome-containing phase. In both scenarios, we could expect a common origin for LGT genes. However, phylogenetic analyses revealed a much more complex picture. A common origin was suggested for genes coding for AS and APSK (components of the sulfate activation pathway, which is present in *M. balamuthi* hydrogenosomes and in *E. histolytica* mitosomes) and for components of the NIF system. The phylogenies of the NIF subunits further suggests that organellar NifS and NifU in *E. histolytica* have lost their targeting sequences and replaced their cytosolic versions (Nyvltova et al. 2013). In contrast, our phylogenetic analyses of the duplicated components of extended glycolysis (ACS, PFO, and hydrogenase) in *M. balamuthi* revealed evolutionary histories different than those in *E. histolytica*. Three paralogs of ACS (ACS-M1, ACS-C2, and ACS-C3) in *M. balamuthi* were likely acquired by LGT from Archaea, whereas two further independent LGTs led to ACS acquisition in diplomonads (*Giardia*, *Spironucleus*) and in a diverse range of eukaryote lineages including *E. histolytica*. [FeFe]-hydrogenases that are present in multiple copies in *M. balamuthi* and in *E. histolytica* appear in distinct clusters within a large clade of eukaryotic [FeFe]-hydrogenases. Moreover, the cytosolic and hydrogenosomal hydrogenases of *M. balamuthi* clustered together, indicating the occurrence of recent gene duplication events. Similar patterns were observed with respect to multiple copies of PFO. Cytosolic and hydrogenosomal forms of *M. balamuthi* homologs clustered together in positions of the tree that are distinct from those occupied by the cytosolic PFO sequence of *E. histolytica*.

Collectively, these findings suggest that a common ancestor of *M. balamuthi* and *E. histolytica* possessed duplicated NIF systems and an organellar sulfate activation pathway, whereas components of extended glycolysis were duplicated only in *M. balamuthi*.

The complex phylogenetic histories of extended glycolysis enzymes in *M. balamuthi* and *E. histolytica* are reminiscent of the complex patterns observed for the glycolytic enzymes (Embden-Meyerhof-Parnas pathway) in these organisms (Liapounova et al. 2006). In anaerobic eukaryotes including the Archamoebae, two ATP-consuming enzymes, phosphofructokinase (PFK) and pyruvate kinase are replaced by inorganic pyrophosphate (PPi)-dependent PFK and pyruvate phosphate dikinase (PPDK), respectively, that increase the ATP yield of glycolysis (Mertens 1993; Slamovits and Keeling 2006). In addition, the class I fructose-bisphosphate aldolase (FBA) that is present in most eukaryotes is replaced by unrelated enzyme class II FBA (Sanchez et al. 2002). Previous phylogenetic analyses indicated that PPDK and FBA class II were present in the common ancestor of *M. balamuthi* and *E. histolytica* (Sanchez et al. 2002; Slamovits and Keeling 2006). PPDK was most likely acquired from gamma proteobacteria, while the phylogeny of class II FBA suggested an LGT event between *T. vaginalis* and the amoebae (Liapounova et al. 2006). In contrast, the phylogeny of PPi-PFK suggested that *M. balamuthi* and *E. histolytica* acquired this enzyme from different LGT events (Liapounova et al. 2006). To obtain more detailed insights into the history of LGT in Archamoebae, we will need genomic information about basal lineages such as *Pelomyxa* or *Rhizomastix* (Ptackova et al. 2013).

In conclusion, we found that *M. balamuthi* possesses dual pathways for extended glycolysis and the ACS-dependent synthesis of ATP; these pathways occur in parallel in the cytosol and in hydrogenosomes. Based on phylogenetic analyses of key components of extended glycolysis and the FeS cluster assembly machinery, we propose that LGT, gene duplication,

and the acquisition of MTS played a key role during the evolution of the *M. balamuthi* hydrogenosome.

Materials and Methods

Cell Cultivation

Mastigamoeba balamuthi (ATCC 30984) was a kind gift of M. Müller (Rockefeller University, USA) and was maintained axenically in PYGC medium at 24 °C (Chavez et al. 1986). *Saccharomyces cerevisiae* strain YPH499 was grown in a rich medium or selective medium as previously described (Lithgow et al. 1994).

Gene Cloning and Expression

Genes coding for *M. balamuthi* ACS, AS, APSK, IPP, and D-LDH were obtained using TBLAST searches of the draft *M. balamuthi* genome sequence http://www.ebi.ac.uk/ena/data/view/CBKX00000000 (January 22, 2015) using the orthologous sequences of *E. histolytica* as queries, except for D-LDH, for which we used the protein sequence of *Chlamydomonas reinhardtii* D-LDH as the query. Coding sequences without introns were obtained by polymerase chain reaction (PCR) using specific primers (see supplementary table S1A, Supplementary Material online, for sequences) and cDNA as the template. Total RNA was isolated from an exponential culture of *M. balamuthi* using Tri-Reagent (Sigma) and used as template for cDNA synthesis using SuperScript III (Invitrogen). Gene sequences were deposited in the NCBI database under the following accession numbers: ACS-M (KF927023), ACS-C (KF927022), AS (KF927024), APSK (KF927026), IPP-1 (KF927025), and D-LDH (KF941316).

To obtain recombinant MDH, ACS, AS, and IPP-1, the corresponding genes were amplified by PCR (primer sequences are given in supplementary table S1A, Supplementary Material online) using cDNA as template, subcloned into the pET42b+ vector (Novagen), and expressed with a 6xHis tag in *Escherichia coli* BL21 (DE3). The proteins were purified by affinity chromatography under denaturing conditions according to the manufacturer's protocol (Qiagen) and used to immunize rats (ACS, AS, IPP) or rabbits (MDH).

In the cellular localization studies, 600-bp gene fragments corresponding to the N-terminal segments of ACS-M1, ACS-C2, D-LDH-M, D-LDH-C, and hydrogenase 10-M were expressed with C-terminal GFP tags in *S. cerevisiae*. The gene fragments were cloned into the pUG35 plasmid using the restriction enzymes XbaI and HindIII (Niedenthal et al. 1996).

Cellular Fractionation

Subcellular fractions of *Mastigamoeba* were obtained by differential and OptiPrep (Axis-Shield PoC AS) gradient centrifugation of the cell homogenate. All steps were carried out at 4 °C in the presence of protease inhibitors (Complete Mini EDTA-free cocktail tablets, Roche). The cells were grown for 6 days in a tissue culture flask (Nunc). To harvest the cells, the growth medium was exchanged for fresh ice-cold medium, and the cells were carefully detached from the flask walls using a cell scraper. The cells were collected by centrifugation at 1,200 × g for 15 min, washed, and resuspended in SDM buffer (250 mM sucrose, 10 mM MOPS-KOH, 10 mM DTT, pH 7.4). The washed cells were disrupted using sonication on ice. The homogenate was centrifuged at 1,000 × g for 15 min to remove unbroken cells, membrane fragments, and nuclei, and the supernatant was carefully decanted. The supernatant was loaded onto a discontinuous OptiPrep gradient (15–40%) and five cell fractions were separated after centrifugation at 100,000 × g overnight.

Alternatively, the supernatant was centrifuged at 9,000 × g for 30 min to remove cell debris and lysosomes. The mitochondrion-enriched fraction was obtained by centrifugation of the supernatant at 100,000 × g for 25 min. The organellar fraction (pellet) was washed, resuspended, and centrifuged again at 100,000 × g for 25 min. The supernatant represented the cytosolic fraction.

Immunofluorescence Microscopy and Immunoblot Analysis

For immunofluorescence microscopy, *M. balamuthi* cells were fixed in 1% formaldehyde for 30 min, washed, and treated in 1% Triton TX-100 for 10 min. Fixed cells were stained on slides using polyclonal rat α-SDHB, α-AS, α-IPP, α-PFL, or mouse α-GCSH, or rabbit α-MDH and α-Cpn60 Abs (Nyvltova et al. 2013) and secondary Alexa Fluor 488 donkey α-rabbit or Alexa Fluor 594 donkey α-rat or α-mouse Abs (Life Technologies), respectively. For the colocalization of rabbits α-MDH and α-Cpn60, 250 µl of the MDH Ab was conjugated with 10 µg Alexa Fluor 488 and purified on Sephadex G-25.

Saccharomyces cerevisiae expressing GFP-tagged proteins were incubated for 15 min with the mitochondrial marker MitoTracker Red CMXRos (Molecular Probes), washed twice in PBS, and stabilized in 1% agarose. All slides were observed using an Olympus IX-81Cell R system. Colocalization of probes was evaluated using Fiji image analysis software and Coloc 2 plug-in (http://fiji.sc/Fiji, January 22, 2015) and quantified for individual cells using Pearson correlation coefficient (PCC) that is expressed as a mean ± standard deviation, where *n* is the number of individually measured cells. To analyze cellular fractions by immunoblotting, the proteins were resolved using 13.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes, and probed with the corresponding antibody; the specific signals were visualized using a LAS-4000 chemiluminescent reader. Polyclonal rabbit Ab raised against mitochondrial Hsp70 of *Neocallimastix* was kindly provided by M. Embley. We raised polyclonal rabbit Ab against a peptide of Cpn60 *M. balamuthi* (CKQISSQDEVRQVATV, EZBiolab USA), and rabbit polyclonal antibodies against *S. cerevisiae* Tim17 and Tom20 were a kind gift from T. Lithgow and K. Gabriel, respectively. Monoclonal mouse Ab raised against human GCS-H was obtained from Abnova (H00002653).

Enzyme Assays

PFO (EC 1.2.7.1), hydrogenase (EC 1.18.3.1), $NAD(P)^+$-dependent malic enzyme (EC 1.1.1.37 and EC 1.1.1.40),

MDH (EC 1.1.1.37), and NADH oxidase were assayed spectrophotometrically as described (Drmota et al. 1997; Rasoloson et al. 2001). Acid phosphatase (EC 3.1.3.2) was measured according to (Barrett 1972). PNO (EC 1.2.1.51) was assayed using the method described in Inui et al. (1987). D-LDH (EC 1.1.1.28) was measured spectrophotometrically at 340 nm in 50 mM Tris-KCl buffer, pH 7.4, using 1 mM NAD^+ as an electron acceptor and 2 mM D-lactate as a substrate. TDH (EC 1.1.1.103) was assayed spectrophotometrically at 340 nm in 50 mM Tris-KCl buffer, pH 7.4, 1 mM $MgCl_2$, using 1 mM NAD^+ as an electron acceptor and 5 mM threonine as a substrate (Boylan and Dekker 1981). Protein concentration was assayed according to Lowry's method.

Native Gel Electrophoresis

Nondenaturing PAGE was carried out in 6% polyacrylamide gels in the absence of SDS in a BioRad Mini-Protean apparatus. Cellular fractions were diluted with a minimal volume of loading dye containing 20% glycerol and bromophenol blue reagent. Electrophoresis was performed at 110 V for 2.5 h at 4 °C. Enzymatic activity was detected using a procedure modified from that of (Meinecke et al. 1989). Gel slides were washed in assay buffer (50 mM HEPES, 2 mM TPP, 2.5 mM $MgCl_2$) for 10 min. For the PFO reaction, the gel was incubated in assay buffer containing 2.5 mM CoA, 2.5 mM pyruvate, and 2 mg/ml NBT. For the PNO activity assays, 1 mM NADP was added to the incubation mixture. The reaction was stopped by washing with water.

ATP Production

ATP production was determined according to Drew and Leeuwenburgh (2003) using a luminometer and an ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) containing luciferase. Several substrates were added to the reaction mixture (2 mM pyruvate, 2 mM ADP, 2.5 mM CoA, and 1.5 mM acetyl-CoA). ATP standard curves were generated using 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM solutions. The organellar fraction obtained after differential centrifugation was used to analyze the ATP content and ATP production rate. To test the functionality of the ATP determination assay, 2.5 mM of the inhibitor P1,P5 –di (adenosine-5′) pentaphosphate was added to freshly isolated organelles, and the mixture was incubated for 10 min. The rate of inhibition of ATP production was determined by comparing the inhibitor-treated organelles to an equal portion of freshly isolated organelles.

Electron Microscopy

To examine the organellar fractions using electron microscopy, OptiPrep fractions were centrifuged and fixed in 0.1 M cacodylate buffer containing 8% glutaraldehyde at 4 °C and in 1% osmium tetroxide for 1 h, stained in block with uranyl acetate, and dehydrated in acetone. The sections were viewed using a JEOL 1011 transmission electron microscope.

Phylogenetic Analysis and Homology Modeling

Mastigamoeba PFO homologs were added to the alignment of Stairs et al. (2014). For all other proteins analyzed, *Mastigamoeba* amino acid sequences were used as queries to retrieve eukaryotic and at least 1,000 prokaryotic homologs using BLASTP against the nonredundant (nr) database in GenBank. These data sets were filtered by finding all sequences with greater than 98% sequence identity to another sequence and then randomly deleting one of them. Data sets were aligned using MAFFT (Katoh and Toh 2008), and regions of ambiguous alignment were automatically trimmed using BMGE (-blosum30; Criscuolo and Gribaldo 2010). Initial phylogenetic analyses were performed using FastTree (Price et al. 2010). The sequences of closely related, "redundant" species were removed by visual inspection of the FastTree output to generate the final data sets. To select the optimal substitution model for the final phylogenetic analyses, we performed model testing, as implemented in RAxML version 8.0.19 (Stamatakis 2014). In addition, we compared the likelihood scores of trees generated using the LG4X and LG4M models (Le et al. 2012) and the optimal model selected by RAxML. In all but one case, the LG4X model was favored over all other models according to the Akaike Information Criterion (the GCSP data set favored the LG4M model). For each gene, the percent bootstrap support from 500 bootstrap replicates was mapped onto the best-scoring ML tree (of 100 heuristic searches). Bayesian inference was conducted using PhyloBayes 3.2 with four Monte Carlo Markov Chains under the site-heterogeneous catfix C20 model of evolution (C20+poisson and gamma 4; Le et al. 2008; Lartillot et al. 2009). Chains were run until convergence (maxdiff < 0.3) and/or 300,000 generations were run for each chain. Every 100 trees were sampled after a manually determined burn-in of trees was removed. PPs were subsequently mapped onto the ML tree using Dendropy (Sukumaran and Holder 2010).

Homology models for SDH were generated using the Swiss-MODEL automated workspace server (Ye and Godzik 2003; Arnold et al. 2006).

Supplementary Material

Supplementary figures S1–S4 and table S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work was supported by the Czech Grant Foundation (P305/11/1061), the Biomedicine Center of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0109) from the European Regional Development Fund and GAUK101710 from Charles University in Prague. C.W.S. is supported by a Natural Science and Engineering Research Council (NSERC) Alexander Graham Bell Canadian Graduate Scholarship and a Killam Graduate Scholarship. A.J.R. acknowledges the Canadian Institute for Advanced Research Program in Microbial Biodiversity, in which he is a Senior Fellow, and the Canada Research Chairs Program (CIHR; MOP-62809). Access to computing and storage

facilities was provided by ELIXIR CZ and the National Grid Infrastructure MetaCentrum.

References

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, et al. 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306:79–86.

Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: a web- based environment for protein structure homology modelling. *Bioinformatics* 22:195–201.

Atteia A, van LR, Tielens AG, Martin WF. 2013. Anaerobic energy metabolism in unicellular photosynthetic eukaryotes. *Biochim Biophys Acta.* 1827:210–223.

Barbera MJ, Ruiz-Trillo I, Tufts JY, Bery A, Silberman JD, Roger AJ. 2010. *Sawyeria marylandensis* (Heterolobosea) has a hydrogenosome with novel metabolic properties. *Eukaryot Cell.* 9:1913–1924.

Barrett AT. 1972. Lysosomal enzymes. In: Dingle JT, editor. Lysosomes. Amsterdam: North-Holland Publishing Company. p. 46–135.

Bird MI, Nunn PB, Lord LA. 1984. Formation of glycine and aminoacetone from L-threonine by rat liver mitochondria. *Biochim Biophys Acta.* 802:229–236.

Boylan SA, Dekker EE. 1981. L-threonine dehydrogenase. Purification and properties of the homogeneous enzyme from *Escherichia coli* K-12. *J Biol Chem.* 256:1809–1815.

Burki F, Corradi N, Sierra R, Pawlowski J, Meyer GR, Abbott CL, Keeling PJ. 2013. Phylogenomics of the intracellular parasite *Mikrocytos mackini* reveals evidence for a mitosome in rhizaria. *Curr Biol.* 23: 1541–1547.

Chavez LA, Balamuth W, Gong T. 1986. A light and electron microscopical study of a new, polymorphic free-living amoeba, *Phreatamoeba balamuthi n. g., n. sp. J Protozool.* 33:397–404.

Criscuolo A, Gribaldo S. 2010. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol Biol.* 10:210.

de Bari L, Atlante A, Guaragnella N, Principato G, Passarella S. 2002. D-lactate transport and metabolism in rat liver mitochondria. *Biochem J.* 365:391–403.

de Graaf RM, Duarte I, van Alen TA, Kuiper JW, Schotanus K, Rosenberg J, Huynen MA, Hackstein JH. 2009. The hydrogenosomes of *Psalteriomonas lanterna*. *BMC Evol Biol.* 9:287.

de Graaf RM, Ricard G, van Alen TA, Duarte I, Dutilh BE, Burgtorf C, Kuiper JW, van der Staay GW, Tielens AG, Huynen MA, et al. 2011. The organellar genome and metabolic potential of the hydrogen-producing mitochondrion of *Nyctotherus ovalis*. *Mol Biol Evol.* 28: 2379–2391.

Dolezal P, Likic V, Tachezy J, Lithgow T. 2006. Evolution of the molecular machines for protein import into mitochondria. *Science* 313: 314–318.

Drew B, Leeuwenburgh C. 2003. Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria of Fischer-344 rats with age and caloric restriction. *Am J Physiol Regul Integr Comp Physiol.* 285:R1259–R1267.

Drmota T, Tachezy J, Kulda J. 1997. Isolation and characterization of cytosolic malate dehydrogenase from *Trichomonas vaginalis*. *Folia Parasitol.* 44:103–108.

Embley TM, Martin W. 2006. Eukaryotic evolution, changes and challenges. *Nature* 440:623–630.

Fritz-Laylin LK, Prochnik SE, Ginger ML, Dacks JB, Carpenter ML, Field MC, Kuo A, Paredez A, Chapman J, Pham J, et al. 2010. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* 140: 631–642.

Gill EE, Diaz-Trivino S, Barbera MJ, Silberman JD, Stechmann A, Gaston D, Tamas I, Roger AJ. 2007. Novel mitochondrion-related organelles in the anaerobic amoeba *Mastigamoeba balamuthi*. *Mol Microbiol.* 66:1306–1320.

Hao HX, Khalimonchuk O, Schraders M, Dephoure N, Bayley JP, Kunst H, Devilee P, Cremers CW, Schiffman JD, Bentz BG, et al. 2009. SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science* 325:1139–1142.

Herrmann G, Jayamani E, Mai G, Buckel W. 2008. Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. *J Bacteriol.* 190:784–791.

Hug LA, Stechmann A, Roger AJ. 2010. Phylogenetic distributions and histories of proteins involved in anaerobic pyruvate metabolism in eukaryotes. *Mol Biol Evol.* 27:311–324.

Inui H, Ono K, Miyatake K, Nakano Y, Kitaoka S. 1987. Purification and characterization of pyruvate:NADP+ oxidoreductase in *Euglena gracilis*. *J Biol Chem.* 262:9130–9135.

Jerlstrom-Hultqvist J, Einarsson E, Xu F, Hjort K, Ek B, Steinhauf D, Hultenby K, Bergquist J, Andersson JO, Svard SG. 2013. Hydrogenosomes in the diplomonad *Spironucleus salmonicida*. *Nat Commun.* 4:2493.

Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform.* 9:286–298.

Kikuchi G. 1973. The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Mol Cell Biochem.* 1: 169–187.

Lartillot N, Lepage T, Blanquart S. 2009. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* 25:2286–2288.

Le SQ, Dang CC, Gascuel O. 2012. Modeling protein evolution with several amino acid replacement matrices depending on site rates. *Mol Biol Evol.* 29:2921–2936.

Le SQ, Lartillot N, Gascuel O. 2008. Phylogenetic mixture models for proteins. *Philos Trans R Soc Lond B Biol Sci.* 363:3965–3976.

Liapounova NA, Hampl V, Gordon PM, Sensen CW, Gedamu L, Dacks JB. 2006. Reconstructing the mosaic glycolytic pathway of the anaerobic eukaryote *Monocercomonoides*. *Eukaryot Cell.* 5:2138–2146.

Lindmark DG, Müller M, Shio H. 1975. Hydrogenosomes in *Trichomonas vaginalis*. *J Parasitol.* 61:552–554.

Lindmark DG, Paolella P, Wood NP. 1969. The pyruvate formate-lyase system of *Streptococcus faecalis*. I. Purification and properties of the formate-pyruvate exchange enzyme. *J Biol Chem.* 244:3605–3612.

Lithgow T, Junne T, Suda K, Gratzer S, Schatz G. 1994. The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. *Proc Natl Acad Sci U S A.* 91:11973–11977.

Lucattini R, Likic VA, Lithgow T. 2004. Bacterial proteins predisposed for targeting to mitochondria. *Mol Biol Evol.* 21:652–658.

Maralikova B, Ali V, Nakada-Tsukui K, Nozaki T, van der Giezen M, Henze K, Tovar J. 2009. Bacterial-type oxygen detoxification and iron-sulfur cluster assembly in amoebal relict mitochondria. *Cell Microbiol.* 12:331–342.

Martin WF. 2011. Early evolution without a tree of life. *Biol Direct.* 6:36.

Meinecke B, Bertram J, Gottschalk G. 1989. Purification and characterization of the pyruvate-ferredoxin oxidoreductase from *Clostridium acetobutylicum*. *Arch Microbiol.* 152:244–250.

Mertens E. 1993. ATP versus pyrophosphate: glycolysis revisited in parasitic protists. *Parasitol Today.* 9:122–126.

Mi-ichi F, Abu YM, Nakada-Tsukui K, Nozaki T. 2009. Mitosomes in *Entamoeba histolytica* contain a sulfate activation pathway. *Proc Natl Acad Sci U S A.* 106:21731–21736.

Müller M, Mentel M, van Hellemond JJ, Henze K, Woehle C, Gould SB, Yu RY, van der Giezen M, Tielens AG, Martin WF. 2012. Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol Mol Biol Rev.* 76:444–495.

Niedenthal RK, Riles L, Johnston M, Hegemann JH. 1996. Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. *Yeast* 12:773–786.

Nyvltova E, Sutak R, Harant K, Sedinova M, Hrdy I, Paces J, Vlcek C, Tachezy J. 2013. NIF-type iron-sulfur cluster assembly system is duplicated and distributed in the mitochondria and cytosol of *Mastigamoeba balamuthi*. *Proc Natl Acad Sci U S A.* 110:7371–7376.

Price MN, Dehal PS, Arkin AP. 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490.

Ptackova E, Kostygov AY, Chistyakova LV, Falteisek L, Frolov AO, Patterson DJ, Walker G, Cepicka I. 2013. Evolution of Archamoebae: morphological and molecular evidence for pelobionts including *Rhizomastix*, *Entamoeba*, *Iodamoeba*, and *Endolimax*. *Protist* 164:380–410.

Rasoloson D, Tomková E, Cammack R, Kulda J, Tachezy J. 2001. Metronidazole-resistant strains of *Trichomonas vaginalis* display increased susceptibility to oxygen. *Parasitology* 123:45–56.

Riordan CE, Langreth SG, Sanchez LB, Kayser O, Keithly JS. 1999. Preliminary evidence for a mitochondrion in *Cryptosporidium parvum*: phylogenetic and therapeutic implications. *J Eukaryot Microbiol.* 46:52S–55S.

Sanchez L, Horner D, Moore D, Henze K, Embley T, Müller M. 2002. Fructose-1,6-bisphosphate aldolases in amitochondriate protists constitute a single protein subfamily with eubacterial relationships. *Gene* 295:51–59.

Slamovits CH, Keeling PJ. 2006. Pyruvate-phosphate dikinase of oxymonads and parabasalia and the evolution of pyrophosphate-dependent glycolysis in anaerobic eukaryotes. *Eukaryot Cell.* 5: 148–154.

Spalding MD, Prigge ST. 2010. Lipoic acid metabolism in microbial pathogens. Microbiol. *Mol Biol Rev.* 74:200–228.

Stairs CW, Eme L, Brown MW, Mutsaers C, Susko E, Dellaire G, Soanes DM, van der Giezen M, Roger AJ. 2014. A SUF Fe-S cluster biogenesis system in the mitochondrion-related organelles of the anaerobic protist *Pygsuia*. *Curr Biol.* 24:1176–86.

Stairs CW, Roger AJ, Hampl V. 2011. Eukaryotic pyruvate formate lyase and its activating enzyme were acquired laterally from a Firmicute. *Mol Biol Evol.* 28:2087–2099.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.

Stechmann A, Hamblin K, Perez-Brocal V, Gaston D, Richmond GS, van der Giezen M, Clark CG, Roger AJ. 2008. Organelles in *Blastocystis* that blur the distinction between mitochondria and hydrogenosomes. *Curr Biol.* 18:580–585.

Sukumaran J, Holder MT. 2010. DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26:1569–1571.

Tachezy J, Dolezal P. 2007. Iron-sulfur proteins and iron-sulfur cluster assembly in organisms with hydrogenosomes and mitosomes. In: Martin WF, Müller M, editors. Origin of mitochondria and hydrogenosomes. Berlin: Springer. p. 105–133.

Tovar J, Fischer A, Clark CG. 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. *Mol Microbiol.* 32:1013–1021.

Tovar J, Leon-Avila G, Sanchez LB, Sutak R, Tachezy J, van der Giezen M, Hernandez M, Müller M, Lucocq JM. 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation. *Nature* 426:172–176.

van der Giezen M, Cox S, Tovar J. 2004. The iron-sulfur cluster assembly genes *iscS* and *iscU* of *Entamoeba histolytica* were acquired by horizontal gene transfer. *BMC Evol Biol.* 4:7.

Williams BAP, Hirt RP, Lucocq JM, Embley TM. 2002. A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. *Nature* 418:865–869.

Yarlett N, Hackstein JHP. 2005. Hydrogenosomes: one organelle, multiple origins. *BioScience* 55:657–667.

Ye Y, Godzik A. 2003. Flexible structure alignment by chaining aligned fragment pairs allowing twists. *Bioinformatics* 19(Suppl 2): ii246–ii255.

Zimorski V, Major P, Hoffmann K, Bras XP, Martin WF, Gould SB. 2013. The N-terminal sequences of four major hydrogenosomal proteins are not essential for import into hydrogenosomes of *Trichomonas vaginalis*. *J Eukaryot Microbiol.* 60:89–97.

7 Conclusions

7.1 Characterization of mitochondrial organelles in Mastigamoeba balamuthi

Functional analysis of putative mitosomes in *M. balamuthi* revealed that unlike mitosomes, these organelles are metabolically active and possess the hydrogen and ATP-generating pathway that is typical for anaerobic energy metabolism in hydrogenosomes (Müller, 1993). This pathway (extended glycolysis) includes the enzymes pyruvate:ferredoxin oxidoreductase (PFO), [FeFe]-hydrogenase and ADP-forming acetyl-CoA synthetase (ACS). An identical pathway is present in hydrogenosomes of *Spironuleus salmonicida* (Jerlstrom-Hultqvist et al., 2013) including ACS which synthesizs ATP, whereas in most hydrogenosomes, ATP synthesis requires the activity of two enzymes: acetate:succinate CoA transferase (ASCT) and succinyl-CoA synthetase (SCS) (Müller, 1993). Based on metabolic activities, we re-named the *M. balamuthi* organelles as hydrogenosomes. In addition to typical hydrogenosomal features, the *M. balamuthi* organelles share some characteristics with anaerobic mitochondria such as (i) presence of the TCA cycle enzyme malate dehydrogenase, (ii) succinate dehydrogenase (respiratory Complex II), and (iii) electron transfer flavoprotein (ETF) and electron transferring flavoprotein dehydrogenase (ETFDH), which are involved in the fatty acid oxidation reaction. ETFDH can transfer the electrons from ETF proteins via ubiquinone (UQ) or rhodoquinone (RQ) to Complex II. However presence of either UQ or RQ has not been confirmed in *Mastigamoeba*, thus far.

There are some more peculiarities of carbohydrate metabolism. The malic enzyme, that is usually present in most hydrogenosomes, was not identified in *M. balamuthi* organelles and its activity was detected exclusively in the cytosol. However, mastigamoeba organelles contain NAD-dependent D-lactate dehydrogenase (D-LDH), which has not been described in any hydrogenosome or mitochondrion so far. The function of D-LDH in these organelles is unclear.

Mastigamoeba organelles possess amino acid metabolic pathways such as a complete glycine cleavage system (proteins P, L, H, and T), serine hydroxymethyltransferase and a putative lipoamide protein ligase, which can provide lipoamide for the glycine cleavage system. We also identified genes for threonine dehydrogenase and α-amino-β- ketobutyrate CoA ligase (AKL), which are components of another typical mitochondrial amino acid

metabolic pathway - threonine degradation. Surprisingly, these enzymes seem to reside in the cytosol.

Functional and genomic analyses revealed that two key pathways, extended glycolysis and NIF system for FeS cluster biogenesis are duplicated in *M. balamuthi* and localize to two cellular compartments, into the cytosol and the hydrogenosomes. Each component of these pathways is encoded by at least two gene copies of which at least one copy encodes a protein with an N-terminal mitochondrion targeting sequence (MTS). The dual localization of both pathways was demonstrated by enzymatic assays using subcellular fractions, western blot analysis of subcellular fractions using specific antibodies, and by immunofluorescence microscopy. The essential role of the MTS for protein delivery to the organelles was verified by heterologous expression of selected *M. balamuthi* genes (such as mitochondrial and cytosolic variants of ACS, LDH, NifS and NifU genes) in *Saccharomyces cerevisiae*. Phylogenetic analyses revealed that genes for extended glycolysis and FeS cluster assembly were mostly acquired by lateral gene transfer (LGT) from bacteria. Based on these results we proposed that during the evolution of ancestral mitochondria to mastigamoeba hydrogenosomes, the ancestral cells acquired genes of extended glycolysis and FeS cluster assembly by LGT, and the gene products originally operated in the cytosol. After subsequent gene duplication and acquisition of MTSs both pathways were duplicated in mitochondria, whereas most of the mitochondrial pathways were lost.

Sulfate activation is the only pathway that is common to *Mastigamoeba* hydrogenosomes and *Entamoeba* mitosomes. Both organelles possess ATP sulfurylase, inorganic pyrophosphatase and APS kinase. Phylogenetic analysis of ATP sulfurylase and APS kinase showed that similarly to the NIF system, these two components were acquired by LGT before the split of the *Mastigamoeba* and *Entamoeba* lineages. However, unlike extended glycolysis and the NIF system, the sulfate activation pathway is not duplicated in *Mastigamoeba*. Results of these studies were published in the article: Nývltová et al., Mol. Biol. Evol., 2015.

The replacement of mitochondrial ISC type FeS cluster assembly by the bacterial NIF system is particularly interesting. In most eukaryotes, it is mediated by ISC machinery that consists of over 10 components. The mitochondrial ISC system most likely evolved from bacterial ISC machinery that was present in the mitochondrial ancestor, and ISC machinery also mediates synthesis of FeS clusters in the majority of contemporary bacteria. In nitrogen fixing bacteria, the NIF system has the specialized function of nitrogenase maturation. Exceptionally, the NIF system has been found in non-nitrogen-fixing anaerobic ε-

proteobacteria, such as *Helicobacter,* in which the two NIF components (NifU and NifS) function instead of the ISC system. In eukaryotes, the substitution of the multicomponent mitochondrial ISC system with the simple NIF machinery was a unique evolutionary event in the archamoebal lineage. Our phylogenetic analysis strongly suggests that the NIF system was acquired by a common ancestor of *M. balamuthi* and *E. histolytica* from ε-proteobacteria. However, whereas in *M. balamuthi* the NIF system has a dual localization, we did not confirm presence of the NIF system in mitosomes of *E. histolytica.* The mechanism of how the elaborated mitochondrial ISC system was replaced by the simple NIF system is an evolutionary puzzle. These results were summarized in the publication: Nývltová et al., PNAS, 2013.

7.2 Hydrogen production in Naegleria gruberi

In the recent genomic and transcriptomic study of *Naegleria gruberi* (Fritz-Laylin et al. 2010), the authors have identified the full sequence of the [FeFe]-hydrogenase gene. Based on a predictable N-terminal MTS they suggested that the enzyme localizes inside the aerobic mitochondria, which would be the first evidence of an anaerobic enzyme hydrogenase in an aerobically living eukaryote. Using gas chromatography, we confirmed that aerobically grown *N. gruberi* indeed produces molecular hydrogen. Next we investigated the localization of hydrogenase using immunofluorescence microscopy and studied the presence of hydrogenase in subcellular fractions. Unexpectedly, hydrogenase appeared exclusively in the *Naegleria* cytosol. Specific antibodies against *N. gruberi* and *T. vaginalis* hydrogenases labeled the *Naegleria* cytosol and do not co-localize with the mitochondrial marker dye MitoTracker. In subcellular fractions, the enzymatic activity of hydrogenase was detected exclusively in the cytosol together with the cytosolic marker enzyme hexokinase. No hydrogenase activity was detected in the mitochondrial fraction in which we detected activity of the mitochondrial marker enzyme glutamate dehydrogenase. We obtained the same results using western blot analysis of the subcellular fractions. Therefore, we did not confirm previous predictions that hydrogenase is present in the mitochondria of *Naegleria gruberi*. Nevertheless, we confirmed that *N. gruberi* is able to produce molecular hydrogen. Moreover, although hydrogenase is believed to be highly sensitive to oxygen, *N. gruberi* is able to produce hydrogen and possesses an active hydrogenase when it is grown under aerobic conditions. These results were published in the article: Tsaousis et al., GBE, 2014.