

The Organellar Genome and Metabolic Potential of the Hydrogen-Producing Mitochondrion of *Nyctotherus ovalis*

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Abstract

It is generally accepted that hydrogenosomes (hydrogen-producing organelles) evolved from a mitochondrial ancestor. However, until recently, only indirect evidence for this hypothesis was available. Here, we present the almost complete genome of the hydrogen-producing mitochondrion of the anaerobic ciliate *Nyctotherus ovalis* and show that, except for the notable absence of genes encoding electron transport chain components of Complexes III, IV, and V, it has a gene content similar to the mitochondrial genomes of aerobic ciliates. Analysis of the genome of the hydrogen-producing mitochondrion, in combination with that of more than 9,000 genomic DNA and cDNA sequences, allows a preliminary reconstruction of the organellar metabolism. The sequence data indicate that *N. ovalis* possesses hydrogen-producing mitochondria that have a truncated, two step (Complex I and II) electron transport chain that uses fumarate as electron acceptor. In addition, components of an extensive protein network for the metabolism of amino acids, defense against oxidative stress, mitochondrial protein synthesis, mitochondrial protein import and processing, and transport of metabolites across the mitochondrial membrane were identified. Genes for MPV17 and ACN9, two hypothetical proteins linked to mitochondrial disease in humans, were also found. The inferred metabolism is remarkably similar to the organellar metabolism of the phylogenetically distant anaerobic Stramenopile *Blastocystis*. Notably, the *Blastocystis* organelle and that of the related flagellate *Proteromonas lacertae* also lack genes encoding components of Complexes III, IV, and V. Thus, our data show that the hydrogenosomes of *N. ovalis* are highly specialized hydrogen-producing mitochondria.

Key words: hydrogenosome, mitochondrion, horizontal gene transfer, evolution, adaptation, *Nyctotherus*.

Introduction

Mitochondria are essential organelles in aerobic eukaryotes. They play a central role in ATP production as well as in many other cellular processes, such as apoptosis, cellular proliferation, heme synthesis, steroid synthesis, and Fe/S cluster assembly (Miller 1995; Lill and Kispal 2000; Scheffler 2001; Duchon 2004). Aerobic mitochondria perform oxidative phosphorylation, that is, they use oxygen as terminal electron acceptor, oxidizing NADH and FADH₂ while producing ATP with the aid of an ATP synthase that exploits the proton gradient generated by the electron transport chain. Eukaryotes living under anaerobic circumstances have evolved a spectrum of organelles that are related

to mitochondria and have names, such as anaerobic mitochondria (both temporarily and permanently anaerobic), hydrogenosomes, mitosomes, mitochondrial remnants, modified mitochondria, or mitochondria-like organelles, reflecting their metabolic peculiarities (Tielens et al. 2002; Barbera et al. 2007; Tachezy and Dolezal 2007; Tachezy and Smid 2007; Tielens and van Hellemond 2007; Hjort et al. 2010).

Mitochondria that produce ATP via oxidative phosphorylation have a mitochondrial genome, as the protein complexes of proton-pumping electron transport chains contain essential subunits whose genes were never translocated to the nuclear genome during evolution but always

remained part of the mitochondrial genome. The canonical (textbook) mitochondrion uses oxygen as final electron acceptor, but there are also anaerobically functioning mitochondria that use compounds other than oxygen as final electron acceptor. Most organisms with anaerobic mitochondria use an endogenously produced electron acceptor, such as fumarate (Tielens et al. 2002). Some anaerobic functioning mitochondria possess a hydrogenase besides a proton-pumping electron transport chain, and therefore, they can use protons as final electron acceptors resulting in the production of hydrogen. These organelles are the so-called hydrogen-producing mitochondria and examples are found in *Nyctotherus ovalis* and *Blastocystis* sp. Hydrogen production in the latter organelles, however, has not been demonstrated so far (Lantsman et al. 2008; Stechmann et al. 2008). These organelles and those of *Proteromonas*, for which currently nothing is known about hydrogen production, are considered to be bona fide mitochondria as they use a proton-pumping electron transport chain and hence possess a mitochondrial genome encoding essential parts of it (Perez-Brocal et al. 2010).

Hydrogenosomes are mitochondrion-related organelles that, by definition, produce ATP and hydrogen using protons as electron acceptors. In contrast to hydrogen-producing mitochondria, hydrogenosomes have no mitochondrial genome, do not possess a proton-pumping electron transport chain, and produce ATP exclusively via substrate-level phosphorylation. Hydrogenosomes are found in various unrelated anaerobic eukaryotes, such as anaerobic flagellates, chytridiomycete fungi, and several anaerobic ciliates (Hackstein et al. 2001; Embley et al. 2003; Embley and Martin 2006; Hackstein, Baker, et al. 2008; Hackstein, de Graaf, et al. 2008; de Graaf, Duarte, et al. 2009).

Mitosomes produce neither hydrogen nor ATP, but in general have retained components of a Fe/S cluster synthesizing machinery, which is now believed to be the reason why mitochondria, hydrogenosomes, and mitosomes are essential to eukaryotic life (Henze and Martin 2003; Yarlett 2004; Tachezy and Dolezal 2007; Goldberg et al. 2008). Even for *Entamoeba* (Aguilera et al. 2008) that has been regarded as the potential exception to this pattern, evidence is now mounting that its mitosomes also assemble Fe/S clusters (Maralikova et al. 2010), although Mi-ichi et al. (2009) could not confirm this finding using a proteomics approach. All eukaryotic organisms studied so far in sufficient molecular and ultrastructural detail appear to possess either mitochondria, hydrogenosomes or mitosomes. Furthermore, no species has been found that contains both mitochondria and mitosomes or mitochondria and hydrogenosomes, supporting the hypothesis that these organelles have a common origin. Despite this common origin, mitochondria, hydrogenosomes, and mitosomes are very diverse: not only in the size of their proteomes but also by virtue of their protein composition and function (Gabaldón and Huynen 2004). However, classical aerobic mitochondria share two characteristics: They have retained a genome, allowing unambiguous documentation of their descent from an α -pro-

teobacterium by phylogenetic analyses (Lang et al. 1999), and they contain an electron transport chain with Complexes I through IV as well as an ATPase (Complex V). In the evolution of mitochondria, hydrogenosomes and mitosomes from an α -proteobacterium, a large number of genes and proteins have been lost, gained from various sources, or retargeted to other organelles, thus shaping the huge diversity of current mitochondria and their homologs in various species (Gabaldón and Huynen 2004). Within the evolutionary “gap” between species with genome-containing mitochondria and species with mitosomes or hydrogenosomes (both lacking an organellar genome), the species *N. ovalis* provides an interesting link. *Nyctotherus ovalis* is an anaerobic ciliate that lives in the hindgut of various cockroach species. It has numerous hydrogen-producing organelles that are intimately associated with endosymbiotic methane-producing archaea that use the hydrogen produced by the organelles. Despite the hydrogenosomal metabolism of this organelle, we have found that it actually has a genome (Akhmanova, Voncken, van Alen, et al. 1998), that similar to mitochondrial genomes (Gray 2005), encodes elements of an electron transport chain (Boxma et al. 2005). Phylogenetic analyses of the organellar genome of *N. ovalis* showed that it has evolved from the genome of an aerobic mitochondrion of a ciliate ancestor (Boxma et al. 2005), providing direct evidence that hydrogenosomes can evolve from mitochondria. This blurs the distinction between mitochondria and hydrogenosomes. A comparable situation is found in the mitochondrion-like organelles of the phylogenetically only distantly related Stramenopile *Blastocystis* that is clearly distinct from the ciliates, which belong to the Alveolata. *Blastocystis* also retains a mitochondrial genome and parts of an electron transport chain (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Because this organelle also hosts a [FeFe] hydrogenase, it can be regarded as a hydrogenosome with genome, although thus far no hydrogenase activity has been demonstrated in this species (Lantsman et al. 2008; Stechmann et al. 2008).

Also for other (genome-less) types of hydrogen-producing organelles evidence is accumulating that they have evolved from mitochondria (Embley and Martin 2006). In the absence of an organellar genome, the evidence for homology is based on phylogenetic analyses of proteomes. Based on phylogenetic analyses of Complex I subunits, the hydrogenosomes of the parabasalid *Trichomonas* are inferred to share a common ancestor with mitochondria (Hrdý et al. 2004). Also, the hydrogenosomes of anaerobic chytrids seem to have evolved from the mitochondria of their aerobic ancestors (Akhmanova, Voncken, Harhangi, et al. 1998). It is therefore likely that hydrogenosomes arose repeatedly by evolutionary tinkering as an adaptation to the particular requirements of hosts, which thrive in rather different anaerobic environments.

Here, we describe the isolation and sequence analysis of the major part (41,666 bp) of the organellar genome of *N. ovalis* and compare it with the mitochondrial genomes of the ciliates *Paramecium aurelia*, *Tetrahymena* spp., and

Euplotes minuta as well as with the mitochondrial/hydrogenosomal genome of the Stramenopiles *Blastocystis* sp. and *Proteromonas lacertae*. We show that the organellar genome of *N. ovalis* is a typical ciliate mitochondrial genome except for the obvious absence of genes encoding components of Complexes III, IV, and V. The lack of evidence for the presence of these genes in *N. ovalis* coincides with the definitive absence of these genes from the organellar genomes of *Blastocystis* sp. and *P. lacertae* (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008; Perez-Brocal et al. 2010). By comparing the hydrogenosomal proteins and metabolic pathways of *N. ovalis* with the various hydrogenosomes, mitosomes, mitochondria-like organelles, and mitochondria, we show that the *N. ovalis* organelle is as complex as some mitochondria.

Materials and Methods

Cell Isolation

Nyctotherus ovalis cells were isolated from the hindgut of the cockroach *Blaberus* sp. var. Amsterdam. Total DNA was isolated by dissolving living cells in 8 M guanidinium chloride and separation on a hydroxyapatite column using standard procedures (de Graaf, van Alen, et al. 2009).

The organellar DNA was isolated from the total DNA by pulsed field gel electrophoresis. This organellar DNA was used for a partial Sau 3A I digest and cloning in the Bam H1 restriction site from the vector pUC18c. From this library, 500 clones were sequenced in both directions and additional 500 in one direction. Less than 3% of these clones contained a mitochondrial sequence. Finally, larger pieces of the organellar genome were reconstructed, and the gaps were filled by long range polymerase chain reaction (PCR) with specific primers resulting in a contig of 41,666 bp representing the major part of the organellar genome (accession number: GU057832). Due to the very limited amount of DNA available, we did not attempt to sequence the chromosome ends.

Identification of *N. ovalis* sequences likely encoding hydrogenosomal proteins

Nyctotherus ovalis sequences translated in the six frames were compared with the mitochondrial proteomes of human, yeast, rat, and *Tetrahymena*. The mitochondrial proteomes were retrieved from the mitoproteome (<http://www.mitoproteome.org>), the SGD database (Cherry et al. 1998), the supplementary material from Sickmann et al. (2003), from Forner et al. (2006), and from Smith et al. (2007). The accession numbers of the *N. ovalis* sequences are displayed in [supplementary table S1, Supplementary Material](#) online. *Nyctotherus ovalis* sequences with a reciprocal best hit (BH) with one of the mitochondrial proteins (Smith Waterman, $E < 0.01$) were selected for further phylogenetic analysis to confirm orthology with a mitochondrial protein. Each nucleotide sequence selected was compared by the SWX algorithm with the

165 complete proteomes, and the first 100 hits were kept and used together with the *N. ovalis* sequence to produce a multiple alignment with Muscle v3.7 (default parameters; Edgar 2004). Positions of the alignments that did not contain gaps were automatically selected, and a tree was subsequently derived using PhyML (Guindon and Gascuel 2003) using the JTT model and an estimated number of invariable sites with four substitution rate categories. Hundred bootstraps were performed.

Phylogenetic Position

To determine the phylogenetic position of the *N. ovalis* hydrogen-producing mitochondrion, we composed a concatenated alignment phylogeny of the Complex I proteins that tend to be encoded on the mitochondrial genome (nad1, nad2, nad3, nad4, nad4L, nad5, and nad6), using the gamma-proteobacterium *Escherichia coli* and the α -proteobacterium *Rickettsia prowazekii* as outgroups. The sequences in each protein family were first aligned with Muscle (Edgar 2004). We then concatenated the alignment blocks, where gaps were inserted in the rare cases that a protein was missing from a certain species (e.g., nad6 from *N. ovalis*). Given the length of the alignment, we opted to compute a bootstrapped Maximum Likelihood (ML) phylogeny. For this, we first selected the best-fit model of amino acid replacement, using the Akaike information criterion (AIC) as goodness of fit measure, as implemented in ProtTest (version 2.2) (Abascal et al. 2005). Accordingly, a ML phylogeny, 100 times bootstrapped, was computed with PhyML (version 3.0.1) (Guindon and Gascuel 2003) using the previously selected LG model of amino acids substitution, with a discrete gamma distribution approximated by 4 rate-categories (+4G), estimated proportion of invariable sites (+I), and observed amino acid frequencies (+F).

Identification of Sequences Derived From HGT

In order to identify horizontal gene transfers (HGTs) present in the *N. ovalis* genome, we built phylogenies for all the proteins that had BHs with noneukaryotic proteins in a Blast search. We carefully selected the homologous data set from the 250 BHs in the nonredundant database, supplemented with ten phylogenetically well distributed, best Blast hits from eukaryotes only, aligned the sequences using ClustalW (version 2.0.10) (Thompson et al. 1994), and subsequently pruned the alignment for redundancy. Each alignment was then submitted to ProtTest (version 2.4) (Abascal et al. 2005) in order to statistically infer the best-fit model of amino acid substitution, according to the AIC. Finally, a ML phylogeny was computed with PhyML (version 3.0.1) (Guindon and Gascuel 2003) employing the previously chosen model and bootstrapped 100 times.

Subcellular Localization

Mitop2 (Andreoli et al. 2004) was used when the N-terminal part of the *N. ovalis* sequence was present to examine the presence of a putative Mitochondrial Import Signal.

N. ovalis cDNA and gDNA

Nyctotherus ovalis cDNA and genomic DNA (gDNA) libraries were constructed as described earlier (Ricard et al. 2008). The macronuclear gDNA clones were obtained after amplification of total ciliate DNA with telomere primers. In general, only gDNA clones with at least one telomere were used in order to exclude contamination by bacterial sequences.

Results and Discussion

Gene Content and Structure of the Organellar Genome of *N. ovalis*

In 2005, Boxma et al. showed that a 14 Kb fragment of the organellar genome of the anaerobic hydrogen-producing ciliate *N. ovalis* that had been isolated from the cockroach *Blaberus* sp. var. Amsterdam was of mitochondrial origin (Boxma et al. 2005). Here, we describe an analysis of the nearly complete organellar genome. Pulsed field gel electrophoresis followed by Southern blot hybridization with two different ³²P-labeled probes, *nad7* and 12S (*rns*), indicated that the size of the organellar genome of *N. ovalis* exceeds 48 kb (supplementary fig. S1, Supplementary Material online).

Mitochondrial genes present in the organellar library were identified by Blast analysis (Altschul et al. 1997), and in the case of nonoverlap, subjected to long-range PCR to fill the gaps between the fragments. Using this approach, the major part of this genome (41,666 bp) has been sequenced and reconstructed as a single contig. This part of the organellar genome contains an almost complete set of genes found in the mitochondrial genomes of other ciliates (table 1, fig. 1). In addition, we found a stretch larger than 11 kb that possesses seven open reading frames (*orfs*) with no significant sequence similarity to any known genes.

The organellar genome contains nine genes encoding elements of mitochondrial Complex I: *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad7*, *nad9*, and *nad10*. This set of genes is also found in other ciliates (table 1). The *nad1* gene is split in the mitochondrial genomes of all ciliates studied so far into a larger (*nad1a*) and a smaller (*nad1b*) part (de Graaf, van Alen, et al. 2009). In *N. ovalis*, we could identify the larger *nad1a* gene piece while the *nad1b* gene is likely encoded by *orf224* (fig. 1). The other Complex I genes have a similar size as in the *Tetrahymena* species and *P. aurelia*. This is in contrast to the situation in *E. minuta* and *E. crassus* where many Complex I genes have extended 5' ends (de Graaf, van Alen, et al. 2009). The *nad4* gene of *N. ovalis* is a bit shorter than in other ciliates, and it is very likely that *orf110* represents an additional part of the *nad4* gene (fig. 1).

Phylogenetic analysis of seven concatenated Complex I genes reveals that the Complex I of *N. ovalis* is closely related to that of *E. crassus* and *E. minuta* and that it clusters together with the Complex I genes of *Tetrahymena* and *Paramecium* (fig. 2), whereas it is—as expected—only distantly related to Complex I of *Blastocystis* and *Proteromonas*.

Organellar genes encoding components of Complexes III, IV, or V could not be identified. These genes are consistently present in the mitochondrial genomes of the aerobic ciliates *P. aurelia*, *Tetrahymena* spp., and *E. minuta* that have a size around 48 kb (table 1; de Graaf, van Alen, et al. 2009). Moreover, as a rule, all mitochondrial genomes sequenced to date host genes belonging to Complexes III, IV, and V (Burger et al. 2003). The loss of genes encoding Complexes III, IV, and V from the mitochondrial genome has been regarded as indicative of a loss from the species rather than a transfer to the nuclear genome in the mitochondrion-like organelles of *Blastocystis* (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). The absence of Complex III and IV genes in the organellar genome of *N. ovalis* is consistent with the observation that activity of Complexes III and IV could not be observed in inhibitor studies (Boxma et al. 2005).

A total of 15 unique *orfs* were identified, seven of which form a cluster with a total length greater than 11 kb with no significant sequence similarity to any known genes. No open reading frames with sequence similarity to *orfs* of *Tetrahymena* spp., *P. aurelia*, and *E. minuta* were identified. We detected three transfer RNA genes in the organellar genome (*trnF*, *trnY*, and *trnW*), similar to the mitochondrial genome of *P. aurelia* in which four transfer RNA (tRNA) genes were found (Pritchard et al. 1990). It is possible that additional tRNA genes are present in the missing part of the organellar genome. A large complex repeat region is present in the organellar genome between *orf479* and *ssu/rns* genes. This region contains 12 repeats of 34 nt followed by a (not perfectly) duplicated stretch of around 400 bp (supplementary fig. S2, Supplementary Material online). It is unlikely that this repeat region plays the same role in initiation of transcription as suggested for the mitochondrial genome of the *E. minuta* and *E. crassus* (de Graaf, van Alen, et al. 2009) because all genes found on the 41,666 bp part of the organellar genome of *N. ovalis* are oriented in the same direction, both before and after the repeat. In *E. minuta* and *E. crassus*, the repeat separates the open reading frames into two blocks, each having a single direction of transcription, from the repeat toward the ends of the chromosome.

We identified seven ribosomal proteins in the organellar genome of *N. ovalis*. This seems to be an average number for ciliates because nine ribosomal proteins were found in *Tetrahymena* spp., seven in *P. aurelia*, and six in *E. minuta* (de Graaf, van Alen, et al. 2009). Three of these ribosomal proteins were found in all four ciliate species with sequenced mitochondrial genomes: Rps12, Rpl2, and Rpl14. In contrast, the *rps8* gene is unique for *N. ovalis* (table 1). Notably, as in *E. minuta*, all organellar ribosomal protein genes of *N. ovalis* have an N-terminal extension that is similar to a mitochondrial targeting signal (not shown, cf. Ueda et al. 2008; de Graaf, van Alen, et al. 2009). The overall A-T content of the organellar genome of *N. ovalis* (58.5%) is identical to the A-T content of the mitochondrial genome of *P. aurelia*. The organellar genes are not tightly packed, and intergenic spacers represent 11.7% of the genome. In

Table 1. Various Genes Encoded by the Organellar Genomes of *Nyctotherus ovalis* (Nov), *Blastocystis* sp. (Blas), *Proteromonas lacertae* (Pro), *Euplotes minuta* (Emi), and *Plasmodium falciparum* (Pfa).

Gene	Nov	Blas	Pro	Emi	Pfa	Gene	Nov	Blas	Pro	Emi	Pfa
<i>nad1</i>	■	■	■	■	□	<i>rps12</i>	■	■	■	■	□
<i>nad2</i>	■	■	■	■	□	<i>rps13</i>	□	■	□	□	□
<i>nad3</i>	■	■	■	■	□	<i>rps14</i>	■	■	■	□	□
<i>nad4</i>	■	■	■	■	□	<i>rps19</i>	□	■	■	□	□
<i>nad4L</i>	■	■	■	■	□	<i>rpl2</i>	■	■	■	■	□
<i>nad5</i>	■	■	■	■	□	<i>rpl5</i>	□	■	■	□	□
<i>nad6</i>	□	■	■	□	□	<i>rpl6</i>	■	■	■	■	□
<i>nad7</i>	■	■	■	■	□	<i>rpl14</i>	■	■	■	■	□
<i>nad9</i>	■	■	■	■	□	<i>rpl16</i>	□	■	■	■	□
<i>nad10</i>	■	□	□	■	□	<i>trnA</i>	□	■	□	□	□
<i>nad11</i>	□	■	■	□	□	<i>trnC</i>	□	■	□	□	□
<i>rnl</i>	■	■	■	■	■	<i>trnD</i>	□	■	■	□	□
<i>rns</i>	■	■	■	■	■	<i>trnE</i>	□	■	■	■	□
<i>cob</i>	□	□	□	■	■	<i>trnF</i>	■	■	■	■	□
<i>cox1</i>	□	□	□	■	■	<i>trnH</i>	□	■	□	■	□
<i>cox2</i>	□	□	□	■	□	<i>trnI</i>	□	■	■	□	□
<i>cox3</i>	□	□	□	□	■	<i>trnK</i>	□	■	■	□	□
<i>atp9</i>	□	□	□	■	□	<i>trnL</i>	□	■	□	□	□
<i>ccmF/yejR</i>	□	□	□	■	□	<i>trnM</i>	□	■	■	■	□
<i>rps2</i>	□	□	■	□	□	<i>trnN</i>	□	■	■	□	□
<i>rps3</i>	□	■	□	■	□	<i>trnP</i>	□	■	■	□	□
<i>rps4</i>	□	■	■	□	□	<i>trnQ</i>	□	□	■	■	□
<i>rps7</i>	□	■	□	□	□	<i>trnS</i>	□	□	■	□	□
<i>rps8</i>	■	■	■	□	□	<i>trnV</i>	□	□	■	□	□
<i>rps10</i>	□	■	■	□	□	<i>trnW</i>	■	■	■	■	□
<i>rps11</i>	□	■	□	□	□	<i>trnY</i>	■	■	■	■	□

NOTE.—■: present in the organellar genome; □: absent in the organellar genome.

contrast, the genomes of the other ciliates contain no more than about 4% of intergenic spacers. The A-T content of these spacers is almost identical to the overall A-T content: 57.8%. Spacer lengths vary from 0 to 2,159 bp with an average size of 320 bp. In six cases, genes have an overlap, ranging from 3–61 bp. The 11 kb fragment that harbors the cluster of seven *orfs* with no homology to other known genes contains large open reading frames with small intergenic spacers ranging from –3 to 9 bp. These seven genes do not appear to be the remains of genes encoding components of Complex III, Complex IV, and Complex V. Neither Blast searches against mitochondrial encoded proteins nor profile-based searches against domain databases (PFAM, SMART) produced hits, even at an insignificant *E* value cutoff of 10. Because none of the proteins has a *pl* > 10, it is also unlikely that they are ribosomal proteins.

Thus, the sequenced fragment of the organellar genome of *N. ovalis* exhibits all characteristics of a ciliate mitochondrial genome, except for the absence of genes encoding components of the mitochondrial Complexes III, IV, and V.

Nuclear Encoded Organellar Proteins

As in other mitochondria, most of the organellar proteins in *N. ovalis* are encoded by the nuclear genome, synthesized in the cytoplasm, and imported into the organelle. The signal to direct these proteins into the hydrogen-producing mitochondrion resembles mitochondrial targeting signals (Boxma et al. 2005) and can be an indication that a certain protein is targeted to the organelle. Furthermore, because mitochondrial proteins tend to preserve their cellular location in evolution (Calvo et al. 2006; Szklarczyk

and Huynen 2009), the orthology of nuclear encoded mitochondrial proteins from species like *Homo sapiens*, *Saccharomyces cerevisiae*, and *T. thermophila* with *N. ovalis* proteins can be used to predict hydrogenosomal proteins. Here, we describe in more detail nuclear encoded proteins that are probably targeted to the hydrogen-producing mitochondrion of *N. ovalis*.

After our initial analysis (Boxma et al. 2005), we have obtained ~4,500 additional *N. ovalis* gDNA and cDNA sequences (Ricard et al. 2008). To identify new proteins involved in the hydrogenosomal metabolism, we first performed a Bidirectional BH analysis. Thousand nine hundred and fourteen nonredundant cDNAs and 2,841 nonredundant gDNAs from *N. ovalis* were compared with the mitochondrial proteomes of human, yeast, and rat. This allowed us to identify orthologs of mitochondrial proteins for which 161 phylogenetic trees were constructed with PhyML and subsequently analyzed by hand to determine orthology relations (not shown). This phylogenetic analysis allowed us to predict a number of new hydrogenosomal proteins as well as to provide stronger evidence for some that had been predicted previously (Boxma et al. 2005). From the phylogenetic trees, we observed that most of the *N. ovalis* sequences with close relatives among the mitochondrial proteins also cluster with their counterparts from the aerobic ciliates *T. thermophila* and *P. tetraurelia*. This indicates their recent descent from proteins functioning in aerobic mitochondria (data not shown). Furthermore, to compare the metabolic complexity of the *N. ovalis* hydrogen-producing mitochondrion with that of mitochondria, genome-less hydrogenosomes, and mitosomes, we analyzed

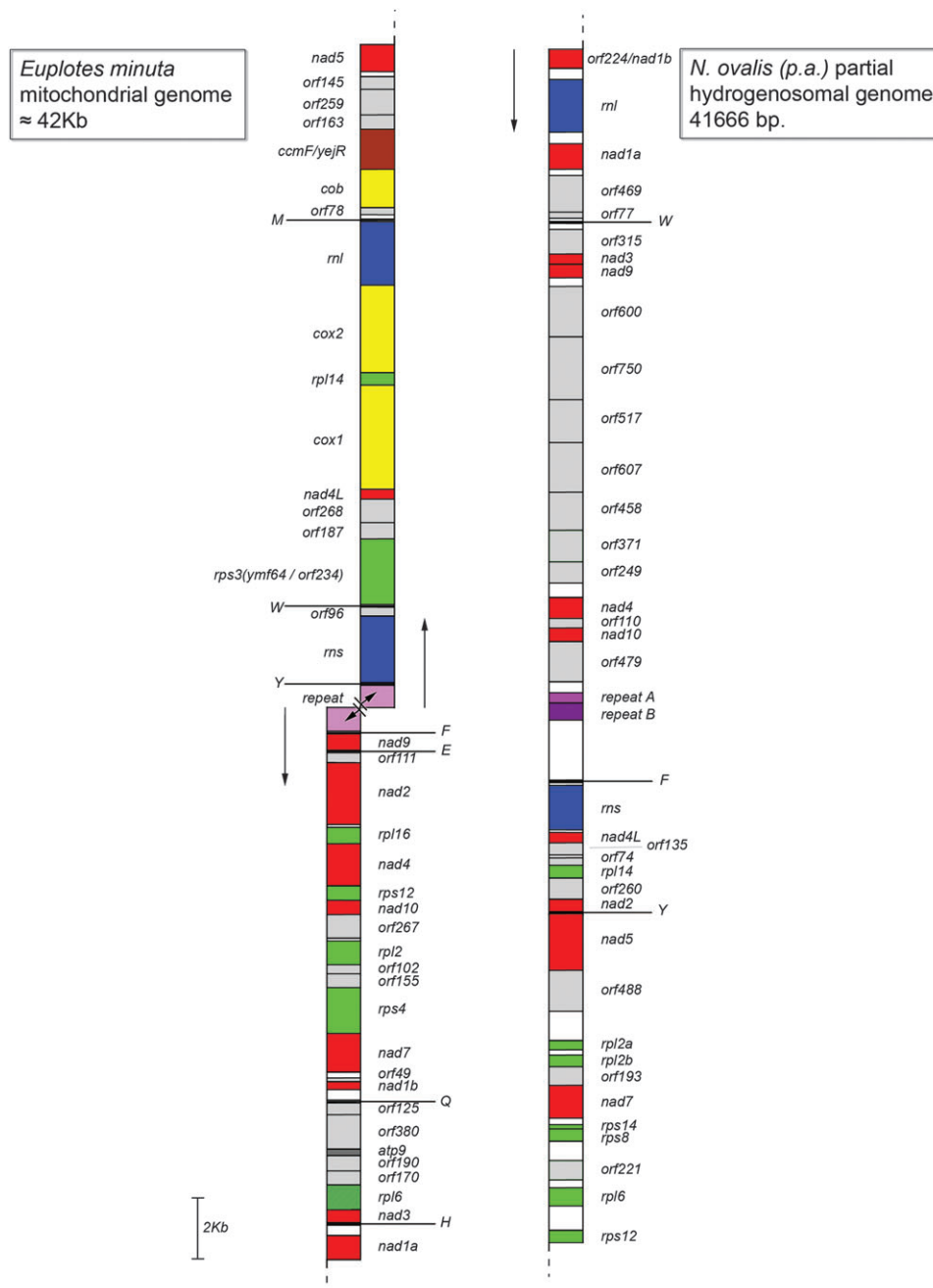


Fig. 1. Organellar gene maps of *Nyctotherus ovalis* and *Euplotes minuta*. Red: Complex I genes, blue: rRNA genes, green: ribosomal proteins, yellow: Complex III and IV genes, gray: unidentified open reading frames, pink: repeat region, dark gray: *atp9* gene, and white: intergenic spacers. Capital letters indicate the various tRNA genes. Arrows: direction of transcription.

the distribution of *N. ovalis* proteins with sequence similarity to organellar proteins in other species. These were the mitochondria-possessing species *Homo*, *Saccharomyces*, *Reclinomonas*, *Plasmodium*, *Tetrahymena*, and *Paramecium*; the species possessing a mitosome *Cryptosporidium*, *Encephalitozoon*, *Giardia*, and *Entamoeba*; and the species with a hydrogenosome *Piromyces*, *Trichomonas*, *Psalteriomonas*, and *Blastocystis* (supplementary table S1, Supplementary Material online). Here, we describe some of the proteins and their roles. Figure 3 shows how they might function together, shaping the organellar metabolism:

Pyruvate Dehydrogenase

The PDH complex converts pyruvate and CoA into acetyl-CoA while reducing NAD^+ to NADH. In addition to the three subunits, which have already been described (E1 α , E1 β , and E2), we found a fourth subunit, a dihydrolipoyl dehydrogenase, also known as subunit E3, thereby completing the complex. The presence of a complete PDH complex contrasts strongly with the pyruvate catabolizing enzymes in other hydrogenosomes. Although the genome analysis of *Blastocystis* revealed a PDH and a pyruvate:ferredoxin oxidoreductase (PFO) (Stechmann et al. 2008), Lantsman et al. (2008) measured pyruvate: NADP^+

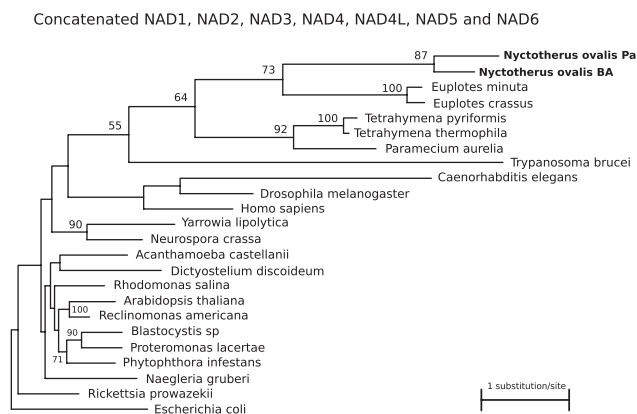


Fig. 2. ML phylogeny of the *Nyctotherus ovalis* hydrogen-producing mitochondrion, based on a concatenated alignment of seven mitochondrial Complex I encoded proteins (Nad1, Nad2, Nad3, Nad4, Nad4L, Nad5, and Nad6). Only bootstrap values of 50% and higher are indicated.

oxidoreductase activity. Thus, the situation in *Blastocystis* is unclear. In *Trichomonas* pyruvate is decarboxylated by PFO (Hrdý et al. 2007). In contrast, in anaerobic chytridiomycete fungi (*Piromyces* sp. E2 and *Neocallimastix* sp. L2), pyruvate is catabolized by pyruvate-formate lyase (PFL) and not by PFO (Akhmanova et al. 1999). Furthermore, a large cDNA library of *Piromyces* constructed by the Department of Energy (DOE) Joint Genome Institute does not contain a single PFO but many PFL sequences (data not shown). The acetyl-CoA generated in *N. ovalis* by PDH is further metabolized by an acetate:succinate CoA-transferase (ASCT) belonging to the subfamily 1A with sequence similarity to the ASCT from *Trypanosoma brucei* (Tielens et al. 2010). Finally, ATP is generated by the action of succinyl-CoA synthetase (SCS), an enzyme that is also part of the tricarboxylic acid (TCA) cycle, but this cycle is not operative in *N. ovalis* (fig. 3). Also in *Blastocystis* and *Trichomonas* acetyl-CoA is metabolized to acetate by the cyclic action of ASCT and SCS. However, *Blastocystis* contains genes for ASCTs of the subfamilies 1B and 1C, and *Trichomonas* contains a gene for an ASCT of the subfamily 1C that does not exhibit sequence similarity to the subfamily 1A ASCT of *N. ovalis* (supplementary table S1, Supplementary Material online; Stechmann et al. 2008; Tielens et al. 2010).

TCA Cycle

Based on experiments with ^{14}C labeled glucose (Boxma et al. 2005), the TCA cycle in *N. ovalis* is not complete, using only the malate–fumarate–succinate part in a reductive direction (fig. 3). Thus far, however, we have only found succinyl-CoA synthetase α and β subunits (the same enzyme as mentioned above) as well as the a and b subunit of succinate dehydrogenase (SDH)/fumarate reductase. These enzymes are also present in the mitochondrion-like organelle of *Blastocystis* in which succinyl-CoA synthetase, SDH, fumarate hydratase, and malate dehydrogenase have been identified (supplementary table S1, Supplementary Material online) (Stechmann et al. 2008; Wawrzyniak et al. 2008). These enzymes are universal in species with mitochondria. Note that

among the gDNA sequences of *N. ovalis*, we did encounter a malate dehydrogenase that is orthologous to cytosolic malate dehydrogenases from other species and was therefore considered to be also cytoplasmic in *N. ovalis* as well.

Electron Transport Chain

Complex I (NADH-quinone oxidoreductase) consists of 14 subunits in eubacteria and of 33–45 subunits in mitochondria (Friedrich and Böttcher 2004; Gabaldon et al. 2005). We found 12 subunits of Complex I in *N. ovalis* (Nad1, Nad2, Nad3, Nad4, Nad4L, Nad5, Nad7, Nad9, Nad10, 24kDa*, 51kDa*, and 75kDa*), among them three that are encoded by the nuclear genome (marked by an asterisk); the others are encoded by the hydrogenosomal genome (fig. 1). All the *N. ovalis* Complex I proteins are part of the 14 proteins that compose the core bacterial Complex I. Also in *Blastocystis*, ten Complex I genes have been identified that are located on the organelle genome, and an additional six that are nuclear encoded (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). In *Proteromonas*, ten Complex I proteins are encoded by the organelle genome (Perez-Brocal et al. 2010).

Of complex II, we detected SDHa and SDHb. In aerobic mitochondria, the four subunits of Complex II catalyze the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol. In *N. ovalis*, SDHa and SDHb have been proposed to function as fumarate reductase, reversing the reaction, and allowing the oxidation of quinols (rhodoquinol) (Boxma et al. 2005). In *Blastocystis*, all four components of Complex II have been identified (Stechmann et al. 2008).

Previous biochemical analyses have failed to detect the presence of Complex III/IV activity in the *N. ovalis* hydrogen-producing mitochondrion (Boxma et al. 2005), and accordingly, the analysis of its genome did not provide any evidence for the presence of Complex III/IV genes (fig. 1). Surprisingly, however, we did identify a nuclear-encoded homolog of cytochrome c1, an electron transporter related to Complexes III and IV. We sequenced the complete gene-sized-piece as well as the complete cDNA, confirming that the cytochrome c protein is transcribed. However, the cDNA appears to contain a stop codon (TGA), albeit one that is very rarely used. Therefore, with our current knowledge, we cannot exclude (Ricard et al. 2008) that the cDNA is translated notwithstanding the presence of a potential stop codon. Accordingly, an alignment of the cytochrome c sequence with homologs from various species reveals that the sequence is highly conserved but that the cysteines that normally hold the heme in cytochrome c1 are not present in the *N. ovalis* sequence, probably rendering the protein nonfunctional in electron transfer (supplementary fig. S3, Supplementary Material online). Therefore, if the gene is translated, it should exhibit an alternative function. This is the first report of a cytochrome c1 that has lost the two crucial cysteines as well as the histidine directly following the second cysteine. Only some *Trypanosoma*, *Leishmania*, and *Euglena* species miss the first cysteine, whereas the second cysteine and the histidine are universally conserved among species (Priest and Hajduk 1992).

dehydrogenases, for example, dehydrogenases that oxidize amino acids. It transfers electrons to the electron transport chain via an ETF-ubiquinone oxidoreductase. The presence of ETF underscores the importance of the *N. ovalis* electron transport chain in its mitochondrial catabolism.

With the notable exception of *Blastocystis* and *Proteromonas*, complete functional Complexes I and II appear to be absent from hydrogenosomal species, such as *Trichomonas*, which has only retained two subunits of Complex I (the 24 and the 51 kD subunits) (Hrdý et al. 2004; Carlton et al. 2007; Stechmann et al. 2008). The electron transport chain is also absent from all mitosomal species. All these species lack a mitochondrial genome, whereas *N. ovalis*, *Blastocystis*, and *Proteromonas* possess elements of the electron transport chain as well as a mitochondrial genome. This supports the conclusion that all mitochondrial genomes carry genes encoding proteins of the electron transport chain (Burger et al. 2003), and likewise, that all organisms with an electron transport chain have a mitochondrial genome.

Propionate Metabolism

The predicted mitochondrial proteome contains a propionyl CoA carboxylase (EC: 6.4.1.3), that catalyzes the carboxylation reaction of propionyl CoA to D-methylmalonyl CoA, and a methylmalonyl CoA mutase (also found in *Blastocystis*, Stechmann et al. 2008) that converts methylmalonyl CoA to succinyl CoA. Both propionyl CoA carboxylase and methylmalonyl CoA mutase are absent from *Paramecium* and *Tetrahymena* (supplementary table S1, Supplementary Material online), indicating that the hydrogen-producing mitochondrion of *N. ovalis* does not just possess a subset of proteins present in aerobic mitochondria.

Fatty Acid Metabolism

In the fatty acid activation pathway, we detected a long-chain-fatty acid-CoA ligase, which catalyzes the reversible reaction:

$$\text{ATP} + \text{a long-chain carboxylic acid} + \text{CoA} \rightleftharpoons \text{AMP} + \text{diphosphate} + \text{acyl-CoA}.$$

This enzyme is present in all the 11 organisms in supplementary table S1, Supplementary Material online for which complete genomes are available, regardless of whether these organisms possess mitochondria, hydrogenosomes, or mitosomes. In contrast, the glycerol kinase is only present in the seven organisms that possess mitochondria or hydrogenosomes. Organisms with mitosomes lack glycerol kinase, with the exception of *G. lamblia* (supplementary table S1, Supplementary Material online).

Amino Acid Metabolism

Besides the glycine cleavage system (GCS, see below), we found several other enzymes involved in the metabolism of amino acids and specifically in the degradation of valine, methionine, and isoleucine. These were a branched-chain aminotransferase (EC: 2.6.1.42), the 2-oxoisovalerate dehydrogenase α and β (EC: 1.2.4.4) subunits, and

a branched-chain α -keto acid dihydrolipoyl acyltransferase (EC: 2.3.1.-). The organelle of *Blastocystis* also contains enzymes involved in valine, leucine, and isoleucine metabolism (Stechmann et al. 2008). In addition, we identified a glutamate dehydrogenase and a cystathionine β -synthase. In general, amino acid metabolism appears much reduced in mitosomes and genome-less hydrogenosomes relative to mitochondria (supplementary table S1, Supplementary Material online).

GCS

The dihydrolipoyl dehydrogenase of PDH is also involved in the GCS that catalyzes the reversible reaction:

$$\text{glycine} + \text{tetrahydrofolate} + \text{NAD}^+ \rightleftharpoons \text{5,10-methylene-tetrahydrofolate} + \text{NH}_3 + \text{CO}_2 + \text{NADH} + \text{H}^+$$

It provides the 5,10-methylene-tetrahydrofolate that is required for nucleotide synthesis. Of the GCS, we detected: the H-protein, a lipoic acid containing protein that carries the aminomethyl moiety of glycine that is bound to the sulfhydryl group by an S-C bond; the T-protein, which degrades reversibly the aminomethyl moiety attached to the H-protein to methylene-tetrahydrofolate and ammonia, using tetrahydrofolate in the process; and the L-protein, a dihydrolipoamide dehydrogenase that catalyzes the oxidation of the dihydrolipoyl group of the H-protein. We did not detect the substrate determining pyridoxal phosphate-containing protein (P-protein) of this complex, casting some doubt on whether the system actually uses Glycine. Nevertheless, we did find other components linked to this pathway: a cystathionine β -synthase, a serine hydroxymethyl transferase that converts serine to glycine, and a 5,10-methenyltetrahydrofolate synthetase. Interestingly, two proteins of the GCS have also been identified in the hydrogenosome of *Trichomonas* (L- and H-proteins) (Carlton et al. 2007), whereas the L, T, and H proteins also appear to be present in the genome of the minimal mitochondria-containing *Plasmodium falciparum* (supplementary table S1, Supplementary Material online). The mitochondrion-like organelle of *Blastocystis* contains all four components of the GCS, including the P-protein (Stechmann et al. 2008). The GCS is completely absent from mitosome-harboring species, such as *Encephalitozoon cuniculi*, *E. histolytica*, *G. lamblia*, and *C. parvum*.

Fe/S Cluster Synthesis

The only characteristic known so far that is shared by all mitochondria, mitosomes, and hydrogenosomes is the synthesis of Fe/S clusters (Lill and Mühlhoff 2005; Tachezy and Dolezal 2007). Unfortunately, we found only a mitochondrial type I [2Fe2S] ferredoxin, which might provide reducing equivalents for Fe/S cluster synthesis as well as for other processes. Furthermore, we found the mitochondrial Hsp70, which has a role in Fe/S assembly and in other processes, such as protein import and folding. Although we have no reason to doubt their presence, we did not detect genes specific for Fe/S cluster assembly like *Isu1/2*, *Nfs1*, *Isd11*, *Isa1/2*, or *frataxin* and therefore have no specific evidence for canonical mitochondrial Fe/S cluster synthesis in *N. ovalis*.

Mitochondrial Protein Synthesis and Turnover

We found eight mitochondrial ribosomal proteins in *N. ovalis*: Rps 8, Rps 12, Rps 14; Rpl 2, Rpl 6, Rpl 14, Rpl 11, Rpl 15, and Rpl 20; the latter three are nuclear encoded. In agreement with Smits and coworkers (Smits et al. 2007), we did not find these mitochondrial ribosomal proteins in species lacking a mitochondrial genome, such as *G. lamblia*, *E. cuniculi*, *Ent. histolytica*, *C. parvum*, or *T. vaginalis*. Notably, 16 ribosomal proteins are encoded in the genome of the mitochondrion-like organelle of *Blastocystis* (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). In *N. ovalis*, we found, besides the ribosomal proteins, FtsJ (MRM2), a well-conserved heat shock protein that is responsible for methylating 23S (*rnl*) rRNA.

Mitochondrial Carriers

We detected three mitochondrial carrier proteins: one orthologous to the 2-oxoglutarate/malate carrier that imports malate into the organelle and exports oxoglutarate out of the mitochondrial matrix, one orthologous to an ADP/ATP carrier, and one carrier that does not specifically cluster with any carrier of known specificity (PET8). The oxoglutarate/malate carrier (Coll et al. 2003) also transports other dicarboxylates and tricarboxylates (such as oxaloacetate, malate, malonate, succinate, citrate, isocitrate, *cis*-aconitate, and *trans*-aconitate). This carrier may also play an important role in several metabolic functions requiring organic acid flux to or from the mitochondria, such as nitrogen assimilation, transport of reducing equivalents into the mitochondria, and fatty acid elongation (Picault et al. 2002). *Nyctotherus ovalis* may use this carrier to import malate into the hydrogen-producing mitochondrion, which is transformed into fumarate and used by Complex II enzymes. In the sequenced genomes in [supplementary table S1, Supplementary Material](#) online, the oxoglutarate/malate carrier is present in several mitochondria-bearing species but absent from yeast, species with mitosomes, and species with hydrogenosomes without an organellar genome ([supplementary table S1, Supplementary Material](#) online). The oxoglutarate/malate carrier and a putative ADP/ATP carrier are also found in *Blastocystis* (Stechmann et al. 2008).

Additional mitochondrial proteins that are lacking in *Trichomonas*, and all species with mitosomes are MPV17 and ACN9, which have homologues in human and yeast mitochondria (not shown in [fig. 3](#)). They are, however, present in *Blastocystis*. Consistent with its presence in *N. ovalis*, MPV17 has been associated with mitochondrial genome stability (Spinazzola et al. 2006), while ACN9 has been associated with respiration (Steinmetz et al. 2002).

Protein Import and Processing

We detected the mitochondrial import machinery components TOM 34, MAS 5, MPP, FtsH, peptidyl-prolyl *cis-trans* isomerase, and protein disulfide isomerase. Hsps 10, 60, 70, and 90 were also identified.

Mitochondrial Division

One gene, a dynamin-related protein, has been found that might be engaged in mitochondrial division because it is an

ortholog of the plant dynamin ADL2 that is known to be involved in mitochondrial division (Arimura et al. 2004).

ROS Defense

To our surprise, we found three systems used to cope with the detrimental effects of oxygen radicals. First of all, we found thioredoxins that act as antioxidants by facilitating the reduction of cysteines in other, oxidized proteins by thiol-disulfide exchange. Second, of the glutathione system, we found a glutathione reductase (EC 1.8.1.7) that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH. Finally, we found a peroxiredoxin.

The presence of enzymes supposed to react against oxygen radicals in an anaerobic environment might seem strange, but Reactive oxygen species (ROS) can be produced endogenously. Complex I is a major source of ROS, although in mitochondria Complex III seems to be the principal site of $O_2^{\bullet-}$ (superoxide) formation (Chen et al. 2003).

HGT

To detect HGT, we followed the same procedure as previously described (Ricard et al. 2006). After clustering the sequences to remove redundancy, we determined the BH of 1,914 cDNAs and 2,841 gDNAs against a set of 165 complete proteomes. We identified 31 cDNAs and 25 gDNAs with a bacterium as BH and 5 cDNAs and 6 gDNAs with an archaeon as BH. We analyzed the 67 *N. ovalis* sequences that have a bacterium or an archaeon as BH further, by reconstructing the evolution of the proteins in a phylogenetic tree generated with PhyML. When requiring that the *N. ovalis* sequence clustered with a high bootstrap value with only noneukaryotic sequences, four genes could be concluded to likely have been acquired through HGT ([table 2; supplementary fig. S4a–d, Supplementary Material](#) online). These genes are an acetyl-CoA synthase (AMP-forming), a β lactamase, a glycosylhydrolase, and an ornithine carbamoyltransferase. None of the proteins encoded by these genes has however an N-terminal targeting signal. In contrast, for the [FeFe] hydrogenase evidence for HGT and organellar location have been documented before (Boxma et al. 2007). This [FeFe] hydrogenase is remarkable because it is fused with the 24 and 51 kD subunits of a bacterial Complex I. This allows the use of NADH as electron donor. That we found only one gene obtained by HGT (the hydrogenase) that unequivocally encodes an enzyme located in the hydrogen-producing mitochondrion, suggests that the role of HGT in the transformation to hydrogen-producing mitochondrion is limited.

Conclusions

With the bioinformatic analysis of the organellar genome and of the gDNAs and cDNAs, we are beginning to get a comprehensive view of the metabolism of the hydrogen-producing mitochondrion of *N. ovalis*. First of all, the organelle genome is a typical ciliate mitochondrial genome, with the exception of the notable lack of components of Complexes III, IV, and V of the electron transport chain. The latter feature is shared with the genome of the

Table 2. *Nyctotherus ovalis* Genes That Likely Have Been Acquired by HGT From Bacteria/Archaea.

HGT (genus BH, id BH, sequence origin)	Accession Number	E.C. No	Hsa	Sce	Tth	Pte	Pfa	Cpa	Tva	Bla	Pla	Psp	Ehi	Gla	Ecu
Acetyl-CoA synthetase (<i>Archaeoglobus</i> , YP_003399995, gDNA incl telomeres)	AJ871315 AM890088	6.2.1.1	Y	Y	Y	Y	Y	Y	Y	Y			N	N	Y
β lactamase (<i>Pseudoalteromonas</i> , YP_339054.1, gDNA incl telomere)	AM894317 AM891292	3.5.2.6	Y	N	Y	Y	N	Y	Y	N			N	N	N
Glycosylhydrolase (glycosidase) (<i>Geobacillus</i> , YP_003244808.1, gDNA)	AM890556	3.2.1.-	N	N	N	N	N	N	N	N			N	N	N
Ornithine carbamoyltransferase (<i>Aeromonas</i> , YP_858515.1, cDNA incl. polyA)	AM896448	2.1.3.3	Y	Y	N	N	Y	N	Y	Y			N	Y	N
[FeFe] hydrogenase (<i>Spirochaeta</i> , YP_003873858, gDNA, telomeres)	AY608627	1.12.7.2	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	N

NOTE.—The proteins associated with the genes were selected based on having a BH with Bacteria or Archaea, followed by a phylogenetic analysis using the 250 BHs in the complete nonredundant database supplemented with the ten BHs in Eukaryotes and requiring a highly supported clustering of the *N. ovalis* gene with noneukaryotic genes, see Materials and Methods for details. With each HGT candidate are indicated the genus name of the species with the BH, the *National Center for Biotechnology Information*-identifier of the best hit, the source of the genetic material for the *N. ovalis* gene (cDNA or gDNA), and the extra evidence that the sequence is indeed derived from *N. ovalis* (the presence of telomeres specific for *N. ovalis* genes or of a poly A tail for a cDNA). Y indicates the presence of an ortholog of the mitochondrial protein in the corresponding species, N its absence. A blank cell is inserted if no answer is possible due to the incomplete genome. Species with mitochondria: (Hsa) *Homo sapiens*, (Sce) *Saccharomyces cerevisiae*, (Tth) *Tetrahymena thermophila*, (Pte) *Paramecium tetraurelia*, (Ram) *Reclinomonas americana*, and (Pfa) *Plasmodium falciparum*; species with hydrogenosomes: (Tva) *Trichomonas vaginalis*, (Bla) *Blastocystis*, (Pla) *Psalteriomonas lanterna*, and (Psp) *Piromyces* sp.; and species with a mitosome: (Cpa) *Cryptosporidium parvum*, (Ehi) *Entamoeba histolytica*, (Gla) *Giardia lamblia*, and (Ecu) *Encephalitozoon cuniculi*.

unrelated mitochondrion-like organelles of *Blastocystis* and *Proteromonas*. This phenomenon is a striking example of convergent evolution. Consequently, given the situation with *Nyctotherus*, *Blastocystis*, and *Proteromonas*, we can no longer use the production of ATP via oxidative phosphorylation as a defining feature of mitochondria, rather what sets them apart from mitosomes and hydrogenosomes is the proton-pumping electron transport chain of their mitochondrion-like organelles.

Moreover, the organelle of *N. ovalis* appears to host, besides an incomplete TCA cycle, other “classical” mitochondrial pathways, such as amino acid metabolism and fatty acid metabolism. A number of these proteins are typical for mitochondria and are not present in species with mitosomes or hydrogenosomes without a genome. These include, of course, proteins of the electron transport chain and of the mitochondrial ribosome but also enzymes, such as succinyl-CoA synthetase (however present in *Trichomonas*), pyruvate dehydrogenase, malate/ α ketoglutarate antiporter, and 5,10-methenyltetrahydrofolate synthetase (see also Ginger et al. 2010). What emerges is a metabolism that is in some aspects (Complex III, Complex IV, and Complex V) reduced relative to the metabolism of aerobic ciliate mitochondria and that is strikingly similar to the metabolism of the mitochondrion-like organelle of *Blastocystis*. However, *N. ovalis* also possesses some extra proteins relative to aerobic ciliates. A few of these appear to have been acquired by HGT. The case of the [FeFe] hydrogenase is well documented (Boxma et al. 2007), but our analysis has also unearthed a few others, like an acetyl-CoA synthetase (AMP-forming). Nevertheless, their role in the adaptation to the anaerobic ecological niche of *N. ovalis* is not obvious. Not all *N. ovalis* proteins that we found and that are absent from the aerobic ciliates have been gained by HGT from bacteria. We also observe a pathway for propionate metabolism that contains a propionyl CoA carboxylase and a methylmalonyl CoA mutase. This pathway has not yet been observed in ciliates, and besides its presence in metazoa, has a patchy distribution among the eukaryotes, suggesting multiple loss events rather than HGT. Another example is a hybrid

cluster protein (prismane) that is absent from the sequenced aerobic ciliates and that among the eukaryotes is mainly present in (facultatively) anaerobic species. However, the *N. ovalis* sequence does not specifically cluster with bacterial sequences (data not shown).

By definition, the organelle of *N. ovalis* is a hydrogenosome because it generates hydrogen with the aid of an [FeFe] hydrogenase (Lindmark and Müller 1973). We have shown here that this organelle is a mitochondrion that produces hydrogen, representing an intermediate state between mitochondria and genome-less hydrogenosomes. This allows us to outline a hypothetical scheme for the evolution of hydrogenosomes. A possible scenario for eukaryotes with a textbook mitochondrion genome and metabolism to evolve into an organism with hydrogenosomes could be as follows (where the order of events could be different):

1. Evolution of a reverse action of the TCA cycle (fumarate respiration) as shown in a number of anaerobic mitochondria (Tielens et al. 2002; Tielens and van Hellemond 2007).
2. Recruitment of a hydrogenase like that shown in *Naegleria gruberi* (Fritz-Laylin et al. 2010) and *N. ovalis*.
3. Loss of Complexes III, IV, and V of the electron transport chain as shown in *N. ovalis*, *Blastocystis*, and *Proteromonas*.
4. Loss of the remaining part of the organellar genome as shown, for example, in *Trichomonas*, *Psalteriomonas*, and other organisms with hydrogenosomes.

It is clear that hydrogenosomes, hydrogen-producing mitochondria, and mitochondrion-like organelles evolved several times from different aerobic progenitors. The eukaryotic cell is clearly characterized by the presence of a mitochondrion that has the capacity to adapt to anaerobic environments by reductive evolution to yield hydrogenosomes (and mitosomes).

Supplementary Material

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

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References

- Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21:2104–2105.
- Aguilera P, Barry T, Tovar J. 2008. Entamoeba histolytica mitochondria: organelles in search of a function. *Exp Parasitol* 118:10–16.
- Akhmanova A, Voncken FG, Harhangi H, Hosea KM, Vogels GD, Hackstein JH. 1998. Cytosolic enzymes with a mitochondrial ancestry from the anaerobic chytrid *Piromyces* sp. E2. *Mol Microbiol* 30:1017–1027.
- Akhmanova A, Voncken FG, Hosea KM, Harhangi H, Keltjens JT, op den Camp HJ, Vogels GD, Hackstein JH. 1999. A hydrogenosome with pyruvate formate-lyase: anaerobic chytrid fungi use an alternative route for pyruvate catabolism. *Mol Microbiol* 32:1103–1114.
- Akhmanova A, Voncken F, van Alen T, van Hoek A, Boxma B, Vogels G, Veenhuis M, Hackstein JH. 1998. A hydrogenosome with a genome. *Nature* 396:527–528.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
- Andreoli C, Prokisch H, Hortnagel K, Mueller JC, Munsterkotter M, Scharfe C, Meitinger T. 2004. MitoP2, an integrated database on mitochondrial proteins in yeast and man. *Nucleic Acids Res* 32:D459–D462.
- Arimura S, Aida GP, Fujimoto M, Nakazono M, Tsutsumi N. 2004. Arabidopsis dynamin-like protein 2a (ADL2a), like ADL2b, is involved in plant mitochondrial division. *Plant Cell Physiol* 45:236–242.
- Barbera MJ, Ruiz-Trillo I, Leigh J, Hug LA, Roger AJ. 2007. The diversity of mitochondrion-related organelles amongst eukaryotic microbes. In: Martin WF, Müller M, editors. *Origin of mitochondria and hydrogenosomes*. Berlin, Heidelberg (Germany): Springer-Verlag. p. 239–275.
- Boxma B, de Graaf RM, van der Staay GW, et al. (15 co-authors). 2005. An anaerobic mitochondrion that produces hydrogen. *Nature* 434:74–79.
- Boxma B, Ricard G, van Hoek AH, et al. (17 co-authors). 2007. The [FeFe] hydrogenase of *Nyctotherus ovalis* has a chimeric origin. *BMC Evol Biol* 7:230.
- Burger G, Gray MW, Lang BF. 2003. Mitochondrial genomes: anything goes. *Trends Genet* 19:709–716.
- Calvo S, Jain M, Xie X, Sheth SA, Chang B, Goldberger OA, Spinazzola A, Zeviani M, Carr SA, Mootha VK. 2006. Systematic identification of human mitochondrial disease genes through integrative genomics. *Nat Genet* 38:576–582.
- Carlton JM, Hirt RP, Silva JC, et al. (65 co-authors). 2007. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. *Science* 315:207–212.
- Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. 2003. Production of reactive oxygen species by mitochondria—central role of complex III. *J Biol Chem* 278:36027–36031.
- Cherry JM, Adler C, Ball C, et al. (12 co-authors). 1998. SGD: *Saccharomyces* Genome Database. *Nucleic Acids Res* 26:73–79.
- Coll O, Colell A, Garcia-Ruiz C, Kaplowitz N, Fernandez-Checa JC. 2003. Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion. *Hepatology* 38:692–702.
- de Graaf RM, Duarte I, Van Alen TA, Kuiper JW, Schotanus K, Rosenberg J, Huynen MA, Hackstein JHP. 2009. The hydrogenosomes of *Psalteriomonas lanterna*. *BMC Evol Biol* 9:287.
- de Graaf RM, van Alen TA, Dutilh BE, Kuiper JW, van Zoggel HJ, Huynh MB, Görtz HD, Huynen MA, Hackstein JH. 2009. The mitochondrial genomes of the ciliates *Euplotes minuta* and *Euplotes crassus*. *BMC Genomics* 10:514.
- Duchen MR. 2004. Roles of mitochondria in health and disease. *Diabetes* 53(Suppl 1):S96–S102.
- Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797.
- Embley TM, Martin W. 2006. Eukaryotic evolution, changes and challenges. *Nature* 440:623–630.
- Embley TM, van der Giezen M, Horner DS, Dyal PL, Bell S, Foster PG. 2003. Hydrogenosomes, mitochondria and early eukaryotic evolution. *IUBMB Life* 55:387–395.
- Förner F, Foster LJ, Campanaro S, Valle G, Mann M. 2006. Quantitative proteomic comparison of rat mitochondria from muscle, heart, and liver. *Mol Cell Proteomics* 5:608–619.
- Friedrich T, Böttcher B. 2004. The gross structure of the respiratory complex I: a Lego System. *Biochim Biophys Acta* 1608:1–9.
- Fritz-Laylin LK, Prochnik SE, Ginger ML, et al. (24 co-authors). 2010. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* 140:631–642.
- Gabaldón T, Huynen MA. 2004. Shaping the mitochondrial proteome. *Biochim Biophys Acta* 1659:212–220.
- Gabaldon T, Rainey D, Huynen MA. 2005. Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (Complex I). *J Mol Biol* 348:857–870.
- Ginger ML, Fritz-Laylin LK, Fulton C, Cande WZ, Dawson SC. 2010. Intermediary metabolism in protists: a sequence-based view of facultative anaerobic metabolism in evolutionarily diverse eukaryotes. *Protist* 161:642–671.
- Goldberg AV, Molik S, Tsaousis AD, Neumann K, Kuhnke G, Delbac F, Vivares CP, Hirt RP, Lill R, Embley TM. 2008. Localization and functionality of microsporidian iron-sulphur cluster assembly proteins. *Nature* 452:624–628.
- Gray MW. 2005. Evolutionary biology: the hydrogenosome’s murky past. *Nature* 434:29–31.
- Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704.
- Hackstein JHP, Akhmanova A, Voncken FGJ, et al. (12 co-authors). 2001. Hydrogenosomes: convergent adaptations of mitochondria to anaerobic environments. *Zoology* 104:290–302.
- Hackstein JHP, Baker SE, van Hellemond JJ, Tielens AG. 2008. Hydrogenosomes of anaerobic chytrids: an alternative way to adapt to anaerobic environments. In: Tachezy J, editor. *Hydrogenosomes and mitochondria of anaerobic eukaryotes*. Berlin, Heidelberg (Germany): Springer-Verlag. p. 147–162.
- Hackstein JHP, de Graaf RM, van Hellemond JJ, Tielens AG. 2008. Hydrogenosomes of anaerobic ciliates. In: Tachezy J, editor. *Hydrogenosomes and mitochondria of anaerobic*

- eukaryotes. Berlin, Heidelberg (Germany): Springer-Verlag. p. 97–112.
- Henze K, Martin W. 2003. Evolutionary biology: essence of mitochondria. *Nature* 426:172–176.
- Hjort K, Goldberg AV, Tsaousis AD, Hirt RP, Embley TM. 2010. Diversity and reductive evolution of mitochondria among microbial eukaryotes. *Philos Trans R Soc Lond B Biol Sci*. 365:713–727.
- Hrdý I, Hirt RP, Dolezal P, Bardonova L, Foster PG, Tachezy J, Embley TM. 2004. Trichomonas hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. *Nature* 432:618–622.
- Hrdý I, Tachezy J, Müller M. 2007. Metabolism of trichomonad hydrogenosomes. In: Tachezy J, editor. Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Berlin, Heidelberg (Germany): Springer-Verlag. p. 113–145.
- Lang BF, Gray MW, Burger G. 1999. Mitochondrial genome evolution and the origin of eukaryotes. *Annu Rev Genet*. 33:351–397.
- Lantsman Y, Tan KS, Morada M, Yarlett N. 2008. Biochemical characterization of a mitochondrial-like organelle from Blastocystis sp. subtype 7. *Microbiology* 154:2757–2766.
- Lill R, Kispal G. 2000. Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem Sci*. 25:352–356.
- Lill R, Mühlhoff U. 2005. Iron-sulfur-protein biogenesis in eukaryotes. *Trends Biochem Sci*. 30:133–141.
- Lindmark DG, Müller M. 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Trichomonas foetus*, and its role in pyruvate metabolism. *J Biol Chem*. 248:7724–7728.
- Maralikova B, Ali V, Nakada-Tsukui K, Nozaki T, van der Giezen M, Henze K, Tovar J. 2010. Bacterial-type oxygen detoxification and iron-sulfur cluster assembly in amoebal relict mitochondria. *Cell Microbiol*. 12:331–342.
- 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 U S A*. 106:21731–21736.
- Miller WL. 1995. Mitochondrial specificity of the early steps in steroidogenesis. *J Steroid Biochem Mol Biol*. 55:607–616.
- Perez-Brocal V, Clark CG. 2008. Analysis of two genomes from the mitochondrion-like organelle of the intestinal parasite Blastocystis: complete sequences, gene content, and genome organization. *Mol Biol Evol*. 25:2475–2482.
- Perez-Brocal V, Shahar-Golan R, Clark CG. 2010. A linear molecule with two large inverted repeats: the mitochondrial genome of the stramenopile *Proteromonas lacertae*. *Genome Biol Evol*. 2:257–266.
- Picault N, Palmieri L, Pisano I, Hodges M, Palmieri F. 2002. Identification of a novel transporter for dicarboxylates and tricarboxylates in plant mitochondria. Bacterial expression, reconstitution, functional characterization, and tissue distribution. *J Biol Chem*. 277:24204–24211.
- Priest JW, Hajduk SL. 1992. Cytochrome c reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c1. *J Biol Chem*. 267:20188–20195.
- Pritchard AE, Seilhamer JJ, Mahalingam R, Sable CL, Venuti SE, Cummings DJ. 1990. Nucleotide sequence of the mitochondrial genome of *Paramecium*. *Nucleic Acids Res*. 18:173–180.
- Ricard G, de Graaf RM, Dutilh BE, et al. (13 co-authors). 2008. Macronuclear genome structure of the ciliate *Nyctotherus ovalis*: single-gene chromosomes and tiny introns. *BMC Genomics*. 9:587.
- Ricard G, McEwan NR, Dutilh BE, et al. (17 co-authors). 2006. Horizontal gene transfer from Bacteria to rumen Ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. *BMC Genomics*. 7:22.
- Scheffer IE. 2001. Mitochondria make a come back. *Adv Drug Deliv Rev*. 49:3–26.
- Sickmann A, Reinders J, Wagner Y, et al. (13 co-authors). 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci U S A*. 100:13207–13212.
- Smith DG, Gawryluk RM, Spencer DF, Pearlman RE, Siu KW, Gray MW. 2007. Exploring the mitochondrial proteome of the ciliate protozoon *Tetrahymena thermophila*: direct analysis by tandem mass spectrometry. *J Mol Biol*. 374:837–863.
- Smits P, Smeitink JAM, van den Heuvel LP, Huynen MA, Ettema TJG. 2007. Reconstructing the evolution of the mitochondrial ribosomal proteome. *Nucleic Acids Res*. 35:4686–4703.
- Spinazzola A, Viscomi C, Fernandez-Vizarrá E, et al. (20 co-authors). 2006. MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. *Nat Genet*. 38:570–575.
- 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.
- Steinmetz LM, Scharfe C, Deutschbauer AM, et al. (11 co-authors). 2002. Systematic screen for human disease genes in yeast. *Nat Genet*. 31:400–404.
- Szklarczyk R, Huynen MA. 2009. Expansion of the human mitochondrial proteome by intra- and inter-compartmental protein duplication. *Genome Biol*. 10:R135.
- 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, Heidelberg (Germany): Springer Verlag. p. 105–133.
- Tachezy J, Smid O. 2007. Mitosomes in parasitic protists. In: Tachezy J, editor. Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Berlin, Heidelberg (Germany): Springer Verlag. p. 201–230.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22:4673–4680.
- Tielens AG, Rotte C, van Hellemond JJ, Martin W. 2002. Mitochondria as we don't know them. *Trends Biochem Sci*. 27:564–572.
- Tielens AG, van Grinsven KW, Henze K, van Hellemond JJ, Martin W. 2010. Acetate formation in the energy metabolism of parasitic helminths and protists. *Int J Parasitol*. 40:387–397.
- Tielens AGM, van Hellemond JJ. 2007. Anaerobic mitochondria: properties and origin. In: Martin WF, Müller M, editors. Origin of mitochondria and hydrogenosomes. Berlin, Heidelberg (Germany): Springer Verlag. p. 84–101.
- Ueda M, Fujimoto M, Arimura S, Tsutsumi N, Kadowaki K. 2008. Presence of a latent mitochondrial targeting signal in gene on mitochondrial genome. *Mol Biol Evol*. 25:1791–1793.
- Wawrzyniak I, Roussel M, Diogon M, Couloux A, Texier C, Tan KS, Vivares CP, Delbac F, Wincker P, El Alaoui H. 2008. Complete circular DNA in the mitochondria-like organelles of Blastocystis hominis. *Int J Parasitol*. 38:1377–1382.
- Yarlett N. 2004. Anaerobic protists and hidden mitochondria. *Microbiology* 150:1127–1129.