

# A Nonmitochondrial Hydrogen Production in *Naegleria gruberi*

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## 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.

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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 (uracil) promoter, so the cells are growing on the uracil-depleted medium (for selection); the protein expression is unregulated because it is immediately 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 resuspended 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 immunofluorescence 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 (Nylvitova et al. 2013), *M. balamuthi* HydE (Nylvitova 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<sup>+</sup>-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<sup>+</sup>, 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<sup>+</sup>, 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,  $1 \times 10^7$  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-4S] 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. 1B) as well as in a lysate of *Escherichia coli* cells heterologously expressing a fragment of *N. gruberi*'s hydrogenase (fig. 1C). Surprisingly, immunofluorescent microscopy of *N. gruberi* cells using  $\alpha$ -hydrogenase Ab showed a cytosolic distribution of this enzyme, and the hydrogenase labeling do not colocalize with the MitoTracker (fig. 1A). The same cytosolic localization we also observed for hydrogenase maturase HydE (fig. 1A 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. 1A). Signal of the  $\alpha$ -Nbp35 antibody had shown the same distribution as the  $\alpha$ -hydrogenase and  $\alpha$ -HydE Abs: it did not colocalize with MitoTracker and consequently showed a pattern corresponding to the cytoplasm of *Naegleria* (fig. 1A).

The localization of [FeFe]-hydrogenase and HydE observed by immunofluorescent 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  $\alpha$ -hydrogenase Ab and  $\alpha$ -HydE Ab, we have detected the signal for hydrogenase and HydE, respectively exclusively in the cytosolic fraction (fig. 1B). In contrast, SdhB was detected in the mitochondrial fraction.

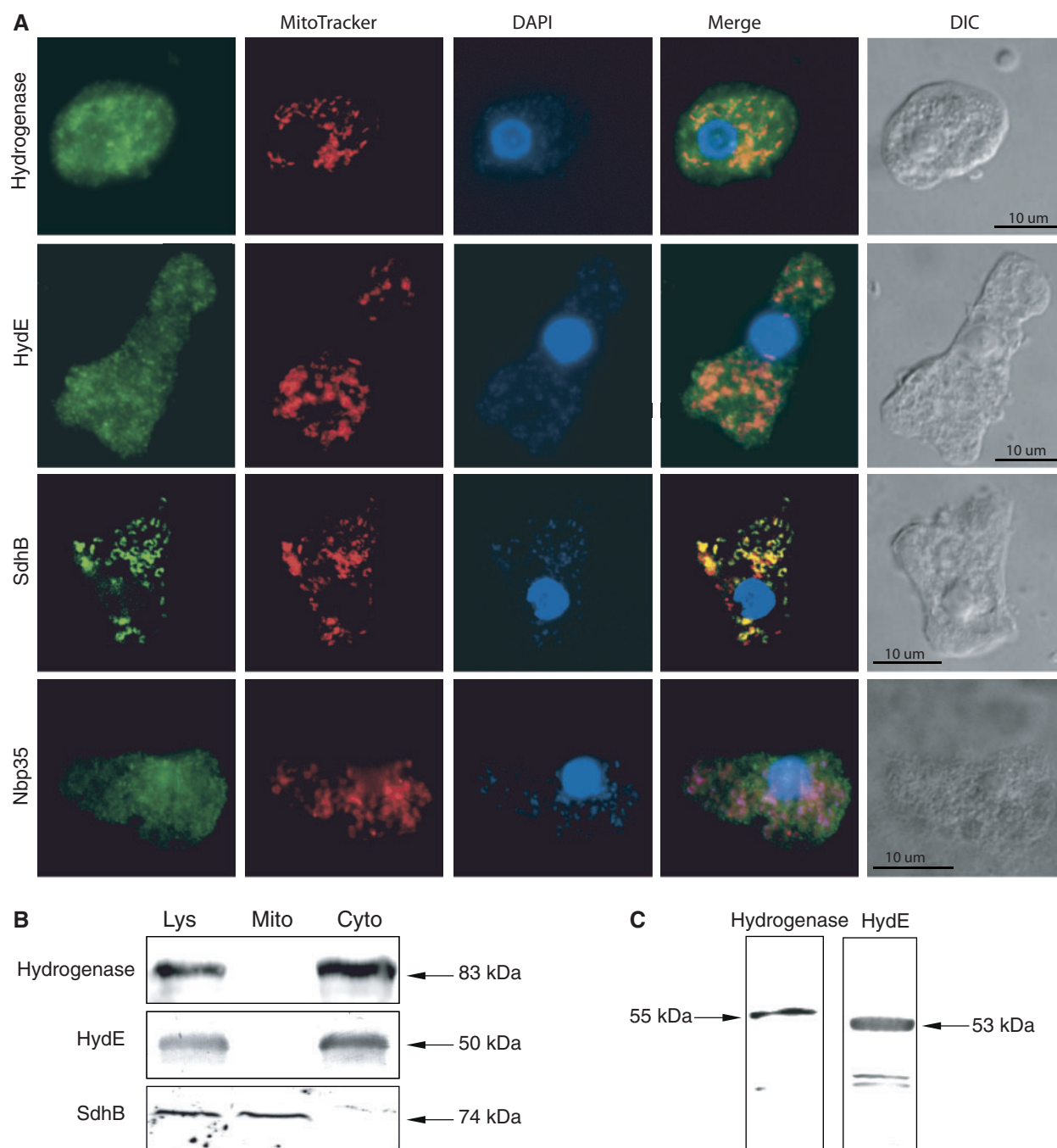
Furthermore, we have decided to employ an alternative approach to evaluate the localization of the [FeFe]-hydrogenase and examine the function of predicted MTS. We expressed the 5'-end region of *N. gruberi*'s [FeFe]-hydrogenase (1,251 bp) and HydE (690 bp) in *S. cerevisiae* and investigated whether 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. 2A), whereas the *N. gruberi* [FeFe]-hydrogenase-GFP and HydE-GFP were found to be present exclusively in the cytosol of the cell (fig. 2A), 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. 2B). 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. 2B). As a control for mitochondrial integrity,  $\alpha$ -Tom20 was used as the outer membrane marker (digested with trypsin without the addition of detergent), and  $\alpha$ -Tim17 was used as an inner membrane marker (not digested with trypsin without the addition of detergent; fig. 2B). 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.

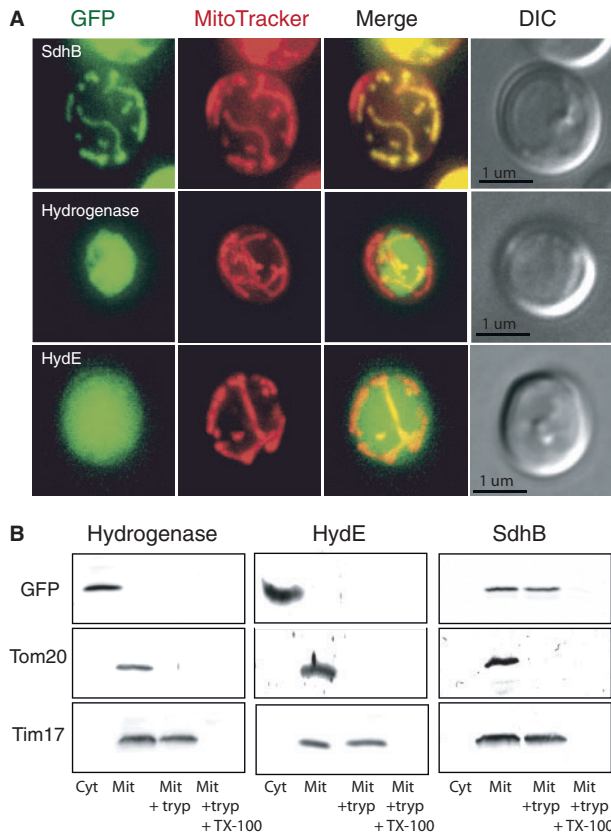




**Fig. 1.**—Cellular localization of hydrogenase and HydE in *N. gruberi* cells. (A) Immunofluorescent microscopy. [FeFe]-Hydrogenase, HydE, and Nbp35 (cytosolic marker) were detected in the cytosol of the cell. The signals for these proteins do not colocalize with the MitoTracker red. The Ab against 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  $\mu$ m. (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*  $\alpha$ -SdhB,  $\alpha$ -HydE and *T. vaginalis*  $\alpha$ -hydrogenase, antibodies, respectively. (C) Specificity of  $\alpha$ -[FeFe]-hydrogenase and  $\alpha$ -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



**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).

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<sup>7</sup> cells (table 2). This level is about three times higher than hydrogen production observed in microaerophilic organisms *G. intestinalis* (2 nmol/min/10<sup>7</sup> cells; Lloyd et al. 2002) and about five times lower than in *T. vaginalis* (Sutak et al. 2012).

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 <sup>7</sup> cells)	
<i>Naegleria gruberi</i>	5.814 ± 0.57	This study
<i>Trichomonas vaginalis</i>	29.098 ± 1.549	This study
		Ellis et al. (1992)
<i>Giardia intestinalis</i>	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/>).

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