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Invited Review

Naegleria gruberi metabolism

Fred R. Opperdoesa,⇑, Johan F. De Jonckheerea,b, Aloysius G.M. Tielensc

a Research Unit for Tropical Diseases, De Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgium
b Scientific Institute of Public Health, B-1050 Brussels, Belgium
c Department of Medical Microbiology and Infectious Diseases, Erasmus MC, University Medical Centre Rotterdam, 3015 CE Rotterdam, The Netherlands

Abstract

The completion of the genome project for Naegleria gruberi provides a unique insight into the metabolic capacities of an organism, for which there is an almost complete lack of experimental data. The metabolism of Naegleria seems to be extremely versatile, as can be expected for a free-living amoeboflagellate, but although considered to be fully aerobic, its genome also predicts important anaerobic traits. Other predictions are that carbohydrates are oxidised to carbon dioxide and water when oxygen is not limiting and that in the absence of oxygen the end-products will be succinate, acetate and minor quantities of ethanol and lactate. The hybrid mitochondrion/hydrogenosome has both cytochromes and an [Fe] hydrogenase, but seems to lack pyruvate-ferredoxin oxidoreductase. Genomic information also provides the possibility to identify drugs with a possible mode of action in the fatal primary amebic meningoencephalitis caused by the closely related opportunistic pathogen Naegleria fowleri.

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1. Introduction

Members of the genus Naegleria are ubiquitous freshwater amoeboflagellates present on all continents (De Jonckheere, 2004). They are found in mud and soil, as well as in rivers, lakes and swamps where they occupy habitats rich in organic matter (Fulton, 1970). The amoebic stage feeds on bacteria although some strains have been adapted to axenic growth. The best studied species is the non-pathogenic Naegleria gruberi, but the genus is notorious because the thermophilic species Naegleria fowleri causes primary amebic meningoencephalitis (PAM) in humans, which almost invariably leads to death. Cases of PAM are reported worldwide and are related to swimming in warm water, whereby the pathogen enters the brain via the nose (De Jonckheere, 2002). Most Naegleria spp. transform from dividing trophozoites (amoebic stages) into non-dividing flagellates with two or more flagella and they all form cysts. The trophozoite stage is characterised by a large nucleus with nucleolus, the presence of numerous mitochondria and food vacuoles, a contractile vacuole and traces of an endoplasmic reticulum (Fulton, 1970). The trophozoites are phagotrophic. They feed mainly on bacteria, but they can grow on other food as well, ranging from mammalian cell debris to non-particulate axenic media. Until now almost nothing was known about the metabolism of Naegleria. Although some individual metabolic enzymes have been described and characterised, few metabolic studies have been carried out to date. Some information exists on the organism’s mitochondria (Weik and John, 1979) and since 2000, the sequence of the mDNA has been available in GenBank (Accession No. AF288092). A chemically defined medium supporting the growth of the NEG-M strain of N. gruberi has been described (Nerad et al., 1983; Fulton et al., 1984). With the recent completion of the genome project of the axenically growing N. gruberi strain NEG-M, a unique insight into the metabolic capacities of this organism is provided (Fritz-Laylin et al., 2010). However, due to an almost total lack of experimental data related to N. gruberi’s metabolic capacities the predictions presented in our review and in recent genome papers (Fritz-Laylin et al., 2010, 2011; Ginger et al., 2010) are highly speculative and need verification by experimental studies. In the present paper we specifically focus on the predicted metabolic capacities of N. gruberi.

2. Nutrition

Naegleria gruberi feeds on bacteria by phagocytosis (Fulton, 1970). Using electron microscopy bacteria and their degradation products can be seen in the numerous food vacuoles present in the cytoplasm. In the laboratory various types of bacteria (such as Aerobacter and Escherichia coli) are routinely used as food sources. Despite the fact that for many years the N. gruberi NEG-M...
strain has been grown in axenic culture (Fulton, 1970), its genome still reflects its capacity to digest bacterial prey. (Acid)phosphatas- 

tases, (phospho)lipases and esterases are all encoded in the genome and are likely constituents of Naegleria's phago-lysosomal system. Naegleria gruberi is also predicted to cope with bacterial peptidoglycans using the enzymes chitinase and lysozyme and to 

degrade storage polysaccharides such as starch, cellobiose and gly- 

cope to maltose and glucose with the aid of α- and β-glucosidases and a set of (gluco)amylases. It is not likely that glycogen serves as a 


carbohydrate store, as none of the specific glycogen-forming en- 


yzmes were found. The presence of a recently identified etherase (Veiga-da-Cunha et al., 2009) together with α-lactate dehydro- 


gnase is an indication that Naegleria is also able to degrade bacterial cell walls. The etherase permits hydrolysis of the ether bond in 


N-acetylmuramate 6-phosphate, a constituent of cell wall murein, thus forming N-acetylglucosamine 6-phosphate and α-lactate, whereas the dehydrogenase converts α-lactate to pyruvate.

Naegleria probably uses the disaccharide α,α-trehalose, a prot- 


tectant against desiccation, for osmoregulation and cyst formation (Elbein et al., 2003), since the enzymes UTP:glucose-1-phosphate 


uridylyltransferase, α,α-trehalose-P synthase and trehalase phosphatase are all present.

3. Carbohydrate metabolism

The presence of the enzymes glucokinase, fructokinase/ 


carbohydrate kinase, pentulose/hexulose kinase and ribokinase (Supplementary Table S1) all suggest that Naegleria utilises a variety of monosaccharides for its carbohydrate needs. Glucose is known to be an important nutrient for Naegleria; its presence stimulates growth and reduces cellular division time of 


N. fowleri (Fulton, 1970), its genome still reflects its capacity to digest bacterial prey. (Acid)phospha-


3.1. Pentose-phosphate pathway

The pentose-phosphate shunt, or hexose-monophosphate path- 


geway, generates NADPH for both biosynthetic purposes and oxida-


tive stress protection, while at the same time ribose moieties are 


formed for the synthesis of nucleic acids. All enzymes of both the oxidative and non-oxidative branches of the pathway plus the en- 


zymes ribokinase and xylulokinase were detected, indicating the 


presence of a separate fructosekinase. The predicted N. gruberi glucokinase is 


related to the glucokinases of the trypanosomatids Trypanosoma cruzi and Leishmania major and of Trichomonas vaginalis and Giardia intestinalis, all closely related to the glucokinase of bacteria (Henze et al., 2001; Wu et al., 2001; Cáceres et al., 2007). Second, a classical ATP-dependent phosphofructokinase (PKF) is absent in 


N. gruberi and the second phosphorylation step of glycolysis is 


catalysed by a pyrophosphate- (PPi)-dependent PKF, with 82% identity to the sequence published for N. fowleri (Mertens et al., 1993). All other glycolytic enzymes were detected in the N. gruberi genome. In addition to a pyruvate kinase, the last enzyme of the pathway, N. gruberi has a pyruvate-phosphate dikinase (PPDK), which catalyses the conversion of phosphoenolpyruvate to pyruvate, similar to pyruvate kinase except that PPDK utilises PP, plus AMP, rather than ADP, as phosphor acceptor (Bringaud et al., 1998; Varela-Gómez et al., 2004). The advantage of using a PPDK is that this enzyme catalyses a reversible reaction and we predict that, in combination with the above-mentioned and equally reversible PPi-dependent PKF catalysed reaction, N. gruberi, which 


seems to lack a fructose-1,6-bisphosphatase (FBPase), uses these 


two enzymes in the reverse direction to carry out gluconeogenesis. Interestingly, the closely related N. fowleri has a FBPase gene (Q77706) and therefore could either use this FBPase or the PPi- 


PKF for gluconeogenesis.

Interestingly, N. gruberi is capable of forming a potent regulator of 


glycolysis/gluconeogenesis, fructose 2,6-bisphosphate (F2,6P2), 


coded in its genome. In most other eukaryotes F2,6P2 regulates the activities of both ATP-dependent PFK and fructose-bisphospha-


tase, which catalyse opposed reactions and, if unregulated, would result in a futile cycle leading to the hydrolysis of all cellular ATP 


(Van Schaftingen, 1987). In Naegleria the ATP-dependent PFK has 


been replaced by a PPi-dependent enzyme and it is therefore not clear how futile cycling is prevented.

Naegleria's metabolic end products have not been directly determined but they can be predicted based on comparison with other, biochemically better characterised protists. In the presence of molecular oxygen, glycolytically produced NADH and pyruvate are oxidised by the mitochondria. A dihydroxyacetone-phos- 


phate/glucose-3-phosphate cycle seems to be operational, consist- 


ing of a cytosolic NAD-linked dehydrogenase and a mitochondrial flavine adenine dinucleotide (FAD)-linked glycerol-3-phosphate dehydrogenase. Alternatively an NADH dehydrogenase (NAD2), likely to be located at the external face of the mitochondrial inner membrane, may take care of the reoxidation of some of the cyto-


solic NADH.

When oxygen is absent, the possibilities of reoxidation of cyto-


colic NADH, produced by the glycolytic pathway, are limited. Homolactic fermentation is not likely to occur. The only lactate 


dehydrogenase predicted by the genome is specific for the α- rather than for the L-enantiomer of lactate and this enzyme is probably 


involved in the degradation of bacterial murein, or plays a role in the 


methylglyoxal bypass, which inactivates any toxic methylgly-


oxyl, formed by spontaneous dephosphorylation of glycolytic dihy-


droxyacetone-phosphate (DHAP), to α-lactate (Wyllie and 


Fairlamb, 2011). The presence of two of the three enzymes of the pathway, glyoxalase I (lactoyl-glutathionyl lyase) and glyoxalase II (hydroxacyl glutathione hydrolyase), is indicative of its presence. However, the first enzyme of the bypass, methylglyoxal synthase, was not found, which suggests that the pathway functions in detoxification of any spontaneously formed methylglyoxal, rather than being involved in a metabolic function. (Fig. 1). Alcoholic fer-


mentation is also not a likely pathway for the reoxidation of 


cytosolic NADH, since alcohol dehydrogenase (Supplementary 


Table S1) is predicted to be a mitochondrial enzyme. Therefore 


the most likely alternative would be via a cytosolic malate dehy-


drogenase which may function as part of a hypothetical malate di-


mutation pathway (see Section 8.2).

4. Peroxisomes

No morphological or biochemical evidence exists for the pres- 


ence of peroxisomes in Naegleria. In general a peroxosomal targeting 


signal at the C-terminal (PTS1) or N-terminal (PTS2) end of proteins and the presence of so-called peroxins, proteins involved in the biogenesis of peroxisomes, are diagnostic for the presence of
peroxisomes in an eukaryotic organism (Galland and Michels, 2010; Rucktäschel et al., 2011). Homologues of several peroxins (Rucktäschel et al., 2011) were detected in *N. gruberi* (Table 1) and when each of the 15,753 protein sequences was scanned for the presence of a PTS1 (Opperdoes and Szikora, 2006), many potential peroxisomal candidates were identified (Table 2). From the high number of PTS-containing proteins involved in typical peroxisomal functions it can be inferred that there should be bona fide peroxisomes in *Naegleria*, which function in fatty acid metabolism and protection against reactive oxygen species (ROS). The presence of both fatty acid β-oxidation and monoamine oxidase suggest the formation of hydrogen peroxide (H₂O₂) inside the organelles. However, although *Naegleria* has both a Cu/Zn- and a Mn-superoxide dismutase, the presence of both a Cu/Zn- and a Mn-superoxide dismutase is still unclear.

**Table 1**

Predicted peroxisomal proteins in *Naegleria gruberi*. Peroxins or PEX proteins were identified by homology with corresponding peroxins in other organisms.

<table>
<thead>
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<td>PEX6</td>
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<td>PEX7</td>
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<tr>
<td>PEX10</td>
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<td>D2V791</td>
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<tr>
<td>PEX19</td>
<td>D2W353</td>
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</table>

**Fig. 1.** Energy and intermediate metabolism in *Naegleria gruberi*. Boxed metabolites are nutrients (in blue). P5P, pentose 5-phosphate; ACP, acyl-carrier protein; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; Fru-1,6-P₂, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone-phosphate; GAP, glyceraldehyde 3-phosphate; G-1,3-P₂, glycerate 1,3-bisphosphate; 3-PGA, glycerate 3-phosphate; 2-PGA, glycerate 2-phosphate; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid; GTP, guanosine-triphosphate; GDP, guanosine-diphosphate; FAD, flavin adenine dinucleotide; FADH₂, reduced FAD; FAS, fatty acid synthase. Enzymes: 1, α- and β-amylase; 2, α- and β-glucosidase and glucosamylase; 3, sugar transporter; 4, glucokinase; 5, phosphoglucone isomerase; 6, ribokinase and xylulokinase; 7, fructokinase; 8, PPI-dependent phosphofructokinase; 9, fructose-bisphosphate aldolase; 10, triosephosphate isomerase; 11, glyceraldehyde-3-phosphate dehydrogenase; 12, phosphoglycerate kinase; 13, phosphoglycerate mutase; 14, enolase; 15, pyruvate kinase; 16, pyruvate phosphate dikinase; 17, glycerol kinase; 18, glyceraldehyde-3-phosphate dehydrogenase; 19, n-lactate dehydrogenase; 20, phosphoenolpyruvate carboxykinase; 21, aspartate aminotransferase; 22, malate dehydrogenase (cytosolic); 23, adenylate kinase; 24, pyruvate dehydrogenase; 25, citrate synthase; 26,aconitate; 27, isocitrate dehydrogenase (NAD); 28, 2-oxoglutarate dehydrogenase; 29, succinyl-CoA synthetase; 30, succinate dehydrogenase; 31, fumarate hydratase; 32, malate dehydrogenase (mitochondrial); 33, pyruvate dehydrogenase; 34, alcohol dehydrogenase; 35, acetate:coenzyme A transferase; 36, malic enzyme (decarboxylating); 37, glutamate dehydrogenase; 38, Fe-hydrogenase; 39, nitrate reductase; 40, citrate lyase; 41, acyl-CoA carboxylase; 42, [acyl-carrier-protein] S-acetyltransferase; 43, [acyl-carrier-protein] S-malonyltransferase; 44, type-1 fatty acid synthase; 45, Acyl-CoA synthetase; 46, Acyl-CoA dehydrogenase/oxidase; 47/48, trifunctional enzyme; 49, thiolase; 50, NADH-dependent fumarate reductase; 51, isocitrate dehydrogenase (NADP).
dismutase and the peroxisomal marker enzyme catalase, none of these predicted proteins carries a PTS. The presence of a PTS1-containing NADP–isocitrate dehydrogenase also points into the direction of peroxisomal ROS protection (Yoshihara et al., 2001; Lu and McAlister-Henn, 2009). However, enzymes of the glyoxylate cycle, such as dihydroxy acid oxidase and 2-hydroxy acid oxidase, were not detected in the Naegleria genome.

5. Dithiol metabolism

Biochemical evidence for the presence of the dithiol trypanothione–catalase combination in the trypanosomatid parasite Trypanosoma brucei (Gray, 1993; Kurland and Andersson, 2000; Gray et al., 2004; Embley and Martin, 2006) suggests that the trypanosomatid mitochondria have evolved into an enzyme with TR activity. Further experimental confirmation of a trypanosome-like dithiol metabolism will be required.

6. Purine biosynthesis

Naegleria is incapable of de novo purine synthesis, as only adenylsuccinate lyase, one of the enzymes required to make inosine monophosphate (IMP) from phosphoribosyl pyrophosphate, was identified. However, this enzyme also plays a role in urea salvage in the urea-nucleotide cycle by converting IMP and aspartate to AMP and fumarate (Van den Berghe et al., 1992). Apparently Naegleria acquires the purine nucleobases and nucleosides from its host bacteria on which it feeds. This is also suggested by the presence of a nucleoside transporter gene in its genome. Most members of the order trypanosomatids are equipped to function both under aerobic and under anaerobic conditions.

7. Pyrimidine biosynthesis

The Naegleria genome encodes all six enzymatic activities necessary for the synthesis of uridine monophosphate (UMP). The first three enzymes, carbamoyl-phosphate synthase (D2VZ04), aspartate transcarbamylase (D2VZ3) and dihydroorotase (D2V9M3) are encoded by separate genes, while in most other eukaryotes these genes have been fused together (Nara et al., 2000). The fourth gene encodes a mitochondrial dihydroorotase dehydrogenase (D2LZZ6), typically found in all eukaryotes except in trypanosomatids and some fungi, where this enzyme is a cytosolic dehydrogenase. The last two activities of the pathway are catalysed by a fusion protein of orotate phosphoribosyltransferase–orotate decarboxylase (OPRT–ODC, D2VR8) similar to most other eukaryotes. Thus in conclusion, the pyrimidine biosynthetic pathway is very similar to that of other eukaryotes but apparently Naegleria may have already separated from the other eukaryotes before a fusion of the first three enzymes occurred (Nara et al., 2000; Opperdores and Michels, 2007).

8. Mitochondrial energy metabolism

The mitochondrion of Naegleria is an organelle of endosymbiotic origin, derived from a member of the α-proteobacteria and its genome is the highly reduced remnant of the genome of the symbiotic bacterium (Gray, 1993; Kurland and Andersson, 2000; Gray et al., 2004; Embley and Martin, 2006). Mammalian mitochondria encode just 13 protein-coding genes plus some RNA-coding genes, and retain only faint similarities to the genomes of living prokaryotes (Calvo and Mootha, 2010). The Naegleria mitochondrial genome (GenBank, Accession No. AF288092) has much more functionality. It contains 49,843 bp and encodes 69 genes, including some 46 protein-coding genes. The protein coding capacity of the mitochondrial genome of Naegleria is intermediate between that of the jakobid Reclinomonas americana (Lang et al., 1997), the largest mitochondrial genome known to date with almost 100 genes and that of higher eukaryotes. The Naegleria genome reveals the presence of an extremely flexible mitochondrial, well equipped to function both under aerobic and under anaerobic conditions.

8.1. Aerobic mitochondrial functions

Naegleria's complement of nuclear and mitochondrial encoded genes suggests that in the presence of molecular oxygen carbohydrates are completely oxidised to carbon dioxide and water via a mitochondrial pyruvate dehydrogenase complex, a fully functional citric acid cycle (all enzymes of the cycle were detected) and respiratory chain (Fritz-Laylin et al., 2010). Reducing equivalents in the form of NADH are oxidised by complex I which consists of 13 nuclear encoded NADH dehydrogenase subunits in addition to 11 mitochondrially encoded subunits (Table 3). The complex has a predicted mass of 780 kDa, larger than the corresponding bacterial complex and that of the euglenozoan Trypanosoma brucei (Opperdores and Michels, 2008) but smaller than the typical mitochondrial complex I of higher eukaryotes (Brandt, 2006). All subunits known to be involved in electron transport as well as the membrane subunits, supposed to be involved in proton extrusion, are present. This suggests that the Na. gruberi complex I couples electron transport to the generation of a proton gradient over the mitochondrial membrane.

Interestingly, γ-carbonic anhydrase, a typical constituent of the complex I of plants (Gabaldón et al., 2005), was also detected in the Na. gruberi genome. Other reducing equivalents, such as

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Table 2

<table>
<thead>
<tr>
<th>PTS1-containing enzymes in Naegleria gruberi.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme name</td>
</tr>
<tr>
<td>Glutamine-dependent NAD synthase</td>
</tr>
<tr>
<td>NADP-dependent malic enzyme</td>
</tr>
<tr>
<td>A(3,5)-A(2,4)-Dienyl CoA isomerase</td>
</tr>
<tr>
<td>Acetyl CoA synthase</td>
</tr>
<tr>
<td>Acyl CoA dehydrogenase</td>
</tr>
<tr>
<td>Fatty acid oxidation complex subunit alpha (trifunctional enzyme)</td>
</tr>
<tr>
<td>DHAP acyltransferase/G3P acyltransferase</td>
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<tr>
<td>Serine palmitoyltransferase</td>
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<tr>
<td>NADP-dependent fumarate reductase</td>
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<tr>
<td>NADP-isocitrate dehydrogenase</td>
</tr>
<tr>
<td>2,4-Dienoyl CoA reductase</td>
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<tr>
<td>Retinal dehydrogenase</td>
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<tr>
<td>3,2-Transenoyl CoA isomerase</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>Dihydothiemolin-4-reductase</td>
</tr>
</tbody>
</table>

PTS1, peroxisomal targeting signal type 1, formed by the protein's last three C-terminal amino acids – SKL, or a variant thereof.
FADH$_2$/ubiquinone and succinate are oxidised via a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase, two nuclear-encoded subunits of succinate dehydrogenase, respectively the FAD-containing subunit and the iron–sulphur-containing subunit, several subunits of complex III (Rieske-iron sulphur protein, QCR7 and RIP1), mitochondrially encoded apocytochrome b, and the cytochromes c and c$_1$. The three mitochondrially encoded subunits of cytochrome oxidase, essential and sufficient for catalytic activity, are present, but contrary to other eukaryotes there is no evidence in Naegleria for the presence of any nuclear encoded subunits. Moreover, there is a plant-like alternative oxidase (AOX) (Fritz-Laylin et al., 2010) as well as an alternative NADH dehydrogenase and aspartate amino transferase genes. The function and not for other metabolic purposes.

8.2. Anaerobic mitochondrial functions

In the absence of molecular oxygen, electrons from NADH may be passed onto an alternative electron acceptor such as nitrate since a plant-like cytochrome b5 containing nitrate reductase (D2V2C0) is encoded in the genome. Since further enzymes of the assimilatory denitrification pathway were not found, it is likely that nitrate serves only as an electron sink for anaerobic respiration and not for other metabolic purposes.

CytoSolic NADH is most likely reoxidised via malate formation which results in redox balance in the cytosol (Tielens et al., 2002; Van Hellemond et al., 2003). Cytosolic phosphoenolpyruvate is first carboxylated to oxaloacetate by phosphoenolpyruvate carboxykinase and subsequently reduced to malate by malate dehydrogenase. This malate could then be subject to malate dismutation (Tielens et al., 1987), as all enzymes seem to be present. After import into the mitochondria, part of the malate is then oxidised by malic enzyme and pyruvate dehydrogenase resulting in the formation of mitochondrial acetyl-CoA; the other part is reoxidised by cyanide plus the alternative oxidase inhibitor salicylhydroxamic acid (SHAM).

Under aerobic conditions, electron transport is coupled to the generation of a membrane potential. A proton gradient is being generated at the level of the respiratory complexes I, III and IV. In addition there are several nuclear encoded and five mitochondrially encoded subunits of the mitochondrial ATP synthase, which uses the proton-motive force to generate mitochondrial ATP. Under conditions of nutrient abundance the need for ATP can probably be met by the glycolytic pathway alone and the cytosolically produced NADH can then be reoxidised by the combined action of the glycolytic pathway and the cytosolically produced NADH can then be reoxidised by the combined action of the mitochondrial respiratory complexes I, III and IV.

Table 3

<table>
<thead>
<tr>
<th>Peptide ID</th>
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$^a$ Uniprot accession number.
$^b$ Uniprot accession number.
substrate of the mitochondrial hydrogenase (see Section 12.1). Which reducing equivalents are transferred to ferredoxin, the usual substrate for the formation of phospholipids were also identified.

Thus, Naegleria also appears to encode a "classical" malate dismutation pathway for anaerobic ATP synthesis as is typical of many facultative anaerobic mitochondria (Tielens et al., 2010). The lic heterolobosean amoeboflagellate Psalteriomonas lanterna (Davidson et al., 2002; De Graaf et al., 2009). The mitochondrial HMG-CoA followed by its cleavage into acetyl-CoA and hydrogenase and the 2-ketoacid dehydrogenase complexes. The early steps of the ether-lipid biosynthesis pathway (although peroxisomal targeting signals were not identified) may also be located in the predicted peroxisomes, indicating that Naegleria is able to form and incorporate ether lipids in its membranes. Most of the enzymes required for the formation of phospholipids were also identified.

Hydroxymethylglutaryl coenzyme A (HMG-CoA), may serve as substrate for the formation of the isoprenoids squalene, ubiquinone and dolichol and the sterols lanosterol and ergosterol (Raederstorff and Rohmer, 1987). HMG-CoA synthase and HMG-CoA reductase were identified. Moreover, the conversion of squalene into sterols requires molecular oxygen as indicated by the presence of a squalene monoxygenase.

8.4. Mitochondrial transport

On the basis of genome analysis, Naegleria possesses a full set of mitochondrial solute transporters (Laloi, 1999). For both pyruvate, the end-product of glycolysis, and phosphate there are specific mitochondrial carriers. Some of the carriers are homologs of the dicarboxylate and tricarboxylate exchanger described in other organisms (Palmieri et al., 2000). These carriers are probably involved in transport of dicarboxylic acid (TCA) cycle intermediates and of aspartate and glutamate across the mitochondrial membrane. Furthermore an ATP/ADP exchanger, a possible folate carrier and a mitochondrial ornithine carrier were found.

9. Lipid metabolism

As a predator of bacteria, Naegleria requires a full complement of lipid degrading enzymes. Indeed, Naegleria has the phospholipases A1, A2, C and D, as well as lyso phospholipase, together allowing for the degradation of membrane phospholipids in their constituent components. There are also lipases for the hydrolysis of triglycerides. The resulting glycerol and free fatty acids may serve as energy substrates. Glycerol, after its phosphorylation by a glycerol kinase and oxidation to DHAP, enters the glycolytic pathway, while fatty acids are most likely activated inside the predicted peroxisomes where they are degraded by β-oxidation (Fig. 1). All of the necessary enzymes of this pathway were detected (see Section 4).

De novo synthesis of fatty acids is possible from mitochondrial-generated acetyl-CoA. After being exported to the cytosol, citrate is cleaved by citrate lyase into oxaloacetate and acetyl-CoA and the latter is carboxylated to malonyl-CoA for chain elongation by the multifunctional type-I fatty acid synthase, which was identified (Fig. 1). Enzymes of the type-II fatty acid synthesis pathway are present in the mitochondrion and may be involved in the synthesis of lipidic acid, an essential component of the pyruvate dehydrogenase.
directly to glycine and acetaldehyde through the action of serine hydroxymethyl transferase, which also acts on threonine. There are two isoenzymes, one of which is most likely mitochondrial. Acetaldehyde is converted to acetyl-CoA. Glycine, in addition to its cleavage by GCS can also be converted to serine by the same enzyme system and to pyruvate by a serine dehydratase.

Lysine is most likely broken down via the saccharopin pathway leading to the formation of HMG-CoA and acetoacetate, since two corresponding dehydrogenases, \( \alpha \)-aminoadipate semialdehyde dehydrogenase (D2UYT1) and \( \alpha \)-aminoadipate aminotransferase (D2W5S9), were both found in the genome.

Most enzymes of the classical oxidative pathway for aromatic amino-acid oxidation are present. Phenylalanine can be converted to tyrosine by a phenylalanine-4-hydroxylase and enzymes such as hydroxyphenylpyruvate dioxygenase, homogentisate 1,2-dioxygenase and fumarylacetoacetate hydrolase, required to convert the latter into fumarate and acetoacetate are all present. For the oxidation of tryptophane all of the enzymes (including 2-hydroxyymuconic semialdehyde dehydrogenase, D2JV7T) are also present.

The presence of a tyrosine aminotransferase and an aspartate aminotransferase with broad substrate specificity, acting also on aromatic amino acids, suggests that phenylalanine and tyrosine can be converted to their corresponding ketoacids. No evidence was found for their further metabolism via the anaerobic degradation pathway.

Serine can be converted to cysteine, while the latter is converted to pyruvate by a serine dehydratase. There is no evidence for the presence of other enzymes of the urea cycle in Naegleria: ornithine carbamoyl transferase, argininosuccinate synthase or lyase, arginine decarboxylase are all absent. Ornithine, after decarboxylation, can be used for the biosynthesis of the polyamines spermidine and putrescine.

**Fig. 2.** Amino acid metabolism and some associated pathways in *Naegleria gruberi*. Metabolic steps indicated in black have been identified by the presence of the corresponding enzymes. Steps in grey represent reactions for which no enzymes could be identified. Question marks represent enzyme catalysed steps for which no unambiguous gene identification could be made. AcAc, acetoacetate; AdoMet, adenosylmethionine; H2F, dihydrofolate; H4F, tetrahydrofolate; HMG-CoA, hydroxymethylglutaryl coenzyme A; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; 3PGA, glyceral 3-phosphate; IMP, inosine monophosphate; TCA, tricarboxylic acid.
10.2. Amino acid biosynthesis

Alanine, aspartate, asparagine, glutamate and glutamine are all formed by transamination of pyruvate, oxaloacetate and 2-keto-glutarate, respectively. Proline is formed from glutamate, since γ-glutamyl kinase and 1-pyrroline-5-carboxylic reductase are present. Ornithine can be formed directly from proline by the action of an ornithine cyclodeaminase, or from the proline-pathway intermediate glutamate-semialdehyde. However, ornithine cannot serve as a precursor for arginine because, apart from the urea-cycle enzyme arginase, all other enzymes of this cycle are absent. It is not clear whether serine can be formed from ν-3-phosphoglycerate. Although the first enzyme committed to the synthesis of serine: 3-phosphoglycerate dehydrogenase is present, no specific phosphoserine phosphatase homologue was detected. However, serine can be formed from threonine. Serine cannot be used for the synthesis of cysteine and methionine and threonine cannot be synthesised from aspartate via homoserine. Glycine and serine are interconverted in each other by hydroxymethyl transferase and the latter can be formed from threonine as well. A pathway for the synthesis of lysine was not found. Also, the pathways for the synthesis of the branched amino acids valine, leucine, isoleucine, for the aromatic amino acids phenylalanine, tyrosine and tryptophane and nine enzymes necessary for the synthesis of histidine are all missing.

Fulton et al. (1984) carried out studies about the amino acid requirements of Naegleria gruberi when grown in a chemically defined medium. It is satisfying to see that our predictions derived from genomic information are entirely in agreement with these early observations.

In summary, Naegleria has a full capacity for the degradation of all 20 natural L-amino acids it may encounter in prey bacteria and even some ν-amino acids present in bacterial cell walls. However it lacks the possibility of synthesising long chain, branched chain and aromatic amino acids, which have to be supplemented in culture medium (Fulton et al., 1984).

11. Cofactors and vitamins

Naegleria is dependent on a number of exogenous cofactors and/ or vitamins as it does not seem to be capable of forming thiamine (vitamin B1), biotin, vitamin B12 and folate. However, nicotinamide is most likely converted to NAD and NADP, whereas coenzyme A can be formed from pantothetic acid. Pyridoxin (vitamin B6) is converted to pyridoxal-phosphate and riboflavin (vitamin B2) to flavin mononucleotide. The presence of a guaninolactone oxidase homologue suggests that Naegleria may be able to form ascorbic acid, although a typical ascorbate peroxidase, as present in the Trypanosomatidae, is absent. Naegleria is unable to synthesise its own haeme. None of the enzymes of the haeme biosynthesis pathway were detected except ferrochelatase, required for the insertion of the Fe²⁺ ion into preformed haeme, in agreement with the fact that Naegleria requires haemin as an essential growth factor (Nerad et al., 1983; Fulton et al., 1984). Thus Naegleria utilises the haeme intact from its prey bacteria.

12. Conclusions and perspectives

12.1. Is Naegleria capable of a true anaerobic lifestyle?

The mitochondria of Naegleria are intermediates in mitochon- drial evolution that unite biochemical properties of both aerobic and anaerobic mitochondria and hydrogenosomes (Shiflett and Johnson, 2010). They contain a hydrogenase typical of hydrogenosomes as well as ubiquinone and cytochromes typical of aerobic mitochondria. The enzyme content of this mitochondrin/hydrogenosome-like organelle predicts a possibility for adaptation to both an aerobic and an anaerobic life style. However, Naegleria has always been considered to be a truly aerobic organism, typical of fresh water habitats. Nevertheless, Naegleria has been isolated from sediments and clays as deep as 6 m (Kofoid, 1915), typical anaerobic habitats. Thus the organism seems able to survive in the absence of oxygen, although it cannot be excluded that the cysts, which are not very metabolically active, rather than its trophozoites or flagellates, are endowed with this capacity.

From its genome some predictions can be made. Carbohydrates may be oxidised completely to carbon dioxide and water when oxygen is not limiting. Nitrate may replace oxygen as terminal electron acceptor. In the absence of both molecular oxygen and ni- trate Naegleria has the capacity to break down carbohydrates to succinate, acetate and (possibly) molecular hydrogen and maybe minor quantities of ethanol and ν-lactate. Formation of ethanol or ν-lactate would result in the production of the limited amount of 2 mol of ATP per mol of hexose sugar consumed, while malate dismutation, leading to the secretion of succinate and acetate, via the acetate:succinate CoA-transferase/succinyl-CoA synthetase cy- cle, would generate extra ATP. The anaerobic formation of molecular hydrogen together with acetate, if at all possible in Naegleria, would result in a theoretical yield of four ATPs per hexose consumed.

The absence of a mitochondrial PFO is intriguing since in most anaerobic protists with hydrogenosomes this enzyme is directly responsible for the transfer of reducing equivalents from pyruvate to protons using a ferredoxin cofactor. It cannot be excluded that in N. gruberi another, as yet unknown, enzyme or co-factor fulfills such transfer, since in their absence the formation of H₂ from NADH + H⁺ (ΔG° = +4.5 kcal/mol) is thermodynamically feasible only at extrem- ely low partial pressures of H₂. Such a condition can only be generated if Naegleria would live in close association with metha- nogenic bacteria with a sufficiently high affinity for molecular hydrogen to keep the partial pressure of H₂ as low as 10⁻⁴ atm. (Gottschalk, 1985). Many anaerobic and microaerophilic hydrogenase-containing protists, such as those present in rumen, or tricho- monads maintain in their cytoplasm H₂-consuming endosymbiotic bacteria. Indeed, there is some indication that an as yet unidenti- fied Naegleria spp., may be capable of such an anaerobic lifestyle when maintained in bioreactors in the presence of methanogenic bacteria (Priya et al., 2008).

12.2. Identification of potential drug targets

Naegleria fowleri is responsible for primary amoebic meningoen- cephalitis in humans (see Section 1), an infection which leads almost invariably to death. The recommended drug against the disease is amphotericin B, but due to the fulminant nature of the infection few successful treatments have been documented (Schuster and Visvesvara, 2004). The genomic information now available may provide insight in the mode of action of some known drugs or aid in the identification of potential new drugs and drug targets.

As described above Naegleria utilises the HMG-CoA pathway for the formation of the isoprenoids squalene, dolichol and ubiqui- none, and the sterols lanosterol and ergosterol, in agreement with earlier observations (Raederstorf and Rohmer, 1987). Thus ergos- terol, and not cholesterol, is expected to be the major sterol present in the Naegleria’s plasma membrane, similar to the situation found in plants, fungi and trypanosomatids. The presence of this pathway in Naegleria explains the inhibitory effect of the sterol-complexing polyene antibiotics nystatin and amphotericin B and that of keto- conazole, an inhibitor of sterol 14-demethylase (DZVC12), involved in the conversion of lanosterol to ergosterol, on the growth of
Naegleria (Raederstorff and Rohmer, 1987). Since amphotericin B has been successful in the treatment of some cases of PAM caused by *N. fowleri* (Vivesvara et al., 2007) there may also be promise in the use of other drugs interfering with sterol formation such as the antifungal azole drugs miconazole, fluconazole, itraconazole and ketoconazole. Miconazole (Seidel et al., 1982), fluconazole (Var-gas-Zepeda et al., 2005) and ketoconazole (Pourvare and Jariya, 1991) are indeed drugs that have been used, although in combination with amphotericin B (and rifampin), in successful treatment of patients.

The genome encodes a bacterial-type mitochondrial nitroreduc-
tase of type-I (NTR1, D2VQ76). Amongst eukaryotes NTR1 is fur-
ther only present in the Trypanosomatidae, where it has been identified as the activator of the prodrugs benznidazole and nif-
tirimox which, once reduced, kill the parasite. Both compounds are the drugs of choice for treatment of American trypanosomiasis or Chagas’ disease (Wilkinson et al., 2008; Hall et al., 2010; Bot et al., 2010) and nifurtimox is now also undergoing phase III clinical trials for African sleeping sickness. This suggests that nifurti-
rimox and other drugs belonging to the class of nitrofurans and nitrimidazoles may also be effective in the treatment of *N. fowleri* infections.

The alternative oxidase AOX found in the *Naegleria* genome is an enzyme that is absent in humans. In African trypanosomes AOX, rather than cytochrome oxidase, functions as the mitochon-
drial terminal oxidase when trypanosomes infect the mammalian host. In the case that *N. fowleri*, when proliferating in the human brain, would also depend exclusively on AOX, this enzyme may be a potential drug target. Specific inhibitors of trypanosome AOX, such as salicyl hydroxamic acid (Oppedoers et al., 1976) and the antibiotic ascofuran (Yabu et al., 2006) have been used to cure trypanosome infections in experimental animals and thus may be potential drug candidates for the treatment of PAM.

12.3 Questions and experiments

The genome of *Naegleria* now invites numerous experiments on the basic biochemistry of the organism to test many, if not all, of the predictions made in this review. Remaining questions are: which are the sugars other than glucose that are able to sustain long-term growth? Which are the polysaccharides that can be formed by gluconeogenesis and are they used as food stores? Is tre-
haloase formed during cyst formation? How are glycolysis and glu-
coneogenesis and are they used as food stores? Is tre-
halose formed during cyst formation? How are glycolysis and glu-
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Nara, T., Hishimoto, T., Aoki, T., 2000. Evolutionary implications of the mosaic pyrimidine-biosynthetic pathway in... 


