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Invited Review

Naegleria gruberi metabolism

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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 D-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 amoebic meningoencephalitis caused by the closely related opportunistic pathogen *Naegleria fowleri*.

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 amoebic 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 experimentation. 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

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strain has been grown in axenic culture (Fulton, 1970), its genome still reflects its capacity to digest bacterial prey. (Acid)phosphatases, (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 glycogen 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 enzymes were found. The presence of a recently identified etherase (Veiga-da-Cunha et al., 2009) together with D-lactate dehydrogenase 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 D-lactate, whereas the dehydrogenase converts D-lactate to pyruvate.

Naegleria probably uses the disaccharide α,α-trehalose, a protectant 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 trehalose 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* (Nerad et al., 1983). Although a full set of enzymes of the glycolytic pathway is present (Fritz-Laylin et al., 2010), the pathway clearly differs from that found in most other eukaryotes (Fig. 1). First, *Naegleria* lacks the enzyme hexokinase as the first enzyme of the pathway. Instead it has a glucokinase gene homologue. Contrary to hexokinase, glucokinase is specific for glucose and does not phosphorylate other hexose sugars. This explains the presence of a separate fructokinase. 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 (PFK) is absent in *N. gruberi* and the second phosphorylation step of glycolysis is catalysed by a pyrophosphate- (PPi-)dependent PFK, 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_i plus AMP, rather than ADP, as phosphoryl 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 PP_i-dependent PFK 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 (Q27706) and therefore could either use this FBPase or the PPi-PFK for gluconeogenesis.

Interestingly, *N. gruberi* is capable of forming a potent regulator of glycolysis/gluconeogenesis, fructose 2,6-bisphosphate ($F2,6P_2$), as a phosphofructose-2-kinase, responsible for its synthesis is encoded in its genome. In most other eukaryotes $F2,6P_2$ regulates the activities of both ATP-dependent PFK and fructose-bisphosphatase, 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 PP_i-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-phosphate/glycerol-3-phosphate cycle seems to be operational, consisting 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 cytosolic NADH.

When oxygen is absent, the possibilities of reoxidation of cytosolic 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 D- 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 methylglyoxyl, formed by spontaneous dephosphorylation of glycolytic dihydroxyacetone-phosphate (DHAP), to D-lactate (Wyllie and Fairlamb, 2011). The presence of two of the three enzymes of the pathway, glyoxalase I (lactoyl-glutathionyl lyase) and glyoxalase II (hydroxyacyl glutathione hydrolase), 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 fermentation 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 dehydrogenase which may function as part of a hypothetical malate dismutation pathway (see Section 8.2).

3.1. Pentose-phosphate pathway

The pentose-phosphate shunt, or hexose-monophosphate pathway, generates NADPH for both biosynthetic purposes and oxidative 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 enzymes ribokinase and xylulokinase were detected, indicating the capacity to feed on sugars other than hexoses. In addition to plants and algae the genome of some unicellular eukaryotes, such as the Trypanosomatidae, ciliates and fungi, encode the Calvin-cycle enzyme sedoheptulose-1,7-bisphosphatase (Hannaert et al., 2003). Although it is not yet clear why these non-photosynthetic microbes have this enzyme, we searched for its presence in *Naegleria*, but no orthologous candidate gene was detected.

4. Peroxisomes

No morphological or biochemical evidence exists for the presence of peroxisomes in *Naegleria*. In general a peroxisomal 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

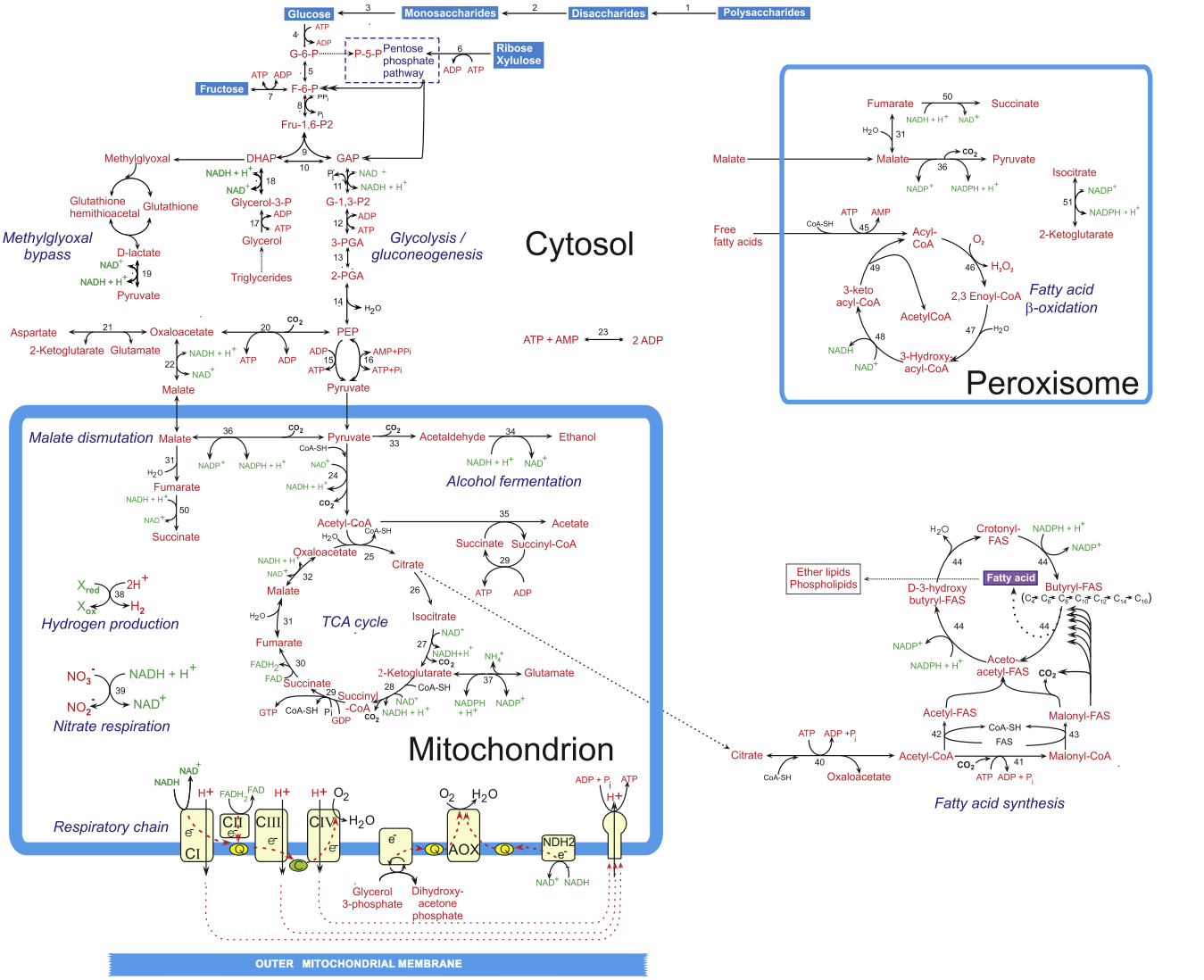

Fig. 1. Energy and intermediate metabolism in *Naegleria gruberi*. Boxed metabolites are nutrients (in blue). P5P, pentose 5-phosphate; ACP, acyl-carrier protein; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone-phosphate; GAP, glyceraldehyde 3-phosphate; G-1,3-P_2, glycerate 1,3-bisphosphate; 3-PGA, glycerate 3-phosphate; 2-PGA, glycerate 2-phosphate; PEP, phospho*enol*pyruvate; TCA, tricarboxylic acid; GTP, guanosine-triphosphate; GDP, guanosine-diphosphate; FAD, flavine adenine dinucleotide; $FADH_2$, reduced FAD; FAS, fatty acid synthase. Enzymes: 1, α- and β-amylase; 2, α- and β-glucosidase and glucoamylase; 3, sugar transporter; 4, glucokinase; 5, phosphoglucose 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, glycerol-3-phosphate dehydrogenase; 19, D-lactate dehydrogenase; 20, phosphoenolpyruvate carboxykinase; 21, aspartate aminotransferase; 22, malate dehydrogenase (cytosolic); 23, adenylate kinase; 24, pyruvate dehydrogenase; 25, citrate synthase; 26, aconitase; 27, isocitrate dehydrogenase (NAD); 28, 2-oxoglutarate dehydrogenase; 29, succinyl-CoA synthetase; 30, succinate dehydrogenase; 31, fumarate hydratase; 32, malate dehydrogenase (mitochondrial); 33, pyruvate decarboxylase; 34, alcohol dehydrogenase; 35, acetate:succinate CoA-transferase; 36; malic enzyme (decarboxylating); 37, glutamate dehydrogenase; 38, (Fe) hydrogenase; 39, nitrate reductase; 40, citrate lyase; 41, acetyl-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).

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 (H_2O_2) inside the organelles. However, although *Naegleria* has both a Cu/Zn- and a Mn-superoxide

Table 1
Predicted peroxisomal proteins in *Naegleria gruberi*. Peroxins or PEX proteins were identified by homology with corresponding peroxins in other organisms.

Peroxins	Uniprot accession
PEX1	D2VAR6
PEX3	D2VHW1
PEX4	D2V3A8
PEX5	D2V581
PEX6	D2UYE2
PEX7	D2VFM6
PEX10	D2VA32
PEX12	D2V791
PEX19	D2W353

Table 2
PTS1-containing enzymes in *Naegleria gruberi*.

Enzyme name	PTS1
Glutamine-dependent NAD synthase	−SKL
NADP-dependent malic enzyme	−SKL
Δ(3,5)-Δ(2,4)-Dienyl CoA isomerase	−NKL
Acetyl CoA synthase	−SKL
Acyl CoA dehydrogenase	−SKL
Fatty acid oxidation complex subunit alpha (trifunctional enzyme)	−SKL
DHAP acyltransferase/G3P acyltransferase	−SKL
Serine palmitoyltransferase	−SKM
NADP-dependent fumarate reductase	−SKL
NADP-isocitrate dehydrogenase	−SKL
2,4-Dienoly CoA reductase	−SKL
Retinal dehydrogenase	−NKL
3,2-Transenoyl CoA isomerase	−SKL
Monoamine oxidase	−SKL
Dihydroflavonol-4-reductase	−SKL

PTS1, peroxisomal targeting signal type 1, formed by the protein's last three C-terminal amino acids −SKL, or a variant thereof.

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, as well as the typical peroxisomal marker enzymes D-amino 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 and the corresponding enzyme trypanothione reductase (TR) has been reported for *N. fowleri* (Ondarza et al., 2006). However, when we used the sequence of the *Leishmania* TR in a reciprocal protein BLAST search against the *N. gruberi* proteome, only homologues of thioredoxin reductases or dihydrolipoamide dehydrogenase were detected. Also, a homologue of the trypanosome glutathionyl spermidine synthase, an enzyme involved in the synthesis of trypanothione in trypanosomes (Oza et al., 2002), was not detected. As all of these enzymes belong to a wide family of homologous proteins it cannot be excluded that by convergent evolution in *Naegleria* one of the other members of this family has 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 adenylosuccinate lyase, one of the 10 enzymes required to make inosine monophosphate (IMP) from phosphoribosyl pyrophosphate, was identified. However, this enzyme also plays a role in purine salvage in the purine-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 the bacteria on which it feeds. This is also suggested by the presence of a nucleoside transporter gene in its genome. Most of the enzymes for the necessary interconversion of purine bases and nucleosides are also present, indicating that purine-salvage mechanisms are operational.

7. Pyrimidine biosynthesis

The *N. gruberi* genome encodes all six enzymatic activities necessary for the synthesis of uridine monophosphate (UMP). The first three enzymes, carbamoyl-phosphate synthase (D2VZ04), aspartate transcarbamylase (D2VZY3) 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 dihydroorotate dehydrogenase (D2UZD6), 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, D2VRR8) 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 occured (Nara et al., 2000; Opperdoes and Michels, 2007). Finally, UMP may be converted to thymidine monophosphate (TMP) by a bifunctional dihydrofolate reductase–thymidylate synthase (DHFR–TS) enzyme (D2W4C0) also present in plants, alveolates and euglenozoa. In bacteria, animals and fungi these two activities are present on two separate genes (Stechmann and Cavalier-Smith, 2002).

8. Mitochondrial energy metabolism

The mitochondrion of eukaryotes 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 mitochondrion, 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* (Opperdoes 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 *N. 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 *N. gruberi* genome. Other reducing equivalents, such as

Table 3
Predicted composition of *Naegleria gruberi* mitochondrial complex I.

Peptide ID	Accession	Description	Peptide Mass (kDa)
NU1M|NuoH	AAG17776[a]	ND1, quinone and rotenone-binding sites	37.6
NU2M|NuoN	AAG17811	ND2	58.5
NU3M|NuoA	AAG17813	ND3	14.7
NU4M|NuoM	AAG17809	ND4	55.8
NU4LM|NuoK	AAG17780	4L	10.0
NU5M|NuoL	AAG17789	ND5	77.7
NU6M|NuoJ	AAG17793	ND6	24.0
NUCM|ndus2|NuoC	AAG17816	ND7, 4Fe-4S protein	45.5
NUIM|ndus8|NuoI|TYKY	AAG17788	ND8, 2 [4Fe-4S] N6a and N6b clusters	18.4
NUGM|ndus3|NuoD	AAG17821	ND9	22.8
NUAM|ndus1|NuoG	AAG17786	Subunit 11, [2Fe-2S] N1b, [4Fe-4S] N4 and [4Fe-4S] N5 clusters	81.7
NUBM|nduv1|NuoF	D2VGS8[b]	[4Fe-4S] cluster N3 and NAD and FMN binding sites	53.2
ACPM|SDAP	D2VIF8	Acyl carrier protein	13.2
NUEM|ndua9	D2VT52		38.7
NB4 M|ndua6	D2VT73	Alpha subcomplex subunit 6 (LYR protein)	20.7
NUHM|nduv2|NuoE	D2VLA0	[2Fe-2S] N1a cluster	29.4
NUKM|ndus7|NuoB|PSST	D2VFY8	[4Fe-4S] N2 cluster	21.1
ndua2	D2VMU6		14.7
ndua5	D2V410		20.0
ndua9	D2V7R4		42.2
nduac	D2V7S7		21.1
ndus4	D2VHM0		18.1
PL2	D2VQC1	UMP8 |Gamma carbonic dehydratase|plant2	28.1
PL6	D2VZA5	Adrenodoxin|ferredoxin|plant6	13.2

[a] GenBank accession number.
[b] Uniprot accession number.

$FADH_2$/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 (NDH2). The presence of an AOX explains why cyanide gives only partial inhibition of mitochondrial respiration (Weik and John, 1979) and therefore it is predicted that total inhibition can only be obtained 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 NDH2 and AOX, which is encoded by three homologous genes. A phylogenetic analysis showed that the *Naegleria* AOX sequences are equally distant from both plants and fungal AOX sequences. The *N. gruberi* NDH2 is most related to its homologs of *Dictyostelium* and the NDH1 and NDH2 of fungi. Mitochondrial oxidation of cytosolic NADH via a malate-aspartate shuttle over the mitochondrial membrane is suggested by the presence of multiple malate dehydrogenase and aspartate amino transferase genes. The functioning of such a shuttle in combination with a fully functional respiratory chain and an ATP synthase predicts a theoretical ATP yield similar to that of the mitochondria of higher eukaryotes. Thus, to summarise this section, *Naegleria* appears to encode everything that an obligate aerobe requires for oxygen-dependent growth.

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 dinitrification 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 reduced to succinate by the concerted action of fumarate hydratase and fumarate reductase. Indeed multiple isoenzymes of malate dehydrogenase, malic enzyme and NADH-dependent fumarate reductase were identified, with a mitochondrial transit peptide predicted for at least one of each. The acetyl-CoA produced in the oxidative branch may be converted via a cycle comprising acetate:succinate CoA-transferase (of family type 1B, homologous to the recently identified *Fasciola hepatica* enzyme by Van Grinsven et al., 2009; Tielens et al., 2010) and succinyl-CoA synthetase. The resulting acetate is excreted. This pathway leads to the production of 1 mol of ATP per mole of acetate, similar to what has been described for trichomonads (Steinbüchel and Müller, 1986) and trypanosomatids (Van Hellemond et al., 1998).

Interestingly the *N. gruberi* genome has a gene coding for the canonical hydrogenosomal hydrogenase (Fritz-Laylin et al., 2010, 2011; Ginger et al., 2010). Its sequence is most closely related

(43% and 50% identity, respectively) to the hydrogenase sequences of the rumen fungus *Neocallimastix frontalis* and the microaerophylic heterolobosean amoeboflagellate *Psalteriomonas lanterna* (Davidson et al., 2002; De Graaf et al., 2009). The *Naegleria* sequence has a large N-terminal extension with a mitochondrial targeting signal. It is surprising that a pyruvate:ferredoxin oxidoreductase (PFO), involved in the transfer of reducing equivalents from pyruvate to the enzyme hydrogenase and typically found in some anaerobic protists capable of producing molecular hydrogen, was not detected (Fritz-Laylin et al., 2010). Therefore, it is not clear which reducing equivalents are transferred to ferredoxin, the usual substrate of the mitochondrial hydrogenase (see Section 12.1).

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).

8.3. Iron–sulphur cluster synthesis

Iron–sulphur clusters are redox prosthetic groups for a wide range of proteins, many of which are localised in the mitochondrion (Lill and Mühlenhoff, 2008; Lill, 2009). The *Naegleria* genome appears to encode enzymes required for the synthesis of these molecules. There are a cysteine desulphurase which may be targeted to the mitochondrion, the mitochondrial iron chaperone frataxin, involved in iron–sulphur cluster regeneration and a mitochondrial iron–sulphur cluster NifU-like protein. They all suggest that the organelle is indeed involved in the generation of elemental sulphur to be used for incorporation into Fe–S cluster proteins.

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 tricarboxylic 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 lysophospholipase, 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 mitochondrially produced citric acid. 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 lipoic acid, an essential component of the pyruvate dehydrogenase 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 monooxygenase.

10. Amino acid metabolism

10.1. Amino acid catabolism

Full capacity for the catabolism of amino acids into TCA-cycle intermediates seems to be present in *Naegleria* (Fig. 2). Thus in the absence of carbohydrates, amino acids may serve as important energy substrates. Not only L-amino acids, but also their D-enantiomers present in the peptidoglycan or murein cell walls of bacteria (Vollmer et al., 2008; Silhavy et al., 2010), can be utilised as food sources, as are deduced from the presence of muramidase (lysozyme), alanine- and serine racemases and diaminopimelate (DAP) epimerase and decarboxylase (D2VIK3). The latter two enzymes convert the murein component DAP to lysine. D-Alanine (interconverted to L-alanine by the above racemase) and aspartate are transaminated, respectively to pyruvate and oxaloacetate, and then oxidised via the TCA cycle. Asparagine and glutamine are deaminated to aspartate and glutamate. After their conversion to, respectively, oxaloacetate and 2-ketoglutarate, they are also oxidised by the TCA cycle. Aspartate may also be converted to fumarate via the purine-nucleotide cycle. The enzymes of this cycle, adenylosuccinate synthase, adenylosuccinate lyase and AMP-deaminase, are all present (see Section 6).

Proline can be oxidised via a mitochondrial proline oxidase, followed by a Δ-1-pyrroline-5-carboxylate dehydrogenase, resulting in the formation of glutamic acid. An arginase converts arginine to ornithine and the latter is transaminated to glutamate 5-semialdehyde, which is converted to glutamate by the already mentioned Δ-1-pyrroline-5-carboxylate dehydrogenase. Histidine is also converted to glutamate, via the enzymes histidine aminolyase, uroconase, imidazolonepropionase and formimidoyl glutamase. Glycine can be split into carbon dioxide and formic acid by the action of the glycine cleavage system (GCS).

Methionine is first converted to cysteine and 2-ketobutyrate and the latter is converted in the mitochondrion via propionyl-CoA to the TCA-cycle intermediate succinyl-CoA. Cysteine is converted to pyruvate (see Section 10, under serine).

The branched amino acids isoleucine and valine, after their transamination in the cytosol to the corresponding 2-ketocarboxylic acids by a branched chain aminotransferase, can be further oxidised in the mitochondrion via a branched chain α-keto acid dehydrogenase and a hydratase, to succinyl-CoA and acetyl-CoA. The latter two compounds enter the TCA cycle or under anaerobic conditions can be converted to acetate and ATP via a cycle catalysed by succinyl-CoA synthetase and acetate:succinate CoA-transferase. Leucine is converted by a similar pathway to mitochondrial HMG-CoA followed by its cleavage into acetyl-CoA and acetoacetate.

Threonine is broken down either by threonine dehydrogenase to glycine and acetyl-CoA via the amino acetone pathway, or

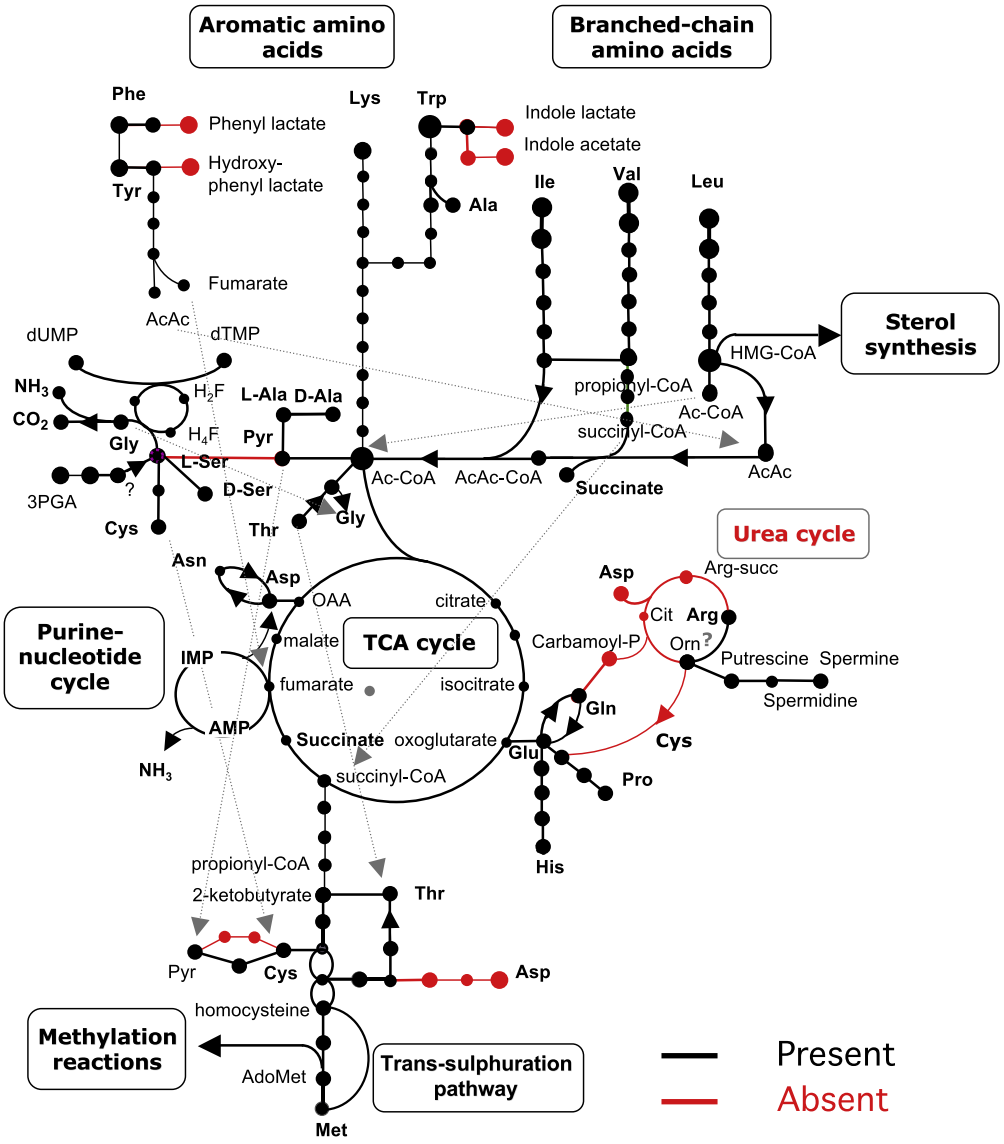

Fig. 2. Amino acid metabolism and some associated pathways in *Naegleria gruberi*. Metabolic steps indicated in black have been identified by the presence of the corresponding enzymes. Steps in grey represent reactions for which no enzymes could be identified. Question marks represent enzyme catalysed steps for which no unambiguous gene identification could be made. AcAc, acetoacetate; AdoMet, adenosylmethionine; H_2F, dihydrofolate; H_4F, tetrahydrofolate; HMG-CoA, hydroxymethylglutaryl coenzyme A; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; 3PGA, glycerate 3-phosphate; IMP, inosine monophosphate; TCA, tricarboxylic acid.

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, α-aminoadipate semialdehyde dehydrogenase (D2UYT1) and α-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-hydroxymuconic semialdehyde dehydrogenase, D2VJV7) 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 for further metabolism to acetyl-CoA via the intermediate mercaptopyruvate. Although no specific cysteine transaminase was detected a mercaptopyruvate sulphurtransferase, converting cyanide and mercaptopyruvate to pyruvate and thiocyanate, with an *E*-value of 6e-17 (D2VT08), was found in the genome.

Apart from arginase and ornithine decarboxylase 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.

10.2. Amino acid biosynthesis

Alanine, aspartate, asparagine, glutamate and glutamine are all formed by transamination of pyruvate, oxaloacetate and 2-ketoglutarate, respectively. Proline is formed from glutamate, since γ-glutamyl kinase and 1-pyrroline-5-carboxylate 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 D-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 *N. 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 D-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, while coenzyme A can be formed from pantothenic acid. Pyridoxin (vitamin B6) is converted to pyridoxal-phosphate and riboflavin (vitamin B2) to flavin mononucleotide. The presence of a gulonolactone 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 Fe^{2+} ion into preformed haeme, in agreement with the fact that *Naegleria* requires haematin 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 mitochondrial 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 mitochondrion/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 nitrate *Naegleria* has the capacity to break down carbohydrates to succinate, acetate and (possibly) molecular hydrogen and maybe minor quantities of ethanol and D-lactate. Formation of ethanol or D-lactate would result in the production of the limited amount of 2 mol of ATP per mole of hexose sugar consumed, while malate dismutation, leading to the secretion of succinate and acetate, via the acetate:succinate CoA-transferase/succinyl-CoA synthetase cycle, 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 fulfils such transfer, since in their absence the formation of H_2 from NADH + H^+ ($\Delta G^{\circ\prime}$ = +4.5 kcal/mol) is thermodynamically feasible only at extremely low partial pressures of H_2. Such a condition can only be generated if *Naegleria* would live in close association with methanogenic bacteria with a sufficiently high affinity for molecular hydrogen to keep the partial pressure of H_2 as low as 10^{-4} atm. (Gottschalk, 1985). Many anaerobic and microaerophilic hydrogenase-containing protists, such as those present in rumen, or trichomonads maintain in their cytoplasm H_2-consuming endosymbiotic bacteria. Indeed, there is some indication that an as yet unidentified *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 meningoencephalitis 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 ubiquinone, and the sterols lanosterol and ergosterol, in agreement with earlier observations (Raederstorff and Rohmer, 1987). Thus ergosterol, 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 ketoconazole, an inhibitor of sterol 14-demethylase (D2VC12), 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* (Visvesvara 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 (Vargas-Zepeda et al., 2005) and ketoconazole (Poungvarin 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 nitroreductase of type-I (NTR1, D2VQ76). Amongst eukaryotes NTR1 is further only present in the Trypanosomatidae, where it has been identified as the activator of the prodrugs benznidazole and nifurtimox 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 nifurtimox and other drugs belonging to the class of nitrofurans and nitroimidazoles 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 mitochondrial 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 (Opperdoes et al., 1976) and the antibiotic ascofuranon (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 trehalose formed during cyst formation? How are glycolysis and gluconeogenesis regulated by F2,6P_2? Which are the end-products of carbohydrate metabolism? Are there true peroxisomes? Does symbiosis of *Naegleria* with methanogenic bacteria result in the generation of molecular hydrogen? Just to mention a few.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.04.004.

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