Biochemical characterization of a mitochondrial-like organelle from Blastocystis sp. subtype 7

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A mitochondrial-like organelle (MLO) was isolated from isotonic homogenates of Blastocystis. The organelle sedimented at 5000 g for 10 min, and had an isopycnic density in sucrose of 1.2 g ml⁻¹. Biochemical characterization enabled the demonstration of several key enzymes that allowed the construction of a metabolic pathway consisting of an incomplete Krebs cycle linked to the oxygen-sensitive enzymes pyruvate : NADP⁺ oxidoreductase (PNO), acetate : succinate CoA transferase (ASCT) and succinate thiokinase (STK), which cumulatively are responsible for recycling CoA and generating ATP. The organelle differs from typical aerobic mitochondria in possessing an oxygen-sensitive PNO that can use FAD⁺ or FMN⁺ as electron acceptor but is inactive with NAD⁺. Spinacia oleracea ferredoxin or Clostridium pasteurianum ferredoxin. A gene with 77 % sequence similarity to the PNO mitochondrial precursor cluster from Euglena gracilis sp[Q941N5] was identified in the Blastocystis genome database. A second cluster with 56 % sequence similarity to the pyruvate : ferredoxin oxidoreductase (PFOR) from Trichomonas vaginalis was also identified, which is in agreement with the concept that the PNO gene arose through the fusion of a eubacterial gene for PFOR with the gene for NADPH : cytochrome p450 reductase. Hydrogenase activity was not detected under the conditions used in this study. The Blastocystis oranelle therefore demonstrates significant biochemical differences from traditional mitochondria and hydrogenosomes, but possesses features of both. Based upon the results of this study, the Blastocystis organelle falls into the category of a MLO.

INTRODUCTION

The protozoon Blastocystis is an inhabitant of the intestinal tract of mammals and some reptiles. Blastocystis is the most frequently reported protozoan in the human intestinal tract (Lavier, 1952; Lee, 1991) and has been reported to cause intestinal disease in a number of cases, resulting in abdominal cramps, diarrhoea and significant discomfort (Weg et al., 1987). In some cases the parasite has been reported to cause acute illness (Russo et al., 1988; Vannatta et al., 1985) and can be life threatening when present in immunocompromised individuals (Prasad et al., 2000). Endoscopic and permeability studies of the intestine in a cohort study of Blastocystis-infected immunocompetent individuals failed to find any significant intestinal inflammation or impairment of the intestinal permeability barrier (Zuckerman et al., 1994). Fresh Blastocystis isolates, though, have been demonstrated to cause significant cytopathology of Chinese hamster ovary cells (CHO), adenocarcinoma HT-29 cells and rat intestinal epithelia (IEC-6) cells in culture (Puthia et al., 2006; Walderich et al., 1998). Metronidazole, paromomycin and ketoconazole have been used as therapeutic agents with mixed success (Zierdt et al., 1983; Dunn & Boreham, 1991; Haresh et al., 1999; Nanba et al., 2003). Although there have been some advances made concerning the morphology and cytochemistry of the organism, little is known of the biochemistry. The presence of mitochondrial-like organelles (MLO) has been inferred, based largely upon microscopic evidence (Zierdt et al., 1988); however, key mitochondrial proteins such as Krebs cycle enzymes and cytochromes were not detected (Zierdt, 1986; Zierdt et al., 1988). These studies were hampered by difficulties in obtaining large amounts of the organism and problems associated with obtaining pure organelles such as MLO, which tend to clump upon fractionation (Zierdt et al., 1988). Nasirudeen & Tan (2004) described a method for the
isolation of MLO from this organism and observed the presence of DNA in the organelles. Adaptation to anaerobic environments has resulted in the evolution of modified energy-generating organelles, such as hydrogenosomes in the trichomonads (Lindmark & Müller, 1973, 1974), rumen protists (Yarlett et al., 1981), rumen fungi (Yarlett et al., 1986) and protists inhabiting the cockroach hind gut (Akhanova et al., 1998). Other recently described modified mitochondria include the mitosome of Entamoeba (Tovar et al., 1999), the cryptic organelle of Giardia sp. (Martí et al., 2003), the relit organelle in Cryptosporidium parvum (Keithly et al., 2005) and the microsporidians Antonospora locustae and Encephalitozoon cuniculi (Burri et al., 2006). These organelles have one or more of the following features in common with the mitochondrion – recognition of specific targeting sequences (Dacks et al., 2006), production of ATP (Embley et al., 2003; van Weelden et al., 2005) and iron–sulfur cluster assembly (Tachezy et al., 2001) – leading to the suggestion that these organelles all arose from a common ancestor. In this study we demonstrate the presence of key mitochondrial enzymes in Blastocystis and show they are localized in organelles previously described as MLO.

METHODS

Culture and preparation of homogenates. Blastocystis isolate B, designated Blastocystis sp. group VII (Noel et al., 2005) or subtype 7 (Stensvold et al., 2007) in later phylogenetic or terminology standardization studies, respectively, was used. The parasite was grown in 10 ml pre-reduced Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated horse serum. Cultures were incubated for 48 h in anaerobic jars using an Oxoid nitrogen before centrifugation at 500 g for 20 min using a Sorvall RC-2B. The supernatant was further fractionated by centrifugation to give a large granular fraction (5000 g for 10 min) and a small granular fraction (25 000 g for 20 min) and a final supernatant. Density gradients were prepared from 15 and 65% (w/w) sucrose in 10 mM potassium phosphate, 74 mM NaCl, 0.6 mM CaCl2 and 1.6 mM KCl, pH 7.4 and resuspended in 50 mM Tris/HCl (pH 7.2).

Fractionation. Cells were broken under a stream of nitrogen gas by a large granular fraction (4 mg protein) and the small granular fraction (2 mg protein) were carefully layered on top of the gradients and centrifuged in a 6×12 ml swing-out rotor (Beckman OTD 65 ultracentrifuge) at 4 °C for 30 min at 46 000 g. Fractions (approx. 2 ml) from the gradient were collected under nitrogen and assayed immediately for enzyme activity. The density of the fractions was determined using a refractometer.

Enzyme analysis

Unless stated otherwise, enzyme assays were performed under anaerobic conditions using buffers, substrates and cofactors that had the air removed under vacuum and replaced with nitrogen. Cuvette additions were made in an anaerobic chamber (Coy Laboratories) and Blastocystis protein (40–60 μg) was added to start the reaction by injection through a rubber septum.

(a) Enzymes characteristic of hydrogenosomes. With the exception of formate dehydrogenase, assays performed for these enzymes used 40 μg Trichomonas vaginalis protein obtained as described by Lindmark & Müller (1973), as a positive control. Hydrogenase (EC 1.12.7.2) and pyruvate:ferredoxin oxidoreductase (PFOR; EC 1.2.7.1) were assayed by measuring the change in absorbance at 600 nm due to the reduction of methyl viologen [extinction coefficient (ε)=8.25 mM−1 cm−1] as described by Lindmark & Müller (1973). Hydrogenase was assayed using 100 mM potassium phosphate (pH 7.4), 20 mM methyl viologen and 250 mM 2-mercaptoethanol under a stream of hydrogen. PFOR was assayed using 100 mM potassium phosphate (pH 7.4), 20 mM methyl viologen, 250 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.25 mM coenzyme A (CoA) and 2.5 mM pyruvate. Malic enzyme (EC 1.1.1.40) was assayed using the method of Lindmark & Müller (1974) in 6 mM triethanolamine (pH 6.8), 1 mM NADP+, 0.66 mM MnCl2 and 0.1% Triton X-100. The reaction was started by the injection of 33 mM malate through a rubber septum and the change in absorbance at 340 nm (ε NADP+=6.3 mM−1 cm−1) was measured. Succinate thiokinase (STK; EC 6.2.1.5) was assayed as described by Lindmark & Müller (1974) in Tris/succinate buffer (pH 7.4), 0.1 mM MgCl2, 1 mM CoA and 1 mM ATP. The reaction was started by the addition of protein and the change in absorbance at 235 nm (ε ATP=40 mM−1 cm−1) compared to controls lacking ATP. Formate dehydrogenase (EC 1.2.1.2) was determined by the method of Naniba et al. (2003). The assay contained 40 mM phosphate buffer (pH 7.2), 0.5 mM sodium formate (pH 7.0), 5 mM NAD+, and protein to start the reaction. The absorbance change at 340 nm (ε NAD+=6.3 mM−1 cm−1) was recorded for 10 min at 30 °C.

(b) Enzymes characteristic of mitochondria. With the exception of pyruvate: NADP+ 2-oxo-oxidoreductase (PNO), assays for this group of enzymes used 30 μg rat liver homogenate (Fansler & Lowenstein, 1969) as a positive control, PNO (EC 1.2.1.51) was determined in the forward direction using the method of Inui et al. (1987). The assay consisted of 100 mM potassium phosphate (pH 7.4), 2.5 mM pyruvate, CoA, 1 mM NADP+. The change in absorbance at 340 nm was measured at 37 °C for 10 min (ε NADP+=6.3 mM−1 cm−1). The activity of PNO was also determined by replacing NADP+ with 1 mM FAD+ (ε FAD+=11.7 mM−1 cm−1 at 446 nm), 1 mM FMN+ (ε FMN+=12.5 mM−1 cm−1 at 450 nm), 0.5–5.0 mM NAD+ (ε NAD+=6.3 mM−1 cm−1 at 340 nm), 0.1–1.0 mg Chlorotrichia pseudariae ferredoxin (ε FMN+=17.6 mM−1 cm−1 at 390 nm) or 0.1–1.0 mg Spinacia oleracea ferredoxin (ε Fe–2S ferredoxin=10.8 mM−1 cm−1 at 425 nm) as electron acceptor. Citrate synthase (EC 2.3.3.1) was assayed under aerobic conditions as described by Parvin (1969) using 2.5 mM 5',5'-dithiobis-2-nitrobenzoic acid (DTNB) dissolved in 20 mM Tris/HCl (pH 8.0) as indicator (ε DTNB=13.6 mM−1 cm−1 at 414 nm). The reaction contained 0.1 M Tris (pH 8.0), 0.25 mM DTNB, 0.2 mM oxaloacetate, 0.1 mM acetyl-CoA and Blastocystis protein. The change in absorbance was monitored at 414 nm for 10 min. Aconitate (EC 4.2.1.3) was determined by the method of Fansler & Lowenstein (1969). The homogenate was activated by incubation under nitrogen for 30 min at 30 °C with a 1:1 mixture of protein and 10 mM thiamol containing 2 mM ferrous ammonium sulfate in Tris (pH 7.8), previously made anaerobic. The assay contained 20 mM Tris (pH 7.6), 50 mM NaCl, 2 mM cis-aconitate, and protein in a final volume of 1 ml. The change in absorbance was recorded at 240 nm (ε cis-aconitate=3.5 mM−1 cm−1) for 15 min at 37 °C. Isocitrate dehydrogenase NADP+ (EC 1.1.1.42) was assayed using the coupled assay described by Cook & Sanwal (1969). The assay
Enzyme analysis

In an attempt to characterize the MLO from Blastocystis, whole-cell extracts and subcellular fractions were examined for activity of enzymes characteristic of mitochondria, hydrogenosomes and other MLO. Two of the enzymes characteristic of hydrogenosomes, PFOR and hydrogenase, were undetectable in whole-cell extracts of Blastocystis (limits of detection 2 nmol min⁻¹) assayed under anaerobic conditions and using a pH of 5.4–7.6. Using T. vaginalis as a positive control, the activity of these enzymes was consistent with the published specific activities (Lindmark & Müller, 1973). Malic enzyme was detected using NADP⁺ as electron acceptor with a specific activity consistent with that found in other organisms (Lindmark et al., 1989; Yarlett et al., 1981). The activity of this and other NAD⁺- and NADP⁺-utilizing enzymes was consistently higher when assayed under anaerobic conditions (described in Methods) due to recycling of the reduced nucleotide by NADH/NADPH oxidase. The activity of NAD⁺- and NADP⁺-utilizing enzymes presented in Table 1 were therefore determined under an oxygen-free atmosphere. The activity of an enzyme converting pyruvate to acetyl-CoA was detected in Blastocystis extracts using NADP⁺ as an electron acceptor. The enzyme was characterized based upon substrate and cofactor requirements to be PNO (EC 1.2.1.51). The Blastocystis PNO could use FAD⁺ (5 mM) or FMN⁺ (5 mM) as an electron acceptor at 30% and 74%, respectively, of the rate observed with NADP⁺ (1 mM). The enzyme was, however, inactive using 0.5–5.0 mM NAD⁺, 0.1–1.0 mg Clostridium pasteurianum (4Fe–4S) or 0.1–1.0 mg Spinacia oleracea (2Fe–2S) ferredoxin as electron acceptor. The enzyme was 100% inhibited by incubation under a stream of air for 5 min, and 60% inhibited by incubation under a
stream of CO$_2$ for 5 min compared to controls measured under N$_2$. The enzyme activity could also be determined using methyl viologen as electron acceptor, which resulted in 30% of the activity obtained using 0.1 mM NADP$^+$. The acetyl-CoA formed by PNO was converted to acetate by ASCT (EC 2.8.3.8). The activity of the Blastocystis ASCT was confirmed using extracts of the procyclic stage of Trypanosoma brucei brucei as positive control. The presence of some of the enzymes characteristic of the Krebs cycle were determined using whole-cell extracts of Blastocystis and compared to a rat liver homogenate as a positive control. Citrate synthase (EC 2.3.3.1) and succinate dehydrogenase (EC 1.3.99.1) were not detected using a pH range of 5.7–7.6 (limits of detection 2 nmol min$^{-1}$). However, other Krebs cycle enzymes were present at levels comparable to control cells; aconitase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.42), $\alpha$-ketoglutarate dehydrogenase (EC 1.2.4.2), STK (EC 6.2.1.5), fumarate hydratase (EC 4.2.1.2) (Table 1), enabling construction of a partial pathway leading from the conversion of citrate to succinyl-CoA (Fig. 1).

### Subcellular localization

Sedimentation of Blastocystis using increasing centrifugal force to produce a large granular fraction (5000 g for 10 min), a small granular fraction (25 000 g for 20 min) and a non-sedimentable fraction was performed under strict anaerobic conditions. The subcellular fractions were analysed for activity of the enzymes (Table 1). Integrity of organelles was confirmed by latency of enzyme activities using 2% Triton X-100. Malic enzyme, PNO and ASCT had 60%, 92% and 83% increased activity with the addition of Triton X-100. Based upon the ratio of the

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**Table 1. Activity of enzymes in homogenates of Blastocystis**

Enzymes were assayed as described in Methods. ND, No detectable activity within the limits of detection stated in Results. MLO, SGF and cytosol indicate activity recovered in the fractions identified as the mitochondria-like organelle, small granular fraction and cytosolic, respectively. Genomic information was obtained from the Blastocystis genome database (http://pepdb.pub.bcm.umontreal.ca/pathway//BH/server.html). Activity is presented as the mean ± SD of triplicate determinations. One unit of enzyme activity represents 1 nmol substrate converted to product in 1 min.

**SDH, succinate dehydrogenase; GPDH, glyceraldehyde-3-phosphate dehydrogenase.**

<table>
<thead>
<tr>
<th align="left">Enzyme Activity [units (mg protein)$^{-1}$]</th>
<th align="left">Gene ID</th>
<th align="left">Gene name</th>
<th align="left">Localization</th>
</tr>
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<tbody>
<tr>
<td align="left">*Malic enzyme (EC 1.1.1.40)</td>
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<td align="left">MLO</td>
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<td align="left">BHL00000444/BHL0000306</td>
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<td align="left">*ASCT (EC 2.8.3.8) acetyl-CoA hydrolase/ transferase related cluster</td>
<td align="left">50.8 ± 5.3</td>
<td align="left">BHL00000508/BHL00000285</td>
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<tr>
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<td align="left">BHL00000157/BHL00000234</td>
<td align="left">MLO</td>
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<tr>
<td align="left">*$\alpha$-Ketoglutarate dehydrogenase (EC 1.2.4.2) Dihydrolipoamide DH-related cluster (EC 1.8.1.4)</td>
<td align="left">29.8 ± 4.9</td>
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<tr>
<td align="left">*Dihydrolipoyl DH-related cluster</td>
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<td align="left">MLO</td>
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<td align="left">Cytosol</td>
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<td align="left">MDHM</td>
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<td align="left">Pyruvate kinase (EC 2.7.1.40)</td>
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<td align="left">pckA</td>
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<td align="left">*Lactate dehydrogenase (EC 1.1.1.27)</td>
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<td align="left">MAN2B1; MA2B1; MANA</td>
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</tbody>
</table>

*Enzymes assayed under oxygen-free conditions as described in Methods.*
specific activity of the enzyme in the fraction to that in the homogenate (relative specific activity), it was concluded that malic enzyme, PNO, ASCT, STK, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase, (7) aconitase, (8) fumarate hydratase, (9) malate dehydrogenase, (10) PEPCK, (11) pyruvate kinase, (12) lactate dehydrogenase. Proposed end products of the pathway are in bold. Reactions shown within the box occur in the large granule fraction which contains the MLO.

**DISCUSSION**

The results of this study demonstrate that the MLO from Blastocystis lack a typical pyruvate dehydrogenase; instead they possess an oxygen-sensitive PNO which produces acetyl-CoA from pyruvate. This enzyme has similar properties to that previously described for the pyruvate-metabolizing enzyme from the mitochondrion of Euglena gracilis (Inui et al., 1987) and Cryptosporidium parvum (Rotte et al., 2001). These biochemical findings are in agreement with a search of the Blastocystis EST database (http://pepdbpub.bcm.umontreal.ca/pathway/BH/server.html) that reveals the presence of a gene with 77% sequence identity to the PNO mitochondrion precursor cluster from E. gracilis sp[Q941N5] (accession number BHL00002376). The PNO gene from E. gracilis is believed to have arisen through fusion of a eubacterial gene for PFOR with the gene for NADPH cytochrome p450 reductase (Rotte et al., 2001). In agreement with these findings, a second cluster with 56% sequence identity to the PFOR cluster typical of T. vaginalis (accession number BHL00001771) was also found, although activity of this enzyme could not be demonstrated. In common with other PNOs, the enzyme was oxygen sensitive, being completely inactivated when cell-free extracts were exposed to air. The Blastocystis PNO was capable of transferring electrons to FAD+ or FMN+ at 30% and 74%, respectively, of the rate observed with NADP+; several other electron acceptors, including NAD+ and Clostridium or spinach ferredoxin, were inactive. In hydrogenosomes the electron acceptor is a 2 iron:2 sulfur ferredoxin (Gorrell et al., 1984), whereas in mitochondria the electrons are transferred from the NADH to the cytochrome chain, resulting in the formation of ATP (Krayl et al., 2007). The electron transport proteins in Blastocystis are unknown, but may be similar to the NADPH:cytochrome p450 reductase in Euglena and Cryptosporidium parvum (Rotte et al., 2001) or the flavoprotein:rhodoquinone complex as described...
in the anaerobic mitochondria of parasitic nematodes (Ma et al., 1993). The efficacy of metronidazole in the treatment of Blastocystis infections indicates the presence of low-potential electron-acceptor proteins in this parasite. Typically, metronidazole reduction involves the transfer of electrons from pyruvate by the action of PFOR (Edwards, 1993). The ability of PNO to reduce metronidazole has not been definitively demonstrated but the enzyme may function in this capacity. It has been demonstrated that the electrons for metronidazole activation by T. vaginalis can originate from malate which is oxidatively decarboxylated by malic enzyme (Hrdy et al., 2005). The electrons released from malate reduce NAD$^+$ and are subsequently transferred to ferredoxin by the NADH:ferredoxin oxidoreductase activity of the NADH dehydrogenase module of mitochondrial respiratory complex I (Hrdy et al., 2004). Future studies will be aimed at identifying the terminal electron acceptor involved in metronidazole reduction by Blastocystis.

The presence of ASCT and STK results in the formation of acetate and ATP. In addition, an incomplete Krebs cycle was detected that results in the formation of succinyl-CoA. The proposed metabolic pathway (Fig. 1) retains key features of typical mitochondria and hydrogenosomes that couple the energy of the thioester bond of CoA to the generation of ATP. The inability to detect Krebs cycle enzymes in earlier seminal studies of Blastocystis by C. H. Zierdt (Zierdt, 1986; Zierdt et al., 1988) that paved the way for these and other studies of this parasite may be due to oxygen inactivation of enzymes involved in the pathway during processing of homogenates.

In common with other anaerobic protists (Lindmark et al., 1989), the fumarate hydratase of Blastocystis is of low activity and localized solely in the cytosol. There are two distinct classes of fumarate hydratase. Class I enzymes are of α-proteobacterial origin and include fumA and fumB; these are homodimeric, thermolabile, iron–sulfur enzymes with a molecular mass of 120 kDa (Woods et al., 1988; Gerbod et al., 2001). Class II enzymes are of archaeal origin (fumC) and are homotetrameric, thermostable, iron-independent enzymes with a molecular mass of 200 kDa (Colombo et al., 1994; Gerbod et al., 2001). They are both present in eukaryotic cells, but are differentially localized with class I fumarate hydratase present in the mitochondria (Woods et al., 1988), whereas class II enzymes are localized in the cytosol (Suzuki et al., 1989). It is unknown what type of fumarate hydratase is present in Blastocystis, but a
search of the *Blastocystis* database (http://pepdbpub.bcm.umontreal.ca/pathway/BH/server.html) reveals the presence of several genes with homology to both class I fumarate hydratases (accession numbers BHL00001095, BHL00001685, BHL00001701) and class II fumarate hydratases (accession numbers BHL00001811, BHL00001165). The *Trichomonas fetus* fumarate hydratase has been shown to be of the class II type, leading to the suggestion that they have a different origin from those of mitochondria-containing cells (Gerbod et al., 2001).

The transformation of energy-rich substrates such as glucose to metabolic end products involves multiple steps that can vary considerably between different organisms. However, there are certain key points that are common to all, such as the steps involving electron transfer and the conservation of high-energy bonds for the ultimate formation of ATP. To this end a significant metabolic feature of the proposed pathway in the *Blastocystis MLO* is the transformation of pyruvate to acetate and the conservation of the high-energy thioester bond to form...
ATP. In common with trichomonad and Neocallimastix hydrogenosomes (Steinbuelch & Muller, 1986; Yarlett et al., 1986), and mitochondria from the promastigote Leishmania mexicana, Leishmania infantum, Phytomonas sp. and procyclic Trypanosoma brucei, as well as the parasitic helminth Fasciola hepatica (Van Hellemont et al., 1998), Blastocystis utilizes a two-step mechanism that couples succinate: succinyl CoA cycling with acetate formation to conserve the energy of the thioester bond to drive subcellular ATP formation (Fig. 1). This finding is consistent with the proposed common origin of hydrogenosomes and mitochondria (Martin, 1999). The absence of detectable succinate dehydrogenase activity suggests that the primary function of the partial Krebs cycle in Blastocystis is to provide precursors for the synthesis of amino acids or acetyl-CoA for fatty acid biosynthesis as occurs in E. gracilis (Hoffmeister et al., 2005). The presence of a partial Krebs cycle has been reported in the mitochondria from procyclic Trypanosoma brucei (Van Hellemont et al., 2005; van Weelden et al., 2005), which is proposed to function to export acetyl-CoA to the cytoplasm for fatty acid biosynthesis. The enzyme transferring the CoA moiety from acetyl-CoA to succinate in T. vaginalis hydrogenosomes is ASC, which has been identified in the T. vaginalis genome (van Grinsven et al., 2008). The enzyme has high similarity to Saccharomyces cerevisiae acetyl-CoA hydrolase and Clostridium kluveri succinyl-CoA:CoA-transferase (van Grinsven et al., 2008). A BLAST search (http://tbestdb.bcm.umontreal.ca searches/login.php) of the Blastocystis database using the ASCT sequence from T. vaginalis G3 (EST DS113258) reveals the presence of a protein with 78% identity (BHL 0000444) to the T. vaginalis protein.

The adaptability of the mitochondrion is evident from the literature and we therefore conclude that, despite the lack of cytochromes or other identifiable electron-transport proteins, the organelle from Blastocystis, which is characterized as a DNA-containing, double-membrane-bound structure with a partial Krebs cycle and an active ATP-generating metabolism, is best described as an MLO.

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