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

Yelena Lantsman,^{1†} Kevin S. W. Tan,³ Mary Morada¹ and Nigel Yarlett^{1,2}

Correspondence

Nigel Yarlett

nyarlett@pace.edu

¹Haskins Laboratories, Pace University, New York, NY 10038, USA

²Department of Chemistry and Physical Sciences, Pace University, New York, NY 10038, USA

³Laboratory of Molecular and Cellular Parasitology and Infectious Disease Programme, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 117597, Singapore

A mitochondrion-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 mitochondrion 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* organelle 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.

Received 22 February 2008

Revised 10 April 2008

Accepted 14 April 2008

INTRODUCTION

The protozoon *Blastocystis* is an inhabitant of the intestinal tract of mammals and some reptiles. *Blastocystis* is the most frequently reported protozoon in the human intestinal tract (Lavie, 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 mitochondrion-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

[†]Present address: The University of Moscow Medical School, Moscow, Russia.

Abbreviations: ASCT, acetate, succinate CoA transferase; CoA, coenzyme A; MLO, mitochondrion-like organelle(s); PEPCK, phosphoenolpyruvate carboxykinase; PFOR, pyruvate, ferredoxin oxidoreductase; PNO, pyruvate, NADP⁺ oxidoreductase; STK, succinate thiokinase.

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 (Akhmanova *et al.*, 1998). Other recently described modified mitochondria include the mitosome of *Entamoeba* (Tovar *et al.*, 1999), the cryptic organelle of *Giardia* sp. (Marti *et al.*, 2003), the relict organelle in *Cryptosporidium parvum* (Keithly *et al.*, 2005) and the microsporidians *Antonosporea 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 (Noël *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 Anerogen pack at 37 °C. Two-day-old cultures were centrifuged at 1600 g for 10 min, washed once in a buffer consisting of 30 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 40 strokes in a 10 ml Potter–Elvehjem tissue homogenizer at 4 °C in an isotonic buffer consisting of 200 mM sucrose (pH 7.2) containing 30 mM phosphate, 15 mM mercaptoethanol, 30 mM NaCl, 0.6 mM CaCl₂ and 0.6 mM KCl. The broken cells were diluted with isotonic buffer that had been degassed under vacuum and the air replaced with nitrogen before centrifugation at 500 g for 10 min using a Sorvall RC-2B. The supernatant was further fractionated by centrifugation to give a large granular fraction (5000 g for 10 min), 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 Tris/HCl buffer (pH 7.2), containing 0.7 mM EDTA and 15 mM mercaptoethanol. The 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) 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 Nanba *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-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 NAD⁺ = 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 *Clostridium pasteurianum* ferredoxin (ϵ 4Fe–4S ferredoxin = 17.6 mM⁻¹ cm⁻¹ at 390 nm) or 0.1–1.0 mg *Spinacia oleracea* ferredoxin (ϵ 2Fe–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. Aconitase (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 thiomalate 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

contained 0.16 M Tris/acetate (pH 7.6), 0.18 mM dichlorophenolindophenol ($\epsilon=34.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 600 nm), 0.5 mM NADP^+ , 0.27 mM AMP, 0.83 mM isocitrate (pH 7.6) and protein to start the reaction. Blanks lacking enzyme were subtracted from the rate. The decrease in absorbance at 600 nm was measured for 10 min. α -Ketoglutarate dehydrogenase (EC 1.2.4.2) was assayed as described by Sanadi (1969), and contained 60 mM phosphate buffer (pH 7.6), 0.10 mM CoA, 35 mM cysteine (pH 7.0), 0.35 mM NAD^+ ($\epsilon=6.3 \text{ mM}^{-1} \text{ cm}^{-1}$) and 0.5 mM α -ketoglutarate. The absorbance change at 340 nm was monitored for 10 min after the addition of protein. Acetate:succinate CoA transferase (ASCT; EC 2.8.3.8) activity was using the radioactive method described by Van Hellemond *et al.* (1998), in a mixture containing 50 mM succinate (pH 7.4), 1 mM $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ (0.2 MBq), 50 mM Tris/HCl (pH 7.4), 10 mM MgCl_2 and 0.05 % (v/v) Triton X-100. The reaction was started by the addition of protein and incubated for 20 min at 30 °C. The reaction was stopped with 10 % trichloroacetic acid and cooled on ice. The $[1\text{-}^{14}\text{C}]\text{acetate}$ was separated by HPLC using a reverse-phase C-18 Percosil column ($4.6 \times 250 \text{ mm}$), 10 μm particle size (Perkin Elmer), with a linear gradient starting with 80 % 0.1 M NaH_2PO_4 (pH 3.25) containing 8 mM octanesulfonic acid changing to 100 % 0.1 M NaH_2PO_4 (pH 5.5) containing 8 mM octanesulfonic acid and 75 % (v/v) methanol in 30 min. Signals were recorded and integrated using a β -Ram model-2 radiometric detector (IN/US Inc.). Malate dehydrogenase (EC 1.1.1.37) was assayed by measuring the decrease in absorbance at 340 nm due to NADH oxidation ($\epsilon \text{ NAD}^+=6.3 \text{ mM}^{-1} \text{ cm}^{-1}$) with oxaloacetate. The assay contained 50 mM triethanolamine.HCl (pH 7.4), 5 mM EDTA, 0.12 mM oxaloacetate (pH 7.0), 0.15 mM NADH, and was started by addition of protein (Englard, 1969). Fumarate hydratase (EC 4.2.1.2) was assayed under aerobic conditions as described by Genda *et al.* (2006) in 100 mM phosphate buffer (pH 7.6), 50 mM malate and protein to start the reaction. The absorbance change at 250 nm ($\epsilon=1.45 \text{ mM}^{-1} \text{ cm}^{-1}$) was monitored for 10 min. Succinate dehydrogenase (EC 1.3.99.1) was assayed using 3.8 mM dichlorophenolindophenol ($\epsilon=34.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 600 nm), 50 mM phenazine methosulfate, 20 mM KCN, in 100 mM potassium phosphate buffer (pH 7.4), and protein (Singer & Kearney, 1963). Sodium succinate (200 mM) was added to start the reaction and the absorbance at 600 nm monitored for 10 min at 37 °C.

(c) Cytosolic enzymes. Assays for this group of enzymes used 40 μg of *T. vaginalis* protein obtained as described by Lindmark & Müller (1973), as a positive control. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was assayed by measuring the change in absorption at 340 nm due to the reduction of 0.5 mM NAD^+ ($\epsilon=6.3 \text{ mM}^{-1} \text{ cm}^{-1}$) with 0.1 mM glyceraldehyde 3-phosphate in 0.14 M phosphate buffer (pH 7.6) (Duggleby & Dennis, 1974). Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Yarlett *et al.* (1986) using 25 mM phosphate buffer (pH 7.4), 0.1 mM NADH ($\epsilon=6.3 \text{ mM}^{-1} \text{ cm}^{-1}$), 0.5 mM pyruvate (pH 7.0). The absorbance change at 340 nm was monitored for 10 min. Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) activity was determined under aerobic conditions by the method of Fukuda *et al.* (2004) using 50 mM NaHCO_3 , 5 mM MnCl_2 , 10 mM phosphoenolpyruvate (PEP), and 2 mM GDP in 100 mM imidazole buffer (pH 7.2). The reaction was started by addition of protein and incubated at 37 °C for 30 min, stopped by addition of 50 mM EDTA and cooled to 4 °C. The oxaloacetate formed was determined by measuring the absorbance change at 340 nm due to the addition of 0.2 mM NADH ($\epsilon=6.3 \text{ mM}^{-1} \text{ cm}^{-1}$) and 3 units of yeast malate dehydrogenase in 100 mM imidazole buffer (pH 7.2). Pyruvate kinase (EC 2.7.1.40) was assayed under aerobic conditions by measuring the ATP produced from ADP (0.1 mM) in 80 mM triethanolamine buffer (pH 7.5) containing 1.3 mM EDTA, 2.0 mM MgSO_4 and 1.0 mM PEP. Protein was added to start the reaction and

the ATP formed after 15 min was quantified using a luminometer and 0.1 mg firefly luciferase (Boyer, 1962).

(d) Enzymes characteristic of lysosomes. Assays for this group of enzymes used 30 μg rat liver homogenate (Fansler & Lowenstein, 1969) as a positive control. α -Galactosidase (EC 3.2.1.22) was assayed under aerobic conditions by measuring the hydrolysis of 0.1 mg *o*-nitrophenyl α -D-galactopyranoside (ϵ for *p*-nitrophenol at 400 nm= $17.5 \text{ mM}^{-1} \text{ cm}^{-1}$) in 0.1 M acetate buffer (pH 4.5) and protein for 10 min at 37 °C. The reaction was stopped using 1 ml 1 M borate (pH 10) and the absorbance at 420 nm recorded (Borooah *et al.*, 1961). α -Mannosidase (EC 3.2.1.24) was determined under aerobic conditions using a fluorescent assay containing 10 mM 4-methylumbelliferyl α -D-mannopyranoside in 25 mM sodium acetate (pH 4.0) and protein for 10 min at 37 °C. The assay was stopped with 2 ml 0.25 M glycine/KOH (pH 10.3) and the fluorescence determined using an excitation wavelength of 355 nm and an emission wavelength of 460 nm and compared to a 4-methylumbelliferone standard (Prencz & Natowicz, 1992).

Protein determination. Protein was determined by the Lowry method.

RESULTS

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

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://pepdpub.bcm.umontreal.ca/pathway//BH/ server.html>). Activity is presented as the mean $\pm$ 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.

Enzyme	Activity [units (mg protein) ⁻¹]	Gene ID	Gene name	Localization
*Malic enzyme (EC 1.1.1.40)	28.9 $\pm$ 1.7			MLO
*PNO (EC 1.2.1.51)	82.4 $\pm$ 6.1	BHL00002376/BHL00001250	PNO/Pyr fd/fld oxred	MLO
*ASCT (EC 2.8.3.8) acetyl-CoA hydrolase/transferase related cluster	50.8 $\pm$ 5.3	BHL00000444/BHL00000306	ACHT	MLO
*STK (EC 6.2.1.5)	317. $\pm$ 11	BHL00000088/BHL00000825	SUCLA2	MLO
* α -Ketoglutarate dehydrogenase (EC 1.2.4.2)	29.8 $\pm$ 4.9			MLO
Dihydrolipoamide DH-related cluster (EC 1.8.1.4)		BHL00001496		
Dihydrolipoyl DH-related cluster		BHL00001057		
*Isocitrate dehydrogenase (EC 1.1.1.42)	5.47 $\pm$ 0.1			MLO
*Aconitase (EC 4.2.1.3)	8.26 $\pm$ 0.7			MLO
Fumarate hydratase (EC 4.2.1.2)	0.30 $\pm$ 0.1	BHL00001701/BHL00001685/ BHL00001095	fumB	Cytosol
		BHL00001165/BHL00001811	fumC	
*Malate dehydrogenase (EC 1.1.1.37)	14.0 $\pm$ 1.8	BHL00002139	MDHM	Cytosol
PEPCK (EC 4.1.1.49)	2.64 $\pm$ 0.6	BHL00001570/BHL00002324	<i>pckA</i>	Cytosol
Pyruvate kinase (EC 2.7.1.40)	26.8 $\pm$ 3.2			Cytosol
*Lactate dehydrogenase (EC 1.1.1.27)	16.0 $\pm$ 1.7			Cytosol
*GPDH (EC 1.2.1.12)	11.4 $\pm$ 2.3			Cytosol
*Formate dehydrogenase (EC 1.2.1.2)	3.56 $\pm$ 0.8			Cytosol
*PFOR (EC 1.2.7.1)	ND	BHL00001771	<i>porA</i>	
*Hydrogenase (EC 1.12.7.2)	ND			
Citrate synthase (EC 2.3.3.1)	ND			
*Succinate dehydrogenase (EC 1.3.99.1)	ND	BHL00001490/BHL00001794	SDHfp/DHSA	
SDH ubiquinone cluster (EC 1.3.5.1)				
α -Galactosidase (EC 3.2.1.22)	23.5	BHL00002178	AGAL	SGF
α -Mannosidase (EC 3.2.1.24)	17.9	BHL00000629; BHL00001607; BHL00003253	MAN2B1; MA2B1; MANA	SGF

*Enzymes assayed under oxygen-free conditions as described in Methods.

stream of CO₂ for 5 min compared to controls measured under N₂. 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), α -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

Fig. 1. Proposed pathway of metabolism in the MLO of *Blastocystis*. (1) Malic enzyme, (2) PNO, (3) ASCT, (4) STK, (5) α-ketoglutarate dehydrogenase, (6) 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.

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 and aconitase localized to the large granular fraction (Fig. 2A–G), whereas PEPCK, malate dehydrogenase, fumarate hydratase, pyruvate kinase and lactate dehydrogenase localized to the non-sedimentable fraction (Fig. 2H–L). α-Galactosidase and α-mannosidase activity were detected in the small granular fraction which is typical of lysosomes (Fig. 2M, N). Isopycnic gradient centrifugation of the large granular fraction and assay of the enzymes malic enzyme, PNO, ASCT, STK, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase and aconitase localized to a band having a density of 1.20 g ml⁻¹ (Fig. 3A), which is the same density as that reported for hydrogenosomes and mitochondria (Hart & Oppendoes, 1984; Woods *et al.*, 1988). Isopycnic gradient centrifugation of the small granular fraction and assay of α-galactosidase and α-mannosidase resulted in these enzymes localizing to a band having a density of 1.16 g ml⁻¹ (Fig. 3B), typical of lysosomal organelles (Hart & Oppendoes, 1984; Woods *et al.*, 1988).

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://pepdpub.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:rhoquinone complex as described

Fig. 2. Subcellular localization of enzymes in *Blastocystis*. Homogenates were subjected to differential centrifugation in anaerobic buffers with increasing *g*-force from left to right. Enzymes were assayed as described in Methods. Relative specific activities (the ratio of the specific activity in fractions to that in the cell-free extract) are plotted against cumulative percentage of protein recovered in each fraction. Percentage recoveries (based on enzyme units in the cell-free extract) were: (A) PNO, 68%; (B) malic enzyme, 84%; (C) ASCT, 72%; (D) aconitase, 74%; (E) isocitrate dehydrogenase, 79%; (F) α -ketoglutarate dehydrogenase, 81%; (G) STK, 83%; (H) fumarate hydratase, 84%; (I) malate dehydrogenase, 79%; (J) pyruvate kinase, 79%; (K) PEPCK, 87%; (L) lactate dehydrogenase, 78%; (M) α -galactosidase, 74%; (N) α -mannosidase, 68%.

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

Fig. 3. Localization of sedimentable enzymes in fractions after isopycnic density fractionation in sucrose gradients. (A) The large granular fraction was layered onto a sucrose gradient (1.02–1.29 g ml⁻¹) as described in Methods. The direction of sedimentation was from left to right. The specific activities of enzymes in nmol min⁻¹ (mg protein)⁻¹ at their maxima, and percentage recoveries (in parentheses), were as follows: PNO, 380 (68 %); ASCT, 240 (73 %); malic enzyme, 152 (87 %); STK, 1540 (71 %); α-ketoglutarate dehydrogenase, 143 (86 %); isocitrate dehydrogenase, 27.5 (79 %), and aconitase, 42 (73 %). Protein recovery was 102 %. (B) The small granular fraction was layered onto a sucrose gradient (1.01–1.27 g ml⁻¹). The direction of sedimentation was from left to right; the units of enzyme activity are as in (A). The specific activities of enzymes defined as as nmol min⁻¹ (mg protein)⁻¹ at their maxima, and percentage recoveries (in parentheses), were as follows: α-galactosidase, 148 (81 %); α-mannosidase, 110 (74 %). Protein recovery was 89 %.

search of the *Blastocystis* database (<http://pepdpub.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 (Steinbuchel & Müller, 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 Hellemond *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 Hellemond *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 ASCT, 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 kluyveri* 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 00000444) 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.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Institutes of Health NCDDG AI40320 (N.Y.) and the Biomedical Research Council BMRC 05/1/21/19/378 (K.S.W.T.). Y.L. acknowledges the support of a travel award from Pace University. The authors thank Dr Thomas Gorrell for critical reading of the manuscript.

REFERENCES

Akhmanova, A., Voncken, F., van Alen, T., van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M. & Hackstein, J. H. (1998). A hydrogenosome with a genome. *Nature* **396**, 527–528.

Boroah, J., Leaback, D. H. & Walker, P. G. (1961). Studies on glucosaminidase. 2. Substrates for *N*-acetyl- β -glucosaminidase. *Biochem J* **78**, 106–110.

Boyer, P. D. (1962). Pyruvate kinase. In *The Enzymes*, pp. 95–113. Edited by P. D. Boyer, K. H. Lardy & K. Myrback. New York: Academic Press.

Burri, L., Williams, B. A., Bursac, D., Lithgow, T. & Keeling, P. J. (2006). Microsporidia mitosomes retain elements of the general mitochondrial targeting system. *Proc Natl Acad Sci U S A* **103**, 15916–15920.

Colombo, S., Grisa, M., Tortora, P. & Vanoni, M. (1994). Molecular cloning, nucleotide sequence and expression of a *Sulfolobus solfataricus* gene encoding a class II fumarase. *FEBS Lett* **337**, 93–98.

Cook, R. A. & Sanwal, B. D. (1969). Isocitrate dehydrogenase (NAD-specific) from *Neurospora crassa*. *Methods Enzymol* **13**, 42–47.

Dacks, J. B., Dyal, P. L., Embley, T. M. & van der Giezen, M. (2006). Hydrogenosomal succinyl-CoA synthetase from the rumen-dwelling fungus *Neocallimastix patriciarum*; an energy-producing enzyme of mitochondrial origin. *Gene* **373**, 75–82.

Duggleby, R. G. & Dennis, D. T. (1974). Nicotinamide adenine dinucleotide-specific glyceraldehyde 3-phosphate dehydrogenase from *Pisum sativum*. *J Biol Chem* **249**, 167–174.

Dunn, L. A. & Boreham, P. F. (1991). The *in vitro* activity of drugs against *Blastocystis hominis*. *J Antimicrob Chemother* **27**, 507–516.

Edwards, D. I. (1993). Nitroimidazole drugs-action and resistance mechanisms. II. Mechanisms of resistance. *J Antimicrob Chemother* **31**, 201–210.

Embley, T. M., van der Giezen, M., Horner, D. S., Dyal, P. S. & Foster, P. (2003). Mitochondria and hydrogenosomes are two forms of the same fundamental organelle. *Philos Trans R Soc Lond B Biol Sci* **358**, 191–201.

Englard, S. (1969). Extramitochondrial L-malate dehydrogenase of beef heart. *Methods Enzymol* **13**, 123–129.

Fansler, B. & Lowenstein, J. M. (1969). Aconitase from pig heart. *Methods Enzymol* **13**, 26–30.

Fukuda, W., Fukui, T., Atomi, H. & Imanaka, T. (2004). First characterization of an archaeal GTP-dependent phosphoenolpyruvate carboxykinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J Bacteriol* **186**, 4620–4627.

Genda, T., Watabe, S. & Ozaki, H. (2006). Purification and characterization from *Corynebacterium glutamicum*. *Biosci Biotechnol Biochem* **70**, 1102–1109.

Gerbod, D., Edgcomb, V. P., Noël, C., Vanacova, S., Wintjens, R., Tachezy, J., Sogin, M. L. & Viscogliosi, E. (2001). Phylogenetic relationships of class II fumarase genes from trichomonads. *Mol Biol Evol* **18**, 1574–1584.

Gorrell, T. E., Yarlett, N. & Müller, M. (1984). Isolation and characterization of *Trichomonas vaginalis* ferredoxin. *Carlsberg Res Commun* **49**, 259–268.

Hareesh, K., Suresh, K., Khairul Anus, A. & Saminathan, S. (1999). Isolate resistance of *Blastocystis hominis* to metronidazole. *Trop Med Int Health* **4**, 274–277.

Hart, D. T. & Oppendoes, F. R. (1984). The occurrence of glycosomes (microbodies) in the promastigote stage of four major *Leishmania* species. *Mol Biochem Parasitol* **13**, 159–172.

Hoffmeister, M., Piotrowski, M., Nowitzki, U. & Martin, W. (2005). Mitochondrial trans-2-enoyl-CoA reductase of wax ester fermentation from *Euglena gracilis* defines a new family of enzymes involved in lipid synthesis. *J Biol Chem* **280**, 4329–4338.

Hrdy, I., Hirt, R. P., Dolezal, P., Bardonova, L., Foster, P. G., Tachezy, J. & Embley, T. M. (2004). *Trichomonas* hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. *Nature* **432**, 618–622.

- Hrdy, I., Cammack, R., Stopka, P., Kulda, J. & Tachezy, J. (2005). Alternative pathway of metronidazole activation in *Trichomonas vaginalis* hydrogenosomes. *Antimicrob Agents Chemother* **49**, 5033–5036.
- Inui, H., Ono, K., Miyatake, K., Nakano, Y. & Kitaoka, S. (1987). Purification and characterization of pyruvate:NADP⁺ oxidoreductase in *Euglena gracilis*. *J Biol Chem* **262**, 9130–9135.
- Keithly, J. S., Langreth, S. G., Buttle, K. F. & Mannella, C. A. (2005). Electron tomographic and ultrastructural analysis of the *Cryptosporidium parvum* relict mitochondrion, its associated membranes, and organelles. *J Eukaryot Microbiol* **52**, 132–140.
- Krayl, M., Lim, J. H., Martin, F., Guiard, B. & Voos, W. (2007). A cooperative action of the ATP-dependent import motor complex and the inner membrane potential drives mitochondrial preprotein import. *Mol Cell Biol* **27**, 411–425.
- Lavier, G. (1952). *Blastocystis* spp. *Ann Parasitol Hum Comp* **27**, 339–356.
- Lee, M. J. (1991). Pathogenicity of *Blastocystis hominis*. *J Clin Microbiol* **29**, 2089.
- Lindmark, D. G. & Müller, M. (1973). Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Trichomonas foetus*, and its role in pyruvate metabolism. *J Biol Chem* **248**, 7724–7728.
- Lindmark, D. G. & Müller, M. (1974). Biochemical cytology of anaerobic flagellates. II. Subcellular distribution of oxidoreductases and hydrolases in *Monocercomonas* sp. *J Protozool* **21**, 374–378.
- Lindmark, D. G., Eckenrode, B. L., Halberg, L. A. & Dinbergs, I. D. (1989). Carbohydrate, energy and hydrogenosomal metabolism of *Trichomonas foetus* and *Trichomonas vaginalis*. *J Protozool* **36**, 214–216.
- Ma, Y.-C., Funk, M., Dunham, W. R. & Komuniecki, R. (1993). Purification and characterization of electron-transfer flavoprotein: rhydroquinone oxidoreductase from anaerobic mitochondria of the adult parasitic nematode *Ascaris suum*. *J Biol Chem* **268**, 20360–20365.
- Marti, M., Regos, A., Li, Y., Schraner, E. M., Wild, P., Muller, N., Knopf, L. G. & Hehl, A. B. (2003). An ancestral secretory apparatus in the protozoan parasite *Giardia intestinalis*. *J Biol Chem* **278**, 24837–24848.
- Martin, W. (1999). A briefly argued case that mitochondria and plastids are descendants of endosymbionts, but that the nuclear compartment is not. *Proc Biol Sci* **266**, 1387–1395.
- Nanba, H., Takaoka, Y. & Hasegawa, J. (2003). Purification and characterization of formate dehydrogenase from *Ancylobacter aquaticus* strain KNK607M, and cloning of the gene. *Biosci Biotechnol Biochem* **67**, 720–728.
- Nasirudeen, A. M. & Tan, K. S. (2004). Isolation and characterization of the mitochondrion-like organelle from *Blastocystis hominis*. *J Microbiol Methods* **58**, 101–109.
- Noël, C., Dufernez, F., Gerbod, D., Edgcomb, V. P., Delgado-Viscogliosi, P., Ho, L. C., Singh, M., Wintjens, R., Sogin, M. L. & other authors (2005). Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *J Clin Microbiol* **43**, 348–355.
- Parvin, R. (1969). Citrate synthase from yeast. *Methods Enzymol* **13**, 16–19.
- Prasad, K. N., Nag, V. L., Dhole, T. N. & Ayyagari, A. (2000). Identification of enteric pathogens in HIV-positive patients with diarrhea in northern India. *J Health Popul Nutr* **18**, 23–26.
- Prenc, E. M. & Natowicz, M. R. (1992). Diagnosis of alpha mannosidosis by measuring alpha mannosidase in plasma. *Clin Chem* **38**, 501–503.
- Puthia, M. K., Sio, S. W., Lu, J. & Tan, K. S. (2006). *Blastocystis ratti* induces contact-independent apoptosis, F-actin rearrangement, and barrier function disruption in IEC-6 cells. *Infect Immun* **74**, 4114–4123.
- Rotte, C., Stejskal, F., Zhu, G., Keithly, J. S. & Martin, W. (2001). Pyruvate:NADP⁺ oxidoreductase from the mitochondrion of *Euglena gracilis* and from the apicomplexan *Cryptosporidium parvum*: a biochemical relic linking pyruvate metabolism in mitochondriate and amitochondriate protists. *Mol Biol Evol* **18**, 710–720.
- Russo, A. R., Stone, S. L., Taplin, M. E., Snapper, H. J. & Doern, G. V. (1988). Presumptive evidence for *Blastocystis hominis* as a cause of colitis. *Arch Intern Med* **148**, 1064.
- Sanadi, D. R. (1969). α -Ketoglutarate dehydrogenase from pig heart. *Methods Enzymol* **13**, 52–55.
- Singer, T. P. & Kearney, E. B. (1963). Succinate dehydrogenase. In *The Enzymes*, pp. 383–445. Edited by P. D. Boyer, K. H. Lardy & K. Myrback. New York: Academic Press.
- Steinbuechel, A. & Müller, M. (1986). Anaerobic pyruvate metabolism of *Trichomonas foetus* and *Trichomonas vaginalis* hydrogenosomes. *Mol Biochem Parasitol* **20**, 57–65.
- Stensvold, C. R., Suresh, G. K., Tan, K. S., Thompson, R. C., Traub, R. J., Viscogliosi, E., Yoshikawa, H. & Clark, C. G. (2007). Terminology for *Blastocystis* subtypes – a consensus. *Trends Parasitol* **23**, 93–96.
- Suzuki, T., Sato, M., Yoshida, T. & Tuboi, S. (1989). Rat liver mitochondrial and cytosolic fumarases with identical amino acid sequences are encoded from a single gene. *J Biol Chem* **264**, 2581–2586.
- Tachezy, J., Sanchez, L. B. & Müller, M. (2001). Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as indicated by the phylogeny of IscS. *Mol Biol Evol* **18**, 1919–1928.
- Tovar, J., Fischer, A. & Clark, C. G. (1999). The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. *Mol Microbiol* **32**, 1013–1021.
- van Grinsven, K. W., Rosnowsky, S., van Weelden, S. W., Pütz, S., van der Giezen, M., Martin, W., van Hellemond, J. J., Tielens, A. G. & Henze, K. (2008). Acetate:succinate CoA-transferase in the hydrogenosomes of *Trichomonas vaginalis*: identification and characterization. *J Biol Chem* **283**, 1411–1418.
- Van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. (1998). Trypanosomatidae produce acetate via a mitochondrial acetate:succinate CoA transferase. *Proc Natl Acad Sci U S A* **95**, 3036–3041.
- Van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. (2005). The extraordinary mitochondrion and unusual citric acid cycle in *Trypanosoma brucei*. *Biochem Soc Trans* **33**, 967–971.
- van Weelden, S. W. H., van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. M. (2005). New functions for parts of the krebs cycle in procyclic *Trypanosoma brucei*, a cycle not operating as a cycle. *J Biol Chem* **280**, 12451–12460.
- Vannatta, J. B., Adamson, D. & Mullican, K. (1985). *Blastocystis hominis* infection presenting as recurrent diarrhea. *Ann Intern Med* **102**, 495–496.
- Walderich, B., Bernauer, S., Renner, M., Knobloch, J. & Burchard, G. D. (1998). Cytopathic effects of *Blastocystis hominis* on Chinese hamster ovary (CHO) and adenocarcinoma HT29 cell cultures. *Trop Med Int Health* **3**, 385–390.
- Weg, A. L., Soave, R. & Jacobson, I. M. (1987). The significance of intestinal *Blastocystis hominis* infection. *Gastroenterology* **92**, 1688.
- Woods, S. A., Schwartzbach, S. D. & Guest, J. D. (1988). Two biochemically distinct classes of fumarase in *Escherichia coli*. *Biochim Biophys Acta* **954**, 14–26.
- Yarlett, N., Hann, A. C., Lloyd, D. & Williams, A. G. (1981). Hydrogenosomes in the rumen protozoan *Dasytricha ruminantium* Schuberg. *Biochem J* **200**, 365–372.

Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C. & Greenwood, C. A. (1986). Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. *Biochem J* **236**, 729–739.

Zierdt, C. H. (1986). Cytochrome free mitochondria of an anaerobic protozoan – *Blastocystis hominis*. *J Protozool* **33**, 67–69.

Zierdt, C. H., Swan, J. C. & Hosseini, J. (1983). In vitro response of *Blastocystis hominis* to antiprotozoal drugs. *J Protozool* **30**, 332–334.

Zierdt, C. H., Donnelly, C. T., Muller, J. & Constantopoulos, G. (1988). Biochemical and ultrastructural study of *Blastocystis hominis*. *J Clin Microbiol* **26**, 965–970.

Zuckerman, M. J., Watts, M. T., Ho, H. & Meriano, F. V. (1994). *Blastocystis hominis* infection and intestinal injury. *Am J Med Sci* **308**, 96–101.

Edited by: J. Tachezy