***Giardia intestinalis*, a eukaryote without hydrogenosomes, produces hydrogen**

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The microaerophilic flagellated protist *Giardia intestinalis*, the commonest protozoal agent of intestinal infections worldwide, is of uncertain phylogeny, but is usually regarded as the earliest branching of the eukaryotic clades. Under strictly anaerobic conditions, a mass spectrometric investigation of gas production indicated a low level of generation of dihydrogen (2 nmol min⁻¹ per 10⁷ organisms), about 10-fold lower than that in *Trichomonas vaginalis* under similar conditions. Hydrogen evolution was O₂ sensitive, and inhibited by 100 µM metronidazole. Fluorescent labelling of *G. intestinalis* cells using monoclonal antibodies to typical hydrogenosomal enzymes from *T. vaginalis* (malate enzyme, and succinyl-CoA synthetase α and β subunits), and to the large-granule fraction (hydrogenosome-enriched, also from *T. vaginalis*) gave no discrete localization of epitopes. Cell-free extracts prepared under anaerobic conditions showed the presence of a CO-sensitive hydrogenase activity. This first report of hydrogen production in a eukaryote with no recognizable hydrogenosomes raises further questions about the early branching status of *G. intestinalis*; the physiological characterization of its hydrogenase, and its recently elucidated gene sequence, will aid further phylogenetic investigations.

Keywords: early branching eukaryotes, diplomonads, membrane inlet mass spectrometry, hydrogenase

INTRODUCTION

The ability of eukaryotes to produce dihydrogen is somewhat limited, despite the widespread occurrence of genes encoding hydrogenase (Horner *et al.* 2000). Dihydrogen is produced by some green algae in the light under anaerobic conditions, e.g. in *Chlamydomonas reinhardtii* (Happe *et al.*, 1994), and in *Scenedesmus obliquus* (Schnackenberg *et al.*, 1993). Other lower eukaryotes that generate hydrogen gas include the microaerophilic protozoa (Yarlett *et al.*, 1982) and chytrid fungi, e.g. *Neocallimastix patriciarum* (Yarlett *et al.*, 1986, 1987; Rees *et al.*, 1998) isolated from the rumen. Like the microaerophilic pathogen *Trichomonas vaginalis* (Paget & Lloyd 1990), and the cattle parasite *Tritrichomonas foetus* (Lloyd *et al.*, 1983), these protists all possess hydrogenosomes, organelles specialized to produce dihydrogen from pyruvate (Lindmark & Müller, 1973). Similar organelles have been shown in the free-living ciliates *Plagiopyla frontata* and *Metopus contortus* (Fenchel & Finlay, 1992; Biagini *et al.*, 1997a).

Hydrogenosomes have inner and outer membranes

(Finlay & Fenchel, 1989), generate a transmembrane electrochemical potential (Humphreys *et al.*, 1994, 1998) and have intra-organellar Ca²⁺-containing granules (Benchimol *et al.*, 1982; Chapman *et al.*, 1985; Biagini *et al.*, 1997b); these are all characteristics shared by mitochondria (Lloyd, 1974). It is now generally believed that hydrogenosomal convergence in disparate descendant phyla (Lloyd *et al.*, 1983) represents the emergence of secondarily derived mitochondria (Biagini *et al.*, 1997c). Evolutionary selection pressures that favoured the necessary modifications (loss of respiratory chains that use cytochromes, and the proton-translocating systems that generate ATP by oxidative phosphorylation) were life under anoxic conditions (Fenchel & Finlay, 1995) or under conditions of low O₂ in microaerobic refuges (e.g. in sediments or in parasitic or symbiotic niches; Lloyd & Williams, 1993).

One of the lower eukaryotic groups adapted to these conditions, the diplomonads, includes *Giardia intestinalis*, an important human pathogen that causes an estimated 2.8 × 10⁸ infections each year. Although this organism can be life-threatening in the immunocompro-

mised, the very young or the very old (Adam, 1991), it is of uncertain phylogeny. It has been commonly assumed that this 'anaerobic amitochondriate' protist is 'ancient and primitive' on account of its many bacterial characteristics (pyrophosphate-biased energy metabolism, arginine dihydrolase pathway of energy production, etc.; Brown *et al.*, 1998), and on the basis of RNA trees, that it represents an extant survivor typical of the earliest branch of the eukaryotes (Sogin, 1991; Leipe *et al.*, 1993; Madigan *et al.*, 2000). However, this assumption has recently been questioned (Embley & Hirt, 1998), as genes for several typically mitochondrial proteins have been shown to be present in *G. intestinalis* (Soltys & Gupta, 1994; Horner *et al.*, 1996; Roger *et al.*, 1998) and in *T. vaginalis*. Although open to various interpretations, this startling discovery might place the diplomonad as a recent derivative of an aerobic lineage. Furthermore, electron-transport activities (Ellis *et al.*, 1993), and transmembrane electrochemical potential generation are associated with distinctive sites in this organism (Lloyd *et al.*, 2002). These observations may be interpreted as suggesting that *G. intestinalis* is not an early branching eukaryote, but having been derived from an antecedent with mitochondria, should be allocated to a crown taxon (Lake, 1994). Here we show, despite earlier claims to the contrary, that *G. intestinalis* possesses hydrogenase and produces dihydrogen, even if it does not possess identifiable hydrogenosomes.

METHODS

Organisms and cultures. *Giardia intestinalis* Portland-1 strain ATCC 30888, was a gift of Michael R. Edwards, University of New South Wales, Sydney, Australia. Cultures were maintained in liquid N₂ after cryopreservation in the presence of 10% (v/v) dimethyl sulphoxide. Trophozoites were cultured axenically and anaerobically in screw-capped Nunclon tubes (Life Technologies) at 37 °C on Diamond's modified TY1-S-33 medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) glucose, 0.106% (w/v) arginine, 0.2% (w/v) NaCl, 0.1% (w/v) cysteine, 0.1% (w/v) bovine bile, 0.02% (w/v) ascorbic acid, 0.0023% (w/v) ammonium ferric citrate, 3% (v/v) minimal essential medium with Earle's salt (Keister, 1983; Edwards *et al.*, 1989), supplemented with 10% (v/v) heat-inactivated fetal calf serum. Subculturing was performed routinely at 48 h intervals by replacing the spent medium with fresh, chilling on ice for 20 min, and inverting gently to detach the monolayer. Cells were counted on a Fuchs Rosenthal haemocytometer slide, using 0.4% (v/v) Trypan blue as a viability indicator; typically this gave cell numbers of 2×10^6 ml⁻¹. *Trichomonas vaginalis* was grown exactly as described by Diamond (1957). All cultures were monitored routinely to confirm axenic growth.

Harvesting. After chilling for 20 min in an ice bucket, tubes were shaken gently to dislodge adherent organisms. Cell numbers were counted and then centrifugation was at 1000 g (3000 r.p.m.) for 4 min at room temperature in a bench centrifuge (MSE Minor). After washing once with phosphate-buffered saline (pH 7.4, PBS) and recentrifugation, organisms were finally resuspended in PBS or 0.31 M mannitol solution (both at pH 7.4) and kept at 4 °C.

Mass spectrometry. Dissolved gases were measured using a Hal series quadrupole mass spectrometer (Hiden Analytical)

fitted with an inlet probe covered by a gas-permeable polymer membrane (1.56 mm outside diameter, 0.5 mm internal diameter, 1 cm length) sealed into a 1 cm length of quartz-glass tube. The inlet orifice was 100 µm diameter, and the membrane used was either silicone rubber (permeable to low-molecular-mass volatiles as well as gases) or Mylar (polyethylene terephthalate), a polymer permeable to H₂ and CO₂, but only much less so to other gases and not penetrated by ethanol. Further details of the mass spectrometer are described elsewhere (Lloyd & Scott, 1982; Lloyd *et al.*, 1992). The probe was used to measure gases dissolved in a 5 ml working volume held in a perspex vessel with magnetic stirring at 200 r.p.m. and 37 °C; the system was open for gas flow over the stable liquid vortex. A gas mixer (Lundsgaard & Degn, 1973) was used to give step changes from argon to 5% oxygen in nitrogen in the gas phase above the stirred vortex. Mass-to-charge ratios used to measure the concentrations of H₂, ethanol, O₂ and CO₂ were 2, 31, 32 and 44 respectively. Solubilities of H₂ and O₂ at 37 °C were 750 and 1328 µM respectively (Wilhelm *et al.*, 1977). CO₂ and ethanol calibrations employed additions of 10 mM NaHCO₃ or C₂H₅OH.

Preparation of cell-free extracts. Packed organisms were resuspended in 5 vols 0.31 M mannitol, then mixed with an equal volume of 0.1 mm diameter acid- and distilled-water-washed glass beads in screw-cap sealed 2 ml Nunclon tubes under argon. They were shaken at 4000 Hz for 20 s in a Braun MSK cell disintegrator. Beads and unbroken cells were removed by centrifugation at 1000 g for 2 min. Assays were performed using the supernatant.

Immunolocalization. Following the method of Brugerolle *et al.* (2000), washed cell suspensions were fixed in PBS, pH 7.4, containing 3.5% formaldehyde for 20 min at room temperature. The cells were then permeabilized in PBS containing 0.5% Triton X-100 for 10 min. After treatment with 0.1 M glycine in PBS for 15 min, the cells were resuspended in 200 µl of the primary antibody (undiluted mAb supernatant) and incubated for 2 h at room temperature. The antibodies used (kindly provided by Dr Guy Bruggerole, Clermont-Ferrand) were anti-malic enzyme (15 D7), anti- α -subunit-succinyl-CoA synthetase (1C8), anti- β -subunit-succinyl-CoA synthetase (5H6) and anti-granule (XIV D2). After this period the cells were washed three times in PBS (3 × 30 min) and then incubated for 1 h with anti-mouse Ig, IgG/M antibody conjugated with fluorescein isothiocyanate. After final washes in PBS (3 × 30 min), the cells were mounted in 1:1 (v/v) glycerol/PBS containing 10 mg 1,4-diazabicyclo-(2,2,2)-octane (DABCO) ml⁻¹. Cells were viewed using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope. Control specimens were treated with the secondary antibody only.

Confocal laser scanning microscopy. Cells prepared for immunofluorescence were incubated with 0.31 M mannitol instead of PBS and viewed using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope. Specimens were scanned using a 25 mW argon laser with appropriate excitation and emission filters for fluorescein (488/510 nm). Specimens were examined using oil-immersion objectives, × 60 (50 µm confocal aperture) and × 100 (100 µm confocal aperture). Series sections through samples were taken at 1 µm intervals (512 × 512 pixels; ~ 0.5 µm thick), and three-dimensional constructs were prepared using Molecular Dynamics Volume Workbench running in Silicon Graphics UNIX workstation.

Materials. Gases were from Air Products; traces of O₂ were removed from research-grade N₂ using an 'Oxytrap' column

(Alltech Associates). Tryptone was purchased from Becton-Dickinson, and trypticase from BioMérieux. Fetal calf serum was supplied by GibcoBRL, and Nunclon screw-capped culture tubes and cryopreservation vials were supplied through Life Technologies. All other chemicals were supplied by Sigma-Aldrich-Fluka.

RESULTS

Fig. 1 shows the responses of a washed intact cell suspension of *G. intestinalis* to successive cycles of anoxia and aerobiosis after addition of 20 mM glucose, as monitored by membrane inlet mass spectrometry using a silicone rubber interface. After equilibration to the anaerobic state, dissolved CO_2 and ethanol (a major fermentation product under anoxic conditions), attained steady states. Switching the mobile gas phase to trace O_2 (using a gas mixture containing 0.4 kPa O_2 in N_2) gave accelerated fermentation, even though the level of dissolved O_2 was less than the detection limit of the mass spectrometer ($0.1 \mu\text{M O}_2$); this was indicated by elevated ethanol production and an increased CO_2 concentration. The response time of the system is longer for ethanol than for fixed gases. Monitoring at $m/z = 2$ (for H_2^+) indicated an increase, but calibrations in parallel with those at $m/z = 31$ confirmed that this signal is derived by the fragmentation of ethanol in the vacuum system (Fig. 2a). The fact that the $m/z = 2$ trend analysis exactly follows that at $m/z = 31$ confirms that it does not arise from dihydrogen; in this experimental configuration (open to gas flow) the latter behaves as do all 'fixed gases', i.e. they reach steady states and do not accumulate as does a low-molecular-mass volatile (e.g. ethanol).

In order to avoid the interference of ethanol at $m/z = 2$ while measuring H_2 , a chemical trap for ethanol (alcohol

Fig. 1. Gas exchange measured in an open reaction vessel in a suspension of *G. intestinalis*. Washed organisms (6×10^7 organisms ml^{-1}) were incubated in PBS with 20 mM glucose. The temperature was 37°C and the membrane interface was silicone rubber.

dehydrogenase plus semicarbazide) was included in the cell suspension, but this did not obviate the problem, as the mass spectrometer was able to compete with the enzyme for ethanol and still gave signals at both mass numbers.

A solution to this problem involved using a more selective gas-permeable membrane. Of several polymers (polyethylene, polypropylene, Teflon and polyethylene terephthalate), the last (Mylar) proved most successful with respect to restricted ethanol permeability. Fig. 2(b) shows that even at very high concentrations, ethanol gives no responses, but H_2 can penetrate rapidly to give a mass spectrometric response with a short half-time. Fig. 3 shows an experiment on glucose utilization by whole cells of *G. intestinalis* in PBS using this Mylar inlet system to monitor gases continuously in a closed vessel. Initially, the organisms consumed dissolved O_2 , and this became undetectable by 8 min. Only after this, when almost complete anoxia was attained, did the signal at $m/z = 2$ increase as H_2 was produced and accumulated continuously. Injection of air-saturated PBS (final $[\text{O}_2] = 8 \mu\text{M}$) gave an almost immediate cessation of H_2 generation. Resumption of H_2 evolution only occurred when added O_2 had been depleted again. Finally, addition of $100 \mu\text{M}$ metronidazole gave complete arrest of hydrogenogenesis.

Experiments with cell-free extracts

Fig. 4 shows the absorbance changes at 600 nm during incubation of cell-free extract with methyl viologen in closed cuvettes under an atmosphere of either N_2 or H_2 in a buffer containing 40 mM dithiothreitol. No dye reduction occurred in the presence of heat-inactivated cell-free extract. The presence of hydrogenase in the active extract was indicated by dye reduction under H_2 at a rate proportional to the volume of extract added. No reaction occurred under a gas phase of N_2 or when air was admitted to the reaction mixture during the hydrogenase reaction. CO ($3 \mu\text{M}$) inhibited the reaction by 20% when H_2 was present at $820 \mu\text{M}$; $16 \mu\text{M}$ CO gave 70% inhibition.

Immunocytology

Incubation of fixed *G. intestinalis* with fluorescent-labelled monoclonal antibodies to hydrogenosomes isolated from *T. vaginalis* or to typical hydrogenosomal enzymes (also purified from *T. vaginalis*) as described by Bruggerole *et al.* (2000) gave no discrete localization as revealed by examination using confocal laser scanning microscopy. The fluorescent hydrogenosome labels (fluorescein $\lambda_{\text{excit}} = 488 \text{ nm}$, $\lambda_{\text{emiss}} = 510 \text{ nm}$) gave positive results for the characteristic distribution of these organelles and for the hydrogenosomal location of malate enzyme and succinyl-CoA synthetase in *T. vaginalis*, but in *G. intestinalis* only faint yellow-green fluorescence was observed throughout the organisms (not shown). Thus no discrete localization of typical hydrogenosomal epitopes was found in the diplomonad.

Fig. 2. Specificity of the permeability of silicone rubber and Mylar. (a) Calibrations for O₂, CO₂ and ethanol and H₂ at *m/z* 32, 44 and 31 and 2, respectively, using silicone rubber. (b) Calibrations for CO₂, ethanol and H₂ at *m/z* 44, 31 and 2, respectively, using a Mylar membrane.

Fig. 3. Gas exchange measured in a suspension of *G. intestinalis*. Washed organisms (6×10^7 organisms ml⁻¹) were incubated in PBS with 20 mM glucose in a closed reaction vessel. The temperature was 37 °C and the membrane interface was Mylar. See text for details. Mz, metronidazole.

Fig. 4. Reduction of methyl viologen by H₂ in an extract of *G. intestinalis*. Absorbance changes at 600 nm were measured; protein concentration was (a) 0.75 and (b) 1.5 mg ml⁻¹; temperature 22 °C.

DISCUSSION

The lack of mitochondria and peroxisomes, and the possession of only a rudimentary Golgi body in the trophozoite may not necessarily point to a 'primitive' status for *G. intestinalis*, but rather to the secondary loss of completely functional organelles during evolution as a parasite (Lloyd *et al.*, 1983; Embley & Hirt, 1998).

Our observation that this organism produces dihydrogen, despite its apparent lack of a discrete structural entity that can be recognized as a hydrogenosome, even by the criterion of immunological cross-reaction with proteins isolated from the hydrogenosomes of another

microaerophilic lower eukaryote, *T. vaginalis* (Bruggerole *et al.*, 2000), provides a further reason for a new emphasis on its uncertain taxonomic position. Dihydrogen production rates (2 nmol min^{-1} per 10^7 organisms) were about 10-fold lower than in *T. vaginalis* (Ellis *et al.*, 1992).

The hydrogenase responsible for the dihydrogenase generation in *G. intestinalis* shows characteristics typical of an (iron-only) [Fe] hydrogenase. Thus its activity, as measured by H_2 -dependent reduction of methyl viologen, is extremely O_2 sensitive and is also inhibited by low concentrations of CO. Other classes of hydrogenase include those containing Ni, or Ni and Se as well as acid-labile sulphur; these hydrogenases are relatively CO insensitive (Cammack *et al.*, 1985).

Other microaerophilic protists containing hydrogenosome-localized hydrogenase include the parasitic species, the trichomonads (Lindmark & Müller, 1973), six different species of the symbiotic rumen protists examined (see Yarlett *et al.*, 1981, 1983a, b, 1984; Hillman *et al.*, 1985; Ellis *et al.*, 1991a, b, c), chytrid fungi (Yarlett *et al.*, 1986, 1987; Rees *et al.*, 1998) and the highly anaerobic free-living sediment-dwelling ciliate *Metopus contortus* (Biagini, 1997a). Clearly, these organisms are widely separated phylogenetically (Lloyd *et al.*, 1983). Molecular analysis, as well as likelihood ratio tests and parametric bootstrapping, suggest that the [Fe] hydrogenases in microaerophilic lower eukaryotes are not monophyletic, even though those from organisms not bearing hydrogenosomes, *Entamoeba histolytica* and *Spironucleus barkhanus*, together with a newly described third hydrogenase from *T. vaginalis*, might possibly be (Horner *et al.*, 2000).

The complete sequence of the *G. intestinalis* hydrogenase gene (<http://www.ncbi.nlm.nih.gov:80/>, GenBank accession no. AF242293), available only after the experiments described here were completed (J. E. Nixon, J. Field, J. Samuelson & M. L. Sogin, unpublished), places *Giardia* close to *E. histolytica* and *S. barkhanus*. The *Spironucleus* sequence is also complete (Horner *et al.*, 2000), and neither that gene nor the one for *Giardia* hydrogenase shows any obvious N-terminus motif that would target it to an organelle. This observation correlates with our unsuccessful attempts to locate hydrogenosomes in *G. intestinalis* reported here.

Monoclonal antibodies for hydrogenosomal malic enzyme, α and β -subunits of succinyl-CoA synthetase, and granules were incubated with *T. vaginalis* (positive control) and *G. intestinalis* to see if this organism contained any of the hydrogenosomal enzymes. All of the *T. vaginalis* tests showed discrete localizations within the cells and no fluorescence when the cells were incubated with fluorescently labelled secondary antibody only (negative control). All of the localizations in the trichomonad were discrete, and in organelles of the same dimensions and distributions. However, incubation of *Giardia* with the same antibodies did not result in any discrete localization within the cells. This suggests

that *Giardia* does not contain any of the hydrogenosomal proteins screened for by this assay; however, this does not necessarily indicate that these are not present. Monoclonal antibodies are highly specific, and even a change of one amino acid of an enzyme can result in a different epitope; then no binding of the antibody would occur.

Extensive mechanistic research on hydrogenases indicates that evolutionary relationships can be elucidated. This first report of a hydrogenase in *G. intestinalis* and the progress of the *Giardia* genome project (Adam, 2000) paves the way for molecular analyses (Horner *et al.*, 2000) that may help phylogenetic studies on *G. intestinalis* as has been the case for *E. histolytica* (Rosenthal *et al.*, 1997). These studies may also have important implications for hypotheses regarding the origin of eukaryotes (Gupta & Golding, 1996; López-García & Moreira, 1999).

Further work is in progress to determine the specific location of the hydrogenase in *G. intestinalis*, especially with regard to its membrane association. Possible functions include intracellular redox balancing by this 'output hydrogenase'. Previous metabolic analysis of fermentation pathways reveals tight control of apportionment of electron flux between pathways divergent from the pyruvate pool to alanine, or ethanol or acetate + CO_2 (Paget *et al.*, 1990, 1993) most likely via the redox balance of the NADH/NAD⁺ couple (Paget *et al.*, 1993). Intracellular O_2 concentration is presumably sensed in order to provide the homeodynamic modulation at this important branch point (Lloyd *et al.*, 2002). The O_2 sensitivity of hydrogenase reported here strongly suggests that dihydrogen serves as an alternative product, the generation of which can serve as a process for the dissipation of excess reducing power, only necessary under highly anaerobic conditions. Clearly, control of intracellular redox state is paramount in an environment that has fluctuating redox and O_2 levels. Other possible functions may also come to light if the hydrogenase turns out to be a reversible transmembrane enzyme with a dual hydrogen-uptake/output role, also capable of the generation of a proton-translocating transmembrane electrochemical potential.

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REFERENCES

- Adam, R. D. (1991). The biology of *Giardia* spp. *Microbiol Rev* 55, 706–732.
- Adam, R. D. (2000). The *Giardia* genome project. *Int J Parasitol* 30, 475–484.
- Benchimol, M., Elias, C. A. & DeSouza, W. (1982). *Trichomonas foetus*: ultrastructural localisation of calcium in the plasmamembrane and in the hydrogenosome. *Exp Parasitol* 54, 277–284.

- Biagini, G. A., Hayes, A. J., Suller, M. T. E., Winters, C., Finlay, B. J. & Lloyd, D. (1997a). Hydrogenosomes of *Metopus contortus* physiologically resemble mitochondria. *Microbiology* **143**, 1623–1629.
- Biagini, G. A., van der Giesen, M., Hill, B., Winters, C. & Lloyd, D. (1997b). Ca^{2+} accumulation in the hydrogenosomes of *Neocallimastix frontalis* L2, a mitochondria-like physiological role. *FEMS Microbiol Lett* **149**, 227–232.
- Biagini, G. A., Finlay, B. & Lloyd, D. (1997c). Evolution of the hydrogenosome. *FEMS Microbiol Lett* **153**, 133–140.
- Brown, D. M., Upcroft, J. A., Edwards, M. R. & Upcroft, P. (1998). Anaerobic bacterial metabolism in the ancient eukaryote *Giardia duodenalis*. *Int J Parasitol* **28**, 149–164.
- Brugerolle, G., Bricheux, G. & Coffe, G. (2000). Immunolocalization of two hydrogenosomal enzymes of *Trichomonas vaginalis*. *Parasitol Res* **86**, 30–35.
- Cammack, R., Hall, D. O. & Rao, K. K. (1985). Hydrogenases: structure and applications in hydrogen production. In *Microbial Gas Metabolism*, pp. 75–102. Edited by R. K. Poole & C. S. Dow. London: Academic Press.
- Chapman, A., Hann, A. O., Linstead, D. & Lloyd, D. (1985). Dispersive X-ray microanalysis of membrane associated inclusions in hydrogenosomes isolated from *Trichomonas vaginalis*. *J Gen Microbiol* **131**, 2933–2939.
- Diamond, L. S. (1957). The establishment of various trichomonads in animals and man in axenic culture. *J Parasitol* **43**, 488–490.
- Edwards, M. R., Gilroy, F. V., Jimenez, B. M. & O'Sullivan, W. J. (1989). Alanine is a major end product of metabolism by *Giardia lamblia*: a proton nuclear magnetic resonance study. *Mol Biochem Parasitol* **37**, 19–26.
- Ellis, J. E., McIntyre, P. S., Saleh, M., Williams, A. G. & Lloyd, D. (1991a). Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the rumen ciliate *Polyplastron multivesiculatum*. *Appl Environ Microbiol* **57**, 1400–1407.
- Ellis, J. E., McIntyre, P. S., Saleh, M., Williams, A. G. & Lloyd, D. (1991b). Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the rumen ciliate *Dasytricha ruminantium*. *J Gen Microbiol* **147**, 1409–1417.
- Ellis, J. E., McIntyre, P. S., Saleh, M., Williams, A. G. & Lloyd, D. (1991c). The influence of minimal concentrations of O_2 on the fermentative metabolism of the rumen entodimorphid ciliate, *Eudiplodinium maggii*. *Curr Microbiol* **23**, 245–251.
- Ellis, J. E., Cole, D. & Lloyd, D. (1992). Influence of oxygen on the fermentative metabolism of metronidazole-sensitive and resistant strains of *Trichomonas vaginalis*. *Mol Biochem Parasitol* **56**, 79–88.
- Ellis, J. E., Williams, R., Cole, D., Cammack, R. & Lloyd, D. (1993). Electron transport components of the parasitic protozoan, *Giardia lamblia*. *FEBS Lett* **325**, 196–200.
- Embley, T. M. & Hirt, R. P. (1998). Early branching eukaryotes? *Curr Opin Genet Dev* **8**, 624–629.
- Fenchel, T. & Finlay, B. J. (1992). Production of methane and hydrogen by anaerobic ciliates containing symbiotic methanogens. *Arch Microbiol* **157**, 475–480.
- Fenchel, T. & Finlay, B. J. (1995). *Ecology and Evolution in Anoxic Worlds*. Oxford: Oxford University Press.
- Finlay, B. J. & Fenchel, T. (1989). Hydrogenosomes in some anaerobic protozoa resemble mitochondria. *FEMS Microbiol Lett* **65**, 311–314.
- Gupta, R. S. & Golding, G. B. (1996). The origin of the eukaryotic cell. *Trends Biochem Sci* **21**, 166–171.
- Happe, T., Mosler, B. & Naber, J. D. (1994). Induction, localization and metal content of hydrogenase in the green alga *Chlamydomonas reinhardtii*. *Eur J Biochem* **222**, 769–774.
- Hillman, K., Lloyd, D., Scott, R. I. & Williams, A. G. (1985). The effect of O_2 on H_2 production by rumen holotrich protozoa as determined by membrane inlet mass spectrometry. In *Gas Metabolism*, pp. 271–277. Edited by R. K. Poole & C. S. Dow. London: Academic Press.
- Horner, D., Hirt, R. P., Kilvington, S., Lloyd, D. & Embley, T. M. (1996). Molecular data suggest an earlier acquisition of the mitochondrial endosymbiont. *Proc R Soc B Biol Sci* **263**, 1053–1059.
- Horner, D. S., Foster, P. G. & Embley, T. M. (2000). Iron hydrogenases and the evolution of anaerobic eukaryotes. *Mol Biol Evol* **17**, 1695–1709.
- Humphreys, M., Ralphs, J., Durrant, L. & Lloyd, D. (1994). Hydrogenosomes in trichomonads are Ca^{2+} -stores and have a transmembrane electrochemical potential. *Biochem Soc Trans* **22**, 324S.
- Humphreys, M., Ralphs, J., Durrant, L. & Lloyd, D. (1998). Confocal laser scanning microscopy of trichomonad hydrogenosomes store calcium and show a membrane potential. *Eur J Protistol* **34**, 356–362.
- Keister, D. B. (1983). Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans R Soc Trop Med Hyg* **77**, 487–488.
- Lake, J. A. (1994). Reconstructing evolutionary trees from DNA and protein sequences: paralogous distances. *Proc Natl Acad Sci USA* **91**, 1455–1459.
- Leipe, D. D., Gunderson, J. H., Nerad, T. A. & Sogin, M. L. (1993). Small subunit ribosomal RNA of *Hexamita inflata* and the quest for the first branch in the eukaryotic tree. *Mol Biochem Parasitol* **59**, 41–48.
- Lindmark, D. G. & Müller, M. (1973). Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus* and its role in pyruvate metabolism. *J Biol Chem* **248**, 7724–7728.
- Lloyd, D. (1974). *The Mitochondria of Microorganisms*. London: Academic Press.
- Lloyd, D. & Scott, R. I. (1982). Direct measurement of dissolved gases using membrane inlet mass spectrometry. *J Microbiol Methods* **1**, 313–320.
- Lloyd, D. & Williams, A. G. (1993). Biological activities of symbiotic and parasitic protists in low O_2 environments. *Adv Microb Ecol* **13**, 211–262.
- Lloyd, D., Williams, A. G., Yarlett, N. & Hillman, K. (1983). Similarities between rumen ciliates and trichomonads: both possess hydrogenosomes. *Abstr Int Soc Evol Protistol*, 5th Meeting, Banyuls-sur-Mer, abstract 26.
- Lloyd, D., Ellis, J. E., Hillman, K. & Williams, A. G. (1992). Membrane inlet mass spectrometry: probing the rumen ecosystem. *J Appl Bacteriol Symp Suppl* **73**, 1555–1635.
- Lloyd, D., Harris, J. C., Biagini, G. A. & 8 other authors (2002). Oxygen homeodynamics in *Giardia*. In *Giardia, a Cosmopolitan Parasite*, edited by P. Wallis. Wallingford: CAB International (in press).
- López-García, P. & Moreira, D. (1999). Metabolic symbiosis at the origin of eukaryotes. *Trends Biochem Sci* **24**, 88–93.
- Lundsgaard, J. & Degn, H. (1973). Digital regulation of gas flow rates and composition of gas mixtures. *IEEE Trans Biomed Eng* **20**, 384–387.

- Madigan, M. T., Martinko, J. M. & Parker, J. (2000).** *Biology of Microorganisms*, 9th edn. Upper Saddle River, NJ: Prentice Hall.
- Paget, T. A. & Lloyd, D. (1990).** *Trichomonas vaginalis* requires traces of oxygen and high concentrations of carbon dioxide for growth. *Mol Biochem Parasitol* **41**, 65–72.
- Paget, T. A., Rayner, M. H., Shipp, D. W. E. & Lloyd, D. (1990).** *Giardia lamblia* produces alanine anaerobically but not in the presence of O₂. *Mol Biochem Parasitol* **42**, 63–68.
- Paget, T. A., Kelly, M. L., Jarroll, E. L., Lindmark, D. G. & Lloyd, D. (1993).** The effects of oxygen on fermentation in *Giardia lamblia*. *Mol Biochem Parasitol* **57**, 65–72.
- Rees, E. M. R., Lloyd, D. & Williams, A. G. (1998).** The effects of differing concentrations of CO₂ and O₂ on the fermentative metabolisms of the rumen fungi *Neocallimastix patriciarum* and *Neocallimastix frontalis* L2. *Can J Microbiol* **44**, 819–824.
- Roger, A. J., Clark, C. G. & Doolittle, W. F. (1998).** A possible mitochondrial gene in the early branching amitochondriate protist *Trichomonas vaginalis*. *Proc Natl Acad Sci USA* **95**, 229–234.
- Rosenthal, B., Zhiming, M., Caplivski, D., Ghosh, S., de la Vega, H., Graf, T. & Samuelson, J. (1997).** Evidence for the bacterial origin of genes encoding fermentation enzymes of the amitochondriate parasite *Entamoeba histolytica*. *J Bacteriol* **179**, 3736–3745.
- Schnackenberg, J., Schulz, R. & Senger, H. (1993).** Characterization and purification of a hydrogenase for the green alga *Scenedesmus obliquus*. *FEBS Lett* **327**, 21–24.
- Sogin, M. L. (1991).** Early evolution and the origin of eukaryotes. *Curr Opin Genet Dev* **1**, 457–463.
- Soltys, B. J. & Gupta, R. S. (1994).** Presence and cellular distributions of a 60 kDa protein related to mitochondrial HSP-60 in *Giardia lamblia*. *J Parasitol* **80**, 580–590.
- Wilhelm, E., Battino, R. & Wilcock, R. J. (1977).** Low pressure solubilities of gases in liquid waters. *Chem Rev* **77**, 219–362.
- Yarlett, N., Hann, A. C., Lloyd, D. & Williams, A. G. (1981).** Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium*. *Biochem J* **200**, 365–372.
- Yarlett, N., Lloyd, D. & Williams, A. G. (1982).** Respiration of the rumen ciliate *Dastricha ruminantium* Schuberg. *Biochem J* **206**, 259–266.
- Yarlett, N., Hann, A. C., Lloyd, D. & Williams, A. G. (1983a).** Hydrogenosomes in a mixed isolate of *Isotricha prostoma* and *Isotricha intestinalis* from ovine rumen contents. *Comp Biochem Physiol* **74B**, 357–364.
- Yarlett, N., Scott, R. I., Williams, A. G. & Lloyd, D. (1983b).** A note on the effects of oxygen on hydrogen production by the rumen protozoon *Dastricha ruminantium* Schuberg. *J Appl Bacteriol* **55**, 359–361.
- Yarlett, N., Coleman, G. S., Williams, A. G. & Lloyd, D. (1984).** Hydrogenosomes in known species of rumen entodiniomorphid protozoa. *FEMS Microbiol Lett* **21**, 15–19.
- 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.
- Yarlett, N., Rowlands, C., Yarlett, N. C., Evans, J. C. & Lloyd, D. (1987).** Respiration in the hydrogenosome-containing fungus *Neocallimastix patriciarum*. *Arch Microbiol* **148**, 25–28.

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