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Mitochondria-derived organelles in the diplomonad fish parasite *Spironucleus vortens*

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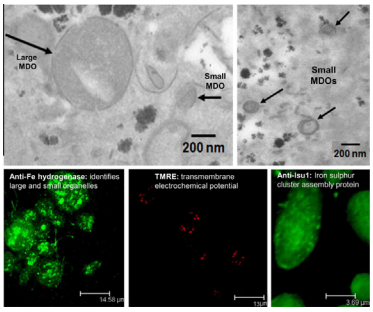
HIGHLIGHTS

- *Spironucleus vortens* has 200–1000 and 100–150 nm double-membraned organelles.
- The larger organelles take up membrane potential-sensitive lipophilic probes.
- Anti- Fe-hydrogenase localizes to the large, autofluorescent organelles.
- Anti-frataxin and Isu1 localize to the smaller organelles.
- Putative presence of mitochondria-derived organelles in *S. vortens*.

GRAPHICAL ABSTRACT



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ABSTRACT

In some eukaryotes, mitochondria have become modified during evolution to yield derived organelles (MDOs) of a similar size (hydrogenosomes), or extremely reduced to produce tiny cellular vesicles (mitosomes). The current study provides evidence for the presence of MDOs in the highly infectious fish pathogen *Spironucleus vortens*, an organism that produces H_2 and is shown here to have no detectable cytochromes. Transmission electron microscopy (TEM) reveals that *S. vortens* trophozoites contain electron-dense, membranous structures sometimes with an electron-dense core (200 nm–1 μm), resembling the hydrogenosomes previously described in other protists from habitats deficient in O_2. Confocal microscopy establishes that these organelles exhibit autofluorescence emission spectra similar to flavoprotein constituents previously described for mitochondria and also present in hydrogenosomes. These organelles possess a membrane potential and are labelled by a fluorescently labeled antibody against Fe-hydrogenase from *Blastocystis hominis*. Heterologous antibodies raised to mitochondrial proteins frataxin and Isu1, also exhibit a discrete punctate pattern of localization in *S. vortens*; however these labelled structures are distinctly smaller (90–150 nm) than hydrogenosomes as observed previously in other organisms. TEM confirms the presence of double-membrane bounded organelles of this smaller size. In addition, strong background immunostaining occurs in the cytosol for frataxin and Isu1, and labelling by anti-ferredoxin antibody is generally distributed and not specifically localized except for at the anterior polar region. This suggests that some of the functions traditionally attributed to such MDOs may also occur elsewhere. The specialized parasitic life-style of *S. vortens* may necessitate more complex intracellular compartmentation of redox reactions than previously recognized. Control of infection requires biochemical characterization of redox-related organelles.

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Abbreviations: MDO, mitochondria-derived organelles; TEM, transmission electron microscopy; PBS, phosphate-buffered saline at pH 7.2; TMRE, tetramethyl rhodamine ethyl ester.

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Introduction

Spironucleus spp., diplomonad protists (Family Hexamitidae, see Brugerolle et al. (1973), (1980), cause severe pathologies in companion and farmed animals and are of considerable concern in veterinary and agricultural medicine. Other *Spironucleus* spp. are of particular importance to aquaculture, as they can lead to devastating outbreaks of systemic infections in both ornamental and food fish (Williams et al., 2011). These small (typically 10–20 μm in length) parasitic flagellates occur in cold, temperate and tropical waters, and infect a wide range of freshwater and marine fish, as well as shellfish and crustaceans. Fish farmed in aquaculture, an industry of growing importance as wild fish stocks become increasingly depleted, are particularly susceptible to disease outbreaks caused by diplomonads. For instance, *Spironucleus salmonicida* (formerly *Spironucleus barkhanus*, see Jorgensen and Sterud, (2006)), has caused massive outbreaks of systemic infections in farmed Norwegian (*Salmo salar*) Atlantic salmon, and British Columbian (*Oncorhynchus tshawytscha*) Chinook salmon. In the ornamental fish industry,*Spironucleus* spp. cause severe infections in several economically important species including cichlids, and is generally associated with poor husbandry (Paull and Matthews, 2001). *Spironucleus vortens* has been proposed as the causative agent of hole-in-the-head, a disease linked to systemic infection with parasites recovered from internal organs, the lateral line, and skin lesions (Paull and Matthews, 2001). Its anaerobic growth in culture is extremely rapid (doubling time about 2 h; Millet et al., 2011a) and in addition to CO_2 it produces ethanol, acetate, alanine and lactate (Millet et al., 2011b). Therapy is limited to treatment with 5-nitroimidazoles (e.g., metronidazole), recently shown to disrupt proteome constituents affecting redox balance (Williams et al., 2012).

Spironucleus vortens was recently found to produce large amounts of H_2 (Millet et al., 2010). This is a characteristic of many parasitic and free-living organisms growing in habitats where O_2 tension is low. Under these conditions, secondary modifications of mitochondria (organelles that are typically composed of 1000–1500 proteins) have led to the evolution of specialized mitochondria-derived organelles (MDOs, Yarlett, 2004; Shiflett and Johnson, 2010). Thus, the hydrogenosomes of *Trichomonas vaginalis* with 569 identified proteins (Schneider et al., 2011) are similar to mitochondria in size, are bounded by double membranes, accumulate Ca^{2+} (Chapman et al., 1985) and are able to generate a transmembrane electrochemical potential (Humphreys et al., 1994; Emelyanov and Goldberg, 2011). However, trichomonad hydrogenosomes do not possess a genome, a cytochrome-mediated electron transport chain (Lloyd et al., 1979a), or F_1–F_0 ATP synthase (Lloyd et al., 1979b), and cardiolipin, present in *Tritrichomonas foetus* (Rosa et al., 2006), is undetectable in*T. vaginalis* (Guschina et al., 2009). Whereas mitochondria in most aerobic animal and fungal cells, are the sole redox active organelles providing ATP through oxidative phosphorylation, in anaerobic and some microaerophilic eukaryotes, hydrogenosomes generate energy only through substrate-level phosphorylation (Muller and Lindmark, 1978; Muller et al., 2012).

Even more extensive evolutionary loss in *Giardia intestinalis* (Lloyd et al., 2002; Tovar et al., 2003; van der Giezen and Tovar, 2005), another diplomonad parasite, has yielded mitosomes, tiny (<150 nm dia.) double-membrane bounded MDOs first described in *Entamoeba histolytica* (Tovar et al., 1999). Also having lost their energy-generating, oxidative phosphorylation functions during adaptation to the anaerobic lifestyle, mitosomes have retained only their most basic functions e.g. as a site for the maturation of Fe-S clusters (Muhlenhoff and Lill, 2000; Muhlenhoff et al., 2002; Tovar et al., 2003; Lill, 2009). They also possess essential translocase functions (Dolezal et al., 2005; Dagley et al., 2009; Schneider et al., 2011) but only have about 20 confirmed proteins (Jedelsky et al., 2011). Mitosomes have been identified in several other parasitic eukaryotes (Vivares et al., 2002) such as the microsporidians *Trachipleistophora hominis* (Williams et al., 2002) and *Encephalitozoon cuniculi* (Tsaousis et al., 2008). Heterogeneity of protein composition in vesicular organelles of mitosomal size in the microsporidian parasite of humans, *E. cuniculi*, implies further evolution of functional diversity (Katinka et al., 2001; Ghosh et al., 2012).

Early ultrastructural investigations of the *Spironucleus* genus using electron microscopy described highly complex intracellular organization, but no evident mitochondria (Brugerolle et al., 1973, 1980; Poynton and Sterud, 2002). The present investigation of the organelles of *S. vortens* was initiated as a consequence of the lack of evidence for cristate mitochondria in published electron micrographs and the rapid H_2 production exhibited by the organism at $[O_2]$ <5 μM (Millet et al., 2010). Here we present evidence for the occurrence of MDOs in *S. vortens* using electron and optical microscopy.

2. Materials and methods

2.1. Organisms and cultures

Spironucleus vortens, ATCC 50386, was obtained from Prof. J. Kulda (Charles University, Prague, Czech Republic) and cultured axenically at 20 °C in Keister's modified TYI-S-33 medium as described (Millet, 2009; Millet et al., 2011a). Log phase cultures were harvested by centrifugation at 800*g* for 3 min at room temperature in a bench centrifuge (MSE minor). *G. intestinalis*, JKH strain, was a gift from Drs. Victoria Hough and Timothy Paget (Medway, School of Pharmacy, UK). Trophozoites were cultured axenically at 37 °C in Diamond's modified medium Diamond's (1957) containing per L: tryptone (BBL), 20 g; yeast extract (oxoid), 10 g; glucose, 5 g; arginine, 1.06 g, NaCl, 1 g; K_2HPO_4, 1 g; KH_2PO_4, 0.6 g; cysteine, 1 g; ascorbic acid, 0.2 g; ammonium ferric citrate, 22.8 mg; bovine bile, 1 g; distilled water, 840 ml; heat inactivated newborn calf serum, 100 ml, pH was adjusted to 6.9–7 prior to filter sterilization (0.22 μm pore size). After 10 min incubation of the culture tubes on ice, detached trophozoites were harvested by centrifugation (800*g*, 5 min), at 4 °C in a Beckman Coulter Avanti J-E centrifuge (Fullerton, California, USA). *T. vaginalis* (ATCC 30001: C-1:NIH) cultures were grown at 37 °C in TYM medium. Log phase cultures were harvested by centrifugation at 800*g* for 2 min at room temperature in a bench centrifuge (MSE minor). *Saccharomyces cerevisiae* IFO-2033 (IFO, Institute of Fermentation, Osaka, Japan), and *Schizosaccharomyces pombe* 972 h^- ATCC 24843 were grown at 30 °C with rotary shaking at 150 rev/min in a medium containing 2% Bactopeptone, 1% yeast extract, 2% (w/v) glucose (Lemar et al., 2002). Where necessary organisms were washed twice by centrifugation after resuspension in phosphate-buffered saline (PBS), pH 7.2). Imaging of live *S. vortens* was limited by their strong motility and even the use of 5% (w/v) agarose or methyl cellulose was ineffective at complete immobilization.

2.2. Low temperature spectrophotometry

In order to detect respiratory pigments, $\sim 10^8$ *S. vortens*, *S. cerevisiae* and *S. pombe* (the latter two were used as positive controls) cells were washed 3× in PBS (pH 7.2) and loaded into two perspex cuvettes with 2 mm path length in a specially constructed

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