Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 38 (2008) 177–190
www.elsevier.com/locate/ijpara

First molecular characterisation of hydrogenosomes in the protozoan parasite *Histomonas meleagridis*

Muriel Mazet [a], Marie Diogon [a], John F. Alderete [b], Christian P. Vivarès [a], Frédéric Delbac [a,*]

[a] *Equipe Parasitologie Moléculaire et Cellulaire, LBP, UMR CNRS 6023, Université Blaise Pascal, 24 Avenue des Landais, 63177 Aubière Cedex, France*
[b] *Department of Microbiology, MC7758, UTHSCSA, 77003 Floyd Curl Dr.San Antonio, TX 78229-3900, USA*

Received 27 April 2007; received in revised form 11 June 2007; accepted 27 June 2007

Abstract

Histomonas meleagridis is a trichomonad species that undergoes a flagellate-to-amoeba transformation during tissue invasion and causes a serious disease in gallinaceous birds (blackhead disease or histomoniasis). Living in the avian cecum, the flagellated form can be grown in vitro in the presence of an ill-defined bacterial flora. Its cytoplasm harbours numerous spherical bodies which structurally resemble hydrogenosomes. To test whether these organelles may be involved in anaerobic metabolism, we undertook the identification of *H. meleagridis* genes encoding some potentially conserved hydrogenosomal enzymes. The strategy was based on several PCR amplification steps using primers designed from available sequences of the phylogenetically-related human parasite *Trichomonas vaginalis*. We first obtained a C-terminal sequence of an iron-hydrogenase homologue (Hm_HYD) with typical active site signatures (H-cluster domain). Immunoelectron microscopy with anti-Hm_HYD polyclonal antibodies showed specific gold labelling of electron-dense organelles, thus confirming their hydrogenosomal nature. The whole genes encoding a malic enzyme (Hm_ME) and the α-subunit of a succinyl coenzyme A synthetase (Hm_α-SCS) were then identified. Short N-terminal presequences for hydrogenosomal targeting were predicted in both proteins. Anti-Hm_ME and anti-Hm_α-SCS antisera provided immunofluorescence staining patterns of *H. meleagridis* cytoplasmic granules similar to those observed with anti-Hm_HYD antiserum or mAb F5.2 known to react with *T. vaginalis* hydrogenosomes. Hm_ME, Hm_α-SCS and Hm_HYD were also detected as reactive bands on immunoblots of proteins from purified hydrogenosomes. Interestingly, anti-Hm_α-SCS staining of the cell surface in non-permeabilised parasites suggests a supplementary role for SCS in cytoadherence, as previously demonstrated in *T. vaginalis*.
© 2007 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: *Histomonas meleagridis*; Hydrogenosome; Iron-hydrogenase; Malic enzyme; α-Succinyl coenzyme A synthetase; Cell adhesion

1. Introduction

Histomoniasis, also known as ‘‘blackhead’’ disease, is a serious parasitic infection due to the microaerophilic flagellated protozoan *Histomonas meleagridis* that causes significant damage to the ceca and liver in gallinaceous birds (turkeys, chickens, quail, etc.). Untreated enterohepatitis may cause an especially high rate of mortality in farm flock turkeys, entailing heavy economic losses to the poultry industry (McDougald, 2003). Some heterocyclic compounds, including nifursol and dimetridazole, were successfully used as feed additives for preventive treatment or as curative drugs (McDougald, 2005). However, due to potential health risks to human consumers, these drugs were withdrawn from the market in Europe, leading to a recrudescence of histomoniasis. The life cycle of *H. meleagridis* commonly involves an intermediate host, the cecal worm *Heterakis gallinarum*, which transmits the parasite from bird to bird within its eggs (Graybill and Smith, 1920; Tyzzer, 1924). Histomoniasis in turkeys has also been shown to occur in the absence of cecal worms, suggesting possible direct, lateral

* Corresponding author. Tel.: +33 4 73 40 78 68; fax: +33 4 73 40 76 70.
E-mail address: frederic.delbac@univ-bpclermont.fr (F. Delbac).

0020-7519/$30.00 © 2007 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2007.06.006

transmission (Hu and McDougald, 2003). Two different forms of *H. meleagridis* can be observed in infected hosts, (i) a flagellated form with one anterior flagellum, residing in the cecal lumen and (ii) an amoeboid form invading the intestinal mucosa and liver (Bishop, 1938; Lund et al., 1967). No cyst has so far been identified. In vitro cultures were obtained in the presence of an undefined bacterial flora taken from the turkey cecum (Lund, 1969).

Electron microscopy studies of *H. meleagridis* have revealed morphological similarities with trichomonads, notably the presence of many mastigont structures with four kinetosomes (Rybicka et al., 1972). In the taxonomic system of Cavalier-Smith (1998), *H. meleagridis* is placed in the phylum Parabasala, class Trichomonadea, family Monocercomonadidae. Comparative analysis of small subunit rRNAs suggests a close phylogenetic relationship with *Dientamoeba fragilis* (Gerbod et al., 2001) an atypical intestinal trichomonad that lacks flagella throughout its life cycle. Thus, *Histomonas* and *Dientamoeba* may be representative of a reductive evolution marked by more or less extensive losses of trichomonad cytoskeletal structures.

Typical trichomonads harbour hydrogenosomes instead of mitochondria, these organelles being the sites of a fermentative oxidation of pyruvate that yields acetate, carbon dioxide and hydrogen with concomitant production of ATP, as discovered in the cattle parasite *Tritrichomonas foetus* (Lindmark and Müller, 1973) and subsequently in the human parasite *Trichomonas vaginalis* (Lindmark et al., 1975). The effective treatment of human trichomoniasis is based on the use of 5-nitroimidazole drugs such as metronidazole, that are activated to cytotoxic anion radicals through their reduction in *T. vaginalis* hydrogenosomes (Hrdý et al., 2005). Similar to *Trichomonas*, *H. meleagridis* is sensitive to 5-nitroimidazoles (McGuire et al., 1964; McDougald and Jinghui, 2004). Membrane-bounded dense bodies have been observed in the cytoplasm of the avian parasite (Schuster, 1968) but the molecular characteristics of the presumed hydrogenosomes have yet to be explored. It should be stressed that some hydrogenosomal enzymes of *T. vaginalis* may have an alternate location related to an additional function. More precisely, these enzymes can be targeted to the cell surface in order to mediate the adherence of an amoeboid form of the parasite to vaginal epithelial cells (Alderete et al., 2001; Garcia et al., 2003; Moreno-Brito et al., 2005). A search for hydrogenosomal enzymes in *H. meleagridis* was therefore of interest in gaining a better understanding of its anaerobic metabolism and putative adhesins.

Here, we have determined the sequences of three *H. meleagridis* genes encoding homologues of the following proteins: (i) an iron-only hydrogenase (Fe-hydrogenase), the key enzyme for molecular hydrogen production in hydrogenosomes (Horner et al., 2000), (ii) the alpha-subunit of a succinyl-coenzyme A synthetase (α-SCS) (Jenkins et al., 1991; Alderete et al., 2001) and (iii) a nicotinamide adenine dinucleotide phosphate (NADP)-dependent hydrogenosomal malic enzyme (ME) (Drmota et al., 1996). Immunolocalisation experiments using antibodies raised against corresponding recombinant polypeptides provide strong evidence for the hydrogenosomal nature of *H. meleagridis* cytoplasmic bodies and indicate that α-SCS may also be transferred to parasite cell surface.

2. Materials and methods

2.1. Parasite culture and nucleic acid preparation

The non-axenic *H. meleagridis* strain HmZL, isolated from a chicken orally infested by *Heterakis gallinarum* eggs, was kindly provided by Dr. L. Zenner (Ecole Nationale Vétérinaire, Lyon, France). Parasites were grown using small culture tubes (4 ml) maintained in a vertical position, each tube containing 3 ml of medium 199 (Gibco) supplemented with 5% horse serum (Gibco) and 5% rice starch (Sigma) at 39 °C (Dwyer, 1970). Cells were collected by centrifugation at 250*g* for 2 min, washed four times in PBS to remove bacteria and then stored at −20 °C. Genomic DNA and total RNA were prepared using the ELU-Quick DNA purification Kit (Schleicher & Schuell) and the TRI Reagent® (Molecular Research Center, Inc.), respectively.

2.2. PCR amplifications

Primers used for the amplification of the *H. meleagridis* [Fe]-hydrogenase, α-SCS and ME gene fragments were first designed from *T. vaginalis* (Tv) genes. In subsequent steps of the protocol, other primers were specific to *H. meleagridis* (Hm) sequences.

Primers Tv1 (5′-ccatgcacagccaagaagga-3′) and Tv2 (5′-gcagccacctgggcaggccat-3′) allowed the amplification of a 460-bp fragment of the [Fe]-hydrogenase gene. Identification of the 3′ end was realised from reverse transcribed mRNAs. Reverse transcription was performed with the adaptator oligo-dT primer (5′-gactcactatagggcatgct$_{17}$-3′) using 15 U of the AMV reverse transcriptase (Pharmacia). PCR reaction was then performed using the adaptator primer (5′-gactcactatagggcatgc-3′) in combination with Hm1 (5′-acaaggatccaaaggttgc-3′). After denaturing the 1:2 dilution of cDNA at 94 °C for 3 min, 35 cycles were run as follows: denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and elongation at 72 °C for 1 min, with a final extension of 10 min at 72 °C.

For the α-*scs* gene, primers Tv3 (5′-cttatcggtccaaactgcc-cag-3′) and Tv4 (5′-agctgtgcccttgccgccag-3′) amplified a fragment of ~400 bp. The lacking 5′ end was identified using primers Tv5 (5′-atgctctcctcttccttc-3′) and Hm3 (5′-gttggaataattcccatc-3′). Amplification of the 3′ end was performed from reverse transcribed mRNAs using the adaptator primer in combination with Hm2 (5′-ggaaaaatcggtattgtttcc-3′). One whole α-*scs* gene was finally amplified from genomic DNA using primers Hm4 (5′-cat-tgccatctgaagacc-3′) and Hm5 (5′-ttagatgcggcccaactt-3′).

An ~800 bp-fragment of the ME gene was obtained with primers Tv6 (5′-gtattctcggtctcggcgatc-3′) and Tv7 (5′-ttggtgttgggttggaaagtg-3′). Identification of the 5′ end was performed using primers Tv8 (5′-atgctcacatcttcagtc-3′) and Hm7 (5′-accaactgggataccaag-3′). The 3′ end was identified after reverse transcription with the adaptator primer in combination with Hm6 (5′-ttgcgcaccacgtatcagg-3′). A complete gene was then amplified from genomic DNA using primers Tv8 (5′-atgctcacatcttcagtc-3′) and Hm8 (5′-ttagtaaacgtgttcatattc-3′).

PCR amplifications were performed in a 50 μl reaction using a Perkin-Elmer DNA thermal cycler 2400 apparatus. After denaturing DNA at 94 °C for 3 min, 35 cycles were run with 20 s of denaturation at 94 °C, 30–45 s of annealing at 48–55 °C and 1 min of extension at 72 °C, followed by a last extension step for 10 min at 72 °C. All PCR products were analysed by electrophoresis in 1% agarose gel and purified with a Qiaquick gel extraction kit (Qiagen). They were then cloned in a pGem-T easy vector (Promega) and sequenced.

2.3. Sequence analyses

Molecular masses and isoelectric points were calculated using ExPASy Proteomics tools (http://www.expasy.org/tools/). Amino acid sequence alignment was performed with ClustalW program (http://www.ebi.ac.uk/clustalw/). The search for conserved protein domains was undertaken with Pfam (http://www.sanger.ac.uk/Software/Pfam/).

2.4. Expression of recombinant *Histomonas meleagridis* proteins in *Escherichia coli*

As a prerequisite to heterologous expression, some parts of the three studied *H. meleagridis* genes were PCR-amplified with the insertion of one BamHI and one EcoRI restriction site in 5′ and 3′ of the amplified fragment, respectively. Corresponding protein regions and primers were as follows: (i) a C-terminal part of Fe-hydrogenase, primers *hyd-D* (5′-cgggatccatcgaaagaccacaattctc-3′) and *hyd-R* (5′-cggaattcgacatctgcaacctttggatc-3′), (ii) α-SCS region 129–273, primers α-*scs*-D (5′-cgggatcccttattggtccaaactgc-3′) and α-*scs*-R (5′-cggaattcagcagtaccctttccacc-3′) and (iii) ME region 165–243, primers *me*-D (5′-cgggatcccgtattctcggtctcggt-3′) and *me*-R (5′-cggaattcgattgttgggttggaaag-3′). Amplifications were performed in a 50 μl reaction according to standard conditions (Eurobio). PCR-products were digested with both BamHI and EcoRI enzymes and cloned into a prokaryotic expression vector pGEX-4T1 (Pharmacia). This vector has previously been modified to include an 8-His tag at the C-terminus of the expressed protein, which is useful to purify the recombinant protein by affinity chromatography on Ni-NTA columns (Qiagen). Resulting recombinant plasmids pGEX-His-Hyd, pGEX-His-αSCS and pGEX-His-ME were introduced into *E. coli* BL21^{+} strain. After induction with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h, bacterial proteins were solubilised in 2.5% SDS, 100 mM dithiothreitol (DTT). Recombinant proteins were also purified on an Ni-NTA column according to the recommendations of the manufacturer (Qiagen). Protein samples were then analysed on 10% polyacrylamide gels.

2.5. Antibody production

Polyclonal antibodies to *E. coli*-expressed recombinant proteins were produced in SWISS mice from protein bands separated by SDS–PAGE. The animal house (agreement C63014.19) and the experimental staff (agreement 63-146) had been approved by the French Veterinary Services, and experiments were conducted according to ethical rules. Protein bands were excised from Coomassie blue-stained gels and crushed in PBS with a Potter tissue homogeniser. Mice were then injected i.p. with samples homogenised with FCA for the first injection and Freund's incomplete adjuvant for the next injections (at days 14, 21 and 28). Sera were collected 1 week after the last injection and stored at −20 °C.

2.6. SDS–PAGE and Western blotting

Histomonas meleagridis proteins were solubilised with 1% Nonidet P-40 in PBS in the presence of 0.2 mM cysteine proteinase inhibitor *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64) (Sigma). Protein samples were then analysed by 10% SDS–PAGE. After electrophoresis, proteins were stained with Coomassie blue or transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). For immunological detection, membranes were saturated in PBS-5% skimmed milk and incubated for 3 h with an appropriate dilution of mouse antibodies (1:100–1:1000). After washing, membranes were reacted with horseradish phosphatase alcaline-conjugated goat anti-mouse IgG (1:10,000 dilution, Promega) and revealed by NBT-BCIP (Promega).

2.7. Indirect immunofluorescence assays

Parasites were fixed with 100% methanol for 30 min at room temperature. In some experiments, fixation was done with 4% formaldehyde and 0.25% glutaraldehyde for 1 h at room temperature then washed in PBS-50 mM NH_4Cl. Fixed cells were washed once in PBS, resuspended in PBS and placed on polylysine-treated cover glasses. Slides were saturated with PBS-5% skimmed milk then incubated for further 1 h with the different antisera diluted in PBS-0.1% Triton X-100. After washing, slides were stained for 1 h with a 1:1000 dilution of Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes). Preparations were examined with a DMR Leica epifluorescence microscope.

2.8. Transmission electron microscopy (TEM)

For TEM observations of *H. meleagridis* cells, parasite pellets were fixed for 30 min with 2% glutaraldehyde and

0.05% ruthenium red in 0.1 M cacodylate buffer (pH 7.4), and then post-fixed for 1 h with 1% OsO_4 in the same buffer. After dehydration through a graded ethanol series and infiltration in propylene oxide, cells were embedded in Epikote resin (Agar Scientific). Ultrathin sections were obtained with an UltracutS Leica ultramicrotome, double stained with uranyl acetate and lead citrate, and then examined under a JEOL 1200X transmission electron microscope.

For immunogold labelling, parasites were fixed with 4% formaldehyde–0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 30 min. Cells were washed in PBS, dehydrated and embedded in Unicryl resin (British BioCell International). Ultrathin sections blocked for 1 h with 1% BSA in PBS were incubated for 3 h with a 1:50 dilution of anti-Hm_HYD mouse antisera then for 1 h with a 1:100 dilution of goat anti-mouse IgG conjugated with 10 nm colloidal gold particles (Sigma). Preparations were stained with 4% uranyl acetate, prior to examination.

2.9. Preparation of a hydrogenosome-enriched fraction

Histomonas meleagridis cells were harvested by centrifugation (250*g*, 2 min), washed three times in PBS and once in an isotonic medium (225 mM sucrose, 20 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2). The washed parasites were resuspended in isotonic medium and disrupted by numerous strokes in a Potter tissue homogeniser for 5 min. The homogenate was centrifuged at 500*g* for 10 min to remove nuclei and large cell debris. A crude hydrogenosome fraction was further pelleted at 14,000*g* for 20 min and examined by TEM. Proteins of this cell fraction were solubilised in Laemmli buffer containing 100 mM DTT and 2.5% SDS, separated by SDS–PAGE and transferred onto PVDF membranes. Mouse antisera were then applied as described above.

3. Results

3.1. Some observations on xenically cultured Histomonas meleagridis

Histomonas meleagridis was cultured in medium 199 supplemented with horse serum and rice starch, the last ingredient being essential for in vitro development of the parasite. In contrast to the original procedure of Dwyer (1970), chicken embryo extract was not added because we failed to detect any significant stimulating effect by this component. Light microscopy observations showed the predominance of roundish flagellated forms, about 15–20 μm in diameter (Fig. 1A). The *H. meleagridis* generation time was estimated to be ~6 h. As shown in Fig. 1, the cultures also contain bacteria that should mainly originate from the turkey ceca. Some preliminary studies to identify these bacteria have been undertaken. Sequencing data from PCR-amplified 16S rDNA supported the preponderance of the anaerobic species *Bacteroides pyogenes*. Our attempts to fully remove the bacterial population with the help of different antibiotics (ampicillin, penicillin, streptomycin, gentamicin) were unsuccessful. TEM examination of such xenic cultures clearly indicate that *H. meleagridis* cells can ingest some bacteria as well as rice starch granules via phagocytosis (Fig. 1C, D), but whether such bacteria are absolutely required for parasite nutrition remains an open question. It seems likely that the microaerophilic culture conditions are converted into nearly anaerobic ones, as a result of the profuse growth of bacteria depleting the oxygen from the medium.

In addition to large food vacuoles, the *H. meleagridis* cytoplasm exhibits numerous spherical bodies, 300–600 nm in diameter, characterised by an electron-dense matrix (Fig. 1E, F) and resembling the hydrogenosomes of *T. vaginalis* and *T. foetus.* Thus, it may be that, in common with *T. vaginalis* and *T. foetus*, *H. meleagridis* has a compartmentalisation of some similar enzymes involved in anaerobic metabolism. Given the phylogenetic affinities between *Histomonas* and *Trichomonas* genera, a first step in our PCR amplification strategy for putative genes encoding hydrogenosomal enzymes in the avian parasite was based on the use of primers designed from available *T. vaginalis* sequences.

3.2. Evidence for Histomonas meleagridis iron-hydrogenase

Fe-hydrogenase is specific to the hydrogenosomal metabolism. Trying to identify such an enzyme in *H. meleagridis* was therefore important to ascertain whether its hydrogenosomes really have the capacity to associate protons and electrons for producing molecular hydrogen. The alignment between four *T. vaginalis* Fe-hydrogenases allowed the design of primers in two conserved regions of the presumed hydrogen-activating site (PCTAKKD and MACPGGC, see Figs. 2 and 3). These primers (Tv1 and Tv2) resulted in the amplification of a 460-bp fragment from *H. meleagridis* genomic DNA. The lacking 3′ region of the gene was then amplified by RT-PCR of *H. meleagridis* mRNAs. After reverse transcription with an oligo-dT primer, the PCR reaction was performed using the primer corresponding to the adaptator sequence linked to oligo-dT and the primer Hm1 designed from the 460-bp DNA sequence (Fig. 2a). A 3′ untranslated region (3′ UTR) of only 18 nucleotides long was revealed. Unfortunately, we did not succeed in identifying the 5′ end part of the gene. The deduced partial protein sequence (Hm_HYD) represents the last 223 amino acids of a complete enzyme and has 62% identity with a 51-kDa Fe-hydrogenase of *T. vaginalis* (Fig. 3). As expected, the C-terminal region harbours conserved motives specific to the H-cluster domain involved in catalysis and three of the four cysteine residues required for its association with a complex metal cofactor.

A part of the obtained *H. meleagridis* sequence (see Fig. 3) was cloned in the pGEX-4T1-His vector for expression of a recombinant polypeptide in *E. coli*. After IPTG induction, SDS–PAGE analysis of the bacterial lysate

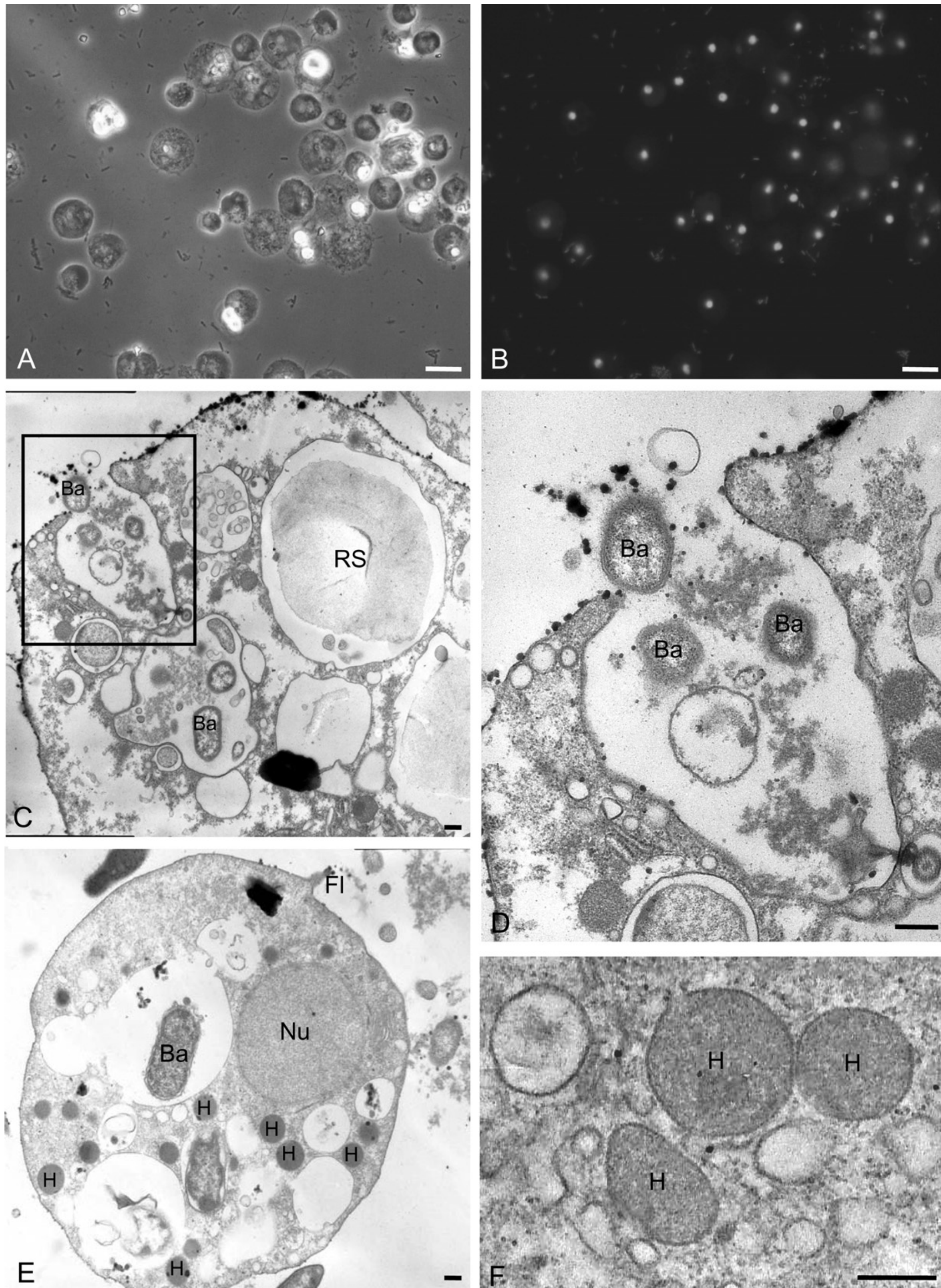

Fig. 1. Light and electron micrographs of in vitro cultivated *Histomonas meleagridis*. As viewed in phase contrast (A) or after DAPI staining (B), the cell population is represented by spheroid protozoan cells (their flagella are out of the plane of focus) and rod-shaped bacteria. Large refractile inclusions correspond to ingested starch granules. (C) Ultrathin cross-section through *H. meleagridis* cytoplasm, showing several food vacuoles that contain rice starch (RS) and bacteria (Ba). (D) Higher magnification of a peripheral area (boxed in C) characterised by an early step of phagocytosis. (E, F) Micrographs illustrating the presence of electron-dense hydrogenosome-like organelles (H) scattered between digestive vacuoles of various sizes. Fl: flagellum, Nu: nucleus, RS: rice starch. Bars, 10 μm for (A–B) and 500 nm for (C–F).

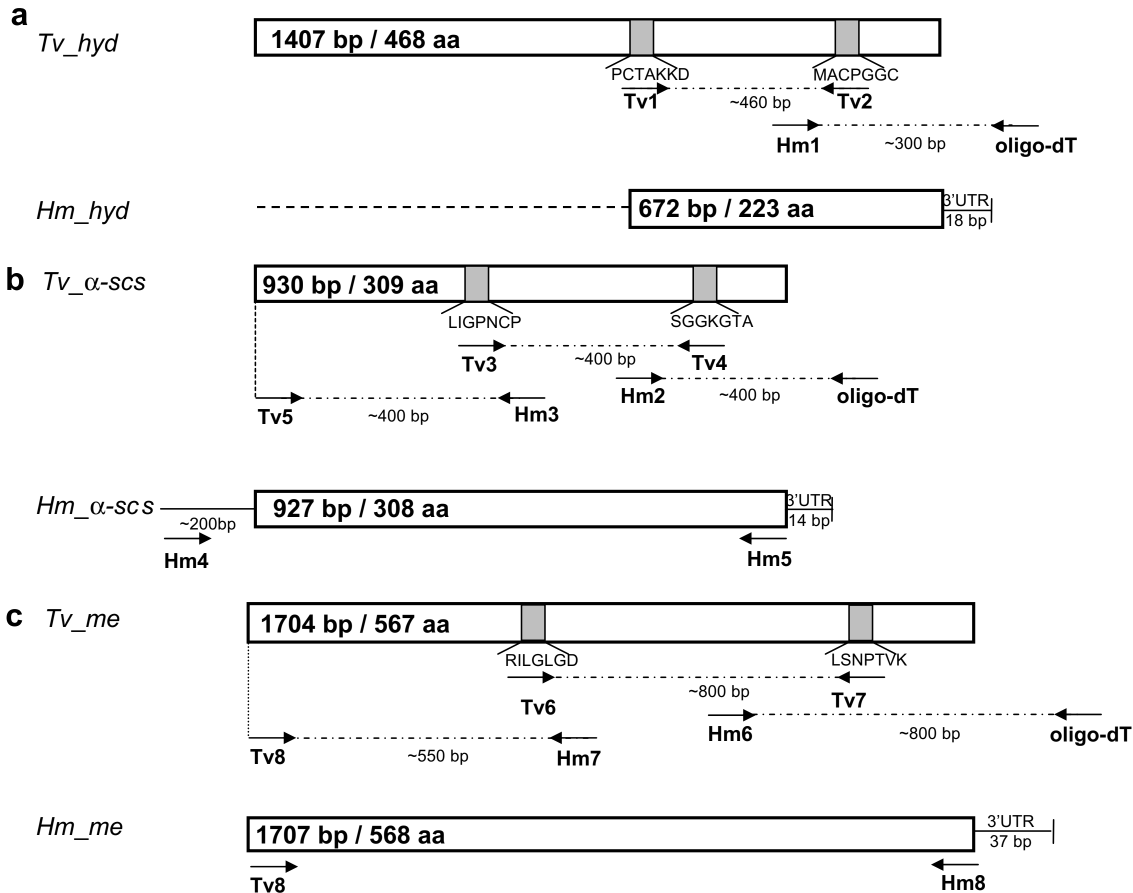

Fig. 2. PCR strategy used for the amplification of three *Histomonas meleagridis* genes encoding Fe-hydrogenase (*Hm_hyd*, (a), α-succinyl coenzyme A synthetase (*Hm_α-scs*, (b) and malic enzyme (*Hm_me*, (c). The first-round PCR was performed using primer pairs (Tv1-Tv2, Tv3-Tv4 and Tv6-Tv7) designed from conserved regions in *Trichomonas vaginalis* open reading frames (ORFs) (*Tv_hyd*, *Tv_α-scs* and *Tv_me*, respectively). The 3′ end region of each gene, including the 3′ untranslated region, was then determined by RT-PCR with an oligo-dT primer and a forward Hm primer designed from a previously amplified *H. meleagridis* sequence. For *Hm_α-scs* and *Hm_me*, a 5′ end region was PCR-amplified using a Tv-Hm primer pair. The full ORFs of the last two genes were finally validated through amplification with either Hm4-Hm5 for *α-scs* or Tv8-Hm8 for *me*.

```
Tv_HYD      MLASSATAMKGFANSLRMKDYSSTGINFDMTKCINCQSCVRACTNIAGQNVLKSLTVNGK   60

Tv_HYD      SVVQTVTGKPLAETNCISCGQCTLGCPKFTIFEADAINPVKEVLTKKNGRIAVCQIAPAI  120

Tv_HYD      RINMAEALGVPAGTISLGKVVTALKRLGFDYVFDTNFAADMTIVEEATELVQRLSDKNAV  180
                   ●
Tv_HYD      LPMFTSCCPAWVNYVEKSDPSLIPHLSSCRSPMSMLSSVIKNVFPKKIGTTADKIYNVAI  240
              ●
Tv_HYD      MPCTAKKDEIQRSQFTMKDGKQETGAVLTSRELAKMIKEAKINFKELPDTPCDNFYSEAS  300
Hm_HYD      -PCTAKKDEIERPQFSTK-GFKETDYVITTRELMRMIKKHKIDFKNLPDSEFDVPFSEAS
             ******** * **   * *  **   * * ***   ***   ** ** ***   *   ****

Tv_HYD      GGGAIFCATGGVMEAAVRSAYKFLTKKELAPIDLQDVRGVASGVKLAEVDIAGTKVKVAV  360
Hm_HYD      GAGAIFCGSGGVMEAAVRTAYNIVTGKELPGTDLVAVRGHKDGIKVASVDIEGTKVGVAV
            * *****  ********* **    * ***    *    *** *** *    *** **** ***
                                                  ●  ●
Tv_HYD      AHGIKNAMTLIKKIKSGEEQFKDVKFVEVMACPGGCVVGGGSPKAKTKKAVQARLNATYS  420
Hm_HYD      AQGIANAMKLIKMIKDKDPKVADVKFVEVMACPGGCVCGGGSTKAKTKKATDARVDAVYK
            * ** *** ***   *        ****************** ******   **   *

Tv_HYD      IDKSSKHRTSQDNPQLLQLYKESFEGKFGGHVAHHLLHTHYKNRKVNP              468
Hm_HYD      IDTQSKVRCSHQNQQLKELYDRLLE-KPNSHLAHELLHTHYTNKKK--
            **  ** * *  * **  **     * *      ** ******** *
```

Fig. 3. Alignment between the partial sequence of an *Histomonas meleagridis* [Fe]-hydrogenase (Hm_HYD) and the C-terminal region of a 51-kDa [Fe]-hydrogenase of *Trichomonas vaginalis* (Tv_HYD; GenBank Accession No. XP 001330775.1). Underlined segments are specific to the conserved H-cluster domain required for catalysis. The four cysteine residues involved in linkage of a complex metal cluster correspond to Cys187, Cys243, Cys392 and Cys396 (●) in the *T. vaginalis* enzyme. Beginning with a proline residue just upstream of the Cys243 counterpart, the partial Hm_HYD sequence contains a major part of the H-cluster domain. The region selected for the expression of a recombinant polypeptide in *Escherichia coli* is shaded. Amino acids are numbered on the right.

revealed a highly expressed fusion protein with the expected size (not shown). This protein was purified on Ni-NTA columns and then used for mouse immunisation. In indirect immunofluorescence assay (IFA) experiments, the anti-Hm_HYD antisera obtained provided strong fluorescence signals associated with small bodies inside *H. meleagridis* cells (Fig. 4C). Immunoelectron microscopy clearly showed specific gold labelling of spheroid cytoplasmic organelles (Fig. 4D), which reinforces the idea that such organelles are hydrogenosomes. Three major bands were detected at 65, 51 and 48 kDa on Western blots (Fig. 4E). Thus, several [Fe]-hydrogenases, including an orthologue of the 51-kDa [Fe]-hydrogenase from *T. vaginalis*, are possibly expressed in *H. meleagridis*.

3.3. Amplification of Histomonas meleagridis whole gene sequences coding for α-SCS and ME

Eukaryotic SCSs are multimeric enzymes located in mitochondria and hydrogenosomes. The substrate-level phosphorylation reaction catalysed by hydrogenosomal SCSs produces ATP rather than GTP. The two kinds of organelles are also known to contain NAD(P)-dependent MEs catalysing the decarboxylation of malate to pyruvate. In *T. vaginalis*, α-SCS and ME both appear as moonlighting proteins, able to switch between a metabolic function and a role in parasite adherence when associated with either the hydrogenosome or the cell surface, respectively (Alderete et al., 2001). We obtained the sequences of two *H. meleagridis* genes encoding the aforementioned enzymes, through different PCR amplification steps (Fig. 2).

For α-SCS, *T. vaginalis*-specific primers (Tv3 and Tv4) were determined in two conserved amino acid regions (LIGPNCP and SGGKGTA), deduced from the alignment of various α-SCSs. Corresponding PCR amplification provided a *H. meleagridis* gene region of ~400 bp. The 3′ end of coding region was then identified by RT-PCR using an oligo-dT primer and its sequencing showed a 3′ UTR only 14 nucleotides in length (Fig. 2b). The 5′ end region was then determined using the primer Tv5 which was designed at the translation initiation codon of the *T. vaginalis α-scs* gene (Fig. 2b). However, the PCR product size was larger than expected (~600 bp instead of ~400 bp). Sequencing of this amplicon showed a complete N-terminal coding region preceded by ~200 nucleotides upstream of the ATG, which indicates that the hybridisation with the primer Tv5 occurred upstream of the start codon.

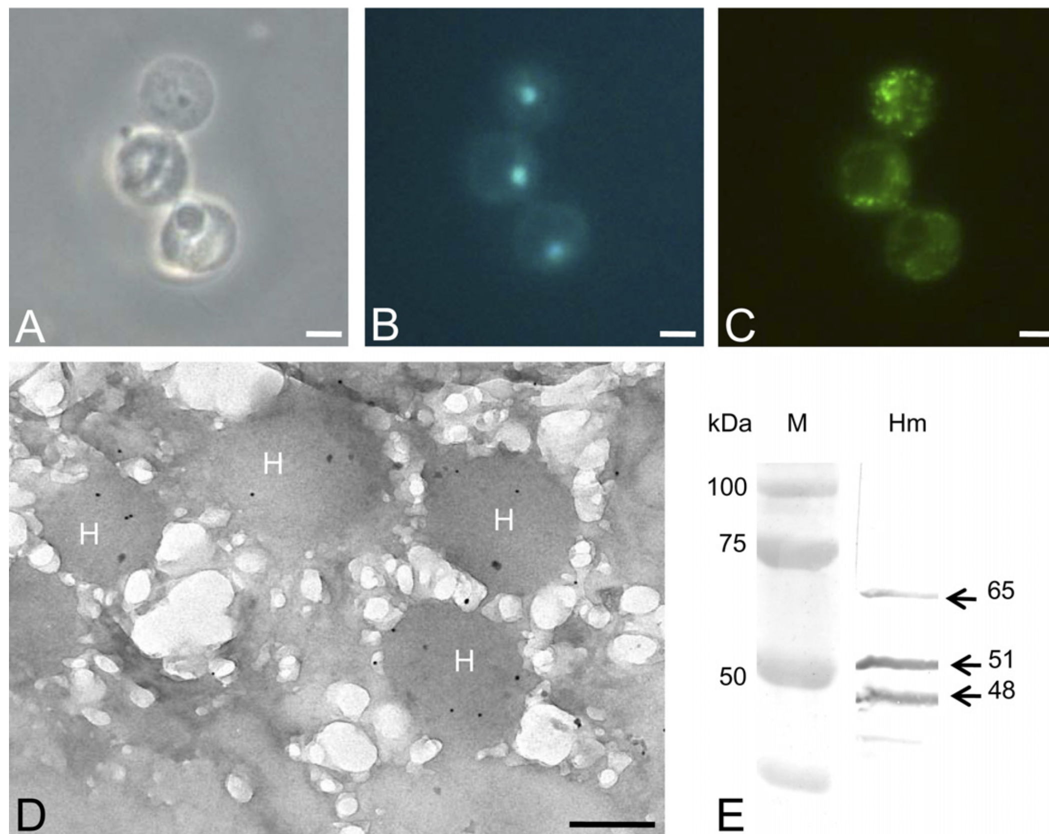

Fig. 4. Immunodetection of *Histomonas meleagridis* [Fe]-hydrogenase using anti-Hm_HYD antisera. Methanol fixed parasites were observed in phase contrast microscopy (A), after DAPI staining of nuclei (B) and after labelling with anti-Hm_HYD (1:100 dilution) in an immunofluorescence assay (C). Some cytoplasmic granules are reactive to the antibodies. Electron immunogold labelling (D) shows that these granules correspond to hydrogenosomes (H). Bars, 10 μm in (A–C) and 200 nm in (D). (E) Western blotting of *H. meleagridis* proteins solubilised with 1% Nonidet P40 (Hm), using anti-Hm_HYD at a 1:500 dilution. Three major reactive bands should represent potential [Fe]-hydrogenases with different sizes. Molecular mass standards (M) are in kDa.

Amino acid sequence alignment between various protozoan and mammalian MEs of mitochondrial or hydrogenosomal origin was helpful to determine primers in the two conserved regions RILGLGD and LSNPTVK (Fig. 2c). As for *hyd* and α-*scs*, two *T. vaginalis*-specific primers (Tv6 and Tv7) were used instead of some degenerate primers deduced from these peptides. An ~800-bp fragment was amplified and the lacking 3′ end region was further obtained by RT-PCR using an oligo-dT primer. The 3′ UTR was also highly reduced (37 nucleotides). The 5′ region was subsequently amplified using the primer Tv8 designed at the start codon of the *T. vaginalis me* gene.

For both enzymes, the whole coding regions were finally amplified from *H. meleagridis* genomic DNA. One primer was designed either 183 bp upstream of the start codon of α-*scs* (Hm4) or at the start codon of *me* (Tv8). The other primer was determined at the stop codon (Hm5 and Hm8 for α-*scs* and *me*, respectively). Sequencing of PCR products confirmed the assembly of previous partial sequences and the size of each open reading frame (ORF): 927 bp for α-*scs* and 1707 bp for *me* (Fig. 2). Similar to their *T. vaginalis* counterparts, the two *H. meleagridis* genes are intronless.

3.4. Amino acid sequence analysis of Hm_α-SCS and Hm_ME

Hm_α-scs ORF encodes a protein of 308 amino acids in length with a theoretical molecular weight of 32,243 Da and a predicted p*I* of 9.44. Unsurprisingly, this protein has the highest identity (81%) with the *T. vaginalis* homologue, justifying the amino acid sequence alignment of the two sequences shown in Fig. 5a. The Hm_α-SCS protein also has significant identity (52%) with α-SCS from *Neocallimastix patriciarum*, an anaerobic fungus that contains hydrogenosomes. Pfam (http://www.sanger.ac.uk/Software/Pfam/) analysis reveals the conservation of some typical signatures for α-SCS including the presence of CoA and ligase-CoA domains. The three zones which may contribute to the nucleotide-binding domain and the region surrounding the active site histidine are also present (Fig. 5a). MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html), iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/) and PSORTII (http://psort.nibb.ac.jp/form2.html) programs failed to predict a presequence for targeting of protein to mitochondria or mitochondria-like organelles. However, it is clear that Hm_α-SCS possesses a very short N-terminal extension (8 amino acids) that is highly similar to those found in *T. vaginalis* hydrogenosomal proteins (Lahti et al., 1994; Alderete et al., 1995). In both species, this peptide starts with Met-Leu-Ala/Ser/Thr and has an arginine at position −2 to the putative cleavage site.

Hm_me ORF codes for a protein of 568 amino acids in length, with a predicted molecular mass of 64,012 Da and pI of 8.25. The highest identity (77%) is found with subunit A of the hydrogenosomal *me* from *T. vaginalis* (Fig. 5b). The Hm_ME sequence exhibits conserved residues involved in catalytic activity of malic enzymes. This is the case for the lysine residue in position 183, shown to be the key residue for catalysis (Kuo et al., 2000). Residues involved in metal–ion binding, corresponding to Glu255, Asp256 and Asp279 (Drmota et al., 1996) are also conserved (Fig. 5b). A short N-terminal extension (12 amino acids) resembling *T. vaginalis* hydrogenosomal presequences is also present in Hm_ME and thus appears as a putative targeting signal.

3.5. α-SCS and ME are also localised into Histomonas meleagridis hydrogenosomes

Recombinant polypeptides of Hm_α-SCS and Hm_ME were expressed in *E. coli*, affinity purified and injected into mice. The constructs corresponded to regions 129–273 for Hm_α-SCS and 165–423 for Hm_ME (see Fig. 5). In Western blot assays with protein extracts from *H. meleagridis* cells, anti-Hm_ME antibodies specifically recognised a band close to 65 kDa and Hm_α-SCS antibodies reacted with a 33-kDa band (Fig. 6, lanes 2 and 3). These apparent molecular weights are in agreement with the values deduced from amino acid sequences. Western blots were also realised using the mAb F5.2 directed against the epitope SEEDAAEW of *T. vaginalis* α-SCS, the same epitope being present in the Hm_α-SCS sequence (see alignment Fig. 5). As expected, mAb F5.2 cross-reacts with the 33-kDa band that was stained with the mouse antisera raised against recombinant Hm_α-SCS (Fig. 6, lane 4).

IFAs were then performed to determine the localisation of Hm_ME and Hm_α-SCS proteins in *H. meleagridis* cells. The mouse antisera strongly reacted with numerous cytoplasmic granules in permeabilised parasites (Fig. 7A, E). The hydrogenosomal nature of these elements was confirmed by the observation of a similar staining pattern with mAb F5.2, known to recognise *T. vaginalis* hydrogenosomes (Fig. 7C). Given that in *T. vaginalis*, α-SCS and ME may play a supplementary function as AP33 and AP65 adhesins, respectively, we also evaluated the potential of both proteins to be localised at the cell surface of *H. meleagridis*. When using non-permeabilised parasites (aldehyde fixation) for IFA, the anti-Hm_α-SCS antiserum stained the cell surface (Fig. 7G), suggesting a dual localisation for α-SCS. In contrast, no fluorescence signal was detected with anti-Hm_ME antisera (Fig. 7K).

A hydrogenosome-enriched fraction was prepared by differential centrifugation of *H. meleagridis* cell homogenate according to the protocol described for *T. vaginalis* (Drmota et al., 1996). TEM observations confirmed the preponderance of membrane-bound ovoid structures viewed as hydrogenosomes (Fig. 8A). A partial extraction of electron-dense matrix contents occurred during organelle isolation and allowed a better visualisation of the two limiting membranes (Fig. 8B). Hm_α-SCS and Hm_ME proteins were present in the considered subcellular fraction, as shown after SDS–PAGE and Western

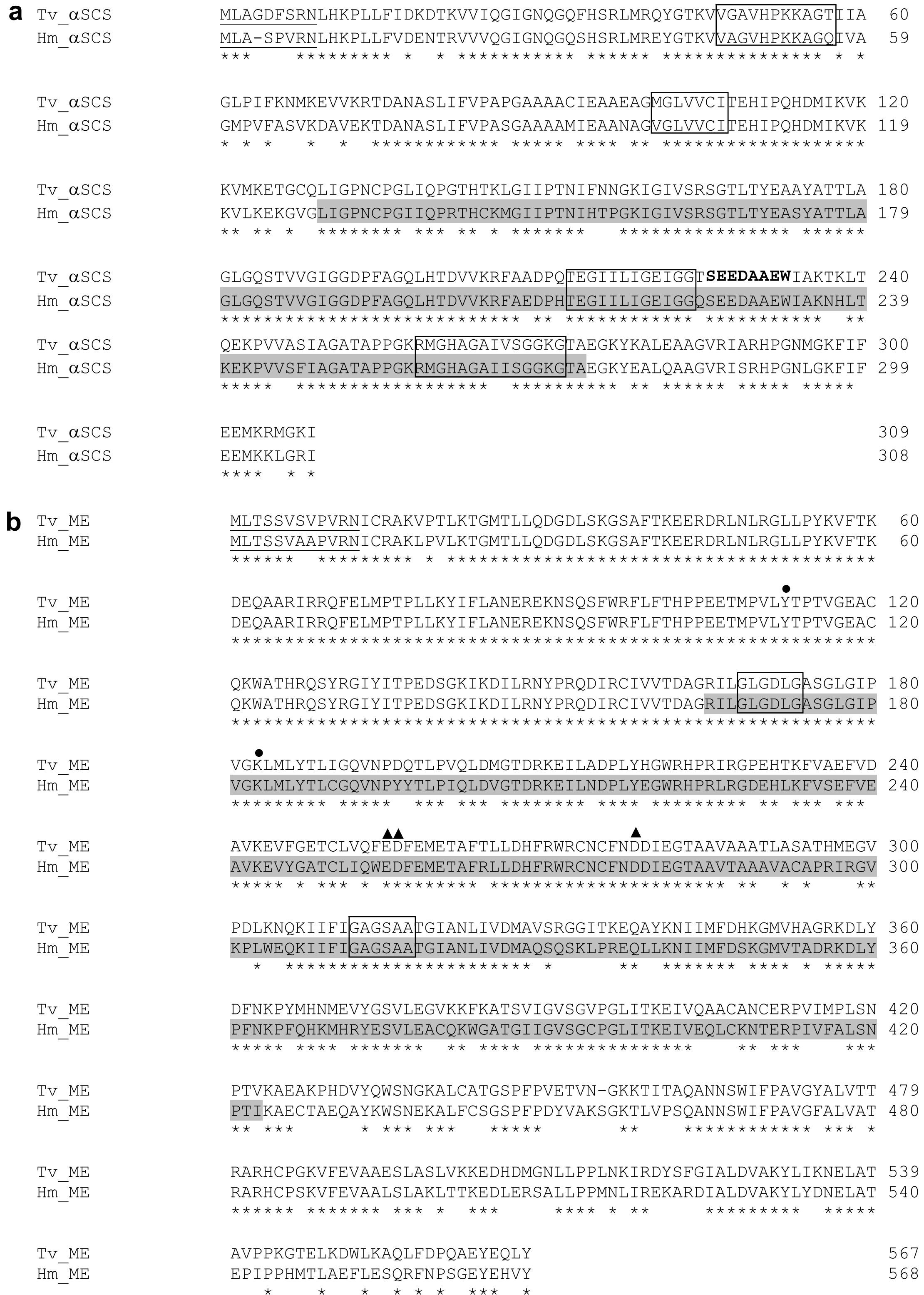

Fig. 5. Amino acid sequence alignment of α-SCS (a) and ME (b) from *Histomonas meleagridis* (Hm) and *Trichomonas vaginalis* (Tv). Underlined regions represent putative peptides for hydrogenosomal targeting. Shaded regions were selected for the expression of recombinant polypeptides in *Escherichia coli*. (a) Boxed areas in α-SCS sequences denote three conserved regions which may contribute to the nucleotide-binding domain (residues 45–56, 100–106 and 212–223) and the region surrounding the active site histidine (residues 258–271). The octapeptide epitope recognised by mAb F5.2 (residues SEEDAAEW) is indicated in bold. (b) Conserved residues involved in catalytic activity of malic enzymes (●) and the three residues involved in metal-ion binding (▲) are also found in the *H. meleagridis* protein. Boxed regions represent dinucleotide-binding signature motifs. Identical residues are indicated by asterisks. Amino acids are numbered on the right. GenBank Accession Nos. for Tv_α-SCS and Tv_ME are P53399 and AAA92714.1, respectively.

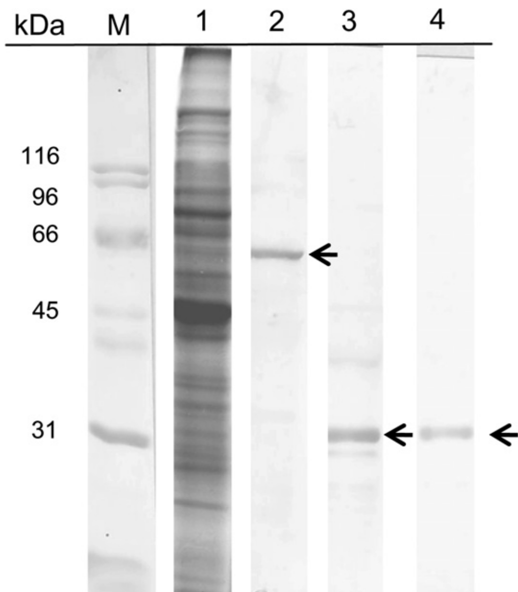

Fig. 6. Immunodetection of malic enzyme and α-SCS in *Histomonas meleagridis* proteins solubilised with 1% Nonidet P40. Lane 1: Coomassie blue-stained profile after SDS–PAGE (10%). Lane 2: Western blot showing the reactivity of anti-Hm_ME antiserum (1:500 dilution) with a 65-kDa protein band. Lanes 3, 4: A 33-kDa protein band is recognised by anti-Hm_α-SCS antiserum (1:500 dilution, lane 3) and mAb F5.2 (1:100 dilution, lane 4). Molecular mass standards (M) are in kDa.

blotting with corresponding antisera or with mAb F5.2 (Fig. 8C).

4. Discussion

Mitochondrion-related remnant organelles have been reported in several unicellular eukaryotes that live in microaerophilic or anaerobic environments and are distributed among diverse lineages. All these organelles are unable to perform oxidative phosphorylation and are currently assigned to two main categories: (i) the hydrogenosomes, found in trichomonads, anaerobic chytridiomycete fungi and several taxa of anaerobic ciliates, and (ii) the mitosomes, revealed in diplomonads, entamoebids, pelobionts and microsporidia (reviews: Müller, 1993; Dyall and Johnson, 2000; Williams and Keeling, 2003; van der Giezen and Tovar, 2005; Hackstein and Yarlett, 2006; Embley and Martin, 2006). Hydrogenosomes can generate ATP via some substrate-level phosphorylations and excrete molecular hydrogen, which is not the case with mitosomes. However, the hydrogenosome–mitosome subdivision may be an oversimplification. Multiple adaptations to anaerobic environments may justify some functional diversity of mitochondria-related organelles, as exemplified by the unusual case of *Cryptosporidium parvum* (Putignani et al., 2004; Henriquez et al., 2005).

Most advances in the knowledge of trichomonad hydrogenosomes were derived from studies in *T. vaginalis.* In addition to their central role in terminal reactions of carbohydrate metabolism and ATP production, the hydrogenosomes of this human pathogen are involved in iron-sulfur cluster biosynthesis (Sutak et al., 2004) and serine-glycine interconversion (Mukherjee et al., 2006), two metabolic pathways shared with mitochondria. Moreover, bioinformatics analysis of the recently determined *T. vaginalis* genome sequence suggests new functions for the hydrogenosome (Carlton et al., 2007). Although harbouring only one flagellum, *H. meleagridis* is also viewed as a trichomonad. In contrast with *T. vaginalis*, there has been little investigation of this bird parasite at the molecular level, as indicated by the availability of only 18S and 5.8S rRNA gene sequences in databases. Therefore, we believe the present study represents the first contribution to the identification of *Histomonas* protein-coding genes required for hydrogenosomal carbon metabolism. *Histomonas meleagridis* electron-dense bodies correspond to true hydrogenosomes. This is supported by the following data: (i) successful PCR-amplification of *H. meleagridis* gene fragments based on the use of primers specifically derived from *T. vaginalis* genes encoding three hydrogenosomal enzymes, (ii) strong sequence conservation of the C-terminal domain obtained for a [Fe]-hydrogenase, (iii) prediction of short N-terminal leader sequences (8–12 amino acids) in the other two enzymes considered (Hm_α-SCS and Hm_ME), as known for *Trichomonas* hydrogenosomal proteins (Häusler et al., 1997), (iv) IFA and ultracytochemical evidence for the localisation of Hm_HYD within *H. meleagridis* cytoplasmic granules and (v) localisation of Hm_α-SCS and Hm_ME in the same granules, as documented by IFA and Western blotting after organelle isolation.

Hydrogenases are a vast ensemble of prokaryotic and eukaryotic enzymes that catalyse the reversible oxidation of molecular hydrogen and are distributed into three major classes: [Fe]-hydrogenases, [Ni–Fe]-hydrogenases and metal-free hydrogenases (Vignais et al., 2001). In *T. vaginalis*, two closely related [Fe]-hydrogenases (TvhydA and TvhydB with respective sizes of 51.5 and 50 kDa) were initially identified and shown to be localised in hydrogenosomes (Bui and Johnson, 1996). Anti-TvhydA polyclonal antibodies reacted with both proteins co-migrating as a single 50-kDa band in blots of whole cell extracts and purified hydrogenosomes. A *T. vaginalis* gene encoding a potential 64-kDa [Fe]-hydrogenase characterised by a longer N-terminal domain (clostridial-type), was reported by other authors (Horner et al., 2000). The targeting of this protein to hydrogenosomes is supported by the prediction of an N-terminal peptide similar to known hydrogenosomal-type presequences. It should also be noted that the draft genome sequence of *T. vaginalis* indicates the presence of different genes encoding four [Fe]-hydrogenase families (Carlton et al., 2007). There is no doubt that the partial hydrogenase sequence obtained in *H. meleagridis* is representative of a C-terminal region specific to [Fe]-hydrogenases. This sequence matches a large segment of the [Fe]-hydrogenase active site (H-cluster domain). Conserved cysteine residues should serve as ligands for a [Fe–Fe] center linked to a

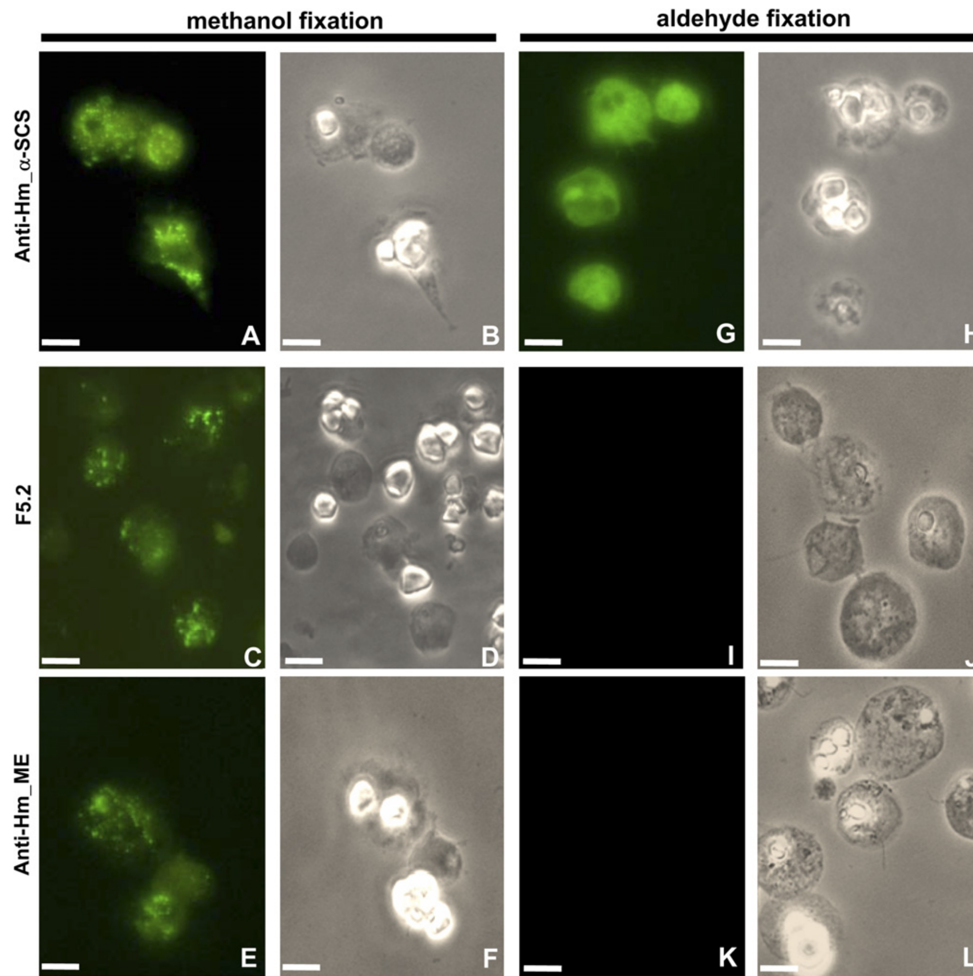

Fig. 7. Immunofluorescence assays with mouse antisera (anti-Hm_α-SCS, anti-Hm_ME) and mAb F5.2 applied to either permeabilised *Histomonas meleagridis* cells after methanol fixation (A, C and E) or non-permeabilised *H. meleagridis* cells fixed with aldehydes (G, I and K). Fluorescent signals provided by anti-Hm_α-SCS are detected intracellularly in permeabilised parasites (A) and on the cell surface of non-permeabilised parasites (G). The mAb F5.2 specific to *Trichomonas vaginalis* hydrogenosomal α-SCS reacts with cytoplasmic granules of *H. meleagridis* (C), not with its cell surface (I). This mAb has previously been shown to only stain the hydrogenosomes in *T. vaginalis* (Alderete, unpublished data). The anti-Hm_ME antiserum also stains *H. meleagridis* granules (E) and offers no signal with non-permeabilised cells (K). Brightfield photomicrographs of the same fields accompany the fluorescence pictures (B, D, F, H, J, L). Anti-Hm_ME and Anti-Hm_α-SCS antisera were used at a 1:100 dilution. Secondary antibody was Alexa 488-conjugated goat anti-mouse IgG (1:1000 dilution). Bars, 15 μm.

[4Fe–4S] cluster (Vignais et al., 2001). The N-terminal region of Hm_HYD is expected to contain accessory domains for the binding of additional [4Fe-4S] clusters. As anti-Hm_HYD antisera are strongly reactive with protein bands at 48, 51 and 65 kDa, it may be hypothesised that *H. meleagridis* [Fe]-hydrogenases exist as "short" and "long" forms similar to those of *T. vaginalis*.

Among eukaryotic organisms, both ME and SCS may be compartmentalised in ATP-producing organelles such as mitochondria and hydrogenosomes. These enzymes are frequently encoded by multigene families, as is the case in *T. vaginalis* (Alderete et al., 1995; Engbring and Alderete, 1998). Sequencing of PCR amplification products from *H. meleagridis* DNA indicated the presence of multiple clones with minor variations for both enzymes (not shown). Although multicopy genes made our strategy highly time-consuming, we succeeded in the identification of two complete *H. meleagridis* gene sequences.

With regard to MEs, it is noteworthy that *T. vaginalis* displays two distinct forms assigned to different families: (i) a cytosolic enzyme that is a dimer of 42-kDa subunits with a strict specificity for $NADP^+$, as in prokaryotes, and (ii) a larger hydrogenosomal enzyme existing as a tetramer of four 60-kDa subunits that preferentially uses NAD^+ to $NADP^+$ and is closely related to MEs found in mitochondria and plastids as well as in the cytosol of eukaryotes (Dolezal et al., 2004). The Hm_ME sequence corresponds to a large-subunit size (65 kDa) and is highly similar to the *T. vaginalis* hydrogenosomal ME subunit A. The protein appears to be strictly localised in *H. meleagridis* hydrogenosomes, as expected from the presence of a potential targeting signal and the lack of a signature residue (Lys 362) for $NADP^+$ specificity (Kuo et al., 2000; Dolezal et al., 2004). Thus, it seems likely that Hm_ME preferentially mediates the oxidative decarboxylation of malate to provide pyruvate as a substrate for a putative

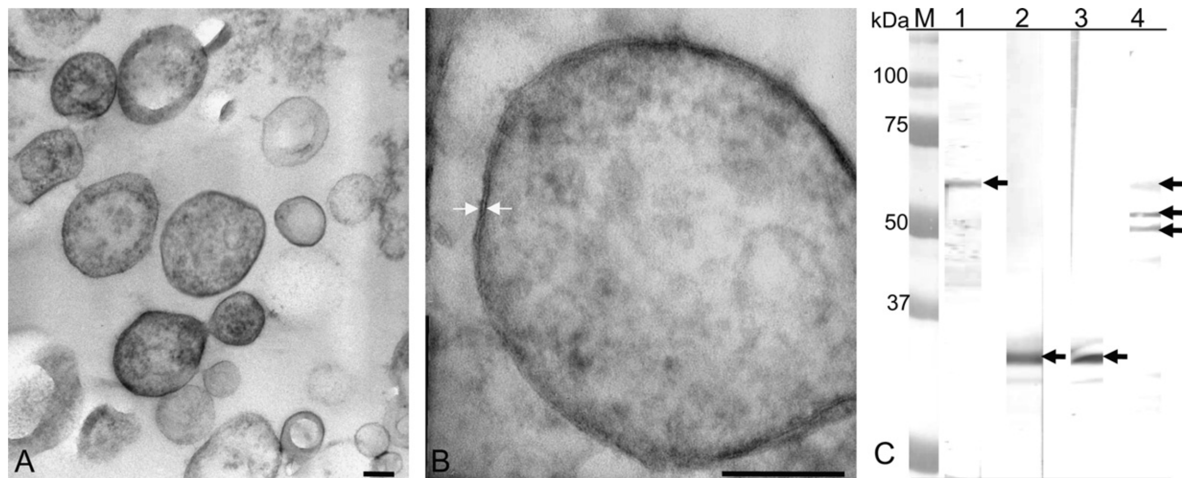

Fig. 8. Analysis of a subcellular fraction enriched in *Histomonas meleagridis* hydrogenosomes. (A, B) TEM appearance of the isolated organelles. A high magnification reveals that the hydrogenosome is bounded by two closely adjacent membranes (B, arrows). Bars, 100 nm. (C) Immunodetection of Hm_ME and Hm_α-SCS. After solubilisation in the presence of 2.5% SDS and 100 mM dithiothreitol, proteins of the hydrogenosome-enriched fraction were separated by SDS–PAGE (10%) and transferred onto polyvinylidene fluoride membranes. Protein bands that are stained with anti-Hm_ME (lane 1), anti-Hm_α-SCS (lane 2), mAb F5.2 (lane 3) and anti-Hm_HYD (lane 4) are indicated by arrows. Molecular mass standards (M) are in kDa.

pyruvate:ferredoxin oxidoreductase inside the hydrogenosome.

The tricarboxylic acid cycle in aerobic mitochondria comprises one substrate-level phosphorylation step catalysed by SCS, a multimeric enzyme composed of α and β subunits. A similar step has been conserved in trichomonad hydrogenosomes. In *T. vaginalis*, the conversion of succinyl-CoA to succinate and coenzyme A with ATP formation is mediated by a tetrameric $\alpha_2\beta_2$ enzyme and is typically coupled with the reaction catalysed by an acetyl-CoA transferase (Lahti et al., 1992, 1994). Hm_α-SCS shares the highest sequence identity with the *T. vaginalis* homologue and contains a candidate presequence for targeting the protein to hydrogenosomes. Such localisation was proved through IFA observations on whole parasites and Western blot detection in the hydrogenosome-enriched fraction. That SCS may be located on the surface of *H. meleagridis* cells was not unexpected, due to similar data about some *T. vaginalis* proteins involved in cytoadherence. The sequences of three *T. vaginalis* adhesins, named AP65, AP51 and AP33, corresponded to hydrogenosomal ME, beta and alpha SCS subunits, respectively (Arroyo et al., 1992; Alderete et al., 1995, 1998; O'Brien et al., 1996; Addis et al., 1997; Engbring and Alderete, 1998). Such adhesins may be released into the extracellular environment (Addis et al., 1997). Their expression on the *Trichomonas* cell surface was thoroughly demonstrated to be stimulated under high-iron growth conditions or upon contact with vaginal epithelial cells (Garcia et al., 2003). In the same study, two-dimensional electrophoresis data confirmed the correspondence between adhesins from surface-labelled parasites and proteins from purified hydrogenosomes. It is therefore possible that SCS may play a role in the adherence of *H. meleagridis*.

In further investigations on *Histomonas* hydrogenosomal proteins, we expected to obtain whole sequences of [Fe]-hydogenases with the help of 5′ RACE PCR experiments. Within the context of *Histomonas* cell differentiation and host–parasite interactions, anti-Hm_ME and anti-Hm_SCS antibodies may be useful for comparative immunolocalisation studies on flagellate and amoeboid stages, as an initial approach to the evaluation of developmentally regulated changes in the amount of putative adhesins expressed by the parasite.

Acknowledgements

M.M. was supported by a grant from the Association Nationale de la Recherche Technique (ANRT) and by AGORA GER2 S.A.R.L. We are grateful to Lionel Zenner (UMR INRA/DGER 958, Ecole Nationale Vétérinaire, Lyon, France) for kindly providing the *H. meleagridis* strain (HmZL). We particularly thank G. Méténier for his assistance in the drafting of this manuscript and A. Guedon for technical assistance.

References

Addis, M.F., Rappelli, P., Cappuccinelli, P., Fiori, P.L., 1997. Extracellular release by *Trichomonas vaginalis* of a $NADP^+$ dependent malic enzyme involved in pathogenicity. Microb. Pathog. 23, 55–61.

Alderete, J.F., O'Brien, J.L., Arroyo, R., Engbring, J.A., Musatovova, O., Lopez, O., Lauriano, C., Nguyen, J., 1995. Cloning and molecular characterization of two genes encoding adhesion proteins involved in *Trichomonas vaginalis* cytoadherence. Mol. Microbiol. 17, 69–83.

Alderete, J.F., Engbring, J., Lauriano, C.M., O'Brien, J.L., 1998. Only two of the *Trichomonas vaginalis* triplet AP51 adhesins are regulated by iron. Microb. Pathog. 24, 1–16.

Alderete, J.F., Millsap, K.W., Lehker, M.W., Benchimol, M., 2001. Enzymes on microbial pathogens and *Trichomonas vaginalis*: molecular mimicry and functional diversity. Cell. Microbiol. 3, 359–370.

Arroyo, R., Engbring, J., Alderete, J.F., 1992. Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. Mol. Microbiol. 6, 853–862.

Bishop, A., 1938. Histomonas meleagridis in domestic fowls (*Gallus gallus*). Cultivation and experimental infection. Parasitology 30, 181–194.

Bui, E.T., Johnson, P.J., 1996. Identification and characterization of [Fe]-hydrogenases in the hydrogenosome of *Trichomonas vaginalis*. Mol. Biochem. Parasitol. 76, 305–310.

Carlton, J.M., Hirt, R.P., Silva, J.C., Delcher, A.L., Schatz, M., Zhao, Q., Wortman, J.R., Bidwell, S.L., Alsmark, U.C., Besteiro, S., Sicheritz-Ponten, T., Noel, C.J., Dacks, J.B., Foster, P.G., Simillion, C., Van de Peer, Y., Miranda-Saavedra, D., Barton, G.J., Westrop, . G.D., Muller, S., Dessi, D., Fiori, P.L., Ren, Q., Paulsen, I., Zhang, H., Bastida-Corcuera, F.D., Simoes-Barbosa, A., Brown, M.T., Hayes, R.D., Mukherjee, M., Okumura, C.Y., Schneider, R., Smith, . A.J., Vanacova, S., Villalvazo, M., Haas, B.J., Pertea, M., Feldblyum, T.V., Utterback, T.R., Shu, C.L., Osoegawa, K., De Jong, P.J., Hrdy, I., Horvathova, L., Zubacova, Z., Dolezal, P., Malik, S.B., Logsdon, J.M., Henze Jr., K., Gupta, A., Wang, C.C., Dunne, R.L., Upcroft, J.A., Upcroft, P., White, O., Salzberg, S.L., Tang, P., Chiu, C.H., Lee, Y.S., Embley, T.M., Coombs, G.H., Mottram, J.C., Tachezy, J., Fraser-Liggett, C.M., Johnson, P.J., 2007. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. Science 12, 207–212.

Cavalier-Smith, T., 1998. A revised six-kingdom system of life. Biol. Rev. Camb. Philos. Soc. 73, 203–266.

Dolezal, P., Vanacova, S., Tachezy, J., Hrdý, I., 2004. Malic enzymes of *Trichomonas vaginalis*: two enzyme families, two distinct origins. Gene 31, 81–92.

Drmota, T., Proost, P., Van Ranst, M., Weyda, F., Kulda, J., Tachezy, J., 1996. Iron–ascorbate cleavable malic enzyme from hydrogenosomes of *Trichomonas vaginalis*: purification and characterization. Mol. Biochem. Parasitol. 83, 221–234.

Dwyer, D.M., 1970. An improved method for cultivating *Histomonas meleagridis*. J. Parasitol. 56, 191–192.

Dyall, S.D., Johnson, P.J., 2000. Origins of hydrogenosomes and mitochondria: evolution and organelle biogenesis. Curr. Opin. Microbiol. 3, 404–411.

Embley, T.M., Martin, W., 2006. Eukaryotic evolution, changes and challenges. Nature 440, 623–630.

Engbring, J.A., Alderete, J.F., 1998. Characterization of *Trichomonas vaginalis* AP33 adhesin and cell surface interactive domains. Microbiology 144, 3011–3018.

Garcia, A.F., Chang, T.H., Benchimol, M., Klumpp, D.J., Lehker, M.W., Alderete, J.F., 2003. Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. Mol. Microbiol. 47, 207–224.

Gerbod, D., Edgcomb, V.P., Noel, C., Zenner, L., Wintjens, R., Delgado-Viscogliosi, P., Holder, M.E., Sogin, M.L., Viscogliosi, E., 2001. Phylogenetic position of the trichomonad parasite of turkeys, *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. J. Eukaryot. Microbiol. 48, 498–504.

Graybill, H.W., Smith, T., 1920. Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. J. Exp. Med. 31, 647–655.

Hackstein, J.H., Yarlett, N., 2006. Hydrogenosomes and symbiosis. Prog. Mol. Subcell. Biol. 41, 117–142.

Häusler, T., Stierhof, Y.D., Blattner, J., Clayton, C., 1997. Conservation of mitochondrial targeting sequence function in mitochondrial and hydrogenosomal proteins from the early-branching eukaryotes *Crithidia*, *Trypanosoma* and *Trichomonas*. Eur. J. Cell. Biol. 73, 240–251.

Henriquez, F.L., Richards, T.A., Roberts, F., McLeod, R., Roberts, C.W., 2005. The unusual mitochondrial compartment of *Cryptosporidium parvum*. Trends Parasitol. 21, 68–74.

Horner, D.S., Foster, P.G., Embley, T.M., 2000. Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol. Biol. Evol. 17, 1695–1709.

Hrdý, 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.

Hu, J., McDougald, L.R., 2003. Direct lateral transmission of *Histomonas meleagridis* in turkeys. Avian Dis. 47, 489–492.

Jenkins, T.M., Gorrell, T.E., Müller, M., Weitzman, P.D., 1991. Hydrogenosomal succinate thiokinase in *Tritrichomonas foetus* and *Trichomonas vaginalis*. Biochem. Biophys. Res. Commun. 179, 892–896.

Kuo, C.C., Tsai, L.C., Chin, T.Y., Chang, G.G., Chou, W.Y., 2000. Lysine residues 162 and 340 are involved in the catalysis and coenzyme binding of NADP(+)-dependent malic enzyme from pigeon. Biochem. Biophys. Res. Commun. 270, 821–825.

Lahti, C.J., d'Oliveira, C.E., Johnson, P.J., 1992. Beta-succinyl-coenzyme A synthetase from *Trichomonas vaginalis* is a soluble hydrogenosomal protein with an amino-terminal sequence that resembles mitochondrial presequences. J. Bacteriol. 174, 6822–6830.

Lahti, C.J., Bradley, P.J., Johnson, P.J., 1994. Molecular characterization of the α-subunit *Trichomonas vaginalis* hydrogenosomal succinyl CoA synthetase. Mol. Biochem. Parasitol. 66, 309–318.

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.

Lindmark, D.G., Müller, M., Shio, H., 1975. Hydrogenosome in *Trichomonas vaginalis*. J. Parasitol. 63, 552–554.

Lund, E.E., Augustine, P.C., Chute, A.M., 1967. *Histomonas meleagridis* after one thousand *in vitro* passages. J. Protozool. 14, 349–351.

Lund, E.E., 1969. Histomoniasis. Adv. Vet. Sci. Comp. Med. 13, 355–390.

McDougald, L.R., 2003. Other protozoan diseases of the intestinal tract-histomoniasis (blackhead). In: Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, L.R., McDougald, L.R., Swayne, D.E. (Eds.), Diseases of Poultry. Iowa State University press, Ames, IA, pp. 1001–1006.

McDougald, L.R., Jinghui, H., 2004. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. Vet. Parasitol. 121, 233–238.

McDougald, L.R., 2005. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis. 49, 462–476.

McGuire, W.C., Moeller, M.W., Morehouse, N.F., 1964. The effect of dimetridazole on the growth and prevention of histomoniasis in poultry. Poult. Sci. 43, 864–871.

Moreno-Brito, V., Yanez-Gomez, C., Meza-Cervantez, P., Avila-Gonzalez, L., Rodriguez, M.A., Ortega-Lopez, J., Gonzalez-Robles, A., Arroyo, R., 2005. A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron. Cell. Microbiol. 7, 245–258.

Mukherjee, M., Brown, M.T., McArthur, A.G., Johnson, P.J., 2006. Proteins of the glycine decarboxylase complex in the hydrogenosome of *Trichomonas vaginalis*. Eukaryot. Cell 5, 2062–2071.

Müller, M., 1993. The hydrogenosome. J. Gen. Microbiol. 139, 2879–2889.

O'Brien, J.L., Lauriano, C.M., Alderete, J.F., 1996. Molecular characterization of a third malic enzyme-like AP65 adhesin gene of *Trichomonas vaginalis*. Microb. Pathog. 20, 335–349.

Putignani, L., Tait, A., Smith, H.V., Horner, D., Tovar, J., Tetley, L., Wastling, J.M., 2004. Characterization of a mitochondrion-like organelle in *Cryptosporidium parvum*. Parasitology 129, 1–18.

Rybicka, K., Honigberg, B.M., Holt, S.C., 1972. Fine structure of the mastigont system in culture forms of *Histomonas meleagridis* (Smith). Protistologica 1, 107–120.

Schuster, F.L., 1968. Ultrastructure of *Histomonas meleagridis* (Smith) Tyzzer, a parasitic amebo-flagellate. J. Parasitol. 54, 725–737.

Sutak, R., Dolezal, P., Fiumera, H.L., Hrdy, I., Dancis, A., Delgadillo-Correa, M., Johnson, P.J., Muller, M., Tachezy, J., 2004. Mitochondrial-type assembly of FeS centers in the hydrogenosomes of the amitochondriate eukaryote *Trichomonas vaginalis*. Proc. Natl. Acad. Sci. USA 28, 10368–10373.

Tyzzer, E.E., 1924. The chicken has a carrier of *Histomonas meleagridis* (Blackhead): the protozoon in its flagellated state. J. Med. Res. 44, 676–678.
van der Giezen, M., Tovar, J., 2005. Degenerate mitochondria. EMBO Rep. 6, 525–530.
Vignais, P.M., Billoud, B., Meyer, J., 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. 25, 455–501.
Williams, B.A., Keeling, P.J., 2003. Cryptic organelles in parasitic protists and fungi. Adv. Parasitol. 54, 9–68.