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Microsporidian Mitochondrial Proteins: Expression in *Antonospora locustae* Spores and Identification of Genes Coding for Two Further Proteins

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ABSTRACT. Microsporidia are obligate intracellular parasites, phylogenetically allied to the fungi. Once considered amitochondriate, now a number of mitochondrion-derived genes have been described from various species, and the relict organelle was recently identified in *Trachipleistophora hominis*. We have investigated the expression of potential mitochondrial targeted proteins in the spore stage to determine whether the organelle is likely to have a role in the spore or early infection stage. To investigate whether the *Antonospora locustae* genome codes for a different complement of mitochondrial proteins than *Encephalitozoon cuniculi* an EST library was searched for putative mitochondrial genes that have not been identified in the *E. cuniculi* genome project. The spore is the infectious stage of microsporidia, but is generally considered to be metabolically dormant. Fourteen genes for putatively mitochondrion-targeted proteins were shown to be present in purified spore mRNA by 3′-rapid amplification of cDNA ends and EST sequencing. Pyruvate dehydrogenase E1α and mitochondrial glycerol-3-phosphate dehydrogenase proteins were also shown to be present in *A. locustae* and *E. cuniculi* spores, respectively, suggesting a role for these proteins in the early stages of infection, or within the spore itself. EST sequencing also revealed two mitochondrial protein-encoding genes in *A. locustae* that are not found in the genome of *E. cuniculi*. One encodes a possible pyruvate transporter, the other a subunit of the mitochondrial inner membrane peptidase. In yeast mitochondria, this protein is part of a trimeric complex that processes proteins targeted to the inner membrane and the intermembrane space, and its substrate in *A. locustae* is presently unknown.

Key Words. Amitochondriate, *Antonospora*, *Encephalitozoon cuniculi*, inner membrane peptidase, mitosome, *Paranosema*.

MICROSPORIDIA are a group of intracellular eukaryotic parasites that infect all major lineages of animals, including humans (Canning 1993). Once thought to be ''primitive'' eukaryotes branching at the base of the eukaryotic tree, they are now widely acknowledged to be fungi with a distinctive cell structure adapted to suit their parasitic lifestyle (Edlind et al. 1996; Hirt et al. 1999; Keeling 2003; Keeling and Doolittle 1996; Van de Peer, Ben Ali, and Meyer 2000). As early branching eukaryotes, microsporidia were once hypothesised to be primitively amitochondriate, having diverged from the main eukaryotic lineage before the mitochondrial endosymbiosis (Cavalier-Smith 1983). Their relationship with fungi is not consistent with this, however, and molecular data hinting at a mitochondrial ancestry soon began to accumulate. Initially, nuclear-encoded genes for the mitochondrial heat shock protein 70 (mtHSP70) and pyruvate dehydrogenase (PDH) were identified (Arisue et al. 2002; Fast and Keeling 2001; Germot, Philippe, and Le Guyader 1997; Hirt et al. 1997; Peyretaillade et al. 1998), and the complete genome of *Encephalitozoon cuniculi* was subsequently shown to contain 19 genes for putatively mitochondrion-targeted proteins (Katinka et al. 2001). The ultrastructure of this organelle was subsequently revealed in *Trachipleistophora hominis* by the localisation of the mtHSP70 (Williams et al. 2002). The microsporidian mitochondrion, or mitosome, is a small, double membrane-bounded structure that lacks cristae or other notable features. The organelle is abundant in the intracellular meront stage, but no localisation to the spore stage has been reported, raising an interesting possibility that the organelle may be further reduced or less active in certain stages of the parasite's life cycle.

Mitochondrial function in microsporidians has been modeled based on the handful of genes found in the *E. cuniculi* genome (Katinka et al. 2001), but the nature and roles of the biochemical pathways of these mitochondria are not completely clear. Furthermore, as *E. cuniculi* has a relatively small genome, it is unclear if its mitosome is representative of other microsporidia, or whether the organelle was reduced along different lines in other lineages. One part of this puzzle is that the *E. cuniculi* genome contains genes coding for incomplete mitochondrial pathways. For example, genes coding for alpha and beta subunits of PDH E1 are present, but the E2 and E3 proteins that otherwise invariably make up the PDH complex are absent (Fast and Keeling 2001; Katinka et al. 2001). Another example of particular interest is the mitochondrial import system. Very few genes coding for components of the mitochondrial protein import machinery have been identified to date in microsporidia. The mtHSP70 gene is present in many microsporidia, but its normal interaction partner, mtHSP40 (Bukau and Horwich 1998) is absent in *E. cuniculi*. Similarly, translocase of the inner membrane subunit 22 (TIM22) and translocase of the outer membrane subunit 70 (TOM70) were found, but each of these is normally part of a large inner and outer membrane complex whose additional components have not been identified. Significantly, there is also no evidence of a mitochondrial processing peptidase, which could cleave the N-terminal transit peptides normally used to target proteins to mitochondria. There has been some debate over whether microsporidian mitochondrial proteins even encode such leaders (Fast and Keeling 2001; Katinka et al. 2001; Williams et al. 2002).

Here we have investigated the complement and expression of mitochondrial proteins in the locust parasite, *Antonospora locustae*. This parasite was originally described as *Nosema locustae* but has recently been independently redescribed as *Antonospora* or *Paranosema*, largely on the basis of it phylogenetic distance from true *Nosema* species (Slamovits, Williams, and Keeling 2004; Sokolova et al. 2003). Transcripts for 14 putative mitochondrial protein-coding genes were shown to be present in spores, and PDH E1 alpha and mitochondrial glycerol-3-phosphate dehydrogenase (mtG3PDH) proteins were also shown to be present in spores of *A. locustae* and *E. cuniculi* respectively, by immunoblotting. We also show that *A. locustae* has genes coding for two mitochondrial proteins that are absent from *E. cuniculi*, one of which, inner membrane peptidase 2 (IMP-2), is a component of the mitochondrial protein import machinery.

MATERIALS AND METHODS

DNA extraction, library construction, PCR. Commercially purified *Antonospora locustae* spores were obtained from M&R Durango, Inc. (Bayfield, CO). Spore purity was confirmed by light microscopy. Genomic DNA was isolated with the Plant DNeasy minikit (Qiagen, Mississauga, ON). Genomic library construction was as previously described (Fast and Keeling 2001). Degenerate

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PCR of manganese superoxide dismutase (MnSOD) was carried out using primers designed to a nucleotide alignment of several bacterial and eukaryotic MnSOD sequences and was successfully amplified using the following primer combination 5′-TAT SCDTAYGATGC-3′ and 5′-TGGAABGTHGTWAAYTGGG-3′. Amplified products were cloned and sequenced as below and used to design 3′-rapid amplification of cDNA ends (RACE) primers. Available *A. locustae* databases were blasted with previously identified microsporidian mitochondrial protein encoding genes. The databases searched were NCBI, and the *A. locustae* Genome Project, Marine Biological Laboratory at Woods Hole (http://www.ncbi.nlm.nih.gov/ and http://gmod.mbl.edu/perl/site/antonospora01). 3′-RACE primers were designed from the gene sequences of ATM1a (a mitochondrial ABC transporter), cysteine desulphurase (ISCS), NADPH-ferredoxin oxido-reductase (NADPH-FOR), Frataxin, mtG3PDH, mt HSP70, MnSOD, ISCU, PDH E1 alpha and beta, TIM22, and IMP-2. Primer sequences are as follows:

mtG3PDH F1, 5′-agctcgagttgaagagagaaataaa-3′, F2, 5′-gaactat atttcaaactcagcacttttg-3′; Hsp70 F1, 5′-gtctctaagtcttggaatcgagactgt t-3′, F2, 5′-aacacaacacttcctgttaaggagacac-3′; MnSOD F1, 5′-tgtcg aaaaagtaggctgcaataac-3′, F2, 5′-actgtctgagatgattaacagcaccttt-3′; ATM1 F1, 5′-gtcaatctccaaataaacaagtctga-3′, F2, 5′-atgaatacgacgg aataataagcatc-3′; ISCS F1, 5′-gaaaagccataagaggggacaccg-3′, F2, 5′-aatctcaatcatggctgtgaacaac-3′; NADPH-FOR F1, 5′-cagacagagt gatacggtggtacaat-3′, F2, 5′-gagaaattcgaggagttcaaaaagc-3′; Frataxin F1, 5′-gttctgtcatggaaagtagacggtgt-3′, F2, 5′-gagtacgtgttcaata agcagacac-3′; IMP-2 F1, 5′-atgataaaatgctttcaagagatgg-3′, F2, 5′-acgctgagtggtgggtgctttttctc-3′; ISCU F1, 5′-tgcggtgacgtaatgaagctt caga-3′, F2, 5′-agacgctgtgttcagaacatttggt-3′; Tim22 F1, 5′-aaaattt gctatgggtgggagtgata-3′, F2, 5′-cgaatggctctagagaagtaccag-3′; PDH alpha F1, 5′-tcgtgcagatcgacacctac-3′, F2, 5′-atttggtgcagagcacat tg-3′; PDH beta F1, 5′-ctcttgaaaggagcggtgtc-3′, F2, 5′-tctgcactcat caacgaagc-3′.

RNA extraction and 3′-RACE. Spores (3.5×10^9) of *A. locustae* were ground under liquid nitrogen with a mortar and pestle. The lysate was resuspended in 500 µl of TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA was purified according to the manufacturer's protocol. The mRNA was then either used for 3′-RACE or polyA purified using the ambion polyA pure mRNA purification kit (Ambion, Austin, TX) before being used for 3′-RACE. Reverse transcription and 3′-RACE were carried out with the First Choice RLM-RACE kit (Ambion). Total RNA from *A. locustae* spores was used as a template for first-strand synthesis with a poly(dT) 3′-adaptor oligonucleotide according to manufacturer's instructions. Second-strand synthesis was carried out using Amplitaq Gold (Applied Biosystems, Foster City, CA) or ready-to-go PCR beads (Amersham, Little Chalfont, England) using two rounds of PCR with one set of nested primers (3′-RACE outer and inner primers supplied in RLM-RACE kit). Cycling conditions were as follows: 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min. The annealing temperature was altered according to the melting temperature of the primers. Reaction products were gel isolated and cloned in TOPO pCR 2.1 (Invitrogen). Positive clones were sequenced with ABI Big Dye v 3.1 Chemistry (Applied Biosystems). New genes sequences were submitted to Genbank under Accession numbers AY952279–AY952292.

Antibody preparation and Western blotting. Rabbit polyclonal antibodies were prepared commercially (AbCam, Cambridge, UK) against a mixture of two synthetic peptides for both *E. cuniculi* mitochondrial glycerol-3-phosphate dehydrogenase (CKMIEKPSEDWEPASR and CEKRHRGERRLPPQEK) and *A. locustae* pyruvate dehydrogenase E1 alpha (CKIRYDDVEK LYRKML and CSTTDGIVYRDETEVR). Proteins were released from *A. locustae* and *E. cuniculi* spores by grinding them under liquid nitrogen as described above for RNA extraction. Approximately 2×10^9 crushed *A. locustae* and 1.5×10^8 *E. cuniculi* spores were each resuspended in 200 µl of 0.15 M NaCl and stored at −20 °C in an equal vol. of protein sample buffer (62.5 mM Tris-HCl [pH 6.8], 0.01% (w/v) bromophenol blue, 2% (w/v) sodium dodecyl sulfate, 5% (v/v) beta-mercaptoethanol, 10% (v/v) glycerol). Proteins were heated in protein sample buffer at 95 °C for 5 min and then separated on a sodium dodecyl sulfate–10% polyacrylamide resolving gel, with a 4% stacking gel. Samples were transferred to Hybond-P polyvinylidene difluoride membranes (Amersham) by electroblotting and incubated with respective antibodies and subsequently a secondary horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Bio-Rad, Hercules, CA). Blots were developed with ECL Western blotting reagents (Amersham).

Phylogenetic methods. The amino acid sequence of the *A. locustae* IMP-2 was aligned to homologues of prokaryotic type 1 signal peptidases and eukaryotic IMP-1 and IMP-2s using CLUSTAL X (Thompson, Higgins, and Gibson 1994). This produced a 32 taxon alignment that was manually edited resulting in a data set of 63 unambiguously aligned characters. Parameters were calculated using TREE-PUZZLE 5.0 (Strimmer and von Haesler 1996) by using the WAG substitution matrix and modeling site-to-site rate variation on a gamma distribution approximated with eight rate categories and an invariable-sites category. The shape parameter and the proportion of invariable sites were estimated from the data. Using these parameters maximum likelihood trees were constructed with PHYML (Version 2.1b1) using the JTT substitution model (Guindon and Gascuel 2003). One-thousand Bootstrap datasets were created and were analysed using both PHYML and FITCH. Maximum likelihood trees and bootstraps were inferred using PHYML with the JTT substitution model and gamma parameter and proportion of invariant sites as calculated above (Guindon and Gascuel 2003). For Fitch-Margoliash bootstraps, distances were calculated from 1,000 bootstrap data sets using PUZZLEBOOT (shell script by A. Roger and M. Holder; www.tree-puzzle.de) using the parameters estimated above, and trees were inferred using FITCH (Version 3.6a) (Felsenstein 1993). Analyses were also carried out with a reduced data set composed of only eukaryotic IMP-1s and IMP-2s, which allowed the number of alignable characters to increase to 89. This data set was analysed as above.

RESULTS

Further microsporidian mitochondrial proteins. To begin to assess the potential role of mitochondria in the spores of microsporidia, we have first identified two further genes that are not known from the *E. cuniculi* genome. A single transcript of 1,042 bases identified by EST sequencing was found to encode two putative mitochondrion-targeted proteins (many *A. locustae* mRNA transcripts, including frataxin, encode a second gene or fragment of a second gene: Williams B. A. P., Slamovits C. H., Patron N. J., Fast N. M., Keeling P. J., unpubl. data). The first open reading frame of 310 bases, which is truncated at the 5′ shared similarity with the *Saccharomyces cerevisiae* ORF, YIL006W coding for an inner mitochondrial membrane protein transporting pyruvate. The second open reading frame of 621 bases encodes a 207-amino acid protein with homology to mitochondrial inner membrane peptidase (IMP). The transporter and the IMP genes are separated by an intergenic region of 64 nucleotides, but they are not co-transcribed as they are encoded on opposite strands of DNA. The transporter is in sense strand and the IMP in the antisense strand, so this transcript likely results in translation of the mitochondrial importer, but not the IMP. Nevertheless, the transcript includes the full-length sequence of the

IMP gene and examination using both MitoprotII and PSORT (Claros and Vincens 1996; Nakai and Horton 1999) predicts the translation of the IMP gene to be targeted to the mitochondrion.

In the global phylogeny of bacterial signal peptidases and mitochondrial IMP-1 and 2 (Fig. 1A), the *A. locustae* gene branched within the clade of other eukaryotes (67% FITCH/74% PHYML bootstrap support), and the IMP-1 and 2 types were resolved as discrete clades but with no convincing bootstrap support. Unfortunately, the signal peptidases are short proteins (about 180 amino acids), and are not highly conserved throughout their entire length, so the unambiguously alignable amino acids amount to only 63 characters when all signal peptidases are included. Restricting this analysis to mitochondrial IMP homologues increases the alignment to 89 characters, so the position of the *A. locustae* gene within the mitochondrial IMP clade was also analysed, excluding bacterial signal peptidases. Here (Fig. 1B), IMP-1 proteins and IMP-2 proteins form discrete clades (69% FITCH/63% PHYML bootstrap support). Within IMP-2, *A. locustae* branches within the fungal clade, albeit with very poor support (< 50%). This phylogeny suggests a homologous origin of the microsporidian IMP with other mitochondrial IMPs, and might reasonably be supposed to have a homologous function in the microsporidian mitochondrion. The closer relationship of the *A. locustae* protein sequence to the IMP-2 isoforms leads to the designation of the gene as an IMP-2 gene sequence.

Expression of putative mitochondrion targeted proteins in the spore. With the characterisation of IMP and the putative pyruvate transporter, there are 21 putatively mitochondrion-targeted proteins known in any microsporidian. 3′-RACE was used to identify transcripts for 12 of these proteins from *A. locustae* spores: mitochondrial glycerol-3-phosphate dehydrogenase, HSP70, pyruvate dehydrogenase E1 alpha and beta subunits, MnSOD, a mitochondrial ABC transporter ATM1 gene, ISCU, ISCS, frataxin, NADPH-FOR, IMP-2 and Tim22. The mRNA transcripts for a second mitochondrial ABC transporter gene (ATM 1b) and the putative pyruvate transporter gene were discovered through EST sequencing. For some genes, more than one size of EST fragment was amplified and sequenced. In some cases this may represent non-specific binding to adenine-rich areas within the mRNA transcript or alternative polyadenylation points for the amplified transcript. In these cases RACE was repeated using polyA purified mRNA. In a single case, ATM1 a, the result is inconclusive as the genomic data shows a polyA tract in the same position as the polyA tail in the sequenced RACE product. This allows the possibility that the product is amplified from DNA contamination. However, an identical transcript was amplified from polyA-purified mRNA, and furthermore an AT-rich putative polyadenylation signal lies 14 bases upstream of this polyA site. Thus, an alternative possibility is that the polyA site is coincidentally the site of a polyA tract.

This represents more than half of the known microsporidian mitochondrial proteins (Table 1), and demonstrates the presence of transcripts in the spore stage of the microsporidia. Ferredoxin, TOM70, and ERV1 were not detected in any available *A. locustae* data, and attempts to amplify *A. locustae* homologues by degenerate PCR were unsuccessful. These are, however, genes with a low level of sequence conservation in microsporidia and therefore difficult to amplify with degenerate primers. Thus, their current absence does not really indicate that they are absent from the *A. locustae* genome.

To determine if mitochondrial proteins were also present in the spore, two proteins were identified in purified spores by immunoblotting. Specific peptide antibodies were raised against the *A. locustae* PDH E1 alpha subunit and the *E. cuniculi* mtG3PDH, and these antibodies used to probe protein preparations from purified spores (Fig. 2). The predicted sizes of these proteins from the gene sequences were 38.1 kDa in the case of *A. locustae* PDH El alpha and 68.6 kDa for the *E. cuniculi* mtG3PDH. These corresponded closely to observed bands of apparent molecular weight of 38 and 67 kDa, respectively, indicating that mitochondrial proteins, as well as transcripts are present in the spores. The antibody to PDH E1 alpha reacts with a second smaller band, this could represent processing of an N-terminal mitochondrial targeting signal, though there is no evidence for an N-terminal extension of this protein.

DISCUSSION

Implications of a microsporidian IMP-2. The discovery of an inner membrane peptidase implies the presence of N-terminal cleavage of some microsporidia mitochondrial proteins. IMP-2 and its interaction partner IMP-1 are homologues of the bacterial leader peptidase that processes proteins translocated across the cytoplasmic membrane. In yeast mitochondria, the catalytic IMP-1 and IMP-2 form a complex with the non-catalytic SOM1 and process a subset of proteins destined for either the intermembrane space or the inner mitochondrial membrane (Jan et al. 2000). In *Saccharomyces cerevisiae*, the proteins specifically known to be cleaved by IMP-1 are the nuclear-encoded NADH cytochrome *b*5 reductase and precursors to cytochrome *b*2, mitochondrial glycerol-3-phosphate dehydrogenase, and the mitochondrial-encoded subunit II of cytochrome *c* oxidase. IMP-2 has been shown to process the nuclear-encoded cytochrome *c*1 (Esser et al. 2004; Nunnari, Fox and Walter 1993). Although the IMP-1 and the IMP-2 proteins have discrete substrates, they are known to interact (Jan et al. 2000). Therefore, an interesting question is whether IMP-2 acts on its own in *A. locustae* or the remaining subunits of the complex are also present and remain to be discovered. The *A. locustae* genome does encode mitochondrial glycerol-3-phosphate dehydrogenase, a substrate for the IMP complex. However, this gene is also encoded by the *E. cunculi* genome in which the IMP genes are absent. This suggests that the mitochondrial glycerol-3-phosphate dehydrogenase is not the substrate of IMP and that *A. locustae* has a different complement of mitochondrial proteins (or perhaps the mitochondrial transporter identified in this study is the substrate). This gene shares homology with the yeast ORF YIL006W which functions in the inner mitochondrial membrane importing pyruvate (Hildyard and Halestrap 2003). Both *A. locustae* and *E. cuniculi* genomes encode the alpha and beta subunits of mitochondrial pyruvate dehydrogenase E1, proteins almost certainly requiring pyruvate as a substrate (Fast and Keeling 2001; Katinka et al. 2001), but it is not known how pyruvate enters their mitochondria. It has been hypothesised that pyruvate is broken down into acetyl CoA or acetate in the microsporidian mitochondrion, although how this fits into microsporidian metabolism is not clear (Keeling and Fast 2002; Vivares et al. 2002). The presence of a pyruvate transporter has interesting implications that fit with the presence of a mitochondrial PDH, but it is curious that the *E. cuniculi* genome does not encode a homologue, suggesting that it has an alternative pyruvate import system.

The IMP-2 and putative pyruvate transporter both mark differences between the biochemical composition of the mitochondrion in *A. locustae* and *E. cuniculi*, suggesting that different species of microsporidia may exhibit different degrees of reduction of the mitosome.

Significance of mitochondrial protein expression for infection process. The spore is a pivotal stage in the microsporidian life cycle, since it is the only extracellular stage, and is responsible for infecting new host cells. Spores are generally considered to be dormant. Most spores can remain viable for at least a year outside the host environment, and the spores of some species are viable up to ten years when stored in ideal conditions (Cali and Takvorian

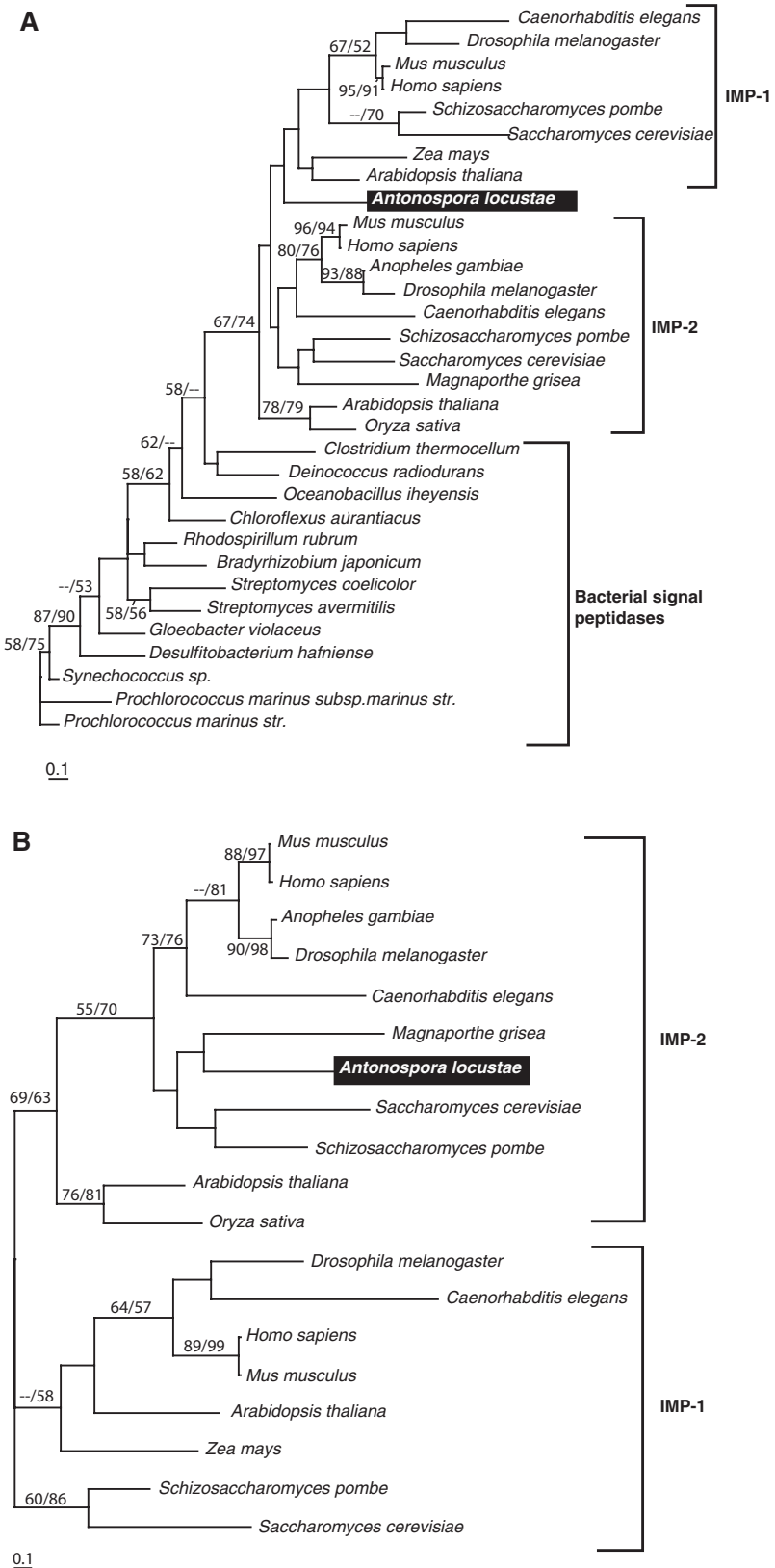

Fig. **1.** Maximum likelihood phylogeny of signal peptidases based on protein sequences. (**A**) Global signal peptidase phylogeny including mitochondrial inner membrane protease-1 and inner membrane protease-2 sequences (bracketed) along with bacterial signal peptidases. (**B**) Phylogeny of IMP-1 and IMP-2 alone, showing position of *Antonospora locustae* homologue within the IMP-2 clade. In both (A) and (B) the *A. locustae* sequences are boxed and bootstrap values over 50% are shown for FITCH and PHYML analyses respectively, with FITCH bootstrap values shown to the left and PHYML values to the right.

Table **1.** Summary of known mitochondrial protein encoding genes and their expression in microsporidia.

	Present in genome[a]	Spore mRNA present[a]	Spore protein present[a]	Best BlastX hit (Altschul et al. 1990) and score for *A. locustae* gene
ATM1 (ABC transporter)	*A. locustae*, *E. cuniculi* (+five other putative mitochondrial *ATM1* genes)	***A. locustae***		ABC transporter (mitochondrial type) *E. cuniculi* e-113
ATM1 b (ABC transporter)	*A. locustae*, *E. cuniculi*	***A. locustae***		ABC TRANSPORTER (mitochondrial type) *E. cuniculi* 8e-49
Cysteine Desulphurase (ISCS)	*A. locustae*, *E. cuniculi*	***A. locustae***		NIFS-like (cysteine desulphurase) *E. cuniculi* e-148
ERV1	*E. cuniculi*			
Ferredoxin	*E. cuniculi*			
NADPH-FOR	*A. locustae*, *E. cuniculi*	***A. locustae***		NADPH adrenodoxin oxidoreductase *E. cuniculi* 2e-25
Frataxin	*A. locustae*, *E. cuniculi*	***A. locustae***		Frataxin *E. cuniculi* 2e-15
Mt G3PDH	*A. locustae*, *E. cuniculi*	***A. locustae***	***E. cuniculi***	mitochondrial glycerol-3-phosphate dehydrogenase *E. cuniculi* e-148
Mt HSP70	*A. locustae*, *E. cuniculi*, *E. hellem*, *T. hominis*, *Vairimorpha necatrix*, *Glugea plecoglossi*	***A. locustae***, *T. hominis*	*T. hominis*	mitochondrial Hsp70 homolog *V. necatrix* e-161
IMP-2	***A. locustae***	***A. locustae***		Inner mitochondrial membrane peptidase 2 *Mus musculus* 9e-21
MnSOD	***A. locustae***, *E. cuniculi*	***A. locustae***		Superoxide dismutase, Mn *Enterococcus faecalis* 3e-46
ISCU	*A. locustae*, *E. cuniculi*	***A. locustae***		NIFU-like protein *E. cuniculi* 4e-47
PDH-E1α	*A. locustae*, *E. cuniculi*	***A. locustae***	***A. locustae***	Pyruvate dehydrogenase e1 component alpha subunit *E. cuniculi* 9e-67
PDH-E1β	*A. locustae*, *E. cuniculi*	***A. locustae***		Pyruvate dehydrogenase e1 component beta subunit *E. cuniculi* e-114
Possible pyruvate importer	***A. locustae***	***A. locustae***		Pvruvate transporter of the mitochondrial inner membrane *S. cerevisiae* 9e-16
TIM22	*A. locustae*, *E. cuniculi*	***A. locustae***		Similarity to Translocase TIM22/17 *E. cuniculi* 2e-13
TOM70	*E. cuniculi*			

[a] Names in boldface indicate data reported in this paper.

1999). After this period of perceived dormancy, spores may be reactivated and host cell infection is rapid. In *A. locustae*, polar tube eversion and sporoplasm expulsion takes 5–10 s (Undeen and Epsky 1990), and activated intracellular stages quickly begin to replicate. Therefore, spores must contain all the components necessary both to maintain the cell through its dormancy and to allow rapid reactivation when host infection is triggered. Mitochondria-like structures have been detected in the sporont and meront stages of microsporidia, but as yet have not been detected in the spore stage. It is difficult to imagine how the mitochondrion could be totally absent from spores (because they are not generated de novo). Nevertheless, they may be harder to identify in the more complex spore, or they may be further reduced in spores. The presence of mtG3PDH and PDH El alpha proteins in spores suggests that at least these two proteins are part of mitochondrial biochemical pathways that are present and potentially active in the spore, perhaps related to the energy requirements of spore maintenance and viability. Moreover, RACE cloning shows that transcripts for many of the known microsporidian mitochondrial proteins are also present in *A. locustae* spores. This could represent active transcription and subsequent translation of these genes, implying that the proteins are also synthesised and integrated into the mitochondria in spores. Perhaps more plausibly, these mRNAs could also represent transcripts stored within the spore for rapid translation upon activation and germination. Spores are known to contain an abundance of ribosomes, many in complex paracrystalline arrangements, and it would be of great interest to know

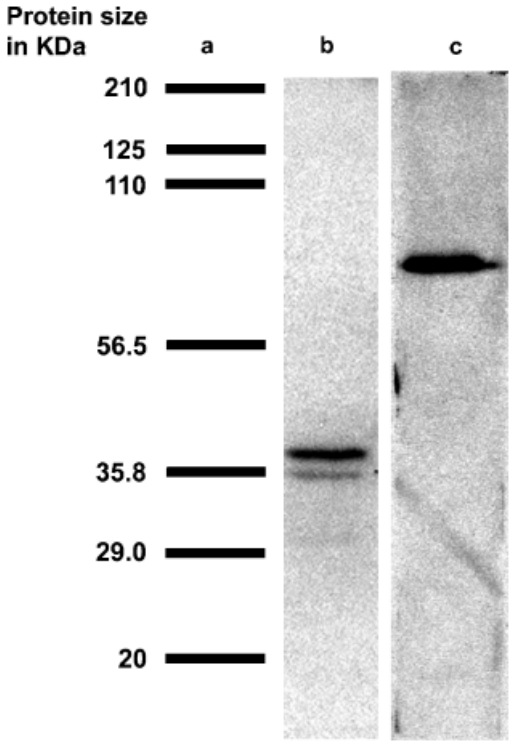

Fig. **2.** Western blots of spore proteins from *Encephalitozoon cuniculi* and *Antonospora locustae*. Lane **a**: protein standard sizes. Lane **b**: Spore protein of *A. locustae* exposed to an anti-pyruvate dehydrogenase-alpha antibody. Lane **c**: Spore proteins of *E. cuniculi* exposed to an anti-mitochondrial glycerol-3-phosphate dehydrogenase antibody. Band sizes in lanes b and c closely correspond to sizes expected based on gene sequences.

whether these were ''charged'' with an mRNA molecule and, if so, how the mRNAs are stabilised. Regardless, the data indicate that the reduced mitochondrion is an important organelle in the infection process or the early stages of infection.

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