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Alpha and beta subunits of pyruvate dehydrogenase E1 from the microsporidian *Nosema locustae*: mitochondrion-derived carbon metabolism in microsporidia☆

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Abstract

Microsporidia are highly adapted eukaryotic intracellular parasites that infect a variety of animals. Microsporidia contain no recognisable mitochondrion, but recently have been shown to have evolved from fungi and to possess heat shock protein genes derived from mitochondria. These findings make it clear that microsporidian ancestors were mitochondrial, yet it remains unknown whether they still contain the organelle, and if so what its role in microsporidian metabolism might be. Here we have characterised genes encoding the alpha and beta subunits of pyruvate dehydrogenase complex E1 (PDH, EC 1.2.4.1) from the microsporidian *Nosema locustae*. All other amitochondriate eukaryotes studied to date have lost the PDH complex and replaced it with pyruvate:ferredoxin oxidoreductase (PFOR). Nevertheless, molecular phylogeny shows that these *Nosema* enzymes are most closely related to mitochondrial PDH from other eukaryotes, demonstrating that elements of mitochondrial metabolism have been retained in microsporidia, and that PDH has not been wholly lost. However, there is still no evidence for a mitochondrion in microsporidia, and neither PDH subunit is predicted to encode an amino terminal leader sequence that could function as a mitochondrion-targeting transit peptide, raising questions as to whether these proteins function in a relic organelle or in the cytosol. Moreover, it is also unclear whether these proteins remain part of the PDH complex, or whether they have been retained for another purpose. We propose that microsporidia may utilise a unique pyruvate decarboxylation pathway involving PDH, demonstrating once again the diversity of core metabolism in amitochondriate eukaryotes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Microsporidia; Mitochondria; Pyruvate dehydrogenase; Evolution; Metabolism

1. Introduction

The microsporidia are a group of obligately intracellular parasites that infect a broad variety of animal hosts, as well as a small number of protists. Outside of their host, microsporidia exist as highly durable but largely dormant spores that contain an extremely elaborate and highly specialised suite of structures that mediate infection [1]. When a spore is triggered, the most dramatic event is the eversion of a tube (the polar tube) that otherwise lays coiled within the spore. This tube can be many times the length of the spore, and when everted in the presence of a potential host, it can adhere to or penetrate the membrane of the host cell, which in turn allows the infectious sporoplasm to pass though the tube and into the host [2].

Microsporidia are one of the more highly adapted groups of eukaryotes known: practically every major feature that distinguishes microsporidia from other eukaryotes is an adaptation to their parasitic lifestyle. Probably the single characteristic that best defines microsporidia is the severe reduction that permeates every level of the cell, from morphology, to biochemistry, to molecular biology. At the level of morphology, microsporidian spores are remarkably small and structurally they contain little that is not directly related to

infection. They lack flagella (or any $9 + 2$ structure) as well as discernable mitochondria and peroxisomes throughout their life cycle, and have only a simplified endomembrane system with no evidence of highly ordered Golgi cisternae [1]. Biochemically, microsporidia are thought to be anaerobic and to lack evidence of electron transport chains and oxidative phosphorylation [3]. Although a variety of enzyme activities have been characterised, little is known about exactly how central metabolism occurs except that it is likely simplified and reduced [3]. It is also likely that microsporidian anabolic metabolism is extremely limited as they are dependent upon their host for a number of metabolites [3]. The biochemical reduction of microsporidia is also evident from the fact that microsporidian genomes are tiny by eukaryotic standards, the smallest being smaller than many bacterial genomes [4].

Their cellular simplicity, in particular the lack of mitochondria, led to the proposal that microsporidia could be one of the earliest eukaryotic lineages, perhaps descended from primitive nucleated cells that lived before typical eukaryotic characters arose [5]. This conclusion was supported by early molecular phylogenies including microsporidian data [6–8], however, it is now clear that this is not the case, and that microsporidia are instead highly derived and extremely divergent fungi. This fungal origin of microsporidia was first proposed based on the relationship between microsporidia and fungi in tubulin phylogenies [9,10], and is now supported by the majority of phylogenetic analyses using a variety of genes (e.g. Refs. [11–15]). Furthermore, many of the original molecular data sets that placed microsporidia at the base of the eukaryotic tree have recently been reanalysed with more sophisticated methods (in particular, taking into account site-to-site rate variation, which can at least partially mitigate the high rates of substitution often seen in microsporidian gene sequences), and the resulting trees do not support an early origin for microsporidia (e.g. Refs. [14,15]).

A fungal origin for microsporidia reveals that otherwise common eukaryotic features lacking in microsporidia, such as the mitochondrion, have been lost and do not reflect a primitive absence. Indeed, in the case of the mitochondrion, a gene encoding a protein derived from the mitochondrial endosymbiont has been characterised from three microsporidia: *Encephalitozoon*, *Vairimorpha*, and *Nosema* [16–18]. This gene encodes HSP70, a heat shock protein involved in folding other proteins during import into the mitochondrion. The presence of this gene reconfirms the mitochondrial ancestry of microsporidia, but it does not prove that the organelle still exists and does little to reveal what such an organelle might be doing in modern microsporidia. Indeed, no mitochondrion has

been physically identified in microsporidian cells, and conflicting conclusions have been drawn as to whether the amino termini of the three microsporidian mitochondrion-derived HSP70 proteins include mitochondrion-targeting transit peptides [16–18]. Altogether, it is presently clear that microsporidia evolved from a mitochondrion-containing lineage and have retained some genes that are derived from the organelle, but it is unclear whether microsporidia have retained a mitochondrion in some altered form or have completely lost it. Moreover, if microsporidia have retained their mitochondrion, there is no evidence as to what metabolic role it could play in these highly reduced, probably anaerobic parasites.

Here we have characterised the first genes encoding mitochondrion-derived metabolic proteins from microsporidia, the two subunits of pyruvate dehydrogenase (PDH) E1. This enzyme is part of the pyruvate dehydrogenase complex, consisting of E1 (PDH or pyruvate decarboxylase, EC 1.2.4.1), E2 (dihydrolipoamide acetyl transferase, EC 2.3.1.12), and E3 (dihydrolipoamide dehydrogenase, EC 1.6.4.3). In nearly all mitochondriate eukaryotes, this complex oxidatively decarboxylates pyruvate as it enters the mitochondrion, resulting in carbon dioxide, NADH, and acetyl-CoA, the latter of which then enters the Krebs cycle. E1 is a heterodimeric enzyme which begins this series of reactions by converting pyruvate and thiamine pyrophosphate (TPP) to 2-alpha-hydroxyethyl-TPP (also known as "active aldehyde") and carbon dioxide [19]. Interestingly, in amitochondriate protists studied to date (i.e. protists lacking typical mitochondrial metabolism, such as *Trichomonas*, *Giardia*, and *Entamoeba*) PDH appears to be absent, having been replaced by pyruvate:ferredoxin oxidoreductase (PFO or PFOR, EC 1.2.7.1) [20]. PFOR is a key enzyme in the metabolism of these protists, and also plays a central role in the activity of metronidazole, a drug commonly used against these parasites [20]. Here we show that microsporidia apparently differ from other amitochondriates in this aspect of core metabolism, since genes encoding both alpha and beta subunits of PDH complex E1 are encoded in the genome of the microsporidian, *Nosema locustae*. Molecular phylogenetic analyses of both subunits individually and combined show that these genes are derived from the mitochondrion, but neither gene possesses evidence of a mitochondrion-targeting transit peptide. Together these genes provide the first evidence for mitochondrion-derived metabolic activity in microsporidia, but it is still unclear whether they are involved in core energy metabolism in a mitochondrion as in other eukaryotes, or if they have been conscripted into some other pathway during the unusual course of evolution in microsporidia.

2. Materials and methods

2.1. Strains, DNA isolation, library construction, and sequencing

Purified *N. locustae* ATCC 30860 spores were generously provided by M&R Durango Inc. (Bayfield, CO). Two lots of approximately 5×10^9 spores were harvested and ground under liquid nitrogen until light microscopy confirmed that 80–90% of spores were disrupted. DNA was purified from ground material using the Plant DNeasy mini isolation kit with an additional AW1 buffer wash (Qiagen), resulting in approximately 7 μg of purified DNA. One lot of DNA was partially digested with *Sau*3A1, size selected for fragments between 2 and 6 Kbp, and cloned into *Bam*HI-digested, CIP-treated pBluscript SK + . This library was used to transform *E. coli* (strain Top10) by electroporation. The other lot was randomly sheared by nebulisation, size selected for fragments between 2 and 5 Kbp, CIP-treated, and cloned using the TOPO blunt-end kit (Invitrogen). This library was used to transform *E. coli* (strain Top10) by heat shock. Random clones from both libraries and an additional library described earlier [21] were picked and sequenced using ABI dye-terminator chemistry as part of a *N. locustae* random sequence survey. Sequences were compared against one another and public databases using the software ESTid (generously provided by M.A. Reith). Two clones were identified that contain sequences homologous to the alpha and beta subunits of PDH. These clones were used to search against all other random *Nosema* sequences generated, and all clones identified that contain similarity to these clones were sequenced and assembled into a single contiguous fragment using Sequencher 4.1 (GeneCodes).

2.2. Primary sequence characterisation and phylogenetic analysis

Open reading frames were identified using Sequencher, and all examples exceeding 300 base pairs in length were translated and compared to public databases using gapped-BLASTP. Intergenic regions were also compared to public databases using gapped-BLASTN and gapped-BLASTX. The amino termini of both alpha and beta subunits of PDH were also examined for the presence of mitochondrion-targeting transit peptides using iPSORT [22]. The alpha and beta subunits of PDH were also translated and the inferred amino acid sequences were aligned with known homologues from public databases using Clustal X. In addition to mitochondrial PDH genes, the alignment included PDH from plastids, eubacteria, and archaebacteria, as well as three closely related proteins from the 2-oxo acid dehydrogenase family from a variety of eubacteria. The alignment was edited manually, and unambiguously alignable sites were chosen for phylogenetic inference (295 and 308 sites from alpha and beta, respectively). Distances were calculated by PUZZLE 5.0 [23] using the WAG substitution matrix and site-to-site rate variation modelled on a gamma distribution with invariant sites and eight rate categories, and the shape parameter was estimated from the data (the estimated parameters for alpha and beta subunits were 1.85 and 1.54, respectively, while 0.02 and 0.01% of sites were estimated to be invariant). Trees were constructed from these distances by weighted neighbour-joining using WEIGHBOR [24] and Fitch–Margoliash using FITCH [25] in the latter case considering the data to include 20 possible characters (other values were also used, and did not alter the position of *Nosema* with the mitochondrial homologues). Bootstrap resampling was carried out using the shell script PUZZLEBOOT (by M. Holder and A. Roger: http://www.tree-puzzle.de) and the settings described above. Alpha and beta subunits were also concatenated into a single data set (603 characters) and subjected to the same analysis (for this combined data set the shape parameter alpha was estimated to be 1.65 and 0.02% of sites were estimated to be invariant). In this case a protein maximum likelihood analysis was also conducted using ProML [25]. Here, site-to-site rate variation was corrected using the R option by entering the nine categories estimated by PUZZLE and their respective frequencies (eight rates plus invariant sites). In addition, global rearrangements were carried out and the sequences randomly added ten times. No bootstrap resamplings were carried out with maximum likelihood since the time taken for the analysis is prohibitive.

3. Results and discussion

3.1. Cloning and primary characteristics of the Nosema pyruvate dehydrogenase E1 genomic fragment

As part of a genome sequence survey to examine genome reduction and metabolic diversity in microsporidia, random genomic clones have been end-sequenced from *N. locustae*, a locust parasite with a genome of 5.4 Mbp [26]. Comparing these sequences to one another and to public databases revealed that several clones contained sequences homologous to the alpha and beta subunits of PDH complex E1, or PDH, an enzyme normally active in the mitochondrion of eukaryotes. These clones were completely sequenced and assembled with all other overlapping clones in the sequence survey, resulting in a contiguous sequence of 7079 bp from four individual overlapping clones.

Surprisingly, both the alpha and beta subunits of PDH were found to be encoded on the same fragment

of the genome, in fact, the two subunits are immediately adjacent to one another and are separated by an intergenic space of only 11 bp. The two genes are, however, encoded on opposite strands of the DNA, so there is no possibility that the two genes are co-transcribed. Why two genes encoding subunits of the same enzyme would be encoded together is uncertain. Other genes of related function have been shown to be linked in other microsporidian genomes [27], however, there is no evidence that this is not just the result of chance, perhaps due to the fact that functionally related genes are more likely to be adjacent in a reduced genome. Five other open reading frames over 300 bp in length were also predicted on this fragment; they are encoded on both strands and range in size from 660 to 1233 bp. These putative genes are tightly packed, with intergenic spaces ranging from 110 bp to as little as 11 bp, in keeping with observations of extreme genome compaction in other microsporidia with smaller genomes [28]. In addition to PDH alpha and beta, two other genes share recognisable similarity to known homologues. These are the checkpoint serine–threonine protein kinase, *chk*1, from yeast and other eukaryotes, and a second gene encoding a protein with a calmodulin-dependent protein kinase domain at its carboxy terminus. Intergenic spacers were also searched against DNA databases and assessed for the presence of tRNA genes, but no genes were identified. A relatively long region (513 bp) of apparently non-coding DNA at one end of the clone was also searched for coding potential, but it was not found to contain any detectable protein or RNA gene. Nevertheless, this region is considerably larger than the other intergenic regions, and we suspect that some part of this region likely encodes the end of an unrecognised gene.

3.2. Phylogeny of alpha and beta subunits of pyruvate dehydrogenase

Amino acid alignments of both subunits of PDH were constructed including a number of mitochondrial homologues as well as representatives of alpha-proteobacteria (the closest relatives of mitochondria), other eubacteria and archaebacteria, and a number of other prokaryotic genes from closely related members of the 2-oxo acid dehydrogenase complex gene families. These include acetoin [2,6-dichlorophenolindophenol] oxidoreductase (AOR), TPP-dependent acetoin dehydrogenase (ADH), and branched-chain alpha-keto acid dehydrogenase E1 [2-oxoisovalerate dehydrogenase] (AKADH). These alignments were used to infer unrooted phylogenetic trees to determine if the *Nosema* genes are more closely related to PDH than to any other 2-oxo acid dehydrogenase, and if they are indeed derived from the mitochondrion. In all analyses of both subunits (Fig. 1A and B) the *Nosema* sequence falls

within a clade composed exclusively of eukaryotic mitochondrial PDH genes (with bootstrap support between 70 and 97%), which is itself related to the alpha-proteobacterial PDH clade (bootstrap support between 95 and 100%). This position for the microsporidian sequence is exactly what one would expect for a mitochondrial PDH gene. The *Nosema* sequences are both more divergent than other mitochondrial homologues, but not as divergent as many microsporidian genes. Probably as a result of this divergence, the *Nosema* gene does not branch with those from fungi, as would be expected, but instead branches at the base of animals and fungi (and *Trypanosoma* in PDH alpha). This is not unusual with microsporidian genes, which often branch deeply due to their divergence [14,15], and in the poorly sampled phylogeny of mitochondrial PDH it would be unwise to make much of the relative positions of the eukaryotic taxa.

The mitochondrial and alpha-proteobacterial clades are in turn related to PDH genes from cyanobacteria and plastids, chlamydias, and *Acidithiobacillus*, and all remaining sequences in both trees are divided among two strongly supported clades. The first consists of PDH genes from a variety of eubacteria and archaebacteria, as well as genes from Gram positives and *Thermoplasma* annotated as AKADH. The second clade consists almost exclusively of genes annotated as AOR or ADH from various eubacteria and archaebacteria. The actual biochemical activity of many of the products of these genes is unknown as their annotation depends largely on their similarity to known homologues. However, the level of congruence between the phylogenies of the alpha and beta subunits is striking, suggesting that the assignment of alpha and beta subunits to particular complexes is indeed accurate. The strong support for the separation of these clades also indicates that many of the non-PDH genes within this family diverged from PDH early in evolution, although more specific conclusions cannot be drawn from an unrooted phylogeny.

The congruence between the phylogenies of the alpha and beta subunits suggested that both subunits might be combined to further address the support for the mitochondrial origin of the *Nosema* PDH genes. Here (Fig. 2), the same overall tree topology is recovered, and the support for the mitochondrial clade increased markedly (93 and 97%). Again, the *Nosema* PDH is clearly derived from the mitochondrion, but its exact position within the mitochondrial clade is unclear, as there is no support for the branches separating *Nosema* from plants, animals, or fungi.

3.3. Potential cellular and biochemical roles for microsporidian pyruvate dehydrogenase

The broad outlines of "amitochondriate" metabolism have only been revealed for a few parasitic protists
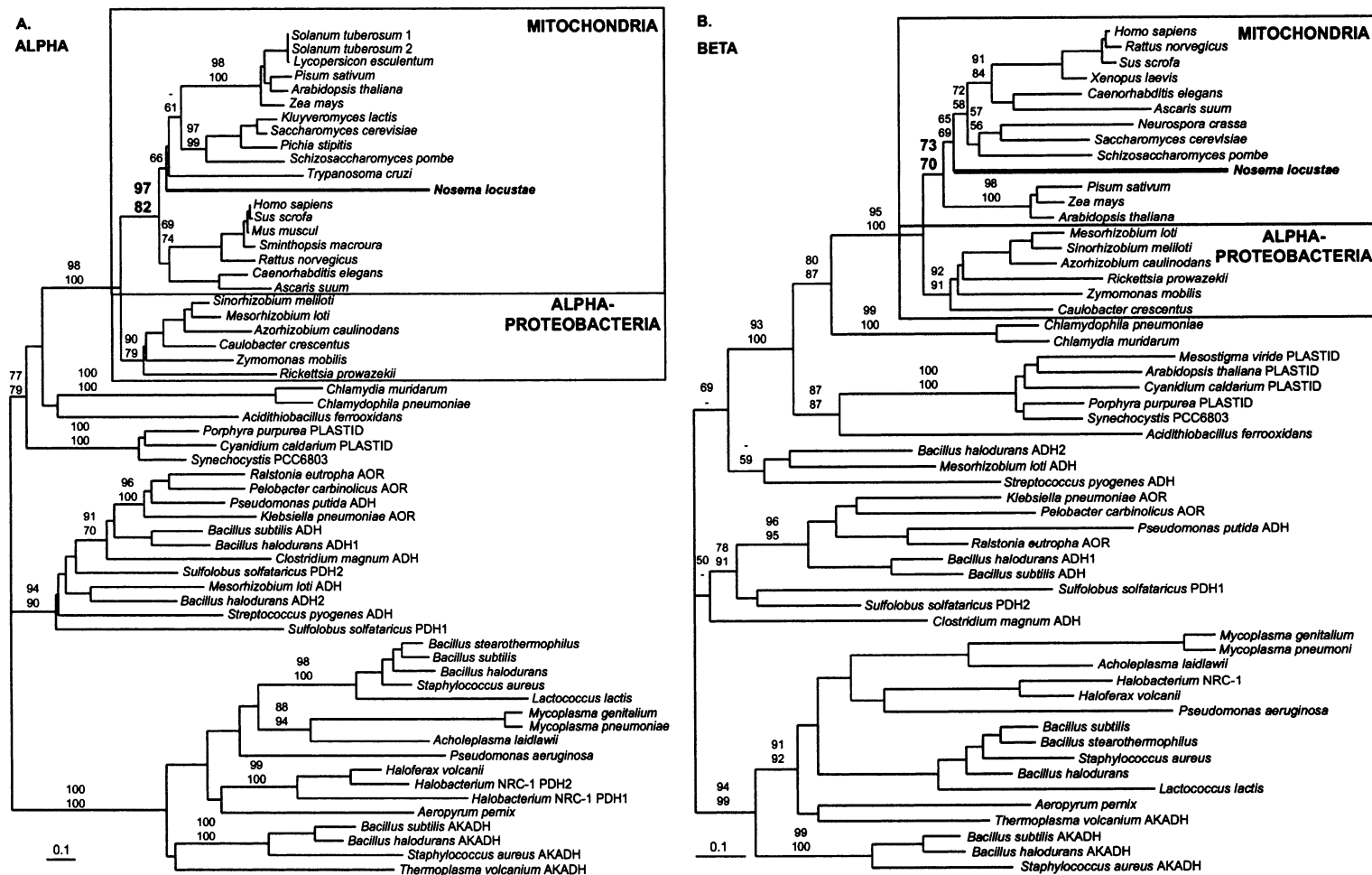
Fig. 1. Molecular phylogeny of PDH subunits alpha (A) and beta (B), and closely related homologous proteins. Fitch–Margoliash trees of gamma-corrected distances with bootstraps for Fitch–Margoliash (top) and weighted neighbor-joining (bottom). Scale bar shows 0.1 (corrected) changes per site. Mitochondrial homologues of PDH are boxed at the top of the tree, and the *Nosema* sequences are shown in bold. PDH genes are labelled with only the name of the organism (unless two copies are known from one organism in which case the copy number is indicated), while non-PDH homologues are distinguished as the following: AOR, ADH and AKADH.

(particularly *Trichomonas*, *Giardia*, *Entamoeba*, and increasingly *Cryptosporidium*), and all in recent years. The emerging picture is that core metabolism has been assembled independently in each taxon in an ad hoc fashion using enzymes from a variety of sources (resident enzymes, lateral transfers from bacteria, etc.). Several amitochondriates have adapted different solutions to the same general metabolic challenges, so there are few generalities that apply to all "amitochondriate" eukaryotes [20]. Nevertheless, one feature common to all groups studied so far is the apparent loss of the PDH complex and the use of PFOR or a derivative of PFOR in its place [20,29]. However, microsporidia are not particularly sensitive to drugs that target PFOR [30–32], suggesting that microsporidia may have adopted some other system. Here we show that the

microsporidia have evolved differently, as they have retained at least the E1 enzyme (PDH) from the PDH complex. The presence of PDH in *Nosema* raises two questions: (1) What is the current relationship of microsporidian PDH to the mitochondrion, and (2) is it involved in synthesising acetyl-CoA?

Until recently, the prevailing notion was that microsporidia were primitively amitochondriate, or that they evolved before the mitochondrion originated [5]. Since we now know that the microsporidia evolved from a fungus [9–11], and that microsporidian genomes contain HSP70 genes derived from the mitochondrion [16–18], a mitochondrial ancestry for microsporidia is confirmed. However, clues as to possible metabolic roles for a microsporidian mitochondrion, or even if the organelle still exists, are currently lacking. As an anal-
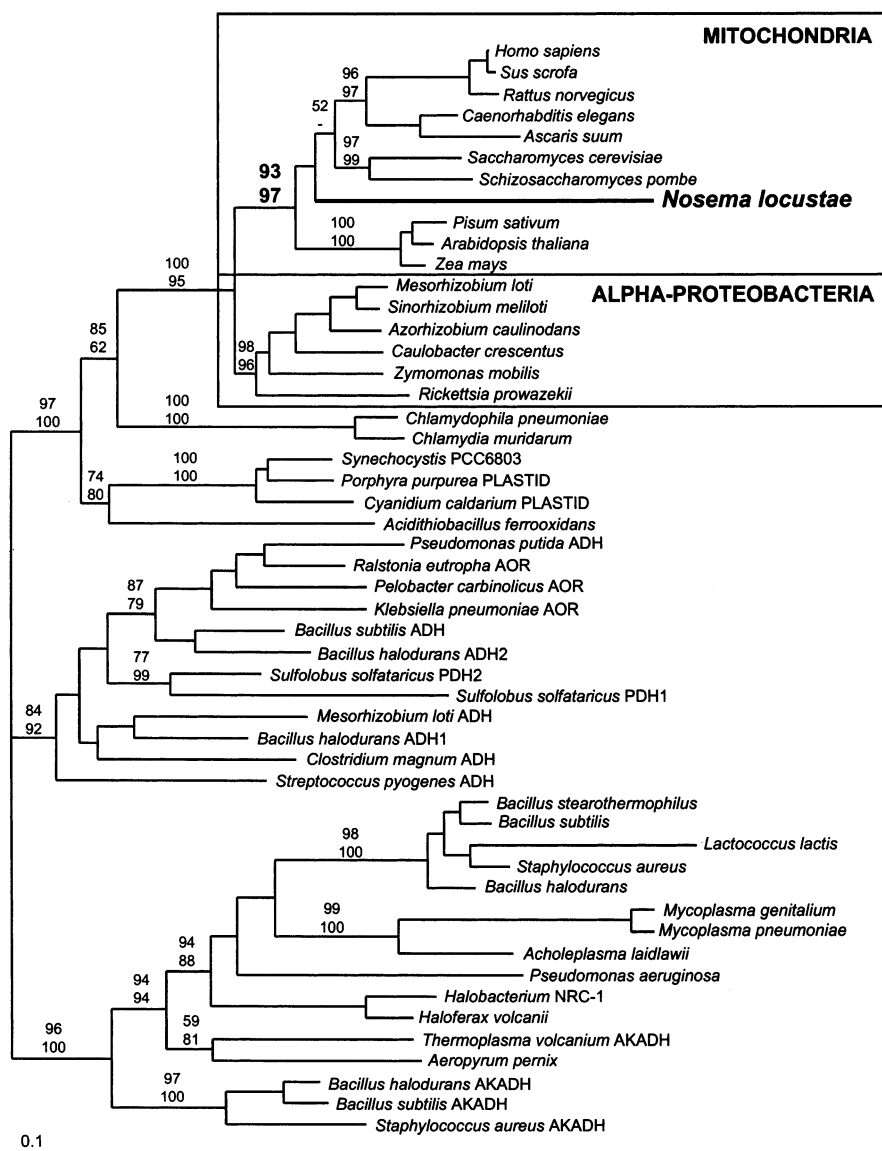

Fig. 2. Combined molecular phylogeny of alpha and beta subunits of PDH. Protein maximum likelihood phylogeny with bootstraps from distance analyses as in Fig. 1. Notation of taxon names and type of gene is also as in Fig. 1.

PDH-alpha
Saccharomyces [+33] [+36] IEGSDTVQIELPESSFESYMLEPPDLSYETSKATLLQMYKDMVIIRRMEMACDALYKAKKIRGF
Nosema   MDFYEEKVDFIAHNISLGNLSCKIRYDDVEKLYRKMLCMRYMDESISKMYSRGLIRGF
Rickettsia   MDIKPEKYKPIKEEYIKSFKDMLLLRRFEEKCGQLYGMGKIGGF

PDH-beta
Saccharomyces [+22] RPSAAAAALRF SSTKTMTVREALNSAMAEELDRDDDVFLIGEEVAQYNGAYKVSKGLLDRFGERRVVDT
Nosema   MESEWITVREAINKALDEELCRDKNVIVLGEEVAKSGGAHQVTKGLLAKYGNCRVMDT
Rickettsia   MQITVREALRDAMQEEMLRDEKVFVIGEEVAEYQGAYKVTQGLLEQFGSKRVIDT

HSP70
Saccharomyces MLAAKNILNRSSLSSSFRIATRL QSTKVQGSVIGIDLGTTNSAVAIMEGKVPKIIENAEGSRTTP
Nosema   MGAEVEKSTIIGIDLGTTNSCVSVIKDRYPKIIRNRTGKRTTP
Vairimorpha   MTGKEISSRIIGIDLGTTNSCVSLMHNNVPHIIENEEGTRTTP
Encephalitozoon   MSNADAPSRKFSSSIIGIDLGTTNSCVSVIKDGKPVIIENQEGERTTP
Rickettsia   MGKVIGIDLGTTNSCVAVMEGKEPKVIDNAEGERTTP

Fig. 3. Leader sequences of *Nosema* PDH alpha and beta. The amino termini of microsporidian PDH alpha (top), PDH beta (centre), and mitochondrial HSP70 (bottom) aligned to homologues from the *Saccharomyces* mitochondrion and the alpha-proteobacterium *Rickettsia*. The *Saccharomyces* leaders are quite long and are not shown in entirety, but the number of amino acids not shown is indicated in square brackets. *Saccharomyces* transit peptides are boxed (in the case of PDH alpha, the upstream sequence not shown exceeds the transit peptide, so the two bracketed numbers correspond to the transit peptide and the remainder of the mature protein). In all cases the microsporidian proteins are substantially shorter than those of *Saccharomyces* (or any other mitochondrial sequence) but slightly larger than those of *Rickettsia*. More importantly, the site of transit peptide cleavage in *Saccharomyces* corresponds well to the start of the microsporidian proteins (except for PDH alpha where the mature *Saccharomyces* protein is even longer than the microsporidian proteins). In addition, none of the microsporidian sequences was predicted to encode a transit peptide by iPSORT.

ogy, two mitochondrial protein-coding genes (cpn60 and hsp70) have been found in the diplomonad *Giardia* [33,34], but localisation experiments suggest that at least one of these is not confined to an organelle [33,35]. This implies that diplomonads may have lost the mitochondrion, but kept certain mitochondrion-derived proteins that now act in the cytosol [33]. Here we have provided a new piece of the puzzle of the microsporidian mitochondrion: the presence of PDH in *Nosema* indicates that microsporidia have not just kept heat shock proteins, but have also maintained elements of the core carbon metabolic machinery of the mitochondrion. But does the presence of PDH confirm that microsporidia have maintained their mitochondrion? The amino termini of all three mitochondrion-derived microsporidian proteins were analysed for evidence that they are targeted to an organelle, and no such evidence was found. Fig. 3 shows the amino-termini of the *Nosema* PDH alpha and beta subunits as well as the *Nosema*, *Vairimorpha* and *Encephalitozoon* HSP70, all aligned with homologues from yeast mitochondria and the closely related alpha-proteobacterium *Rickettsia*. In each case, the microsporidian proteins lack any obvious amino-terminal extension. What is more, the microsporidian proteins each begin at approximately the same position as the yeast transit peptide is cleaved to yield the mature protein (in the case of PDH alpha there is considerable amino-terminal length heterogeneity and the mature yeast protein is actually longer than the *Nosema* protein). When the HSP70 genes were first characterised, no consensus was reached as to whether they encoded transit peptides [16–18]. Now, with three different proteins to query, we have analysed the amino-termini of all mitochondrion-derived microsporidian genes (PDH alpha, beta, and three

HSP70s) for characteristics commonly associated with transit peptides using iPSORT, and none were predicted to encode a transit peptide. At face value this suggests that these proteins function in the cytosol, but without experimental evidence for the localisation of the PDH and HSP70 proteins, any conclusion regarding the presence or absence of a mitochondrion in microsporidia can only be made tentatively, especially since leaders in other unusual mitochondrion-derivatives can be very short [36,37].

With respect to the metabolic role of the *Nosema* PDH, in every other system studied E1 is part of the PDH complex that synthesises acetyl-CoA to be used in the Krebs cycle and as an active compound in acetylation reactions. It is entirely possible that *Nosema* also has E2 and E3 proteins to make up a complete PDH complex, however, bearing in mind the unusual nature of microsporidian metabolism [3], we can take little for granted about the role of PDH. Indeed, we cannot be absolutely certain that E1 is used in acetyl-CoA synthesis, or that the entire PDH complex is present in microsporidia. In the reaction carried out by the PDH complex, E1 is responsible for the critical TPP-dependent decarboxylation of pyruvate, resulting in 2-alpha-hydroxyethyl-TPP. Normally, this is converted to acetyl-CoA by E2 and E3, but it is also possible that 2-alpha-hydroxyethyl-TPP is an essential intermediate in another pathway in *Nosema*. We will discuss two possibilities, however there are many potential roles involving this pathway that have not yet been characterised.

First, hydroxyethyl-TPP serves as an intermediate in the biosynthesis of valine, leucine, and isoleucine [19]. Yeast PDH alpha mutants are leucine auxotrophs, suggesting that this compound may be synthesised by the

uncoupling of E1 activity from the PDH complex. However, this auxotrophy could also reflect a negative regulatory effect resulting from the build-up of free CoA [38] and it is also clear from yeast studies that PDH E1 is not the only source of 2-alpha-hydroxy-yethyl-TPP in the cell [38]. Whether amino acid biosynthesis could account for the presence of PDH in *Nosema* remains to be seen, but it seems unlikely that microsporidia would actively synthesise amino acids when they could more easily be scavenged from their hosts.

Perhaps a more interesting possibility stems from similarities in the activities of PDH and PFOR, the enzyme that replaced the PDH complex in other amitochondriate eukaryotes studied to date. The recent isolation of a crystal structure for PFOR and its comparison with the PDH complex identified structural similarities between the active sites of the two enzymes [39,40]. Furthermore, hydroxyethyl-TPP has been found to be an intermediate in the activity of PFOR [40–42]. In this reaction, hydroxyethyl-TPP is first created by the oxidative decarboxylation of pyruvate, then electrons are transferred from hydroxyethyl-TPP to an iron–sulphur cluster within the PFOR enzyme. This leaves the cluster reduced and creates an acyl-TPP intermediate that is cleaved by CoA to yield acetyl-CoA [42]. It is an intriguing possibility that PDH in *Nosema* is generating hydroxyethyl-TPP to transfer electrons to an iron–sulphur cluster in a second enzyme, in effect mimicking the PFOR reaction in the absence of PFOR. This is obviously speculative, but does generate the testable predictions that *Nosema* will contain proteins with iron–sulphur centres as well as ferredoxin and perhaps ferredoxin:NADP oxidoreductase. This would represent a completely novel approach to core carbon metabolism never before seen in any organism, and would further the general observation that amitochondriate eukaryotes have each assembled their core metabolism in different ways [20]. Moreover, this also explains the apparent lack of sensitivity to metronidazole seen in microsporidia [30–32], since they are predicted to lack PFOR and therefore cannot activate the drug.

In summary, the presence of the alpha and beta subunits of PDH E1 in *N. locustae* provides the first evidence of mitochondrion-derived metabolism in microsporidia, and also the first evidence for pyruvate utilisation. Although this is clearly a mitochondrion-derived enzyme, there is still no clear evidence as to whether microsporidia maintain a mitochondrion or, if they do, whether these proteins actually function in the organelle. It also remains to be seen whether this enzyme is part of a complete PDH complex that synthesises acetyl-CoA, or whether it is involved in other pathways independently of E2 and E3. Whatever the answers to these questions may be, these results show conclusively that microsporidia, unlike other amitochondriates studied to date, have preserved some metabolic functionality from their mitochondrion even if they are found to have lost the organelle entirely.

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