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agarose gels (10 μg of DNA per lane). Total RNA was separated on 1.2% agarose–formaldehyde gels (15 μg of RNA per lane). Gels were blotted to Hybond N^+ membranes (Amersham). Different cDNAs, used as probes (see Figs 4 and 5), were labelled by polymerase chain reaction (PCR) with α-[^{32}P]-dATP. Hybridization was performed in 0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 1% BSA, 1 mM EDTA at 60°C. Filters were washed stringently with 50 mM sodium phosphate buffer, 0.5% SDS at 60°C.

Sequence analysis

Sequences were analysed with the help of the GCG sequence analysis package (Devereux *et al.*, 1984). Phylogenetic trees were constructed with the aid of the programs in PHYLIP V3.5c (Felsenstein, 1993).

Enzyme assays

Subcellular fractionation of *Piromyces* sp. E2 was performed essentially as described by Marvin-Sikkema *et al.* (1993a), but the homogenization medium was substituted for 20 mM HEPES, pH 7.4, 250 mM sucrose and 2 mM dithiothreitol. Hexokinase was assayed as described by Bergmeyer *et al.* (1983). Malic enzyme activity was determined according to the method of Kremer *et al.* (1989). Hydrogenase activity was measured as described by Marvin-Sikkema *et al.* (1993a). MDH was assayed according to Stams *et al.* (1984). Aconitase was assayed as described by Reeves *et al.* (1971). The specificity of the aconitase enzyme assay was supported by the complete inhibition of the aconitase activity by 10 mM fluorocitrate (see Table 1). Acetohydroxyacid reductoisomerase activity was measured as described by Magee and Hereford (1969), using α-acetolactate as substrate. α-Acetolactate was prepared by the hydrolysis of ethyl-2-acetoxy-2-methylacetoacetate (Aldrich) with NaOH (Park *et al.*, 1995). In order to permeabilize the membrane-bound hydrogenosomes, Triton X-100 was added to a final concentration of 0.2% (v/v). All enzyme assays were performed at 39°C.

Proteinase K protection assay

Isolated hydrogenosomes (0.4 mg of protein) were incubated anaerobically (N_2 gas phase) in 0.5 ml of homogenization medium with or without 50 μg of proteinase K. The hydrogenosomes were permeabilized by the addition of Triton X-100 (2% v/v). After 30 min incubation on ice, the proteolytic digestion was terminated by the addition of 20 mM phenylmethylsulphonyl fluoride (PMSF). Aconitase and malic enzyme activities were assayed as described above in the presence of 20 mM PMSF and Triton X-100 (2% v/v).

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van der Giezen, M., Kiel, J.A.K.W., Sjollema, K.A., and

but, beyond the hypothesis of Martin and Müller (1998), it would implicate the evolution of a hydrogenosome from a compartment different from the mitochondrion. In the first model, the subcellular compartment and the mitochondrial import machinery are maintained. This should also allow the import of all mitochondrial proteins after a loss or repression of respiratory functions. Evidence in favour of this scenario is provided by the genes encoding hydrogenosomal malic enzyme and the β-subunit of succinyl-CoA synthetase (Brondijk *et al.*, 1996; van der Giezen *et al.*, 1997b, 1998).

The second scenario assumes that the mitochondria have been lost during the transition from aerobiosis to anaerobiosis leading to 'type I' anaerobes according to the hypothesis of Martin and Müller (1998). In this scenario, however, hydrogenosomes of chytrids must have evolved *de novo*, perhaps from microbody-like organelles as postulated by Marvin-Sikkema *et al.* (1993b). This scenario is supported by the data on the hydrogenosomal adenylate kinase of *Neocallimastix* and *Piromyces*. This hydrogenosomal enzyme has a mitochondrial ancestry. It lacks an N-terminal extension, but contains a carboxy-terminal sequence, the tripeptide SKL, which is known as a peroxisomal targeting signal. Expression of the hydrogenosomal adenylate kinase in the heterologous host *Hansenula polymorpha* revealed that this tripeptide is functional in sorting this enzyme to peroxisomes (F. G. J. Voncken *et al.*, submitted).

As shown in this paper, the chytrid MDH, aconitase and acetohydroxyacid reductoisomerase also have a mitochondrial ancestry. However, they lack mitochondrial targeting information and are located in the cytosol. This phenomenon might fit into both scenarios. Under the assumption that mitochondria evolved into hydrogenosomes while maintaining a mitochondrial import machinery, one can speculate that the presence of MDH, aconitase and acetohydroxyacid reductoisomerase interfered with the evolving hydrogenosomal metabolism. Consequently, the exclusion of these enzymes from the hydrogenosomes might have required the removal of all targeting information.

If the mitochondria were completely lost in anaerobic chytrids, hundreds of nuclear genes encoding mitochondrial proteins would have remained functional. In the absence of a suitable subcellular compartment, the encoded proteins would stay in the cytosol, unless their synthesis is downregulated. Also, the N-terminal leader peptides that are normally removed by specific mitochondrial peptidases would be retained in the mature protein. The presence of such N-terminal extensions could potentially interfere with protein folding and enzyme activity (Danpure, 1997). Thus, the second assumption can also provide arguments for the absence of mitochondrial leader sequences from the chytrid MDH, aconitase and acetohydroxyacid reductoisomerase.

It is evident that more information about the subcellular localization of 'mitochondrial' enzymes and the nature of the chytrid hydrogenosomal import system(s) will be required to discriminate between the two scenarios described above. The origin of chytrid hydrogenosome remains elusive, as we are far from understanding the meaning of the data available so far. However, regardless of whether the mitochondrial compartment has been lost or, alternatively, become transformed into a hydrogenosome, the adaptation to anoxic environments by the chytrid ancestors must have involved a loss of the mitochondrial genome, a loss of mitochondrial functions, the recompartmentalization of mitochondrial enzymes and, perhaps also, the acquisition of novel enzymes. The data presented here show that such an adaptation required dramatic changes in subcellular sorting and import.

Experimental procedures

Organism and growth conditions

Piromyces sp. E2 (ATCC 76762) was cultured in medium M2, supplemented with 0.5% fructose as a carbon source (Teunissen *et al.*, 1991). Biomass for the preparation of DNA, RNA or homogenates for subcellular fractionation and enzyme assays was harvested after 40–48 h of growth at 39°C.

RNA and DNA isolation

RNA was prepared by the guanidinium-chloride method (Chirgwin *et al.*, 1979). For the preparation of poly(A)$^+$ RNA, an mRNA purification kit (Pharmacia) was used according to the manufacturer's protocol. *Piromyces* sp. E2 genomic DNA was prepared as described by Brownlee (1994).

Construction and random sequencing of the Piromyces *sp. E2 cDNA library*

The *Piromyces* sp. E2 cDNA library in the vector lambda ZAPII was constructed with the help of the ZAP-cDNA synthesis kit (Stratagene). As starting material, 7.5 μg of poly(A)$^+$ RNA, isolated from *Piromyces* sp. E2 grown on 0.5% fructose, was used. The titre of the primary library was 2×10^6 plaque-forming units ml^{-1}. An aliquot of this library was converted to pBluescript SK– clones by mass excision with the ExAssist helper phage (Stratagene). Clones were picked at random and sequenced with the M13 reverse primer to determine the sequence of the 5′ part of the cDNAs. The sequences of interesting cDNA clones were completed by generating shorter subclones in pUC18 and by using internal sequencing primers. Sequencing was performed with the ABI Prism model 310 automatic sequencer, using a dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems).

Southern and Northern blotting

Piromyces sp. E2 genomic DNA was digested by several restriction enzymes (see Fig. 4) and separated on 0.7%

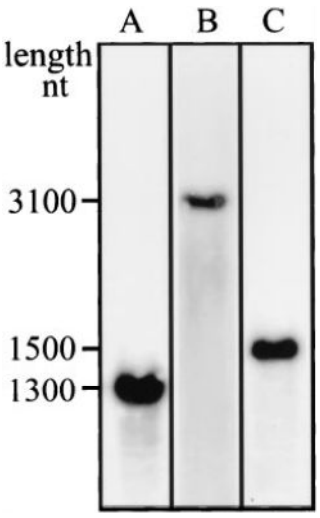


Fig. 5. Northern blots with total RNA from *Piromyces* sp. E2, probed with cDNAs encoding MDH (A), aconitase (B) and acetohydroxyacid reductoisomerase (C). In (A), (B) and (C), the same blot was used.

these transcripts were in good agreement with the lengths of the corresponding cDNAs.

Discussion

In this study, we have identified cDNA clones that encode MDH, aconitase and acetohydroxyacid reductoisomerase from anaerobic chytrid *Piromyces* sp. E2. Phylogenetic analysis of the deduced protein sequences (Fig. 1) demonstrated that these enzymes are closely related to the corresponding mitochondrial isoforms from aerobic eukaryotes. DNA sequence analysis has revealed the absence of N-terminal extensions, which function as a targeting signal in the mitochondrial homologues (Fig. 2). The absence of such N-terminal leader sequences correlates with the cytosolic localization of these enzymes in *Piromyces* sp. E2 (Tables 1 and 2), and one has to conclude that certain mitochondrial enzymes of the aerobic ancestor became localized in the cytoplasm as a consequence of the adaptation of chytrids to anaerobic environments.

Anaerobic chytrids lack mitochondria and several key enzymes of the TCA cycle (Yarlett *et al.*, 1986; O'Fallon *et al.*, 1991; Mountfort, 1994; Yarlett, 1994). However, they must still rely on the various TCA cycle intermediates and enzymes that are necessary for the biosynthesis of compounds that permit their growth on simple sugars and ammonia as sole source of carbon and nitrogen (Teunissen *et al.*, 1991; Dijkerman *et al.*, 1997). It has been shown previously that some important enzymes involved in biosynthetic reactions, such as phosphoenolpyruvate carboxykinase, are localized in the cytosol of anaerobic chytrids (Marvin-Sikkema *et al.*, 1993a; Mountfort, 1994; Yarlett, 1994). Also, a part of the TCA cycle – working in the reverse (reductive) direction from oxaloacetate to malate, fumarate and, finally, succinate – is located in the cytosol (Marvin-Sikkema *et al.*, 1993a). This metabolic pathway plays an important role in the synthesis of intermediates that are essential for several anabolic reactions. The MDH is also playing a major role in main cellular carbon flow by converting oxaloacetate to malate, the sole substrate of hydrogenosomal metabolism (Marvin-Sikkema *et al.*, 1993a; 1994).

As anaerobic chytrids can grow on glucose and ammonium as the sole carbon and nitrogen source (Dijkerman *et al.*, 1997), they must contain a full set of enzymes necessary for the biosynthesis of all amino acids, purines and pyrimidines. In glucose-grown yeast, the disruption of the gene encoding the mitochondrial aconitase results in glutamate auxotrophy, in addition to respiratory defects (see McAlister-Henn and Small, 1997 and references therein). Therefore, it is likely that, in *Piromyces* sp. E2, after the adaptation to anaerobiosis and the 'loss' of functional mitochondria and a complete TCA cycle, a cytosolic aconitase and a cytosolic isocitrate dehydrogenase (results not shown) must maintain the cellular pool of α-ketoglutarate. This intermediate is used to synthesize glutamate and other amino acids. Acetohydroxyacid reductoisomerase is crucial for the biosynthesis of branched amino acids (see Petersen and Holmberg, 1986 and references therein). Thus, several biochemical pathways necessary for amino acid synthesis are localized in the cytosol in anaerobic chytrids.

We have shown here that MDH, aconitase and acetohydroxyacid reductoisomerase, enzymes normally localized in the mitochondria of aerobic fungi, are expressed and active in the cytosol of the *Piromyces* sp. E2. Therefore, we have to conclude that many, if not all, of the 'mitochondrial' biosynthetic reactions are localized in the cytosol of this anaerobic chytrid. These aspects and the fact that part of the chytrid's carbohydrate metabolism is running 'backwards' might be of some relevance to the hypothesis of Martin and Müller (1998) for the evolution of the eukaryotic cell. They have postulated that the presence of biosynthetic and catabolic reactions in the same subcellular compartment might result in futile cycling. Consequently, one might conclude that the cytoplasmic localization of MDH and aconitase is a necessary consequence of the evolution of a compartmentalized hydrogenosomal metabolism that uses malate and retains a ('mitochondrial') succinyl-thiokinase (Brondijk *et al.*, 1996).

The adaptation to anoxic environments apparently involved the retargeting of several 'mitochondrial' enzymes to the cytosol. There are several possible scenarios for the adaptation of the chytrids to anaerobiosis. The first scenario assumes a conversion of mitochondria into hydrogenosomes as postulated by Martin and Müller (1998). The second scenario would postulate a loss of mitochondria

Table 1. Enzyme activities in different subcellular fractions of *Piromyces* sp. E2.

	Specific activity (μmol min^{-1} mg^{-1} protein)		
	Cytosol	Hydrogenosomes	
Enzyme	+Triton X-100	−Triton X-100	+Triton X-100
Hydrogenase	1.90	11.49	172.45
Malic enzyme (NADP)	0.19	4.42	20.60
Hexokinase	0.83	ND	ND
Malate dehydrogenase (NAD)	0.24	ND	ND
Acetohydroxyacid reductoisomerase (NADP)	0.01	ND	ND
Aconitase	0.17	0.09	0.08
Aconitase + fluorocitrate	ND	ND	ND

ND, not detectable within the limits of the assay sensitivity (1 nmol min^{-1} mg^{-1} protein).

sp. E2 (see Table 1). Aconitase activity was found in both the cytosolic and the hydrogenosomal fractions. However, a proteinase K protection assay revealed that no aconitase activity is present inside the hydrogenosomes (Table 2). After incubation of isolated hydrogenosomes with proteinase K, no aconitase activity could be detected, regardless of whether the detergent Triton X-100 was added or not. These data indicated that the aconitase activity measured in the hydrogenosomal fraction (Table 1) results from the adhesion of cytosolic aconitase to the outside of the isolated hydrogenosomes. In the control experiment, the hydrogenosomal malic enzyme (van der Giezen *et al.*, 1997b) could only be digested by proteinase K in the presence of the detergent Triton X-100 (Table 2).

Table 2. Proteinase K protection assays for hydrogenosomal enzymes of *Piromyces* sp. E2.

	Malic enzyme			Aconitase		
Proteinase K	−	+	+	−	+	+
Triton X-100	−	−	+	−	−	+
Specific activity (μmol mg^{-1} min^{-1})	3.79	3.99	0.23	0.10	ND	ND

ND, not detectable within the limits of the assay sensitivity (1 nmol min^{-1} mg^{-1} protein).

Thus, the three enzymes analysed here, i.e. MDH, aconitase and acetohydroxyacid reductoisomerase, are located exclusively in the cytosol of *Piromyces* sp. E2.

Gene copy number and expression of MDH, aconitase and acetohydroxyacid reductoisomerase at the mRNA level

The copy number of the MDH-, aconitase- and acetohydroxyacid reductoisomerase-encoding genes was determined by Southern blotting (Fig. 4). Hybridization with the MDH probe revealed the presence of one gene copy with a high similarity to the probe (Fig. 4A). For aconitase, similar results were found (Fig. 4B). The acetohydroxyacid reductoisomerase-encoding gene is apparently present in several copies (Fig. 4C). It is unclear whether more than one of these gene copies is expressed. The two acetohydroxyacid reductoisomerase cDNAs isolated in our random screening were identical in the overlapping region (400 bp) and are most probably derived from the same gene. Northern blotting revealed the presence of single transcripts for MDH, aconitase and acetohydroxyacid reductoisomerase (see Fig. 5). The estimated lengths of

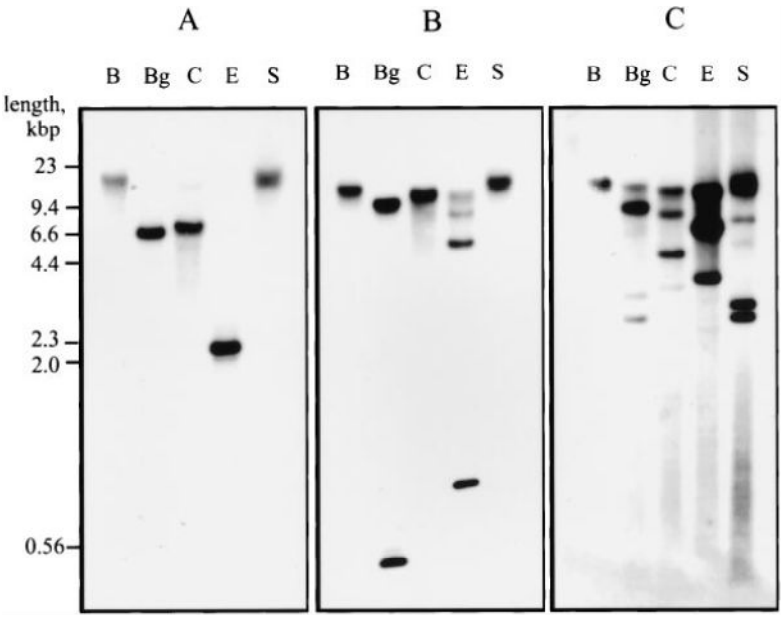


Fig. 4. Southern blots with genomic DNA from *Piromyces* sp. E2 probed with cDNAs encoding MDH (A), aconitase (B) and acetohydroxyacid reductoisomerase (C). DNA was digested with the following restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; S, *Sst*I; K, *Kpn*I; X, *Xba*I. The presence of several bands in (B) in the lanes with genomic DNA, digested with *Bgl*II and *Eco*RI, results from the presence of internal *Bgl*II and *Eco*RI restriction sites in the aconitase cDNA.

1995). Apparently, the N-terminal extension, which functions as a targeting signal in mitochondrial orthologues, is absent from the genes described here.

It has been reported previously that the peroxisomal MDH from yeast also lacks an N-terminal extension, although it probably has a mitochondrial ancestry (see Fig. 1; Ocheretina and Scheibe, 1997). Instead, it contains a conserved carboxy-terminal peroxisomal targeting signal (the tripeptide SKL), which is responsible for the peroxisomal sorting of this isoform (McAlister-Henn *et al.*, 1995). Sequence analysis of the deduced carboxy-terminal ends from the chytrid MDH-, aconitase- and acetohydroxyacid reductoisomerase-encoding genes did not provide evidence for the presence of putative peroxisomal targeting signals.

Finally, for the yeast mitochondrial MDH, it has been shown that the N-terminal leader sequence is not the only determinant of mitochondrial import. Deletion of the N-terminal extension still allows mitochondrial import of this MDH isoform (Thompson and McAlister-Henn, 1989). This import relies on three positively charged residues near the N-terminus of the mature protein (Lys-25, Arg-30 and Lys-38). Their alteration, together with a simultaneous removal of the N-terminal presequence, abolishes mitochondrial import of this MDH protein (Small and McAlister-Henn, 1997). In the deduced *Piromyces* sp. E2 MDH sequence, the corresponding positions are occupied by uncharged amino acids, suggesting that this cryptic mitochondrial targeting signal is also absent.

Localization of the MDH, aconitase and acetohydroxyacid reductoisomerase activities

Absence of the N-terminal leader sequences from the deduced MDH, aconitase and acetohydroxyacid reductoisomerase sequences suggests that their gene products are localized in the cytosol. To test this hypothesis, the activities of these enzymes were assayed in the cytosolic and hydrogenosomal fractions from *Piromyces* sp. E2 homogenates.

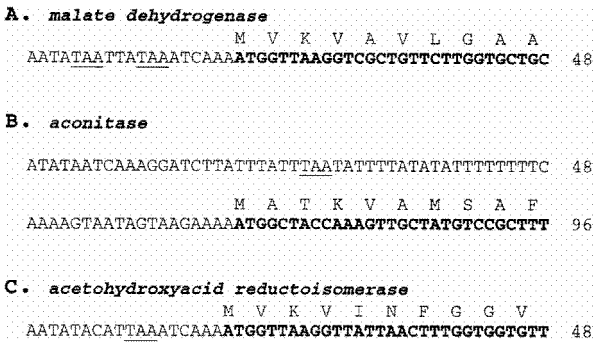


Fig. 3. 5′ sequences of cDNAs encoding MDH, aconitase and acetohydroxyacid reductoisomerase from *Piromyces* sp. E2. The putative coding regions are shown in bold, with the encoded amino acids indicated above the nucleotide sequence. Stop codons present upstream of the translation start codon and in the same translation frame as it are underlined. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers Y16748, Y16747 and Y16743.

The quality of the subcellular fractionation was controlled by measuring the activities of cytosolic and hydrogenosomal marker enzymes. As expected, the cytosolic marker enzyme hexokinase was present exclusively in the cytosolic fraction of *Piromyces* sp. E2 (Table 1). The activities of the hydrogenosomal marker enzymes hydrogenase and malic enzyme could be detected in both the cytosolic and the hydrogenosomal fractions (Table 1). However, they were significantly enriched in the hydrogenosomal fraction of *Piromyces* sp. E2, which indicated that the majority of the isolated hydrogenosomes was intact (Marvin-Sikkema *et al.*, 1993a). The addition of Triton X-100, a detergent permeabilizing the hydrogenosomal membrane, resulted in a considerable increase in the measured hydrogenosomal enzyme activities (Table 1).

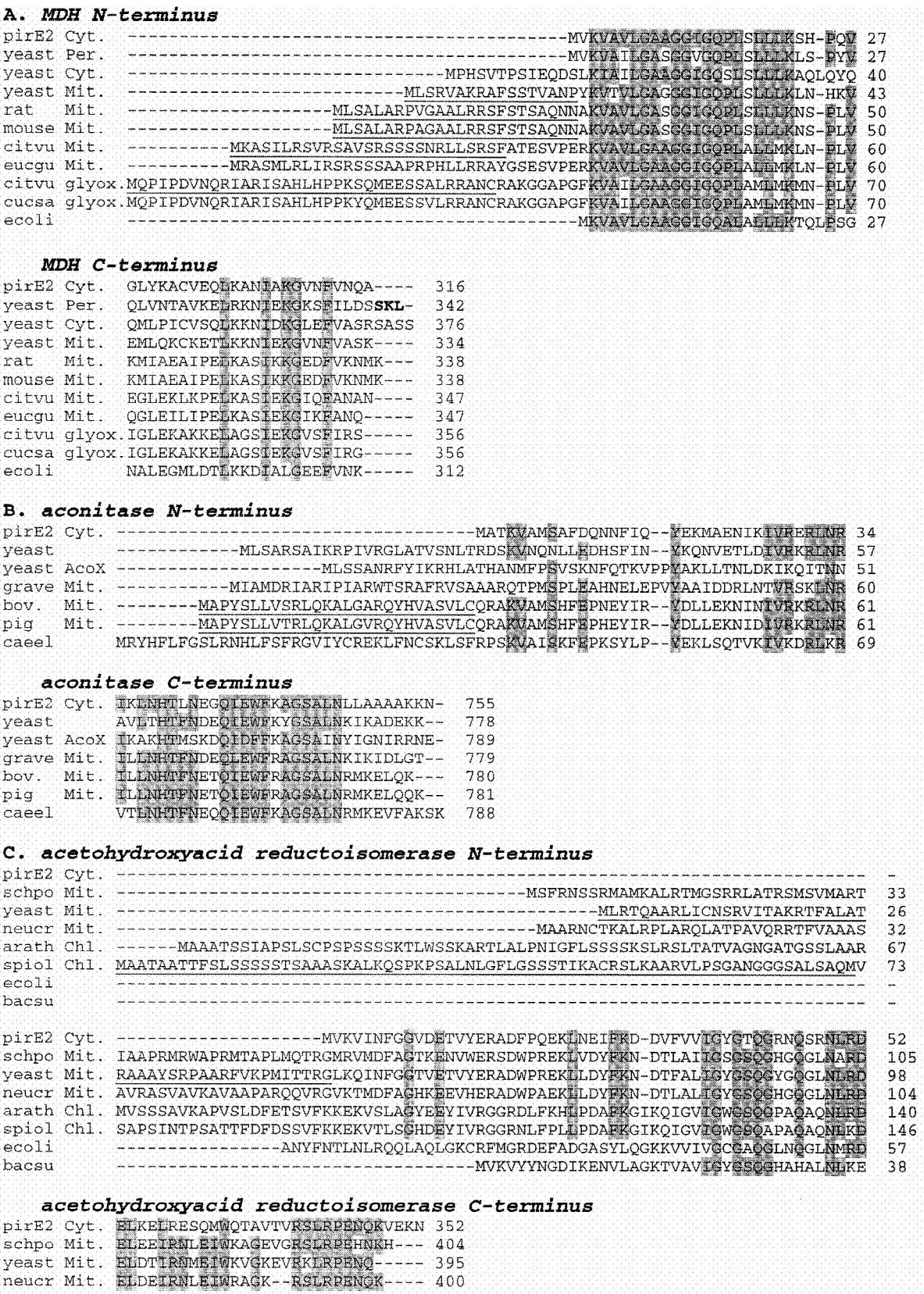
MDH and acetohydroxyacid reductoisomerase activity was found exclusively in the cytosolic fraction of *Piromyces*

Fig. 2. Multiple alignments of the N- and C-terminal sequences of malate dehydrogenases (A), aconitases (B) and acetohydroxyacid reductoisomerases (C). Amino acids identical in more than 60% of the sequences are shaded in grey. Gaps are indicated by dashes. N-terminal transit peptides shown experimentally to be removed from the corresponding mature proteins are underlined. The PTS1 peroxisomal targeting signal of the yeast peroxisomal MDH is shown in bold. As the C-terminal domains of different acetohydroxyacid reductoisomerase proteins are poorly conserved, only the C-termini of the fungal sequences are shown in (C). The source sequences and data bank accession numbers are listed below.

A. Malate dehydrogenases: pirE2, *Piromyces* sp. E2, this study; yeast Per., *S. cerevisiae*, peroxisomal (P32419); yeast Cyt., *S. cerevisiae*, cytosolic (P22133); yeast Mit., *S. cerevisiae*, mitochondrial (P17505); rat Mit., *Rattus norvegicus*, mitochondrial (P04636); mouse Mit., *Mus musculus*, mitochondrial (P08249); citvu Mit., *Citrullus vulgaris*, mitochondrial (P17783); eucgu Mit., *Eucalyptus gunnii*, mitochondrial (P46487); citvu glyox., *Citrullus vulgaris*, glyoxysomal (P19446); cucsa glyox., *Cucumis sativus*, glyoxysomal (P46488); ecoli, *Escherichia coli* (P06994).

B. Aconitases: pirE2 aconitase, *Piromyces* sp. E2, this study; yeast, *Saccharomyces cerevisiae* (P19414); yeast AcoX, *S. cerevisiae* (P39533); grave Mit., *Gracilaria verrucosa*, mitochondrial (P49609); bov. Mit., *Bos taurus*, mitochondrial (P20004); pig Mit., *Sus scrofa*, mitochondrial (P16276); caeel, *Caenorhabditis elegans* (P34455).

C. Acetohydroxyacid reductoisomerases: pirE2, *Piromyces* sp. E2, this study; schpo Mit., *Schizosaccharomyces pombe* (P78827); yeast Mit., *S. cerevisiae* (P06168); neucr Mit., *Neurospora crassa* (P38674); arath Chl., *Arabidopsis thaliana*, chloroplast (Q05758); spiol Chl., *Spinacia oleracea*, chloroplast (Q01292); ecoli, *Escherichia coli* (P05793); bacsu, *Bacillus subtilis* (P37253).


A. MDH N-terminus
pirE2 Cyt. -----------MVKVAVLGAAGGIGQPLSLLLKSH-PQV 27
yeast Per. -----------MVKVAILGASGGVGQPLSLLLKLS-PYV 27
yeast Cyt. -----------MPHSVTPSIEQDSLKIAILGAAGGIGQSLSLLLKAQLQYQ 40
yeast Mit. -----------MLSRVAKRAFSSTVANPYEVTVLGAGGIGQPLSLLLKLN-HKV 43
rat Mit. -----------MLSALARPVGAALRRSFSTSAQNNAKVAVLGASGGIGQPLSLLLKNS-PLV 50
mouse Mit. -----------MLSALARPAGAALRRSFSTSAQNNAKVAVLGASGGIGQPLSLLLKNS-PLV 50
citvu Mit. ----------MKASILRSVRSAVSRSSSSNRLLSRSFATESVPERKVAVLGAAGGIGQPLALLMKLN-PLV 60
eucgu Mit. ----------MRASMLRLIRSRSSSAAPRPHLLRRAYGSESVPERKVAVLGAAGGIGQPLALLMKLN-PLV 60
citvu glyox. MQPIPDVNQRIARISAHLHPPKSQMEESSALRRANCRAKGGAPGFKVAILGAAGGIGQPLAMLMKMN-PLV 70
cucsa glyox. MQPIPDVNQRIARISAHLHPPKYQMEESSVLRRANCRAKGGAPGFKVAILGAAGGIGQPLAMLMKMN-PLV 70
ecoli -----------MKVAVLGAAGGIGQALALLLKTQLPSG 27
MDH C-terminus
pirE2 Cyt. GLYKACVEQLKANIAKGVNFVNQA---- 316
yeast Per. QLVNTAVKELRKNIEKGKSFILDSSKL- 342
yeast Cyt. QMLPICVSQLKKNIDKGLEFVASRSASS 376
yeast Mit. EMLQKCKETLKKNIEKGVNFVASK---- 334
rat Mit. KMIAEAIPELKASIKKGEDFVKNMK--- 338
mouse Mit. KMIAEAIPELKASIKKGEDFVKNMK--- 338
citvu Mit. EGLEKLKPELKASIEKGIQFANAN---- 347
eucgu Mit. QGLEILIPELKASIEKGIKFANQ----- 347
citvu glyox. IGLEKAKKELAGSIEKGVSFIRS----- 356
cucsa glyox. IGLEKAKKELAGSIEKGVSFIRG----- 356
ecoli NALEGMLDTLKKDIALGEEFVNK----- 312
B. aconitase N-terminus
pirE2 Cyt. ----------MATKVAMSAFDQNNFIQ--YEKMAENIKIVRERLNR 34
yeast ----------MLSARSAIKRPIVRGLATVSNLTRDSKVNQNLLEDHSFIN--YKQNVETLDIVRKRLNR 57
yeast AcoX ----------MLSSANRFYIKRHLATHANMFPSVSKNFQTKVPPYAKLLTNLDKIKQITNN 51
grave Mit. ----------MIAMDRIARIPIARWTSRAFRVSAAARQTPMSPLEAHNELEPVYAAIDDRLNTVRSKLNR 60
bov. Mit. --------MAPYSLLVSRLQKALGARQYHVASVLCQRAKVAMSHFEPNEYIR--YDLLEKNINIVRKRLNR 61
pig Mit. --------MAPYSLLVTRLQKALGVRQYHVASVLCQRAKVAMSHFEPHEYIR--YDLLEKNIDIVRKRLNR 61
caeel MRYHFLFGSLRNHLFSFRGVIYCREKLFNCSKLSFRPSKVAISKFEPKSYLP--YEKLSQTVKIVKDRLKR 69
aconitase C-terminus
pirE2 Cyt. IKLNHTLNEGQIEWFKAGSALNLLAAAAKKN- 755
yeast AVLTHTFNDEQIEWFKYGSALNKIKADEKK-- 778
yeast AcoX IKAKHTMSKDQIDFFKAGSAINYIGNIRRNE- 789
grave Mit. ILLNHTFNDEQLEWFRAGSALNKIKIDLGT-- 779
bov. Mit. ILLNHTFNETQIEWFRAGSALNRMKELQK--- 780
pig Mit. ILLNHTFNETQIEWFRAGSALNRMKELQQK-- 781
caeel VTLNHTFNEQQIEWFKAGSALNRMKEVFAKSK 788
C. acetohydroxyacid reductoisomerase N-terminus
pirE2 Cyt. ---------- -
schpo Mit. ----------MSFRNSSRMAMKALRTMGSRRLATRSMSVMART 33
yeast Mit. ----------MLRTQAARLICNSRVITAKRTFALAT 26
neucr Mit. ----------MAARNCTKALRPLARQLATPAVQRRTFVAAAS 32
arath Chl. ------MAAATSSIAPSLSCPSPSSSSKTLWSSKARTLALPNIGFLSSSSKSLRSLTATVAGNGATGSSLAAR 67
spiol Chl. MAATAATTFSLSSSSSTSAAASKALKQSPKPSALNLGFLGSSSTIKACRSLKAARVLPSGANGGGSALSAQMV 73
ecoli ---------- -
bacsu ---------- -
pirE2 Cyt. ----------MVKVINFGGVDETVYERADFPQEKLNEIFKD-DVFVVIGYGTQGRNQSRNLRD 52
schpo Mit. IAAPRMRWAPRMTAPLMQTRGMRVMDFAGTKENVWERSDWPREKLVDYFKN-DTLAIIGSGSQGHGQGLNARD 105
yeast Mit. RAAAYSRPAARFVKPMITTRGLKQINFGGTVETVYERADWPREKLLDYFKN-DTFALIGYGSQGYGQGLNLRD 98
neucr Mit. AVRASVAVKAVAAPARQQVRGVKTMDFAGHKEEVHERADWPAEKLLDYFKN-DTLALIGYGSQGHGQGLNLRD 104
arath Chl. MVSSSAVKAPVSLDFETSVFKKEKVSLAGYEEYIVRGGRDLFKHLPDAFKGIKQIGVIGWGSQGPAQAQNLRD 140
spiol Chl. SAPSINTPSATTFDFDSSVFKKEKVTLSGHDEYIVRGGRNLFPLLPDAFKGIKQIGVIGWGSQAPAQAQNLKD 146
ecoli ----------ANYFNTLNLRQQLAQLGKCRFMGRDEFADGASYLQGKKVVIVGCGAQGLNQGLNMRD 57
bacsu ----------MVKVYYNGDIKENVLAGKTVAVIGYGSQGHAHALNLKE 38
acetohydroxyacid reductoisomerase C-terminus
pirE2 Cyt. ELKELRESQMWQTAVTVRSLRPENQKVEKN 352
schpo Mit. ELEEIRNLEIWKAGEVGRSLRPEHNKH--- 404
yeast Mit. ELDTIRNMEIWKVGKEVEKLRPENQ----- 395
neucr Mit. ELDEIRNLEIWRAGK--RSLRPENGK---- 400

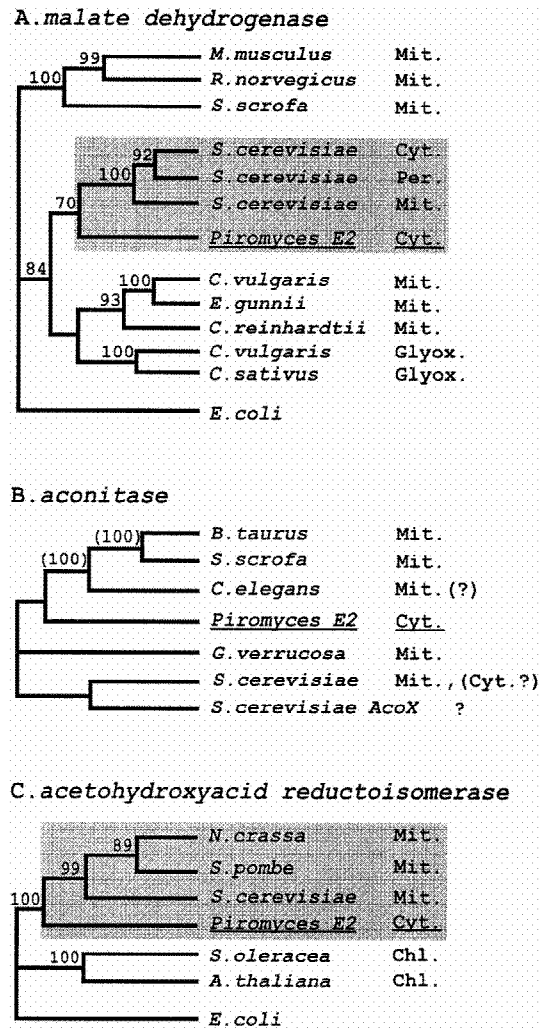


Fig. 1. Phylogenetic relations between malate dehydrogenases (A), aconitases (B) and acetohydroxyacid reductoisomerases (C). Phylogenetic trees were obtained using the neighbour-joining method (A and C) or the maximum likelihood method (B). Bootstrap values of 70% or more are indicated. For the aconitase tree (B), bootstrap values obtained for the indicated clades using the neighbour-joining method are shown in parentheses. The clades including fungal sequences are shaded in grey. Cellular localization of the gene products is indicated: Cyt., cytosol; Chl., chloroplast; Mit., mitochondrion; Glyox., glyoxysome; Per., peroxisome. Uncertain localization is indicated by a question mark. For the accession numbers of the source sequences, see the legend to Fig. 2. Additional sequences not included in Fig. 2 were: pig MDH, *Sus scrofa*, mitochondrial (P00346/Q95308); *Chlamydomonas reinhardtii* MDH, mitochondrial (Q42686).

MDHs (Ocheretina and Scheibe, 1997). Comparison of the *Piromyces* sp. E2 MDH sequence with the yeast MDHs revealed that the chytrid MDH is more closely related to the yeast mitochondrial MDH (62% identity) than to the yeast peroxisomal and cytosolic MDHs (56% and 55% identity respectively).

The second cDNA clone (pa14) contained one ORF, encoding a protein of 755 amino acids with high similarity to aconitases. Similar to MDH, the aconitase-encoding genes also belong to two different lineages. The first lineage represents the mammalian cytosolic aconitases, the plant mitochondrial and cytosolic aconitases and some eubacterial aconitases (Peyret *et al.*, 1995; Zhou and Ragan, 1995; Gruer *et al.*, 1997). The mitochondrial aconitases from mammals, red algae and fungi constitute the other lineage. Pairwise comparison revealed that the chytrid aconitase showed the highest similarity (66–70% identity) to the aconitases from the mitochondria of mammals, algae and fungi. Also, phylogenetic analysis showed clustering of the chytrid aconitase within the mitochondrial clade (see Fig. 1B). The exact branching order within this group could not be determined, because of the small number of available aconitase sequences and their high degree of identity.

The cDNA clones pa1 and pa62 encoded an ORF with high similarity to acetohydroxyacid reductoisomerases. One of these clones (pa1) was sequenced completely. The ORF encoded a protein of 352 amino acids. Pairwise comparison has shown that the deduced chytrid sequence displayed the highest similarity (57–64% identity) with the mitochondrial acetohydroxyacid reductoisomerases from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa*. Phylogenetic analysis also revealed clustering of the *Piromyces* sp. E2 acetohydroxyacid reductoisomerase sequence with the mitochondrial homologues from aerobic fungi (see Fig. 1C).

Malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase of Piromyces *sp. E2 lack N-terminal leader sequences*

Many nuclear-encoded mitochondrial matrix proteins are sorted to their subcellular compartment by an N-terminal leader sequence. This leader sequence is enriched in positively charged and hydroxylated amino acids and cleaved off upon import into the mitochondrial matrix (Hendrick *et al.*, 1989; von Heijne *et al.*, 1989). Also, mitochondrial malate dehydrogenases, aconitases and acetohydroxyacid reductoisomerases are sorted to the mitochondrial matrix with the aid of N-terminal leader sequences (see Fig. 2).

Analysis of the deduced MDH, aconitase and acetohydroxyacid reductoisomerase sequences of *Piromyces* sp. E2 has shown that they lack N-terminal extensions. Their coding sequences start around the processing site of the mitochondrial N-terminal leader sequences of the mitochondrial isoforms from aerobic eukaryotes (see Fig. 2). It is very unlikely that translation of the analysed cDNAs starts upstream of the first ATG codon, as the upstream sequences of all three cDNAs contain in frame stop codons and are highly AT rich, as is characteristic of untranslated regions of anaerobic chytrids (see Fig. 3) (Reymond *et al.*, 1992; Durand *et al.*, 1995; Fanutti *et al.*,

in more detail. They postulated that the evolution of a hydrogenosome from a mitochondrion (or its progenitor) would entail the loss of the respiratory pathway, and they emphasized that losses probably occurred several times in the evolution of different lineages of eukaryotes (Martin and Müller, 1998).

The lack of a genome in the hydrogenosomes of the chytrids *Piromyces* and *Neocallimastix* makes it impossible to reconstruct their ancestry directly (Müller, 1993; van der Giezen *et al.*, 1997a; cf. Margulis, 1993; Embley *et al.*, 1997; Hackstein *et al.*, 1997, 1998). Only the phylogeny of the genes that encode hydrogenosomal proteins, the type of hydrogenosomal targeting signals and the hydrogenosomal import machinery can provide indirect evidence for the evolutionary history of the hydrogenosome (Sogin, 1997). The molecular basis of the changes that accompany the transition from aerobic, mitochondrial to anaerobic hydrogenosomal energy metabolism are still poorly understood. A crucial event in the adaptation to anoxic environments and the evolution of hydrogenosomes is the expression or acquisition of a hydrogenase and a pyruvate:ferredoxin oxidoreductase. However, the questions why these enzymes become compartmentalized, whereas others are targeted to a different subcellular compartment, and why these changes eventually cause the loss of the hydrogenosomal genome have not been answered yet. Similarly, potential changes in enzyme compartmentalization and losses of enzyme activities that might be required to cope with the new environment on the one hand and to avoid an interference with the metabolic routes that evolve in response to the novel evolutionary constraints on the other hand are far from being understood. Moreover, as Martin and Müller (1998) have pointed out, a total loss of mitochondria can also be envisaged under conditions of adaptation to anaerobic environments.

Regardless of whether the mitochondrion became modified or lost during the adaptation to anoxic environments, the repression or factual loss of the tricarboxylic acid (TCA) cycle in anaerobic chytrids (aerobic chytrids do possess a complete TCA cycle; see Ragan and Chapman, 1978) and of enzymes involved in the oxidative phosphorylation must have serious consequences for chytrid cellular metabolism, as mitochondria, besides their function in energy metabolism, provide intermediates for the biosynthesis of cellular components. Also, the synthesis of branched-chain amino acids that is known to be located in the mitochondria of aerobic fungi might be affected by the modification or the loss of mitochondria (Ryan and Kohlhaw, 1974). Moreover, in mammals and apicomplexan parasites, mitochondria are involved in purine and pyrimidine biosynthesis (Gutteridge *et al.*, 1979; Prapunwattana *et al.*, 1988; Krungkrai *et al.*, 1991). Therefore, a loss (or repression) of these functions could potentially result in auxotrophy of the organisms.

However, it has been shown that anaerobic chytrids are still capable of growth on monosaccharides and ammonia as the sole carbon and nitrogen sources (Teunissen *et al.*, 1991; Dijkerman *et al.*, 1997). Therefore, anaerobic chytrids must be able to synthesize all amino acids and nucleotides, including those that normally require functional mitochondria for their biosynthesis in the absence of functional mitochondria. This suggests that at least some of the mitochondrial functions have been retained in the amitochondriate chytrids – in the absence of functional mitochondria.

To address these questions, we have performed random sequencing of *Piromyces* sp. E2 cDNAs in order to identify genes with a mitochondrial ancestry. Here, we describe the cDNAs encoding TCA cycle enzymes malate dehydrogenase (MDH) and aconitase and a cDNA that encodes acetohydroxyacid reductoisomerase, an enzyme involved in the biosynthesis of isoleucine and valine. We show that these enzymes are of mitochondrial origin, lack mitochondrial targeting signals and are active in the cytosol of *Piromyces* sp. E2.

Results

cDNAs encoding malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase

In order to identify highly expressed genes from the anaerobic chytrid *Piromyces* sp. E2, a cDNA library was constructed. Randomly selected cDNA clones were sequenced. Clones with a high similarity to malate dehydrogenase- (clone pa49), aconitase- (clone pa14) and acetohydroxyacid reductoisomerase-encoding genes (clones pa1 and pa62) were identified. These cDNA clones were analysed in detail.

The first cDNA clone (pa49) contained one open reading frame (ORF), encoding a protein of 316 amino acids with high similarity to malate dehydrogenases (MDHs). MDH-encoding genes can be grouped into a 'mitochondrial' and a 'cytosolic' lineage (Joh *et al.*, 1987; Cendrin *et al.*, 1993; Ocheretina and Scheibe, 1997). The 'mitochondrial' lineage includes the mitochondrial MDHs from animals, plants and fungi and the glyoxysomal MDHs from plants. Cytosolic MDHs from plants and animals and the chloroplast MDH constitute the 'cytosolic' lineage. Comparison of the deduced MDH sequence with MDHs from both lineages revealed that it displayed 61–67% identity to mitochondrial MDH sequences. When compared with the MDHs from the 'cytosolic' lineage, it displayed only 25–27% identity. Phylogenetic analysis of the mitochondrial MDH sequences grouped the *Piromyces* sp. E2 MDH sequence in one clade together with the yeast MDHs (see Fig. 1A). It should also be noted that, in yeast, the cytosolic and peroxisomal MDHs are derived from mitochondrial

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Cytosolic enzymes with a mitochondrial ancestry from the anaerobic chytrid *Piromyces* sp. E2

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Summary

The anaerobic chytrid *Piromyces* sp. E2 lacks mitochondria, but contains hydrogen-producing organelles, the hydrogenosomes. We are interested in how the adaptation to anaerobiosis influenced enzyme compartmentalization in this organism. Random sequencing of a cDNA library from *Piromyces* sp. E2 resulted in the isolation of cDNAs encoding malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase. Phylogenetic analysis of the deduced amino acid sequences revealed that they are closely related to their mitochondrial homologues from aerobic eukaryotes. However, the deduced sequences lack N-terminal extensions, which function as mitochondrial leader sequences in the corresponding mitochondrial enzymes from aerobic eukaryotes. Subcellular fractionation and enzyme assays confirmed that the corresponding enzymes are located in the cytosol. As anaerobic chytrids evolved from aerobic, mitochondria-bearing ancestors, we suggest that, in the course of the adaptation from an aerobic to an anaerobic lifestyle, mitochondrial enzymes were retargeted to the cytosol with the concomitant loss of their N-terminal leader sequences.

Introduction

Anaerobic chytrids are important symbionts in the gastrointestinal tract of many herbivorous mammals (see Trinci *et al.*, 1994 and references therein). They are highly adapted to these intestinal environments and, during their whole life cycle, they thrive under anoxic conditions (for review, see Orpin, 1994). The life cycle of all anaerobic fungi studied to date consists of an alternation between motile, flagellated zoospore stages and a vegetative phase in which a complex rhizomycelial system is formed. The hyphae of the rhizomycelial system attach to plant-derived particles of the digesta, and the intestinal chytrids contribute significantly to the digestion of plant polymers by the production and excretion of a broad spectrum of (cellulolytic and other) enzymes (Teunissen *et al.*, 1991; Orpin, 1994).

These highly specialized anaerobic chytrids evolved from mitochondria-bearing aerobic ancestors, as phylogenetic analysis of their rRNA genes has shown unequivocally that yeasts and fungi belong to a 'crown group' of eukaryotic microorganisms that includes the aerobic and anaerobic chytrids (Sogin, 1991; Knoll, 1992). DNA sequence analysis reveals a clustering of both aerobic and anaerobic chytrids (Dore and Stahl, 1991; Bowman *et al.*, 1992; Li and Heath, 1992). Also, an analysis of biochemical (Ragan and Chapman, 1978) and morphological traits (Li *et al.*, 1993) consistently establishes a close relationship between chytrids and other fungi. Moreover, for the aerobic chytrids, a congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees has been demonstrated (Paquin *et al.*, 1995). Lastly, the complete nucleotide sequence from the mitochondrial genome of *Allomyces macrogynus*, an aerobic chytrid, has been determined (Paquin and Lang, 1996). This genome exhibits all the traits characteristic of a eufungal mitochondrial genome, albeit an ancestral one. Consequently, there is no doubt that the chytrids that live in the gastrointestinal tract of herbivorous mammals have adopted an anaerobic life style secondarily.

In contrast to their aerobic relatives, anaerobic chytrids such as *Neocallimastix* and *Piromyces* lack mitochondria. Instead, they possess membrane-bound organelles, called hydrogenosomes (Yarlett *et al.*, 1986; Müller, 1993). These organelles are involved in anaerobic energy metabolism. Malate is imported into the organelles, where it undergoes oxidative decarboxylation to pyruvate. The reduction equivalents formed in this process are excreted as molecular hydrogen via an H_2:ferredoxin oxidoreductase (Marvin-Sikkema *et al.*, 1993a). As evidence has accumulated that the hydrogenosomes of the parabasalid *Trichomonas* and mitochondria share a common ancestor (see reviews by Biagini *et al.*, 1997; Embley *et al.*, 1997; Müller, 1997; Sogin, 1997), it is possible that the hydrogenosomes of anaerobic chytrids also evolved from the mitochondria of their aerobic ancestors. Recently, Martin and Müller (1998) discussed the evolution of aerobic and anaerobic eukaryotes

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