Plant Molecular Biology **53:** 175–188, 2003.

Bifunctional aldehyde/alcohol dehydrogenase (ADHE) in chlorophyte algal mitochondria

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Received 26 July 2003; accepted in revised form 1 September 2003

Key words: chlorophytes, evolution, OXPHOS complexes, pH regulation

Abstract

Protein profiles of mitochondria isolated from the heterotrophic chlorophyte *Polytomella* sp. grown on ethanol at pH 6.0 and pH 3.7 were analyzed by Blue Native and denaturing polyacrylamide gel electrophoresis. Steady-state levels of oxidative phosphorylation complexes were influenced by external pH. Levels of an abundant, soluble, mitochondrial protein of 85 kDa and its corresponding mRNA increased at pH 6.0 relative to pH 3.7. N-terminal and internal sequencing of the 85 kDa mitochondrial protein together with the corresponding cDNA identified it as a bifunctional aldehyde/alcohol dehydrogenase (ADHE) with strong similarity to homologues from eubacteria and amitochondriate protists. A mitochondrial targeting sequence of 27 amino acids precedes the N-terminus of the mature mitochondrial protein. A gene encoding an ADHE homologue was also identified in the genome of *Chlamydomonas reinhardtii*, a photosynthetic relative of *Polytomella*. ADHE reveals a complex picture of sequence similarity among homologues. The lack of ADHE from archaebacteria indicates a eubacterial origin for the eukaryotic enzyme. Among eukaryotes, ADHE has hitherto been characteristic of anaerobes since it is essential to cytosolic energy metabolism of amitochondriate protists such as *Giardia intestinalis* and *Entamoeba histolytica.* Its abundance and expression pattern suggest an important role for ADHE in mitochondrial metabolism of *Polytomella* under the conditions studied. The current data are compatible with the view that *Polytomella* ADHE could be involved either in ethanol production or assimilation, or both, depending upon environmental conditions. Presence of ADHE in an oxygen-respiring algal mitochondrion and co-expression at ambient oxygen levels with respiratory chain components is unexpected with respect to the view that eukaryotes acquired ADHE genes specifically as an adaptation to an anaerobic lifestyle.

Abbreviations: ADHE, aldehyde/alcohol dehydrogenase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; BN-PAGE, blue native polyacrylamide gel electrophoresis; Fe-ADH, iron-dependent alcohol dehydrogenase; MTS, mitochondrial targeting sequence; OXPHOS, oxidative phosphorylation; PFL, pyruvate formate-lyase

Introduction

The colorless chlorophytes of the genus *Polytomella* are members of a single monophyletic clade, the *Reinhardtii* clade (Pröschold *et al*., 2001), and share a common ancestor with their photosynthetic relatives, *Chlamydomonas reinhardtii* and *Volvox carterii* (Melkonian and Surek, 1995; Nakayama *et al*., 1996). *Polytomella* is found in various habitats including freshwater ponds and greenhouse soils (Prinsgsheim, 1955). In the laboratory the algae can be grown on a great variety of carbon sources and under a wide range of pH values (Lwoff, 1941; Wise, 1955, 1959; Atteia

The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AJ495765

et al., 2000). The growth of this alga is often associated with significant changes in the pH of the culture medium (Atteia *et al*., 2000). The ability of the alga to adapt to different habitats implies a tight regulation of the intracellular concentration of solutes and protons.

Polytomella sp. is able to grow on ethanol at pH below 7.0 whereby its metabolism tends to acidify the growth medium, although it can also be grown under conditions where the pH is maintained constant (Atteia *et al*., 2000). Studies on *Polytomella* sp. cells grown on ethanol in the presence of non-metabolizable buffers have shown that the external pH influences the function and biogenesis of mitochondria. The rates of oxygen uptake in the presence of substrates like succinate, malate or ethanol are 20–25% higher in mitochondria isolated from cells grown at pH 3.7 than in mitochondria from cells grown at pH 6.0 (Atteia *et al*., 2000). The steady-state accumulation of mitochondrial proteins is also affected by the external pH. Mitochondria from cells grown at pH 3.7 contained more polypeptides of 30 kDa or less, one of which was cytochrome *c* (Atteia *et al*., 2000), relative to mitochondria from cells grown at pH 6.0. At present, the identity of the different mitochondrial protein patterns of cells grown at pH 6.0 and pH 3.7 is not known.

The aim of this study was to further characterize the influence of the external pH on the mitochondrial protein content in *Polytomella* sp. grown on ethanol and to identify proteins that exhibit a pH-dependent accumulation. Here we report changes in the levels of oxidative phosphorylation (OXPHOS) complexes in mitochondria isolated from *Polytomella* sp. cells grown on ethanol at pH 6.0 and pH 3.7 and in the levels of an 85 kDa soluble protein that we identified, on the basis of its amino acid sequence, as a bifunctional aldehyde/alcohol dehydrogenase (ADHE). An ADHE homologue is also present in *C. reinhardtii*, a photosynthetic relative of *Polytomella*.

Materials and methods

Isolation and subfractionation of Polytomella sp. *mitochondria*

Polytomella sp. (198.80, E.G. Pringsheim) was grown in Erlenmeyer flasks with cotton stoppers allowing for ample gas exchange at room temperature on ethanol at pH 3.7 and at pH 6.0, or on acetate at pH 6.0 (Atteia *et al*., 2000). Mitochondria were isolated as described (Atteia *et al*., 2000). Mitochondria, resuspended in 0.2 M mannitol, 5 mM potassium phosphate pH 7.2 at a concentration of 10–12 mg/ml protein in the presence of 0.5 mM PMSF and 2 mM amino caproic acid, were sonicated three times for 10 s, and centrifuged for 1 h at 100 000 × g.

Protein analysis

Mitochondria and mitochondrial subfractions were freshly prepared for Blue Native (BN)-PAGE analysis. The soluble mitochondrial fraction was supplemented with 1% dodecyl maltoside (*n*-dodecyl β-D-maltoside) and 0.25% Coomassie Serva Blue G. Mitochondria and mitochondrial membranes were washed twice in 250 mM sorbitol, 15 mM Bis-Tris pH 7.0; for solubilization, the proteins were resuspended at 15 mM Bis-Tris, 750 mM amino caproic acid pH 7.0 containing 2% dodecyl maltoside at a final protein concentration of 5 mg/ml. The sample was centrifuged for 20 min at 40 000 × g; the solubilized material was then supplemented with Coomassie Serva Blue G (one half of the volume of added dodecyl maltoside). All the samples were loaded on BN-PAGE with acrylamide gradients of 5–12% or 5–15% (Schägger and von Jagow, 1991). Staining for NADH dehydrogenase activities on BN-PAGE was performed as described (Kuonen *et al*., 1986). Electroblotting of BN-PAGE lanes was done as described (Jänsch *et al*., 1996). Immunodetection was carried out by the enhanced chemiluminescence peroxidase method (ECL^{TM}, Amersham-Pharmacia Biotech) with antisera raised against the β subunit of the bovine mitochondrial ATP synthase, the COXIIA subunit of *Polytomella* sp. cytochrome *c* oxidase, and the core I subunit of *Neurospora crassa* bc_1 complex. Entire lanes of BN-PAGE were used to resolve the proteins in a 2D-Tricine-SDS-PAGE (15% acrylamide) (Jänsch *et al*., 1996). For sequence determination, a lane of BN-PAGE with soluble mitochondrial proteins (1 mg of protein) was resolved on 2D-SDS-PAGE; after electrophoresis, the proteins were electrotransferred onto ProBlot membrane and stained with Coomassie blue R-250 (Atteia *et al*., 1997). The proteins of interest were excised and subjected to N-terminal or internal sequencing as described (van Lis *et al*., 2003). Protein concentrations were determined according to Markwell *et al.* (1978). Pre-stained molecular mass markers (Invitrogen) were used.

Isolation of Polytomella sp. *ADHE cDNA*

Two oligodeoxynucleotides, 5′-GAGCAGAAGTCC-AAGTCYGAYGAGG-3′ and 5′-CTTCTCRGCRTC-RGCGGARGG-3′, were designed from the N-terminal sequence (residues Glu-6 to Glu-13 of the mature protein) and the internal sequence *IS2* (Pro-688 to Lys-694) of *Polytomella* sp. mature ADHE pro-

tein. PCR amplification was carried out with *Taq* DNA polymerase (Qiagen). Total *Polytomella* sp. DNA was denatured for 5 min at 94 °C, then subjected to three cycles of 1 min denaturation at 94 °C, 45 s annealing at 60 °C, and 3 min extension at 72 °C; and subjected to 27 cycles of 1 min denaturation at 94 °C, 45 s annealing at 62 °C, and 3 min extension at 72 °C. The obtained 2 kb PCR product (p*AdhE*) was cloned into pGEM-T Easy Vector (Promega) and sequenced. p*AdhE* was further used to screen a λZAPII *Polytomella* cDNA library. The sequence of the longest cDNA isolated (1.6 kb) from 5000 p.f.u. screened overlapped the 3′ end of p*AdhE* PCR product by 300 bp. The 5′-end sequence of *Polytomella* sp. ADHE cDNA was determined by the RNA ligase-mediated rapid amplification of cDNAs ends method (RLM-RACE, Ambion), as indicated by the provider, and with total RNA from cells grown on acetate at pH 6.0. The gene-specific primers used were 5′-GGCTGTAAACGAACTCGGAGGCGAAG-3′ (corresponding to residues Phe-81 to Ser-88 of the mature protein) and 5′-GCGGCGCGGAAGATCTTGTCG-3′ (residues Asp-42 to Ala-47). The PCR steps were carried out at 60 °C. Sequencing was done at the Unidad de Biología Molecular (IFC-UNAM) and at MWG Biotech. Inc. (USA). The *Polytomella* sp. ADHE cDNA sequence can be found in the DDBJ/ EMBL/GenBank databases under the accession number AJ495765.

DNA and RNA analysis

Total *Polytomella* sp. DNA isolated according to Newman *et al*. (1990) was digested with restriction enzymes, separated on a 1% agarose gel, and transferred onto Hybond-N^+ membranes (Amersham Pharmacia Biotech.) according to standard protocols (Sambrook *et al*., 1989). Membranes were hybridized overnight at 65 °C with the p*AdhE* PCR product and washed for 2×20 min at 65 °C in 0.2× SSC and 0.5% SDS. Total RNA from *Polytomella* sp. cells was isolated with Trizol Reagent (Gibco-BRL), separated on a 1% agarose gel, and transferred onto Hybond-N^+ membranes. Hybridization was carried out overnight as previously described (Atteia *et al*., 2000). The membranes were washed for 2×20 min, at 42 °C in 1× SSC and 0.5% SDS. DNA probes p*AdhE* (see above) and *TubB1* from *Polytomella agilis* (Conner *et al.*, 1989; Atteia *et al.*, 2000) were labeled with [α-^{32}P]dCTP with the Random Primer labeling kit (Gibco-BRL).

Sequence analysis

EST clones of *C. reinhardtii* were obtained from the ChlamyEST database at http://www.biology.duke.edu/chlamy_genome/cgp.html with the WU-TBLASTN program. Searching for an ADHE gene in the *C. reinhardtii* genome was done with the site http://genome.jgi-psf.org/cgi-bin/browserLoad/3e7f2c99428ddae9031d6856. Protein sequence data were retrieved from Swiss-Prot + TrEMBL (Bairoch and Apweiler, 2000) and GenBank, non-redundant protein sequence databases (Benson *et al*., 2000), with the gapped BLASTP program with default gap penalties and the BLOSUM 62 substitution matrix (Altschul *et al.*, 1997). Molecular mass and pI were calculated with the Compute pI/MW tool (Bjellqvist *et al*., 1994). Motif searching was done with the Integrated Protein Classification Database (iProClass) (pir.georgetown.edu) software. Sequences were aligned with ClustalW (Thompson *et al*., 1997). Protein logdet distances (Lockhart *et al*., 1994) were calculated with the LDDist program available at the website http://artedi.ebc.uu.se/molev/software/LDDist.html and used for constructing neighbor-joining trees (Saitou and Nei, 1987) and planar networks. Planar networks were constructed with NeighborNet (Bryant and Moulton, 2002) and SplitsTree (Huson, 1998).

Results

Identification of the major OXPHOS complexes from Polytomella *sp.*

Mitochondria from *Polytomella* sp. cells grown on ethanol at pH 6.0 were solubilized with dodecyl maltoside and analyzed on BN-PAGE. As shown in Figure 1A, the pattern of *Polytomella* sp. mitochondrial protein complexes contrasts with the well-characterized pattern of beef heart mitochondrial complexes (Schägger and von Jagow, 1991). The major *Polytomella* OXPHOS complexes were identified by immunoblot analysis and specific activity staining. An antiserum against subunit β of bovine complex V (F_0F_1-ATP synthase) detected a single band of at least 1600 kDa on BN-PAGE (Figure 1B). Thus, like in *C. reinhardtii* (van Lis *et al*., 2003), *Polytomella* sp. complex V runs as a dimer. The incubation of a BN-PAGE lane with nitroblue tetrazolium and NADH (Kuonen *et al*., 1986) led to the detection of two bands of ca. 980 and 250 kDa exhibiting NADH dehydrogenase activity (Figure 1B). Based on its mobility on BN-PAGE and on its polypeptide composition (see Figure 2B), the 980 kDa band was identified as complex I (NADH:Q oxidoreductase); the band of 250 kDa was not identified. The 500 kDa protein complex was assigned

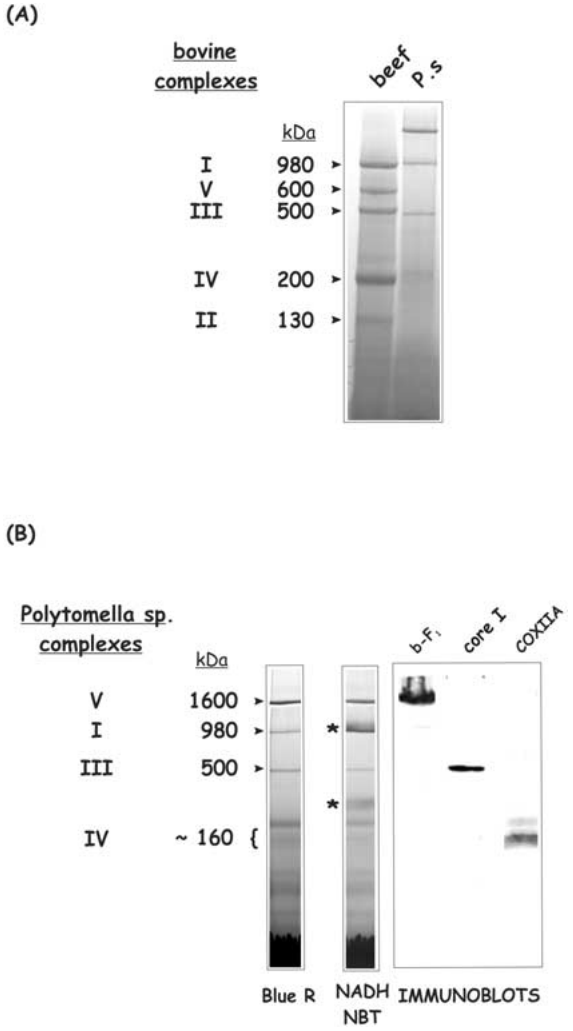

Figure 1. Identification of mitochondrial protein complexes from *Polytomella* sp. by BN-PAGE. A. 5–15% BN-PAGE of mitochondrial proteins from bovine heart (beef) (400 μg) and from *Polytomella* sp. cells grown on ethanol at pH 6.0 (P.s.) (600 μg). The most prominent bands of bovine mitochondria correspond to the OXPHOS components: complex I (NADH:Q oxidoreductase), complex II (succinate:Q oxidoreductase), complex III (QH_2:cyt *c* reductase), complex IV (cytochrome *c* oxidase) and complex V (FOF1-ATP synthase). The apparent molecular masses of the bovine protein complexes are from Schägger and von Jagow (1991) and Carroll *et al.* (2002). B. Identification of *Polytomella* sp. major OXPHOS complexes. NADH-NBT: a BN-PAGE lane was incubated in the presence of NADH and nitroblue tetrazolium; * indicates the protein complexes that exhibit NADH dehydrogenase activity. Immunoblots: BN-PAGE lanes were transferred to nitrocellulose and probed with the following antisera: b-F_1, against subunit β of bovine F_0F_1-ATPase; core I, against *N. crassa* core I; COXIIA, against *Polytomella* sp. COXIIA subunit. The apparent molecular masses of *Polytomella* sp. respiratory complexes were estimated using the bovine OXPHOS complexes as markers.

to *Polytomella* sp. complex III (QH_2:cyt *c* oxidoreductase) on the basis of its detection with an antiserum against *N. crassa* core I subunit. The position of complex IV (cytochrome *c* oxidase) was determined with an antiserum against the COXIIA subunit (Pérez-Martínez *et al.*, 2001). As shown in Figure 1B, this antibody recognized multiple bands on BN-PAGE in the range of 150 to 180 kDa, none of which coincided with the strong band at 200 kDa (Figure 1B; see below). Therefore, in contrast to complex IV from various sources, including mammals (Schägger and von Jagow, 1991), plants (Jänsch *et al.*, 1996) and *C. reinhardtii* (van Lis *et al.*, 2003), *Polytomella* complex IV does not appear as a major band on BN-PAGE (Figure 1A).

External pH affects accumulation of mitochondrial protein complexes

BN-PAGE patterns of mitochondria isolated from *Polytomella* sp. cells grown on ethanol at pH 6.0 and pH 3.7 were qualitatively similar but the relative abundance of protein complexes differed (Figure 2A). BN-PAGE and 2D-SDS-PAGE (Figure 2B) showed that the levels of complex V were significantly higher in mitochondria from cells grown at pH 6.0 than in mitochondria from cells grown at pH 3.7. In contrast, the levels of respiratory complexes I and III were clearly lower in mitochondria from cells grown at pH 6.0 than in mitochondria from cells grown at pH 3.7.

Besides the proteins of the OXPHOS system, several other proteins showed pH-dependent accumulation. One of them was a protein of 85 kDa which belongs to the aforementioned 200 kDa complex visible on BN-PAGE above complex IV (Figures 1 and 2). Higher levels of the 85 kDa protein were found in mitochondria from cells grown at pH 6.0 (Figure 2, arrow). The N-terminal sequence of the 85 kDa protein is reported in Table 1.

2D-SDS-PAGE analysis of soluble protein complexes in Polytomella sp.

Mitochondria from cells grown on ethanol at pH 6.0 were fractionated into their soluble and membrane-bound components and the protein complexes in the subfractions were further separated on BN-PAGE (Figure 3, left panel). As expected, the OXPHOS complexes were found in the membrane fraction. In the soluble fraction, two major protein complexes of ca. 200 and 100 kDa were detected (Figure 3, left panel). The soluble protein complexes, separated by BN-PAGE, were resolved into their constitutive subunits on a 2D-SDS-PAGE (Figure 3, right panel).

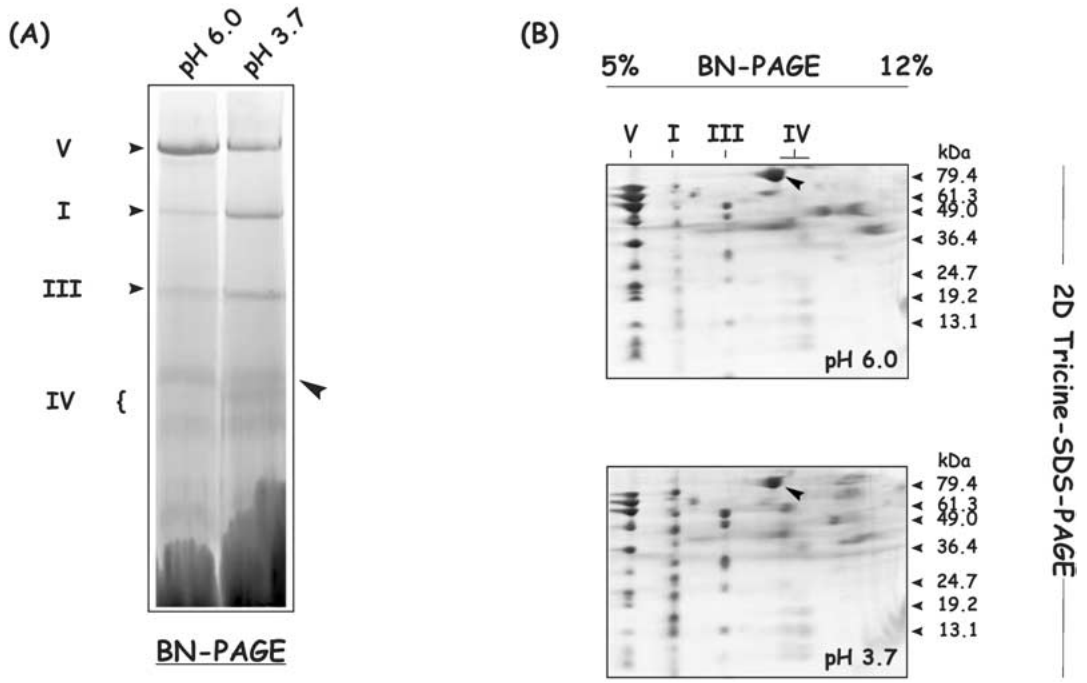

Figure 2. A. BN-PAGE analysis of mitochondria isolated from *Polytomella* sp. cells grown on ethanol at different pH. Protein complexes from mitochondria (800 μg) isolated from cells grown on ethanol at pH 6.0 and pH 3.7 were separated on a 5–12% BN-PAGE and stained with Coomassie blue R. B. Two-dimensional resolution of *Polytomella* sp. mitochondrial protein complexes. BN-PAGE lanes (A) were cut and placed horizontally for subsequent resolution of the protein complexes into their respective subunits on Tricine-SDS-gel (15% acrylamide). 2D-SDS-PAGE were stained with Coomassie Brilliant blue R250. I, III, IV, V refer to the OXPHOS complexes. Oblique arrows point to the 85 kDa protein (ADHE) whose accumulation is pH-dependent.

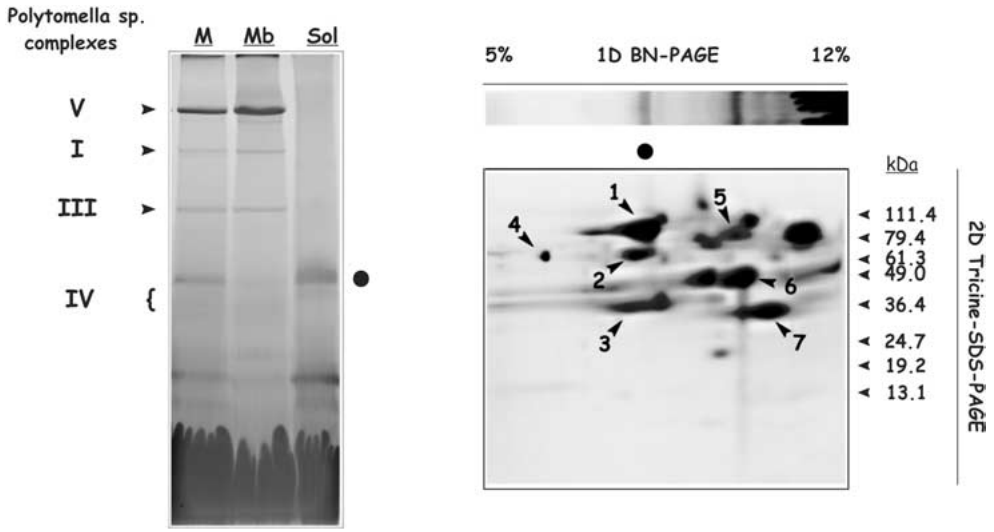

Figure 3. 2D-SDS-PAGE analysis of the soluble mitochondrial protein complexes of *Polytomella* sp. Left panel, mitochondria from cells grown on ethanol at pH 6.0 were fractionated into their soluble and membrane-bound components and all the fractions were analyzed on BN-PAGE. M, mitochondria (800 μg); Mb, membrane-bound proteins (800 μg); Sol, soluble proteins (700 μg). The position of the protein complexes as identified in Figure 1 is indicated. ● indicates the position of the 200 kDa complex. Right panel, BN-PAGE lane of the soluble fraction was transferred horizontally on a SDS-gel (12% acrylamide). The protein spots subjected to Edman degradation are pointed with an arrow. The determined N-terminal sequences are reported in Table 1.

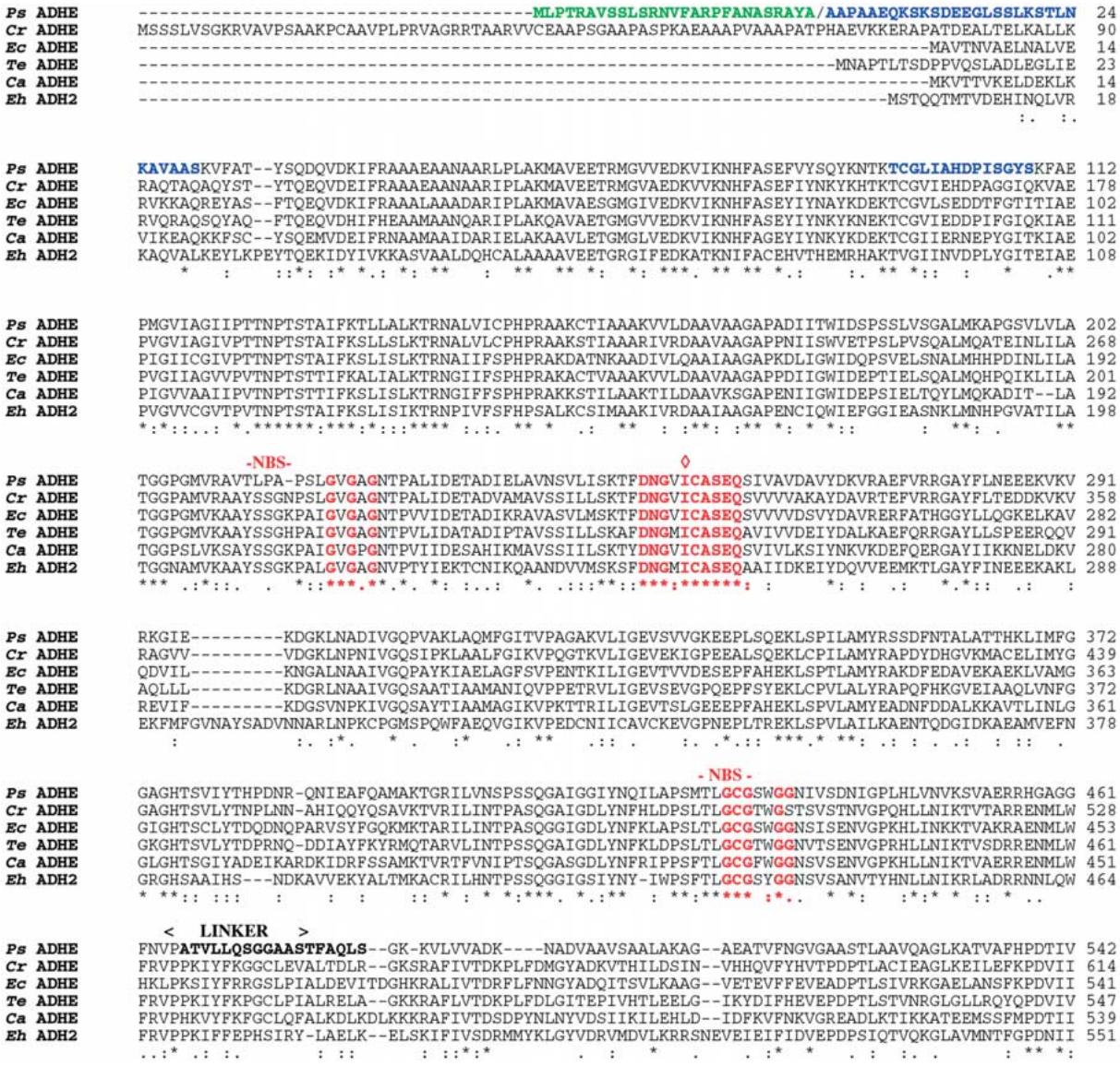

Figure 4. Multiple sequence alignment of P*olytomella* sp. ADHE with homologues from various sources. Sequences are from *Polytomella* sp. (*Ps* ADHE) (this work), *Chlamydomonas reinhardtii* (*Cr* ADHE) (this work), *Escherichia coli* (*Ec* ADHE) (P17547); *Entamoeba histolytica* (*Eh* ADH2) (Q24803); *Clostridium acetobutylicum* (*Ca* ADHE) (P33744) and *Thermosynechococcus elongatus* strain BP-1 (*Te* ADHE) (BAC07780). The cleavable mitochondrial targeting sequence in *Polytomella* ADHE is indicated in green. Amino acid sequences of *Polytomella* sp. ADHE determined by Edman degradation are indicated in blue. Conserved patterns in the CoA-dependent ALDH domain and in the Fe-ADH domain are indicated in red. ◊, Cys nucleophile in the catalytic center that is invariant in all CoA-dependent and CoA-independent ALDH. The position of iron-containing ADH signature 1 (PS00913; ADH IRON 1) and signature 2 (PS00060; ADH IRON 2) are indicated. Note the absence in *Polytomella* sp. ADHE sequence of the His residues in the sequence corresponding to Fe-ADH signature 2 (ADH IRON 2). NBS, potential nucleotide binding site.

Corresponding to the 200 kDa range several proteins were resolved, with a major protein of 85 kDa (spot 1) and two additional proteins of 60 kDa (spot 2) and 35 kDa (spot 3). The N-terminal sequence of the protein in spot 1 was identical to the N-terminal sequence of the 85 kDa protein (Table 1) indicating that this protein is soluble. The N-terminal sequence of two tryptic fragments obtained from the 85 kDa protein (IS1, IS2; see Table 1) did not produce significant hits in database searches. Protein spots 2 and 3 were also subjected to Edman degradation. No N-terminal sequence could be obtained for spot 2, likely because of a blocked N-terminus. Edman degradation of spot 3 gave two amino acids for several cycles (Table 1).

Database searching identified several protein spots on 2D gels as typical mitochondrial proteins (Table 1).

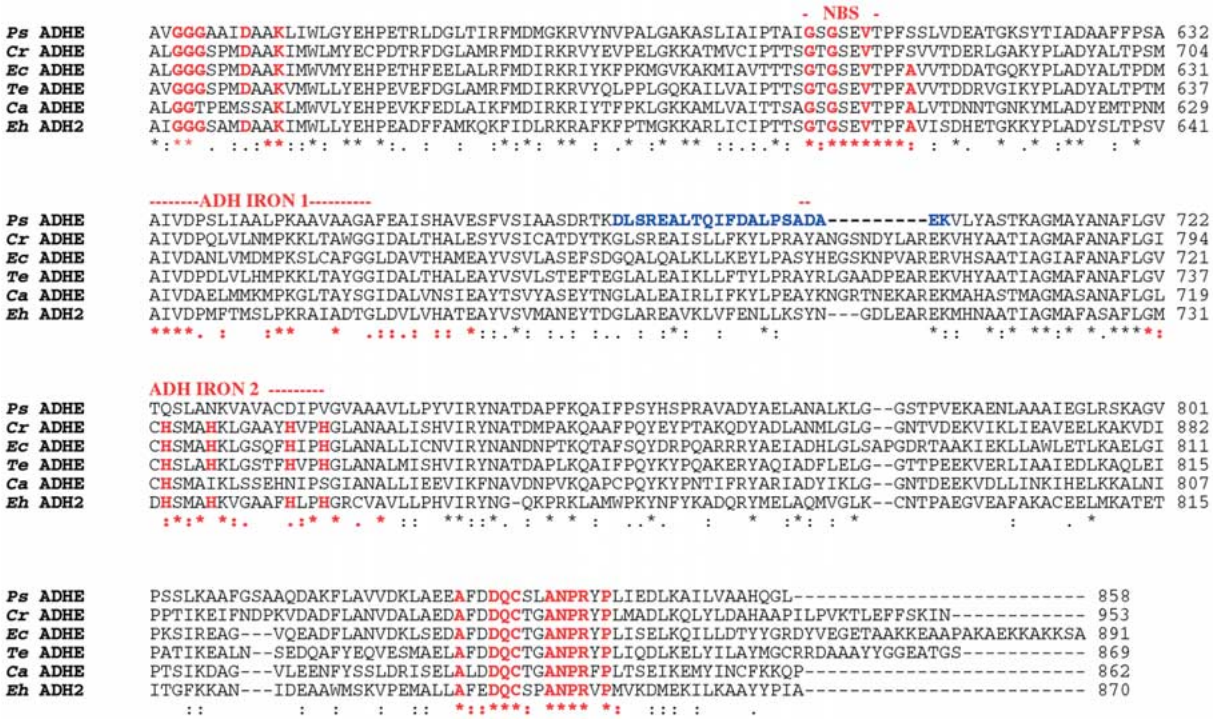

Figure 4. (Continued.)

Spot 4 (60 kDa) and spot 5 (70 kDa) were identified as the mitochondrial heat-shock proteins HSP 60 and HSP 70. Spot 6 (45 kDa) and spot 7 (35 kDa) were identified as malate dehydrogenase and citrate synthase (Table 1) of the tricarboxylic acid cycle.

Identification of a Polytomella sp. *cDNA encoding mitochondrial ADHE*

By means of primers designed from the peptides obtained from spot 1, its corresponding cDNA was isolated through PCR amplification, cDNA library screening and RLM-RACE. Database searching with the cDNA sequence revealed that spot 1 corresponds to a bifunctional aldehyde/alcohol dehydrogenase, ADHE. *Polytomella* ADHE cDNA encodes a 885 amino acid protein encompassing the N-terminal sequence determined from the mature mitochondrial protein, thus revealing the cleavage site of the 27 amino acid N-terminal mitochondrial targeting sequence (MTS) (Figure 4). The MTS lacks acidic residues, has a high content of basic and hydroxylated residues and, conforms well to MTS prediction programs, including MITOPROT II (Claros and Vincens, 1996), PREDOTAR (version 0.5, www.inra.fr/predotar/) and TargetP V1 (Emanuelsson *et al*., 2000). The molecular mass of the mature ADHE was calculated to be 88 547 Da and its pI 6.98. Southern hybridization against total *Polytomella* DNA (data not shown) indicates that ADHE is encoded by a single-copy gene.

Database searching identified the two distinct enzymatic domains typical of ADHE in the *Polytomella* protein: the N-terminal region (residues Lys-20 to Pro-465) is homologous to the acetylating aldehyde dehydrogenase (ALDH) family, a member of the ALDH superfamily (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.10), whereas the C-terminal region (Lys-485 to Ala-854) is homologous to the iron-containing alcohol dehydrogenase family (Fe-ADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1). *Polytomella* ADHE exhibits high similarity (52–69%) to ADHE from cyanobacteria, clostridia and enterobacteria; the algal protein also shows similarity (47–64%) to ADHE from lactobacilli, bacilli and from the amitochondriate eukaryotes *Giardia intestinalis*, *Spironucleus barkhanus*, *Mastigamoeba balmuthii*, and *Entamoeba histolytica* (Yang *et al*., 1994; Rosenthal *et al*., 1997). *Polytomella* ADHE exhibits several features characteristic of ADHE: the conserved sequences found in CoA-acetylating ALDH, $PxG(x_6)P(x_3)P$ (residues 113–126 of the mature protein) (Goodlove *et al*., 1989; Fontaine *et al*., 2002) and $G(x_6)D(x_7)A(x_7)K(x_4)G(x_2)C$ (residues 224–255) (Fontaine *et al*., 2002); the ALDH catalytic center DNGxICASEQ (residues 250–259) (Rosenthal *et al*., 1997); two nucleotide-binding sites GxGxG (residues 220–224) and $GCG(x_2)GG$

Table 1. N-terminal sequences of Polytomella sp. soluble mitochondrial proteins.

Spot number[a]	Mass[b] (kDa)	N-terminal sequence	Assignment[c]
1	65	AAPAAEQKSKSDEEGLSSLKSTLNKAVAAS *IS1*: TCGLIAHDPISGYSK *IS2*: DLSREALTQIFDALPSADAEK	ADHE
2	60	blocked	–
3	37	Agx(N,T)(Q,V)AI(G,L)I(N,T)RF(A,G)RIS	–
4	60	ATKEMRFGQD(A,V)RE(R,E)VLQ	HSP60
5	70	ADEVIGIDLVTTNS	HSP70
6	45	SSxTDLKKTVAELIPAEQDR	citrate synthase
7	31	GSSSGEVGRKVTVLGAAGGIxQPL	malate dehydrogenase

[a]Protein numbers as indicated in Figure 3.
[b]Molecular masses, estimated from SDS-PAGE in Figure 3.
[c]Assignment made on the basis of sequence similarity with known proteins, except for ADHE which was identified from its corresponding cDNA sequence (see text). x indicates amino acids that were not identified. Residues in parenthesis indicate simultaneous detection. *IS1* and *IS2*, two internal tryptic fragments of ADHE.

(residues 428–434) that may be involved in NADH binding (Fontaine *et al*., 2002), and a third nucleotide binding site GxG(x_2)V(x_3)S in the ADH domain (residues 601–610) implicated in NAD(P)H binding (Nair *et al*., 1994; Fontaine *et al*., 2002) (Figure 4). The C-terminal ADH domain of ADHE shows high similarity with Fe-ADH homologues that use iron to polarize the carbonyl group of acetaldehyde during catalysis. *Polytomella* sp. ADHE also exhibits the two iron-binding motifs conserved in Fe-ADH: signature 1 (ADH IRON1; AIVDPSLIAAL-PKAAVAAGAFEAISHAVE; residues 633–661) is highly conserved, while signature 2 (ADH IRON2; GVTQSLANKVAVACDIPVGVAAA; residues 711–732) lacks three histidine residues conserved in most ADHE homologues (Figure 4). The sequence between the two ADH-IRON signatures is shorter in *Polytomella* sp. than in other ADHE sequences as confirmed by the amino acid sequencing of the tryptic fragment IS2 (Asp-674 to Lys-694). *Polytomella* ADHE also contains the conserved patterns for ADH type III enzymes GGG(x_3)D(x_2)K (residues 545–554) and A(x_2)DQC(x_2)ANPRxP (residues 829–842) (Fontaine *et al*., 2002). Finally, unlike bacteria and amitochondriate protists, the linker sequence (Rosenthal *et al*., 1997) that connects the ALDH domain with the ADH domain in *Polytomella* ADHE (residues Ala-466 to Gly-484) is unique in that it lacks charged residues and contains several hydroxylated residues.

Searching of *C. reinhardtii* expressed sequence tag (EST) data revealed the presence of an ADHE homologue in the photosynthetic alga. Overlapping EST clones (AV397610, AV639995, AV644998; BQ808648, BI873972, AV624287, BG855351, BQ810550, BI873402 and BG855598), contigs (20021010.5320.2, 20021010.2041.2) and genomic sequence (scaffold 592) allowed assembly of the *C. reinhardtii* ADHE cDNA sequence. *C. reinhardtii* ADHE is encoded as a 951 amino acids that exhibits an N-terminal extension of ca. 60 residues compared to ADHE in amitochondriate eukaryotes (Figure 4). A mitochondrial localization for *C. reinhardtii* ADHE is predicted with the programs PREDOTAR (version 0.5, www.inra.fr/predotar/) and MITOPROT II (Claros and Vincens, 1996). *C. reinhardtii* and *Polytomella* sp. ADHE sequences are highly similar (56% identity); nevertheless, differences between the sequences of the putative ADH-IRON 2 signature and between the linker sequences were observed (Figure 4). The complete genomic sequence encoding *C. reinhardtii* ADHE spans 6358 bp and contains 15 introns.

Expression of Polytomella *sp. ADHE*

Northern hybridization was performed with RNA isolated from cells grown on ethanol at pH 6.0 or 3.7, and on acetate at pH 6.0. RNA blots were probed with the p*AdhE* PCR product and the *TubB1* probe as an internal control for loading equivalent amounts of RNA. A single *AdhE* transcript of 3.5 kb was detected in all three conditions (Figure 5). While strong hybridization signals were observed with RNA from cells grown at pH 6.0 on acetate or on ethanol, the signal obtained with RNA from cells grown on ethanol at pH 3.7 was significantly weaker, indicating that *Polytomella*

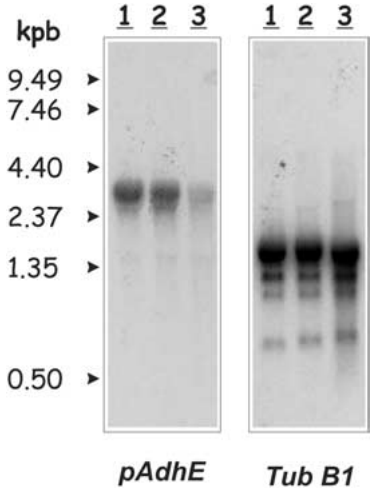

Figure 5. RNA analysis of ADHE levels in *Polytomella* sp. Total RNA was isolated from *Polytomella* sp. cells grown on acetate, pH 6.0 (1), ethanol, pH 6.0 (2) and ethanol, pH 3.7 (3). Equivalent amounts of RNA in each lane (15 μg) were hybridized with PCR amplification product (p*AdhE*) and *Polytomella agilis* β-tubulin 1 gene (*Tub B1*).

ADHE expression is strongly influenced by the pH of the culture medium.

Phylogenetic analyses of ADHE

Phylogenetic analysis was carried out with ADHE sequences available in databases: four from amitochondriate protists and the remainder from eubacteria. No archaebacterial homologues were detected in database searches. As shown in Figure 6a, *Polytomella* sp. and *C. reinhardtii* ADHE cluster closely but in a position distinct from *Entamoeba histolytica* ADHE in the bifurcating NJ tree. The ADHE from *E. histolytica* shares higher amino acid sequence identity with homologues from *Pasteurella multocida* (63%) or *Streptococcus pneumoniae* (62%) whereas ADHE from *Polytomella* sp. and *C. reinhardtii* are more similar to the homologue from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (52% and 66% identity, respectively). Notably, the predicted *C. reinhardtii* ADHE shows fewer positional identities to *Polytomella* sp. ADHE (57%) than to ADHE in the cyanobacterium *T. elongatus* (66%), an affinity that is represented as a shared component of similarity by the NeighborNet planar network in Figure 6b.

Discussion

ADHE in Polytomella *sp. mitochondria*

ADHE catalyzes the fermentative production of ethanol by two sequential NADH-dependent reductions of acetyl-CoA, releasing ethanol and CoA-SH (Goodlove *et al.*, 1989; Bruchhaus and Tannich, 1994; Sánchez, 1998). It is believed that ADHE arose through an ancient gene fusion of an acetyl-CoA-dependent aldehyde dehydrogenase and an iron-dependent alcohol dehydrogenase, probably within the same eubacterial operon (Rosenthal *et al.*, 1997; Sánchez, 1998). In the *Salmonella typhimurium eut* operon, the *EutE* gene encoding an acetyl-CoA ALDH is proximal to the *EutG* gene that encodes a Fe-ADH; the EutE and EutG protein sequences in tandem alignment with ADHE (Stojiljkovic *et al.*, 1995). The ADHE gene occurs in a skewed distribution among phylogenetically disparate lineages. The majority of the prokaryotes do not have the gene and among eukaryotes it has only been found in a few amitochondriate protists.

The presence of ADHE in *Polytomella* sp. was shown by amino acid sequencing of the protein identified in isolated mitochondria. The protein is soluble and appears to be mainly present as a homodimer, in contrast to *Escherichia coli* and *E. histolytica*, where ADHE exists as multimers of 20 to 60 protomers (Bruchhaus and Tannich, 1994; Kessler *et al.*, 1991). Comparison of the N-terminus of mitochondrial ADHE from *Polytomella* to the cDNA sequence reveals that ADHE is encoded as a precursor protein with a typical MTS that is cleaved upon import into the organelle. Based upon sequence similarity and the presence of conserved cofactor-binding signatures, *Polytomella* sp. ADHE is likely to perform the same enzymatic reactions catalyzed by eubacterial ADHE.

Expression of *E. coli* ADHE is anaerobically regulated at both the transcriptional and translation levels (Clark and Cronan, 1980; Leonardo *et al.*, 1996). During aerobic metabolism, ADHE is highly susceptible to metal-catalyzed oxidation. The amino acid chains in ADHE and, in particular the histidine residues in the ADH IRON 2 signature, are thought to be attacked by highly reactive hydroxyl radicals locally generated by the active site Fe^{2+} of the ADH domain (Cabiscol *et al.*, 1994; Tamarit *et al.*, 1998). In contrast to *Chlamydomonas*, *Polytomella* ADHE lacks the conserved histidine residues in the ADH-IRON 2 signature, suggesting that the protein has lost its iron-binding capacity or that iron chelation involves more distant residues, and also suggesting a lower sensitivity to oxygen.

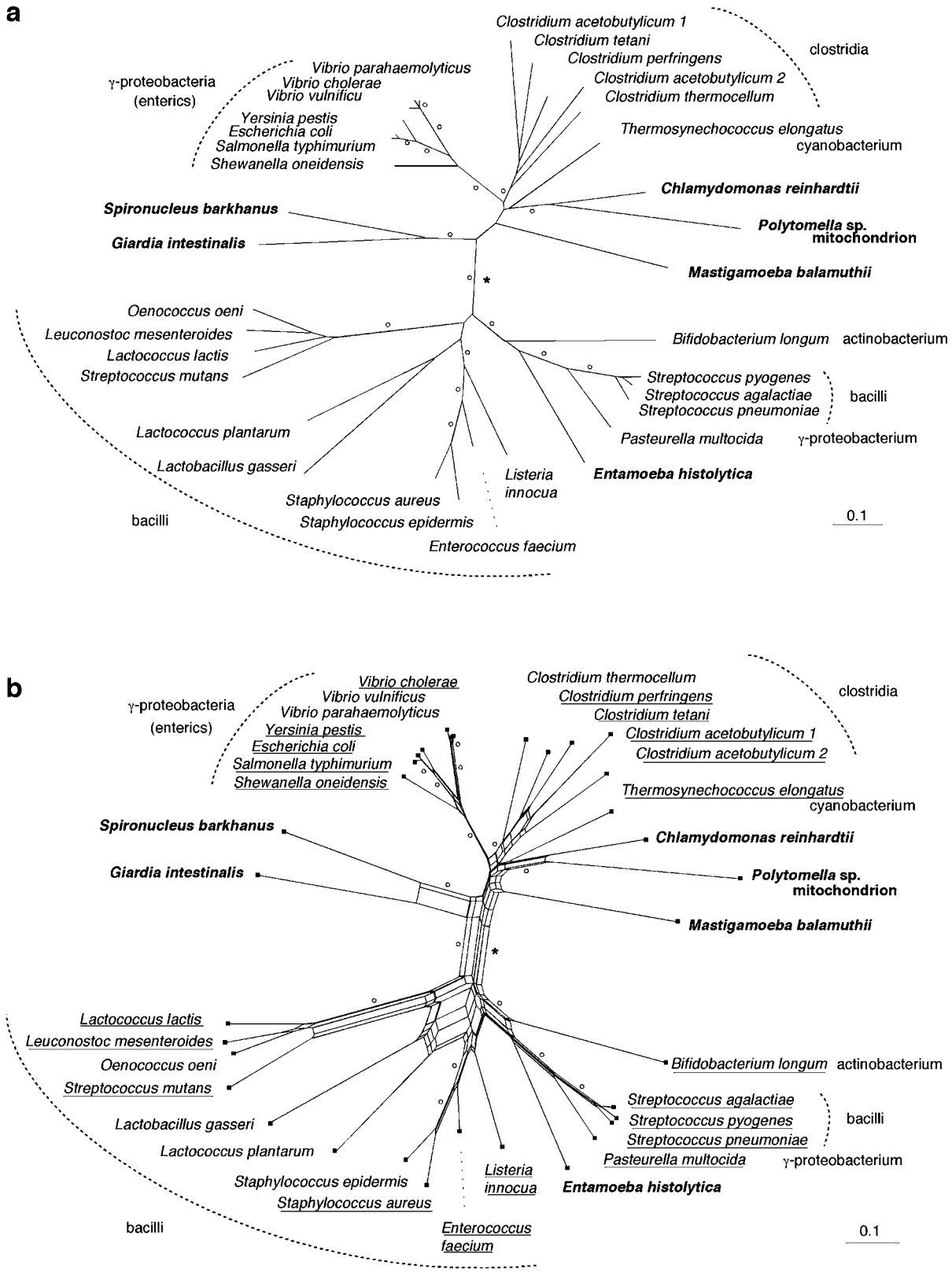

Figure 6. Sequence similarity among ADHE proteins. Open circles indicate branches (or splits) found in >95/100 bootstrap replications. Eubacterial classifications recognized at http://www.ncbi.nlm.nih.gov/Taxonomy/ are indicated. Eukaryotic sequences are indicated in boldface type. Underlined species names indicate that the genome sequence is available at http://www.tigr.org/. Scalebars indicate 0.1 substitution per site. a. Neighbor-joining tree of protein logdet distances. b. NeighborNet graph of protein logdet distances showing multiple conflicting signals. Splits are represented as parallel lines.

Site-directed mutagenesis of *E. coli* ADHE showed that the conversion of Glu-568 in the ADH domain into virtually any non-acidic residue resulted in an enzyme active under both aerobic and anaerobic conditions; the mutated ADHE allowed *E. coli* to grow aerobically on ethanol (Holland-Staley *et al*., 2000). The presence of a glycine residue in the algal sequences (Gly-569 in *Polytomella* sp. and Gly-641 in *C. reinhardtii*) at the equivalent position of the Glu-568 in the *E. coli* sequence or of the Ala-138 in *S. typhimurium* EutG protein (Kofoid *et al*., 1999) suggests that the algal protein may be able to function as an ethanol dehydrogenase. However, Glu-568 in the ADH domain is not an invariable amino acid, for example it is replaced by Ala-578 in the anaerobic protist *E. histolytica*.

External pH and the role of ADHE in Polytomella *sp. mitochondrial metabolism*

External pH influences the levels of OXPHOS complexes in *Polytomella* cells grown on ethanol at acidic pH. The levels of respiratory complexes I, III and IV were shown to be noticeably up-regulated at pH 3.7. At pH 3.7, the respiratory rates are higher than at pH 6.0, which is likely the consequence of a higher content in respiratory complexes and also in cytochrome *c* (Atteia *et al*., 2000). The ratio between the respiratory complexes and the F_0F_1-ATPase is clearly affected by external pH, indicating an influence of pH on core energy metabolism in the colorless alga *Polytomella*.

In amitochondriate protists, ADHE is a cytosolic enzyme that is integral to maintaining redox balance via the NAD(P)H-dependent reduction of acetyl-CoA to ethanol, which is excreted as an end-product (Reeves, 1984; Nair *et al*., 1994; Bruchhaus and Tannich, 1994; Sánchez, 1998). In *E. coli*, the *adhE* promoter is regulated in response to the cytosolic NADH/NAD^+ ratio (Leonardo *et al*., 1996), also suggesting a role in redox balance in that organism. Both OXPHOS complexes and ADHE in *Polytomella* sp. cells grown on ethanol show a pH-dependent expression and accumulation. The expression of ADHE is higher at moderately acidic pH, with either acetate or ethanol as a carbon source (Figure 6). The current data are compatible with the view that *Polytomella* mitochondrial ADHE could be involved either in the maintenance of redox balance (ethanol production) or in ethanol assimilation (producing acetyl-CoA and NADH for respiration), or both, depending upon environmental conditions. Experiments are currently being done to determine the function and specific activity of *Polytomella* ADHE isolated from cells grown at different pH values.

Evolutionary considerations

ADHE genes were found in the genomes of several Gram-positive bacteria belonging to the categories bacilli and clostridia among the Firmicutes, in several γ-proteobacteria (particularly enterics), in one actinobacterium and one cyanobacterium, in addition to the previously characterized sequences from several amitochondriate protists (Yang *et al*., 1994; Rosenthal *et al*., 1997; Andersson *et al*., 2003) (Figure 6). Previous phylogenetic analyses have suggested that the anaerobic eukaryotes *E. histolytica* and *G. intestinalis* acquired the gene for their ADHE enzyme through independent lateral gene transfers, possibly from Gram-positive donors (Rosenthal *et al*., 1997; Field *et al*., 2000; Andersson *et al*., 2003). The similarity of ADHE sequences from the diplomonads *G. intestinalis* and *S. barkhanus* suggests the presence of the ADHE gene in their common ancestor (Andersson *et al*., 2003), but an independent lateral acquisition of *E. histolytica* ADHE seems to be the easiest explanation (Field *et al*., 2000; Fontaine *et al*., 2002; Andersson *et al*., 2003) (see also Figure 6a). The sequences from *Polytomella* sp. mitochondria and *C. reinhardtii* make the picture somewhat more complicated because they cluster close to, but not with the sequences from *Giardia*, *Spironucleus*, and *Mastigamoeba*.

Notably, the clostridial (low-GC Gram positives) sequences cluster much more closely to the homologues from enterics than they do to the homologues from bacilli (low-GC Gram-positives) (Figure 6). The central branch or split (marked by an asterisk in Figure 6) separates available ADHE sequences into two larger groups. The overall picture of ADHE sequence similarity is highly reminiscent of that found for pyruvate kinase (PK), where two clusters (I and II) and a skewed distribution paralleling that found for ADHE were also observed (Schramm *et al*.,. 2000). Schramm *et al.* (2000) noted that the PK dichotomy correlated with allosteric properties of the enzymes, not with phylogeny. Although we were unable to identify in the alignment specific motifs or in the literature regulatory properties of ADHE that might distinguish sequences above and below the asterisk in Figure 6, a pattern of sequence similarity that is largely driven by functional aspects rather than by neutral evolution cannot be excluded *a priori* for this sequence sample. Even if we accept lateral gene transfer from Gram positive donors for the origin of the *Bifidobacterium*, *Pasteurella*, and *Thermosynechococcus* genes, it is difficult to evoke

either lateral gene transfer (from what donor?) or ancient duplication and differential losses (too many) to account for the differentness of ADHE from clostridia and bacilli.

Perhaps more caution is warranted when it comes to evidence for horizontal gene transfer on the basis of an unusual phylogeny of an ancient enzyme, as is the case for ADHE. Methods of phylogenetic reconstruction used in this and prior studies to construct the phylogenies from which horizontal gene transfers for ADHE can be inferred are based upon the rates across sites (RAS) models of protein evolution. But we know of no evidence to indicate that proteins in general or ADHE in particular actually evolve according to a RAS model. From the standpoint of molecular evolutionary theory, RAS models have been argued to be less realistic than covarion models (Lockhart *et al*., 2000; Penny *et al*. 2001) and protein evolution simulations taking into account protein folding produced results highly compatible with a covarion model (Bastolla *et al*., 2002; Bastolla *et al*., 2003). If the model under which a phylogeny is reconstructed deviates strongly from the process by which the protein evolved, the phylogeny can be severely in error (Penny *et al*., 2001).

The neighbornet graph of the ADHE protein sequence similarity shown in Figure 6b indicates that the ADHE data are non-tree-like in many respects. This could be due to convergence, noise, or other conflicting signal (Bryant and Moulton, 2002). In a neighbornet graph such conflicting signals in the data become visible that are not represented in purely bifurcating trees. Notwithstanding very complicated patterns of sequence similarity, eukaryotic ADHE, like most enzymes of eukaryotic core energy metabolism studied to date lacks obvious archaebacterial homologues and thus appears to be of eubacterial origin.

Functional considerations

Earlier biochemical studies of *Chlamydomonas* and related green algae had provided evidence for ADHE activity in the mitochondria of these algae (Kreuzberg, 1984; 1985; Kreuzberg *et al*., 1987), but until now ADHE sequences were only available for the enzyme from the cytosol of anaerobic eukaryotes with an energy metabolic pattern designated as Type I (Müller, 1998). The expression of *Polytomella* ADHE under aerobic conditions (Figure 5) extends the occurrence and expression of this enzyme to aerobic eukaryotes growing under aerobic conditions. In *Polytomella* ADHE is clearly localized to mitochondria, extending the occurrence of the enzyme to oxygen-respiring mitochondria as well. The presence of a seemingly anaerobic-specific enzyme in an oxygen-respiring mitochondrion is not without precedent, because *Euglena* mitochondria contain pyruvate:ferredoxin oxidoreductase, an oxygen-sensitive enzyme otherwise typical of hydrogenosomes (Rotte *et al*., 2001). Furthermore, the oxygen-sensitive assembly of Fe-S clusters occurs in the mitochondrial matrix (Tachezy *et al*., 2001), perhaps because it is the most oxygen-poor compartment in aerotolerant eukaryotes.

In *E. coli*, ADHE harbors an additional enzymatic activity, that of a pyruvate formate-lyase (PFL) deactivase (Kessler *et al*., 1991). This is noteworthy because PFL activity has been measured both in mitochondria of *Chlamydomonas* (Kreuzberg *et al*., 1987) and in whole cells of the green alga *Chlorogonium* (Kreuzberg, 1985). PFL also occurs in chytridomycete fungi, where it is localized to hydrogenosomes (Akhmanova *et al*., 1999) and evidence for the presence of ADHE in PFL-possessing chytrids has been noted (Hackstein *et al*., 1999). PFL also requires an activating enzyme in *E. coli* (Wong *et al*., 1993), and all sequenced prokaryotic genomes surveyed here (underlined in Figure 6b) that possess ADHE also possess both PFL and PFL activase. Database searching indicated the presence of a gene encoding a PFL-activase in the green alga *C. reinhardtii* (W. Martin and A. Atteia, unpublished results).

The presence and expression of ADHE in eukaryotes that can live under fully aerobic conditions and that possess fully developed mitochondria is distinctly at odds with the view that eukaryotes acquired ADHE genes specifically as adaptations to an anaerobic lifestyle (Andersson *et al*., 2003), because *Polytomella* ADHE is expressed under ambient oxygen levels in an oxygen-respiring organelle. Chlorophycean algae are widely distributed in nature and undergo intimate interactions with other organisms. For example, *Chlamydomonas* sp. cells can be parasitized by chytrid fungi (Shin *et al*., 2001), and some species of *Chlamydomonas* are endosymbionts of large miliolid foraminifera (Pawlowski *et al*., 2001). Thus, there has been ample opportunity during evolution for these oxygen respiring algae to have acquired their genes for ADHE via horizontal transfer from yet unidentifiable donors, but the present data are incompatible with the view that if such acquisitions occurred, they did so as an adaptation to an anaerobic lifestyle.

Acknowledgements

EST sequences were obtained from the *Chlamydomonas* genome sequence project. These sequence data were produced by the US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/ and were provided for use in this publication only. We are indebted to Dr J. d'Alayer (Institut Pasteur, Paris, France) for the determination of internal protein sequences; to Dr H.P. Braun (Hannover University, Germany) for providing the anti-core I antibody of *N. crassa*; to M. Vázquez-Acevedo (IFC-UNAM) for the supply of the antibody against *Polytomella* sp. COXIIA subunit; to A. Julián-Sánchez (FM-UNAM) for help with phylogenetic analyses; to J. Wong (Brown University) and to Drs A. Peña, J. Ramírez and G. Dreyfus (IFC-UNAM) for the use of material and equipment. This work was supported by Grant 27754N from CONACyT (México), IN204595 from DGAPA (UNAM) and TW01176 from Fogarty International Center (NIH). R.v.L. received a Ph.D. student fellowship from DGEP-UNAM.

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