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Cloning, expression and biochemical characterization of mitochondrial and cytosolic malate dehydrogenase from *Phytophthora infestans*

Patricia E. LÓPEZ-CALCAGNO[a], *Johanna MORENO*[a], *Luis CEDEÑO*[b], *Luis LABRADOR*[a], *Juan L. CONCEPCIÓN*[c], *Luisana AVILÁN*[a,*]

[a]*Laboratorio de Fisiología, Facultad de Ciencias, Universidad de Los Andes, Mérida, Venezuela*
[b]*Laboratorio de Fitopatología, Instituto de Investigaciones Agropecuarias, Universidad de Los Andes, Mérida, Venezuela*
[c]*Laboratorio de Enzimología de Parásitos, Facultad de Ciencias, Universidad de Los Andes, Mérida, Venezuela*

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ABSTRACT

The genes of the mitochondrial and cytosolic malate dehydrogenase (mMDH and cMDH) of *Phytophthora infestans* were cloned and overexpressed in *Escherichia coli* as active enzymes. The catalytic properties of these proteins were determined: both enzymes have a similar specific activity. In addition, the natural mitochondrial isoenzyme was semi-purified from mycelia and its catalytic properties determined: the recombinant mitochondrial isoform behaved as the natural enzyme. A phylogenetic analysis indicated that mMDH, present in all stramenopiles studied, can be useful to study the relationships between these organisms. MDH with the conserved domain MDH_cytoplasmic_cytosolic is absent in some stramenopiles as well as in fungi. This enzyme seems to be less related within the stramenopile group. The *Phytophthora* cMDHs have an insertion of six amino acids that is also present in the stramenopile cMDHs studied, with the exception of *Thalassiosira pseudonana* cMDH, and is absent in other known eukaryotic cMDHs.

Introduction

Phytophthora infestans of the class oomycetes, is the causal agent of late blight, a disease affecting potato and other members of the Solanaceae family (Kamoun & Smart 2005). Oomycetes had been previously classified as lower fungi. However, it is now accepted that they belong to the Chromista or Stramenopile kingdom that includes diverse organisms such as brown algae and diatoms (Adl *et al.* 2005). Although this kingdom includes photosynthetic organisms, the members of the genus *Phytophthora* do not photosynthesize and their classification inside this group is based on morphological and molecular evidence. In addition, the *Phytophthora* species have genes with strong similarities to genes of photosynthetic organisms (Tyler *et al.* 2006). Despite their economic importance, *Phytophthora* species remain poorly characterized at the metabolic level. Recently, intense research efforts have been made in the comprehension of the mechanisms of invasion and pathogenesis of *P. infestans* (Kamoun 2006; Hardham 2007). Moreover, the recent genome project will make it possible to launch further analyses of different aspects of the biology of *P. infestans*. Among these aspects one that deserves

* Corresponding author. Tel.: +58 274 2401307; fax: +58 274 2401286.
E-mail address: avilan@ula.ve
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attention is energetic metabolism. A better knowledge of metabolism could in the future lead to the discovery of enzyme targets as a step toward the development of compounds for specific disease control. Previous studies on energetic metabolism have been performed in *Phytophthora cinnamoni* specifically on the enzyme pyruvate phosphate dikinase (Marshall *et al.* 2001). This study suggested that the enzyme participates in the glycolytic flux, an aspect that constitutes an important difference with the energetic metabolism in the plant host. Efforts to understand the metabolism of members of the genus *Phytophthora* have also been performed by studying gene expression in different forms of the life cycle of these organisms (Grenville-Briggs *et al.* 2005; Kim & Judelson 2003; Prakob & Judelson 2007; Skalamera *et al.* 2004; Tani *et al.* 2004). However, it is clear that further studies are required to understand the metabolism of *Phytophthora*.

An enzyme with important functions in the energetic metabolism of eukaryotic cells is malate dehydrogenase (MDH). This enzyme catalyzes the interconversion of malate and oxaloacetate with a concomitant reduction of NAD or oxidation of NADH. In most eukaryotic cells, MDH occurs in two isoforms: mitochondrial MDH (mMDH), an essential enzyme in the tricarboxylic acid cycle, and cytosolic MDH (cMDH), which is usually involved in gluconeogenesis or shuttle systems for the transfer of reducing equivalents across the mitochondrial membrane (Goward & Nicholls 1994). MDH belongs to the lactate dehydrogenase/malate dehydrogenase (LDH/MDH) super-family which is subdivided into three main groups: LDH, LDH-like MDH (tetrameric) and dimeric MDH (Madern 2002). In the third group, several subgroups (ie: cytoplasmic and mitochondrial) can be differentiated by specific conserved domains (Madern 2002).

In the present study, mitochondrial and cytosolic malate dehydrogenases were cloned from the *P. infestans* genomic DNA and total RNA, respectively, and overexpressed in *Escherichia coli* to determine their catalytic properties. The recombinant protein made it possible to raise specific antisera that recognized only one band in mycelium protein extracts. In addition the natural mitochondrial enzyme was semi-purified from mycelium and its kinetic properties compared to that of the recombinant enzyme.

Materials and methods

Culture and extracts

The *Phytophthora infestans* isolate SR 1.8-04 (homothallic, originally isolated from potato var. Granola, grown at Santa Rosa, Mérida, Venezuela) was maintained and propagated in petri dishes containing oat meal (4 % w/v) agar (OMA) at 16 °C. To obtain mycelia for experiments, five small plugs were cut from the edge of a colony grown on OMA and placed in culture dishes with 20 ml of 6 % (w/v) pea–2 % (w/v) sucrose medium. The culture was maintained at 25 °C for 10 d. Fresh mycelia were harvested by vacuum filtration onto filter paper, rinsed with distilled water and stored at −20 °C. Extracts of mycelia were obtained by grinding with silicon carbide, 200 mesh (74 μm; Sigma, St Louis, MO, USA) in extraction buffer (50 mM Tris–HCl, pH 7.5, 30 mM NaCl) and centrifuged at 5000 × *g* for 10 min. Rupture was monitored by optical microscopy. The protein concentration of the extract was determined using the Bradford assay (Biorad, Hercules, CA, USA).

Genomic DNA and cDNA from Phytophthora infestans

Genomic DNA was isolated from frozen mycelia. The sample was ground in lysis buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS) followed by chloroform extraction, precipitation with isopropanol and washing with 70 % cold ethanol. The DNA sample was resuspended in sterile water. For reverse transcription (RT), total RNA was isolated from 100 mg of frozen mycelia using 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Invitrogen) prior to reverse transcription according the manufacturers' instructions. To prepare the cDNA, 1 μg of total RNA was used for the reverse transcription reaction in the presence of 0.5 μg of oligo$(dT)_{15}$, 0.5 mM deoxynucleoside triphosphates (dNTPs), 0.5 mM DTT and 100 U of Superscript III reverse transcriptase (Invitrogen), and incubated at 50 °C for 1 h in 20 μl. Subsequently, a fraction (5 μl) of this reaction was used for PCR amplification.

Cloning of MDH *genes*

The sequences of the *mMDH* and *cMDH* genes of *Phytophthora infestans* (PITG_13614.1 and PITG_15476.1, respectively) were retrieved from the *P. infestans* database of the Broad Institute (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html). The *mMDH* gene was amplified directly by PCR from genomic DNA. Since the gene for cMDH contains an intron, the complete sequence of this gene was amplified from the cDNA. For *cMDH* the forward primer was 5′_CGC**CATATG**ACTACCCTCAAGATCGTC_3′ (NdeI site in bold) and the reverse primer was 5′_CG**GAATTC**TCACTGGCG CGACAGGATC_3′ (EcoRI site in bold). To amplify the *mMDH* gene without the signal peptide (mature form) the forward primer was 5′_CG**CATATG**TCGGCCCCTGGCCAGC_3′ (NdeI site in bold), and the reverse primer was 5′_CG**GAATTC**T TAATTGTTCTTGGCAAAGTCCAC_3′ (EcoRI site in bold). The amplification mixture (50 μl) contained either 1 μg of genomic DNA (for mMDH) or 5 μl from the reverse transcription reaction (for cMDH), 1 μM of each primer, 200 μM of each of the four deoxynucleotides, 1.5 mM $MgCl_2$ and 1.5 U of DNA polymerase (goGreen taq, Promega, Madison, WI, USA) with the corresponding PCR buffer. PCR was performed as follows for both MDHs: an initial incubation at 94 °C for 2 min; 36 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s; the final incubation was at 72 °C for 10 min. The PCR product was ligated into the pGEM-T vector (Promega), cloned and sequenced using the T7 and SP6 primers in an automated sequencer. Several PCR products of independent reactions were sequenced. Subsequently, each full-length gene was transferred to the pET28a vector (Novagen, Darmstadt, Germany) and used to transform the *Escherichia coli* strain BL21(DE3)pLys for the production of the recombinant proteins with an N-terminal 20 residue-long extension containing a poly-His-tag.

Phylogenetic analysis and modeling

Sequences for phylogenetic analyses were retrieved from NCBI (http://www.ncbi.nlm.nih.gov), from the genome project databases of *Phytophthora ramorum*, *Phytophthora sojae*, *Aureococcus anophagefferens*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Mycosphaerella fijiensis* (http://genome.jgi-psf.org), *Dictyostelium discoideum* (http://dictybase.org/) and *Phytophthora infestans*. Searches for MDH sequences were also performed in the genome sequencing project and expres-sed sequence tag (EST) databases of *Pythium ultimum* (http://pythium.plantbiology.msu.edu/; http://cpgr.plantbiology.msu.edu/cpgr_ta.shtml), *Hyaloperonospora parasitica* (http://genome.wustl.edu/) and *Aphanomyces euteiches* (http://www.polebio.scv.ups-tlse.fr/aphano). MDH sequences were aligned using the ClustalW alignment program (Thompson *et al.* 1994) with default parameters. In the case of mMDH, mitochondrial targeting sequences were predicted using the Mitoprot program (Claros & Vincens 1996) and removed from the complete sequences. A phylogenetic tree was constructed from the aligned sequences using the neighbor-joining method contained in the MEGA version 4 phylogenic package (Tamura *et al.* 2007). One thousand bootstrap resamplings were carried out. A three-dimensional model of cMDH was built using the Swiss-Model (Arnold *et al.* 2006) using as a template the structure of the pig cMDH (PDB entry code 4mdhA).

Overexpression of MDHs in Escherichia coli

The *E. coli* BL21pLys strain harboring the expression plasmids was grown either at 37 °C in LB medium or at 25 °C for 48 h in the ZYM-5052 autoinduction medium (0.5 % (w/v) yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5 % (w/v) glycerol, 0.05 % (w/v) glucose and 0.2 % (w/v) lactose) (Studier 2005). In both cases the medium was supplemented with 33 $\mu g\,ml^{-1}$ kanamycin and 34 $\mu g\,ml^{-1}$ chloramphenicol. When grown in LB medium, expression was induced at an OD_{600nm} of 0.4 by the addition of 0.4 mM isopropylthio-β-D-galactoside (IPTG) and growth was continued for another 3 h. The bacteria were harvested by centrifugation at 12 000 × *g* for 15 min at 4 °C. The pelleted cells were resuspended in 5 ml lysis buffer (20 mM potassium phosphate, pH 8 and 300 mM KCl) in the presence of a cocktail of protease inhibitors (Sigma) and broken by sonication. The lysate was then centrifuged at 12 000 × *g* for 15 min at 4 °C and the supernatant was applied onto a HisLink resin (Promega), equilibrated with the same lysis buffer. Both MDHs were eluted with 20 mM phosphate buffer pH 6 containing 500 mM imidazole. The imidazole was removed from the samples using a PD-10 column (Amersham Biosciences, Uppsala, Sweden). The purity of the enzymes was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue R-250 staining.

To cleave the N-terminal His-tag extension from the recombinant MDHs, 1 mg protein was incubated with 5 NIH units of human thrombin (Sigma) in cleavage buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl and 2.5 mM $CaCl_2$) for 24 h at room temperature. Finally the protein without the His-tag was recovered from the flow-through of the HisLink resin. Cleavage of the His-tag was verified by Western blotting.

Purification of natural MDH

The extract obtained from fresh mycelia (10 g) was precipitated with $(NH_4)_2SO_4$ between 60 and 80 % saturation. The resulting pellet was resuspended with 50 mM Tris–HCl, pH 8, 1 mM EDTA and 1 mM $MgSO_4$ and loaded onto a phenyl-Sepharose column (4.5 × 1 cm) equilibrated with the same buffer containing 0.5 M $(NH_4)_2SO_4$. A linear gradient from 0.5 to 0 M $(NH_4)_2SO_4$ was applied (50 ml each) followed by elution with the equilibration buffer without $(NH_4)_2SO_4$. MDH activity was only detected after this last step. The fractions containing MDH activity were pooled and concentrated by ultrafiltration using an ICON concentrator (Pierce, Appleton, WI, USA). The sample was then applied onto a cibacron blue column (4.5 × 1.6 cm) equilibrated with 25 mM Tris–HCl, pH 6.8, 1 mM EDTA and 1 mM $MgSO_4$. After washing, the proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. In addition the column was washed with the buffer containing 0.5 M NaCl and 1 mM NADH.

Enzyme activity

MDH activity was measured spectrophotometrically for reduction and oxidation at 340 nm. For the oxaloacetate reduction assay, the reaction mixture contained in 1 ml, unless otherwise specified, the assay buffer (75 mM Tris–HCl, pH 8.5), 0.42 mM NADH and either 1 mM oxaloacetate for mMDH or 1.5 mM oxaloacetate for cMDH. For the malate oxidation assay, the reaction mixture contained in 1 ml the assay buffer (75 mM Tris–HCl, pH 10 for mMDH or pH 9.5 for cMDH), 2.5 mM NAD and 10 mM L-malate. To determine the kinetic parameters, the substrate concentrations were varied from 0 to 4 mM for oxaloacetate, 0 to 0.2 mM for NADH, 0 to 12 mM for L-malate and 0 to 3.5 mM for NAD. The effect of pH was studied between pH 4 and pH 10 using a polybuffer composed of 50 mM each of Mes, Pipes, Tris and glycine adjusted to the appropriate pH with NaOH or HCl.

Antibodies

Polyclonal antisera against the recombinant MDHs were raised in a rabbit by intradermic injection with 200 μg of purified protein in complete Freund's adjuvant (1:1) for the first injection. The immune response of the rabbits was boosted three times, at two-week intervals, using incomplete Freund's adjuvant (1:1). The rabbits were bled by cardiac puncture two weeks after the fourth injection. The sera were stored at −20 °C. The commercial antibodies used were goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma).

Electrophoresis and western blotting

SDS-PAGE was performed according to Laemmli (1970). For Western blot experiments, the proteins were transferred to nitrocellulose membranes as described (Sambrook *et al.* 1989). The membrane was blocked with PBS containing 5 % dried skim milk (Colanta, Medellin, Colombia) and incubated with the appropriate primary antisera for 1 h at room temperature. After washing, the membrane was incubated with

alkaline phosphatase-conjugated secondary antibodies and subsequently revealed by addition of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. To visualize the MDH activity, native PAGE was performed according to Davis (1964). Gels were stained in 10 ml solution containing 150 mM Tris–HCl pH 8.5, 1.5 mg ml^{-1} NAD, 300 mM L-malic acid (disodium salt), 0.2 mg ml^{-1} thiazolyl blue tetrazolium bromide (Sigma) and 0.32 mM phenazine methosulfate (Sigma).

Results

Sequence analysis and cloning of the Phytophthora infestans MDHs

Two putative *MDH* genes were identified through the genome sequencing project of *P. infestans* at the Broad Institute (personal communication from C. Nusbaum). These sequences were analyzed regarding the footprints of the conserved domains using the conserved domain database (CDD) at NCBI (Marchler-Bauer *et al.* 2007). One of the *MDH* sequences (accession code: PITG_13614.1) showed the signature of mitochondrial MDH (conserved domain: MDH_glycosomal_mitochondrial, Cd: 01337) and the other sequence (accession code: PITG_15476.1) the signature for cytosolic MDH (conserved domain: MDH_cytoplasmic_cytosolic, Cd: 01336). The *mMDH* gene is a 1008 bp open reading frame coding for a polypeptide of 335 amino acids with a molecular mass of 35.4 kDa. Analysis of the sequence of this *MDH* gene using the Signal P 3.0 server: http://www.cbs.dtu.dk/services/SignalP (Bendtsen *et al.* 2004) revealed an N-terminal mitochondrial signal peptide (amino acids 1–18), the mature form of mMDH having a predicted molecular mass of 33.4 kDa. In the case of *cMDH*, the gene spans 1072 bp and has an intron located at position 68–128. The coding sequence without the intron is 1011 bp long. The mature polypeptide for this MDH possesses 336 amino acids and a molecular mass of 36.4 kDa. The *P. infestans MDHs* show 22.2 % identity to each other at the protein level. The amino acid sequence for the mMDH was identical to that of the *P. infestans* database. The cMDH had only one change: threonine replaced by valine in position 141. The alignment of the protein sequence derived for both MDHs (not shown) with MDHs from other organisms revealed that the MDHs from *P. infestans* are very close to the corresponding enzyme of *P. ramorum* (92.4 % identity for mMDH and 89.1 % identity for cMDH) and *P. sojae* (94.3 % identity for mMDH and 92.5 % identity for cMDH) (Man In 't Veld *et al.* 2007). The identity with other MDHs of eukaryotes, including stramenopiles, within the same conserved domain ranged from 52 to 89.6 % for mMDH and from 40.6 to 82.5 % for cMDH. All residues essential for enzyme activity or for the binding of the substrates were conserved. In addition, *P. infestans* cMDH possesses an insertion of six residues between position 170 and 176 that is only present in cMDHs from some but not all stramenopile organisms (Fig 1A) when compared with other eukaryotic cMDHs. The enzyme from the stramenopile *Thalassiosira pseudonana* lacks this insert. Modeling of the *P. infestans* cMDH using the pig cMDH as template structure (54.7 % identity) shows that this insertion is located in a loop that protrudes from the main body of the protein (Fig 1B).

Phylogenetic analysis

Sequences for phylogenetic analyses were selected based on the presence of the specific conserved domain MDH_glycosomal_mitochondrial (Cd: 01337) or MDH_cytoplasmic_cytosolic (Cd 01336). Fig 2 shows the consensus tree obtained by the neighbor-joining method for 23 sequences of mMDH and 17 sequences of cMDH. In agreement with previous studies (Madern 2002; McAlister-Henn 1988; Man In 't Veld *et al.* 2007), cMDHs and mMDHs from *Phytophthora infestans* form two separate clades. In both cases, *P. infestans* MDHs branches with those from other *Phytophthora* species. For mMDH, all the sequences representative of the stramenopile group (including photosynthetic and non-photosynthetic organisms) form a clade that is separate from that of fungi, metazoa and plants (Fig 2). In the case of cMDH, the sequences from the *Phytophthora* species also form a different clade distinct from metazoa and plants. However, an MDH sequence with the conserved domain MDH_cytoplasmic_cytosolic is not present in all stramenopile organisms available. Moreover, *Thalassiosira pseudonana,* an organism of this group, possesses a cMDH whose sequence is

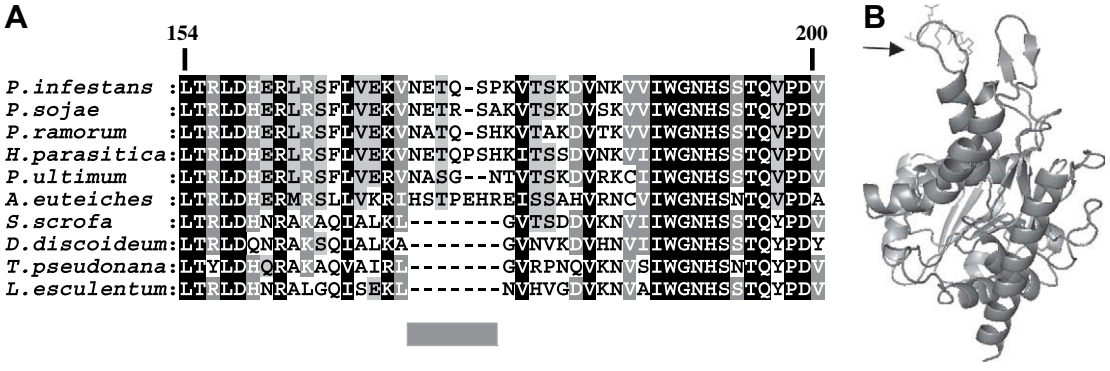

Fig 1 – Insertion of six amino acids in the cMDH of *Phytophthora infestans*. (A) Alignment of a segment of cMDH from *P. infestans*, *Phytophthora sojae*, *Phytophthora ramorum*, *Hyaloperonospora parasitica*, *Pythium ultimum*, *Aphanomyces euteiches*, *Sus scrofa*, *Dictyostelium discoideum*, *Thalassiosira pseudonana and Lycopersicon esculentum*. Horizontal bar signifies additional sequence found only in cMDH of some stramenopile species in comparison with other eukaryotic cMDHs. The numbers correspond to the amino acid positions within the *P. infestans* sequence. (B) Modeling of *P. infestans* cMDH against pig cMDH, indicating the loop (arrow) corresponding to the insertion of six amino acids shown in A.

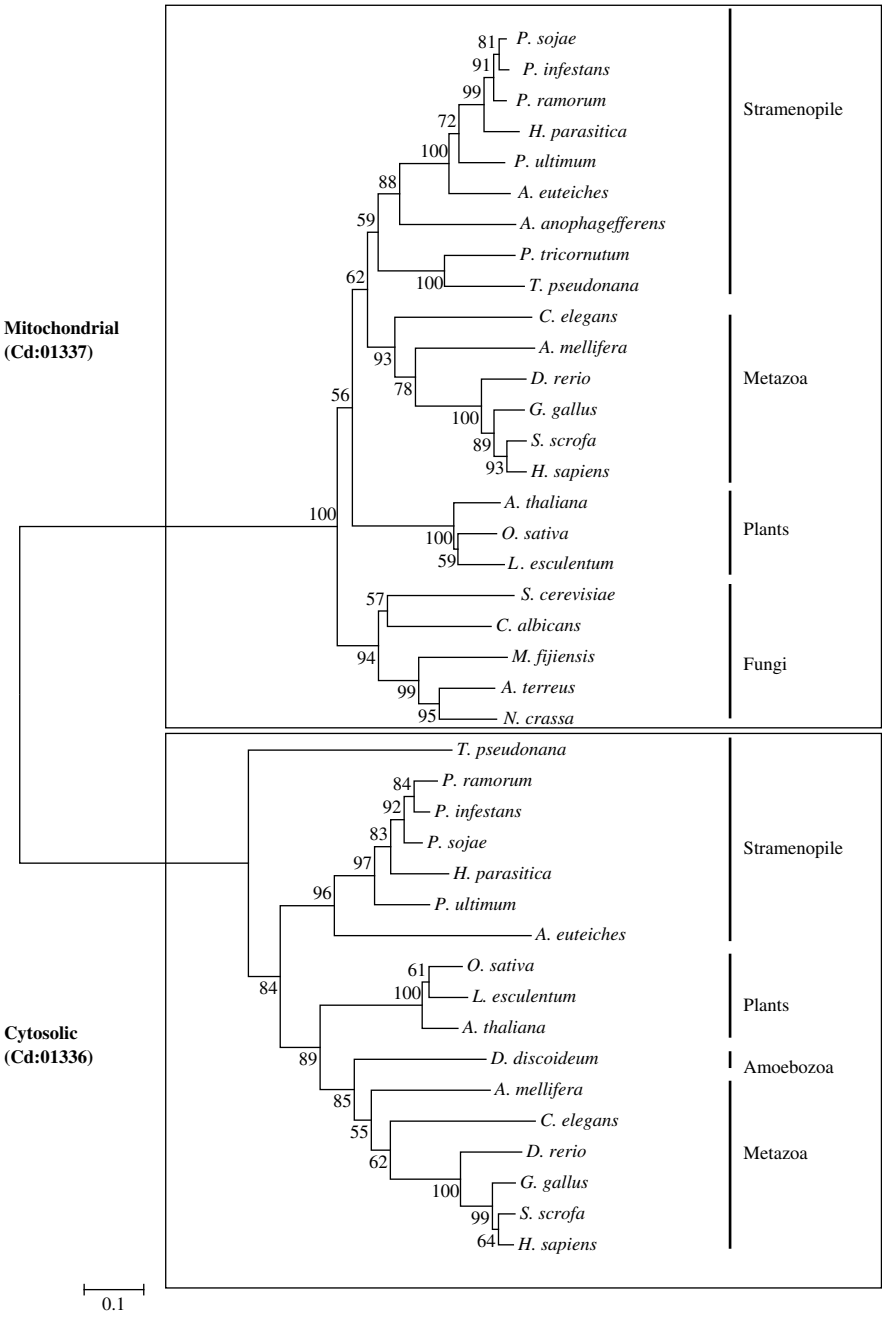

Fig 2 – Phylogenetic tree (neighbor-joining) constructed from MDH sequences. Mitochondrial and cytosolic MDHs possess the specific domains: MDH_glycosomal_mitochondrial and MDH_cytoplasmic_cytosolic, respectively. The mitochondrial sequences are without signal peptide. Bar represents 0.1 substitution per site. MDH sequences for *Phytophthora infestans* (PITG_13614.1 PITG_15476.1) were retrieved from http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html. Sequences from *Phytophthora ramorum* (72140 and 71856), *Phytophthora sojae* (109054 and 109232), *Phaeodactylum tricornutum* (42398), *Aureococcus anophagefferens* (60302), *Thalassiosira pseudonana* (20726 and 41425) and *Mycosphaerella fijiensis* (88245) were retrieved from http://genome.jgi-psf.org. Other sequences were from NCBI: *Apis mellifera* (XP_392478.2 and XP_394487.2), *Danio rerio* (NP_998296.1 and NP_956263.1), *Sus scrofa* (P00346.2 and NP_999039.1), *Gallus gallus* (XP_415765 and Q5ZME2.1), *Arabidopsis thaliana* (AAK00366.1 and AAL59959.1), *Oryza sativa* (AAM00435.1 and AAK26431.1), *Lycopersicon esculentum* (AAU29198.1 and AAU29199.1), *Saccharomyces cerevisiae* (NP_012838.1), *Candida albicans* (XP_722674.1), *Neurospora crassa* (XP_958408), *Aspergillus terreus* (XP_001215536), *Homo sapiens* (NP_005909 and NP_005908), *Caenorhabditis elegans* (NP_498457 and NP_504656). *The Dictyostelium discoideum MDH sequence* (DDB_G0280255) *was from* http://dictybase.org/. Sequences from *Aphanomyces euteiches* (Ae_9AL7510 and Ae_6AL6574) were from http://www.polebio.scv.ups-tlse.fr/aphano. Sequences from *Hyaloperonospora parasitica* and mMDH from *Pythium ultimum* were obtained using the blast program in the genome project of these organisms (*H. parasitica*: http://genome.wustl.edu/ and *P. ultimum*: http://pythium.plantbiology.msu.edu/). The cMDH from *P. ultimum* (TA3418_65071) was from the EST databank: http://cpgr.plantbiology.msu.edu/cpgr_ta.shtml.

not close to any cMDH of other organisms. The phylogenetic analyses did not include bacterial MDHs, but both conserved domains specified above can be found in these organisms.

Expression and purification of recombinant MDHs

The mMDH coding sequence without the nucleotides encoding the mitochondrial signal peptide (mature form) were amplified from mycelium DNA by PCR. The cMDH coding sequence was amplified from total RNA of mycelium by RT-PCR. Recombinant MDHs were expressed as N-terminal fusion proteins with a poly-His-tag in *Escherichia coli* in an autoinduction medium and purified by metal affinity chromatography (Fig 3A,B). The apparent subunit molecular masses of these proteins as estimated by SDS-PAGE were 41 kDa and 42 kDa for mMDH and cMDH, respectively (Fig 3A,B). Both forms were eluted with 500 mM imidazole. cMDH was totally soluble and the same yield was obtained by induction with IPTG (data not shown). In contrast, mMDH formed inclusion bodies upon induction with IPTG and was only obtained in partially soluble form by autoinduction growth. The yield of purified protein was 0.14 mg for mMDH and 2 mg for the cMDH from about 10 mg of bacterial cell-free extract. The purified enzymes were used for their respective kinetic characterization and for molecular mass determination. Gel filtration on a Sephacryl S-200 column (data not shown) indicates that both MDHs have a molecular mass of 90 ± 10 kDa; this value is consistent with the homodimeric form of the enzyme.

The antisera raised against each recombinant protein recognized only one band of the expected size in mycelium extracts (Fig 3C,D). No immunological cross reactivity was present between the recombinant cMDH and mMDH (Fig 3C,D), the antisera generated being specific of each protein. This result indicates that the two MDHs do not share antigenic determinants.

Kinetic characteristics of recombinant MDHs

The values of the kinetic parameters were determined using the oxidation and reduction assays and are summarized in Table 1. The optimum pH for the recombinant mMDH and cMDH enzymes in the oxaloacetate reduction assay was 8.5, and in the malate oxidation assay it was between 9.5 and 10.0. These pH optima correspond to the data obtained for MDH enzymes from different sources (Genda *et al.* 2003; Hayes *et al.* 1991; Tripathi *et al.* 2004; Yueh *et al.* 1989; Zee & Zinkham 1968; Zheng *et al.* 2005). Further kinetic analyses were performed at the optimum pHs. To examine if the His-tag of the recombinant proteins had an effect on the catalytic properties of the enzymes, the tag was removed using thrombin (Fig 3E,F). The catalytic properties of the untagged mMDH were similar to those of the tagged recombinant enzyme (data not shown). However, for cMDH the His-tag has some effect on the catalytic properties. The K_m for oxaloacetate and NADH was three fold lower and the V_{max} two fold higher than those of the tagged enzyme, the untagged enzyme being thus catalytically more efficient. The results presented in Table 1 and Fig 4 for both enzymes correspond to the untagged proteins. Substrate inhibition was observed for the recombinant mMDH and cMDH (Fig 4). The K_i was determined by non-linear regression fitting the data to an equation that includes one productive and one inhibitory binding site for oxaloacetate (Mahmoud *et al.* 1995), the K_i for mMDH being lower than that for cMDH. Substrate inhibition for oxaloacetate has been well studied and has been reported for both mitochondrial and cytosolic MDHs (Agüero *et al.* 2004; Bernstein *et al.* 1978; Maloney *et al.* 2004). For the other substrates, the enzyme displayed standard Michaelis–Menten kinetics. The specific

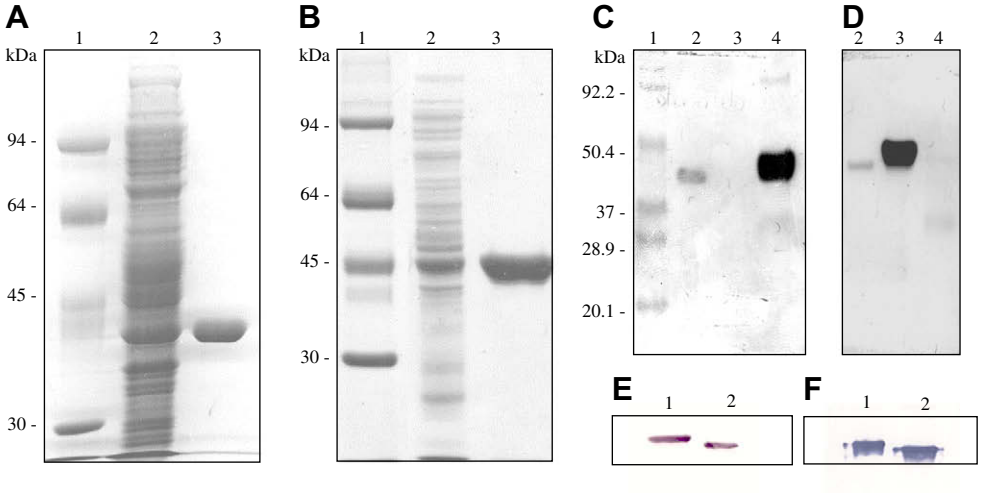

Fig 3 – Purification of recombinant *Phytophthora infestans* MDHs expressed in *Escherichia coli*. Recombinant *P. infestans* mMDH (A) and recombinant *P. infestans* cMDH (B). Lane 1, molecular mass marker; lane 2, cell-free extract; lane 3, eluted with 500 mM imidazole. (C and D) Western blots of mycelium extracts and recombinant proteins using anti-mMDH serum (C) and anti-cMDH serum (D). Lane 1, molecular mass markers; lane 2, mycelium extract (50 μg protein); lane 3, 2 μg recombinant cMDH; lane 4, 2 μg recombinant mMDH. (E) and (F) Treatment with thrombin of mMDH (E) and cMDH (F) as visualized by Western blots using the corresponding antisera. Lane 1, purified His-tag recombinant enzyme; lane 2, after thrombin treatment.

Table 1 – Kinetic characteristics of recombinant *Phytophthora infestans* cMDH and recombinant and natural *P. infestans* mMDH

Enzyme/substrate	V_{max}[a,b] (U mg^{-1})	K_m (mM)	K_i (mM)	V_{max}/K_m (U mg^{-1} mM^{-1})
Recombinant cMDH (untagged)				
Oxaloacetate	1819.3 ± 184	0.176 ± 0.012	12.3 ± 5.6	10 336.9
NADH	857.7 ± 83	0.026 ± 0.001		32 988.4
Malate	257.6 ± 7.2	1.72 ± 0.006		149.8
NAD	226.8 ± 10	0.21 ± 0.002		1080
Recombinant mMDH (untagged)				
Oxaloacetate	1166 ± 199	0.022 ± 0.001	2.25 ± 0.3	53 000
NADH	2015 ± 125	0.086 ± 0.002		23 430
Malate	320 ± 12	0.64 ± 0.03		500
NAD	358.7 ± 20	0.37 ± 0.01		969.5
Natural mMDH				
Oxaloacetate	–	0.035 ± 0.006	2.8 ± 0.4	–
NADH	–	0.21 ± 0.02		–
Malate	–	0.94 ± 0.2		–
NAD	–	0.21 ± 0.05		–

a One unit is defined as the conversion of 1 μmol of substrate per minute at 25 °C.
b The values were obtained from Lineweaver–Burk plots except the K_i value for oxaloacetate that was obtained from non-linear regression using the substrate inhibition equation.

activities of both enzymes were similar in both directions of the reaction. The recombinant mMDH had a lower K_m for both oxaloacetate (8 fold less) and malate (2.6 fold less) than the recombinant cMDH. The cytosolic isoform exhibited a K_m for NADH that was 3.3 fold lower than that of the mitochondrial isoform. The K_m for NAD was similar in both cases. For both recombinant enzymes the highest V_{max} values as well as the catalytic efficiencies (V_{max}/K_m) corresponded to those of the oxaloacetate reduction direction.

Purification of natural mMDH from mycelium and kinetic characterization

A soluble protein preparation obtained from mechanical rupture of mycelia of *Phytophthora infestans* was used to visualize MDH activity by native gel electrophoresis. As expected, two bands were detected (Fig 5) indicating that the two isoforms, cytosolic and mitochondrial, are present in the mycelium. Similar results were obtained previously using mycelia from *P. ramorum* (Man In 't Veld *et al.* 2007). Taking into account the theoretical molecular mass (33.4 kDa for mMDH and 36.4 for cMDH) and the isoelectric points for both proteins (5.92 for mMDH and 6.17 for cMDH), the band with higher mobility corresponds to mMDH. In an attempt to purify both proteins, the homogenate was submitted to $(NH_4)_2SO_4$ precipitation (60–80 %) followed by a hydrophobic chromatography. In this step, both MDHs eluted together from the column (Fig 5A). In the following step, the cibacron blue chromatography step, only the protein with higher mobility in a native gel was eluted using an NaCl gradient (Fig 5B). This protein was confirmed as being the mitochondrial isoform by immunoblotting, since it was recognized by anti-mMDH serum but not by anti-cMDH serum (Fig 5C). Attempts to elute the cytosolic isoform from the cibacron blue column were performed using NADH, but without success. The final preparation of the natural mMDH was not pure (Fig 5D) but allowed us to determine several catalytic characteristics (Table 1). The natural mitochondrial enzyme had

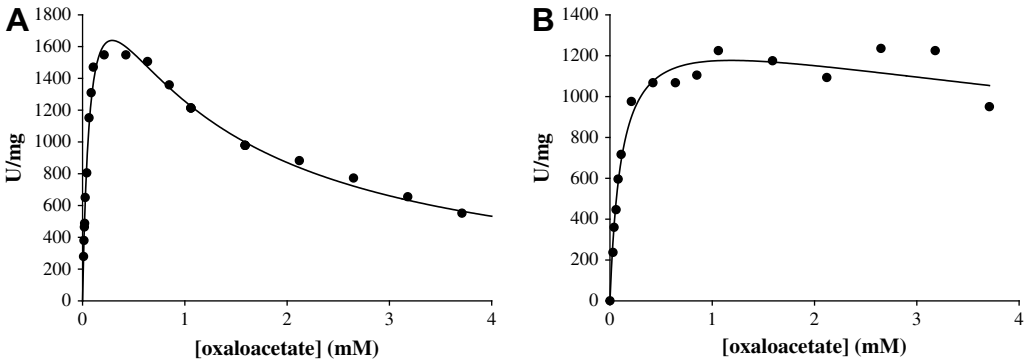

Fig 4 – Kinetic curves for oxaloacetate of *Phytophthora infestans* recombinant MDHs. (A) mMDH and (B) cMDH. The activity (U mg^{-1}) was measured by varying the oxaloacetate concentration at a fixed NADH concentration (0.36 mM). The enzymes used for the assays were the untagged recombinant proteins. The data was fitted to the equation for substrate inhibition (Mahmoud *et al.* 1995).

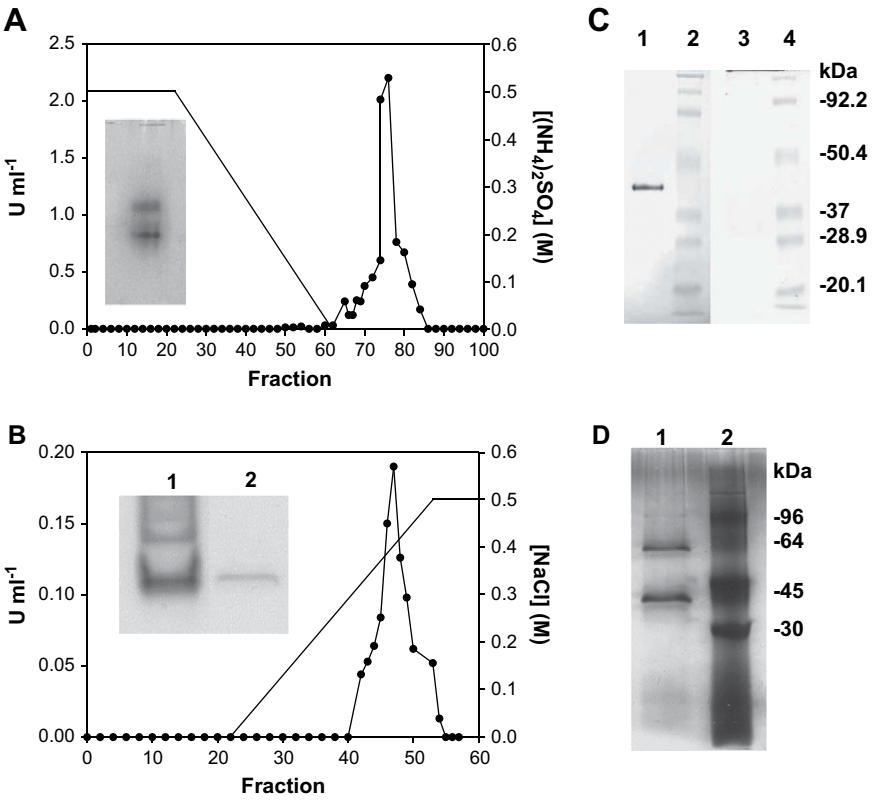

Fig 5 – Purification of the natural *Phytophthora infestans* mMDH. (A) Elution profile obtained with phenyl-Sepharose chromatography. The activity of MDH appeared after the $(NH_4)_2SO_4$ gradient in the absence of salt. Inset: MDH activity of the collected pool of this chromatography as detected by native PAGE. (B) Elution profile obtained by cibacron blue chromatography. The activity of MDH appeared at 0.4 M NaCl. Inset; MDH activity detected by native PAGE. Lane 1, mycelium extract; lane 2, collected pool of this chromatography. (C) Western blot of the collected pool of the cibacron blue chromatography step revealed with anti-mMDH serum (lane 1) or anti-cMDH serum (lane 3). Lanes 2 and 4: molecular mass markers. (D) SDS-PAGE of the semi-purified mMDH stained with silver nitrate. Lane 1, sample from the cibacron blue chromatography step; lane 2, molecular mass markers.

the same catalytic properties as the recombinant enzyme validating the use of this latter enzyme as a model for further studies of this enzyme.

Discussion

The *Phytophthora infestans* genome database reports two sequences for *MDH* gene (cytosolic and mitochondrial). Indeed, mycelium homogenates revealed two MDHs by native gel electrophoresis. In the present study, these enzymes were cloned, overexpressed in *Escherichia coli*, and kinetically characterized. This represents a first step toward understanding energetic metabolism in this pathogen.

Sequence comparisons showed that the two *P. infestans* MDH sequences have a low level of identity (22.2 %). Each *P. infestans* MDH is highly similar to the corresponding MDH (either cytosolic or mitochondrial) reported in the genome of other species of *Phytophthora* (*P. sojae* and *P. ramorum*) (Tyler *et al.* 2006). Phylogenetic analyses revealed a relationship between members of the stramenopile group. For mMDH, the *Phytophthora* species form a clade with other stramenopiles that include organisms of different orders. *Phytophthora* mMDHs are more closely related to the enzyme from *Hyaloperonospora parasitica*, an organism of the same order (Peronosporales). The mMDHs of the stramenopile clade are differentiated from those of plants, metazoa and fungi. Thus the mitochondrial isoform of MDH is useful for studying the relationship between stramenopiles. As previously determined (Kamoun *et al.* 1999) this mMDH presents a greater similarity to plant than to fungal mMDH. The cMDHs from the *Phytophthora* species also form a clade. However, MDH with the conserved domain MDH_cytoplasmic_cytosolic (Cd01336) is not present in all the stramenopiles analyzed. Moreover, the stramenopile *Thalassiosira pseudonana* has a cytosolic MDH (Armbrust *et al.* 2004) but it only shares 40.6 % identity with the *P. infestans* cMDH, less than the identity between *P. infestans* and pig cMDH (Man In 't Veld *et al.* 2007). Because of the limited number of sequences available to date for stramenopile organisms, we cannot assess the relationship between cMDHs within this group. *Phytophthora* cMDHs are also more closely related to the cMDH from *H. parasitica*. This type of MDH seems to be absent from fungi, highlighting another important characteristic that distinguishes oomycetes from fungi. Although MDH is known to be present in the cytosol of fungi such as *Saccharomyces cerevisae* (Minard & McAlister-Henn 1991), it

belongs to MDHs that contain the signature of the mitochondrial enzyme. Thus organisms of the genus *Phytophthora* and other stramenopiles resemble many eukaryotic organisms in having both mitochondrial and cytosolic MDHs within the specific conserved domains MDH_glycosomal_mitochondrial and MDH_cytoplasmic_cytosolic, respectively. However more sequences are required to establish which stramenopiles possess both types of MDH. A characteristic of the cMDH of the *Phytophthora* species and other stramenopile organisms is the insertion of six or seven amino acids; modeling of cMDH from *P. infestans* showed that it is located in an extra loop with respect to the template structure, that of pig cMDH. One may speculate that this loop could be implicated in protein–protein-interaction. The interaction of cMDHs with other proteins has been previously documented (Gibson & McAlister-Henn 2003, Hanss *et al.* 2002). Another possible function for this insert is to be a target for phosphorylation; the insert has serine or threonine residues that could be phosphorylated. In the case of *Phytophthora* and *H. parasitica* this insert has a serine residue lying close to a C-terminal basic residue which might exhibit a preference for phosphorylation by protein kinase C (Kishimoto *et al.* 1985). If this protein is phosphorylated *in vivo*, its activity might be regulated. Moreover, the MDH isoenzyme located in the cytosol in *S. cerevisae* has been shown to be regulated by phosphorylation (Minard & McAlister-Henn 1994). Further investigations are needed to assess the function of this insertion in the *Phytophthora* cMDH.

The catalytic properties of the recombinant enzymes were studied in the oxidation as well as in the reduction reactions. With both recombinant enzymes, the K_m for oxaloacetate and NADH is lower than the K_m for malate and NAD, as is the case of the mitochondrial and cytosolic isoforms from numerous sources (Aranda *et al.* 2006; Ding & Ma 2004; Maloney *et al.* 2004; Trejo *et al.* 1996; Zheng *et al.* 2005). Similarly, the catalytic efficiency (V_{max}/K_m) was higher for the oxaloacetate reduction with both enzymes. The V_{max} for both enzymes (2015 U mg^{-1} for mMDH and 1819 U mg^{-1} for cMDH) are high in comparison to those of other MDHs reported except for those for mMDH of *Talaromyces emersonii* (5200 U mg^{-1}) (Maloney *et al.* 2004) and cMDH of wheat (3118 U mg^{-1}) (Ding & Ma 2004). The K_m values for oxaloacetate and malate vary considerably between MDHs from different sources. The K_m values for these substrates in the MDHs from *P. infestans* are within these values. *P. infestans* mMDH and cMDH presented the following substrate inhibition values: the K_i for cMDH (12.3 mM) was higher than that for mMDH (2.2 mM). For mMDH, this K_i value was similar to those of pig mMDH (Bernstein *et al.* 1978) and pig cMDH (Trejo *et al.* 1996). Substrate inhibition for oxaloacetate seems not to have a physiological significance (Bernstein *et al.* 1978).

The recombinant mMDH appears identical to the natural enzyme isolated from the mycelium. Natural cMDH was not isolated using our purification approach, to test its catalytic properties but its K_m values as well the high value of specific activity obtained suggest that this recombinant protein behaves as the natural protein. In both cases, the recombinant *P. infestans* MDHs can be useful to test the action of different inhibitors. The availability of large quantities of recombinant enzyme will facilitate the discovery of inhibitors for MDHs by compound library screening.

It is well known that mMDH is implicated in the regeneration of oxaloacetate in the tricarboxylic acid cycle in mitochondria. On the other hand, the function of cMDH in the metabolism of *P. infestans* is not clear. cMDHs have several roles in cellular metabolism such as gluconeogenesis and aspartate–malate shuttle between mitochondria and cytosol. A clue to the function of *P. infestans* cMDH can be deduced from the different catalytic efficiency of this enzyme in the oxidation and reduction reactions; it is about 30 fold higher for oxaloacetate reduction than for the malate oxidation, suggesting that oxaloacetate reduction could be more favorable *in vivo*. mMDH presents the same or higher differences between oxidation and reduction but in the tricarboxylic acid cycle malate oxidation is driven by the strong utilization of NADH by the respiratory chain. If cMDH functions by reducing oxaloacetate in the cytosol, it might be involved in malate–aspartate shuttle transferring reducing equivalents from the cytosol to mitochondria. In addition cMDH might produce the substrate for the malic enzyme, malate, which is ultimately oxidized to pyruvate leading to the concomitant production of cytosolic NADPH. The latter coenzyme is essential for biosynthetic processes. Moreover malic enzyme is expressed in this organism as well as in *P. ramorum* (Man In 't Veld *et al.* 2007; Randall *et al.* 2005). However, to assess the function of cMDH further studies on the metabolism of *P. infestans* must be performed.

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REFERENCES

Adl SM, Simpson AGB, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup Ø, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MFJR, 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *The Journal of Eukaryotic Microbiology* **52**: 399–451.

Agüero F, Noé G, Hellman U, Repetto Y, Zaha A, Cazzulo JJ, 2004. Purification, cloning, and expression of the mitochondrial malate dehydrogenase (mMDH) from protoscolices of *Echinococcus granulosus*. *Molecular and Biochemical Parasitology* **137**: 207–214.

Aranda A, Maugeri D, Uttaro AD, Opperdoes F, Cazzulo JJ, C Nowicki, 2006. The malate dehydrogenase isoforms from *Trypanosoma brucei*: subcellular localization and differential expression in bloodstream and procyclic forms. *Internacional Journal for Parasitology* **36**: 295–307.

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A,

Wilkerson FP, Rokhsar DS, 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* **306**: 79–86.

Arnold K, Bordoli L, Kopp J, Schwede T, 2006. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**: 195–201.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S, 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**: 783–795.

Bernstein LH, Grisham MB, Cole KD, Everse J, 1978. Substrate inhibition of the mitochondrial and cytoplasmic malate dehydrogenases. *The Journal of Biological Chemistry* **253**: 8697–8701.

Claros MG, Vincens P, 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. *European Journal of Biochemistry* **241**: 779–786.

Davis BJ, 1964. Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**: 404–407.

Ding Y, Ma QH, 2004. Characterization of a cytosolic malate dehydrogenase cDNA which encodes an isozyme toward oxaloacetate reduction in wheat. *Biochimie* **86**: 509–518.

Genda T, Nakamatsu T, Ozak H, 2003. Purification and characterization of malate dehydrogenase from *Corynebacterium glutamicum*. *Journal of Bioscience and Bioengineering* **95**: 562–566.

Gibson N, McAlister-Henn L, 2003. Physical and genetic interactions of cytosolic malate dehydrogenase with other gluconeogenic enzymes. *The Journal of Biological Chemistry* **278**: 25628–25636.

Goward CR, Nicholls DJ, 1994. Malate dehydrogenase: a model for structure, evolution, and catalysis. *Protein Science* **3**: 1883–1888.

Grenville-Briggs LJ, Avrova AO, Bruce CR, Williams A, Whisson SC, Birch PRJ, van West P, 2005. Elevated amino acid biosynthesis in *Phytophthora infestans* during appressorium formation and potato infection. *Fungal Genetics and Biology* **42**: 244–256.

Hanss B, Leal-Pinto E, Teixeira A, Christian RE, Shabanowitz J, Hunt DF, Klotman PE, 2002. Cytosolic malate dehydrogenase confers selectivity of the nucleic acid-conducting channel. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 1707–1712.

Hardham AR, 2007. Cell biology of plant–oomycete interactions. *Cellular Microbiology* **9**: 31–39.

Hayes MK, Luethy MH, Elthon TE, 1991. Mitochondrial malate dehydrogenase from corn: purification of multiple forms. *Plant Physiology* **97**: 1381–1387.

Kamoun S, 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**: 41–60.

Kamoun S, Hraber P, Sobral B, Nuss D, Govers F, 1999. Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. *Fungal Genetics and Biology* **28**: 94–106.

Kamoun S, Smart CD, 2005. Late blight of potato and tomato in the genomics era. *Plant Disease* **89**: 692–699.

Kim KS, Judelson HS, 2003. Sporangium-specific gene expression in the oomycete phytopathogen *Phytophthora infestans*. *Eukaryotic Cell* **2**: 1376–1385.

Kishimoto A, Nishiyama K, Nakanishi H, Uratsuji Y, Nomura H, Takeyama Y, Nishizuka Y, 1985. Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3′:5′-monophosphate-dependent protein kinase. *The Journal of Biological Chemistry* **260**: 12492–12499.

Laemmli UK, 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Madern D, 2002. Molecular evolution within the L-malate and L-lactate dehydrogenase super-family. *Journal of Molecular Evolution* **54**: 825–840.

Mahmoud YAG, el Souod SMA, Niehaus WG, 1995. Purification and characterization of malate dehydrogenase from *Cryptococcus neoformans*. *Archives of Biochemistry and Biophysics* **322**: 69–75.

Maloney AP, Callan SM, Murray PG, Tuohy MG, 2004. Mitochondrial malate dehydrogenase from the thermophilic, filamentous fungus *Talaromyces emersonii*. *European Journal of Biochemistry* **271**: 3115–3126.

Man In 't Veld WA, Govers F, Meijer HJG, 2007. Correlation of isozyme profiles with genomic sequences of *Phytophthora ramorum* and its *P. sojae* orthologues. *Current Genetics* **52**: 247–257.

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH, 2007. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Research* **35**: D237–D240.

Marshall JS, Ashton AR, Govers F, Hardham AR, 2001. Isolation and characterization of four genes encoding pyruvate, phosphate dikinase in the oomycete plant pathogen *Phytophthora cinnamomi*. *Current Genetics* **40**: 73–81.

McAlister-Henn L, 1988. Evolutionary relationships among the malate dehydrogenases. *Trends in Biochemical Sciences* **13**: 178–181.

Minard KI, McAlister-Henn L, 1991. Isolation, nucleotide sequence analysis, and disruption of the MDH2 gene from *Saccharomyces cerevisiae*: evidence for three isozymes of yeast malate dehydrogenase. *Molecular and Cellular Biology* **11**: 370–380.

Minard KI, McAlister-Henn L, 1994. Glucose-induced phosphorylation of the MDH2 isozyme of malate dehydrogenase in *Saccharomyces cerevisiae*. *Archives of Biochemistry and Biophysics* **315**: 302–309.

Prakob W, Judelson HS, 2007. Gene expression during oosporogenesis in heterothallic and homothallic. *Phytophthora*. *Fungal Genetics and Biology* **44**: 726–739.

Randall TA, Dwyer RA, Huitema E, Beyer K, Cvitanich C, Kelkar H, Fong AMVA, Gates K, Roberts S, Yatzkan E, Gaffney T, Law M, Testa A, Torto-Alalibo T, Zhang M, Zheng L, Mueller E, Windass J, Binder A, Birch PRJ, Gisi U, Govers F, Gow NA, Mauch F, van West P, Waugh ME, Yu J, Boller T, Kamoun S, Lam ST, Judelson HS, 2005. Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. *Molecular Plant–Microbe Interactions* **18**: 229–243.

Sambrook J, Fritsch EF, Maniatis T, 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Skalamera D, Wasson AP, Hardham AR, 2004. Genes expressed in zoospores of *Phytophthora nicotianae*. *Molecular Genetics and Genomics* **270**: 549–557.

Studier W, 2005. Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification* **41**: 207–234.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Tani S, Yatzkan E, Judelson HS, 2004. Multiple pathways regulate the induction of genes during zoosporogenesis in *Phytophthora infestans*. *Molecular Plant–Microbe Interactions* **17**: 330–337.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Trejo F, Costa M, Gelpí JL, Busquets M, Clarke AR, Holbrook JJ, Cortés A, 1996. Cloning, sequencing and functional expression of a DNA encoding pig cytosolic malate dehydrogenase: purification and characterization of the recombinant enzyme. *Gene* **172**: 303–308.

Tripathi AK, Desai PV, Pradhan A, Khan SI, Avery MA, Walker LA, Tekwani BL, 2004. An alpha-proteobacterial type malate dehydrogenase may complement LDH function in *Plasmodium falciparum*. Cloning and biochemical characterization of the enzyme. *European Journal of Biochemistry* **271**: 3488–3502.

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RHY, Aerts A, Arredondo FD, Baxter L, Bensasson D, Beynon JL, Chapman J, Damasceno CM, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M, Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee MK, McDonald WH, Medina M, Meijer HJG, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose JKC, Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BWS, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, Boore JL, 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* **313**: 1261–1266.

Yueh AY, Chung CS, Lai YK, 1989. Purification and molecular properties of malate dehydrogenase from the marine diatom *Nitzschia alba*. *The Biochemical Journal* **258**: 221–228.

Zee DS, Zinkham WH, 1968. Malate dehydrogenase in *Ascaris suum*: characterization, ontogeny, and genetic control. *Archives of Biochemistry and Biophysics* **126**: 574–584.

Zheng N, Xu J, Wu Z, Chen J, Hu X, Song L, Yang G, Ji C, Chen S, Gu S, Ying K, Yu X, 2005. *Clonorchis sinensis*: molecular cloning and functional expression of novel cytosolic malate dehydrogenase. *Experimental Parasitology* **109**: 220–227.