The Multifunctional iso-Propanol Dehydrogenase of Phytomonas sp. could be the Result of a Horizontal Gene Transfer from a Bacterium to the Trypanosomatid lineage.

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RUNNING TITLE

Iso-Propanol Dehydrogenase from Phytomonas sp.
SUMMARY

Iso-propanol dehydrogenase (iPDH) is a dimeric mitochondrial alcohol dehydrogenase (ADH), so far detected within the Trypanosomatidae only in the genus *Phytomonas*. The cloning, sequencing and heterologous expression of the two gene alleles of the enzyme revealed that it is a zinc-dependent medium-chain ADH. Both polypeptides have 361 amino acids. A mitochondrial targeting sequence was identified. The mature proteins each have 348 amino acids and a calculated molecular mass of 37 kDa. They differ only in one amino acid, which can explain the three isoenzymes and their respective isoelectric points previously found. A phylogenetic analysis locates iPDH within a cluster with fermentative ADHs from bacteria, sharing 74% similarity and 60% identity with *Ralstonia eutropha* ADH. The characterization of the two bacterially expressed *Phytomonas* enzymes and the comparison of their kinetic properties with those of the wild type iPDH and of the *R. eutropha* ADH strongly support the idea of a horizontal gene transfer event from a bacterium to a trypanosomatid to explain the origin of the iPDH in *Phytomonas*. *Phytomonas* iPDH and *R. eutropha* ADH are able to use a wide range of substrates with similar $K_m$s, such as primary and secondary alcohols, diols, aldehydes, as well as ketones such as acetone, diacetyl and acetoin. We speculate that, as for *R. eutropha* ADH, *Phytomonas* iPDH acts as a safety valve for the release of excess reducing power.
INTRODUCTION

Alcohol dehydrogenases (ADHs)\(^1\) display a wide range of substrate specificities and fulfil several key physiological functions. They are classified into three major categories: NAD(P)-dependent enzymes, NAD(P)-independent enzymes and irreversible alcohol oxidases. NAD(P)-dependent ADHs are subdivided into groups according to their metal dependence: medium-chain zinc-dependent, short-chain zinc-independent and iron-activated enzymes (1).

Zinc-dependent medium-chain dehydrogenases constitute a large superfamily of enzymes widely found in vertebrates, plants, fungi and bacteria, including polyol dehydrogenases, threonine dehydrogenase, quinone oxidoreductases and other proteins (2). Currently, they can be divided into tetrameric (as NAD-dependent ADHs from fungi or some bacterial NADP-dependent enzymes) and dimeric NAD-linked ADH families. The last in turn, are divided in the classes found in vertebrates (I-VII) or plants (III, C and P), according to substrate preference and amino-acid identity (3, 4).

Although it is not uncommon for ADHs to exhibit a broad substrate spectrum, as a rule they are specific either for primary or for secondary alcohols. One interesting exception is the tetrameric multifunctional *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) ADH. This enzyme is able to use both primary and secondary alcohols, polyols as 2,3-butanediol, acetaldehyde, acetone, diacetyl or acetoin as substrates, using NAD(H) or NADP(H) as cosubstrate (5).

Few trypanosomatid ADHs have been described. These are the cytosolic NADPH-linked aldehyde reductases from *Leishmania donovani* (6) and *Trypanosoma cruzi* (7), which use \(n\)-propanol as the preferred substrate, or the NADH-dependent ADH from *Crithidia fasciculata* (8). Unfortunately, neither sequence information, nor sufficient biochemical data are available to correctly classify these enzymes.
We have previously described the purification and characterization of the mitochondrial ADH from *Phytomonas*, a trypanosomatid isolated from the laticiferous plant *Euphorbia characias*. It is a dimeric NAD-linked enzyme able to use a broad range of both primary and secondary alcohols. We have named the enzyme isopropanol dehydrogenase (iPDH) owing to its high affinity and specific activity with this substrate (9). In order to understand the physiological role of the enzyme and its peculiar properties, we decided to clone the genes and to express the corresponding proteins in a heterologous system. To our knowledge this is the first trypanosomatid ADH so far sequenced. Both its sequence and its kinetic parameters suggest that the *Phytomonas* iPDH is the result of an event of horizontal gene transfer from a strictly aerobic bacterium to a trypanosomatid. The possible physiological role of this multifunctional ADH is discussed.
EXPERIMENTAL PROCEDURES

Materials- The Phytomonas strain was isolated in France from *Euphorbia characias* (10). Phenyl-Sepharose and Blue-Sepharose were from Amersham Pharmacia Biotech. Alcohols, aldehydes, ketones, D,L-3-hydroxybutanoate, acetoacetate and *N,O-bis*- (trimethylsilyl)-trifluoroacetamide (BSTFA) were from Sigma. Restriction enzymes were from New England BioLabs. Hybond-N nylon membrane was from Amersham Pharmacia Biotech.

Purification of Enzymes- Natural iPDH was purified from 1 liter of *Phytomonas* culture as described (9). Briefly, a method was used involving hydrophobic interaction chromatography on Phenyl-Sepharose followed by affinity chromatography on Blue-Sepharose, both at pH 7.8.

Enzymes expressed in *Escherichia coli* were purified from 1 liter of bacterial culture. Cells were washed twice and resuspended in buffer A (25 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol). Extracts were prepared using a French press. Cell debris was removed by centrifugation (10,000 g). Ammonium sulfate and Triton X-100 were added to the supernatant to a final concentration of 1 M and 0.05% (w/v), respectively and the supernatant was clarified by centrifugation at 40,000 g and loaded onto a Phenyl-Sepharose column (1.4 cm x 25 cm) equilibrated in 1 M ammonium sulfate in buffer A. The column was washed with 50 ml of the same buffer, and then with 50 ml of buffer B (50 mM ammonium sulfate in buffer A) and eluted with a gradient of ethylene glycol from 0 to 70% (v/v) in buffer B (300 ml). Fractions of 7 ml were collected, and those containing activity were pooled. The ethylene glycol concentration was adjusted to 45% (v/v) before loading the pooled active fractions onto a Blue-Sepharose column (1 x 7 cm) equilibrated with ethylene glycol 45% (v/v in buffer B). The column was washed with the same buffer followed by a 60-ml gradient of NAD⁺ (0-2 mM).

Protein Determination- Protein concentration was determined by the method of Bradford (11) using bovine serum albumin as a standard.
**SDS-PAGE** - Analytical SDS-PAGE was performed in 10% (w/v) polyacrylamide gels according to Chua (12). Protein was visualized by Coomassie Brilliant Blue R-250 staining.

*Partial Amino-Acid Sequence Determination* - The mitochondrial iPDH was purified by SDS-PAGE. Bands were cut from the gel, concentrated in an agarose-based gel concentration system, and digested with trypsin (13). Peptides were analyzed by nanoelectrospray ionization-tandem MS (MS/MS). Briefly, 2 µl of the acidified and desalted trypsin digest was injected to a LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, Calif.) fitted with a nanoelectrospray probe (14). Spectra were taken in full MS and zoom scan mode to determine parent masses and their charge state. Selected peptides were fragmented by collision-induced dissociation to generate tandem mass spectra. Peptide sequences were derived *de novo* from MS/MS spectra by following the fragmentation path of y-ion series and b-ion series.

*Cloning and sequencing of iso-propanol dehydrogenase* - Available zinc-dependent medium chain ADH amino-acid sequences were aligned and two consensus sequences established. Consensus GHE(G,A)(A,G)G was used to design primer UP2 (5´-ggicaygarggigcigg), and consensus E(V,Y)FIDFT found on fungi ADHs, for primer DW1 (5´-gtraartcratiaagta). Primer UP1 (5´-gtrccsgtbccsaa) was designed on peptide P1 (DEKPVPVPK) obtained from amino-acid sequencing of purified iPDH. A DNA fragment of 600 bp was obtained by PCR on genomic DNA from *Phytomonas*, using primers UP1 and DW1. The fragment was cloned in the pGEM-T Easy vector (Promega), sequenced and used as a hybridization probe to a Southern Blot, carried out with Hybond-N membranes on 4 µg of total DNA from *Phytomonas* sp. digested with different restriction enzymes. A limited genomic library was made with *Phytomonas* total DNA digested with *Pst*I. After electrophoresis in an agarose gel, a portion of the gel corresponding to the region of 3-4 kb was cut, based on
Southern Blot information, and DNA recovered by standard procedures (15). Recovered genomic fragments were ligated to vector pUC18 digested with PstI and introduced into *E. coli* XL1 Blue by electroporation. 378 colonies were screened by colony hybridization (15) with the same probe. Two positive clones (215 and 345) were identified and isolated. Plasmid DNA was obtained from them, digested with different restriction enzymes and subjected to Southern Blot analysis using the same probe. Plasmids from clones 215 and 345 were totally or partially sequenced, respectively.

**Determination of iPDH Isoenzyme Number**—Based on bacterial and *Phytononas* protein ADH sequence multiple aligment, two highly conserved peptide sequences were selected. The corresponding DNA sequences were used to design oligonucleotides UP3 (5′-atyacsaccaccac) and DW2 (5′-cgcatgcgytcctt). Amplification of genomic DNA from *Phytononas* was performed with these primers using total DNA from *Sinorhizobium meliloti* as a positive control and *T. brucei* and *E. coli* DNA as negative controls. Amplified fragments from *Phytononas* of approximately 800 bp were cloned in pGEM-T Easy vector and eight clones were totally sequenced. Sequences were compared with those from clones 215 and 345.

**Phylogenetic Analyses**—Available zinc-dependent medium-chain ADH protein sequences were aligned using the program ClustalX (16). Positions with gaps were removed, and pairwise distances were calculated using a correction for multiple hits and back mutations according to Kimura (17) as implemented in the Protdist program (18). Phylogenetic analyses were carried out by neighbor-joining (NJ) method, using the programs Neighbor and Consense (18) with 10,000 bootstrap samplings, or by maximum likelihood with 1000 quartets puzzle steps using the program TreePuzzle 5.0 (19) and the JTT matrix as the evolutionary model. Both methods gave similar, but not identical trees topologies.
Expression in *E. coli*-Iso-propanol dehydrogenase genes were amplified by PCR from clones 215 and 345 DNA using the oligonucleotide 5´-ggaattccatatgcttcgccgttccacc, which contains a 5´ *NdeI* artificial restriction site (underlined) followed by 18 nucleotides that correspond to the 5´end of the genes, and oligonucleotide 5´-cgggatccttatggcttgatagctacctt, which has 21 nucleotides complementary to the 3´end including the TAA stop codon and an artificial *BamHI* restriction site. The amplified DNA fragments were cloned in the pT7-7.1 expression vector (20) digested with *NdeI* and *BamHI*. The sequences were verified before introduction into *E. coli* BL-21 (DE3) containing the plasmid pCRF1 (21). Transformed cells were grown at 30ºC in Luria Bertani medium (15) supplemented with ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml). Expression was induced at an A600nm of 0.6 by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mM and growth was continued overnight.

**Kinetic Characterization**-Dehydrogenases were assayed spectrophotometrically by following the ketone-dependent oxidation of NADH or the alcohol-dependent reduction of NAD⁺ at 340 nm and 25 ºC. Optimal pH and buffers for forward and reverse reactions were as described previously (9). One unit of activity is the amount of enzyme catalyzing the conversion of 1 µmol of substrate/min at 25 ºC. Relative specific activity with different substrates was referred to that with *D*-2,3-butanediol for the forward reaction and that with acetoin for the reverse reaction.

**GC-Mass Analysis**-NADH-dependent reduction of acetoacetate (1 mM initial concentration) was followed spectrophotometrically, as indicated before, up to exhaustion of the substrate. The reaction mixture (1 ml) was acidified with 200 µl of 3N HCl and extracted twice with one volume of ethylacetate. Organic phases were collected and dried under a stream nitrogen. Pyridine (100 µl) and BSTFA (100 µl) were added and incubated
at 80°C for 1 h. One µl was injected into a Perkin Elmer AutoSystem XL gas chromatograph equipped with a PE1 column (30m x 0.25 mm internal diameter, Perkin Elmer). Gas chromatographic analysis was performed at 180 °C isothermically. GC-MS was carried out using a Perkin Elmer mass detector (model TurboMass) operated at an ionization voltage of 70 eV with a scan range of 20-500 Da. Retention time and mass spectrum of peaks were compared with that of standards (Sigma) and information available in the data base NBS75K (National Bureau of Standards).
RESULTS

Partial Amino-Acid Sequence Determination of Purified Mitochondrial iPDH-
Mitochondrial iPDH was purified from *Phytomonas* cells as described (9). Following gel purification, concentration in agarose and trypsin digestion, peptides were analyzed by nanoelectrospray ionization-MS/MS. This allowed the sequencing of two tryptic peptides (P1 and P2 in Fig. 3). In an initial search of the GenBank™ database, only peptide P1 showed similarity with ADHs, specially with that from yeasts (Fig. 1).

Isolation and Sequence of Allelic Variants of iPDH-Our previous work suggested that *Phytomonas* iPDH belongs to the zinc-dependent medium-chain ADH family. This was based on our previous estimation of the subunit relative molecular mass of the purified enzyme (37-40 kDa), its absolute dependence on NAD(H) for enzymatic activity, and the inhibitory effect of Hg$^{2+}$ (9). Moreover, 2 mM pyrazole completely inhibited the enzyme with a $IC_{50}$ of 67 µM. Culture media containing 50-100 mM ZnSO$_4$ stimulated the expression of iPDH in *Phytomonas* (data not shown). These properties are characteristic of zinc-dependent ADHs (22). Based on this information, a multiple sequence alignment was created with available protein sequences of zinc-dependent ADHs from plants, bacteria, vertebrates and yeasts. This allowed us to determine two consensus sequences useful for the design of degenerate oligonucleotides (UP2 and DW1). A partial alignment (Fig. 1), indicates the position of these consensus sequences and the region of similarity with peptide P1 on which primer UP1 was based. PCR amplifications on genomic *Phytomonas* DNA were carried out using these primers. Amplification of a 600-bp fragment was possible only when using UP1 and DW1 as primers. Sequencing the resulting fragment revealed a high similarity with ADHs from a small group of bacteria. This fragment was used as a hybridization probe in a Southern Blot of *Phytomonas* genomic DNA. As shown in Fig. 2A, the
putative ADH appears to be present in *Phytomonas* in one copy per haploid genome and is contained in a *PstI* restriction fragment of 3.5 kb. A limited size library was made using *PstI* restricted genomic DNA and screened using the same probe. Two positive clones with identical restriction patterns (Fig. 2B) were isolated. The *PstI* insert of clone 215 was completely sequenced and contains 3493 bp. Only one open-reading frame (ORF) of 1086 bp was detected, which encodes a putative protein of 361 amino acids (allele 1 or iPDH1). A hypothetical N-terminal mitochondrial transit peptide was also detected (Fig. 3). This peptide contains 13 amino acids and has a high content of basic amino acids and a high degree of similarity with other mitochondrial transit peptides reported for trypanosomatids (23). A central region of the 3.5 kb insert of clone 345 (from the first *NdeI* to the second *EcoRI* site, Fig. 2B) was sequenced. It contained an ORF that is 99% identical to that present in clone 215. The inferred protein (iPDH2) differs only in one amino acid, lysine instead of the glutamic acid at position 60 in iPDH1 (Fig. 3). When the trypanosomatid sequences were compared with the bacterial ADHs, a high degree of similarity was always observed starting from the methionine at position 14 in the iPDHs. Assuming that Met14 is the N-terminus of the mature proteins, the calculated isoelectric points are 6.7 and 7.5 for iPDH1 and iPDH2, respectively, with a molecular mass of 37 kDa. This is in good agreement with the estimated *M*<sub>r</sub> by SDS-PAGE and with *pI* values obtained for the three dimeric isoenzymes previously isolated from crude extracts of *Phytomonas* sp. (9).

In order to confirm the copy number of the iPDH genes, two highly conserved regions of bacterial and *Phytomonas* ADHs were used to design oligonucleotides UP3 and DW2 (Fig. 3). After PCR amplification on genomic DNA from *Phytomonas* and cloning of a 800-bp fragment, eight clones were chosen at random and sequenced. Five clones contained a sequence identical to that of allele 1 and three others were identical to allele 2. This supports the notion that only two allelic variants of iPDH are present in the genome of *Phytomonas* sp.
**Phylogenetic Relationship Between iPDH and Zinc-Dependent Alcohol Dehydrogenases**

The multiple alignment containing 19 sequences served to create a phylogenetic tree using both distance and maximum likelihood methods (Fig. 4). The *Phytomonas* iPDH appeared to be robustly monophyletic with the ADHs from *Mycoplasma penetrans* and several proteobacteria with which it shared between 60 and 66% identical residues. This clustering is supported by the fact that the *Phytomonas* sequence shared with the other members of this clade the presence of a unique (Thr)$_4$ tract at positions 67-70 in the alignment, as well as a deletion at position 78. The presence of the *Phytomonas* sequence within this bacterial clade can only be explained by a horizontal gene transfer event. Interestingly, a similar event of horizontal gene transfer can be observed in the other robust clade (100% supported by bootstrap analysis) of (facultative) anaerobes. Here again, a prokaryote must have provided the ADH1 gene for the anaerobic protists *Trichomonas vaginalis* and *Entamoeba histolytica*. The two protist enzymes, which share 77% identical residues, also share between 60 and 66% identical residues with the bacterial ADH sequence of this clade.

**Expression in *E. coli* and Purification of iPDHs from bacteria** - The iPDHs derived from clones 215 (iPDH1) and 345 (iPDH2) were ligated into vector pT7-7.1 and expressed in *E. coli*. We used an *E. coli* strain harboring the plasmid pCRF1, which allows the co-expression of the GroESL chaperonin system from *Chromatium vinosum* (21). Although 80% of the expressed protein was trapped in inclusion bodies, a 2-fold increase in specific activity of iPDH was measured in crude extracts of transformed *E. coli/pCRF1* as compared to commonly used *E. coli* expression strains.

**Enzymatic Characterization of Bacterially Expressed and Natural iPDH-Enzymes**

Enzymes purified from *E. coli* presented specific activities of 9.7 and 12.5 U per mg of protein for iPDH1
and iPDH2, respectively. Activity with ethanol for iPDH1 and iPDH2 was 20 ± 2 and 11 ± 2 -fold less than with iso-propanol, respectively. These respective ratios were highly reproducible and therefore could be useful in isoenzyme identification. For the enzyme purified from cultured Phytomonas, the ratio of iso-propanol/ethanol activity was much more variable and ranged from 15 to 21, which suggests a variable expression level of one or both alleles.

The relative specific activity with and affinity for different substrates for both natural and bacterially-expressed iPDHs were compared with the data available from the multifunctional fermentative ADH of R. eutropha (5). As shown in Table 1, a similar pattern of relative activities with the various substrates was found for the Phytomonas and R. eutropha enzymes. Only with acetone as substrate, significant differences were found for the reverse reaction, with iPDH showing the higher relative activity. The Phytomonas enzymes also appear to oxidize acetaldehyde, indicating that iPDH is a multifunctional enzyme able to dismutate this substrate, similar to what has been described for the R. eutropha ADH (5).

The apparent affinities for the various substrates, although more variable, are still comparable. The differences were larger for acetaldehyde reduction and ethanol oxidation with 6-fold higher $K_m$s for iPDH purified from the parasites (Table 2). Unfortunately no data are available for the affinities for acetone or iso-propanol of the R. eutropha ADH to extend the comparison. The Phytomonas enzymes have the lowest $K_m$s for these compounds, indicating that they are the preferred substrates for the reverse and the forward reaction, respectively.

The enzymatic properties for the Phytomonas and bacterially-expressed iPDHs compared very well. Relative activity and affinity of iPDH from the parasite for the different substrates were close to the averaged values for both isoforms produced by the bacteria. It is interesting to note that the heterologously expressed enzymes correspond to homodimers of the respective putative pre-iPDHs and not the mature enzymes. On the other hand, the enzyme purified from
Phytomonas will, most likely, represent a mixture of homodimers and heterodimers of mature polypeptides.

Our finding that the apparent rate of acetoacetate reduction was similar to that observed for acetone (Table 1) was further studied by analyzing the products of the reaction by GC-MS. The expected reaction product, 3-hydroxybutanoate, could not be detected after 1-6 h of incubation, while a progressive loss of acetoacetate did occur. On the other hand, 3-hydroxybutanoate was not a substrate for the oxidation reaction (Table 1). This indicates that the apparent oxidation of NADH by iPDH with acetoacetate as substrate is most likely due to the spontaneous decarboxylation of acetoacetate, a reaction which is strongly stimulated by the presence of proteins (24), followed by a further reduction of the resulting acetone to iso-propanol.
DISCUSSION

We have cloned and sequenced the two allelic variants of the *Phytomonas* iPDH. Sequence analysis indicates that the encoded proteins belong to the extended class of zinc-dependent medium-chain alcohol dehydrogenases (2). The deduced amino-acid sequences reveal a N-terminal mitochondrial transit peptide, in agreement with our previously observed mitochondrial localization of the enzyme (9). Based on sequence similarity with other ADHs and with other mitochondrial targeting signals, we propose that Met14 is the most likely N-terminus for the mature polypeptides. The two putative homodimers have a *Mr* of 74 kDa and a *pI* of 6.7 and 7.5, respectively, and the heterodimer is expected to have a *pI* close to the average of the latter two values. Although we have no direct evidence for the precise N-terminus of the mature protein, the predicted *Mr* and *pI* are in good agreement with the experimentally observed *Mr* and *pI* of the three dimeric isoenzymes found previously in *Phytomonas* (9).

The phylogenetic analysis indicates that *Phytomonas* iPDH belongs to a small group of enzymes sharing amino-acid similarities of 76 to 74 % and identities of 62-58 % with ADHs of strict aerobic bacteria from Pseudomonad, Rhizobial and Burkholderial families. The substrate profile and kinetic parameters are very similar to that of the *R. eutropha* fermentative ADH, which is so far the only characterized enzyme from this group of bacterial ADHs (5). Zinc-dependent enzymes are specific for either primary or secondary alcohols. However, *R. eutropha* fermentative ADH and *Phytomonas* iPDH are multifunctional enzymes able to use both primary and secondary alcohols, polyols, aldehydes and ketones. They dismutate acetaldehyde as well but its rate of reduction is one order of magnitude higher than its rate of oxidation. Amino acids involved in substrate and NADH binding are conserved with respect to other zinc-dependent ADHs (22). Interestingly, residues related to the coordination of the catalytic and structural Zn$^{2+}$ are highly conserved in iPDH, but not in *R. eutropha* ADH, where only those residues involved
to the coordination of the catalytic Zn\(^{2+}\) are present. Both enzymes possess a Ile-(Thr)\(_4\)-Ile amino-acid motif immediately in front of the conserved Cys50 in the substrate-binding domain (Fig. 3). This motif is only present in ADHs belonging to this phylogenetic group. In a preliminary structural study, this threonine tract appears to replace a region of hydrophobic amino acids at the bottom of the active site cleft present in other ADHs (22, 25). The binding of longer secondary alcohols or polyols could be stabilized by hydrogen bonds between threonines and functional groups on the substrates, justifying the broad pattern of substrate specificity which is characteristic for these multifunctional ADHs.

The iPDH amino-acid sequence and its kinetic properties suggest that this enzyme originated in the genus *Phytomonas* most likely as the result of a horizontal gene transfer between a strictly aerobic bacterium as donor organism and an ancestral trypanosomatid as recipient. The fact that this enzyme is now found in all phytomonads analyzed thus far, but not in any other representative of the trypanosomatid family (26), suggests that the acquisition of this gene may have resulted in a selective advantage for a trypanosomatid living in an environment as is present in the phloemic or laticiferous tubes of colonized plants. In this respect it is interesting to note that the two sister taxa of *Phytomonas* are *Sinorhizobium meliloti*, fixing nitrogen for the legumes alfalfa, sweet clover and fenugree, and *Pseudomonas fluorescens*, a plant-growth promoting bacterium that colonizes both leaves and roots of plants. Thus, gene transfer may have been facilitated by a close contact within a common host organism between these plant-colonizing bacteria and the *Phytomonas* ancestor. A similar explanation can be put forward for the appearance of the *Mycoplasma penetrans* ADH within this clade, whereas *M. pneumoniae* clusters with the Clostridia, as expected. Mycoplasmas are well known obligate parasites of eukaryotes, including plants, and could have picked up the ADH gene from the same or another plant-colonizing bacterium, as did the *Phytomonas* ancestor.
iPDH activity was present in 20 isolates of *Phytomonas*, while the enzyme was absent from 17 non-phytomonad species of trypanosomatids which belonged to the genera *Trypanosoma, Leishmania, Leptomonas, Crithidia, Blastocrithidia, and Herpetomonas* (26). This renders iPDH an interesting biochemical marker for the *Phytomonas* genus. Due to the high DNA sequence identity (60%) between iPDH and *R. eutropha* ADH, we expect an even higher percentage of identical residues between iPDH genes from different *Phytomonas* isolates. This would allow a detailed phylogenetic analysis of the genus, as a first approach to establish *Phytomonas* as a species. Work in this direction is in progress.

*Phytomonads* are highly dependent on glycolysis. We have previously shown that they excrete hydrolytic enzymes involved in the degradation of polysaccharide constituents of plants and consume the resulting monosaccharides at a high rate (27). This high glycolytic rate is forced upon the organism by the absence of oxidative phosphorylation as the result of a complete repression of mitochondrial activity. Cytochromes cannot be detected and a functional Krebs’ cycle is missing (27, 28). This high glycolytic rate can only be maintained by an effective regeneration of oxidative power. A mitochondrial cyanide-insensitive glycerol-3-phosphate oxidase is involved in the reoxidation of glycolytic NADH and most likely the iPDH fulfills a similar role in the mitochondrion. In the strictly aerobic *R. eutropha*, the fermentative ADH has the function to regenerate oxidative power in the form of NAD⁺. This is done by the reduction of acetoin produced from pyruvate under limited oxygen supply, and by reduction of acetaldehyde formed from the accumulated acetyl CoA (5). Under these conditions in *R. eutropha* the expression of the enzyme is induced. In *Phytomonas*, oxidative phosphorylation is permanently suppressed, and iPDH is constitutive. As shown previously, one third of the pyruvate is converted to ethanol under aerobic conditions; the remainder is transformed into acetate (29). Apparently the majority of the pyruvate produced from glycolysis enters the mitochondrion
where it is converted to acetyl CoA with concomitant reduction of NAD$^+$ (27, 29). Acetyl CoA may provide an additional molecule of ATP by passing through the acetate:succinate CoA transferase/succinyl CoA synthetase cycle (30). A minor part of the pyruvate is converted to acetaldehyde by a putative pyruvate decarboxylase. The subsequent reduction of the acetaldehyde by iPDH, producing ethanol as an end product of glycolysis, would regenerate part of the NAD$^+$ consumed by the pyruvate dehydrogenase. Under anaerobic conditions essentially all pyruvate is converted to ethanol. When energy sources are non-limiting, excess of acetyl CoA could be converted to acetoacetate which is known to decarboxylate spontaneously to acetone. The latter can then be reduced to iso-propanol by iPDH. For *Phytomonas* iPDH, in contrast to *R. eutropha* ADH, acetone appears to be the best substrate for the reverse reaction, with a $V_{max}$ of 6.8 U/mg and a $K_M$ of 32 µM.

Another potential source of acetone could be the acetoacetate produced by amino-acid catabolism. Although virtually nothing is known about amino-acid metabolism in *Phytomonas*, amino acids are considered to be the principal energy source for the parasite inside the insect vector, due to the lack of carbohydrates in this host. In other trypanosomatids, proline serves as the major energy source for the insect stage, however this amino acid is metabolized via the Krebs’ cycle (31). Since *Phytomonas* lacks a functional Krebs’ cycle, it is expected that in this organism other amino acids than proline fulfil the role of energy substrate. In preliminary experiments, we have found evidence that the ketogenic amino acids leucine, lysine and phenylalanine are able to support growth of *Phytomonas*. If in *Phytomonas* these amino acids are catabolized in a way similar to other eukaryotes (32), iPDH could be essential for the elimination of the resulting acetone with the concomitant regeneration of oxidative power.
Aknowledgments—We wish to thank Mónica Hourcade, Dominique Cottem and Joris van Roy for technical assistance. ADU is member of Carrera del Investigador Científico, CONICET, Argentina. This work was partially supported by Fondo Nacional de Ciencia y Tecnología, FONCyT, SECyT, Argentina, grant PICT 99 Nº 1-7160 and, Ministerio de Educación de la Nación, Argentina, FOMEC 138, by the Fund for Medical Scientific Research (Belgium), by the Belgian Directorate General for Higher Education and Scientific Research.
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FOOTNOTES:

The nucleotide sequence reported in this paper has been submitted to the GenBank™ and were assigned GenBank Accession Number AY283263.

¹The abbreviations used are: ADHs, alcohol dehydrogenases; iPDH, iso-propanol dehydrogenase; PCR, polymerase-chain reaction; ORF, open-reading frame; PAGE, polyacrylamide gel electrophoresis; BSTFA, N,O-bis-(trimethylsilyl)-trifluoroacetamide; MS/MS, tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry.
FIGURE LEGENDS

Fig. 1. Multiple alignment of zinc-dependent ADHs. A limited number of available sequences and only the relevant regions of high similarity are shown. P1 indicates tryptic peptide P1, obtained from purified Phytomonas iPDH; UP1, UP2 and DW1 indicate regions used to design degenerate oligonucleotides. Scer, Saccharomyces cerevisiae ADH2 (GenBank accession number P00331); Spom, Schizosaccharomyces pombe ADH (P00332); Tbro, Thermoanaerobacter brockii ADH (P14941); Cbei, Clostridium beijerinckii (P25984); Bste, Bacillus stearothermophilus ADH3 (P42328); Atha, Arabidopsis thaliana (P06525); Zmay, Zea mays ADH1 (AAF43977.1); Hsap, Homo sapiens ADH A (P07327).

Fig. 2. Southern blot analysis and restriction map of the iPDH genomic region. A, Phytomonas total DNA was digested with the indicated restriction enzymes. After electrophoresis in an agarose gel followed by Southern Blotting, membranes were hybridized with a radiolabeled probe consisting of a 600 bp fragment obtained by PCR on genomic DNA using oligonucleotides UP1 and DW1. B, restriction map of the 3.5 kb PstI fragment containing iPDH. The gray rectangle indicates the ORF coding for iPDH. The black bar indicates the 600 bp fragment used as a probe. A, Aval; E, EcoRI; H, HindIII; Hc, HincII; K, KpnI; N, NdeI; P, PstI; S, SalI; Sc, SacI; X, XhoI.

Fig. 3. Alignment of Phytomonas iPDH and bacterial ADHs. The putative mitochondrial targeting signal is in bold. Consensus sequences used to design degenerate oligonucleotides UP3 and DW2 are underlined. Position of peptides P1 and P2 are indicated, as the E-K amino-acid substitution at position 60 between iPDH1 and iPDH2, respectively; (*) indicates the catalytic
zinc-coordinating amino acids, (+) those coordinating the structural zinc; (·) moieties related to substrate binding, (^) those related to cosubstrate binding.

**Fig. 4. Multiple alignment and phylogenetic tree of ADHs.** A multiple alignment of homologous ADH sequences was constructed using the *Phytomonas* iPDH, 17 sequences which were most closely related to the iPDH in a BlastP search and the sequence of *Methylobacter marinus* formaldehyde dehydrogenase. A, a section from the multiple alignment containing the (Thr)4 tract (boxed) as present in the *Phytomonas* iPDH and its 8 homologous sequences. B, Maximum likelihood and neighbor-joining trees constructed from the complete alignment from which all regions with insertions and deletions were removed. The tree was rooted using the sequence of *Methylobacter marinus* formaldehyde dehydrogenase as an outgroup. The horizontal bar represents 10 accepted amino-acid substitutions per 100 residues. Numbers at the nodes represent quartet-puzzle frequencies (ML, 1000 quartet-puzzling steps) and bootstrap values (NJ, 10,000 samplings), respectively. In the case only one value is given at a branch point this means that the branch point was not the preferred one in NJ. ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; TDH, threonine dehydrogenase; FDH, formaldehyde dehydrogenase.
TABLE I
Comparison of relative activities between *R. eutropha* ADH, iPDH purified from *Phytonomas* and bacterially expressed iPDHs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. eutropha</em> ADH</td>
</tr>
<tr>
<td><strong>Reduction:</strong></td>
<td></td>
</tr>
<tr>
<td>Acetoin</td>
<td>100</td>
</tr>
<tr>
<td>Acetone</td>
<td>46.4</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>133</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>118.6</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>-</td>
</tr>
<tr>
<td><strong>Oxidation:</strong></td>
<td></td>
</tr>
<tr>
<td>D-2,3-butanediol</td>
<td>100</td>
</tr>
<tr>
<td>n-butanol</td>
<td>0.3</td>
</tr>
<tr>
<td>Sec-butanol</td>
<td>257.2</td>
</tr>
<tr>
<td>Iso-butanol</td>
<td>9</td>
</tr>
<tr>
<td>n-propanol</td>
<td>8.3</td>
</tr>
<tr>
<td>Iso-propanol</td>
<td>114.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.9</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.4</td>
</tr>
<tr>
<td>D,L-3-HO-butanoate</td>
<td>0</td>
</tr>
<tr>
<td>Acetoin</td>
<td>0</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>18</td>
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</table>
TABLE II
Comparison of apparent substrate affinities between *R. eutropha* ADH, iPDH purified from *Phytomonas* and bacterially expressed iPDHs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent $K_M$ (mM)</th>
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<tbody>
<tr>
<td></td>
<td><em>R. eutropha</em> ADH</td>
</tr>
<tr>
<td>Reduction:</td>
<td></td>
</tr>
<tr>
<td>Acetoin</td>
<td>0.93</td>
</tr>
<tr>
<td>Acetone</td>
<td>-</td>
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<tr>
<td>Diacetyl</td>
<td>0.56</td>
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<tr>
<td>Acetaldehyde</td>
<td>0.67</td>
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<tr>
<td>Oxidation:</td>
<td></td>
</tr>
<tr>
<td><em>D</em>-2,3-butanediol</td>
<td>22.2-18.2</td>
</tr>
<tr>
<td><em>Iso</em>-propanol</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5</td>
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Figure 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>SCER</th>
<th>SPOM</th>
<th>TBRO</th>
<th>CBEI</th>
<th>BSTE</th>
<th>ATHA</th>
<th>ZMAY</th>
<th>HSAP</th>
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<tbody>
<tr>
<td><strong>UP1</strong></td>
<td>KLEHKDIPVFVKPKNELLINVKYSGVCHTDLH.49</td>
<td>NVKFEEVPVAEPQDEVLVNIKYTGVCHTDLH.51</td>
<td>-----EKRPAPGPDATIPRLAVAPCTSDIH.42</td>
<td>-----EKERPVAGSYDAIVRPLAVSPECTSDIH.42</td>
<td>-----EKERPVAGSYDAIVRPLAVSPECTSDIH.42</td>
<td>-----EKERPVAGSYDAIVRPLAVSPECTSDIH.42</td>
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<td>-----EKERPVAGSYDAIVRPLAVSPECTSDIH.42</td>
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<tr>
<td><strong>UP2</strong></td>
<td>PLPTKLPLVGGHEGAGVVVGMGENVKGKWIGDYA.89</td>
<td>PLPAKLPLIGGHEGAGVVVGMGENVKGKWIGDYA.89</td>
<td>AIGERHNMILGHEAVGEVEVGSEVFKPKPDGV.81</td>
<td>ALGDRKMNILGHEAVGEVEVGSEVFKPKPDGV.81</td>
<td>ALGDRKMNILGHEAVGEVEVGSEVFKPKPDGV.81</td>
<td>ALGDRKMNILGHEAVGEVEVGSEVFKPKPDGV.81</td>
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<td>ALGDRKMNILGHEAVGEVEVGSEVFKPKPDGV.81</td>
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<tr>
<td><strong>DW1</strong></td>
<td>SLAVQYAKAME-YVRLGDGGPGEELFTSLGC.52</td>
<td>HLAVQYAKAMAM-RVVAIDTGGDAELVSKSGAEVFLDFKK-EADMIEAVKAATNG-GAHGTLVLSFK.253</td>
<td>LMAVAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
<td>LMGIAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
<td>LMGIAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
<td>LMGIAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
<td>LMGIAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
<td>LMGIAGAKLRGRGIAAVGRSPVCAAKYYGATDILVNYK--GPIESQIMNLTEKGDVAIAGKD.247</td>
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Figure 2

A

B
Figure 3

<table>
<thead>
<tr>
<th>P1</th>
<th>DEKPVPVPK</th>
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<tr>
<td>iPDH2</td>
<td>M-</td>
</tr>
<tr>
<td>iPDH1</td>
<td>MLRRSTLRCVPKT</td>
</tr>
<tr>
<td>R.eutropha</td>
<td>M-</td>
</tr>
<tr>
<td>S.meliloti</td>
<td>M-</td>
</tr>
<tr>
<td>R.eutropha</td>
<td>IIEKLGSAVTGYREGQRVIAAGAICPFNSYAAQDGVAQDSYLMASGQCCHGYKATAGWRFNMDGTQAEYV</td>
</tr>
<tr>
<td>S.meliloti</td>
<td>VIEKLGSAVRGSEQRVIAAGAICTSGHSNAALCGCHAQDGPGKT-</td>
</tr>
<tr>
<td>iPDH1</td>
<td>IVPDAAANMYPIPDGITDEQVLMCPIPMSTGFSGPESAGVKVDTVVIYAQGPIGLGATAAGKMGATKIVVDR</td>
</tr>
<tr>
<td>R.eutropha</td>
<td>IVPDAAANMYPIPDGITDEQVLMCPIPMSTGFSGPESAGVKVDTVVIYAQGPIGLGATAAGKMGATKIVVDR</td>
</tr>
<tr>
<td>S.meliloti</td>
<td>IVPDAAANMYPIPDGITDEQVLMCPIPMSTGFSGPESAGVKVDTVVIYAQGPIGLGATAAGKMGATKIVVDR</td>
</tr>
<tr>
<td>iPDH1</td>
<td>FPERLALAKKGADAYLDFTKCPNOPIEVEMLTGGVDAIEALQLQSTFESCLRCLPGGVSGLGVYSDDLR</td>
</tr>
<tr>
<td>R.eutropha</td>
<td>FPERLALAKKGADAYLDFTKCPNOPIEVEMLTGGVDAIEALQLQSTFESCLRCLPGGVSGLGVYSDDLR</td>
</tr>
<tr>
<td>S.meliloti</td>
<td>FPERLALAKKGADAYLDFTKCPNOPIEVEMLTGGVDAIEALQLQSTFESCLRCLPGGVSGLGVYSDDLR</td>
</tr>
<tr>
<td>P2</td>
<td>LPNDAAAGLDFK</td>
</tr>
<tr>
<td>iPDH1</td>
<td>LPNDAAAGLDFKIVTTLCPGGKERMRLSSVIESGRVMDRPMVHTFTKLDIEIEKAYDLFNGQRDGVLKVAIKP-</td>
</tr>
<tr>
<td>R.eutropha</td>
<td>LPNDAAAGLDFKIVTTLCPGGKERMRLSSVIESGRVMDRPMVHTFTKLDIEIEKAYDLFNGQRDGVLKVAIKP-</td>
</tr>
<tr>
<td>S.meliloti</td>
<td>LPNDAAAGLDFKIVTTLCPGGKERMRLSSVIESGRVMDRPMVHTFTKLDIEIEKAYDLFNGQRDGVLKVAIKP-</td>
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</table>
Figure 4

ADH T. brockii
ADH T. ethanolicus
ADH T. tengcongensis
ADH C. beijerinckii
ADH M. aceticivorans
ADH T. vaginalis
ADH M. pneumoniae
ADH X. fastidiosa
ADH P. fluorescens
ADH S. meliloti
IPDH Phytomonas sp.
ADH A. vinelandii
ADH R. metallidurans
ADH P. aeruginosa
ADH B. fungorum
ADH R. eutropha
ADH M. penetrans
FDH M. marinus

ADH T. brockii
ADH T. ethanolicus
ADH T. tengcongensis
ADH C. beijerinckii
ADH M. aceticivorans
ADH T. vaginalis
ADH M. pneumoniae
ADH X. fastidiosa
ADH P. fluorescens
ADH S. meliloti
IPDH Phytomonas sp.
ADH A. vinelandii
ADH R. metallidurans
ADH P. aeruginosa
ADH B. fungorum
ADH R. eutropha
ADH M. penetrans
FDH M. marinus

ADH Thermoanaerobacter brockii
ADH Thermoanaerobacter ethanolicus
ADH Thermoanaerobacter tengcongensis
ADH Clostridium beijerinckii
ADH Methanosarcina acetivorans
ADH Mycoplasma pneumoniae
ADH Enthamoeba histolytica
ADH Trichomonas vaginalis
ADH Xylella fastidiosa

ADH Pseudomonas fluorescens
ADH Sinorhizobium meliloti
IPDH Phytomonas sp.
ADH Azotobacter vinelandii
ADH Raistonia metalldurans
ADH Pseudomonas aeruginosa
ADH Burkholderia fungorum
ADH Raistonia eutropha
ADH Mycoplasma penetrans
FDH Methylobacter marinus
The multifunctional iso-propanol dehydrogenase of phytomonas sp. could be the result of a horizontal gene transfer from a bacterium to the trypanosomatid lineage
Sara M. Molinas, Silvia G. Altabe, Fred R. Oppendoes, Mark H. Rider, Paul A. M. Michels and Antonio D. Uttaro

J. Biol. Chem. published online July 9, 2003

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