Infection, Genetics and Evolution 12 (2012) 539–548

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Horizontal gene transfer confers fermentative metabolism in the respiratory-deficient plant trypanosomatid *Phytomonas serpens*

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ARTICLE INFO

Article history:
Received 8 August 2011
Received in revised form 11 January 2012
Accepted 14 January 2012
Available online 25 January 2012

Keywords:
Trypanosomatids
Genealogy
Pyruvate decarboxylase
Horizontal gene transfer
γ-Proteobacteria
Ethanol
NADH oxidation

ABSTRACT

Among trypanosomatids, the genus *Phytomonas* is the only one specifically adapted to infect plants. These hosts provide a particular habitat with a plentiful supply of carbohydrates. *Phytomonas* sp. lacks a cytochrome-mediated respiratory chain and Krebs cycle, and ATP production relies predominantly on glycolysis. We have characterised the complete gene encoding a putative pyruvate/indolepyruvate decarboxylase (PDC/IPDC) (548 amino acids) of *P. serpens*, that displays high amino acid sequence similarity with phytobacteria and *Leishmania* enzymes. No orthologous *PDC/IPDC* genes were found in *Trypanosoma cruzi* or *T. brucei*. Conservation of the *PDC/IPDC* gene sequence was verified in 14 *Phytomonas* isolates. A phylogenetic analysis shows that *Phytomonas* protein is robustly monophyletic with *Leishmania* spp. and *C. fasciculata* enzymes. In the trees this clade appears as a sister group of indolepyruvate decarboxylases of γ-proteobacteria. This supports the proposition that a horizontal gene transfer event from a donor phytobacteria to a recipient ancestral trypanosome has occurred prior to the separation between *Phytomonas*, *Leishmania* and *Crithidia*. We have measured the PDC activity in *P. serpens* cell extracts. The enzyme has a Km value for pyruvate of 1.4 mM. The acquisition of a PDC, a key enzyme in alcoholic fermentation, explains earlier observations that ethanol is one of the major end-products of glucose catabolism under aerobic and anaerobic conditions. This represents an alternative and necessary route to reoxidise part of the NADH produced in the highly demanding glycolytic pathway and highlights the importance of this type of event in metabolic adaptation.

1. Introduction

Kinetoplastids are a remarkable group of protists that includes pathogens of invertebrates, vertebrates and even plants. The genus *Phytomonas* designates trypanosomatids found in latex, phloem, fruits and seeds of different plant species with a wide geographical distribution (reviewed by Dollet, 1984; Camargo, 1999). *Phytomonas* are thought to be transmitted by phytophagous insects and, accordingly, are classified as dixenous trypanosomatids.

Besides *Phytomonas* other trypanosomatids of the *Leptomonas*, *Herpetomonas* and *Crithidia* genera have been isolated from plants (Conchon et al., 1989). These monogenetic organisms are insect-specific and their presence in fruits, seeds and flowers results from accidental inoculation into the insect feeding sites (Conchon et al., 1989; Jankevicius et al., 1989; Serrano et al., 1999a; Fiorini et al., 2001). Genetic markers allow the distinction of the *Phytomonas* genus from the monogenetic insect kinetoplastids (Teixeira et al., 1996; Muller et al., 1997; Serrano et al., 1999b; Sturm et al., 2007).

Multiple horizontal gene transfer (HGT) events have been implicated in the acquisition of structural and biochemical peculiarities of the trypanosomatids that allowed the adaptation of these organisms to a vast range of different hosts (Opperdoes and Michels, 2007). The authors speculate that these genes were probably originated from bacterial donors that could have been endosymbionts or organisms resident in the midgut of an insect host.

Accordingly, profound metabolic adaptations are expected to permit *Phytomonas* to thrive in the phloem and laticiferous tubes of host plants. Due to the facility of *in vitro* cultivation, some characteristics of *Phytomonas* metabolism have been investigated in detail for the tomato isolate *Phytomonas serpens*. These studies concluded that several mitochondrial functions, such as cytochrome-mediated respiration, ATP production and Krebs cycle, are missing, and cell energetics are based predominantly on glycolysis (Maslov et al., 1999; Nawathean and Maslov, 2000). Another distinctive characteristic of *Phytomonas*, shared only by *Crithidia*, is ethanol production

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doi:10.1016/j.meegid.2012.01.016

as one of the major end-products of glycolysis (Cazzulo et al., 1985; Chaumont et al., 1994).

Little genomic and proteomic information regarding members of the genus *Phytomonas* is available. As a first step toward the definition of the *Phytomonas* genome, the molecular karyotypes of isolates from latex and phloem have been reported (Marín et al., 2008, 2009). Aiming to expand basic knowledge on the biology and metabolism of *Phytomonas*, our group undertook a moderate gene survey by means of the generation, sequencing and analysis of *P. serpens* expressed sequence tags (ESTs) (Pappas et al., 2005). A number of enzymes of metabolic pathways were identified, as well as several ESTs that can shed some light on physiological aspects of the parasite lifestyle.

The 540-bp consensus sequence of one EST cluster (cluster 53) showed high sequence similarity at the amino acid level with pyruvate/indole-pyruvate decarboxylases of phytobacteria. Pyruvate decarboxylases (PDC; EC 4.1.1.1) are key enzymes in alcoholic fermentation, functioning as a "gateway" leading from glycolysis to fermentation, while indolepyruvate decarboxylases (IPDCs; EC 4.1.1.74) are key enzymes in the biosynthetic pathway of indole-3-acetic acid of many bacteria (reviewed by Koga, 1995). The two classes of decarboxylases (PDC and IPDC) display high structural similarities, but different affinities for their substrates (Schütz et al., 2003).

The presence of *P. serpens PDC/IPDC* gene displaying high similarity to genes of phytobacteria raised the stimulating hypothesis that a HGT event may have occurred.

In this study we have characterised the complete gene encoding a putative PDC/IPDC of *P. serpens*, shown that this sequence is conserved in *Phytomonas* members and obtained phylogenetic evidence of a probable HGT event providing the molecular basis for alcoholic fermentation in *Phytomonas*.

2. Material and methods

2.1. Trypanosomatid isolates, cultivation and DNA extraction

Phytomonas isolates used in this study were obtained from two cryopreserved collections of the Universidade de São Paulo (USP, São Paulo, Brazil) (Table 1). PCR assays targeted to the spliced-leader region were used to identify *Phytomonas isolates* (Catarino et al., 2001 and references therein). Information on other trypanosomatids used in this study is provided in Supplementary material (Table A1). *P. serpens* and *L. major* stocks were kindly provided, respectively, by Dr. Marta M. G. Teixeira (Instituto de Ciências Biológicas, USP) and Dr. Lucile Floeter-Winter (Instituto de Biociências, USP). Promastigotes of *P. serpens* were grown at 28 °C in Grace's insect medium (Sigma) supplemented with 10% foetal calf serum; promastigotes of *L. major* were grown at 25 °C in 199 medium (Vitrocell) supplemented with 10% foetal calf serum. *P. serpens* and *L. major* genomic DNA was prepared by the conventional phenol-chloroform extraction method. DNA preparations of other trypanosomatids were kindly provided by Dr. Marta M. G. Teixeira (Instituto de Ciências Biológicas, USP).

2.2. PCR amplification

Three pairs of primers were employed for the amplification of *P. serpens PDC/IPDC* gene (Table 2; Fig. 1) designed based on the nucleotide sequence of this gene here determined (GenBank JN400885). For the amplification of *P. serpens* housekeeping gene *glycosomal glyceraldehyde dehydrogenase (gGAPDH)* the pair of primers Ps.GAPDH.For/Ps.GAPDH.Rev (Table 2) was designed based on the nucleotide sequence (GenBank No EU084892). Amplification reactions were performed in 25 μL total volume, containing 50 ng DNA, 1 unit *Taq* DNA polymerase Biolase (Bioline), 1× enzyme buffer, 1.5 mM $MgCl_2$, 50 mM KCl (for Ps.IPDC.N.For/Ps.IPDC.N.Rev and Ps.IPDC.M.For/Ps.IPDC.C.Rev primers), 0.2 μm dNTPs, 0.4 μM of each primer, 5% DMSO (for Ps.IPDC.U.For/Ps.IPDC.U.Rev primers). PCR conditions included 1 min denaturation at 95 °C, followed by 30 cycles at three temperatures: 1 min denaturation at 95 °C; 1 min primer annealing at the temperature specified in Table 2; and 1 min elongation at 72 °C; followed by 9 min elongation at 72 °C. PCR products were separated in agarose gels and stained with ethidium bromide.

2.3. Cloning and sequencing of the PDC/IPDC gene

The 2400 bp insert of clone pBSSK$^+$ p228, belonging to the EST cluster 53 was fully sequenced by standard procedures using subclones obtained with a number of restriction enzymes. Sequencing was carried out with an ABI 377 automated DNA sequencer (Applied Biosystems Inc.).

2.4. Sequencing of the PDC/IPDC intergenic region

The intergenic region was amplified from *P. serpens* genomic DNA with the Ps.IPDC.U.For/Ps.IPDC.U.Rev primers (Table 2; Fig. 1) and *Pfu* DNA polymerase (Fermentas). The amplification

Table 1
Characteristics of *Phytomonas* isolates used in this study.

Isolate	Identification[a]	Host		
		Species	Source	Country
P. serpens	TCC 060	*Lycopersicon esculentum* (tomato)	Fruit	Brazil
P. françai	TCC 064	*Manihot esculenta* (cassava)	Latex	Brazil
P. mcgheei 163	TCC 300	Zea mays (maize)	Seed	Brazil
1G	Trycc 56	*Nezara viridula* (insect)	NA	Brazil
412 U	Trycc 55	*Bixa orellana* (annatto)	Seed	Brazil
492 FG	Trycc 72	*Cajanus flavus* (bean)	Seed	Brazil
Bni	TCC 086	*Blepharodon nitidus*	Latex	Surinam
EC2	TCC 052	*Euphorbia characias*	Latex	France
Epi 053 (=Em1)	TCC 053	*Euphorbia pínea*	Latex	France
Jma	TCC 066	*Jatropha macrantha*	Latex	Peru
Msc 084	TCC 084	*Mandevilla scabra*	Latex	Surinam
Sam 225	TCC 225	*Solanum americanum*	Fruit	Brazil
Ser 226 (Seri 226)	TCC 226	*Solanum erianthum*	Fruit	Brazil
Svi 223	TCC 223	*Solanum viarum*	NA	Brazil

insert NA = not aware
[a] TCC: Trypanosomatids Culture Collection of the Universidade de São Paulo; TryCC: Trypanosomatids Culture Collection of the Department of Parasitology – Universidade de São Paulo.