Biol Res 43: 233-241, 2010

Sera of Chagasic patients react with antigens from the tomato parasite *Phytomonas serpens*

Viviane K. Graça-de Souza[1], Viviane Monteiro-Góes[2], Patrício Manque[3], Tatiana A.C.B Souza[1], Paulo R.C. Corrêa[1], Gregory A. Buck[3], Andréa R. Ávila[2], Lucy M. Yamauchi[1], Phileno Pinge-Filho[4], Samuel Goldenberg[2], Marco A. Krieger[2] and Sueli F. Yamada-Ogatta[1,*]

[1] Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid s/n, Campus Universitário, 86055-900, Londrina, Paraná, Brazil.
[2] Instituto Carlos Chagas, FIOCRUZ, Avenida Algacyr Munhoz Mader 3775, Cidade Industrial, 81350-010, Curitiba, Paraná, Brazil.
[3] Center for the Study of Biological Complexity, Virginia Commonwealth University, Richmond, Virginia 23284-2030 Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298-0678, USA.
[4] Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid s/n, Campus Universitário, 86055-900, Londrina, Paraná, Brazil

ABSTRACT

The genus *Phytomonas* comprises trypanosomatids that can parasitize a broad range of plant species. These flagellates can cause diseases in some plant families with a wide geographic distribution, which can result in great economic losses. We have demonstrated previously that *Phytomonas serpens* 15T, a tomato trypanosomatid, shares antigens with *Trypanosoma cruzi,* the agent of human Chagas disease. Herein, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were used to identify proteins of *P. serpens* 15T that are recognized by sera from patients with Chagas disease. After 2D-electrophoresis of whole-cell lysates, 31 peptides were selected and analyzed by tandem mass spectrometry. Twenty-eight polypeptides were identified, resulting in 22 different putative proteins. The identified proteins were classified into 8 groups according to biological process, most of which were clustered into a cellular metabolic process category. These results generated a collection of proteins that can provide a starting point to obtain insights into antigenic cross reactivity among trypanosomatids and to explore *P. serpens* antigens as candidates for vaccine and immunologic diagnosis studies.

Key terms: *Phytomonas serpens, Trypanosoma cruzi,* antigenic cross-reactivity, trypanosomatids.

INTRODUCTION

Some species of the family Trypanosomatidae are responsible for diseases that affect humans, animals (*Leishmania* and *Trypanosoma*) and plants (*Phytomonas*). *Trypanosoma cruzi* is the etiologic agent of Chagas disease (Chagas, 1909), an illness that affects millions of people, particularly in Latin America (Guhl and Lazdins-Helds, 2007). There is no vaccine against infection by *T. cruzi* and chemotherapy remains the only means of treatment for Chagas disease. Meanwhile, the drugs available for treatment are few and their efficacy is limited, mainly due to the development of resistance and the lack of host specificity (Filardi and Brener, 1987). Despite this scenario, efforts directed at the discovery of new antitrypanosomal agents and/or vaccines are insufficient (Tarleton *et al*., 2007).

Species of the genus *Phytomonas* alternate their biological cycle between phytophagous insects of the order Hemiptera and many species of plants (Jankevicius *et al*., 1989; Batistoti *et al*., 2001). Promastigote forms of the parasite colonize the digestive tract of these insects, cross the intestinal barrier and reach the salivary glands through the hemolymph. These microorganisms are transmitted by the host through the saliva when feeding. In the plant, promastigote forms, and rarely amastigote forms, are found in the phloem, lactiferous tubes, fruits or seeds (Jankevicius *et al*., 1989). However, only the trypanosomatids found in the phloem of some economically important plants cause fatal phytopathological conditions (Camargo, 1999).

The lack of specificity and extensive cross-reactivity among flagellates of the Trypanosomatidae family was previously observed.

* Corresponding author: Sueli Fumie Yamada-Ogatta, Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Microbiologia. Rodovia Celso Garcia Cid, PR 445, km 380. CEP 86055-900. Tel: +55-43-3371-4297; fax: +55-43-3371-4788. e-mail: ogatta@uel.br

Received: November 18, 2009. Accepted: December 12, 2009.

One of the first lines of evidence of antigenic cross-reactivity among these trypanosomatids was reported by Noguchi (1926), when utilizing serologic tests to differentiate trypanosomatids of insects of the genus *Herpetomonas* from various species of *Leishmania*. Since then, several studies have demonstrated immunologic cross-reactivity between *T. cruzi* and monoxenous species of the family Trypanosomatidae. One approach broadly used in those studies was the utilization of sera from chagasic patients to detect common antigens among these trypanosomatids (Lopes *et al*., 1981; Monteón *et al*., 1997). It has also been demonstrated that polyclonal antibodies against cruzipain, the main cysteine peptidase from *T. cruzi*, recognize two peptides of 38 and 40 kDa in *P. serpens*. The 40 kDa protein is located on the cell surface, and both have cysteine peptidase proteolytic activity and other features similar to cruzipain (Santos *et al*., 2007).

Previous works of our research group have also demonstrated that *P. serpens* 15T shares antigens with *T. cruzi*. We have demonstrated that sera from patients with Chagas disease display a strong reactivity with *P. serpens* antigens by indirect immunofluorescence (IIF) assay. When the sera of Chagas disease patients were adsorbed with living *P. serpens* 15T promastigotes, a significant reduction in IIF titers to *T. cruzi* antigens was observed. Moreover, rabbit hyperimmune serum raised against living forms of *P. serpens* 15T or *T. cruzi* was able to recognize both trypanosomatids antigens by IIF assay. It was also observed that there was partial protection against infection of BALB/c mice immunized with living *P. serpens* 15T by the intraperitoneal or oral route and later challenged with a lethal inoculum of blood trypomastigotes of *T. cruzi*. Infected and previously immunized mice showed a reduction in blood trypomastigote counts and in mortality compared to non-immunized animals (Breganó *et al*., 2003). The protection afforded by *P. serpens* immunization is due to nitric oxide production, as observed from the higher parasitemia and mortality of inducible nitric oxide synthase (iNOS) deficient mice when compared to wild-type C57BL/6 mice. Moreover, immunized and infected wild-type mice showed fewer amastigote nests in their hearts, although immunization with *P. serpens* did not induce inflammation in the myocardium (Pinge-Filho *et al*., 2005). In view of these results, a proteomic-based study was carried out to identify proteins of *P. serpens* 15T that are recognized by sera from patients with Chagas disease. The identified proteins may be further explored as antigens for vaccine or immunologic diagnosis studies.

METHODS

Microorganism

P. serpens 15T was isolated from *Lycopersicum esculentum* in Londrina, Paraná, Brazil. Promastigote forms of the flagellate were maintained at 28 ºC in GYPMI medium [10.0 g/l Glucose, 2.5 g/l Yeast extract, 2.5 g/l Peptone, 20% Meat Infusion, 0.001% Hemin, 10.0 g/l KCl, 8.5 g/l NaCl pH 7.0 (Jankevicius *et al*., 1989)]. The genus identification was confirmed by PCR amplification of a genus-specific sequence of the spliced leader gene as described by Serrano *et al*., (1999).

Human sera

Sera from normal individuals and chronic chagasic patients were supplied by the Blood Bank of the University Hospital, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. The presence of antibodies to *T. cruzi* was determined as described in Breganó *et al*., (2003). A pool of 14 sera from chagasic patients was used in a Western blot assay.

Protein solubilization

Aliquots of 2 x 10^8 log-phase promastigotes, cultivated in GYPMI medium, of *P. serpens* were harvested by centrifugation at 800 *g* for 10 min at 4 ºC and washed three times in phosphate-buffered saline (PBS), pH 7.2. Total protein extracts were obtained by lysing the flagellates in a buffer containing 40 mM Tris base, 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]1-propane sulfonate - CHAPS (General Electrics Life Sciences, São Paulo, Brazil) and 1 mM Nα-tosyl-L-lysine chloromethyl ketone hydrochloride - TLCK (Sigma-Aldrich Co, São Paulo, Brazil). After 30 min incubation at room temperature with gentle agitation, lysed flagellates were centrifuged for 30 min at 13,000 *g* and the supernatant was kept at - 80 ºC. The protein concentration was determined using the Bradford reagent, and the proteins were loaded for isoelectric focusing (IEF).

2D electrophoresis

For the first dimension, aliquots of solubilized proteins (500 μg) were diluted to a final volume of 250 μl in rehydration solution (8 M urea, 2% CHAPS, 40 mM dithiothreitol - DTT, 0.5% ampholytes 3-10, 0.002% bromophenol blue). This solution was applied to 13-cm IPG-strips (Amersham Biosciences, Uppsala, Sweden) with a non-linear separation range of gradient pH 3-10 by in-gel rehydration. After 10 h of rehydration at 20 ºC,

IEF was performed on an Ethan™ IPGphor™ unit (Amersham Biosciences), at the same temperature, with the following conditions: 500 V for 1 h, 1000 V for 1 h, 4000 V for 1 h, 6000 V for 2 h, 8000 V for 9 h and 100 V for 1h. Before second dimension electrophoresis, proteins were reduced and alkylated by incubation of the strips as follow: 15 min in equilibration buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 5.0 mg/ml DTT, and for an additional 15 min in the same buffer containing 12.5 mg/ml iodoacetamide instead of DTT. Equilibrated IPGphor strips were separated across 12.5% SDS-PAGE gels, using a vertical system (Hoefer™ SE 600 Ruby™, Amersham Biosciences), at 30 mA/gel constant current at 10 ºC until the dye front reached the lower end of the gel. Proteins were visualized by silver staining according to the following procedures: gels were fixed in 12% v/v acetic acid, 50% v/v ethanol and 0.5 ml/l formaldehyde (37% v/v) for 30 min. After 3 x washes (5, 10 and 15 min) in 50% v/v ethanol, the gels were incubated in 0.02% w/v sodium thiosulfate for 30 s followed by three 5-min washes in water. Another 30-min incubation was carried out in a solution containing 0.2% w/v silver nitrate and 0.75 ml/l formaldehyde. After 3 x washes in water, development was performed by incubation in a solution containing 3% w/v sodium carbonate, 2% w/v sodium thiosulfate and 0.5 ml/l formaldehyde. The reaction was stopped with 50% v/v ethanol and 12% v/v acetic acid.

Western blotting

Parasite protein extracts separated by electrophoresis, as above, were electrotransferred onto Hybond-C membranes (GE Healthcare Life Sciences, São Paulo, Brazil) according to standard procedures (Towbin *et al*., 1979). The membranes were blocked by incubation in 5% skim milk powder in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween-20. The blots were probed using 1: 80 dilution of chagasic sera. Bound antibodies were detected with 1: 7,500 dilution of phosphatase alkaline-conjugated anti-human IgG (Promega, Wisconsin, USA) and developed with 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium.

Protein digestion and mass spectrometric analysis

Some of the *P. serpens* protein spots that reacted with sera from Chagas' disease patients were manually excised from the gel and destained for 15 min with a freshly prepared solution of 30 mM potassium ferricyanide, 100 mM sodium thiosulfate. After several washings in water, the gel pieces were further washed 2 x 30 min in 0.1 M ammonium bicarbonate, pH 8.3, 50% v/v acetonitrile solution. The gels were dried using a Savant Speed Vac" evaporator (TeleChem International, Inc. Sunnyvale, USA) and were rehydrated in 15 μl of 50 mM ammonium bicarbonate. The proteins were enzymatically digested overnight at 37 ºC with sequencing grade porcine trypsin (0.5 μg/ml, Promega). The tryptic peptides were extracted twice with 40 μl of 60% v/v acetonitrile, 0.1% v/v TFA (trifluoroacetic acid) solution in a sonicator for 10 min. The extracts were concentrated under vacuum to an approximate volume of 10 μl. The resulting tryptic peptides were desalted on C8 cartridges (Michrom BioResources Inc., California, USA) and subjected to 2D Nano LC/MS/MS analyses on a Michrom BioResources Paradigm MS4 Multi-Dimensional Separations Module, a Michrom NanoTrap Platform and a LCQ Deca XP plus ion trap mass spectrometer. The mass spectrometer was operated in data-dependent mode and the four most abundant ions in each MS spectrum were selected and fragmented to produce tandem mass spectra. The MS/MS spectra were recorded in the profile mode.

Database search

Proteins were identified by searching MS/MS spectra against the National Center for Biotechnology non-redundant and GeneDB databases, and its reverse complement using Bioworks v3.2. Peptide and protein hits were scored and ranked using the probability-based scoring algorithm incorporated in Bioworks v3.2 and adjusted to a false positive rate of < 1%. Only peptides identified as possessing fully tryptic termini with cross-correlation scores (Xcorr) greater than 1.9 for singly charged peptides, 2.3 for doubly charged peptides and 3.75 for triply charged peptides were used for peptide identification. In addition, the delta correlation scores (D Cn) were required to be greater than 0.1, and for increased stringency, proteins were accepted only if their probability score was <0.0005 and the result was repeated with the same spot picked from a parallel gel.

RESULTS

2D electrophoresis and Western blotting analyses

Proteins from whole-cell lysates of log-phase promastigotes of *P. serpens* 15T were separated by 2DE in pH range 3-10. Approximately 150 spots were detected in the silver-stained gels. There was homogeneous distribution of the peptides

in relation to isoelectric focusing, but with the greatest concentration of peptides with an estimated molecular mass above 30 kDa (Fig. 1A). The 2DE protein spot profiles obtained from 3 independent experiments were highly reproducible in terms of the total number of protein spots and their positions, and the reactivity with sera from chagasic patients.

Despite the intrinsic limitation of the Western analysis used here, more than 50 polypeptide spots reacted strongly with the sera from patients with Chagas disease (Fig. 1B), and most of them showed a molecular mass range of 25-100 kDa. Although some reactive polypeptides were detected in Western assay, they could not be visualized with the 2DE gels. One possible limitation for spot detection resides in the sensitivity of the silver staining method used here, which is compatible with MS analysis. Healthy human sera were also tested, and the result is shown in Fig. 1C. A total of 31 tryptic peptide samples that strongly reacted with sera of chagasic patient and clearly resolved on 2DE gels (shown in the Figs. 1A and 1B) were selected and analyzed by MS/MS.

Protein identification

Automatic and blast searching allowed the identification of 28 spots, resulting in 22 different putative proteins (Table 1). One limitation of this approach is that few genes and proteins of *Phytomonas* spp. have been sequenced and characterized to date, thus we assigned the protein identity by homology with other trypanosomatids. Pappas *et al*. (2005) generated expressed sequence tags (EST) from a cDNA library of *P. serpens* 10T. Most of the protein identified in our study matched with the deduced amino acid sequence from these ESTs (Table 1), validating previous gene predictions.

The results of this analysis showed that two proteins, from spots 6 and 18, identified in this analysis are ortholog genes annotated as hypothetical in *L. braziliensis* and *T. cruzi* databases, respectively. Some proteins were detected in more than one spot: cystathionine beta synthase (4 and 27), fructose-bisphosphate aldolase (9 and 11) glyceraldehyde-3-phosphate dehydrogenase (10 and 12), enolase (16 and 24), and malic enzyme (29, 30 and 31).

Biological functions of the identified peptides

According to the gene ontology annotation of biological processes, the identified proteins of *P. serpens* 15T could be classified into 8 categories (Table 2). Of the 22 proteins identified, 7 (31.8%) were associated with carbohydrate metabolism. Protein synthesis represented the second most

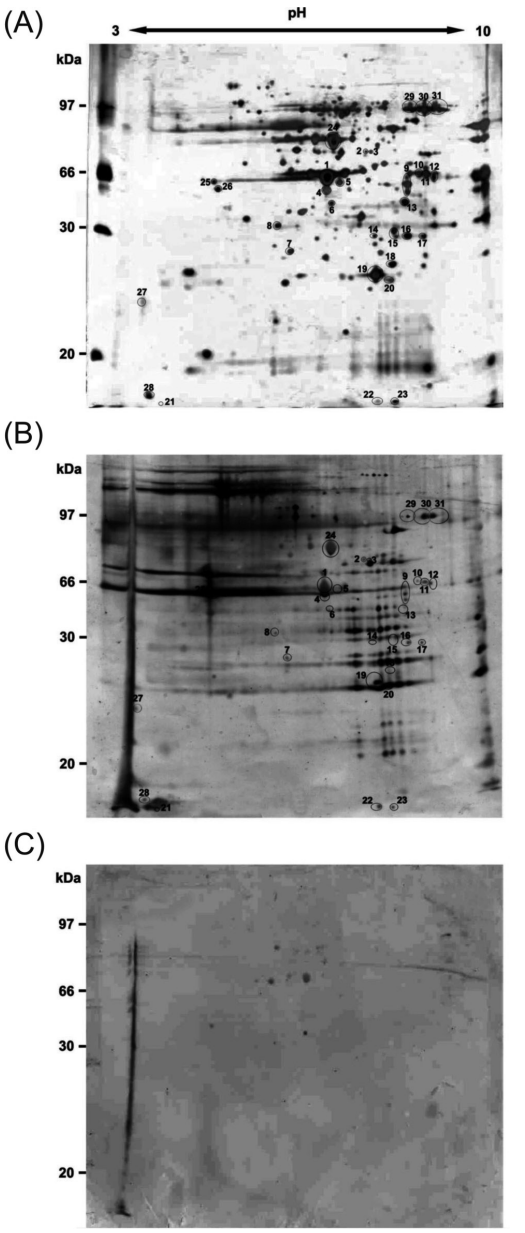

Fig. 1: Sera of Chagasic patients recognized proteins from *P. serpens* 15T. Cells (2 x 10^8) were solubilized directly in sample buffer and the proteins (500 µg) were separated over a non-linear pH gradients 3-10 immobilized strip (IPG). The second dimension was realized in 12.5% SDS-PAGE and the proteins were detected by silver staining. The scale at the top indicates the pH range of the first dimension IPG strip. The molecular weight standard is shown in kDa on the left. The results are representative of three 2DE gel analysis. (A) Promastigotes of *P. serpens* 15T 2D-electrophoresis map. Western blot analysis of *P. serpens* 15T proteome assayed with sera from patients with Chagas disease (B) and with sera from non-chagasic individuals (C). The numbers in (A) and (B) indicate the selected spots for tandem mass spectrometry.

Table 1
***P. serpens* 15T proteins recognized by chagasic patients' sera.**

Spot	Accession number[a]	Protein name	Organism[b]	Peptide sequence[c]	*P* value
1	XP_001566831/ CO723781	Alcohol dehydrogenase	*L. brasiliensis*	KCDYIISLGGGSPIDCAKG	1.16×10^{-09}
2	XP_847200/CO724325	Isocitrate dehydrogenase mitochondrial	*T. brucei*	RHAYGDQYAATDSKF	3.21×10^{-04}
3	XP_001566289	2-Oxoglutarate dehydrogenase	*L. brasiliensis*	KLGLMSPFVKA	4.35×10^{-05}
4	XP_812998/CO723809	Cystathionine beta synthase	*T. cruzi*	RVGKNMVLTAEAEGRL	2.65×10^{-06}
5	XP_001568880	Sterol C24-delta methyltransferase	*L. braziliensis*	RYAQESFYESIMRH	3.13×10^{-05}
6	XP_001565894	Hypothetical protein	*L. braziliensis*	RNGQTGDWVGTFEGHKG	9.96×10^{-06}
7	XP_816099/CO723969	60S ribosomal protein L7	*T. cruzi*	RQAATAGNYYLEAKPKV	2.73×10^{-07}
8	XP_809696/CO723819	40S ribosomal protein S4	*T. cruzi*	RLKDAAGNEFATRA	1.92×10^{-08}
9	XP_822806/CO723885	Fructose-biphosphate aldolase, glycosomal	*T. brucei*	RGKGLLAADESTGSCTKR	3.78×10^{-06}
10	ABW81352	Glyceraldehyde-3-phosphate dehydrogenase, glycosomal	*P. serpens*	KKVVISAPASGGVKT	1.35×10^{-05}
11	XP_809369/CO723885	Fructose-biphosphate aldolase, glycosomal	*T. cruzi*	YISGVILHDETVYQKA	5.40×10^{-06}
12	ABW81352	Glyceraldehyde-3-phosphate dehydrogenase, glycosomal	*P. serpens*	RYDSVHGKPRS	1.91×10^{-06}
15	XP_810966/CO723939	60S Ribosomal protein L13	*T. cruzi*	RKVTSEEQTKNVYKF	1.98×10^{-04}
16	XP_819700	Enolase	*T. cruzi*	KSKLGANAILGCSMAISKA	1.74×10^{-08}
17	XP_802422	40S ribosomal protein SA	*T. cruzi*	RVIAAVENPQDVCVCSARL	4.88×10^{-06}
18	XP_806271	Hypothetical protein	*T. cruzi*	RGNEYLLQTAINALSRC	3.81×10^{-05}
19	XP_811505	Proteasome alpha 2 subunit	*T. cruzi*	RLVQIEYATTAASKG	2.02×10^{-06}
20	XP_843706/CO724310	GTP-binding rtb2	*T. brucei*	RGKICFNCWDTAGQEKF	1.96×10^{-08}
21	XP_001566999/ CO723839	60S acidic ribosomal protein P2	*L. braziliensis*	RVGALFTELEGKSFDELCMEGKS	8.10×10^{-10}
23	XP_805385/CO723840	Histone H2B	*T. cruzi*	KIVNSFVNDLFERI	8.55×10^{-06}
24	AAD45340	Enolase	*T. cruzi*	KSKLGANAILGCSMAISKA	7.09×10^{-09}
25	XP_821118/CO723812	60S ribosomal protein PO	*T. cruzi*	KISKGTVEIVSDRK	6.09×10^{-08}
26	XP_813392	25 kDa Translation elongation factor 1-beta	*T. cruzi*	RDGLLWGDHKL	6.87×10^{-05}
27	AAK16402/CO723809	Cystathionine beta synthase	*T. cruzi*	RTPSALLSEHPDSLFGVANRL	3.77×10^{-11}
28	XP_808090/CO724395 and DQ503481	Calmodulin	*T. cruzi*	RVFDKDGNGFISAAELRH	1.10×10^{-10}
29	XP_814410/CO723957	Malic enzyme	*T. cruzi*	RILGLGDLGCNGVGISIGKS	4.45×10^{-06}
30	XP_814409/CO723957	Malic enzyme	*T. cruzi*	KSSLYVAGAGLQPSRV	4.55×10^{-06}
31	XP_814409/CO723957	Malic enzyme	*T. cruzi*	RFTNKGTAFTMKERK	5.23×10^{-08}

[a] NCBI database: Proteins identified by homology with the trypanosomatids entries([b])/*Phytomonas* spp. similar nucleotide sequence. [c] Using the LC/MS/MS peptide mass fingerprinting profile, SEQUEST algorithm and ProFit Protein software against the NCBI and GeneDB databases. Representative peptide of at least three matched peptides.

Table 2
Proteins identified by MS/MS with known function grouped into functional categories

Categories	Protein
Carbohydrate metabolism	Alcohol dehydrogenase; Enolase; Fructose-biphosphate aldolase, glycosomal; Glyceraldehyde-3-phosphate dehydrogenase, glycosomal; Isocitrate dehydrogenase, mitochondrial; Malic enzyme; 2-Oxoglutarate dehydrogenase
Protein synthesis	60S ribosomal protein L7; 60S ribosomal protein L13; 60S ribosomal protein P0; 60S acidic ribosomal protein P2; 40S ribosomal protein SA; 40S ribosomal protein S4; 25 kDa translation elongation factor 1-beta
Amino acid metabolism	Cystathionine beta synthase
Lipid metabolism	Sterol C24-delta methyltransferase
Nucleosome assembly	Histone H2B
Proteolysis	Proteasome alfa 2 subunit
Endomembrane system	Calmodulin
Intracellular protein/RNA transport	GTP-binding rtb2

abundant category, where 6 (27.3%) different proteins were identified. The other proteins assigned in this study are associated with various activities, including: amino acid and lipid metabolism, nucleosome assembly, proteolysis, endomembrane system, and intracellular protein/RNA transport.

DISCUSSION

In this study, 22 putative proteins from *P. serpens* 15T were identified and most of them were clustered into a cellular metabolic process category. As there is little *Phytomonas* genomic information, the mass spectra derived from reactive polypeptides were searched against all available trypanosomatid sequences at the time to maximize the possibility of identifying the proteins. But we cannot exclude the possibility of some protein misidentification. Because of the hydrophobic nature of membrane proteins, they are usually under-represented in proteomic analyses (Santoni *et al*., 2000). Indeed, in previous 2-D electrophoresis mapping of whole-cell lysates of *T. cruzi,* membrane proteins were not identified (Paba *et al*., 2004, Andrade *et al*., 2008). This could explain why membrane proteins of *P. serpens* were not identified in this study.

Three enzymes of the glycolytic pathway were identified in this study, fructose-bisphosphate aldolase, glycosomal glyceraldehyde-3-phosphate dehydrogenase and cytosolic enolase. We also identified one peptide as isocitrate dehydrogenase and three as mitochondrial malic enzyme. Glyceraldehyde-3-phosphate dehydrogenase is a glycosomal enzyme that has also been detected in the cytosol of trypanosomatids (Hannaert *et al*., 1998), which could explain the isoenzymes found in this study. The presence has been reported of two isoforms of malic enzymes in *Phytomonas* sp., as well in *T. cruzi*, a mitochondrial and a glycosomal isoenzymes (Cannata *et al*., 1979; Uttaro and Opperdoes, 1997). It is probable that at least three isoforms of malic enzymes are expressed in *P. serpens* 15T, as indicated by their different isoelectric points. We also cannot exclude the possibility that some protein spots may have resulted from protein degradation.

Like other trypanosomatids, species of *Phytomonas* degrade carbohydrates via glycolysis, and the first reactions of the classical Embden-Meyerhof pathway occur inside glycosomes (Sanchez-Moreno *et al*., 1992). Since *Phytomonas* spp. lacks a functional citric acid cycle (Sanchez-Moreno *et al*., 1992; Chaumont *et al*., 1994) and the genes for cytochrome mediated respiration are missing in the maxicircle kinetoplast DNA (Maslov *et al*., 1999; Nawathean and Maslov, 2000; Opperdoes and Michels, 2008), they depend on glycolysis to obtain energy. One important aspect of this metabolism is that enzymes from the glycolytic pathway have been selected as targets for drugs against members of Trypanosomatidae family, including fructose-bisphosphate aldolase (Dax *et al*., 2006) and glyceraldehyde-3-phosphate dehydrogenase (de Marchi *et al*., 2004). The same approach has been used for sterol biosynthesis since trypanosomatids synthesize ergosterol and related 24-alkylated sterols, whose structure and biosynthetic pathway show differences compared

to that for cholesterol found in mammalian cells. One principal difference is the reaction catalyzed by S adenosyl-L-methionine C24-delta sterol methyltransferase, which introduces a C24-methyl group to the ergosterol and stigmasterol side chains (Roberts *et al*., 2003). Inhibitors of this enzyme have been shown to have antiproliferative effects in several trypanosomatids (Lorente *et al*., 2004). Another potential target for the development of new drugs in the treatment of diseases caused by trypanosomatids is the metabolic pathway of sulfur-containing amino acids. The cystathionine beta synthase catalyzes the *trans*-sulfuration reaction of homocysteine to cysteine, a sulfur-containing amino acid that plays an important role in the structure, stability and catalytic functions of many proteins. *T. cruzi* cystathionine beta synthase lacks the 90-120 amino acids in the carboxyl terminal, is not activated by the presence of S-adenosylmethionine, and does not contain heme, which differs from the corresponding mammalian enzymes (Nozaki *et al*., 2001).

Of particular interest is a small phosphorylated protein located at the ribosome "stalk," the 60S acidic ribosomal protein P. The biological function of eukaryotic P proteins is still unclear. Their participation in protein synthesis has been shown, and these proteins may also be involved in transcription and DNA repair processes. In addition, P proteins have been implicated in several diseases associated with the immune response, including systemic lupus erythematosus, allergies caused by some filamentous fungi and protozoan infections (Tchórzewski, 2002). Antibodies to ribosomal P proteins are prevalent in patients with chronic Chagas heart disease and they are directed against the carboxy-terminal region of the *T. cruzi* proteins (Levin *et al*., 1989). These antibodies were able to cross-react with the acidic motif present on the second extracellular loop of human cardiac β1 adrenergic receptor (Smulski *et al*., 2006). Anti-P antibodies are believed to take part in the induction of heart dysfunctions, such as arrhythmias and/or other electrical disorders (Lopez Bergami *et al*., 2001). Corroborating this, mice immunized with the recombinant *T. cruzi* P protein (TcP2b) demonstrated a strong response against the C-terminal region of this protein and developed lethal supraventricular tachycardia (Lopez Bergami *et al*., 1997). As mentioned, mice previously immunized with live *P. serpens* 15T and infected with *T. cruzi* did not display inflammation in the myocardium (Pinge-Filho *et al*., 2005). Altogether, these data open perspectives for exploring the role of the 60S acidic ribosomal protein P from *P. serpens* in the protection against *T. cruzi* infection.

The other proteins assigned in this study are associated with various important activities in trypanosomatids. A protein involved in proteolytic activity, the proteasome alpha 2 subunit, was identified in this study. De Diego *et al*., (2001) have reported that the ubiquitin-proteasome pathway has an essential role in protein turnover during *T. cruzi* differentiation. Calmodulin, a universal Ca^{2+}-binding protein that can modulate the activity of other proteins, has been shown to have a role in *T. cruzi* differentiation (Lammel *et al*., 1996) and motility (Ridgley *et al*., 2000). Finally, one peptide was identified as a GTP-binding rtb2 protein by homology with the ortholog protein in *T. brucei*. In this parasite, the protein has homology to Ran, a member of G protein superfamily, which is an essential element in the transport of proteins and RNA across the nuclear membrane (Field *et al*., 1995).

Promastigote forms of *P. serpens*, a trypanosomatid isolated from edible tomatoes, are easily cultivated in *in vitro* conditions (Batistoti *et al*., 2001). This non-human pathogenic flagellate is highly immunogenic and expresses important protein homologs of trypanosomatids that cause human infections. The antigenic and metabolic pathway similarities between *P. serpens* and *T. cruzi* raise important questions about obtaining and utilizing antigens from microorganisms that are innocuous to humans for immunologic diagnosis of Chagas disease, as well as for the development of new strategies of immunization against infection by *T. cruzi*, and studying target molecules for the development of new chemotherapeutic agents against trypanosomatids. Therefore, as pointed out by Santos *et al*., (2007) this flagellate is useful as a model for the immunological and biochemical studies among the Trypanosomatidae family. Now, we are faced with the task of trying to understand the significance of these proteins in *T. cruzi* cross-reactivity and *P. serpens* biology.

ACKNOWLEDGMENTS

This work was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária -Paraná and Pro-Reitoria de Pesquisa e Pós Graduação (PROPPG) of Universidade Estadual de Londrina (UEL). This work was part of the Ph.D. dissertation of V.K. Graça-de Souza. We thank Dr. A. Leyva for English editing of the manuscript and Ediel Clementino da Costa for technical support.

REFERENCES

ANDRADE HM, MURTA SM, CHAPEAUROUGE A, PERALES J, NIRDÉ P, ROMANHA AJ (2008) Proteomic analysis of *Trypanosoma cruzi* resistance to benznidazole. J Proteome Res 7: 2357-2367.

BATISTOTI M, CAVAZZANA MJr, SERRANO MG, OGATTA SF, BACCAN GC, JANKEVICIUS JV, TEIXEIRA MM, JANKEVICIUS SI (2001) Genetic variability of trypanosomatids isolated from phytophagous hemiptera defined by morphological, biochemical, and molecular taxonomic markers. J Parasitol 87: 1335-1341.

BREGANÓ JW, PICÃO RC, GRAÇA VK, MENOLLI RA, ITOW-JANKEVICIUS S, PINGE-FILHO P, JANKEVICIUS JV (2003) *Phytomonas serpens*, a tomato parasite, shares antigens with *Trypanosoma cruzi* that are recognized by human sera and induce protective immunity in mice. FEMS Immunol Med Microbiol 39: 257-264.

CAMARGO EP (1999) *Phytomonas* and other trypanosomatid parasites of plant and fruit. Adv Parasitol 42: 29-112.

CANNATA JJ, FRASCH AC, CATALDI DE FLOMBAUM MA, SEGURA EL, CAZZULO JJ (1979) Two forms of 'malic' enzyme with different regulatory properties in *Trypanosoma cruzi*. Biochem J 184: 409-419.

CHAGAS C (1909) Nova tripanosomiase humana. Estudo sobre a morfolojia e o cyclo evolutivo do *Schizotrypanum cruzi* n. *gen*., n. sp., ajente etiolojico de nova entidade morbida no homem. Mem Inst Oswaldo Cruz 1: 159-218.

CHAUMONT F, SCHANCK AN, BLUM JJ, OPPERDOES FR (1994) Aerobic and anaerobic glucose metabolism of *Phytomonas* sp. isolated from *Euphorbia characias*. Mol Biochem Parasitol 67: 321-331.

DAX C, DUFFIEUX F, CHABOT N, COINCON M, SYGUSCH J, MICHELS PA, BLONSKI C (2006) Selective irreversible inhibition of fructose 1,6-bisphosphate aldolase from *Trypanosoma brucei*. J Med Chem 49: 1499-1502.

DE DIEGO JL, KATZ JM, MARSHALL P, GUTIÉRREZ B, MANNING JE, NUSSENZWEIG V, GONZÁLEZ J (2001) The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. Biochemistry 40: 1053-1062.

DE MARCHI AA, CASTILHO MS, NASCIMENTO PG, ARCHANJO FC, DEL PONTE G, OLIVA G, PUPO MT (2004) New 3-piperonylcoumarins as inhibitors of glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) from *Trypanosoma cruzi*. Bioorg Med Chem 12: 4823-4833.

FIELD MC, FIELD H, BOOTHROYD JC (1995) A homologue of nuclear GTPase ran/TC4 from *Trypanosoma brucei*. Mol Biochem Parasitol 69: 131-134.

FILARDI LS, BRENER Z (1987) Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. Trans R Soc Trop Med Hyg 81: 755-759.

GUHL F, LAZDINS-HELDS JK editors. (2007) *Reporte sobre la enfermedad de Chagas*. Special Programme for Research and Training in Tropical Diseases of World Health Organization (WHO), p. 1-104.

HANNAERT V, OPPERDOES FR, MICHELS PA (1998) Comparison and evolutionary analysis of the glycosomal glyceraldehyde-3-phosphate dehydrogenase from different Kinetoplastidae. J Mol Evol 47: 728-738.

JANKEVICIUS JV, ITOW-JANKEVICIUS S, CAMPANER M, CONCHON I, MAEDA L, TEIXEIRA MM, FREYMULLER E, CAMARGO EP (1989) The life cycle and culturing of *Phytomonas serpens* (Gibbs), a trypanosomatid parasite of tomatoes. J Eukaryot Microbiol 36: 265-271.

LAMMEL EM, BARBIERI MA, WILKOWSKY SE, BERTINI F, ISOLA EL (1996) *Trypanosoma cruzi*: involvement of intracellular calcium in multiplication and differentiation. Exp Parasitol 83: 240-249.

LEVIN MJ, MESRI E, BENAROUS R, LEVITUS G, SCHIJMAN A, LEVY-YEYATI P, CHIALE PA, RUIZ AM, KAHN A, ROSENBAUM MB, TORRES HN, SEGURA EL (1989) Identification of major *Trypanosoma cruzi* antigenic determinants in chronic Chagas' heart disease. Am J Trop Med Hyg 41: 530-538.

LOPES JD, CAULADA Z, BARBIERI CL, CAMARGO EP (1981) Cross-reactivity between *Trypanosoma cruzi* and insect trypanosomatids as a basis for the diagnosis of Chagas'disease. Am J Trop Med Hyg 30: 1183-1188.

LOPEZ BERGAMI P, CABEZA MECKERT P, KAPLAN D, LEVITUS G, ELIAS F, QUINTANA F, VAN REGENMORTEL M, LAGUENS R, LEVIN MJ (1997) Immunization with recombinant *Trypanosoma cruzi* ribosomal P2beta protein induces changes in the electrocardiogram of immunized mice. FEMS Immunol Med Microbiol 18: 75-85.

LOPEZ BERGAMI P, SCAGLIONE J, LEVIN MJ (2001) Antibodies against the carboxyl-terminal end of the *Trypanosoma cruzi* ribosomal P proteins are pathogenic. The FASEB J 15: 2602-2612.

LORENTE SO, RODRIGUES JC, JIMÉNEZ JIMÉNEZ C, JOYCE-MENEKSE M, RODRIGUES C, CROFT SL, YARDLEY V, DE LUCA-FRADLEY K, RUIZ-PÉREZ LM, URBINA J, DE SOUZA W, GONZÁLEZ PACANOWSKA D, GILBERT IH (2004) Novel azasterols as potential agents for treatment of leishmaniasis and trypanosomiasis. Antimicrob Agents Chemother 48: 2937-2950.

MASLOV DA, NAWATHEAN P, SCHEEL J (1999) Partial kinetoplast-mitochondrial gene organization and expression in the respiratory deficient plant trypanosomatid *Phytomonas serpens*. Mol Biochem Parasitol 99: 207-221.

MONTEÓN VM, GUZMÁN-ROJAS L, NEGRETE-GARCIA C, ROSALES-ENCINA JL, LOPES PA (1997) Serodiagnosis of American trypanosomosis by using nonpathogenic trypanosomatid antigen. J Clin Microbiol 35: 3316-3319.

NAWATHEAN P, MASLOV DA (2000) The absence of genes for cytochrome c oxidase and reductase subunits in maxicircle kinetoplast DNA of the respiration-deficient plant trypanosomatid *Phytomonas serpens*. Curr Genet 38: 95-103.

NOGUCHI H. (1926) Comparative studies of Herpetomonads and Leishmanias. Differentiation of the organisms by serological reactions and fermentation tests. J Exp Med 44: 327-337.

NOZAKI T, SHIGETA Y, SAITO-NAKANO Y, IMADA M, KRUGER WD (2001) Characterization of transsulfuration and cysteine biosynthetic pathways in the protozoan hemoflagellate, *Trypanosoma cruzi*. Isolation and molecular characterization of cystathionine b-synthase and serine acetyltransferase from *Trypanosoma*. J Biol Chem 276: 6516-6523.

OPPERDOES FR, MICHELS PA (2008) Complex I of Trypanosomatidae: does it exist? Trends Parasitol 24: 310-317.

PABA J, SANTANA JM, TEIXEIRA AR, FONTES W, SOUSA MV, RICART CA (2004) Proteomic analysis of the human pathogen *Trypanosoma cruzi*. Proteomics 4: 1-8.

PAPPAS GJJR, BENABDELLAH K, ZINGALES B, GONZÁLEZ A (2005) Expressed sequence tags from the plant trypanosomatid *Phytomonas serpens*. Mol Biochem Parasitol 142: 149-157.

PINGE-FILHO P, PERON JP, DE MOURA TR, MENOLLI RA, GRAÇA VK, ESTEVÃO D, TADOKORO CE, JANKEVICIUS JV, RIZZO LV (2005) Protective immunity against *Trypanosoma cruzi* provided by oral immunization with *Phytomonas serpens*: role of nitric oxide. Immunol Lett 96: 283-290.

RIDGLEY E, WEBSTER P, PATTON C, RUBEN L (2000) Calmodulin-binding properties of the paraflagellar rod complex from *Trypanosoma brucei*. Mol Biochem Parasitol 109: 195-201.

ROBERTS CW, MCLEOD R, RICE DW, GINGER M, CHANCE ML, GOAD LJ (2003) Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. Mol Biochem Parasitol 126: 129-142.

SANCHEZ-MORENO M, LASZTITY D, COPPENS I, OPPERDOES FR (1992) Characterization of carbohydrate metabolism and demonstration of glycosomes in a *Phytomonas* sp. isolated from *Euphorbia characias*. Mol Biochem Parasitol 54: 185-200.

SANTONI V, KIEFFER S, DESCLAUX D, MASSON F, RABILLOUD T (2000) Membrane proteomics: use of additive main effects with multiplicative interaction model to classify plasma membrane proteins according to their solubility and electrophoretic properties. Electrophoresis 21: 3329-3344.

SANTOS AL, D'AVILA-LEVY CM, ELIAS CG, VERMELHO AB, BRANQUINHA MH (2007) *Phytomonas serpens*: immunological similarities with the human trypanosomatid pathogens. Microbes Infect 9: 915-921.

SERRANO MG, NUNES LR, CAMPANER M, BUCK GA, CAMARGO EP, TEIXEIRA MM (1999) Trypanosomatidae: *Phytomonas* detection in plants and phytophagous insects by PCR amplification of a genus-specific sequence of the spliced leader gene. Exp Parasitol 91: 268-279.

SMULSKI C, LABOVSKY V, LEVY G, HONTEBEYRIE M, HOEBEKE J, LEVIN MJ (2006) Structural basis of the cross-reaction between an antibody to the *Trypanosoma cruzi* ribosomal P2β protein and the human β1 adrenergic receptor. FASEB J 20: 1396-1406.

TARLETON RL, REITHINGER R, URBINA JA, KITRON U, GÜRTLER RE (2007) The challenges of Chagas Disease - grim outlook or glimmer of hope. PLoS Med 4: 1852-1857.

TCHÓRZEWSKI M. (2002) The acidic ribosomal P proteins. Int J Biochem Cell Biol 34: 911-915.

TOWBIN H, STAEHELIN T, GORDON J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354.

UTTARO AD, OPPERDOES FR (1997) Characterisation of two malate dehydrogenases from *Phytomonas* sp. Purification of the glycosomal isoenzyme. Mol Biochem Parasitol 89: 51-59.