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Proteome profiling of the growth phases of *Leishmania pifanoi* promastigotes in axenic culture reveals differential abundance of immunostimulatory proteins.

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Graphical Abstract

Highlights

1. The genome of *L. pifanoi* has not been sequenced and its molecular and cell biology has been scarcely studied.
2. This is the first insight into the proteome of *L. pifanoi*.
3. Despite the lack of a genome sequence, we were able to identify constantly and differentially regulated proteins by the 2DE-MALDI-TOF/TOF approach.
4. Certain immunostimulatory proteins previously described are over-expressed in late-logarithmic phase or stationary phase promastigotes of *L. pifanoi*.

ABSTRACT

Leishmaniasis is a term that encompasses a compendium of neglected tropical diseases caused by dimorphic and digenetic protozoan parasites from the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). The clinical manifestations of neotropical cutaneous leishmaniasis (NCL) caused by *L. pifanoi* and other species of the “*L. mexicana* complex” mainly correspond to anergic diffuse cutaneous leishmaniasis (ADCL), which is the origin of considerable morbidity. Despite the outstanding advances in the characterization of the trypanosomatid genomes and proteomes, the biology of this species has been scarcely explored. However, the close relation of *L. pifanoi* to the sequenced species *L. mexicana* and others included in the “*L. mexicana* complex” allowed us to perform a two-dimension electrophoresis (2DE) approach to the promastigote proteome at the differential expression level. Protein identifications were performed by matrix-assisted laser desorption-ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF). This insight has revealed similarities and differences between *L. pifanoi* and other species responsible for cutaneous and visceral leishmaniasis. Interestingly, certain proteins that were previously described as

immunostimulatory (elongation factor 1 β , trypanothione peroxidase, heat shock protein 70, enolase, GDP-forming succinyl-CoA and aldehyde dehydrogenase) are more abundant in the final growth stages of promastigotes (late-logarithmic and/or stationary phase) in the case of *L. pifanoi*.

Keywords: *Leishmania pifanoi*; differential gene expression; 2DE; MALDI-TOF/TOF; elongation factor 1 β ; trypanothione peroxidase; hsp70; enolase; aldehyde dehydrogenase.

1. Introduction.

The life cycle of the protozoan parasites from the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) is dimorphic and digenetic because they develop two stages in two different hosts. The extracellular promastigote stage develops within the gut of the hematophagous sand fly vector host (Psychodidae: Phlebotominae). When promastigotes are transmitted to the mammalian host's dermis by the bite of the sand fly, they differentiate to the non-motile obligate intracellular amastigote stage. Most *Leishmania* species are responsible for a compendium of neglected tropical diseases termed leishmaniasis. The main clinical manifestations are cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL). Only VL is life threatening, whereas CL and especially MCL compromise quality of life. The incidence of CL and MCL leishmaniasis is approximately 1.5 million annual cases worldwide (WHO, 2010). The species included in the "*L. mexicana* complex" cause neotropical cutaneous leishmaniasis (NCL). This complex includes *L. mexicana*, *L. amazonensis*, *L. pifanoi*, *L. venezuelensis* and *L. garnhami*. Some authors suggest that *L. pifanoi* is a subspecies of *L. mexicana* (Palatnik et al., 1990; Pan, 1984). The main distribution areas of *L. pifanoi* are the Amazon basin, the Matto Grosso state in Brazil and Venezuela. NCL is a compendium of zoonosis, being rodents the main reservoirs of the parasite. The dermal lesions are localized at the site of the injection of the parasite and their shape resembles a crater, which can eventually ulcerate. However, a disseminated manifestation of the disease that resembles lepromatous lesions and is called anergic diffuse cutaneous leishmaniasis (ADCL) might occur and significantly increase morbidity. So far, *L. pifanoi* has been isolated only from ADCL lesions. In these cases, the initial lesion may ulcerate or even disappear over a span of days to years before ADCL is developed (John

and Petri, 2006). NCL is associated to poverty, being the proximity of reservoirs (WHO, 2010) and environmental changes that allow the proliferation of the *Lutzomyia* spp. sand fly vectors (Killick-Kendrick, 1999) important factors in the geographical dissemination of the disease.

Currently, 14 genomes of the genus *Leishmania* have been sequenced and are available in TriTrypDB (Aslett et al., 2010). This includes two species of the *L. mexicana* complex (*L. mexicana* and *L. amazonensis*), but not *L. pifanoi*. Bibliographical records evidence the little interest this species has awakened relatively. At the time this manuscript was written, just 65 out of 21,159 articles deposited in the NCBI's PubMed database containing the term '*Leishmania*' had mentioned the species '*Leishmania pifanoi*'. None of them provide information about differential gene expression in this species. This sort of studies is possible thanks to the availability of the genome sequences, which helps to improve understanding of the biology of the parasite (de Toledo et al., 2010). Several high-throughput gene expression studies have been accomplished in *L. major*, *L. donovani*, *L. infantum*, *L. mexicana* and *L. amazonensis* (e.g. (Akopyants et al., 2004; Alcolea et al., 2010a; Holzer et al., 2006; Magalhaes et al., 2014; Saxena et al., 2007)), which is starting to pay off in the search of drug targets and vaccine candidates (e.g. (Moreno et al., 2014)). Herein, we describe the first insight into the proteome of *L. pifanoi* promastigotes, which has been performed by the two-dimension electrophoresis (2DE) approach. As a result, constitutive and differentially abundant proteins have been detected. These proteins have been identified by matrix-assisted laser desorption-ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF). The differences and similarities found with other leishmania species causative of cutaneous and visceral leishmaniasis are discussed herein.

2. Materials and methods.

2.1. *L. pifanoi* promastigotes.

Promastigotes of the *L. pifanoi* strain MHOM/VE/60/Ltrod (Colmenares et al., 2002; Pan, 1984; Rainey et al., 1991; Soong et al., 1995; Traub-Cseko et al., 1993) were cultured at 27 °C in RPMI 1640 supplemented with L-glutamine (Life Technologies, Carlsbad, CA), 10% heat inactivated fetal bovine serum (Lonza, Basel, Switzerland) and 100 µg/ml streptomycin – 100 IU/ml penicillin (Life Technologies), pH 7.2. Cultures were initiated at 2×10^6 cells/ml and growth was registered daily at the light microscope with a Neubauer chamber. Aliquots containing 10^8 promastigotes were taken on a daily basis to represent the lag/early-logarithmic, mid-logarithmic, late-logarithmic/early stationary and stationary phases. Once each sample was collected, the parasites were immediately harvested at 2,000 g for 10 min and processed.

2.2. Extraction of total protein.

Promastigote samples were washed once in PBS and resuspended in 150 µl lysis buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 50 mM DTT, 1% Triton X-100, 50 µg/ml DNase and Mini EDTA-free Protease Inhibitor Cocktail according to the manufacturer's instructions –Roche, Mannheim, Germany). Next, lysis was allowed by mild rotation at 4 °C for 30 min. The lysate was then centrifuged at 8,000g for 10 min and proteins from the supernatant were precipitated with methanol/chloroform (Wessel and Flugge, 1984), dried at room temperature for 5 min and resuspended in 2X rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.003% bromophenol blue). Protein concentration was assessed with the *RC DC protein assay kit* (BioRad) according to the manufacturer's instructions and the results were checked by PAGE-SDS following a described procedure (Alcolea et al., 2011b).

2.3. Analysis of gene expression by 2DE proteomics.

An aliquot of each sample containing 50 µg of total protein was brought to a final volume of 140 µl using 2X isoelectrofocusing (IEF) buffer (18.2 M DTT and 0.5% *IPG buffer solution* pH 3-10, BioRad). IEF was carried out on 7 cm IPG strips of pH 3-10 nonlinear gradient (BioRad) in a Protean IEF Cell system (BioRad) applying a 7-step run program as follows: 50 V for 12h, 250 V for 1h, 500 V for 1h, 1000 V for 1h, 2000 V for 1h, linear ramp to 8000 V for 1h and 8000 V up to 3500 V·h. More than 12,000 V·h were reached in each step. The second dimension was developed by 12% SDS-PAGE in a pre-cooled MiniProtean 3 Dodeca Cell system (BioRad) at 0.5 W/gel for 30 min and then at 1.5 W for 5 min after the dye-front reached the bottom edge. Gels were developed with SYPRO Ruby stain (BioRad) and the gel images were acquired with EXQuest Spot Cutter (BioRad) following the manufacturer's instructions.

Spot detection and analysis of differential gene expression were performed with PDQuest 2D Advanced 8.0.1 software (BioRad) according to the manufacturer's instructions. After gel cropping and experiment configuration (three biological replicates for each of the growth curve time points analyzed), spots were automatically detected in all individual member gel images and in the master gel composite image. Undetected spots were manually added with the corresponding tool. Pairwise recognition of each spot between gels was enhanced by using the landmark tool and manually check was performed for all of them with the image analysis tools provided in the software (3D density graphs, single spot quantitation histograms and 2DE gel images). The normalization algorithm was *Total Quantity in Valid Spots* and significant differential protein abundance in three biological replicate cultures was contrasted by the Student's t-test ($\alpha = 0.05$).

2.4. Protein identification by MALDI-TOF/TOF.

The selected spots were excised with EXQuest Spot Cutter (BioRad) and in-gel digestion was performed with porcine trypsin and prepared for MALDI-TOF/TOF mass-spectrometry as described (Alcolea et al., 2011b). The digests were deposited in an OptiTOF™ Plate (Life Technologies), each well containing a mixture of 0.8 µl peptides plus 0.8 µl of 3 µg/µl α-cyano-4-hydroxycinnamic acid (Sigma, Basel, Switzerland). These droplets were allowed to dry at room temperature prior to be run in an ABI 4800 MALDI-TOF/TOF mass spectrometer (Life Technologies) in positive reflector mode at 25 kV for MS and 1 kV for MS/MS. Spectral data were analyzed with ABI 4000 Series Explorer Software 3.6 (Life Technologies) and identifications were performed with MASCOT 2.1 using Global Protein Server Explorer 4.9 (Life Technologies). The *L. mexicana* genome sequence and annotations (<http://tritrypdb.org/common/downloads/release-9.0/LmexicanaMHOMGT2001U1103/fasta/data/>) were used as the reference for identifications. Data were also run against the NCBI nr database. These identifications are available in the PRIDE repository of the ProteomeXchange Consortium (Vizcaino et al., 2014) with the accession number PXD002939.

3. Results.

3.1. Analysis of differential gene expression in cultured *L. pifanoi* promastigotes by 2DE-MALDI-TOF/TOF.

Cultures of *L. pifanoi* promastigotes were set in triplicate and the growth curves were monitored (Figure 1). Total protein was extracted from promastigotes in lag or early-logarithmic phase (day 2), mid-logarithmic phase (day 3), late-logarithmic/early stationary phase (day 5) and stationary phase (day 7). Total protein was comprised between 2 and 5 mg/ml as assessed by two different methods (electrophoresis and a spectrophotometry-based method). 2DE of all time points and replicates was

successfully performed (Figure 2), and differential protein abundance was analyzed (Figure 3A) with the PD Quest software (BioRad). All spots were manually checked with the 3D viewing tools and bar graphs to ensure that every spot considered in the analysis contained protein and was not a stain artifact. The cut-off ratio values were 1.7 for up-regulation and 0.6 for down-regulation respectively, within the significance level inferred with the Student's t test ($p < 0.05$). Ratios were calculated with respect to early-logarithmic phase promastigotes (day 2). According to the cutoff values mentioned, the number of spots containing differentially expressed proteins was 30. Thirty three spots without changes in protein abundance were also selected randomly. The number of spots with positive identifications by MALDI-TOF/TOF (MASCOT score ≥ 52 , significant) is 43 (Table 1). The count of unsuccessfully identified (MASCOT score < 52 , non-significant) is 20 (Table 2), which may be explained by the unavailability of a genome sequence and gene annotations in the case of *L. pifanoi* and the usage of the closely related *L. mexicana* genome retrieved from TriTrypDB. Indeed, low sequence similarity between peptides of the *L. pifanoi* target proteins and the orthologue sequences in *L. mexicana* are responsible for low reliability of identifications in these cases. The TriTrypDB Id., protein names and major MASCOT hits have been indicated between brackets in Table 2. Searches against the NCBI database did not clarify unidentified spots, except in the case of Lpi5101, which has been included in Table 1. In fact, most genome sequences contained in TriTrypDB are not found in NCBI. According to this analysis, 12 proteins are more abundant at the early logarithmic phase (day 2), 11 are increased at day 5 and 11 at day 7 (Table 1).

3.2. Differentially abundant proteins in cultured *L. pifanoi* promastigotes.

The differentially abundant proteins successfully identified (Table 1) are involved in regulation of gene expression (translation factors and the ribonucleoprotein p18),

protein folding (a heat shock protein), redox homeostasis (the trypanothione peroxidase, TryP), metabolism (enzymes of the glycolysis pathway, the Krebs cycle, lipid and amino acid metabolism) and in the cytoskeleton (β -tubulin). These expression profiles at the protein abundance level are discussed below and have been illustrated in Figure 4. The translation factors, the enzymes involved in glycolysis and the TryP described in Table 1 reach their maximum abundance levels in late logarithmic and/or stationary phase promastigotes, i.e. at the final stages of growth of the promastigote cultures.

In the case of unsuccessfully identified spots (statistically not significant) described in Table 2, the “most probable” protein match (highest MASCOT score) of each particular spot is a hypothetical protein or is involved in one of the functions mentioned above (e.g. 40S ribosomal protein S16). The complete set of matches for each individual spot is provided in the ProteomeXChange database (PXD002939). When different spots contained the same kind of protein, the term “protein variant” (Alcolea et al., 2014) has been used to describe differential expression changes. The reason is that this 2DE-MS/MS approach is not valid to distinguish different aggregation states of a particular protein (monomers, dimers,...) or to identify protein processing (e.g. removal of signal peptides) and post-translational modifications other than carbamidomethylation or methionine methylation (PXD002939), which is not the aim of this study.

4. Discussion.

Two elongation factors are differentially regulated in *L. pifanoi* promastigotes, being their expression profiles the opposite. On the one hand, an elongation factor 2 (EF2) variant is up-regulated in late logarithmic and stationary phase and a second variant in the latter. The EF2 was described to be a major immunostimulatory protein in the species *L. donovani* (Gupta et al., 2007), which causes visceral leishmaniasis. On the other hand, the elongation factor 1 β (EF1 β) is down-regulated in the same growth

phases. Surprisingly, it was described that this translational factor bears trypanothione S-transferase activity (Vickers and Fairlamb, 2004; Vickers et al., 2004). The trypanothione is a specific molecule of trypanosomatids with a similar function to glutathione in these and other eukaryotes. In fact, this molecule is composed of two glutathione residues coupled by spermidine. The trypanothione is essential in the trypanothione reductase/tryparedoxin peroxidase system (TryR-TryP), which performs reduction of reactive oxygen species (ROS). Interestingly, the trypanothione S-transferase function of the EF1 β together with the tryparedoxin peroxidase might have a role in the disseminated form of MCL that *L. (Viannia) guyanensis* causes (Walker et al., 2006). *L. (L.) pifanoi* promastigotes over-express a variant of the TryP in stationary phase together with the EF1 β , whereas the abundance of two other TryP variants does not change (Table 1). Therefore, one variant of the TryP and the EF1 β are more abundant at the end of the growth curve (Table 1) and their increased abundance suggests their involvement in the dissemination of CL and/or ADCL. Interestingly, the EF1 β transcript was found to be up-regulated in promastigotes exposed to stress by cadmium, suggesting that it may have a potential role in certain stress situations (Alcolea et al., 2011a), as supported by its trypanothione S-transferase activity.

The heat shock protein hsp70 is up-regulated in late logarithmic and stationary phase promastigotes, which is in contrast with the reported constitutive expression levels throughout the main stages of the life cycle of other *Leishmania* spp. (Wiesgigl and Clos, 2001; Zilberstein and Shapira, 1994). For example, we did not detect differential expression in *L. infantum* promastigotes (Alcolea et al., 2010a; Alcolea et al., 2011b). The hsp70 is another immunostimulatory protein in the case of *L. infantum* and *L. donovani* (Carrillo et al., 2008; Gupta et al., 2007) identified in an immunoproteomic analysis of amastigotes isolated from symptomatic dogs (Coelho et al., 2012). Both

species are responsible for visceral leishmaniasis. Therefore, the higher abundance of the hsp70 in *L. pifanoi* at the end of growth and differentiation in culture does not take place in species responsible for VL. As a difference with the hsp70, the hsp83-1 is constitutive in *L. pifanoi* promastigotes (Table 1), which is in agreement with the constitutive expression levels that were also found in other species (Wiesgigl and Clos, 2001; Zilberstein and Shapira, 1994). The relative abundance of the hsp60 chaperonin does not change across the growth curve of *L. pifanoi* promastigotes either.

The adenosine kinase domain-containing protein nucleoside diphosphate kinase b (NDKB) is also constitutive throughout the growth curve of *L. pifanoi* promastigotes in culture. This protein is more abundant in *L. major*, *L. mexicana*, *L. infantum* and *L. donovani* promastigotes than in amastigotes but it is probably constitutive in these species (Alcolea et al., 2010a; Almeida et al., 2004; Holzer et al., 2006; Rosenzweig et al., 2008a; Rosenzweig et al., 2008b). The NDKB-encoding gene is up-regulated in the procyclic subpopulation within the stationary phase of promastigote growth (Alcolea et al., 2009). In the case of *L. amazonensis*, the NDKB is released to the macrophage at the transcript level, thus preventing its cytolysis in the benefit of parasite's survival as proposed (Kolli et al., 2008).

The glycolytic enzymes phosphoglycerate kinase (PGK) and enolase are up-regulated in late logarithmic phase *L. pifanoi* promastigotes in axenic culture. Two different PGK isoforms have been identified in the spot Lpi9402 thanks to this analysis. One is glycosomal and the other one cytosolic. The analysis does not allow concluding which isoform is actually more abundant. Nevertheless, it is clear that the PGK function presents this expression profile mentioned. The enolase is an additional immunostimulatory protein that was found in *L. donovani* (Gupta et al., 2007) and it was also identified by an immunoproteomic approach in amastigotes isolated from an

asymptomatic dog (Coelho et al., 2012). The *L. infantum* enolase is over-expressed in the procyclic subpopulation of promastigotes within the stationary phase population of *L. infantum* at the transcript level (Alcolea et al., 2009). This is in agreement with the enolase expression profile found in the *L. pifanoi* promastigotes. The enolase is also up-regulated in promastigotes with respect to amastigotes at least in *L. major*, *L. infantum* and *L. donovani*.

The key enzyme of gluconeogenesis glycosomal phosphoenolpyruvate carboxykinase (gPEPCK) is also up-regulated in late-logarithmic phase *L. pifanoi* promastigotes, which does not necessarily contrast with up-regulation of PGK and enolase because they participate in the second phase of glycolysis, a pathway playing a central role in sugar metabolism, and none of the enzymes catalyzing reactions of the first phase have been found as differentially regulated. In addition, the enolase and the PGK catalyze reversible reactions, thus participating in both pathways and their relative expression profile in *L. pifanoi* promastigotes is equal. Hence, the data point in the direction of gluconeogenesis rather than glycolysis when the growth and differentiation stage is advanced but not over (Figure 4). This may be triggered by nutrient depletion caused by growth of the promastigote culture. The gPEPCK is up-regulated in stationary phase promastigotes of *L. infantum* at the transcript level (Alcolea et al., 2010a). This profile is similar but not exactly equal to the corresponding one in *L. pifanoi*. In the case of *L. donovani*, the highest gPEPCK levels are reached in amastigotes (Rosenzweig et al., 2008a; Rosenzweig et al., 2008b).

The aconitase is more abundant in stationary phase *L. pifanoi* promastigotes, as well as the transcript of the *L. infantum* orthologue (Alcolea et al., 2010a). Conversely, the GDP-forming succinyl-CoA ligase (SCL) is down-regulated in mid logarithmic phase promastigotes. The SCL was identified by an immunoproteomic approach performed in

an asymptomatic dog (Coelho et al., 2012). In that analysis, an aldehyde reductase was also detected in amastigotes, although they were isolated from a symptomatic dog in this case. The abundance of the *L. pifanoi* aldehyde dehydrogenase (ALDH) considerably increases at day 5 of the growth curve (Table 1). The *L. pifanoi* cytochrome c oxidase IV has been identified as constitutively expressed, which is in agreement with previous studies performed in other species (e.g. (Alcolea et al., 2010a)).

A variant of the 3-hydroxy-3-methylglutaryl-CoA synthase (3-HMG-CoA-S) is more abundant in *L. pifanoi* stationary phase promastigotes, whereas two other 3-HMG-CoA variants are constitutive. This suggests that isoprenoid biosynthesis increases in a fully grown culture. The 3-HMG-CoA-S gene was found to be up-regulated in intracellular (Alcolea et al., 2010a) and axenic (Alcolea et al., 2010b) *L. infantum* amastigotes at the transcript level, but no expression change was detected in the case of axenically cultured promastigotes. However, the expression levels are higher in *L. infantum* metacyclic promastigotes in culture than in those derived from the sand fly (Alcolea, 2011). The prostaglandin F2 α synthase is constitutive throughout the growth curve of *L. pifanoi*, whereas it is up-regulated in *L. infantum* at the transcript level in the ongoing of the growth curve in logarithmic phase promastigotes (Alcolea et al., 2010a), in procyclic promastigotes within the stationary phase (Alcolea et al., 2009) and in stationary phase promastigotes with respect to amastigotes (Alcolea et al., 2010a). Therefore, the 3-HMG-CoA-S is constitutively expressed in *L. pifanoi* but differentially expressed in *L. infantum* throughout the growth curve.

The cystathionine γ lyase (CGL) is up-regulated in *L. pifanoi* early-logarithmic phase promastigotes, as well as its orthologue in *L. infantum* (Alcolea et al., 2010a). The up-regulation of the CGL gene at an early stage of growth and differentiation of *L. pifanoi*

and *L. infantum* suggests changes in cysteine, serine, threonine and polyamine metabolism at the beginning of these metabolic processes.

4. Conclusions.

2DE-MALDI-TOF/TOF proteome analysis of *L. pifanoi* promastigotes has revealed increased abundance of proteins previously described as immunostimulatory at the final stages of growth of promastigotes in culture: the EF1 β , the TryP, the hsp70, the enolase and the GDP-forming succinyl-CoA ligase and an aldehyde dehydrogenase. The expression profiles of certain proteins such the hsp83-1, the gPEPCK and the SCL are similar in promastigotes of *L. pifanoi* and *L. infantum*, whereas differences have been found in other cases such the hsp70 and the NDKB.

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Figure legends.

Figure 1. Growth curve of *L. pifanoi* promastigotes. Total protein samples were prepared and quantified at day 2 (early logarithmic phase), day 3 (mid logarithmic phase), day 5 (late logarithmic phase/early stationary phase) and day 7 (stationary phase), as indicated by the arrows.

Figure 2. 2DE images of the *L. pifanoi* promastigote growth phases in culture. One out of three biological replicates of the experiment. The pH 3-10 gradients are non-linear. **A.** Day 2 (early logarithmic phase). **B.** Day 3 (mid logarithmic phase). **C.** Day 5 (late logarithmic phase/early stationary phase). **D.** Day 7 (stationary phase).

Figure 3. 2DE physical map of *L. pifanoi* protein spots. **A.** 2DE master gel image generated with the PDQuest software (BioRad). MW (KDa) and pI of every protein spot are provided. **B.** Differential abundance of immunostimulatory proteins (see Discussion,

Table 1 and Figure 3). The EF1 β decreases at days 5 and 7 compared to day 2; the hsp83 gene is constantly expressed at the protein level; the hsp70 is more abundant at days 5 (late logarithmic phase) and day 7 (stationary phase) with respect to day 2 (logarithmic phase); the enolase is more abundant at day 5 compared to day 2; the SCL β is more abundant at day 2; and the ADH increases at day 5.

Figure 4. Constitutive and differentially abundant proteins identified in *L. pifanoi*.

Constitutive and differentially abundant proteins that participate in glycolysis, the Krebs cycle, amino acid metabolism, isoprenoid and sterol biosynthesis, redox homeostasis, regulation of gene expression and protein folding are represented. Colours that represent increased abundance: green, day 2 (lag/early log); orange, day 5 (late log); red, day 7 (stat). The constitutively expressed proteins experimentally detected throughout the promastigote growth curve are highlighted in violet. The big arrow represents that the data (Table 1) point in the direction of gluconeogenesis rather than glycolysis when the growth and differentiation stage is advanced but not over in *L. pifanoi* promastigotes. An asterisk indicates that the protein has been previously described as immunostimulatory. Abbreviations not detailed in the text: PGM, phosphoglycerate mutase; TryR, trypanothione reductase; T[S]₂/T[SH]₂, trypanothione; TXN1, tryparedoxin. A more detailed schema is included in Figure S1.

Table captions.

Table 1. Constitutive and differential expression of identified proteins in *L. pifanoi* promastigotes. A 2DE physical map of spots is provided in Figure 3. The MW and pI values provided have been estimated according to the physical map. Differential abundance ratios are referred to the early logarithmic phase (Day3:Day2; Day5:Day2; Day7:Day2). The assignment of TriTrypDB identifiers and protein names provided in this table is statistically significant in all cases according to MASCOT searches. The outcome of each search yields normally distinct protein identities ordered by score. Only the significant one/s (MASCOT score > 52) for each spot is/are included in this table.

Table 2. Constitutive and differential expression of unidentified proteins in *L. pifanoi* promastigotes. A 2DE physical map of spots is provided in Figure 3. The MW and pI values provided have been estimated according to the physical map. Differential abundance ratios are referred to the early logarithmic phase (Day3:Day2; Day5:Day2; Day7:Day2). The spots described in this table do not contain any significant identification (MASCOT score < 52). The complete list of not significant hits for each spot appears in the ProteomeXChange database (accession number PXD002939). Only the “most significant” or “most probable” hit in the case of each spot (i.e., MASCOT score closer to 52) is included between brackets in this table.

Table 1. Constitutive and differential expression of identified proteins in *L. pifanoi* promastigotes.

Spot	TriTrypDB ID	Protein	MASCOT score (p<0.05)	MW (KDa)	pI	Day3: Day2	Day5: Day2	Day7: Day2
Lpi1					5.			
003	LmxM.13.0450	Hypothetical protein, conserved	87	13.24	4	0.01		
Lpi1	LmxM.36.1430/LmxM.33.0840				4.			
201	/LmxM.33.0820	Translation elongation factor 1-beta, putative	104/93/87	34.92	4		0.47	0.47
Lpi2	LmxM.28.2770/LmxM.28.2820				5.			
803	/LmxM.28.2780	Heat-shock protein hsp70, putative	948/221/185	75.94	2		6.66	4.48
Lpi4		Ribonucleoprotein p18, mitochondrial precursor, putative			5.			
003	LmxM.15.0281/LmxM.15.0275		72/72	36.18	7	0.33		
Lpi4	LmxM.13.0300/LmxM.13.0390				5.			
202	/LmxM.13.0280	Unspecified product/Alpha tubulin	634/618/618	27.20	6	0.04	0.04	0.03
Lpi4					5.			
504	LmxM.14.1160	Enolase	482	77.03	7		116.82	
Lpi5	LmxM.08.1230/LmxM.21.1860		85/84/84/84/8		5.			
002	/LmxM.08.1171	Beta tubulin	1	13.26	8	0.01	0.01	0.029
Lpi5					5.			
101	AF020947 (NCBI ID)	Tryparedoxin peroxidase	72	18.61	9			5.6
Lpi5		6-phosphogluconate dehydrogenase, decarboxylating, putative/Enolase			5.			
402	LmxM.34.3340/LmxM.14.1160		172/128	43.25	9	0.42		
Lpi5					5.			
901	LmxM.36.0180	Elongation factor 2 (Lbra)	55	104.08	8			51.61
Lpi6		3-hydroxy-3-methylglutaryl-CoA synthase, putative (HMGS)			5.			
602	LmxM.24.2110		109	64.21	9			146.12
Lpi6					5.			
901	LmxM.36.0180	Elongation factor 2	250	105.06	9		51.85	63.48
Lpi6	LmxM.36.0180	Elongation factor 2	209	104.94	6.		49.08	

902					0			
Lpi7		Methylthioadenosine phosphorylase,			6.			
302	LmxM.05.0830/LmxM.36.1260	putative/Fructose-1,6-bisphosphate aldolase	86/78	37.80	6	0.01		
Lpi7					6.			
403	LmxM.36.2950	succinyl-CoA ligase [GDP-forming] beta-chain, putative	475	45.81	2	0.03		
Lpi7					6.			
502	LmxM.34.3230	Cystathione gamma lyase, putative	226	71.43	3	0.03		
Lpi7					6.			
904	LmxM.18.0510	Aconitase, putative	197	103.01	2			48.19
Lpi8					9.			
404	LmxM.36.1260	fructose-1,6-bisphosphate aldolase	141	39.14	0			2.53
Lpi8					7.			
604	LmxM.25.1120	Aldehyde dehydrogenase, mitochondrial precursor	87	60.05	5		97.07	
Lpi9		Phosphoglycerate kinase B,			8.			
402	LmxM.20.0110/LmxM.20.0100	cytosolic/Phosphoglycerate kinase C, glycosomal	227/213	47.51	2		1.73	
Lpi9		Glycosomal phosphoenolpyruvate carboxykinase,			8.			
605	LmxM.27.1805/LmxM.27.1810	putative	226/226	71.00	6		130.99	
Lpi4					5.			
203	LmxM.25.2010	Hypothetical protein, conserved	359	31.24	7	–	–	–
Lpi1					4.			
501	LmxM.25.0750	Protein phosphatase, putative	185	70.33	7	–	–	–
Lpi2					5.			
102	LmxM.23.0040	Peroxidoxin, tryparedoxin peroxidase	255	24.51	7	–	–	–
Lpi2	LmxM.13.0300/LmxM.13.0390				5.			
202	/LmxM.13.0280	Unspecified product/Alpha tubulin	551/511/511	32.81	7	–	–	–
Lpi2	LmxM.13.0390/0280/0300/123		218/218/203/1		5.			
301	0/LmxM.21.1860	Alpha tubulin/Unspecified product	67/164	36.41	2	–	–	–
Lpi2					5.			
701	LmxM.36.2030/LmxM.36.2020	Chaperonin HSP60, mitochondrial precursor	317/227	71.05	2	–	–	–
Lpi2	LmxM.32.0312/LmxM.32.0314				5.			
801	/LmxM.32.0316	Heat-shock protein 83-1	391/391/391	87.51	9	–	–	–

Lpi3		Hypothetical protein, conserved/Soluble N-			5.			
202	LmxM.25.2010/LmxM.20.1690	ethylmaleimide sensitive factor, putative	84/57	29.94	5.	—	—	—
Lpi3					5.			
403	LmxM.12.0670	Cytochrome c oxidase subunit IV	240	39.17	5	—	—	—
Lpi3					5.			
502	LmxM.04.1230	Actin	67	73.09	5	—	—	—
Lpi4	LmxM.13.0300/LmxM.13.0390				5.			
402	/LmxM.13.0280	Unspecified product/Alpha tubulin	634/618/618	47.11	7	—	—	—
Lpi4					5.			
501	LmxM.04.1230	Actin (Lmex)	250	73.72	6	—	—	—
Lpi5					5.			
502	LmxM.14.1160	Enolase	475	76.61	9	—	—	—
Lpi6					6.			
003	LmxM.23.0200	Endoribonuclease L-PSP (pb5), putative	118	13.04	0	—	—	—
Lpi6		3-hydroxy-3-methylglutaryl-CoA synthase, putative			6.			
601	LmxM.24.2110	(HMGS)	125	77.81	0	—	—	—
Lpi7					6.			
102	LmxM.15.1040/LmxM.15.1160	Tryparedoxin peroxidase	111/110	21.07	4	—	—	—
Lpi7					6.			
201	LmxM.30.2150	Prostaglandin f2-alpha synthase	75	34.81	4	—	—	—
Lpi7		3-hydroxy-3-methylglutaryl-CoA synthase, putative			6.			
603	LmxM.24.2110	(HMGS)	155	78.40	2	—	—	—
Lpi8					6.			
001	LmxM.31.2951/LmxM.31.2950	Nucleoside diphosphate kinase b	259/259	13.02	5	—	—	—
Lpi8					8.			
004	LmxM.25.0910	Cyclophilin A	166	33.52	0	—	—	—
Lpi8					6.			
005	LmxM.31.1820/LmxM.31.1830	Iron superoxide dismutase, putative	223/222	38.06	5	—	—	—
Lpi8					8.			
301	LmxM.33.0140	Malate dehydrogenase	202	36.64	0	—	—	—

Table 2. Constitutive and differential expression of unidentified proteins in *L. pifanoi* promastigotes.

Spot	TriTrypDB ID	Protein	MASCOT score	MW (KDa)	pI	Day3:D ay2	Day5:D ay2	Day7:D ay2
Lpi19 01	(LmxM.26.0890 /LmxM.26.0880)	(40S ribosomal protein S16, putative)	(33/33)	134.10	4. 7			92.78
Lpi29 01	(LmxM.08_29.0760)	(Heat shock protein 90, LPG biosynthetic protein 3, glucose regulated protein 94)	31	101.25	4. 9		6.66	
Lpi32 01	(LmxM.09.1170)	(Hypothetical protein, conserved)	33	27.95	5. 4			0.47
Lpi46 02	(LmxM.36.5340)	(Hypothetical protein, conserved)	50	67.38	5. 7			7.34
Lpi48 02	(LmxM.23.1460)	(Hypothetical protein, conserved)	44	76.04	5. 5		1.72	
Lpi68 02	(LmxM.36.3930)	(Telomerase reverse transcriptase, putative (TERT))	41	78.17	6. 0		102.71	140.64
Lpi74 01	(LmxM.15.0340)	(Hypothetical protein, conserved)	29	42.71	6. 2			2.08
Lpi80 07	(LmxM.32.2190)	(Hypothetical protein, conserved)	49	12.30	7. 5	0.02	0.02	
Lpi83 02	(LmxM.33.2620)	(RNA helicase, putative, mitochondrial, putative)	36	36.14	8. 9		79.63	
Lpi08 04	(LmxM.05.1061)	(ATPase, putative)	36	102.06	3. 6	—	—	—
Lpi25 01	(LmxM.25.1950)	(Kinesin)	41	69.29	5. 6	—	—	—
Lpi36 01	(LmxM.07.0020/LmxM .29.3380)	(Hypothetical protein, conserved/PAS-domain containing phosphoglycerate kinase, putative)	32/32	52.24	5. 5	—	—	—
Lpi45 03	(LmxM.11.1070)	(Hypothetical protein)	35	45.04	5. 7	—	—	—

Lpi64					6.			
01	(LmxM.18.1130)	(Ras-related protein Rab5A, putative (RAB5A))	41	39.51	0	—	—	—
Lpi64					6.			
02	(LmxM.15.0340)	(Hypothetical protein, conserved)	31	39.57	1	—	—	—
Lpi73					6.			
01	(LmxM.26.2280)	(Nitrilase, putative)	41	37.85	2	—	—	—
Lpi76					6.			
01	(LmxM.31.2570)	(Hypothetical protein, conserved)	42	68.47	2	—	—	—
Lpi76					6.			
05	(LmxM.27.1230)	(Hypothetical protein, conserved)	40	68.43	4	—	—	—
Lpi78					6.			
01	(LmxM.33.1410)	(D-isomer specific 2-hydroxyacid dehydrogenase-like protein)	43	82.72	2	—	—	—
Lpi80					8.			
03	(LmxM.36.4800)	(Hypothetical protein, conserved)	33	15.89	9	—	—	—