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Genome-wide gene expression profile induced by exposure to cadmium acetate in *Leishmania infantum* promastigotes

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Summary. *Leishmania infantum* is the etiological agent of visceral leishmaniasis in Mediterranean areas. The life cycle of the protist is dimorphic and heteroxene, as promastigotes develop inside the gut of sand-fly vectors and amastigotes multiply inside mammalian phagocytic cells. In previous studies, we analyzed the expression profiles of these stages and the modulation of gene expression triggered by temperature increase and acidification, both of which are crucial in the differentiation of promastigotes to amastigotes. Differential expression profiles of translation initiation and elongation factors were detected. Here we report that the presence of 1 mM cadmium acetate in the culture medium leads to a shock response consisting of growth arrest, morphological changes, the absence of motility, and the up-regulation of genes that code for: a heavy metal transporter, trypanothione reductase, a haloacid-dehalogenase-like hydrolase, and a metalloexopeptidase from the M20 family, among others. This response is probably controlled by the differential expression of regulatory genes such as those encoding initiation factors 4E, eukaryotic translation initiation factor 3 subunits 8 and 2α, and elongation factor 1β. The initiation factor 2α gene is induced in anomalous environments, i.e., those outside of the protist's normal life-cycle progression, for example, in response to the presence of cadmium ions, acidification without temperature increase, and vice versa. Our results suggest that the regulation of gene expression is a key component of the shock response. [**Int Microbiol** 2011; 14(1):1-11]

Keywords: *Leishmania infantum* · *L. infantum* promastigotes · cadmium · translation factors · DNA microarrays

Introduction

Trypanosomatid protists from the genus *Leishmania* are the etiological agents of a compendium of neglected diseases termed leishmaniasis, which affect a variety of mammalian hosts including humans. The estimated prevalence is 12 million people worldwide and the incidence is 2 million, although declaration is compulsory only in 32 out of the 88 affected countries, which leads to an underestimation of the real magnitude of the problem. About half a million people are affected by visceral leishmaniasis, which kills over 60,000 people per year [World Health Organization. Leishmaniasis. Burden of disease, http://www.who.int/leishmaniasis/burden/en/]. *Leishmania infantum* is the etiological agent of visceral leishmaniasis in the Mediterranean basin, where coinfection with HIV is increasing [9,18] and domestic dogs are the main reservoir of the protist [6]. The life cycle of *Leishmania* is dimorphic and heteroxene. The motile promastigote stage is fusiform and flagellated and develops inside the gut of female phlebotominae sand-fly vectors. The non-motile round amastigote form is able to multiply inside

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phagocytic cells of the mammalian host, thus evading the immune response (reviewed in [12]). As a consequence, three differentiation processes take place in the *Leishmania* life cycle: metacyclogenesis inside the vector gut, which leads to highly infective promastigotes that are transmitted to the mammalian host [10]; promastigote-to-amastigote differentiation inside phagocytes; and amastigote-to-promastigote transition when a sand-fly feeds from an infected mammal. The gene expression profile of the promastigote-to-amastigote transition has been intensively studied in some species by means of: partial random genomic microarrays [1,21], oligonucleotide microarrays [13,15,19], partial cDNA microarrays [5], serial analysis of gene expression (SAGE) [11,16], and proteomic analysis [20]. However, we have reported a large number of unknown and non-annotated genes in the genome of *L. infantum*. They have been determined by hybridization analysis of complete shotgun genome microarrays in a series of gene expression profile analyses. Namely, we have studied the main differentiation processes of the *L. infantum* life cycle [2,3] and the specific effect on differentiation at the post-transcriptional level of temperature increase and acidification [4]. These are the most significant contributing factors in amastigote differentiation, and their contributions were analyzed simultaneously and individually. As a result, we observed, unexpectedly, that temperature increase has a greater influence than pH decrease in the differentiation of *L. infantum* promastigotes to amastigotes. The expression profile induced by pH decrease alone is very different from the one induced by the combination of both factors. By contrast, the temperature-increase-induced expression profile resembles the profile induced by the combination of temperature increase and acidification (an amastigote-like profile). However, there are substantial differences, such as the expression pattern of eukaryotic elongation initiation factor 2α (eEF2α). eEF2α is independently up-regulated by acidification and temperature increase, but not by a combination of both. The environments defined by increased temperatures or decreases in pH are situations that challenge the normal life-cycle progression of *Leishmania* spp.

In this work, we describe the transcript-level response of *Leishmania infantum* promastigotes to another set of conditions outside its standard life cycle: the presence of the heavy metal cadmium. In spite of cadmium toxicity, the resistance of the protist in the promastigote stage was strong and lasting. Gene expression profiles were compared on the basis of morphological changes in the population that were triggered by cadmium acetate. The functions of some of the genes differentially regulated by cadmium acetate were found to be related to gene expression regulatory mechanisms. These mechanisms may be the key component of cadmium resistance in particular and of stress conditions in general.

Materials and methods

Protist culture. *Leishmania infantum* promastigotes (isolate M/CAN/ES/98/10445, zymodeme MON-1) were cultured in RPMI 1640 supplemented with L-glutamine (Cambrex, Karlskoga, Sweden), 10% heat inactivated fetal bovine serum (HIFBS) (Cambrex) and 100 μg streptomycin/ml –100 IU penicillin/ml (Cambrex) (complete medium) at 27°C at a starting density of 2×10^6 promastigotes/ml from an early passage (5th to 10th) of the culture after extraction from the gut of the sand fly. Three replicate cultures in the absence and presence of cadmium acetate 0.1 mM were started. Cell density was assessed daily and promastigotes were harvested at 2000 g for 10 min at day 4. Aliquots of 10^5 and 2×10^7 promastigotes were used for Giemsa staining and RNA preparation, respectively.

Gene expression profiling by microarray hybridization analysis and qRT-PCR validation. These procedures were described in detail in a previous report [2]. Briefly, total RNA was isolated and its quality and absence of DNA contamination were assessed by capillary electrophoresis. Single-stranded cDNA was synthesized and indirectly labeled with cyanines (Cy5 for cadmium acetate-treated promastigotes and Cy3 for control promastigotes) by the aminoallyl-dUTP procedure. Custom *L. infantum* DNA microarrays were then hybridized with both cDNA samples using equimolar amounts of incorporated cyanin. After scanning and local background subtraction, the medians of the raw intensity values and the Cy5/Cy3 ratios (GenePix scanner and 4.0 software, Axon Instruments) were normalized by applying the locally weighted scatter plot smoothing (LOWESS) algorithm. A paired t-test was performed for three biological replicates to find genes with significant differences in expression levels between cadmium-acetate-treated and untreated cells (AlmaZen). We considered that a given clone contained a gene with significant differential regulation when it fulfilled the following criteria: (i) fold change (F) ≥ 1.7 (Cy5/Cy3 ratio if Cy5 > Cy3) or ≤ –1.7 (–Cy3/Cy5 ratio if Cy3 > Cy5), (ii) total relative fluorescence intensity value > 5000 FU (fluorescence units), and (iii) $p < 0.05$. The selected clones were sequenced, assembled, and mapped against the *L. infantum* genome sequence. Some of the clones were validated by relative quantitative real time PCR (qRT-PCR), applying the SYBR-Green method as described [2,4]. Brifely, non-labeled cDNA samples were synthesized with Superscript III Reverse Transcriptase (Invitrogen). The reactions were carried out in iQ™ SYBR Green Supermix (BioRad) in an MyiQ™ Single-Color real-time PCR detection system (BioRad). The oligonucleotide pairs (0.3 μM each) used for qRT-PCR analyses (5′–3′) were: TR_Fw, ACGGCGAGGTTCTGGGTGTT; TR_Rv, TCCGATGGTGCTGTGGAAGT; DEAD/H_5.360_Fw; DEAD/H_5.360_Rv; mpM20_Fw, CGGATTGACAGCAGCCGTAGT; mpM20_Rw, TCGCACCACAAACTCTTGGAT; 6P1FK_Fw, GCACCAACCTGGCAACTCTT; 6P1FK_Rv, CCTTGATGGGCACGAGGATA; eEF1β_Fw, ACGAGACACCGGGCATGAAT; eEF1β_Rw, CGGACTGCGTGTGCTTCTCT; 3′ NT/Nase_Fw, GGCTGAGGTGCACAACCACT; 3′ NT/Nase_Rv, GGGCGACGTGCTCATAGGAA; 3′ NT/NaseP_Fw, CAACACCACCATTGGGCACA; 3′ NT/NaseP_Rv, TAAATCCAGTGCGATCGGCT; mmc_Fw, GGATAGACCGCCACGGATA; mmc_Rv, CGGAGAGATCGACGGATGAA; drpplp_Fw, AGTCAGCAAACCAGCTCTGCA; drpplp_Rv, CGCGTACCGTAGCCCTCCAT. Quantification was performed by the efficiency-corrected ΔCt method. The gene of reference was 18 S rRNA.

Results and Discussion

Expression profile of *L. infantum* promastigotes in the presence of cadmium acetate. Promastigotes can survive for at least four months when cadmium acetate is added to the culture medium. Nevertheless, round morphologies instead of the typical promastigote shape are observed (Alfredo Toraño, personal communication). The exposure to cadmium acetate 1 mM for 4 days resulted in growth arrest (Fig. 1A) and changes in morphology (Fig. 1B). Growth arrest occurred beginning on day 2 but significant morphological changes appeared somewhat later, from 72 to 84 h of exposure. For the gene expression profile analysis, RNA was extracted from untreated and treated replicate promastigote cultures in parallel with the initial morphological changes. The transformation of promastigotes under cadmium pressure appeared to be sequential. Cadmium-treated and untreated (control) promastigotes were analyzed for gene expression at day 4 by means of shotgun genomic microarrays carried out as previously reported [2].

The quality of the isolated total RNA and amplified mRNA is shown in Fig. 2. Ninety-eight genes were found to be differentially regulated, with 57 up-regulated and 41 down-regulated (Fig. 3, Table 1, 2 and 3). Note that only 12 up-regulated and 12 down-regulated genes had previously been characterized to some extent, by gene function annotation *in silico* and/or experimental characterization. The expression pattern of these genes is illustrated in Fig. 4. In addition, a gene encoding an unknown protein, two genes encoding hypothetical proteins of unknown function, and five genes encoding conserved hypothetical proteins were up-regulated by cadmium, while four genes encoding conserved hypothetical proteins were down-regulated by the metal. Twelve and 14 unresolved clones containing more than one gene annotation were, respectively, up-and down-regulated, while 26 and 11 clones, respectively, did not contain an annotated gene fragment (Tables 2 and 3). Similar observations were reported for the expression profile analyses performed throughout the life cycle, in metacyclic vs. procyclic promastigotes, and in response to temperature and/or pH shifts [2]. The differentiated stages showed a higher rate of up-regulation of uncharacterized and non-annotated genes. Moreover, the number of clones that mapped against the genome sequence but not with any annotated gene was higher at these stages (Table 1). The characterization of these genes will improve our understanding of the physiology of the protist, particularly during differentiation and under environmental pressure.

Genes involved in transport, signal transduction, nucleotide metabolism, and the regulation of gene expression are known to be differentially regulated by exposure to cadmium acetate. Among the signal transduction genes, those encoding an ADP ribosylation factor, a protein kinase (PK), two mitogen activated protein kinases (MAPK), and 3′ nucleotidase/nuclease (3′ NT/Nase) are down-regulated. Down-regulation of MAPK genes is consistent with the growth arrest of the promastigotes (Fig. 1). As noted in previous reports [2–4], signal transduction and cell cycle regulation pathways in the genus *Leishmania* have not been elucidated to date, despite annotation of the sequenced genome and research into the kinomes of trypanosomatids [17]. The gene expression profiles of this study are an initial step in the

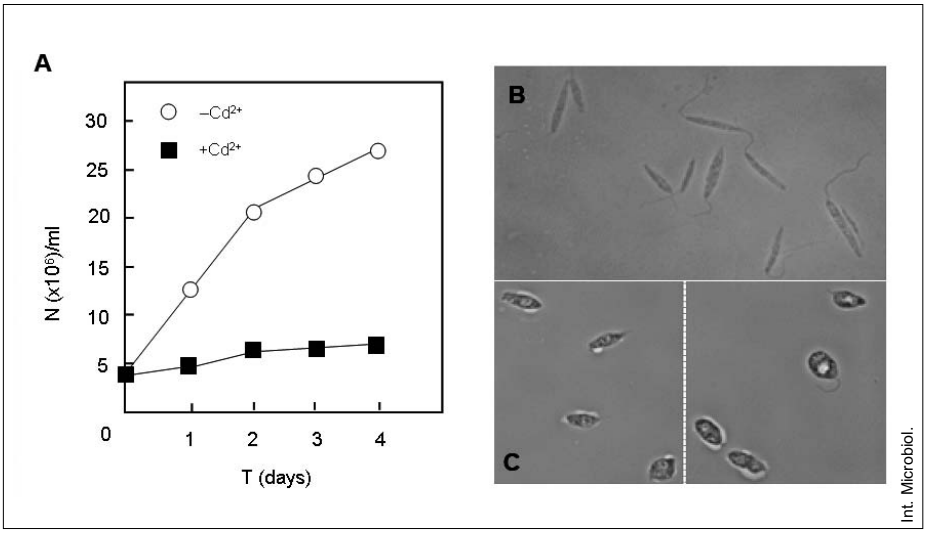

Fig. 1. Growth and morphology of promastigotes in the absence and presence of 1 mM cadmium acetate. (**A**) Average growth curves of three replicates of the cultures performed for each of the conditions assayed. (**B**) Morphology of untreated promastigotes and (**C**) cadmium acetate-treated promastigotes (two fields shown).

Table 1. Annotation status of differentially regulated genes in promastigotes in the presence of 1 mM cadmium acetate

	Number of genes		GenBank (GSS)	
Annotation status	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Function inferred from homology/and or experimentally characterized	12	12	The clones are described in Tables 2 and 3	
Hypothetical protein, conserved	5	4		
Hypothetical protein, unknown function	1	0		
Unknown	1	0		
Unresolved clones	12	14	GS883004–15	GS883040–52
Clone without gene annotation	26	11	GS883016–39	GS883053–65

elucidation of signal transduction pathways, improving our understanding of the protist's ability to sense and respond to environmental challenges. 3′ NT/Nase is also involved in nucleotide metabolism, as is nucleotide diphosphate kinase b (Ndkb). Both genes were up-regulated in stationary phase promastigotes with respect to their expression in amastigotes [3]. In the case of 3′ NT/Nase, its gene was up-regulated in response to temperature increases and pH decreases [4]. Ndkb expression was down-regulated in metacyclic compared to procyclic promastigotes [2]. The down-regulation of genes involved in nucleotide uptake, signaling, and cell cycle control together with the observed growth arrest suggests a non-dividing response triggered by the presence of cadmium acetate.

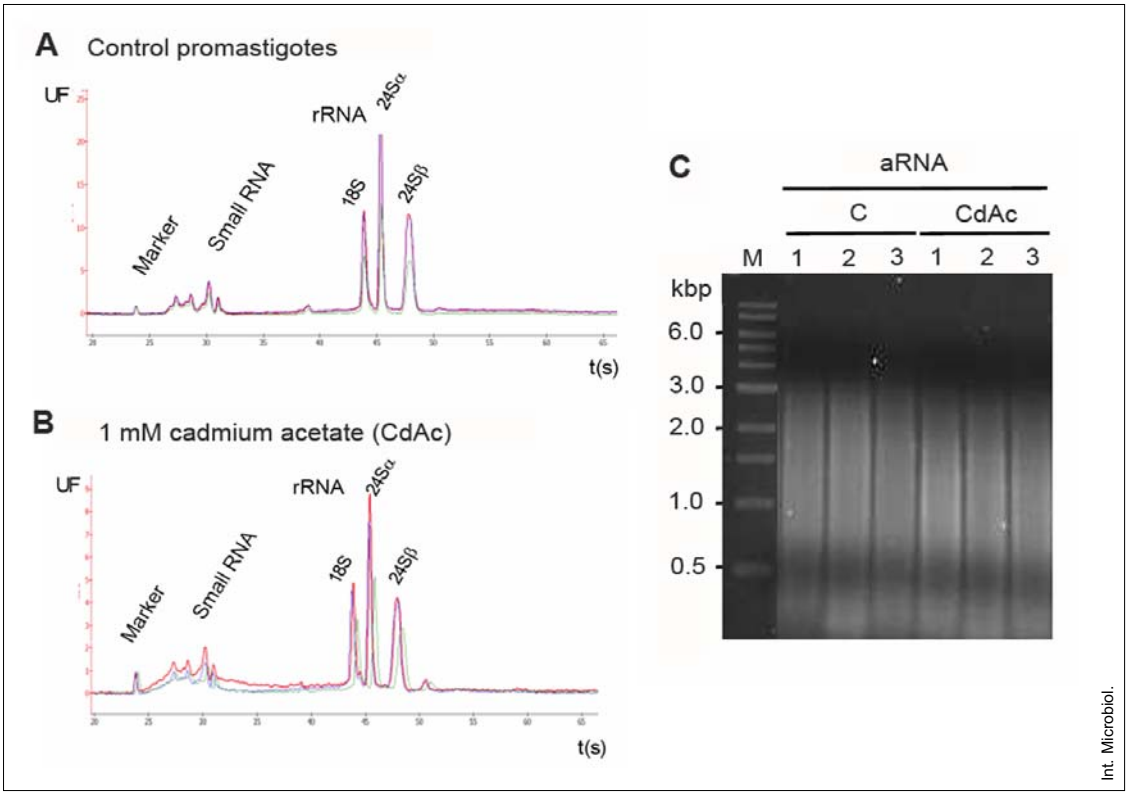

Fig. 2. Analysis of RNA samples. Electropherograms of total RNA samples of cadmium acetate-untreated (**A**) and treated (**B**) promastigotes. The first spike corresponds to RNA6000 Nano Marker (Agilent Technologies). RNA integrity is observed in rRNA 18S and 24S α and β spikes, with a 24S/18S ratio of 2.2; no smear was observed in the RNA samples extracted and processed for the gene expression profile on day 4. (**C**) 1% agarose gel electrophoresis of amplified mRNAs.

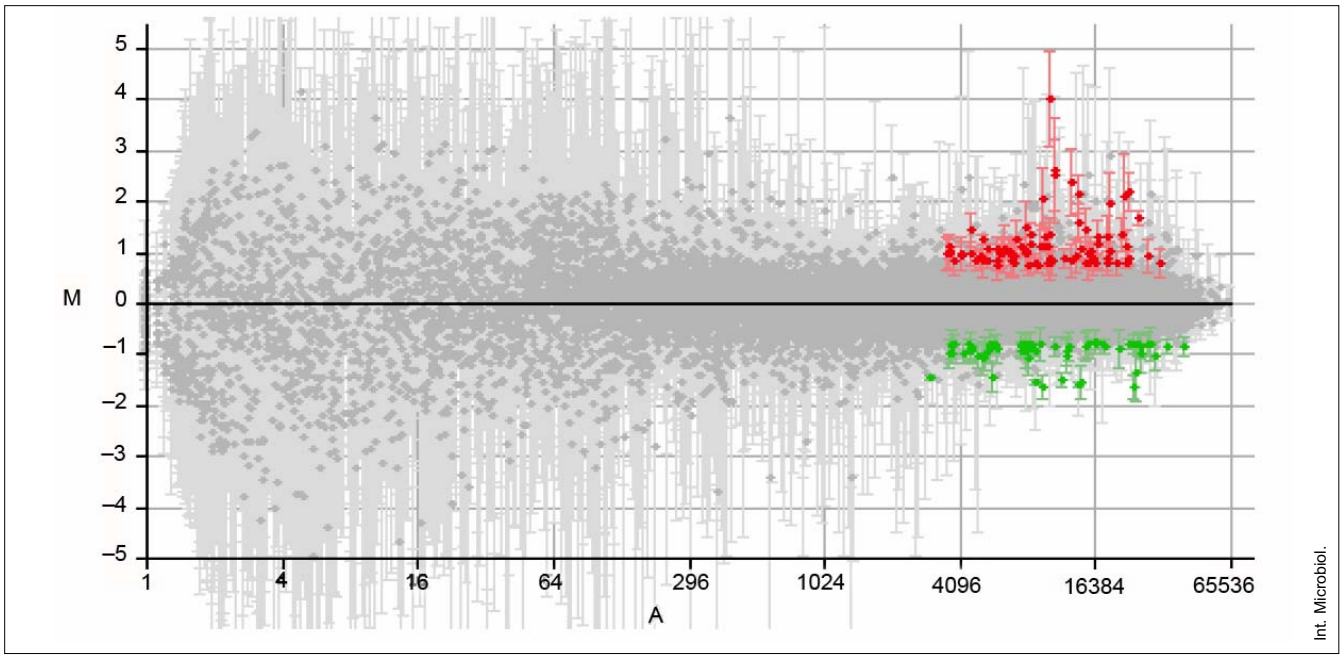

Fig. 3. M/A scatter plot of the microarray hybridization analysis. $M = (\log_2 R_i/\log_2 G_i)$ and $A = [(\log_2 R_i + \log_2 V_i)/2]$, where R and G are, respectively, fluorescence intensity values of red (Cy5-cDNA for CdAc-treated promastigotes) and green (Cy3-cDNA for control promastigotes) dyes.

The down-regulation of branched-chain amino acid aminotransferase following cadmium acetate exposure suggests a decrease in branched-chain amino acid catabolism under these conditions. Two transporter genes were up-regulated in response to cadmium acetate pressure: a zinc transporter containing a ZIP domain (ZnT) and an ABC transporter from the E subfamily (ABCE). Two other transporter genes were down-regulated: a pteridine transporter and an amino acid transporter aATP11 (two copies tandemly located). Trypanosomatids are auxotrophs for pteridines. Shifts in temperature or pH affect pteridine uptake, consistent with our recent finding that the pteridine transporter gene LinJ06_V3.1320 (PT) is down-regulated by an increase in temperature, either independently or in combination with medium acidification [4]. These findings suggest that, under certain environmental conditions, the protists inactivate distinct amino acid and nucleotide biosynthetic pathways. In fact, a vacuolar metalloexopeptidase from ClanMH and the M20 (mpM20) family was up-regulated under exposure to cadmium acetate, suggesting the utilization of exogenous amino acid sources and/or amino acids recycled from the degradation of proteins abundantly expressed in the promastigote stage under normal life-cycle progression. Thus, in *L. infantum* promastigotes, amino acid and nucleotide metabolic changes are reflected by alterations in proliferation, cell motility, and shape.

The calmodulin gene (CaM) was down-regulated under the selective pressure of cadmium acetate; its orthologue in *L. major* was shown to be constitutively expressed in the main stages of the protist's life cycle [15]. The essential functions of CaM include Ca^{2+} homeostasis, which is triggered by its accumulation in the plasma membrane in trypanosomatids, in contrast to other eukaryotes. The accumulation reflects the activation of Ca^{2+}-ATPase by CaM [7]. Consistent with this sequence of events was the identification in the hypothetical protein gene LinJ32_V3.2410 of a calcyclin binding protein domain (CD06468 code from the Conserved Domain database).

Among the cadmium-acetate-sensitive genes involved in the cytoskeleton and flagellum, the gene encoding paraflagellar rod protein 4 (PFR4) was found to be down-regulated. While the flagellum emerged in promastigotes treated with cadmium, movement in fresh preparations of this life stage was not observed, perhaps due to PFR4 down-regulation. Furthermore, the formin gene was also down-regulated, which suggests that actin polymerization priming is diminished in cadmium-acetate-treated promastigotes.

The surface antigen-like protein gene (SALp) was up-regulated in the presence of cadmium acetate. SALp was also shown to be differentially regulated in the life cycle of *L. infantum*, namely, down-regulated in stationary vs. exponential-growth phase promastigotes and up-regulated in both

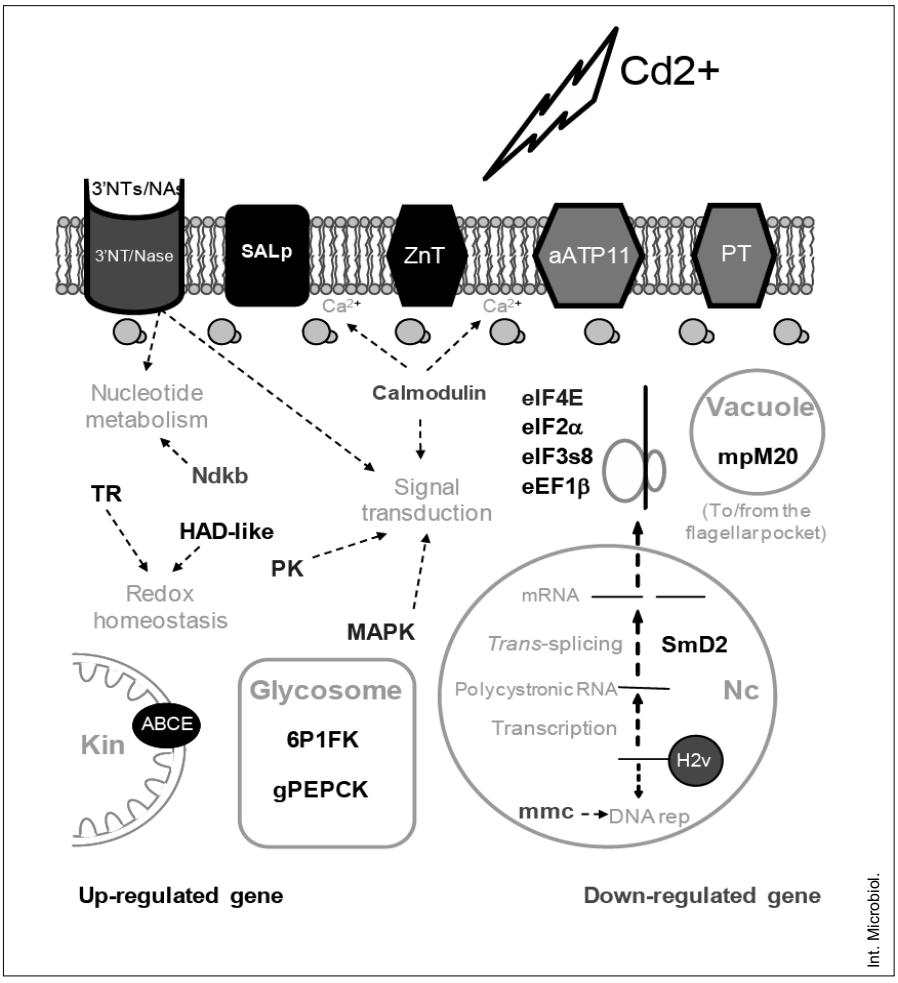

Fig. 4. Differential gene expression profile triggered by cadmium acetate 1 mM.

of these phases in promastigotes vs. amastigotes. In other words, SALp was down-regulated in amastigotes, while its expression reached the highest levels in exponentially growing promastigotes. Consequently, SALp appeared to be a promastigote-specific gene, whereas, as previously shown [2], SALp2 is an amastigote-specific gene.

Finally, for several hypothetical proteins, the respective genes that were up-regulated by cadmium contained conserved domains of known functions. For example, a cytochrome b5 domain (PF00173 from the PFAM database) was identified in the gene LinJ31_V3.1210; the gene LinJ28_V3.0530 contained a tetratricopeptide repeat domain (CD00189) involved in protein-protein interactions (probably an hsp90-like chaperone function); and the gene LinJ06_V3.1290 was similar to the monooxygenase gene of *Trypanosoma cruzi* (BLASTP search: 93% homology, e value 4e-142). To summarize our findings: promastigotes are resistant to cadmium but their lack of movement (down-regulation of PFR4) and metabolic adaptations affect their further development. These results have interesting implications for the development of transmission control agents.

Gene expression regulation: the key to the shock responses and differentiation? Exposure to cadmium acetate led to growth arrest (Fig. 1A), dramatic morphological changes including a decrease in cell volume (Fig. 1B), and the up-regulation of genes encoding: ZnT, trypanothione reductase (TR), glycosomal phosphoenolpyruvate carboxykinase (gPEPCK), 6-phospho-1-fructokinase (FK); a metalloexopeptidase (mpM20) from the ClanMH family, and a haloacid dehalogenase-like hydrolase (HAD-like) (Table 2).

According to GO annotations, ZnT is able to transport heavy metal ions, and mpM20 is vacuolar. HAD-like belongs to the family of dehalogenases, whose members include those involved in detoxification [14]. Taken as a whole, these findings support a sequence of events in which the shock response activated by the protist consists basically of detoxi-

Table 2. Genes up-regulated by 1 mM cadmium acetate. The features described are: clone number; *F*; base-two logarithmic scale *F*, and SD values; *P*. GenBank GSS accession numbers; annotation; annotated gene function; qRT-PCR. When a given clone overlapped with more than one annotation, stage-specific regulation was only demonstrated if the qRT-PCR result was positive (+)

Clones	*F*	$Log_2F \pm$ SD	*P*	GenBank GSS	Annotation	Annotated gene function	qRT-PCR +/–	qRT-PCR *F* ± SD
Lin13D11	1.79	0.8 ± 0.3	0.042	GS882959	LinJ21_V3.0770	ATP-binding cassette, subfamily E		ND
Lin89D9	1.76	0.8 ± 0.2	0.029	GS882960				
Lin166F2	2.19	1.1 ± 0.2	0.012	GS882961				
Lin76E5	1.80	0.8 ± 0.3	0.032	GS882962	LinJ36_V3.7320	Eukaryotic translation initiation factor 3 subunit 8		ND
Lin93A7	2.09	1.1 ± 0.2	0.038	GS882963	LinJ21_V3.0790	Hypothetical protein, conserved		ND
Lin95A3	2.16	1.1 ± 0.1	0.004	GS882964				
Lin101D5	2.46	1.3 ± 0.4	0.035	GS882965	LinJ27_V3.2500	Glycosomal phosphoenolpyruvate carboxykinase		ND
Lin116A5*	1.83	0.9 ± 0.1	0.008	GS882966	LinJ05_V3.0350	Trypanothione reductase	+	6.8 ± 0.2
					LinJ05_V3.0360	ATP-dependent RNA helicase, putative	–	–1.2 ± 0.0
Lin125A5	1.73	0.8 ± 0.1	0.006	GS882967	LinJ32_V3.2410	Hypothetical protein, conserved		ND
Lin139F3*	1.73	0.8 ± 0.2	0.030	GS882968	LinJ29_V3.2470	(Asp) Metallopeptidase, Clan MH, family M20	+	8.7 ± 0.1
Lin144H7	2.03	1.0 ± 0.3	0.028	GS882969	LinJ31_V3.1210	Hypothetical protein, unknown function		ND
Lin177E11*	1.70	0.8 ± 0.3	0.038	GS882970	LinJ29_V3.2620	6-Phospho-1-fructokinase, putative	+	2.5 ± 0.3
Lin295F11	1.82	0.9 ± 0.3	0.041	GS882971				
Lin124C7	1.97	1.0 ± 0.2	0.013	GS882972	LinJ33_V3.3340	Small nuclear ribonucleoprotein SmD2		ND
Lin150H10	2.12	1.1 ± 0.3	0.018	GS882973	LinJ05_V3.1210	Surface antigen-like protein		ND
Lin198E1	1.77	0.8 ± 0.1	0.008	GS882974	LinJ27_V3.2460	Unknown		ND.
Lin221B6	1.77	0.8 ± 0.2	0.015	GS882975	LinJ28_V3.1470/80	Haloacid dehalogenase-like hydrolase, putative		ND
Lin228F5	4.25	2.1 ± 0.3	0.033	GS882976	LinJ06_V3.1290	Hypothetical protein, conserved		ND
Lin234F2	1.76	0.8 ± 0.2	0.021	GS882977	LinJ28_V3.0530	Hypothetical protein, conserved		ND
Lin248G3	2.03	1.0 ± 0.2	0.011	GS882978	LinJ23_V3.0130	Hypothetical protein, conserved		ND
Lin282B6	2.85	1.5 ± 0.5	0.039	GS882979	LinJ03_V3.0960	Elongation initiation factor 2a subunit, putative		ND
Lin283D9	1.74	0.8 ± 0.2	0.019	GS882980	LinJ28_V3.2050	Zinc transporter (ZIP domain), putative		ND
Lin284F5	2.58	1.4 ± 0.3	0.011	GS882981	LinJ36_V3.1490	Translation elongation factor 1β, putative	+	30.6 ± 2.1
Lin309A6	1.92	0.9 ± 0.2	0.10	GS882982	LinJ26_V3.2710	Hypothetical protein, unknown function		ND

*Clone mapping with more genes than shown in the table that have been resolved by qRT-PCR.

fication and the storage of nutrients. These responses may be controlled by the inhibition of DNA replication as well as by the post-transcriptional and translational regulation of gene expression. In fact, some genes involved in the regulation of gene expression were shown to be differentially displayed in response to cadmium acetate. Transcriptionally down-regulated genes included those encoding: a minichromosome maintenance complex protein (*mmc*) involved in DNA replication, a histone H2B variant (H2Bv), a putative splicing factor, eukaryotic translation initiation factor 4E (eIF4E), and a

Table 3. Genes down-regulated by 1 mM cadmium acetate. The features described are: clone number; *F*; base-two logarithmic scale *F*, and SD values; *P*. GenBank GSS accession numbers; annotation; annotated gene function; qRT-PCR. When a given clone overlapped with more than one annotation, stage-specific regulation was only demonstrated if the qRT-PCR result was positive (+). (*) Type c clones [2]

Clones	*F*	$Log_2F \pm$ SD	*P*	GenBank GSS	Annotation	Annotated gene function	qRT-PCR +/–	qRT-PCR *F* ± SD
Lin35A12	–1.74	–0.8 ± 0.2	0.028	GS883041	LinJ30_V3.2370	Zinc finger protein	+	–6.8 ± 0.3
					LinJ30_V3.2380	ADP ribosylation factor	+	–19.0 ± 0.7
					LinJ30_V3.2390	Hypothetical protein, conserved		ND
Lin46H3*	–1.79	–0.8 ± 0.2	0.011	GS883042	LinJ27_V3.1950	Branched chain amino acid aminotransferase	+	–11.6 ± 1.0
					LinJ35_V3.3620	Hypothetical protein, conserved		ND
Lin54F2	–2.05	–1.0 ± 0.3	0.022	GS882983	LinJ03_V3.0600/10	Amino acid transporter aATP11, putative		ND
Lin61A5	–1.73	–0.8 ± 0.3	0.039	GS883043	LinJ27_V3.2210	Hypothetical protein, conserved	+	–5.1 ± 0.2
					LinJ27_V3.2220	Translation elongation regulation factor, putative		ND
Lin60B1*	–2.80	–1.5 ± 0.2	0.004	GS882984	LinJ31_V3.2370/80	3′-Nucleotidase/nuclease/precursor	+	–16.9 ± 1.0
Lin84E8	–2.89	–1.5 ± 0.0	0.000	GS882985				
Lin93D4	–1.72	–0.8 ± 0.2	0.024	GS882986				
Lin157D8	–3.08	–1.6 ± 0.2	0.007	GS882987				
Lin242E2	–2.87	–1.5 ± 0.3	0.016	GS882988				
Lin77B12	–1.71	–0.8 ± 0.1	0.004	GS882989	LinJ27_V3.1520	Eukaryotic translation initiation factor eIF-4E, putative		ND
Lin125B3	–1.71	–0.8 ± 0.1	0.005	GS882990				
Lin84D7	–1.81	–0.9 ± 0.3	0.032	GS882991	LinJ32_V3.3100	Nucleoside diphosphate kinase b		ND
Lin104C3	–1.87	–0.9 ± 0.1	0.006	GS882992	LinJ32_V3.3100/10			
					LinJ32_V3.3120	Minichromosome maintenance protein complex protein (mmc)	+	–54.8 ± 1.6
Lin158H4	–2.11	–1.1 ± 0.3	0.029	GS882993	LinJ32_V3.3110	Nucleoside diphosphate kinase b		ND
Lin83G10	–1.78	–0.8 ± 0.1	0.036	GS882994	LinJ33_V3.0100	Hypothetical protein, conserved		ND
Lin135A4	–1.73	–0.8 ± 0.2	0.016	GS882995	LinJ13_V3.0280	Mitogen activated protein kinase		ND
Lin150E4	–1.76	–0.8 ± 0.1	0.006	GS882996	LinJ13_V3.1060	Calmodulin, putative		ND
Lin173A1*	–3.01	–1.6 ± 0.1	0.000	GS883049	LinJ24_V3.1120	Splicing factor, putative	+	–6.8 ± 0.5
					LinJ24_V3.1130	Formin, putative	+	–8.5 ± 0.4
Lin173F3	–1.74	–0.8 ± 0.2	0.012	GS882997	LinJ25_V3.1520	Hypothetical protein, conserved		ND
Lin187C10	–2.77	–1.5 ± 0.3	0.013	GS882998	LinJ06_V3.1320	Pteridine transporter, putative		ND

Continued on next page

zinc finger protein gene, while the genes encoding small nuclear ribonucleoprotein SmD2 (SmD2), eukaryotic translation initiation factors eEF2α and eIF3s8, and elongation factor eEF1β were up-regulated at the transcript level. H2Bv was found to be significantly up-regulated in mature amastigotes compared to stationary phase promastigotes [20], and *mmc* by the specific influence of an increase in temperature and a decrease in pH [4]. eIF4E, part of the eIF4F initiation

Table 3. *Continued.*

Clones	*F*	$Log_2F \pm$ SD	*P*	GenBank GSS	Annotation	Annotated Gene Function	qRT-PCR +/–	qRT-PCR *F* ± SD
Lin201C10	–1.77	–0.8 ± 0.2	0.015	GS882999	LinJ34_V3.2770	Hypothetical protein, conserved		ND
Lin209B12*	–1.71	–0.8 ± 0.3	0.031	GS883051	LinJ05_V3.0040	Paraflagellar rod protein 4	+	–3.6 ± 0.4
					LinJ05_V3.0050	Hypothetical protein, conserved		ND
Lin227B5	–1.76	–0.8 ± 0.1	0.015	GS883000	LinJ20_V3.0290 (**)	Hypothetical protein, conserved (**)		ND
					LinJ20_V3.0300 (**)	Hypothetical protein, conserved (**)		ND
					LinJ20_V3.0310 (**)	Developmentally regulated phosphoprotein-like protein (**)	–	1.4 ± 0.5
Lin231A1	–1.72	–0.8 ± 0.2	0.019	GS883001	LinJ29_V3.2680	Protein kinase, putative		ND
Lin255E12	–1.76	–0.8 ± 0.2	0.019	GS883002	LinJ28_V3.0210	Histone H2B variant		ND
Lin265E2	–2.04	–1.0 ± 0.3	0.031	GS883052	LinJ36_V3.6760	Mitogen activated protein kinase homologue	+	–5.7 ± 0.4
Lin273A8	–1.86	–0.9 ± 0.2	0.012	GS883003	LinJ12_V3.0650	Hypothetical protein, conserved		ND

**Negative qRT-PCR result. Further quantification by qRT-PCR is required to determine the up-regulated gene mapping with more genes than shown in the table that have been resolved by qRT-PCR.

complex, binds the 5′ cap of mature mRNAs [8]. The down-regulation of this gene was indicative of a general decrease in translation. The orthologue of SmD2 in *L. major* is involved in *trans*-splicing (GO0045291, term from the Gene Ontology database) and is constitutively expressed in amastigotes and exponential phase promastigotes [15]. eIF3s8 and eEF2α were recently found to be down-regulated in amastigotes vs. exponential phase promastigotes [2]. In addition, eEF2α is

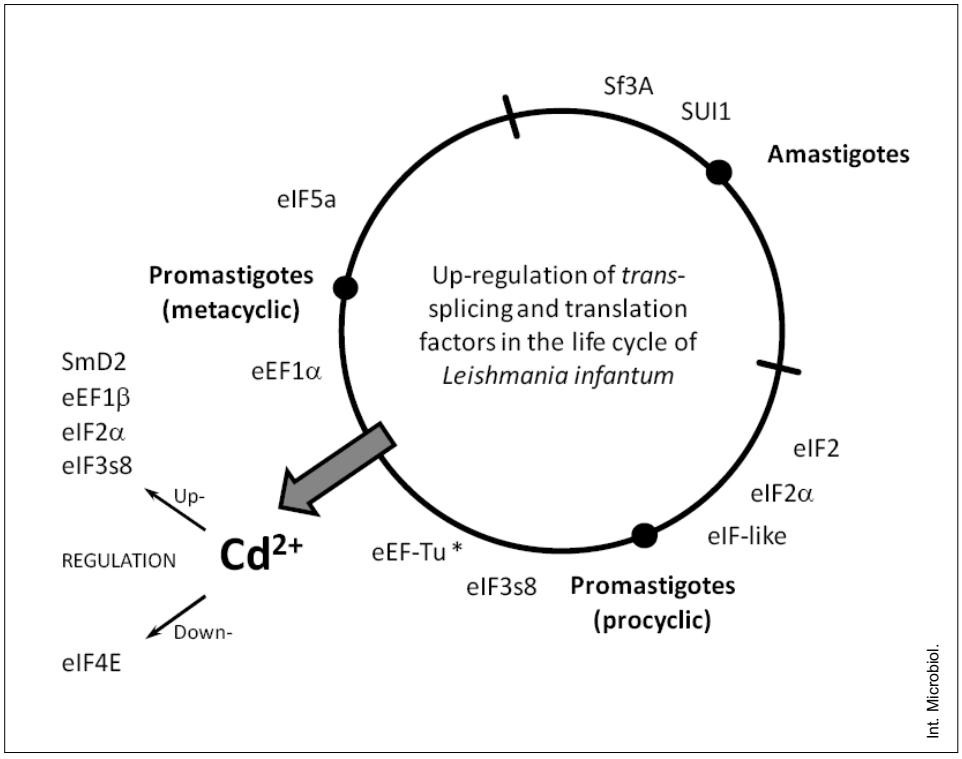

Fig. 5. Differential regulation of genes involved in gene expression modulation. The differential display of splicing and translation factors is observed throughout the life cycle of *L. infantum* and under exposure to cadmium acetate.

up-regulated in amastigotes by the specific influence of either temperature increase or pH decrease whereas the concurrence of both factors had no effect on amastigote-like forms (gp46 negative) [2] or metacyclic promastigotes [2]. These data suggest the induction of eEF2α expression in certain anomalous environments, i.e., those outside of the normal progress of the protist's life cycle, including the presence of heavy metals, temperature increase, and pH decrease.

Differential regulation of eEF1β has not been found in any of the *L. infantum* main stages but its expression was up-regulated by cadmium acetate. By contrast, eEF1α is up-regulated in metacyclic promastigotes [2] and in response to a temperature increase but down-regulated by increased temperature when combined with acidification [2]; however, it was not modulated by cadmium acetate. In summary, each of the translation factors mentioned is specifically modulated by the protist in response to certain challenges, either affecting the life cycle or under abnormal environmental situations (Fig. 5). Consequently, both polycistronic mRNA processing and translational control are probably key targets in the stimulation of the shock response.

In our study, *L. infantum* promastigotes were highly resistant to cadmium acetate, which at concentrations of 1 mM resulted in growth arrest, perhaps related to the down-regulation of two MAPK genes. Despite the emerging flagellum, growth was arrested and the promastigotes were not motile, the latter probably due to the down-regulation of the paraflagellar rod proteinPFR4. Initial morphological changes in the promastigote population occurred 72–84 hours after the addition of cadmium acetate, which also induced a shock response consisting of the up-regulation of ZnT, TR, gPEPCK, FK, mpM20, and HAD-like genes. It can be hypothesized that these expression profiles were triggered by changes in the regulation of gene expression, consistent with the down-regulation of H2v, SmD2, eIF4E, and a putative splicing factor and the up-regulation of eEF2α, eIF3s8, and eEF1β.

Our findings suggest that the modulation of gene expression would be a key component of the shock response of *L. infantum* promastigotes to cadmium acetate. Together with the expression pattern of other translation factors throughout the progression of the *L. infantum* life cycle and in response to temperature and/or pH shifts, the data are consistent with differential regulation of gene expression depending on the environmental challenges. The results are applicable to downstream strategies in the field of transmission control.

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