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Characterization of a gene encoding alcohol dehydrogenase in benznidazole-susceptible and -resistant populations of *Trypanosoma cruzi*

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ABSTRACT

Alcohol dehydrogenases (ADH) are a class of oxidoreductases that catalyse the reversible oxidation of ethanol to acetaldehyde. In the human parasite *Trypanosoma cruzi* the *TcADH* gene was identified through microarray analysis as having reduced transcription in an *in vitro* induced benznidazole (BZ)-resistant population. In the present study, we have extended these results by characterizing the *TcADH* gene from 11 strains of *T. cruzi* that were either susceptible or naturally resistant to benznidazole and nifurtimox or had *in vivo* selected or *in vitro* induced resistance to BZ. Sequence comparisons showed that *TcADH* was more similar to prokaryotic ADHs than to orthologs identified *Leishmania* spp. Immunolocalisation using confocal microscopy revealed that TcADH is present in the kinetoplast region and along the parasite body, consistent with the mitochondrial localization predicted by sequence analysis. Northern blots showed a 1.9 kb transcript with similar signal intensity in all *T. cruzi* samples analysed, except for the *in vitro* selected resistant population, where transcript levels were 2-fold lower. These findings were confirmed by quantitative real-time PCR. In Western blot analysis, anti-TcADH polyclonal antisera recognised a 42 kDa protein in all *T. cruzi* strains tested. The level of expression of this polypeptide was approximately 2-fold lower in the *in vitro* induced benznidazole-resistant strain, than in the susceptible parental strain. The chromosomal location of the *TcADH* gene was variable, but was not associated with the zymodeme or with the drug resistance phenotype. The data presented here show that the TcADH enzyme has a decreased level of expression in the *in vitro* induced BZ-resistant *T. cruzi* population, a situation that has not been observed in the *in vivo* selected BZ-resistant and naturally resistant strains.

1. Introduction

The flagellate protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae), the etiological agent of Chagas disease, currently affects some 13–15 million people in Latin America (World Health Organization, 2007). Two drugs, nifurtimox (4-[5-nitrofurfurylidenamino]-3-methylthiomorpholine-1,1-dioxide; NFX) and benznidazole (*N*-benzyl-2-[2-nitroimidazol-1-yl]acetamide; BZ), are presently utilised in the treatment of Chagas disease, but both have toxic side-effects and have a very low efficacy in treating the chronic phase of the disease (Urbina and Docampo, 2003). The 5-nitrofuran NFX acts by reducing nitro groups to unstable nitro anion radicals, which in turn react to produce highly toxic reduced-oxygen metabolites (i.e. superoxide anions and hydrogen peroxide) (Docampo et al., 1981). The mechanism of action of the 2-nitroimidazole BZ involves a process of reductive stress in which covalent modification of macromolecules, such as DNA, proteins and lipids, by the reduction of nitro intermediates leads to the inhibition of parasite growth (Diaz de Toranzo et al., 1988). Various strains of *T. cruzi* exhibit different levels of susceptibility to NFX and BZ, and this variability may partially explain the observed differences in effectiveness of chemotherapy involving the two drugs (Filardi and Brener, 1987).

The innovative DNA microarray technique is finding increasing application in many areas of microbiological research and has been employed in studies concerning the life cycle and development of parasites, parasite–host relationships, assessment of virulence in different organisms, design of vaccines and studies of responses to

Abbreviations: ADH, Alcohol dehydrogenase; BZ, Benznidazole; NFX, Nifurtimox; GST, Glutathione S-transferase; HGPRT, Hypoxanthine–guanine phosphoribosyltransferase; PCR, Polymerase chain reaction; PFGE, Pulsed field gel electrophoresis; TcADH, *Trypanosoma cruzi* alcohol dehydrogenase; Z, zymodeme.

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Table 1
Trypanosoma cruzi strains used in this study and the chromosomal location of the *TcADH* gene.

T. cruzi	Origin[a]	Host	Sus[b]	Zym[c]	Chromosomal location of *TcADH* (kb)
17WTS	Mex	Triatomine	S	Z1	1890; 1120
17LER	Mex	Triatomine	R	Z1	1890; 1120
Yuyu	BA	*Triatoma infestans*	R	Z1	1270; 1540
Quaraizinho	RGS	*Triatoma infestans*	S	Z1	1120
BZS	SP	Human (acute case)	S	Z2	1420
BZR	SP	Human (acute case)	R	Z2	1420
Berenice	MG	Human (chronic case)	S	Z2	1420
Ernane	GO	Human (chronic case)	S	Z2	1120; 1230
VL-10	MG	Human (chronic case)	R	Z2	1270
Buriti	RGS	*Triatoma infestans*	S	ZB	1270; 1540
CL Brener	RGS	*Triatoma infestans*	S	ZB	1270; 1540

[a] BA, Bahia; RGS, Rio Grande do Sul; SP, São Paulo; MG, Minas Gerais; GO, Goiás (States of Brazil) and Mex, Mexico.
[b] Sus, *in vivo* drug susceptibility as described by Filardi and Brener (1987); S, susceptible; R, resistant.
[c] Zym, zymodeme classification according to Murta et al. (1998).

infections by parasites and micro-organisms (Schena et al., 1995; Gobert et al., 2005). The methodology enables the collection of considerable amounts of valuable information concerning profiles of gene expression, and has been of particular value in studies of differentially expressed genes in *Leishmania* (Guimond et al., 2003) and *Candida albicans* (Rogers and Barker, 2002; Garaizar et al., 2006) correlated with the phenotype of resistance to drugs. With respect to *T. cruzi*, the methodology has been used to assess differential gene expression profiles during trypomastigote-amastigote transformation (Minning et al., 2003) and between *T. cruzi* groups I and II (Baptista et al., 2004), as well as for analysing host transcriptional responses to *T. cruzi* infection (Burleigh, 2004). In a previous study (S.M.F Murta et al., in preparation), we employed DNA microarray methodology and demonstrated the *ca*. 4-fold lower expression of a *T. cruzi* gene encoding for an alcohol dehydrogenase in an *in vitro* induced BZ-resistant population (17LER) than in its BZ-susceptible counterpart (17WTS).

The interconversions of alcohols, aldehydes and ketones are essential metabolic processes in both prokaryotes and eukaryotes. Alcohol dehydrogenases (ADH; EC 1.1.1.1) comprise a class of oxidoreductases that catalyses the reversible oxidation of ethanol to acetaldehyde with the concomitant reduction of NAD (Reid and Fewson, 1994) and they may be categorised as NAD(P)-dependent, NAD(P)-independent or FAD-dependent enzymes. Of the three categories, the NAD(P)-dependent enzymes have been most fully characterized and further subdivided, according to structure and catalytic activity, into groups I and II comprising, respectively, long-chain and short-chain zinc independent ADHs, and group III containing the iron-activated ADHs (Jörnvall et al., 1987). Iron-dependent dehydrogenases are present in various micro-organisms such as *Escherichia coli* and *Clostridium perfringens*, as well as in protozoan pathogens such as *Leishmania, Trichomonas vaginalis, Giardia lamblia*, and *Entamoeba histolytica*. Remarkably, the lack of this type of enzyme in vertebrates makes it an important target for antimicrobial chemotherapy (Chen et al., 2004).

Few ADHs have been described for members of the Trypanosomatidae (Molinas et al., 2003). The presence of ADH (also known as NADP-aldehyde-reductase) in *T. cruzi* was first described by Arauzo and Cazzulo (1989) who partially purified the enzyme and characterized its activity. Recently the complete genome of *T. cruzi* has been sequenced revealing that this parasite contains a gene (*TcADH*) encoding a group III iron-activated ADH (El-Sayed et al., 2005).

In the present paper we report the first characterization of the *ADH* gene in *T. cruzi*. Initially, a phylogenetic analysis was carried out by comparing the amino acid sequence of the TcADH protein with those of ADHs from other organisms. Immunolocalisation assays of the protein inside the parasite were conducted through confocal microscopy. Subsequently, the levels of mRNA, the copy numbers and the chromosomal location of *TcADH* gene and the level of TcADH protein were determined in 11 BZ-susceptible and -resistant populations of *T. cruzi*.

2. Material and methods

2.1. Strains of T. cruzi

The 11 strains of *T. cruzi* employed in this study are listed in Table 1. The BZ-resistant *T. cruzi* population (17LER) derived from the Tehuantepec cl2 susceptible wild-type strain (17WTS) (Nirdé et al., 1995) was obtained *in vitro* by increasing in a stepwise manner the concentration of benznidazole (BZ) (*N*-benzyl-2-nitro-1-imidazolacetamide, Rochagan, Roche Co.). The 17LER parasites are resistant to 220 μM BZ. The BZ-resistant population BZR was derived in a previous study (Murta and Romanha, 1998) from the susceptible Y strain following *in vivo* selection after 25 successive passages in mice treated with a single high dose of BZ (500 mg/kg body weight). The *in vivo* susceptibility to BZ and NFX of the remaining seven strains of *T. cruzi* had been characterized previously (Filardi and Brener, 1987). Of these strains, five were susceptible and two were naturally resistant to both drugs. All *T. cruzi* strains had been classified previously as zymodemes Z1, Z2 or ZB according to their isoenzyme patterns (Murta et al., 1998).

2.2. Phylogenetic analysis

On the basis of one nucleotide (GenBank accession no. XM814171) and two amino acid (GenBank accession no. XP819264; GenBank accession no. XP821876) sequences of *T. cruzi* ADH that were available in GenBank, orthologous genes were identified in *Leishmania major* (GenBank accession no. LmjF30.2090), *L. infantum* (GenBank accession no. LinJ30.2440) and *L. braziliensis* (GenBank accession no. LbrM30v2.2040). The following amino acid sequences were also employed: *Candida albicans* (GenBank accession no. EAK99442), *Chromobacterium violaceum* (GenBank accession no. NP902398), *Clostridium perfringens* (GenBank accession no. NP561365), *Drosophila melanogaster* (GenBank accession no. NP477209), *Entamoeba histolytica* (GenBank accession no. XP652262), *Escherichia coli* (GenBank accession no. NP756272), *Giardia lamblia* (GenBank accession no. XP770830), *Shewanella amazonensis* (GenBank accession no. ZP00585549) and *Trichomonas vaginalis* (GenBank accession no. AAO21494). Selected sequences were compared using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI), and WU-BLAST software from The Institute of Genomic Research (TIGR). Both nucleotide (BLASTX) and amino acid (BLASTP) sequences of TcADH were compared with non-redundant sequences of proteins deposited in GenBank and the *Leishmania* database at Genedb (www.genedb.org). The sequences obtained

were aligned using Clustal X windows interface (Thompson et al., 1997) and the phylogenetic tree was constructed through application of MEGA 3 software (Kumar et al., 2004).

2.3. In silico analysis of subcellular localization of TcADH and immunofluorescence studies

The *in silico* prediction of TcADH localization was carried out using the programs TargetP (Emanuelsson et al., 2000), Mito Prot II (Claros and Vincens, 1996) and Predotar (Small et al., 2004). TargetP, based on neural network programming, was developed to predict targeting of protein sequences to chloroplasts, mitochondria, and the secretory system using a knowledgebase derived from Swiss-Prot sequence entries. MitoProt was developed to predict mitochondrial targeting and presequence cleavage sites based on a set of 47 known characteristics of presequences and cleavage sites. Predotar recognises the N-terminal targeting sequences of classically targeted mitochondrial and chloroplast precursor proteins.

Immunolocalisation assays were conducted through confocal microscopy; briefly epimastigotes of *T. cruzi* (Y strain) were harvested and washed twice in PBS by centrifugation at 500 × *g* for 10 min at 4 °C. The parasite pellet was resuspended in 4% paraformaldehyde in PBS, fixed by incubation for 2 h at 4 °C, adjusted to a concentration of 5×10^6 cells/mL, washed in PBS and finally allowed to attach to poly-L-lysine-coated glass slides. After a 1 h blocking and permeabilisation step involving treatment with PGSN (PBS containing 0.2% Sigma gelatine, 0.1% Sigma saponin and 0.1% sodium azide), samples were incubated with anti-TcADH antibodies (diluted 1:50 in PGSN) and anti-Pol-β (diluted 1:50 in PGSN) for 12 h in a humid chamber. The antiserum anti-TcADH was generated as described in Section 2.7 and the anti-Pol-β was provided by Dr. Carlos Renato Machado (Lopes et al., 2008). After three washes with PBS, the bound antibodies were treated with Sigma goat anti-rabbit Ig conjugated to Cy3 (diluted 1:40) and 10 μM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) in order to visualise nuclei. Following a 1 h incubation, samples were washed three times in PBS and mounted in 0.1 M Tris hydrochloride (pH 8.8)-buffered glycerol containing 0.1% *p*-phenylenediamine (Sigma) as anti-bleaching agent. The epimastigotes were then imaged using a Bio-Rad 1024UV confocal system attached to a Carl Zeiss (Hercules, CA, USA) Axiovert 100 microscope equipped with an NA 1.2 water-immersion planapochromatic 40× objective. Images were processed with Image-J (http://rsb.info.nih.gov/ij/) and Adobe Photoshop software.

2.4. RNA and DNA preparations

Total RNA and genomic DNA from *T. cruzi* samples were extracted as previously described (Nogueira et al., 2006). Southern and northern blots were carried out using protocol previously described (Murta et al., 2006).

2.5. Real-time RT-PCR

The real-time RT-PCR procedure was realized as previously described (Nogueira et al., 2006). The cDNA was used for real-time PCR amplification on an ABI Prism 7000-Sequence Detection System SDS (PE Applied Biosystems, Foster City, CA, USA). The specific primers (forward: 5′ GCAAGAATCTTGTGGCACGAG and reverse 5′ AAGCTGATGAGCCATTGCG) employed were designed from the complete nucleotide sequence of the *TcADH* gene (GenBank accession no. XP819264). The *T. cruzi* housekeeping gene hypoxanthine–guanine phosphoribosyltransferase (*TcHGPRT*) was used to normalise the amount of samples according to (Murta et al., 2006). Standard curves were prepared for each run using known quantities of pCR 2.1-TOPO plasmids (Invitrogen) containing the *TcADH* and *TcHGPRT* genes. Raw products were quantified using Sequence Detection System data analysis software and normalised to the *TcHGPRT* values for each sample.

2.6. Cloning of the TcADH gene

A 1220 bp segment corresponding to the ORF of *TcADH* (obtained from the complete nucleotide sequence corresponding to GenBank accession no. XP819264) was amplified using the forward primer: 5′-*cgcggatcc*CCATGTTTCGCTTCTCACGCCC-3′and the reverse primer: 5′-*ccggaattctaca*TTGACTCACGGTAGAT-3′ in which the sequences lowercase in italics correspond to *Bam*HI and *Eco*RI restriction sites, respectively, inserted in order to facilitate cloning. The PCR product encoding TcADH was restricted with *Bam*HI and *Eco*RI and inserted into the corresponding sites of the pGEX-5X-3 expression vector (Amersham Biosciences, Amersham, UK) containing a glutathione S-transferase gene (*GST*) from *Schistosoma japonicum*.

2.7. Purification of recombinant TcADH protein and production of polyclonal antiserum

Cells of *Escherichia coli* strain BL-21 were transformed with the expression vector carrying the PCR product, cultured for 5 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega, Madison, WI, USA), harvested and lysed. The GST-recombinant protein produced in *E. coli* BL-21 was separated on a glutathione Sepharose 4B column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and purified using an electro-eluter cell (Bio-Rad, Hercules, CA, USA) operated according to the protocol recommended by the manufacturer. Protocols for production of polyclonal antiserum were as previously described (Murta et al., 2006).

2.8. Western blot analysis

The levels of expression of TcADH were determined by western blot analysis according to (Murta et al., 2006), using antiserum raised against recombinant rTcADH at 1:5000 dilution.

2.9. Pulsed field gel electrophoresis (PFGE)

Chromosomes of *T. cruzi* were separated by PFGE using the Gene-Navigator™ system (Pharmacia, Uppsala, Sweden) as previously described (Murta et al., 2006). Electrophoretic conditions were optimised for *TcADH* and the pulse intervals used were: 70 s for 10 h; 90 s for 24 h; 200 s for 15 h and 400 s for 15 h (at 180 V). Following electrophoresis, gels were stained with ethidium bromide (0.5 μg/mL) and the chromosome bands were transferred onto nylon membranes. The *TcADH* gene was identified by incubation of the membranes with ^{32}P-labeled *TcADH* gene probe.

2.10. Densitometric analysis

All Southern and Northern auto-radiograms and Western blot membranes were photographed and analysed using ImageMaster VDS software (Amersham Pharmacia Biotech). Differences were considered significant when the intensity band ratio was ≥2-fold.

3. Results

3.1. Identification of TcADH and sequence alignment

A search on GenBank revealed two published *T. cruzi* amino acid sequences relating to ADH (accession nos. XP819264 and XP821876). Alignment of these indicated 99% sequence identity

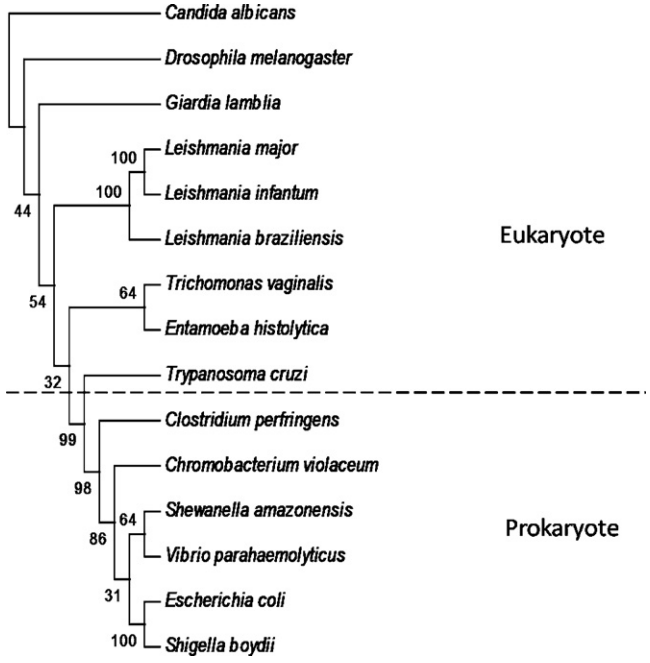

Fig. 1. Neighbour-joining phylogenetic tree of ADH sequences of *Trypanosoma cruzi* and other organisms with a high similarity degree. The numbers shown are bootstrap values. Bootstrap is a method that provides assessments of confidence for each clade of an observed tree, based on the proportion of bootstrap trees showing that same clade (Efron et al., 1996).

with substitutions at residues 299 (asparagine/serine) and 347 (proline/serine). The sequence with accession number XP819264 was subsequently employed in the study. The *TcADH* gene encodes a protein comprising 392 amino acids with a predicted mass of 42 kDa. The TcADH enzyme harbours a single conserved domain corresponding to an iron-containing ADH, and this comprised almost the whole protein (amino acid residues 17–384) in the sequences analysed.

The similarity index between the TcADH sequence and those of ADHs from prokaryotic organisms varied between 52% and 57%, it attained only 11–31% with respect to most other eukaryotic ADHs and just 35% compared with orthologs from the three *Leishmania* species studied (Supplemental Fig. S1). Moreover, comparison between the TcADH gene sequence and database sequences of other species of *Trypanosoma*, including *T. brucei, T. congolense, T. rangeli* and *T. vivax*, revealed that no protein similar to TcADH had been found in these organisms.

In order to compare TcADH amino acid sequence with ADH sequences identified in 12 different prokaryotic and eukaryotic organisms, a neighbour-joining phylogenetic tree was constructed (Fig. 1). Interestingly, the ADH of *T. cruzi* is more closely related to prokaryotic ADHs, even bacterial ADHs, than to the three *Leishmania* species studied.

3.2. In silico analysis of subcellular localization of TcADH and immunofluorescence studies

The three bioinformatics tools, TargetP, Mito ProtII and Predotar, have been used to predict subcellular localization of the TcADH. All three programs indicated a mitochondrial localization for the TcADH with a probability of 78%, predicted by Mito ProtII.

Subsequently, immunolocalisation assays were carried out in order to determine the distribution of TcADH protein in *T. cruzi* Y strain epimastigotes (Fig. 2). The rabbit antiserum anti-TcADH presented a reticular labeling concentrated at the kinetoplast region of parasite, consistent with the mitochondrial distribution pattern (Fig. 2A–D). Fig. 2E–F obtained after optical sectioning and Volume-J renderization (http://rsb.info.nih.gov/ij), shows that TcADH is distributed along the parasite body. *T. cruzi* and others Trypanosomatids possess a single mitochondrion, which extends throughout the cell (Coombs et al., 1986). DAPI staining was used to visualise nucleus and kinetoplast (containing the parasite's mitochondrial DNA network; Fig. 2C). The rabbit antiserum anti-Pol-β (Lopes et al., 2008) was used as a control of the kinetoplast labeling. Fig. 2H shows Pol-β labeling of two dots at the kinetoplast border. These results strongly suggest that the TcADH enzyme is located in the mitochondria, consistent with the mitochondrial localization predicted by bioinformatics analysis.

3.3. Quantification of TcADH RNA levels in parasite populations by Northern blotting and real-time RT-PCR

The levels of *TcADH* mRNA in parasite populations were evaluated by Northern blot analysis. A 1.9 kb transcript was detected in Northern blots of total RNA derived from BZ-susceptible and -resistant *T. cruzi* strains following hybridisation with a ^{32}P-labeled *TcADH* gene-specific probe (Fig. 3A). Quantitative controls using a ribosomal RNA probe are shown in Fig. 3B. Densitometric analyses of transcript profiles of *T. cruzi* strains revealed that *TcADH* mRNA levels were 2-fold lower in the BZ-resistant 17LER population than in the 17WTS population. No differences in the levels of *TcADH* mRNA were detected between the other strains of *T. cruzi* analysed regardless of the drug resistance phenotype.

TcADH mRNA levels were also determined more precisely by quantitative real-time RT-PCR experiments in which total RNA of each strain was normalised by reference to the single copy *T. cruzi* housekeeping gene *TcHGPRT*. The amount of *TcADH* cDNA in the *T. cruzi* strains was determined by linear regression analysis using the PCR threshold cycle (C_T) values obtained from the standard curve generated with known amounts of the *TcADH* plasmid. The levels of transcription of the *TcADH* gene were 2.5-fold lower in the 17LER population compared with the 17WTS population (Fig. 3C). No differences in the levels of transcription of the *TcADH* gene were detected between the other *T. cruzi* BZ-susceptible and -resistant sample pairs, i.e. BZS *versus* BZR and CL Brener *versus* VL-10.

3.4. Copy number ratio of the TcADH gene

The copy number ratio of the *TcADH* gene per genome of pairs 17WTS and 17LER was determined by real-time PCR. On the basis that one *T. cruzi* parasite contains 0.33 pg of DNA (Moser et al., 1989) and that *TcHGPRT* is a single copy gene (Allen and Ullman, 1994), *TcADH* copy numbers were estimated using 200, 100, 50 and 25 ng of genomic DNA. It was found that the copy number ratio of the *TcADH* gene was the same for 17WTS/17LER populations (Supplemental Table S1).

3.5. Expression, purification and analysis of a recombinant TcADH protein

Anti-TcADH polyclonal antibodies were produced in rabbits using GST-tagged rTcADH protein as antigen, and the antibodies were employed in Western blot analyses to evaluate the level of enzyme produced by each strain of *T. cruzi.* SDS-PAGE profiles of the total protein extracts obtained from the *T. cruzi* strains contained proteins ranging from 10 to 100 kDa. In the Western blot, the antibody to TcADH recognised a 42 kDa band in the native protein, and this band was present in all *T. cruzi* samples studied. In order to quantify the level of expression of rTcADH in each strain, the same membrane was incubated with anti-rTcHSP70 polyclonal antibodies (diluted 1:10,000) since the level of expression of the TcHSP70 protein has been observed to be equal in both BZ-susceptible and

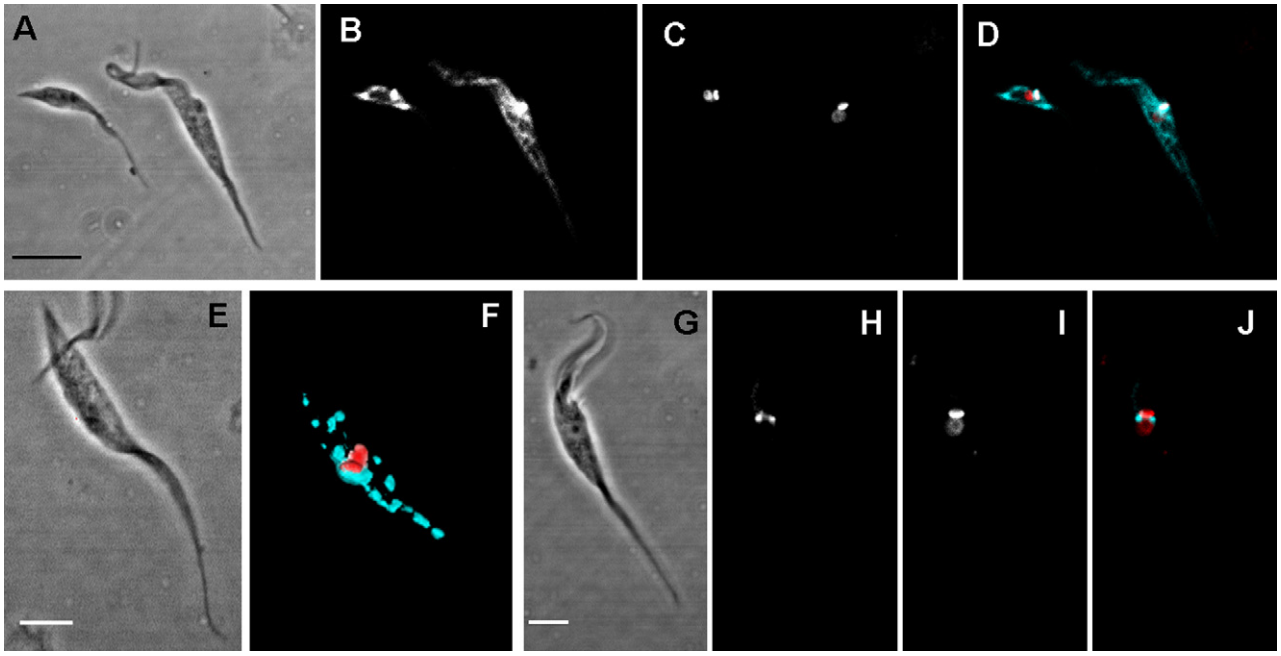

Fig. 2. Distribution of Tc-ADH in *T. cruzi* Y strain epimastigotes. (A–D) Labeling with rabbit antiserum to Tc-ADH: (A) phase contrast image; (B) Tc-ADH (note the reticular labeling concentrated at the kinetoplast region, consistent with the mitochondrial distribution pattern); (C) DAPI labeling of kinetoplasts and nuclei; (D) merged image of Tc-ADH (cyan) and DAPI (red). Bar in A = 10 mm. (E and F) Reticular distribution of Tc-ADH: (E) phase contrast image; (F) Tc-ADH distribution along the parasite body, obtained after optical sectioning and Volume-J renderization (http://rsb.info.nih.gov/ij). (G–J) Distribution of Pol-b in *T. cruzi* epimastigotes. (G) phase contrast image; (H) Pol-b labeling of two dots at the kinetoplast border; (I) DAPI labeling of kinetoplast and nucleus; (J) merged image of Tc-ADH (cyan) and DAPI (red). Bars in I and G = 5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

-resistant *T. cruzi* populations (Murta et al., 2008). Densitometric analysis of the TcADH band using TcHSP70 as a reference showed that the level of expression of the TcADH protein was similar for all samples tested except 17LER, which exhibited a level that was 2-fold lower compared with 17WTS (Fig. 4). This result was confirmed by Western blots analysis using serial dilutions of protein extracts from 17WTS and 17LER populations (data not shown). Moreover, when control Western blot analyses were carried out with Invitrogen anti-GST antibodies (diluted 1:15,000), no components of the total protein extracts from *T. cruzi* strains were recognised (data not shown).

3.6. Chromosomal location of the TcADH gene

The chromosomes of *T. cruzi* were readily separated by PFGE and their number and size (ranging from 800 to 3000 kb) differed markedly between the clones and strains analysed (Fig. 5A). Hybridisation of Southern blots of the chromosomes with a *TcADH* gene-specific probe revealed that the size and number of chromosomes containing this gene were noticeably different and strain specific, although the chromosomal location of *TcADH* was not associated with the drug resistance phenotype. However, in the two *T. cruzi* strains with the ZB zymodeme, the ADH gene was located on the same chromosomes of 1270 and 1540 kb (Fig. 5B).

4. Discussion

In the present paper we characterized a gene encoding alcohol dehydrogenase in 11 *T. cruzi* strains susceptible and resistant to benznidazole. Alcohol dehydrogenases are a class of oxidoreductases that catalyse the reversible oxidation of ethanol to acetaldehyde. Sequence analysis revealed that the *TcADH* gene encodes a protein comprising 392 amino acids with a predicted mass of 42 kDa. Western blot analysis using antisera raised against this protein confirmed the presence this polypeptide in all *T. cruzi* strains tested.

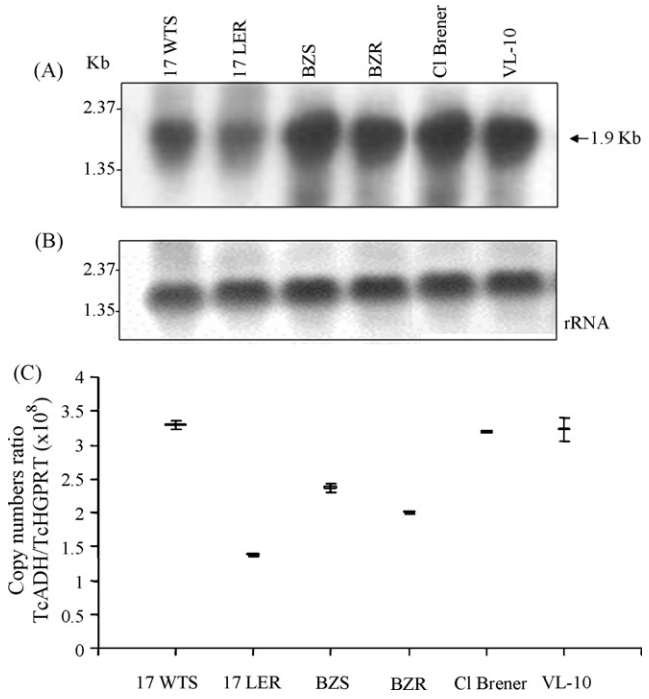

Fig. 3. Levels of transcription of the *TcADH* gene in BZ-resistant and -susceptible *T. cruzi* strains. (A) Northern blot profile of total RNA extracts from the *T. cruzi* strains obtained using a ^{32}P-labeled *TcADH*- specific probe. (B) As quantitative control, the same membrane was then exposed to a *T. cruzi* ribosomal RNA probe. (C) Amplification of *T. cruzi TcADH* and *TcHGPRT* genes by real-time RT-PCR. Values were normalised to those obtained for *TcHGPRT* and represent the means (± S.E.M.) of duplicate real-time RT-PCR analyses from three independent experiments. The characteristics of the strains used are listed in Table 1.

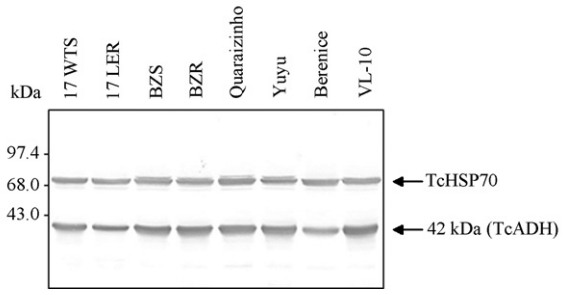

Fig. 4. Western blot analysis of protein derived from *T. cruzi* strains probed with a polyclonal rabbit antiserum raised against the recombinant proteins TcADH and TcHSP70. The drug resistance phenotypes of the *T. cruzi* strains analysed are listed in Table 1.

This enzyme has an iron ion in its structure and thus belongs to ADH group III. Immunolocalisation assays and bioinformatics analysis indicated a mitochondrial localization for the TcADH. Northern blot analysis revealed that *TcADH* gene is expressed as a transcript of 1.9 kb in all *T. cruzi* strains analysed.

Phylogenetic analysis was carried out by comparing the amino acid sequence of the TcADH protein with those of ADHs from other organisms. The results showed that *TcADH* in *T. cruzi* exhibited higher levels of similarity to prokaryotic ADHs than to orthologs identified in species of *Leishmania*. Analysis of the primary structure of *TcADH,* together with a consideration of its phylogeny, indicated that the gene has undergone significant modification throughout the evolutionary process. Within the family Trypanosomatidae, evolutionary modifications have resulted in the absence of the gene in *T. brucei*, *T. rangeli* and other *Trypanosoma* species. A low level of similarity was found in the present study between ADHs from three *Leishmania* species and TcADH, the latter more resembling a prokaryote ADH. On this basis, we suggest that the activity of this gene may play an important role in the specific development of each parasite. This finding is not surprising since El-Sayed et al. (2005), through comparative studies of the genomes of trypanosomatids (i.e. *Leishmania major, T. cruzi* and *T. brucei*), demonstrated that gene insertions, substitutions and deletions are common processes in such organisms, which may result in both physiological and biochemical differences between the parasites. Additionally, Molinas et al. (2003) purified and characterized a mitochondrial isopropyl alcohol dehydrogenase of *Phytomonas* sp., a trypanosomatid isolated from plants. Based on the evidence that this enzyme was more similar to prokaryotic than eukaryotic ADHs, the authors suggested that there may have been a horizontal gene transfer between anaerobic bacteria and the trypanosomatids.

Immunolocalisation assays revealed that the TcADH protein is present in the kinetoplast region and along the parasite body, consistent with the mitochondrial localization predicted by bioinformatics tools TargetP, Mito Prot II and Predotar. This parasite, along with other members of the family Trypanosomatidae, has a single mitochondrion harbouring the mitochondrial DNA. However, in addition to this DNA, which corresponds with that found in other eukaryotic cells, there is a large amount of DNA organised in the form of mini-circles and concentrated at a specific site below the basal corpuscle, which forms an intra-mitochondrial structure known as the kinetoplast. Cytochemical and biochemical investigations have revealed the presence in the kinetoplast of mitochondrial enzymes including cytochrome oxidase, succinate dehydrogenase, isocitrate dehydrogenase, NADPH diaphorase, alpha glycerol phosphate dehydrogenase and beta hydroxybutyrate dehydrogenase. Such enzymes fulfil both metabolic and respiratory functions, providing parasites with alternative adaptations to suit different environments throughout their life cycle (Lukes et al., 2005; Motyka et al., 2006). In *Trichomonas vaginalis*, for example, ADH, together with other enzymes involved in drug metabolism, is located in an organelle known as the hydrogenosome. This organelle shares a common evolutionary origin with mitochondria, and it is the site at which carbohydrate metabolism and the activation of metronidazole occur (Rasoloson et al., 2002).

In a previous study Murta et al. (in preparation) identified through microarray analysis (using total and polysomal RNA) genes that were differentially expressed in populations of *T. cruzi* that were susceptible or resistant to BZ, and observed that a *TcADH* gene showed lower expression (4-fold) in the BZ-resistant 17LER population compared with its BZ-susceptible 17WTS counterpart. In order to confirm these findings, here the levels of TcADH mRNA and protein were compared in 11 BZ-susceptible and -resistant strains of *T. cruzi*. Northern blot hybridisation analyses showed that the TcADH probe recognised a 1.9 kb transcript with similar signal intensity in all *T. cruzi* samples analysed except for the 17LER population, which exhibited intensity values that were 2-fold lower. Western blot analyses also indicated that the level of expression of TcADH was approximately 2-fold lower in the BZ-resistant 17LER population than in the susceptible 17WTS. Benznidazole functions as prodrug and must undergo enzyme-mediated activation within the pathogen to have cytotoxic effects (Wilkinson et al., 2008). Recently,

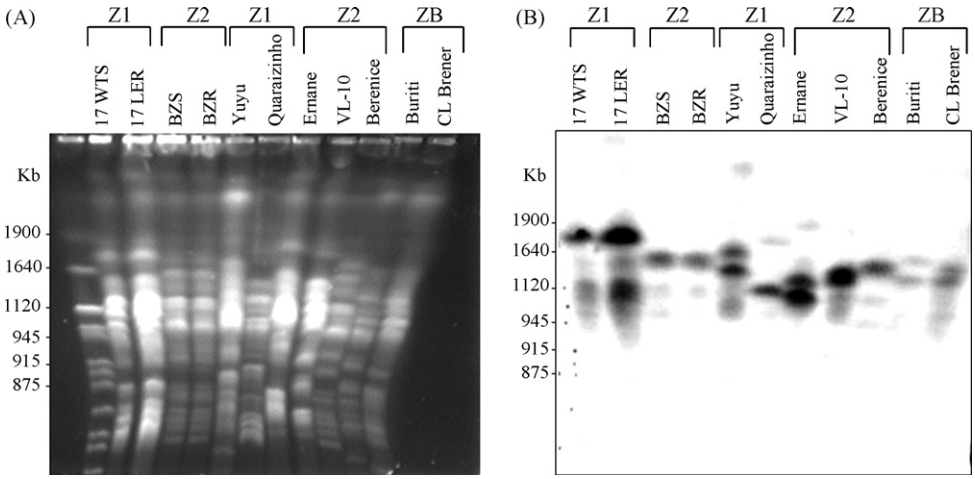

Fig. 5. Chromosomal location of the *TcADH* gene in *T. cruzi* strains with different zymodeme and drug resistance phenotypes. (A) Chromosomal bands from the *T. cruzi* strains were separated by PFGE and stained with ethidium bromide. (B) Southern blots of the chromosomes were hybridised with a ^{32}P-labeled *TcADH*-specific probe. Whole chromosomes from *Saccharomyces cerevisae* were used as molecular weight markers.

the deletion of copies of the genes encoding two different nitroreductases type I: an NAD(P)H-flavin oxidoreductase (known as the old yellow enzyme (TcOYE) or prostaglandin synthase) (Murta et al., 2006) and NTR-1 (Wilkinson et al., 2008), has been associated with *in vitro* BZ and NFX resistance in *T. cruzi*. Interestingly, in *T. vaginalis,* ADHs participate in the oxidoreduction processes associated with the activation of metronidazole, a nitroimidazole derivative similar to BZ (Kulda, 1999) whilst in metronidazole-resistant strains of this parasite, ADH activity was found to be reduced, with a low hydrogenase activity of the enzyme and, consequently, a low rate of production of hydrogen peroxide (Ellis et al., 1992). The data presented in this paper show that the TcADH enzyme has a decreased level of expression in the *in vitro* induced BZ-resistant *T. cruzi* population. Thus we believe that *T. cruzi* susceptible parasites in which enzyme levels are normal, TcADH, together with other enzymes, might be involved in the reduction of either BZ or its metabolites thus leading to its activation and to parasite death.

In the present study it has been demonstrated that the *TcADH* gene is located in different chromosome bands according to the strain of *T. cruzi,* but that its localization exhibits no association with the phenotype of drug resistance in this parasite. In contrast, Nozaki et al. (1996) have previously analysed the karyotype of *in vitro* induced NFX-resistant *T. cruzi* populations and presented evidence that NFX resistance was followed by DNA changes including rearrangements of the chromosomes and aneuploidy.

Many of studies concerning drug resistance mechanisms in parasites were based on models produced by artificial induction of resistance. In contrast, there is very little information available on the biochemical mechanisms underlying drug resistance in field isolates. The data presented in this paper show that the TcADH enzyme has a decreased level of expression in the *in vitro* induced BZ-resistant *T. cruzi* population, a situation that has not been observed in the *in vivo* selected resistant and naturally resistant lines. Interestingly, comparative studies of drug susceptibility between *T. cruzi* strains have found no correlation between drug susceptibility *in vivo* and *in vitro* (Neal and Van Bueren, 1988). This absence of correlation may explain, at least in part, differences in the drug resistance mechanisms found in *T. cruzi* lines naturally resistant to BZ or with *in vivo* selected resistance to BZ compared those with *in vitro* induced resistance. Recently, Villarreal et al. (2005) have assessed the degree of gene expression in *T. cruzi* populations by comparing *in vitro* induced and *in vivo* selected resistance to BZ. In agreement with our results, these authors observed that the mechanisms associated with natural resistance to drugs differ from those in induced resistance. The mechanism of drug resistance, such as that to BZ, is often complex and typically ensues as a result of the concomitant activation of multiple, often overlapping, signalling pathways, including factors associated with the host immune system, which may interfere in the susceptibility of the parasite to the drug (Murta et al., 1999). Studies to determine whether overexpressing TcADH in the *in vitro* induced BZ-resistant population will confer BZ-susceptibility to these parasites are required to confirm our hypothesis that TcADH may is involved in *T. cruzi* resistance to BZ.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2009.02.007.

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