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Δ9 desaturase from *Trypanosoma cruzi*: Key enzyme in the parasite metabolism. Cloning and overexpression

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ABSTRACT

Desaturases, key enzymes in the metabolism of fatty acids, regulate the physical and biochemical properties of membranes. They adjust the composition of saturated and unsaturated fatty acids in response to changes in the environmental.

We demonstrated the existence of Δ9 desaturase activity in epimastigotes of the *Trypanosoma cruzi* Tulahuen strain. In the present study, showed that this enzyme has an approximate molecular mass of 50 kDa and a pI value of approximately 9. In order to characterize the Δ9 desaturase of *Trypanosoma cruzi*, (TcΔ9DES) we have cloned, sequenced and expressed in *Escherichia coli*. The gene consists of 1300 bp and encodes a peptide of 433 amino acids with a molecular weight of 50 kDa. Analysis of the amino acid sequence revealed three clusters of histidine and two hydrophobic regions, characteristic of membrane-bound desaturases.

Gene expression studies showed that TcΔ9DES was overexpressed as an active protein. Fatty acid analysis showed that the expressed protein was confirmed to be functional with Δ9 desaturase activity. This enzyme changed the fatty acid profile of TcΔ9DES-expressing *E. coli*, decreasing the levels of palmitic (16:0) and stearic (18:0) acids and enhancing palmitoleic (16:1Δ9) and monounsaturated 18 carbons fatty acids. When [1–14C]palmitic or [1–14C]stearic acid was used as substrate, TcΔ9DES-expressing *E. coli* exhibited high desaturase activity associated with increased levels of monounsaturated fatty acids, suggesting that the TcΔ9DES enzyme was actively expressed in *E. coli*.

To check the commitment of TcΔ9DES against sterol biosynthesis inhibitors we tested the activity under ketoconazole effect. Native TcΔ9DES, showed a significant activity inhibition.

Since TcΔ9DES has shown active participation under different environmental factors, among them, ketoconazole, we consider that it plays a critical role in the metabolism of the parasite

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1. Introduction

American trypanosomiasis, also known as Chagas disease, is caused by *Trypanosoma cruzi*, a flagellate parasite of the order of the kinetoplastida. The infection is transmitted by the blood-sucking *Hemiptera Reduviidae* insect family, subfamily Triatominae (Miles et al., 2003). Chagas disease is considered endemic to South and Central America, although it has now spread to other continents due to immigration flows. About 6 million to 7 million people are estimated to be infected worldwide, mostly in Latin America, [WHO World Health Organization, 2015. http://www.who.int/entity/mediacentre/factsheets/fs340/en/.]

Current medical treatment of trypanosomiasis is associated with high toxicity and, in some cases, low efficiency. Consequently, there is an urgent priority to develop new chemotherapy components against this disease. Lipids have been considered important chemotherapeutic targets (Urbina, 2009). Among used

Abbreviations: FA, fatty acids; FID, flame ionization detector; FAME, fatty acid methyl esters; FBS, fetal bovine serum; IPTG, isopropyl-β-D-thiogalactopyranoside; SBI, sterol biosynthesis inhibitors; TLC, thin layer chromatography; PMSF, phenylmethylsulphonyl fluoride; LB, Luria-Bertani; TcΔ9DES, Δ9 desaturase of Trypanosoma cruzi; TcΔ9DES, Δ9 desaturase gene of Trypanosoma cruzi.

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drugs against the protozoan parasite *T cruzi,* sterol biosynthesis inhibitors (SBI) cause antiproliferative effects on the parasite. Contreras et al. (1997) showed that, one of the primary effects of the depletion of endogenous sterols induced by SBI in *T. cruzi* is a modification of the cellular phospholipid composition as a consequence of a reduced activity of PE-PC-*N*-methyl transferase and probably of the acyl Δ9 and Δ6 desaturases.

Delving into the development of future drugs, have been identified a number of genes of the sterol biosynthesis pathway (Cosentino and Agüero, 2014).

Desaturases, which are proteins distributed universally among living organisms, are responsible for the biosynthesis of unsaturated fatty acids and play a central role in the regulation of lipid metabolism. Because fatty acids (FA) are the major constituents of membrane phospholipids, modulation of the number and position of double bonds by desaturases plays an important role in maintaining the dynamic state of membranes (Sajbidor, 1997; Denich et al., 2003). It has been shown that Δ9 desaturase activity is increased at low temperature (McCartney et al., 1996). Additionally, *Tetrahymena thermophila* responds to temperature changes modifying, in part, Δ9 desaturase activity (Nakashima et al., 1996).

Previous studies done by our research group contributed to the knowledge of lipid metabolism in *T. cruzi*. The existence of Δ9 and Δ12 desaturases in epimastigotes of *T. cruzi* was determined in our laboratory (de Lema and Aeberhard, 1986) by adding radioactive FA to the culture medium, which were incorporated and metabolised. In addition, our studies on *T. cruzi* epimastigotes revealed that environmental factors modify the proportion of unsaturated to saturated FA through changes in desaturase enzyme activity (Racagni et al., 1995; Villasuso et al., 2005). Moreover, we have shown that *T. cruzi* is able to desaturate different substrates using Δ9 desaturase by modifying the concentration of fetal bovine serum (FBS) suggesting the importance of this enzyme in the metabolism of the parasite (Villasuso et al., 2010).

Despite Δ9 desaturase of *T. cruzi* (TcΔ9DES) being partially biochemically characterized, the associated gene with this activity has not been identified. While Δ9 desaturase is present in *T. cruzi* and its mammalian host, this enzyme appears to have structural differences, suggesting that this enzyme could be targeted in order to design selective therapies (Alloatti et al., 2009).

Isolation and characterization of this enzyme have imposed a difficult task due to its persistent association with cell membranes and its fragility. Early studies on the purification of this enzyme demonstrated that it was extremely labile, even at high purity and stored at −80 °C. These observations suggested the presence of a microsomal protease that might be responsible for the degradation of the enzyme in addition to the potential loss of desaturase activity by denaturation of the enzyme (Heinemann and Ozols, 2002). One way to overcome this inherent limitation is the overexpression of these proteins in heterologous hosts, such as *E. coli*, with subsequent purification and characterization by conventional methods (Schertler Gebhard, 1992). A large number of membrane desaturases, including Δ9 desaturases, such as those of *Pseudoalteromonas sp*. (Li et al., 2009), rat (Strittmatter et al., 1988), *Cyanobacteria Synechocystis* sp. (Maali et al., 2007) and S-ACP-desaturase of *Arabidopsis thaliana* (Yujin et al., 2010) have been characterized by using heterologous expression systems. Trypanosomatide desaturases have been, also, overexpressed. Thus Petrini et al. (2004), have described the isolation and functional characterization of a *Trypanosoma brucei* oleate desaturase by heterologous expression in *Saccharomyces cerevisiae.* In addition Maldonado et al. (2006), reported the molecular characterization of *T. cruzi* oleate desaturase and they also showed the presence of homologous *T. cruzi* oleate desaturase genes in various pathogenic and nonpathogenic trypanosomatids.

Therefore, in this study we describe some biochemical features of the native TcΔ9DES enzyme from epimastigotes of *T. cruzi*, Tulahuen strain, the behavior in the presence of a SBI, ketoconazole and its further isolation and functional characterization by heterologous expression in *E. coli.*

2. Materials and methods

2.1. Microorganisms, plasmids and growth conditions

The microorganisms, plasmids and oligonucleotide primers used in this study are listed in Table 1 (Taliaffero and Pizzi, 1955). *T. cruzi* epimastigote forms were grown at 28 °C in modified Warren's medium (Warren, 1960) as previously described (Racagni et al., 1992). The medium was supplemented with 10% FBS and 33000 UI penicillin per 4×10^7 parasites. Cells in the logarithmic growth phase (5 days old) were harvested by centrifugation at 4500 × *g* for 10 min. The weight of the harvested cells and the number of mobile cells per ml culture medium was measured.

The *E. coli* XL 10 GOLD strain was used in all routine DNA manipulations and cloning procedures. *E. coli* BL21 codon Plus was used as a host for the pET28a (+) plasmid containing the putative *TcΔ9DES*. Recombinant *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth containing the required antibiotics.

2.2. Partial characterization of the native Δ9 desaturase of T. cruzi

2.2.1. Isolation and partial purification

To determine the characteristics of TcΔ9DES, epimastigotes of *T. cruzi* were subjected to differential centrifugation using a previously described method (Villasuso et al., 2010). Thus, different subcellular fractions were collected, and the 105,000 × *g* pellet was used as a protein source in further analysis.

2.2.2. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 10% (w/v)) was carried out (Laemmli, 1970) using a Mini-protean IV Bio-Rad system and Kaleidoscope 195–6.4 D (Bio-Rad, Hercules, CA, USA) as a molecular mass standard. The bands were stained with Coomasie Brilliant Blue R-250.

2.2.3. Isoelectric focusing

Isoelectric focusing (IEF) was carried out as previously described (Agostini et al., 2000) and resolved on polyacrylamide gels over a pH range 3–10 using a Bio-Rad Mini IEF System. Samples were previously desalted with Sephadex G-25. The gels were calibrated using the Bio-Rad pI Calibration Kit (4.45–9.60) (Bio-Rad, Hercules, CA, USA). To detect the protein markers for pI, the gel was immersed in a solution containing 0.04% (w/v) Coomasie Brilliant Blue R-250 and 0.05% (w/v) Crocein Scarlet for 10 min, and the protein bands were stained with silver nitrate.

2.2.4. Immunoblotting

The presence of native TcΔ9DES was determined by Western blotting (Toubin et al., 1979); the samples were subjected to SDS-PAGE and IEF electrophoresis, and the bands were electrotransferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) using a Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA) at a constant voltage of 100 V for 1 h at 4 °C. Blotting was performed using a primary antibody against rat Δ9 desaturase, which was kindly provided by Dr. Omar Rimoldi, INIBIOLP (Instituto de Investigaciones Bioquimicas de La Plata-Argentina). Detection was realised using the amplified alkaline phosphatase Immun-Blot kit (BioRad, Hercules, CA, USA) by SDS

Table 1
Strains, bacterial plasmid and oligonucleotide primers used in this study.

Strain, plasmid and primers	Description	Source or reference
Strain		
T. cruzi	Tulahuen strain of *T. cruzi* epimastigotes	(Taliaferro and Pizzi, 1955)
E. coli XL 10 GOLD	Tet^r *D(mcrA) D(mcrCB-hsdSMR-mrr)173 end A1 suppE44 thi-1 recA1 gyrA96 relA1 lac Hte* (Fí *proAB lacZDM15 Tn10*(Tet^r)*Tn5*	Stratagene
E. coli BL 21 codon Plus	F^- *ompT hsdS* (r_B–m_B–) *dcm+* Tet^r *gal l* (DE3) *endA* Hte *(argU ileY leuW* Cam^r)	Promega
E. coli pET	*E. coli* BL21(DE3) harboring pET plasmid	This study
E. coli pET-des	*E. coli* BL21(DE3) harboring pET-des plasmid	This study
Plasmid		
pCRII-TOPO	plasmid cloning Apr T7 promoter, Gen LacZα	Invitrogen
pCRII-TOPO-*des*	pCRII-TOPO harboring putative *TcΔ9DES*	This study
pET	pET-28a (+) vector expression KNR, T7 promoter, His tag N, C	Novagen
pET- des	pET-28a (+) harboring putative *TcΔ9DES*	This study
Oligonucleotide primers		
Des *T. cruzi* Forward	5'-G˘TCGACATGACGAGTTTAAACA-3' *SalI*	
Des *T. cruzi* Reverse	5'-GC˘GGCCGCTCACTTACGCTT-3' *NotI*	

electrophoresis and chemifluorescence (ECL plus detection System, Amersham Biosciences, Piscataway, NJ, USA) by IEF.

2.3. Identification, cloning and expression of putative Δ9 desaturase gene of T. cruzi in E. coli

Des *T. cruzi*-specific oligonucleotides were designed based on the putative *T. cruzi* CL Brener Δ9 desaturase gene (GenBank 70861362) (Table 1), and the gene was amplified from genomic DNA of the parasite. Total DNA extraction was carried out using the JETFLEX Genomic DNA purification kit (Genomed). PCR amplification was carried out in a total volume of 25 μL containing 1 μL of genomic DNA, 1 μL of Taq polymerase ($1\,U\,\mu L^{-1}$) (Promega, Madison, WI, USA), 2.5 μL of 10× Taq buffer, 1.5 μL of $MgCl_2$ (50 mM), 2 μL of dNTPs (10 mM each) and 5 μL of primers under the following procedure: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplification product of 1300 bp was gel purified, cloned into the pCR®II-TOPO® vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The *TcΔ9DES* sequence data reported in this paper is available in the GenBank database under the accession number JN007035 (http://www.ncbi.nlm.nih.gov/nuccore/353282215). To express the TcΔ9DES recombinant protein in *E. coli*, the putative desaturase gene that was previously cloned into pCRII-TOPO® was released from the vector by digestion with *SalI* and *NotI* and was subsequently cloned into the pET- expression vector. The pET- des construction was used to transform *E. coli* BL21 using the method of Chung et al. (1989), and a kanamycin-resistant *E. coli* colony was used for overexpression assays. This construct directs the production of a protein with a $(His)_6$-tagged at the C-terminus that is under the control of the *T7lac* promoter, thus allowing purification of the recombinant protein by immobilized metal affinity chromatography (IMAC). Cells were grown at 37 °C with shaking in LB medium (25 mL) supplemented with kanamycin ($50\,\mu g ml^{-1}$). When an optical density of 0.6 at 600 nm was reached, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce protein expression for 12 h at 20 °C. Cells were harvested by centrifugation at 6000 × *g*. Control experiments were performed under the same conditions using transformed *E. coli* containing empty pET plasmid. For FA composition experiments and to test the activity of the enzyme, cultures were supplemented with 400 μM 18:0 and were collected after 4 h of induction with IPTG 0.4 mM at 20 °C.

E. coli cells were harvested by centrifugation at 6000 × *g* for 10 min and resuspended in lysis buffer containing 50 mM M Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin and lysozyme at a final concentration of $1\,mg\,ml^{-1}$. DNA was digested by treatment with DNase I at a final concentration of $0.1\,mg\,ml^{-1}$. The cells were disrupted by ultrasonication, and a cell-free extract was obtained by centrifugation at 20,000 × *g* for 25 min. All procedures were performed at 4 °C. Cell-free extract containing the TcΔ9DES recombinant $(His)_6$-tagged protein was applied to a Ni^{2+} ProBond™ Resin (Invitrogen, Carlsbad, CA, USA) for IMAC purification. The column was washed with Tris–HCl buffer, pH 7.6, containing 0.5 M NaCl and 80 mM imidazole, and elution of the $(His)_6$-tagged protein was performed with a similar buffer containing 300 mM imidazole. The purification was performed at 4 °C. The efficiency of recombinant protein expression was analysed by SDS-PAGE using 10% acrylamide and Western blotting as described above, using a primary antibody against rat Δ9 desaturase and an ECL Western blotting detection system (Amersham Biosciences).

2.4. Sequence analysis

Database were screened for homologous sequences to the TcΔ9DES using BLAST (http://www.ncbi.nlm.nih.gov.). Multiple sequence alignments were performed using CLUSTAL W.

A phylogenetic tree was reconstructed using the neighbour-joining method (Saidtou and Nei, 1987) and *p*-distance model in the MEGA4 program (Tamura et al., 2007) with bootstrap values based on 1000 replications (Felsenstein, 1985).

2.5. Assay of recombinant desaturase activity in vivo

2.5.1. Incorporation of radioactive substrates

Total of 0.5 μCi of [1–14C]palmitic (16:0) or [1–14C]stearic acid (18:0) was added to 25 mL of culture. The *E. coli* cultures were incubated at the appropriate temperature with shaking for the appropriate time. The cells were then harvested by centrifugation at 6000 × *g* for 10 min at 4 °C, and the pellets were used in further studies.

2.5.2. Lipid extraction and separation

Lipids were extracted from washed bacteria with chloroform/methanol/water (Bligh and Dyer, 1959). The lower phase, containing lipids, was dried under N_2 and dissolved in an appropriate volume of chloroform/methanol 2:1 (v/v).

FA methyl esters (FAME) were prepared from total lipid extracts with 10% BF_3 in methanol (Morrison and Smith, 1964) and resolved according to the number of double bonds on thin layer chromatography (TLC) plates impregnated with $AgNO_3$ 10% (w/v), using hexane/ethyl ether/acetic acid 94:4:2 (v/v/v) as solvent. FAME

bands were detected under UV light after spraying the plates with dichlorofluorescein and eluting (Henderson and Tocher, 1992) and drying in counting vials. Three millilitres of Optiphase Hisafe 2 (PerkinElmer, USA) were added to each vial, and radioactivity was measured using a liquid scintillation counter (Beckman LS 60001C, USA) (Kates, 1972).

2.6. Analysis of fatty acids by GC-FID

FAME from *E. coli* recombinant cells, prepared as above, were analysed using a Hewlett Packard 5890 II gas chromatograph (GC) equipped with a highly polar column of cyanopropyl, HP 88 (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.2 μm) and a flame ionization detector was used. The gas chromatograph conditions were as follows: injector temperature, 250 °C; detector temperature, 300 °C. Nitrogen was used as the carrier gas. The temperature was programmed at 120 °C for 1 min and then increased by 10 °C min^{-1} to 175 °C for 10 min, 5 °C min^{-1} to 210 °C for 5 min and 5 °C min^{-1} to 230 °C for 5 min. The peak areas of carboxylic acids in the total ions were used to determine the relative amounts. Fatty acids were identified by comparing the retention times to commercial standards (Sigma Chemical Co., St. Louis, MO, USA).

2.7. Effect of ketoconazole on lipids composition

Previously sterilized (0.4 μCi/ml) of [1–14C]acetate, sodium salt, was added to the culture medium to the inoculation time and Ketoconazole (1 μM) was added 24 h later. Parasites were harvested after five days of growth. Lipids were extracted from washed parasites with chloroform/methanol/water (Bligh and Dyer, 1959). The lower phase, containing lipids, was dried under N_2 and dissolved in an appropriate volume of chloroform/methanol 2:1 (v/v).

2.7.1. Separation and analysis of phospholipids and neutral lipids

Aliquots of the total lipid extracts were subjected to TLC to separate the total phospholipid fraction from the neutral lipids, using hexane/ethyl ether/acetic acid 80:20:1 (v/v/v) as solvent. After TLC, the lipids were located by exposing the plates to iodine vapours. The areas corresponding to each of the neutral lipids and phospholipids fraction were scraped from the plate and transferred to the respective scintillation vials. Three millilitres of Optiphase Hisafe 2 (PerkinElmer, USA) were added to each vial, and radioactivity was measured using a liquid scintillation counter (Beckman LS 60001C, USA) (Kates, 1972).

2.8. Assay of Δ9 desaturase activity in vitro under ketoconazole effect

Epimastigotes of *T. cruzi* grown in the presence or absence of Ketoconazole (1 μM), added to the culture medium after 24 h of growth, were subjected to differential centrifugation using a previously described method (Villasuso et al., 2010). Thus, different subcellular fractions were collected, and the 105000 × *g* pellet was used as a protein source in further analysis. Reaction mixture contained [1–14C]palmitic acid and all necessary cofactors (Villasuso et al., 2010). FAME were prepared from reaction products, extracts with 10% BF_3 in methanol (Morrison and Smith, 1964) and resolved according to the number of double bonds on TLC plates impregnated with $AgNO_3$ 10% (w/v), (according to item 2.4.2).

2.9. Statistical analyses

Data were compared by a one-way analysis of variance (ANOVA) test.

3. Results

3.1. Partial characterization of native Δ9 desaturase of *T. cruzi*

Our previous studies have demonstrated that *T. cruzi* epimastigotes have Δ9 desaturase activity. Therefore, we decided to further characterize the native protein. In order to initially identify and confirm the presence of Δ9 desaturase in *T. cruzi* epimastigotes, we have used direct immunodetection in cultured *T. cruzi* epimastigotes subcellular fractions. A band with an approximate molecular mass of 50 kDa was detected in the 105000 × *g* fraction of *T. cruzi* (Fig. 1 AI and AII). This band was classified as a basic protein according to its pI value of approximately 9, using IEF monitoring (Fig. 1 BI

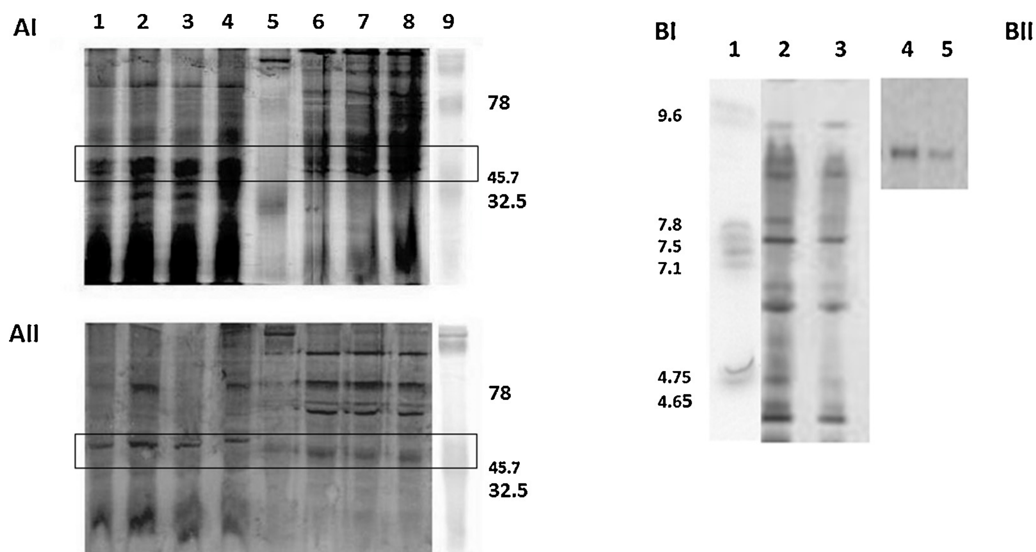

Fig. 1. Partial characterization of the native *T. cruzi* desaturase.
(AI) SDS-PAGE, (AII) Western Blot. Lanes 1, 2, 3 and 4 positive control rat microsomal fraction, lanes 5 and 9 prestained protein molecular mass market, and lanes 6, 7 and 8 microsomal fraction of *T. cruzi* epimastigotes (105,000 × *g* pellet), 10% FBS.
(BI) Isoelectric focusing (pH 3–10) and (BII) Western Blot. Lane 1, unstained pI standards, lanes 2 and 4, positive control rat microsomal fraction, and lanes 3 and 5, 105,000 × *g* fraction of *T. cruzi* epimastigotes grown in 10% FBS.

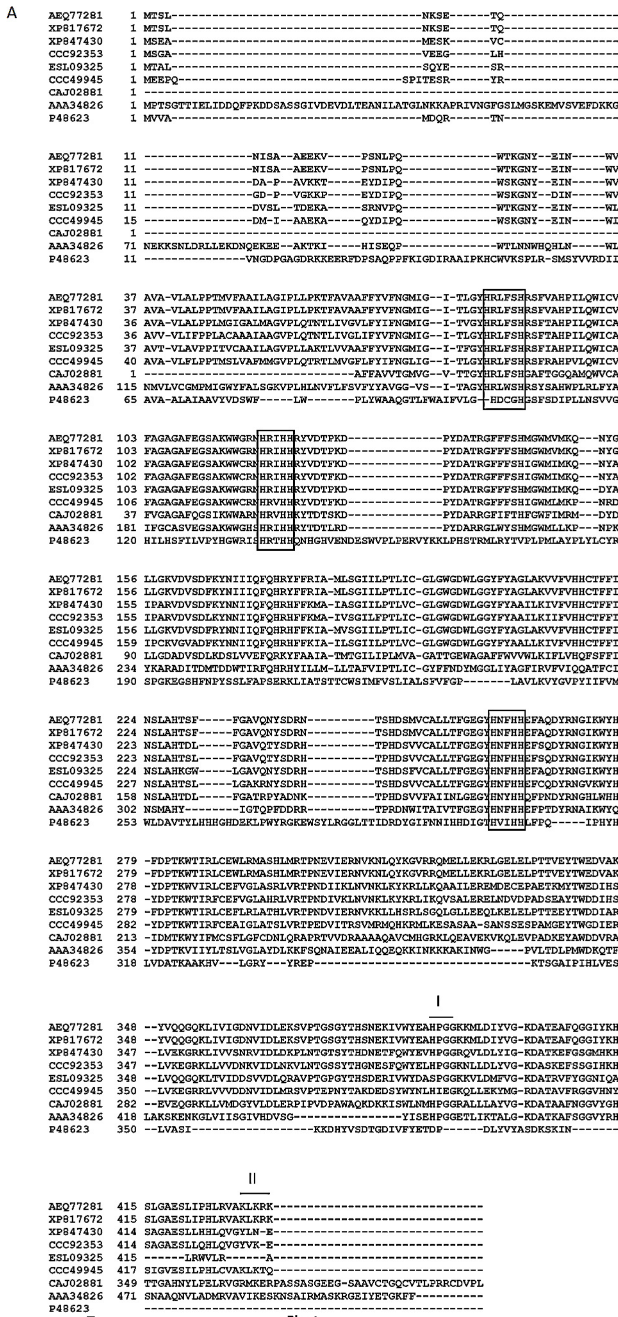

Fig. 2. Sequence analysis of the *T. cruzi* desaturase.
(A) Multiple sequence alignment of desaturase from *T. cruzi* Tulahuen strain (AEQ77281) and other eukaryotic organisms using the Crustal W and Boxshade programs.*T. cruzi* Brener strain (XP817672), *T. brucei* (XP847430), *T. congolense* (CCC92353), *T. rangeli* (ESL09325), *T. vivax* (CCC49945), *Leishmania major* (CAJ02881), *Saccharomyces cerevisiae* (AAA34826) and *Arabidopsis thaliana* (P48623). Three conserved histidine clusters (one HXXXXH and two HXXHH) are indicated by boxes. Bold horizontal lines indicate the (I) C-terminal cytochrome b5 motif and (II) Similar motif to ER found in other organims.
(B) Phylogenetic tree of Δ9 desaturases from different organisms including *T. cruzi* Tulahuen strain (AEQ77281), *T. cruzi* Brener strain (XP817672), *T. brucei* (XP847430), *T. congolense* (CCC92353), *T. rangeli* (ESL09325), *T. vivax* (CCC49945), *Leishmania major* (CAJ02881), *Saccharomyces cerevisiae* (AAA34826), *Arabidopsis thaliana* (P48623), *Rattus norvegicus* (AAH61737), *Drosophila melanogaster* (BAB21540), *Anopheles darlingi* (ETN58291), *Homo sapiens* (EAW49830), *Mortierella alpina* (AGZ84118), *Aspergillus flavus* (EED47805), *Synechococcus. sp* (WP030007857), *Mus musculus* (NP033154), *Takifugu rubripes* (AAU89872), *Culex quinquefasciatus* (EDS40306), *Acinetobacter baumannii* (CUW35772), *Bos taurus* (NP776384) and *Scytonema millei* (WP039717214). Bootstrap values are based on 1000 replications and are shown at branch points. Bar indicates 0.05% sequence divergence.

and BII), suggesting the presence of Δ9 desaturase protein in this fraction.

3.2. Isolation and sequence analysis of the putative T. cruzi desaturase gene

A DNA fragment of approximately 1300 bp in length was amplified from *T. cruzi* using the Des *T. cruzi* forward and reverse primers (Table 1). The amplified DNA fragment showed high sequence identity (100%) to a desaturase gene from the *T. cruzi* CL Brener strain. Deduced amino acid sequence analysis of the open reading frame (ORF) showed a high similarity to a membrane desaturase. Furthermore, the distribution of hydrophobic amino acids generated by Prediction of Transmembrane regions in proteins (PRED-TMR) demonstrated two large hydrophobic regions with five transmembrane regions included between amino acids 35–56, 63–82, 90–109, 181–199 and 201–223. The obtained sequence of *T. cruzi* was aligned with different organism desaturase sequences showing histidine-rich motif characteristics, which are highly conserved among membrane-bound desaturases and proposed to form the potential di-iron active site (Fig. 2A). Therefore, using this information and according to the predictions obtained from databases (ELM RESOURCE), TcΔ9DES protein possesses an motif (KXKXX) at position 429–433 and a cytochrome b5 domain at the C-terminus (339–433), which includes the HPGG motif (339–342) (Fig. 2A). Phylogenetic tree analysis using Δ9 desaturase homologous sequences from a variety of organisms indicated that the Δ9 desaturase could be grouped in 4 major phylogenetic clusters. TcΔ9DES is most closely related to Δ9 desaturases from the trypanosomatids and fungi, and most distantly related to Δ9 desaturases from mammalian and plants (Fig. 2B).

3.3. Functional expression of the putative T. cruzi desaturase gene

To determine the function of TcΔ9DES, the gene was cloned into the pET28a(+) vector and expressed in *E. coli* BL 21 codon Plus. Recombinant TcΔ9DES was expressed as a soluble protein of approximately 50 kDa, corresponding to the expected size of the majority of membrane desaturases. In addition, the recombinant protein was purified by IMAC, and Western blotting confirmed the molecular mass of 50 kDa (Fig. 3, A and B). The activity of the recombinant enzyme was measured in vivo through the incorporation of [1–14C]16:0 or [1–14C]18:0 added to the culture medium. Fig. 4 (A and B) shows the radioactivity distribution in FAME of the *E. coli* pET and pET-des strains. The labeled substrates were incorporated into the cells in the *E. coli* pET and pET-des, and most of the radioactivity remained in the fraction of saturated FA. However, in the strain transformed with the putative desaturase gene, 111% more radioactivity was recovered from the monounsaturated FA fraction than the *E. coli* strain harboring the empty plasmid when [1–14C]16:0 FA was the substrate. When [1–14C]18:0

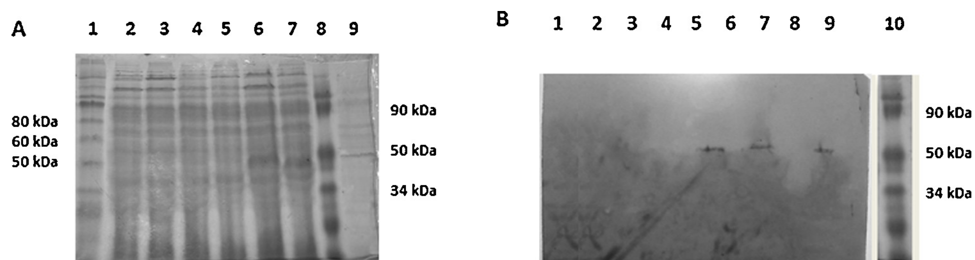

Fig. 3. Analysis of the recombinant protein expressed in *E. coli*.
(A) SDS-PAGE and (B) Western Blotting. IPTG (0.4 mM) was added to the growth medium to induce the desaturase gene, and the culture was incubated at 20 °C for 12 h prior to analysis of cellular proteins. Lanes 1, 8 and 10, prestained protein molecular mass marker; lanes 2 and 3 total proteins from *E. coli* pET (with IPTG), lanes 4 and 5, total proteins from *E. coli* pET-des (without IPTG), lane 6 and 7, total proteins from *E. coli* pET-des (with IPTG), and lane 9, recombinant TcΔ9DES purified by IMAC.

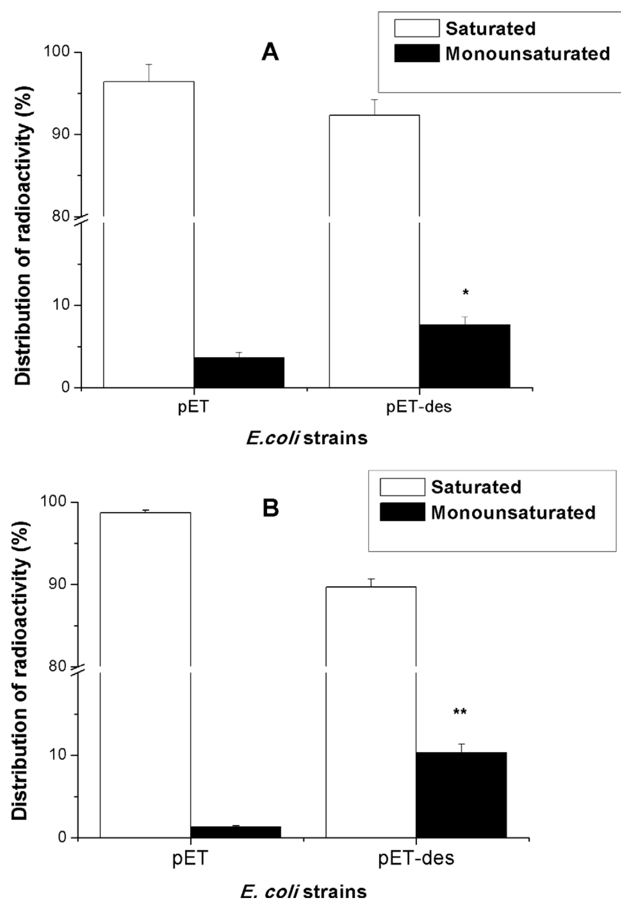

Fig. 4. in vivo TcΔ9DES activity measured in *E. coli* strain expressing *TcΔ9DES*. (A) [1–14C]16:0 acid was added to the cell culture and incubated at 37 °C until an OD_{600} of 0.3–0.5 was reached, after which the expression of the recombinant protein was induced by IPTG at 20 °C for 12 h. Total lipids were extracted, treated with methanol and separated according to unsaturation degree using TLC plates impregnated with 10% $AgNO_3$. The results are expressed as the percentage of total radioactivity incorporated into each FA fraction. (B) Same as panel a, except that the FA added to the culture was [1–14C]18:0. Values represent the mean ± SEM from three independent experiments.
*Difference from control (pET) value statistically significant at P < 0.05 level.

was the substrate, 692% more of radioactivity was found in the monounsaturated fraction of transformed strain with the putative desaturase gene than in cells transformed with the control.

The functionality of the recombinant *T. cruzi* enzyme was carried out by determining the FA profile by GC (Table 2). The FA composition of *E.coli* pET showed three major FA that are normally found in *E. coli*: palmitic (16:0), palmitoleic (16:1Δ9) and cis vaccenic (18:1Δ11). The changes in the pattern of the content of 16 carbons FA (16:0 and 16:1Δ9) between the pET-des and the control strain

Table 2
Fatty acid composition of *Escherichia coli* transformed with empty or recombinant plasmids.

Fatty acids (%)	*E. coli*	
	pET	*pET-des*
14:0	2.13 ± 0.17	2.07 ± 0.7
16:0	50.8 ± 3.2	41.5 ± 0.2*
16:1	17.9 ± 1.1	23.6 ± 1.3*
18:0	2.3 ± 0.75	1.4 ± 0.02*
18:1	26.8 ± 2.4	31.4 ± 0.3*

Strains were grown at 37 °C on LB medium to an OD_{600} of 0.3-0.5, and the expression of recombinant protein was induced by the addition of IPTG. After a 12 h induction, total lipids were extracted, and FA were converted to methyl esters and analysed by GC as described in the text. The percentage of each FA is relative to total FA (defined as 100%). Values represent means ± SEM of three independent experiments.
*Difference from control (pET) value statistically significant at P < 0.05 level.

indicate that the desaturase can use 16:0 as substrate. In the pET-des strain, an increase of 32% monounsaturated FA (16:1Δ9) and a decrease of 18% at the 16:0 level was observed with respect to *E. coli* pET. In addition, an increase of 17% in the 18:1 content was observed in the pET-des strain, while 18:0 content decreased 39%. Due to low availability of substrate, 18:0, in the pET-des strain, the culture medium was supplemented with 18:0. In addition and to promote enzyme activity, this assay was conducted at 20 °C. Under these conditions, the 18:0 content, was reduced (57%) and 18:1 content increased (32%) in cells transformed with the desaturase gene compared with the control (Fig. 5).

3.4. Effect of ketoconazole on physiological and biochemical parameters

Ketoconazole added to the culture medium, provoked a reduction of the *T. cruzi* number of 14% (from 6.7×10^7 to 5.8×10^7). The wet weight decreased a 20% (from 246 mg to 197 mg)

3.4.1. Biosynthesis of lipids under ketoconazole effect

The [1–14C]acetate incorporation in lipids was modified by ketoconazole. Free sterols and sterol esters synthesis decreased 62% and 73% respectively in cultures with ketoconazole compared with the control (Table 3). Contrary to this, the incorporation of [1–14C]acetate in phospholipids increased 19%.

3.4.2. Δ9 desaturase activity under ketoconazole effect

We tested Δ9 desaturase activity using [1–14C]palmitic acid, as it is a more compatible substrate with the enzyme, (Villasuso et al., 2010) and 105000 xg precipitate as enzymatic source, under ketoconazole (1 μM) effect. Thus, this activity was reduced. A decrease of incorporated radioactivity in monounsaturated fraction of 90% (from 150 to 15 pmoles/min/mg of protein) was observed (Fig. 6)

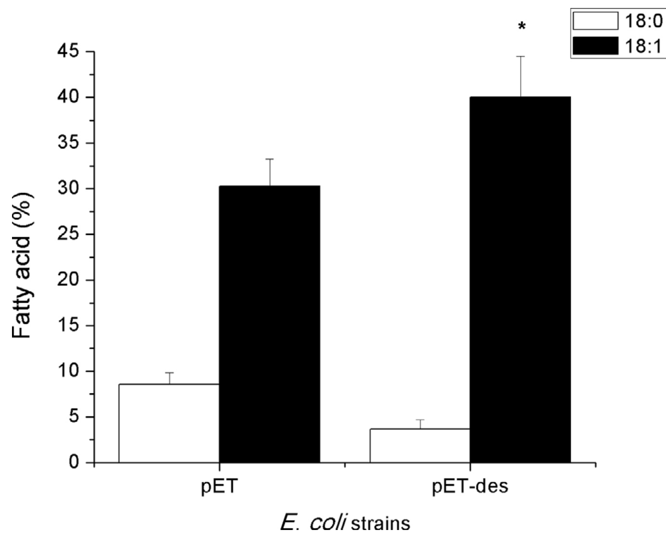

Fig. 5. Fatty acid composition of *E. coli* strain expressing *TcΔ9DES* Strains were grown at 20 °C on LB medium supplemented with 18:0 to an OD_{600} of 0.3-0.5, and the expression of recombinant protein was induced by the addition of IPTG. After a 4 h induction, total lipids were extracted, and FA were converted to FAME and analysed by GC as described in the text. The percentage of each FA is relative to total FA (defined as 100%). Values represent means ± SEM of three independent experiments.
*Difference from control (pET) value statistically significant at P < 0.05 level.

Table 3
Effect of ketoconazole on the incorporation of [1–14C]acetate in total neutral lipids of *T. cruzi* epimastigotes.

Neutral Lipids	*T. cruzi*	
	Control (no drug)	Ketoconazole
Phospholipids	68.6 ± 6.2	81.7 ± 5.7
Sterols	14.5 ± 2.9	5.5 ± 1.7
Free fatty acids	1.6 ± 0.4	2.9 ± 1.6
Triacylglycerols	4.9 ± 2.4	4.8 ± 2.4
Methyl esters	2.6 ± 1.6	3.1 ± 1.8
Sterol esters	7.8 ± 0.3	2.1 ± 1.0

Profile of neutral lipids in *T. cruzi* grown in liquid medium supplemented with 10% FBS (Control) and 10% FBS + 1 μM Ketoconazole (Ketoconazole) was carried out by TLC using a solution hexane/ethyl ether/acetic acid 80: 20: 1 (v/v/v) as solvent. The results correspond to the percentage distribution of radioactivity in each lipid fraction to the total sum regarded as 100%. Values represent means ± SEM of three independent experiments.

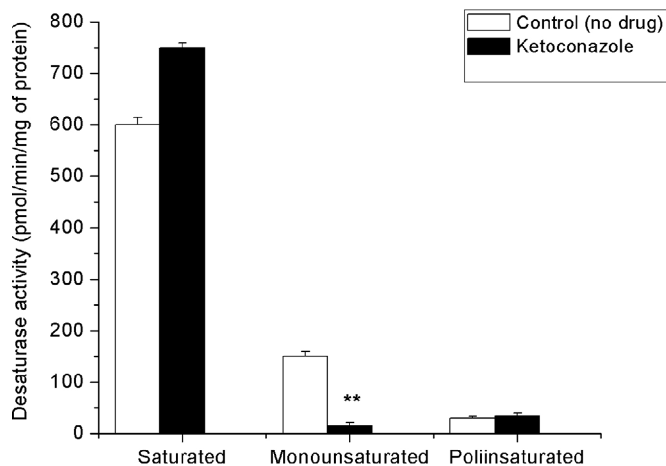

Fig. 6. Effect of ketoconazole on *T. cruzi* desaturase activity.
Parasites were grown with 10% FBS and harvested at the logarithmic phase of growth (5 days). Δ9 desaturase was determined using [1–14C]palmitic acid as substrate. Values (pmol/min/mg of protein) represent means ± SEM of three independent experiments.
*Difference from control (no drug) value statistically significant at P < 0.05 level.

4. Discussion

The trypanosomatids comprise a large number of species that are obligate intracellular parasites. The interaction of *T. cruzi* with its host and its vector brings temperature changes along its life cycle, which implies that the parasite must adapt to these changes to survive. In this context, the lipid composition of the parasite membrane may drastically change depending on environmental variations. Thus, the content of polyunsaturated FA increases as the temperature decreases to maintain the biophysical properties of the membranes. In *T. cruzi*, unsaturated FA are the major components of the plasma membrane (Villasuso et al., 2005). As was indicated, this parasite undergoes drastic temperature changes (from 28 °C, insect vector, to 37 °C, mammalian host) during its life cycle. Therefore, it seems probable that membrane fluidity plays an important role in the differentiation of these parasites, which involves changes in the shape of the cells, the morphology of some organelles and biochemical characteristics (Florin-Christensen et al., 1997). In addition, GPI-anchored mucins, the major surface antigens of *T. cruzi* trypomastigotes, contain unsaturated (C18:1 or C18:2) FA in their GPI moiety, and these FA seem to be, in great part, responsible for its immunological activity (Almeida and Gazzinelli, 2001). Therefore, it can be surmised that FA desaturation must be an important event for *T. cruzi*. Then the knowledge of the behavior of *T. cruzi* desaturases is relevant both physiologically and biologically, even more taking into account that these enzymes are considered as an additional target for selective therapy. It was previously shown that *T. cruzi* (CL Brener strain) desaturases can be considered as drug targets (Alloatti et al., 2009).

Previous studies from our laboratory showed that the ratio of unsaturated to saturated FA increases with growth in culture, as indicated by an increased percentage of linoleic acid (18:2 Δ12), and that carbamoylcholine increases [14C] labeling of triacylglycerols and diacylglycerols (Villasuso et al., 2005). These findings indicate that unsaturated FA are important factors during parasite aging and their response to environmental stimuli. Furthermore, we have previously demonstrated de novo biosynthesis of 16:0 FA in *T. cruzi*, and we later showed that epimastigotes of *T. cruzi* are able to incorporate and metabolise exogenous FA and that 16:0 acid is elongated to 18:0 FA and then desaturated to 18:1Δ9 FA and 18:2Δ12 FA. These data strongly suggest the existence of Δ9 and Δ12 desaturases (Aeberhard et al., 1981; de Lema and Aeberhard, 1986). In addition, we have described the partial biochemical characterization of native TcΔ9DES and TcΔ12DES using subcellular fractions and radioactive FA. In *T. cruzi* epimastigotes, TcΔ9DES is capable of using 16:0 and 18:0 FA as substrates and showed the highest specific activity in the 105000 x g pellet (Villasuso et al., 2010), similar to what was observed in mammals and yeast (Shanklin and Cahoon, 1998). Herein, this fraction showed an immunopositive band, an approximate molecular mass of 50 kDa and a pI value of approximately 9. These characteristics coincide with that of Δ9 desaturases from other organisms, such as porcine stearoyl-CoA desaturase (Ren et al., 2004) and yeast Δ9 fatty acyl-CoA desaturase (Stukey et al., 1989).

In this study, using the *T. cruzi*, Tulahuen strain and molecular studies, we identified and sequenced a putative desaturase gene that shares high identity with desaturase genes from members of the Trypanosomatidae family.

The nucleotide sequence corresponding to *TcΔ9DES* encodes 433 amino acid residues and the polypeptide sequence translated from this gene has an apparent molecular mass of 49.58 kDa and a pI of 9.10. These results are in concordance with the results obtained for the native TcΔ9DES enzyme. The amino acid sequence of TcΔ9DES showed conserved sequences, including the 8 histidine residues comprising the tripartite motifs HXXXXH, HXXHH and HXXHH. These motifs are essential for the activity of the enzyme

because they form ligands for the di-iron cluster in the catalytic site and, therefore, comprise the catalytic centre of the enzyme (Shanklin and Cahoon, 1998; Nakamura and Nara, 2004). Studies on the primary sequence determined that this enzyme belongs to the superfamily of membrane desaturases (cd01060) that is grouped within clusters (cd03505) (www.ncbi.nlm.nih.gov/structure/cdd). Hydropathy examination of the amino acid sequence suggested that in TcΔ9DES two long hydrophobic domains are present and span the membrane several times. Similar analyses carried out on the membrane FA desaturases from *T. cruzi* (oleate desaturase from Y strain, CL-Brener), mammals, fungi, insects, higher plants and cyanobacteria indicate that these enzymes contain up to 2 long hydrophobic domains, which would be long enough to span the membrane bilayer twice (Maldonado et al., 2006; Shanklin et al., 1994). In our model, the 3 histidine motifs of TcΔ9DES were located at the hydrophilic regions. Based on these results, we may assume that TcΔ9DES is compatible with the integral desaturase membrane protein models (Los and Murata, 1998; Hernández et al., 2005). In addition we found in the C-terminal TcΔ9DES the KXKXX motif. Different C-terminal motifs have been described in various organisms which serve as tetrapeptide retention

signal to ER. Thus, in trypanosomatids *(T. brucei*) have been described, divergent and heterogeneous anchoring signals ER, for several proteins, such as MDDL, KQDL EKEL among others (Bangs et al., 1993; Hsu et al., 1989; Wang et al., 2010). C-terminal dilysines, N terminal or internally positioned di-arginines or various C-terminal aromatic amino acid-enriched motifs were found in ER resident membrane proteins, in mammalian cells, yeast and membrane desaturase in plants (McCartney et al., 2004). The discovery of the signal KXKXX C-terminal *T cruzi* delta 9 desaturase, surprising since the same is similar to that described for ER resident membrane proteins in yeast (Zerangue et al., 1999) signal and membrane desaturase from *Brassica napus* (McCartney et al., 2004). The function of this motif in *T. cruzi* is now unknown

The *T. cruzi* motif (KXKXX) is located at position 429–433. In addition, the enzyme has a cytochrome b5 domain at the C-terminus (339–433) like Heme/domain Steroid binding domain (determinated by CD-Search of NCBI), comprising the motif characteristic of the cytochrome b5 domain (HPGG).

Noteworthy, several membrane desaturases contain a cytochrome b5 domain either at their C-terminus, like in the fungal Δ9 desaturase (Mitchell and Martin, 1995) or at their N-terminus, like in the 'front-end' desaturases (Napier et al., 1999). The independent acquirement of a cytochrome b5 domain in different evolutionary branches may have provided a selective advantage by increasing the efficiency of the electron transport required during the unsaturation reaction (Hernández et al., 2005).

The desaturase activity of the recombinant protein could be readily detected when assayed in vivo with radioactive 16:0 or 18:0 FA as substrates, which were both converted to the corresponding monounsaturated FA. This activity was also reflected in the FA composition of the recombinant strain, where an increase in 16:1Δ9 and a parallel decrease of 16:0, indicated that the overexpressed enzyme was a Δ9 desaturase. When the substrate 18:0 was added to the culture medium the strain transformed with the desaturase gene was able to produce more 18:1 (15%) than when the substrate was not added. In turn, the level of 18:0 was reduced, indicating that the enzyme used, further, the external substrate to increase its activity. Thus, our results clearly demonstrated the expression of Δ9 desaturase of *T. cruzi*, key enzyme in the parasite metabolism. TcΔ9DES, the first enzyme in the synthesis of unsaturated FA, can be considered a potential target for attacking parasite transmission based on the product of their action, where 18:1Δ9c acid present in the intestinal extracts of *T. infestans* induces cell differentiation of *T. cruzi* epimastigotes into the infective metacyclic form (Wainszelbaum et al., 2003). In addition, the activity of this enzyme responds to changes in the environment, such as the FBS concentration (Villasuso et al., 2010). On the other hand, this enzyme appears to have structural differences relative to the same enzyme from human guest *T. cruzi* (Alloatti et al., 2009). *T. cruzi* desaturase is more related to yeast Δ9 desaturase (Mitchell and Martin, 1995).

To check whether the Δ9 desaturase of *T. cruzi*, Tulahuen strain, is involved in the response to SBI we submitted to epimastigotes of *T. cruzi* in culture to the action of ketoconazole. The effects found on the reduction in the number and parasites weight, as well as the decrease in endogenous synthesis of sterols coincide with those reported by Contreras et al. (1997).

Previous studies in *T. cruzi* suggested the possible role of the Δ9 desaturase in response to ketoconazole (Contreras et al., 1997). In the present study, we show that ketoconazole highly decreases the activity of this enzyme (90%), indicating the engagement of Δ9 desaturase in response to this SBI. Studies with ketoconazole in *Candida albicans* reported decrease in monounsaturated fatty acids in agreement with our results (Georgopapadakou et al., 1987). Other compounds as thioestearato positional isomers and isoxyl were tested in *T. cruzi*, showed reduce activity of Δ9 desaturase (Alloatti et al., 2009). It is shown that all sterols share a common property: the ability to regulate dynamics in order to maintain membranes in a microfluid state where it can convey important biological processes. (Dufourc, 2008). Further, it can be envisioned that changes in sterol might lead to an alteration in FA composition to re-establish optimal lipid–lipid interaction, which could be the result of a modification in the Δ9 desaturase activity.

Since Δ9 desaturase of *T. cruzi* has shown active participation under different environmental factors, such as concentration of FBS, carbamoylcholine, temperature, age of the culture and ketoconazole, we consider that it plays a critical role in the metabolism of the parasite. Therefore, based on the previous results and the reported in this paper, we validate the TcΔ9DES as a promising target for the development of selective therapy against Chagas disease

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