# Arginine kinase overexpression improves *Trypanosoma cruzi* survival capability

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**Abstract Arginine kinase catalyzes the reversible transphosphorylation between adenosine diphosphate (ADP) and phosphoarginine, which is involved in temporal and spatial adenosine triphosphate (ATP) buffering. Here we demonstrate that the homologous overexpression of the *Trypanosoma cruzi* arginine kinase improves the ability of the transfectant cells to grow and resist nutritional and pH stress conditions. The stable transfected parasites showed an increased cell density since day 10 of culture, when the carbon sources became scarce, which resulted 2.5-fold higher than the control group on day 28. Additional stress conditions were also tested. We propose that arginine kinase is involved in the adaptation of the parasite to environmental changes.**


*Key words:* Arginine kinase; Phosphoarginine; Phosphagen; Guanidino kinase; *Trypanosoma cruzi*

## 1. Introduction

Phosphoarginine and phosphocreatine, generally called phosphagens, play a critical role as energy reserve because the high-energy phosphate can be transferred to adenosine diphosphate (ADP) when the renewal of adenosine triphosphate (ATP) is needed. It has been proposed that phosphoarginine supports bursts of cellular activity until metabolic events such as glycogenolysis, glycolysis and oxidative phosphorylation are switched on [1,2]. Phosphoarginine synthesis also allows the cells to operate with low ATP levels since it may constitute a usable pool of the high-energy phosphate [3]. Phosphagens act as reservoir, not only of ATP, but also of inorganic phosphate that is mostly returned to the medium by the metabolic consumption of ATP [3].

Arginine kinase (ATP:arginine phosphotransferase; EC 2.7.3.3) catalyzes the reversible transphosphorylation between *N*-phospho-L-arginine and ADP [4].

MgATP+guanidino acceptor ⇄ *P*-guanidino acceptor+ MgADP+$H^+$

The molecular and biochemical characterizations of arginine kinases in *Trypanosoma cruzi* and *Trypanosoma brucei*, the etiological agents of Chagas' disease and human sleeping sickness respectively, have been reported by this laboratory [5–7]. It was established that a single-copy gene encodes for a functional arginine kinase in *T. cruzi*. The corresponding protein has 357 amino acids and a calculated molecular weight of 40 kDa [6]. The finding in these parasites of a phosphagen and its biosynthetic pathway, which are totally different from those in mammalian host tissues, points out arginine kinase as a possible chemotherapy target.

We also reported that arginine kinase protein and the associated specific activity increase continuously, about 7-fold, along the parasite growth curve [8]. The arginine kinase expression pattern in epimastigote cells suggests a correlation between the enzyme activity and the nutrient availability or parasite density. In addition, we recently described the existence of a relationship between arginine uptake, arginine kinase activity and the parasite stage and replication capability [9]. In this way, arginine kinase seems to play a critical role as a regulator of energetic reserves and cell growth. Taking into account that the more energy-demanding processes in trypanosomatids are cell division, stage differentiation and environmental stress resistance, the role of arginine kinase activity in the cell energy requirements could be considered as a relevant unresolved issue. Due to limitation in the use of classic genetic methods in studies of trypanosomatids, a transfection-based reverse genetic approach has been considered as a useful alternative to understand the function of new trypanosomatid genes. This article reports a successful overexpression model of a *T. cruzi* endogenous enzyme, such as arginine kinase and the characterization of its biological effects.

## 2. Materials and methods

### 2.1. Parasite cultures and cell extracts

Epimastigotes of the CL Brener strain were cultured at 28°C in 25 $cm^2$ plastic flasks, containing 5 ml of liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum, 10 U/ml penicillin, and 10 mg/l streptomycin [10]. Cultures were started with $10^6$ cells per ml. At the indicated times, cells were counted using a hemocytometric chamber, harvested by centrifugation at 1500×*g* for 10 min and washed three times with phosphate-buffered saline (PBS: 137 mM

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*Abbreviations:* LIT, liver infusion tryptose; pTREX-AK, pTREX vector containing the arginine kinase gene; pTREX-GFP, pTREX vector containing the green fluorescent protein gene; TAU-3AG, triatomine artificial urine medium supplemented with proline, aspartate, glutamate and glucose; UE, μmol $min^{-1}$ $mg^{-1}$

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NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$). Cell pellets were then resuspended in 50 mM HEPES buffer, pH 7.3, containing 0.01 mg/ml leupeptin, 25 U/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride and 14 mM 2-mercaptoethanol, and lysed by six cycles of freezing in liquid $N_2$ and thawing at 4°C. The extracts were then centrifuged at $10\,000 \times g$ for 10 min.

### 2.2. *In vitro* T. cruzi *metacyclogenesis*

Differentiation assays were performed using two different protocols. In the Contreras et al. method [11], epimastigote cells cultured in LIT medium were harvested by centrifugation at $1000 \times g$ for 15 min and resuspended to a concentration of $10^7$ cells per ml in triatomine artificial urine medium (TAU; 190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM phosphate buffer, pH 6.0 and 0.035% sodium bicarbonate) containing 10 mM each of glucose, proline, glutamate and aspartate (TAU-3AG) and incubated at 28°C for 72 h. Alternatively, the Isola et al. protocol [12] was employed. In this case, epimastigotes ($5 \times 10^6$ cells per ml) were incubated in Grace medium in the presence of *Triatoma infestans* intestinal extract, protein concentration 2 mg/ml, for 15 min at 28°C, transferred to modified Grace medium and incubated for 7 days at 28°C. In both methods, aliquots of the parasite suspensions were fixed, stained with Giemsa and trypomastigote cells were counted using a hemocytometric chamber.

### 2.3. *Arginine kinase assay*

The incubation mixture contained 25 mM HEPES buffer, pH 7.3, 2 mM ATP, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 1 mM L-[2,3-$^3$H]arginine (NEN Life Science Products; 0.5 μCi per assay), and enzyme source (0.3–10 μg of protein), in a total volume of 0.2 ml. Incubations were carried out 10 min at 30°C, and reactions were stopped by the addition of 1 ml of 25 mM HEPES buffer, pH 7.3, containing 10 mM L-arginine and 5 mM ethylenediamine tetraacetic acid (EDTA) (stop buffer). The mixtures were then resolved by passage through a strong anion exchange resin, AG1-X4, 200–400 mesh chloride form (Bio-Rad Laboratories), mounted into 1 ml tulip columns equilibrated with stop buffer. After loading the samples, the columns were washed with 3 ml of 25 mM HEPES buffer, pH 7.3, and eluted with 2 ml of 1 M NaCl [6].

### 2.4. *Western, Northern, Southern and chromosome blot analysis*

General molecular biology procedures were carried out as previously described [6]. For Western blots, the transferred membranes were incubated for 2 h with a 1:1000 dilution of the mouse polyclonal anti-arginine kinase antiserum [6]. The values were normalized among the different samples by comparing the bands obtained using anti-β-tubulin antibodies (Amersham Pharmacia Biotech). For Northern blot analysis total RNA was isolated from $10^8$ cells and hybridized with the full-length arginine kinase gene as probe. Southern blot analysis was performed using samples of DNA previously digested with *Eco*RI, *Hin*dIII and *Pst*I and hybridized as described for Northern blots. Fractionation of chromosomal bands by pulsed-field gel electrophoresis (PFGE) was performed in a CHEF electrophoresis cell (Bio-Rad Laboratories) under conditions previously described [6]. Chromosomes from *Saccharomyces cerevisiae* (Life Technologies) were used as molecular mass standards and hybridization conditions were the same as those described for Northern blots.

### 2.5. *Plasmid constructions*

*T. cruzi* arginine kinase gene was amplified, from genomic DNA, using the following oligonucleotides: CGGAATTCAACTCAGTCACGATGGCCT (5′ end carrying an *Eco*RI site) and CGGTCGACTCACCTCGCAGACTTCTCC (3′ end carrying a *Sal*I site). The product was cloned into the pTREX expression vector [13] and the constructions were confirmed by polymerase chain reaction (PCR) and sequencing. This vector carries an mRNA processing signal (HX1) downstream of the RNA polymerase I ribosomal promoter that highly improves expression efficiency (Fig. 1). The HX1 sequence was obtained from the upstream region of the *T. cruzi* TcP2β gene and cloned into the pRIBOTEX vector [14]. Stable transfected cell lines were obtained by integration of the pTREX vector by homologous recombination of plasmid ribosomal promoter with *T. cruzi* ribosomal loci, corresponding to chromosome bands of 1200 and 1400 kb, preferentially in the 1200 kb chromosome. Stable cell lines were achieved after 60 days of treatment with 500 μg/ml G418 (Gibco BRL) and confirmed by Southern blot and chromosome blot analysis.

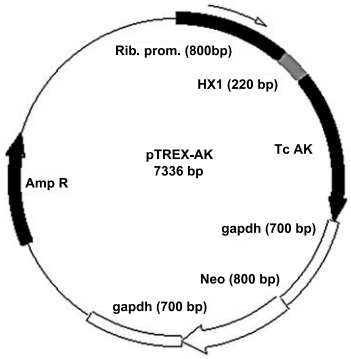


Fig. 1. Diagram of arginine kinase overexpression plasmid. The pTREX-AK plasmid is shown, the black box represents the *T. cruzi* ribosomal RNA polymerase I promoter (Rib. prom.) and the gray box indicates the HX1 trans-splicing region (HX1). The black arrows represent the arginine kinase gene (Tc AK) and the ampicillin resistance gene (Amp R), the white boxes indicates the glyceraldehyde-3-phosphate dehydrogenase 5′ and 3′ intergenic regions (gapdh). The white arrow represents the neomycin (G418) resistance gene (Neo).

Using the pTREX plasmid, the expression level of chloramphenicol acetyl transferase reporter gene was 2760-fold more efficient than of pRIBOTEX [13].

### 2.6. *Parasite transfection*

*T. cruzi* epimastigote cells of CL Brener strain were transfected with the plasmids using a gene pulser electroporator (Bio-Rad Laboratories) under the following conditions: $10^8$ parasites grown in LIT medium at 28°C were harvested by centrifugation, washed with PBS, and resuspended in 0.35 ml of electroporation buffer (PBS containing 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The cell suspension was mixed with 50 μg of plasmid DNA purified by passage through a Qiagen column (Qiagen) in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated with a single discharge of 400 V, 500 μF with a time constant of about 5 ms. After transfection, parasites were left to recover for 48 h at 28°C in LIT medium, followed by the selection in the same medium but containing 500 μg/ml G418 (Gibco BRL). A pTREX-GFP (green fluorescent protein) construction was used as a selection control. Parasites expressing GFP were observed under a fluorescence microscope.

## 3. Results

### 3.1. *General characteristics of the arginine kinase stable transfection*

To further determine whether the overexpression of the enzyme has an effect on high-energy-demanding processes, a 1075 bp full-length arginine kinase gene was cloned into the pTREX expression vector (pTREX-AK). We choose a novel high expression vector such as pTREX [13] instead of pRIBOTEX [14], because of its elevated arginine kinase expression capacity. After electroporation approximately 50% of epimastigote cells survived. In addition, 1 day after transfection with the control pTREX-GFP plasmid, 20% of the epimastigote cells showed GFP expression. Complete selection, evidenced by the total GFP positive control population, was obtained after about 60 days in the presence of the antibiotic G418.

### 3.2. *Arginine kinase expression and activity of the stable transfected population*

In *T. cruzi*, arginine kinase protein and specific activity increase about 7-fold along the epimastigote growth curve, from the initial log phase on day 3 (0.035 μmol $min^{-1}$ $mg^{-1}$

Table 1
Arginine kinase specific activity in the transfected parasites along the growth curve[a]

| Day | pTREX-GFP AK activity (UE) | pTREX-AK AK activity (UE) | Ratio (%) |
|---|---|---|---|
| 3 | 0.036(±0.005) | 4.219(±0.569) | 118.1 |
| 6 | 0.108(±0.011) | 4.408(±0.297) | 40.9 |
| 14 | 0.252(±0.011) | 4.762(±0.713) | 18.9 |

[a]Soluble extracts (0.3–10 μg of protein) from days 3, 6 and 14 of epimastigote cell cultures were assayed for arginine kinase specific activity as described in Section 2. Results are expressed as the mean ± S.D. of at least four experiments.

(UE)), up to the stationary phase on day 14 (0.249 UE) [8]. Stable pTREX-AK transfected cells were assayed for arginine kinase activity. Increments in the enzymatic activity compared with controls fluctuated between 118-fold (4.219 UE) in the beginning of the growth curve on day 3 to 19-fold in the day 14 culture (Table 1 and Fig. 2A). It is important to remark that such differences are due to the continuous increase along the growth curve of arginine kinase protein and specific activity in the control cultures [8]. It is interesting that arginine kinase specific activity from pTREX-AK transfected parasites remained constant along the growth curve, from day 3 to day 14, suggesting that the high expression level reached by this vector could mask the regulation process affecting this enzyme activity. In addition, no significant differences were detected between the pTREX-GFP transfected cells and wild-type non-transfected CL Brener strain cells.

In order to quantify arginine kinase expression levels a Western blot analysis was performed using samples corresponding to the third, seventh and 14th day of culture. As expected, densitometric analysis of the bands revealed that there is a close correspondence between arginine kinase enzymatic activity and enzyme protein levels. These levels were higher than 50-fold in the pTREX-AK transfected population when compared with the control cells corresponding to the same culture day (upper arrow in Fig. 2A). In addition, putative degradation intermediaries of the arginine kinase protein were also detected (lower arrows in Fig. 2A). Quantification of the bands corresponding to these products also shows a 7-fold decrease from day 3 to day 14.

Arginine kinase mRNA levels in the transfected cells overexpressing the enzyme were also analyzed in Northern blots. Quantification of bands revealed an increase higher than 50-fold when compared with the control population mRNA (Fig. 2B).

### 3.3. *Cell growth kinetics of the arginine kinase transfected population*

In order to establish whether the overexpression of the ar-

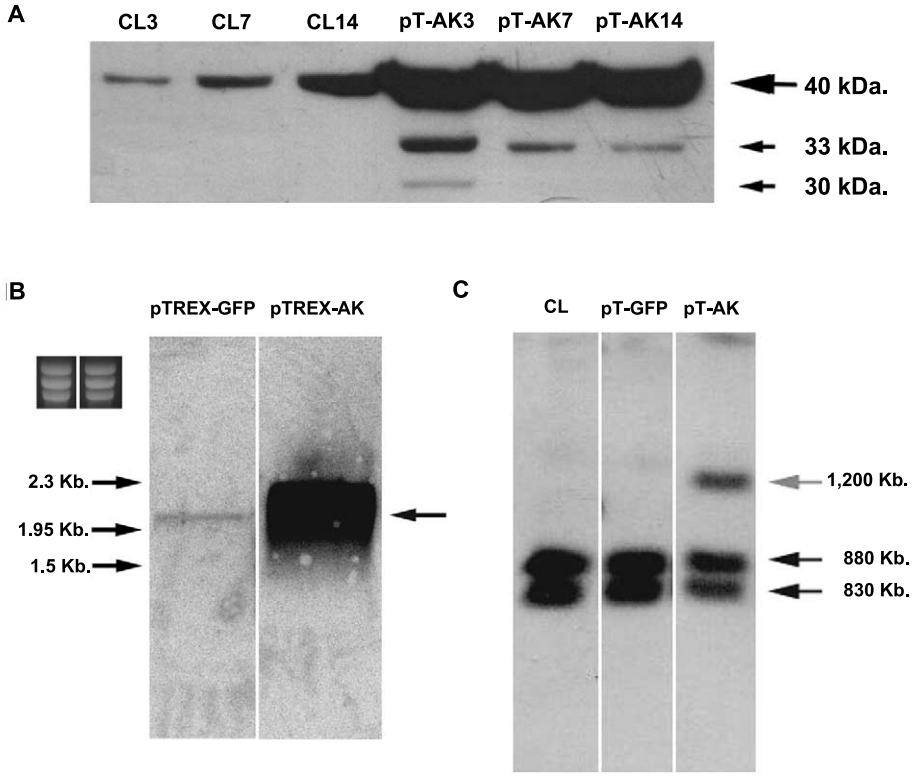


Fig. 2. A: Western blot analysis of the pTREX-AK and pTREX-GFP populations along the growth curve. Western blots were performed using soluble fractions (30 μg) from arginine kinase transfected or wild-type (CL Brener) parasites from days 3 (pT-AK3 or CL3), 7 (pT-AK7 or CL7) and 14 (pT-AK14 or CL14) of the growth curve. Membranes were incubated for 2 h with 1:1000 anti-arginine kinase serum. Upper arrow represents arginine kinase protein and lower arrows its putative degradation products. B: Northern blot analysis of pTREX-AK and pTREX-GFP populations. Total RNA from arginine kinase (pTREX-AK) or GFP (pTREX-GFP) transfected parasites was electrophoresed in a denaturing agarose gel and hybridized with the complete arginine kinase gene as a probe. The molecular weight markers and loading control correspond to the three ribosomal RNA bands (left arrows and inset). C: Chromosome blot analysis of the pTREX-AK and pTREX-GFP populations. Chromosomal DNA bands of arginine kinase and GFP transfected parasites (pT-AK and pT-GFP), and wild-type (CL) cells were separated using PFGE. The gel was stained with ethidium bromide. Approximate sizes were taken from chromosomal markers run on the same gel. Black arrows indicate bands corresponding to the endogenous arginine kinase genes and gray arrow indicates the insertion of the pTREX-AK plasmid.

Table 2
Growing kinetics of the transfected parasites[a]

| Day | pTREX-GFP cells per ml ($\times 10^7$) | pTREX-AK cells per ml ($\times 10^7$) | Ratio (%) |
|---|---|---|---|
| 0 | 0.1 (±0.00) | 0.1 (±0.01) | 100.0 |
| 3 | 0.8 (±0.02) | 0.7 (±0.02) | 87.5 |
| 5 | 1.5 (±0.06) | 1.6 (±0.07) | 106.7 |
| 7 | 2.2 (±0.07) | 2.1 (±0.05) | 95.5 |
| 10 | 4.8 (±0.12) | 5.4 (±0.38) | 112.5 |
| 12 | 5.2 (±0.14) | 6.3 (±0.10) | 121.2 |
| 14 | 5.1 (±0.06) | 7.3 (±0.17) | 143.1 |
| 17 | 5.1 (±0.46) | 9.0 (±0.34) | 176.5 |
| 19 | 6.0 (±0.26) | 9.1 (±0.22) | 151.7 |
| 21 | 5.7 (±0.15) | 8.5 (±0.15) | 149.1 |
| 24 | 4.5 (±0.36) | 8.0 (±0.22) | 177.8 |
| 26 | 2.4 (±0.18) | 4.4 (±0.14) | 183.3 |
| 28 | 1.7 (±0.13) | 4.2 (±0.21) | 247.1 |

[a]Epimastigote cells were cultured at 28°C in LIT medium. At the indicated day aliquots of each culture were counted using a hemocytometric chamber during 28 days. Results are expressed as the mean ± S.D. of at least four experiments.

ginine kinase gene altered cell growth kinetic, the behavior of arginine kinase transfected epimastigote culture cells was analyzed up to the 28th day of culture. Between days 10 and 28 cell density was considerably higher in the pTREX-AK transfected population in comparison with control cells transfected with pTREX-GFP. Maximal differences, about 150% (pTREX-AK, $4.2\times10^7$ cells per ml; pTREX-GFP, $1.7\times10^7$ cells per ml; CL Brener $1.7\times10^7$ cells per ml), were observed on day 28 corresponding to the late-stationary phase of growth (Table 2). No significant differences were observed between the non-transfected parasites and the GFP expressing populations.

### 3.4. Evaluation of the survival capability of the arginine kinase transfected population under stress conditions

To evaluate the stage differentiation capability in the arginine kinase and GFP transfected populations, epimastigote cells were cultured in TAU-3AG [11]. This medium promotes differentiation of epimastigote to trypomastigote forms by stress and emulating some of the conditions present in the insect vector hindgut where this process takes place. Although no significant increase in the population of trypomastigote cells was observed in the differentiation process (up to 10% of total epimastigote), after 72 h of incubation in TAU-3AG medium the pTREX-AK population density was about 54% higher than the control cells (pTREX-AK, $0.63\times10^7$ cells per ml; pTREX-GFP, $0.41\times10^7$ cells per ml; CL Brener $0.43\times10^7$ cells per ml; Table 3). Stage differentiation was also induced in Grace medium according to Isola et al. [12]. After 7 days of treatment the average number of trypomastigote cells obtained was 60.6% (±5.3%), however no significant differences between the pTREX-AK transfected population and the pTREX-GFP control group were observed. To further investigate whether overexpression of arginine kinase affects the parasite survival to other extreme conditions, pH and nutritional stresses were tested. Epimastigote cells were incubated for 5 days in cell-free conditioned LIT medium (from 14 days cultures) or PBS at pH 7.2, 9.0 or 4.0. The pTREX-AK transfected cells showed about 79% increase in the survival capability growing in conditioned medium (pTREX-AK, $1.54\times10^7$ cells per ml; pTREX-GFP, $0.86\times10^7$ cells per ml; CL Brener $0.83\times10^7$ cells per ml), whereas pTREX-AK transfected cells incubated in PBS pH 9.0 presented an increase of 91% (pTREX-AK, $1.49\times10^7$ cells per ml; pTREX-GFP, $0.78\times10^7$ cells per ml; CL Brener $0.77\times10^7$ cells per ml), no differences were observed after incubation at pH 7.2 or 4.0 (Table 3).

### 3.5. Genomic organization of the arginine kinase transfected gene

In order to establish the insertional position of the pTREX-AK plasmid into the *T. cruzi* genome, two strategies were followed. Arginine kinase and GFP transfected populations were compared by Southern blot analysis. As expected, bands attributed to the endogenous, single-copy arginine kinase gene were detected in both DNA samples. Additional bands corresponding to the foreign copy of the arginine kinase gene were observed in the pTREX-AK DNA sample (data not shown). The latter results were corroborated by chromosome blot analysis. As Fig. 2C shows, two bands corresponding to the 830 and 880 kb chromosomes containing the endogenous arginine kinase gene (black arrows), and an additional 1200 kb band were observed in the transfected population (gray arrow). These results are in agreement with one of the reported

Table 3
Growing kinetics of the transfected parasites under medium stress conditions[a]

| Treatment | pTREX-GFP cells per ml ($\times 10^7$) | pTREX-AK cells per ml ($\times 10^7$) | Ratio (%) |
|---|---|---|---|
| LIT control | 5.80 (±0.41) | 5.70 (±0.29) | 98.3 |
| Conditioned LIT | 0.86 (±0.05) | 1.54 (±0.13) | 179.1 |
| PBS (pH 7.2) | 1.12 (±0.07) | 1.48 (±0.06) | 132.1 |
| PBS (pH 9.0) | 0.78 (±0.04) | 1.49 (±0.03) | 191.0 |
| PBS (pH 4.0) | 1.69 (±0.08) | 1.96 (±0.06) | 116.0 |
| TAU-3AG | 0.41 (±0.02) | 0.63 (±0.03) | 153.7 |

[a]Epimastigote cells ($10^7$ cells per ml) were cultured at 28°C in 24-well plates during 5 days in LIT medium (LIT control), cell-free conditioned LIT medium obtained from 14 days cultures (conditioned LIT), and PBS pH 7.2, 9.0 and 4.0. For TAU-3AG treatment epimastigote cells ($10^7$ cell per ml) were grown for 3 days at 28°C. Cell cultures were counted using a hemocytometric chamber. Results are expressed as the mean ± S.D. of at least four experiments.

insertional positions of the pTREX vector into the ribosomal promoter locus [13].

## 4. Discussion

Previous evidence from this laboratory indicated that in *T. cruzi* arginine kinase is involved in the management of the cell energy reserves. It was reported that arginine kinase protein and specific activity increase along the epimastigote growth curve, suggesting that the enzyme would be regulated by cell density, parasite replication or nutritional stress [8]. In the present work, the benefits of the use of a high-efficiency expression vector such as pTREX were established. An expression level of the arginine kinase gene was obtained that led to raise the enzyme activity of the transfectant cells more than 100 times when compared to control cultures. It was recently suggested that arginine kinase would play a role as a stress resistance factor in yeast. In fact, Canonaco et al. [15] reported that recombinant yeast expressing crab muscle arginine kinase showed improved resistance under stress challenges that drain cellular energy, which were transient pH reduction and starvation. It is important to remark that the insect stage of the *T. cruzi* life cycle is frequently exposed to nutritional and pH stress conditions, depending on the feeding status of the vector. For example, the pH of *T. infestans* excreted material, feces and urine, containing trypomastigote cells varies between 5.7 and 8.9 [16]. Although no significant differences in stage differentiation capability were detected using Grace medium, we observed that the pTREX-AK population showed a higher cell density under stress conditions such as TAU-3AG medium and alkaline PBS, revealing an increase in the parasite fitness. In addition, the pTREX-AK population showed improved survival capability when it was cultured in conditioned LIT medium or for long periods of time (28 days), with the consequent nutrient depletion of the medium. On the other hand, no significant differences between the pTREX-AK and the pTREX-GFP populations were observed under stress by glucose deprivation induced by 11 mM 2-desoxyglucose or 20 mM sodium arsenate (data not shown).

This result, and the fact that the arginine kinase specific activity and expression increase during the *T. cruzi* growth phases, led us to also propose this enzyme as a constituent of an adaptive response to nutritional stress conditions. On the other hand, Western blot analysis suggests that arginine kinase has degradation kinetics along the parasite growth curve coincident with an increase of the endogenous enzyme protein and specific activity [8]. This strongly suggests that the enzyme activity might be regulated post-translationally, by proteolysis.

The definition of the processes where arginine kinase is involved would be of relevance, since arginine kinase is a promising therapeutic target because it is totally different from phophagen kinases present in the mammalian hosts.

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