

***Trypanosoma cruzi* amino acid transporter TcAAAP411 mediates arginine uptake in yeasts**

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Abstract

Trypanosoma cruzi, the aetiological agent of Chagas' disease, is exposed to extremely different environment conditions during its life cycle, and transporters are key molecules for its adaptive regulation. Amino acids, and particularly arginine, are essential components in *T. cruzi* metabolism. In this work, a novel *T. cruzi* arginine permease was identified by screening different members of the AAAP family (amino acid/auxin permeases) in yeast complementation assays using a toxic arginine analogue. One gene candidate, TcAAAP411, was characterized as a very specific, high-affinity, L-arginine permease. This work is the first identification of the molecular components involved specifically in amino acid transport in *T. cruzi* and provides new insights for further validation of the TcAAAP family as functional permeases.

Introduction

Chagas' disease is a zoonosis caused by the parasite *Trypanosoma cruzi*, a haematic protozoan transmitted by insects of the *Reduviidae* family. Chagas' disease is closely associated with poverty; the number of infected individuals in Latin America ranges from 12 to 14 million people in 18 endemic countries. Furthermore, Chagas' disease is becoming an important health issue in the United States and Europe (Tarleton *et al.*, 2007). During its life cycle, *T. cruzi* is exposed to different conditions in the insect gut, the mammalian bloodstream and also cell cytoplasm, which required evolutionary adaptations to such environments (Brenner, 1973; Kollien *et al.*, 2001). Among them, transport processes are rapid and efficient mechanisms for supplying metabolites from parasite extracellular media, and also to regulate the first step on metabolic pathways. Trypanosomatids have a metabolism largely based on the consumption of amino acids, which constitute the main carbon and energy sources in the insect stage of the parasite life cycle (Silber *et al.*, 2005). In *T. cruzi*, arginine is an essential amino acid and a key substrate for several metabolic pathways and

it is obtained from the host through different transport systems or by intracellular proteolysis (Pereira *et al.*, 1999; Canepa *et al.*, 2004). Arginine participates in the management of cell energy through an arginine kinase (Pereira *et al.*, 2000; Alonso *et al.*, 2001). This enzyme, which was also found in *Trypanosoma brucei* (Pereira *et al.*, 2002b), catalyses the reversible transphosphorylation between phosphoarginine and ATP, and thus phosphorylated arginine acts as an energy reservoir involved in the renewal of ATP (Pereira *et al.*, 2002a, 2003). As phosphoarginine is completely absent in mammalian tissues, arginine kinase is a possible target for the future development of chemotherapeutic agents. Despite the relevance of amino acids in trypanosomatids, the way in which they are internalized to become available for metabolism remains relatively unexplored. In this sense, the amino acid transporters are the first cell proteins that are in contact with solutes in the surrounding medium, and in several cases they function not only as permeases to carry the solutes into the cytoplasm but also as environmental sensors. One of the major transporter families of amino acids is AAAP (TC 2.A.18), which is largely found in plants (Young *et al.*, 1999). In *T. cruzi*, members of

this family were first identified by our group (Bouvier *et al.*, 2004) and confirmed by the Trityps genome project (Berriman *et al.*, 2005). The *T. cruzi* subfamily, named TcAAAP, has > 30 genes coding for proteins with lengths of 400–500 amino acids and 10–12 predicted transmembrane α -helical spanners. One interesting feature of this permease family is the absence of similar sequences in mammalian organisms; however, the presence of unidentified orthologues could not be rejected (Akerman *et al.*, 2004).

In this work we present the first functional characterization of an amino acid permease from *T. cruzi*. TcAAAP411 was identified as a specific arginine permease and functionally characterized in a yeast model. Kinetics and specificity data suggest that TcAAAP411 is at least one of the components of the *T. cruzi* arginine transport system, mostly studied during the last decade.

Materials and methods

Plasmid constructions

Genes of the TcAAAP family were amplified by PCR from gDNA and cloned into the yeast expression vector pDR196 (Rentsch *et al.*, 1995). The following genes were chosen for the complementation assay: TcAAAP187 (Tc00.1047053510187.540), TcAAAP245 (Tc00.1047053510245.10), TcAAAP411 (Tc00.1047053511411.30), TcAAAP431 (Tc00.1047053510431.30), TcAAAP545 (Tc00.1047053511545.80), TcAAAP507 (Tc00.1047053510507.40), TcAAAP649 (Tc00.1047053511649.100), TcAAAP659 (Tc00.1047053507659.20), TcAAAP707 (Tc00.1047053508707.10), TcAAAP837 (Tc00.1047053503837.20) and TcAAAP069 (Tc00.1047053504069.120). Genes have been named according to the organism *T. cruzi* (Tc), the transporter gene family (AAAP, TCDB 2.A.18) and the three last numbers of the systematic ID from the GeneDB.

Strains and media

The *Saccharomyces cerevisiae* strain S288C (BY4742 MATa *his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0, can1::kanMX4*) was kindly provided by Dr Alejandro Colman Lerner (FBMC, UBA, Argentina). S288C Δ can was maintained on complete yeast extract/peptone/dextrose medium. Ura⁺ transformants were selected on synthetic complete (SC) medium, which is composed of 2% glucose, 0.17% yeast nitrogen base (without amino acids and ammonium sulphate), 0.5% ammonium sulphate, 0.1% histidine, 0.1% leucine, 0.1% lysine and 2% agar. For the recovery of canavanine-sensitive phenotype, 150 mg mL⁻¹ of canavanine was added to the SC plates. Yeasts were transformed with pDR196-TcAAs and an empty vector pDR196 according to Gietz & Woods (2002). Ammonium sulphate was added to media to reduce

the background amino acid transport produced by general permeases (Courchesne & Magasanik, 1983).

Transport assays

Saccharomyces cerevisiae transformants were grown in the media described above, harvested in the logarithmic growth phase and resuspended in phosphate-buffered saline (PBS) to a final OD_{600 nm} of 1. To start the reaction, 100 μ L of this cell suspension was added to 100 μ L of PBS containing labelled L-[³H] arginine (0.1 μ Ci) at the indicated concentrations. Following incubation at the indicated times at 28 °C, the reaction was stopped by five volumes of cold PBS and centrifugation at 8000 g for 30 s; cells were washed twice with 1 mL of ice-cold PBS. Pellets were then resuspended in 0.2 mL of 1% SDS–0.2 N NaOH and counted for radioactivity in liquid scintillation cocktail (Packard Instrument Co., Meriden, CT). Differences in transport rates have been statistically analysed using a *t*-test and a cut-off *P*-value of 0.05.

Bioinformatics

Sequences from the Trityps genome projects were obtained at GeneDB (<http://www.genedb.org/>) and TcruziDB (<http://tcruidb.org/>). Assembly and analysis of the DNA sequence data, including prediction of ORFs, were carried out using the software package VECTOR NTI ver. 10.3.0 (Invitrogen) and the online version of BLAST at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Local or online software was used under default parameters. For the grey-scale scheme of sequence identities, TcAAAP amino acid sequences were aligned using the CLUSTALW method and this information was the input for a short routine programmed in PERL. Amino acids letters were replaced by grey-scale coloured lines, where dark tones indicate a low-identity position.

Results

Identification of an arginine transporter by canavanine selection in yeasts

To identify gene candidates coding for arginine permeases belonging to the TcAAAP family, 11 of about 34 genes, according to the Trityps genome project (Berriman *et al.*, 2005), were tested using a yeast model. All available TcAAAP sequences were first analysed, and haplotypes, incomplete sequences and pseudogenes discarded. Using a phenogram constructed from a global sequence alignment and the CLUSTALW algorithm, about one representative member was selected from each cluster of the tree. This 'rational' approach was applied to reduce the number of genes analysed. After selection in SC medium, the transformants were functionally tested for their ability to grow in a medium containing canavanine, an arginine-toxic analogue.

Canavanine resistance in yeasts results from a deletion in the gene coding for a specific arginine permease (Can1p) (Grenson *et al.*, 1966). As Fig. 1a shows, adding canavanine in the selection medium, one clear candidate gene (named TcAAAP411) restored the canavanine toxicity in all complementation assays performed. However, a second candidate TcAAAP545 presented slight growth differences with control, and was also included for further characterization.

Validation of TcPAT411 by arginine uptake assays

Canavanine sensitization in yeast could result from various aspects of arginine metabolism other than transport systems. To determine whether TcAAAP411 and TcAAAP545 are actually arginine permeases, the accumulation of radiolabelled L-arginine was analysed. Selected transformant yeasts (TcAAAP545 and TcAAAP411) were compared with

those transformed with an empty plasmid (pDR196) or with a permease gene in which the resistance was not reversed (TcAAAP069). The initial rate of arginine transport in pDR196, TcAAAP069 and TcAAAP545 showed similar values (1.50, 1.16 and 1.43 pmol min⁻¹ per 10⁷ cells, respectively), whereas in TcAAAP411 arginine uptake was more than threefold higher and increased linearly over time (4.60 pmol min⁻¹ per 10⁷ cells; Fig. 1b). The expression of TcAAAP411 mRNA was also confirmed by reverse transcriptase-PCR.

Bioinformatic analysis of TcAAAP411

The TcAAAP family includes > 30 sequences, with 34 according to the genome data, but the real gene number is difficult to determine as this genome project remains unfinished and a few putative TcAAAP genes have been classified as 'unknowns', pseudogenes or haplotypes variants. In addition, the first bioinformatic characterization of this family was made before the completion of the *T. cruzi* genome, using only unassembled single-read sequences (Bouvier *et al.*, 2004). Figure 2a is a sequence identity colour-based scheme constructed using all available TcAAAP genes. As Fig. 2a shows, it is evident that the N-terminal domain (about 90 amino acids) represents a highly variable region (5% of consensus), in contrast to the central and C-terminal domains, which have > 70% of consensus amino acid positions. Comparing amino acid identities between 11 TcAAAP analysed, TcAAAP411 is located close to TcAAAP545 in the identity-based phenogram (Fig. 2b). These data correlate perfectly with *in vitro* results where both genes were capable of reversing canavanine resistance in yeasts. However, the *Leishmania donovani* arginine permease LdAAP3 (Shaked-Mishan *et al.*, 2006) is located in a branch distant from TcAAAP411. *In silico* topological analysis of TcAAAP411 using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) predicted 10 transmembrane helices and the variable N-terminal domain outside the cell. Two copies of TcAAAP411 were found in the *T. cruzi* genome database (GeneDB, <http://www.genedb.org/>), one characterized herein, and the other haplotype with three different amino acid positions (GeneDB systematic ID: Tc00.1047053506053.10).

Biochemical properties of TcAAAP411

To define the substrate specificity of the permease, competitive transport studies were undertaken. The initial rate of arginine uptake was measured in the presence of 20 µM arginine and 20-fold excess of unlabelled competing molecule. Considering the participation of other endogenous yeast amino acid permeases, control experiments were also performed using pDR196 yeasts. None of the tested compounds produced a significant decrease on arginine uptake

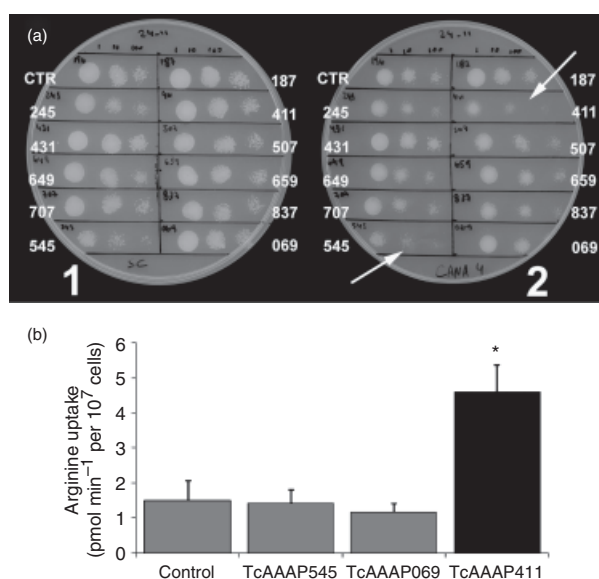
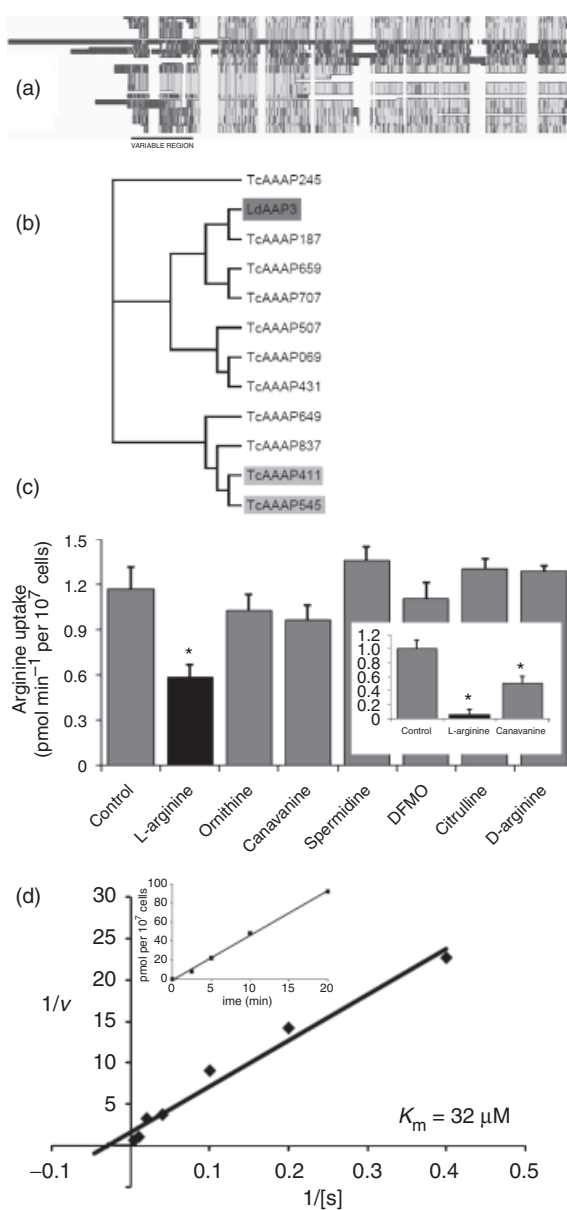


Fig. 1. (a) Impaired TcAAAP411 and TcAAAP545-mediated growth of *Saccharomyces cerevisiae* S288C (BY4742 MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 can1::kanMX4*) in SC canavanine medium. The *S. cerevisiae* strain was transformed with the pDR196 plasmid harbouring the genes TcAAAP187, TcAAAP245, TcAAAP411, TcAAAP431, TcAAAP545, TcAAAP507, TcAAAP649, TcAAAP659, TcAAAP707, TcAAAP837 and TcAAAP069 or an empty pDR196 plasmid, and tested for growth in SC medium (1) or SC medium plus 150 µg mL⁻¹ of the arginine toxic analogue canavanine (2). Transformed strains were placed in drops containing approximately 10 000, 1000 and 100 cells. Plates were photographed after 3 days at 30 °C. (b) L-[³H] arginine uptake into strain of *S. cerevisiae* S288C (BY4742 MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0, can1::kanMX4*) transformed with the constructs pDR196-TcAAAP411, pDR196-TcAAAP545, pDR196-TcAAAP069 and pDR196. Transport experiments with 100 µM [³H]-labelled arginine were performed using transformed cells grown to exponential phase in SC medium and assayed for L-[³H] arginine uptake for 20 min. Control transport rate corresponds to 1.50 pmol min⁻¹ per 10⁷ cells of the pDR196-transformed strain. **P* < 0.05, significantly different in *t*-test.

except unlabelled arginine, as expected (Fig. 2c). To test whether canavanine can enter the cells through TcAAAP411, as occurs in the selection yeast media, the same assay was repeated using a 50-fold excess of canavanine. The inset in Fig. 2c shows that, in these conditions, canavanine produced a significant decrease on arginine uptake of about 50%. Transport of L-arginine by TcAAAP411 yeasts was found to be roughly proportional to an incubation time up to 20 min (Fig. 2d, inset). Data obtained from concentration-dependent arginine influx curves were analysed using Lineweaver–Burk plots and the apparent Michaelis–Menten constant (K_m) value was estimated as about 30 μ M (Fig. 2d).



Discussion

Ten years ago, *T. cruzi* arginine transport, coupled to phosphoarginine synthesis, was identified and biochemically characterized (Pereira *et al.*, 1999). This transport system showed very similar kinetic parameters and substrate specificity to TcAAAP411, suggesting that this permease is at least one component of the previously measured arginine transport system. Recently, a similar arginine transporter (LdAAP3) has been identified in the protozoan parasite *L. donovani* (Shaked-Mishan *et al.*, 2006). Its regulation depends on the availability of the extracellular substrate, as amino acid starvation produces an increase in arginine transport and LdAAP3 abundance (Darlyuk *et al.*, 2009). Interestingly, this mechanism of regulation was described in *T. cruzi* for arginine uptake and also for other amino acid transport processes (Pereira *et al.*, 2008), supporting the hypothesis that previously characterized transport systems in trypanosomatids involve members of the AAAP family. A *T. cruzi* spermidine permease, TcPAT12, was previously characterized by our group (Carrillo *et al.*, 2006). This protein is the most divergent member, in terms of amino acid identity, of the TcAAAP family. Although TcPAT12 is essentially a spermidine transporter, as occurs with other permeases, it is also capable of transporting other metabolites such as putrescine and arginine, but at lower rates compared with spermidine (5.4-fold lower). Therefore, we speculate that some divergent genes, such as TcPAT12, were selected during evolution for the uptake of amino acid-related molecules, as is the case of polyamines.

The importance of finding and further confirmation of the presence of the AAAP family in *T. cruzi* rests on the

Fig. 2. (a) Sequence identity analysis of TcAAAP family. All available TcAAAP amino acid sequences were aligned using the CLUSTAL method and this information was the input for a short routine programmed in PERL. Amino acid letters were replaced by grey-scale coloured lines, where dark tones indicate a low-identity position. (b) Phenogram analysis of TcAAAP genes and *Leishmania donovani* LdAAP3 arginine transporter. Amino acid sequences of TcAAAP187, TcAAAP245, TcAAAP411, TcAAAP431, TcAAAP545, TcAAAP507, TcAAAP649, TcAAAP659, TcAAAP707, TcAAAP837, TcAAAP069 and LdAAP3 were aligned using the CLUSTALW method and a neighbour-joining phylogenetic tree was constructed from the multiple sequence alignment. Dark grey boxes correspond to *L. donovani* LdAAP3 arginine transporter, light grey boxes to TcAAAP411 and TcAAAP545. (c) Competitive inhibition studies. The rate of uptake of L-[³H] arginine (20 μ M) was measured in either pDR196-TcAAAP411 or pDR196 transformants. Competitors were added at a concentration of 0.4 mM (20-fold excess) or 1 mM (50-fold excess) (inset). * $P < 0.05$, significantly different in *t*-test. (d) Kinetic analysis of arginine uptake in TcAAAP411-transformed yeasts. The kinetic parameters were calculated from a Lineweaver–Burk plot, and K_m and regression coefficient are indicated. A time-course arginine uptake experiment was performed for 20 min (inset).

apparent absence of these permeases in mammals. It has been proposed that amino acid transporters could be promising targets for therapeutic drugs. Crystal violet is a 'classic' trypanocidal drug currently used in blood banks in endemic areas in attempts to eliminate *T. cruzi* transmission. It has been proposed that the mechanism of action of this drug is by inhibition of protein synthesis and amino acid transport (Hoffmann *et al.*, 1995). It was demonstrated that the amino acid derivatives canavanine and homoarginine inhibited epimastigote growth and arginine kinase activity (Pereira *et al.*, 2003); interestingly, the same compounds were previously characterized as arginine transport inhibitors (Pereira *et al.*, 1999). Recently, it was reported that epimastigotes incubated with the proline analogue L-thiazolidine-4-carboxylic acid, a competitive inhibitor of proline transport, partially inhibited the epimastigote growth and trypomastigote bursting (Magdaleno *et al.*, 2009). In addition, other amino acid analogues have been extensively tested as trypanocidal compounds (Barrett & Gilbert, 2006). Taken together, these data suggest that amino acid permeases may provide multiple, as yet unexplored targets for portals of therapeutic drugs.

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Authors' contribution

C.C. and G.E.C. contributed equally to this work.

References

- Akerman M, Shaked-Mishan P, Mazareb S, Volpin H & Zilberstein D (2004) Novel motifs in amino acid permease genes from *Leishmania*. *Biochem Bioph Res Co* **325**: 353–366.
- Alonso GD, Pereira CA, Remedi MS, Paveto MC, Cochela L, Ivaldi MS, Gerez de Burgos NM, Torres HN & Flawiá MM (2001) Arginine kinase of the flagellate protozoa *Trypanosoma cruzi*. Regulation of its expression and catalytic activity. *FEBS Lett* **498**: 22–25.
- Barrett MP & Gilbert IH (2006) Targeting of toxic compounds to the trypanosome's interior. *Adv Parasit* **63**: 125–183.
- Berriman M, Ghedin E, Hertz-Fowler C *et al.* (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science* **309**: 416–422.
- Bouvier LA, Silber AM, Galvão Lopes C, Canepa GE, Miranda MR, Tonelli RR, Colli W, Alves MJ & Pereira CA (2004) Post genomic analysis of permeases from the amino acid/auxin family in protozoan parasites. *Biochem Bioph Res Co* **321**: 547–556.
- Brener Z (1973) Biology of *Trypanosoma cruzi*. *Annu Rev Microbiol* **27**: 347–382.
- Canepa GE, Silber AM, Bouvier LA & Pereira CA (2004) Biochemical characterization of a low-affinity arginine permease from the parasite *Trypanosoma cruzi*. *FEMS Microbiol Lett* **236**: 79–84.
- Carrillo C, Canepa GE, Algranati ID & Pereira CA (2006) Molecular and functional characterization of a spermidine transporter (TcPAT12) from *Trypanosoma cruzi*. *Biochem Bioph Res Co* **344**: 936–940.
- Courchesne WE & Magasanik B (1983) Ammonia regulation of amino acid permeases in *Saccharomyces cerevisiae*. *Mol Cell Biol* **3**: 672–683.
- Darlyuk I, Goldman A, Roberts SC, Ullman B, Rentsch D & Zilberstein D (2009) Arginine homeostasis and transport in the human pathogen *Leishmania donovani*. *J Biol Chem* **284**: 19800–19807.
- Gietz RD & Woods RA (2002) Transformation of yeast by the Liac/SS carrier DNA/PEG method. *Method Enzymol* **350**: 87–96.
- Grenson M, Mousset M, Wiame JM & Bechet J (1966) Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine-transporting system. *Biochim Biophys Acta* **127**: 325–338.
- Hoffmann ME, Jang J, Moreno SN & Docampo R (1995) Inhibition of protein synthesis and amino acid transport by crystal violet in *Trypanosoma cruzi*. *J Eukaryot Microbiol* **42**: 293–297.
- Kollien AH, Grospietsch T, Kleffmann T, Zerbst-Boroffka I & Schaub GA (2001) Ionic composition of the rectal contents and excreta of the reduviid bug *Triatoma infestans*. *J Insect Physiol* **47**: 739–747.
- Magdaleno A, Ahn IY, Paes LS & Silber AM (2009) Actions of a proline analogue, L-thiazolidine-4-carboxylic acid (T4C), on *Trypanosoma cruzi*. *PLoS One* **4**: e4534.
- Pereira CA, Alonso GD, Paveto MC, Flawiá MM & Torres HN (1999) L-Arginine uptake and L-phosphoarginine synthesis in *Trypanosoma cruzi*. *J Eukaryot Microbiol* **46**: 566–570.
- Pereira CA, Alonso GD, Paveto MC, Iribarren A, Cabanas ML, Torres HN & Flawiá MM (2000) *Trypanosoma cruzi* arginine kinase characterization and cloning. A novel energetic pathway in protozoan parasites. *J Biol Chem* **275**: 1495–1501.
- Pereira CA, Alonso GD, Ivaldi S, Silber AM, Alves MJ, Bouvier LA, Flawiá MM & Torres HN (2002a) Arginine metabolism in *Trypanosoma cruzi* is coupled to parasite stage and replication. *FEBS Lett* **526**: 111–114.

- Pereira CA, Alonso GD, Torres HN & Flawiá MM (2002b) Arginine kinase: a common feature for management of energy reserves in African and American flagellated trypanosomatids. *J Eukaryot Microbiol* **49**: 82–85.
- Pereira CA, Alonso GD, Ivaldi S, Silber AM, Alves MJ, Torres HN & Flawiá MM (2003) Arginine kinase overexpression improves *Trypanosoma cruzi* survival capability. *FEBS Lett* **554**: 201–205.
- Pereira CA, Carrillo C, Miranda MR, Bouvier LA & Cánepa GE (2008) *Trypanosoma cruzi*: transport of essential metabolites acquired from the host. *Medicina* **68**: 398–404.
- Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S & Frommer WB (1995) NTR1 encodes a high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett* **370**: 264–268.
- Shaked-Mishan P, Suter-Grotemeyer M, Yoel-Almagor T, Holland N, Zilberstein D & Rentsch D (2006) A novel high-affinity arginine transporter from the human parasitic protozoan *Leishmania donovani*. *Mol Microbiol* **60**: 30–38.
- Silber AM, Colli W, Ulrich H, Alves MJ & Pereira CA (2005) Amino acid metabolic routes in *Trypanosoma cruzi*: possible therapeutic targets against Chagas' disease. *Curr Drug Targets Infect Disord* **5**: 53–64.
- Tarleton RL, Reithinger R, Urbina JA, Kitron Z & Gürtler RE (2007) The challenges of Chagas disease – grim outlook or glimmer of hope? *PLoS Med* **4**: e332.
- Young GB, Jack DL, Smith DW & Saier MH Jr (1999) The amino acid/auxin: proton symport permease family. *Biochim Biophys Acta* **1415**: 306–322.