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Selenocysteine in *Trypanosoma evansi*: Identification of the Genes *selb, selc, seld, pstk, seltryp* and the Selenophosphate Synthetase Protein

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Abstract. Selenoproteins have been described in all three domains of life and their function has been mainly associated with oxidative stress defense. Canonical elements required for selenoprotein production have been identified in members of the kinetoplastid group supporting the existence of a complete selenocysteine synthesis pathway in these organisms. Currently, nothing is known regarding the selenocysteine pathway in *Trypanosoma evansi*. In this study, we identified the expression of the elements *selB*, *selC*, *selD*, *PSTK* and *selTRYP* at the mRNA level in *T. evansi*. All translated proteins (selD, PSTK, selTRYP and selB) have the domains predicted and higher identity with *Trypanosoma brucei. gambiense*. The selenophosphate synthetase protein was localized in the cytoplasm. Our results support the existence of an active selenocysteine pathway in *T. evansi*.

Keywords: *Trypanosoma evansi*, selenocysteine, *selB, selC, selD, PSTK, seltTRYP.*

INTRODUCTION

Trypanosoma evansi is the pathogenic trypanosomatid with the widest distribution worldwide, posing a health threat to livestock and causing economic losses in Africa, South America, Asia and Oceania (Desquesnes *et al.* 2013; Salah *et al.* 2015; Kumar *et al.* 2017). Nonspecific mechanical vectors ubiquitous to most environments can transmit *T. evansi*, which it is the etiologic agent of the disease known as *surra*. The disease, characterized by anemia, fever, widespread subcutaneous edema and a marked ataxia in the hindlimbs, affects almost all species of mammals (Silva *et al.* 2002), including humans (Joshi *et al.* 2005), and is distinct from the diseases caused by closely related

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trypanosomes such as *T. brucei* and *T. equiperdum* (Desquesnes *et al.* 2013).

The incorporation of selenium into proteins is an important metabolic pathway described in all three domains of life and its function has been mainly associated with oxidative stress defence (Papp *et al.* 2007). Selenium is used in the form of the amino acid selenocysteine, which is cotranslationally incorporated in a complex, context-specific manner into nascent polypeptides at UGA codons (Mangiapane *et al.* 2014). In bacteria, the biosynthesis and insertion of selenocysteine in proteins is associated with four genes: *selA*, *selB*, *selC* and *selD* (Leinfelder *et al.* 1988). In eukaryotes, among the key elements in this pathway are a signaling nucleotide structure in the messenger RNA (SECIS), a specific tRNA ($tRNA^{Sec}$, product of the *selC* gene) and protein complexes such as selenophosphate synthetase (SPS, product of *selD* gene) and a GTP-dependent elongation factor that is specific for Sec incorporation (EF-Sec, product of *selB* gene). There is no homolog for *selA* in eukaryotes, however, the enzymes O-phosphoseryl-tRNASec kinase (PSTK gene) and O-phosphoseryl-tRNA(Sec) selenium transferase (SepSecS gene) acts in serine phosphorylation and selenocysteylation (Geslain *et al.* 2006). These elements allow the conversion of selenium to its organic form, its aminoacylation into $tRNA^{Sec}$ and the coupling of nucleotidic at the UGA codon for the insertion of selenocysteine into the protein (Allmang *et al.* 2009). Canonical elements required for selenoprotein production have been identified in members of the kinetoplastid group, supporting the existence of a complete selenocysteine synthesis pathway in these organisms (Cassago *et al.* 2006). In *T. brucei* three selenoprotein genes were found; one of them, *seltryp*, has not been found in any other organism so far (Cassago *et al.* 2006; Geslain *et al.* 2006; Lobanov *et al.* 2006; Sculaccio *et al.* 2008; Aeby *et al.* 2009a). For *T. evansi*, due to their great similarity with *T. brucei* the elements of selenoprotein metabolism must be present. Thus, given the relevance of this parasite in health and economics and the importance of this pathway for the parasite, this study aimed to identify elements of the selenocysteine pathway in *T. evansi,* and verifying the occurrence of substantial differences between them.

MATERIALS AND METHODS

Animal infection and parasite purification

The *T. evansi* strain used in this study was isolated from a naturally infected dog in southern Brazil (Colpo *et al.* 2005), characterized (Duarte *et al.* 2014) and stored in liquid nitrogen until a rat (*Rattus norvegicus*) was experimentally inoculated with 108 trypomastigotes by intraperitoneal injection. Parasitemia was estimated daily by blood smear examination under the microscope. Slides were mounted with blood collected from the tail vein, stained by the panoptic method and visualized at a 1000× magnification. When parasitemia reached >80 trypanosomes per field, the rat was anesthetized with Zoletil® (Fort Dodge® Ingelheim am Rhein, Germany) using 20–40 mg/kg and blood containing parasites was collected by cardiac puncture. Parasites were separated from blood by centrifugation with HEPES buffered Percoll® (GE Healthcare® Chicago, USA) according to the technique described by Grab & Bwayo (1982). Trypanosomes were separated from white blood cells using DEAE-Cellulose (Sigma Aldrich® Saint Louis, USA) chromatography with PBS-Glucose by the method previously described by Lanham & Godfrey (1970). Purified parasites were used in the following procedures. The procedures described were approved by the Animal Welfare Committee of Santa Catarina State University (UDESC), number 1.30.11, in accordance to Brazilian laws and ethical principles published by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Gene expression and sequencing

Total RNA was extracted from *T. evansi* using Trizol® reagent (Invitrogen®Carlsbad, USA), according to the manufacturer´s instructions. Complementary strand (cDNA) synthesis was performed using the ProtoScript M-MuLV First Strand cDNA Synthesis® kit (New England Biolabs® Ipswich, USA). PCRs were performed employing specific primers, as shown in Table 1. Primer pairs were designed based on *T. brucei* gene sequences for *selB* (XM_839239), *selC* (DQ508818), *selD* (XM_818071), *PSTK* (XM_818060) and *selTRYP* (XM_839398) deposited in GenBank. PCR products were resolved in 1% agarose gel, cloned into pGEM using the pGEM-T-Easy kit (Promega®, Madison, USA) and sequenced.

Bioinformatic Analysis

The identities of cloned sequences were confirmed using BLAST tool (www.ncbi.nlm.nih.gov/blast) and ORF were translated with the Expasy Translate tool (http://expasy.org/tools/dna.html). *selC* tRNA secondary structure was obtained using ARAGORN software (http://130.235.46.10/aragorn/). Amino acid sequences were aligned with sequences of related species using the ClustalX software (Thompson *et al.* 1997) and domain analysis was performed using Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) tools (data not shown).

The bioinformatic processes used a set of 5 strains downloaded from TryTripDB (http://tritrypdb.org/tritrypdb/): *T. evansi* (STI805), *T. brucei* (TREU927), *T. b. gambiense* (DAL972), *T. vivax* (Y486) and *T. equiperdum* (5694) this last one downloaded from NCBI under the ID number txid5694 (https://www.ncbi.nlm.nih.gov/genome/?term=txid5694).

A FASTA file containing the *selD* (TevSTIB805.10.9990), *selB* (TevSTIB805.4.1890), *PSTK* (TevSTIB805.10.9870) and *selTRYP* (TevSTIB805.4.3500) genes, was used to search for orthologous genes in the above related strains through of Orthovenn software (www.bioinfogenome.net/OrthoVenn/) with parameters E-value 1e-5 and Inflation value 1.5. The orthologous genes on the *selD*, *selB*, *PSTK* and *selTRYP* genes identified had the similarity analysis done through the BLASTp online tool (https://blast.ncbi.nlm.nih.gov/). The orthologous sequences were then aligned using the MEGA7 software using the ClustalW method, with the default parameters of the tool, to construct the phylogenetic tree through the Maximum Likehood method, and this tree was plotted by the Interactive Tree of Life (iTOL) tool.

A search of the PFAM database (https://pfam.xfam.org/) was performed through HMMER (available at https://www.ebi.ac.uk/Tools/hmmer/) to identify the domains of the selD, selB, PTSK and selTRYP proteins.

The identified domains were separated according to the position indicated in the search and the domains of each protein in *T. evansi* were compared by BLAST with other trypanosomatids species.

Immunodetection of selenophosphate synthetase

Immunolocalization of selenophosphate synthetase protein (SPS), which is coded by the *selD* gene in *T. evansi*, was performed using indirect immunofluorescence. Purified parasites were fixed in paraformaldehyde 4% and distributed on immunofluorescence slides. Slides were washed with 0.1% PBS-Tween and 5% skimmed dry milk for 1 hour. Next, the parasites were incubated 1 hour with a mouse anti-SPS primary polyclonal antibody against *T. brucei* SPS (kindly provided by Dr. Otavio H. Thiemman) diluted 1:100 and 15 minutes with the anti-IgG mouse conjugate Alexa Fluor 488® (Invitrogen® Carlsbad, USA) diluted 1:1000. Images were obtained using an Olympus fluorescence microscope model BX40-FL.

Western Blot for SPS detection was performed with the same polyclonal antibody used in the immunofluorescence assay. 50 µg of total protein extract from purified *T. evansi* were separated on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Nonspecific sites on the membrane were blocked for 1 hour with 5% skimmed dry milk in blocking buffer (10 mM Tris-HCl, pH 8; 120 mM NaCl, 0.05% Tween-20). SPS protein was detected by incubating for 2 hours with the anti-SPS-antibody diluted 1:500 and 2 hours with the anti-mouse IgG conjugated with alkaline phosphatase (Sigma Aldrich® Saint Louis, USA) diluted 1:30000. The membrane was incubated with the NBT/BCIP solution (Sigma Aldrich® Saint Louis, USA) in 190mM Tris base, 1 mM $MgCl_2$ until color appeared.

RESULTS

Sequencing, translation and domain analysis of *Trypanosoma evansi* genes selB, selC, selD, PSTK and selTRYP

Expression of key genes of the selenocysteine pathway in *T. evansi* was demonstrated following parasite purification by DEAE-cellulose chromatography, RNA extraction and sequencing. PCR performed on cDNA using specific primer pairs for *selB, selC, selD*, *PSTK* and *selTRYP* revealed bands with the predicted sizes for each gene (Figure 1). Our sequencing results, performed with a brazilian *T. evansi* strain (Colpo *et al.* 2005), did not differ from the deposited at the TritrypDB.

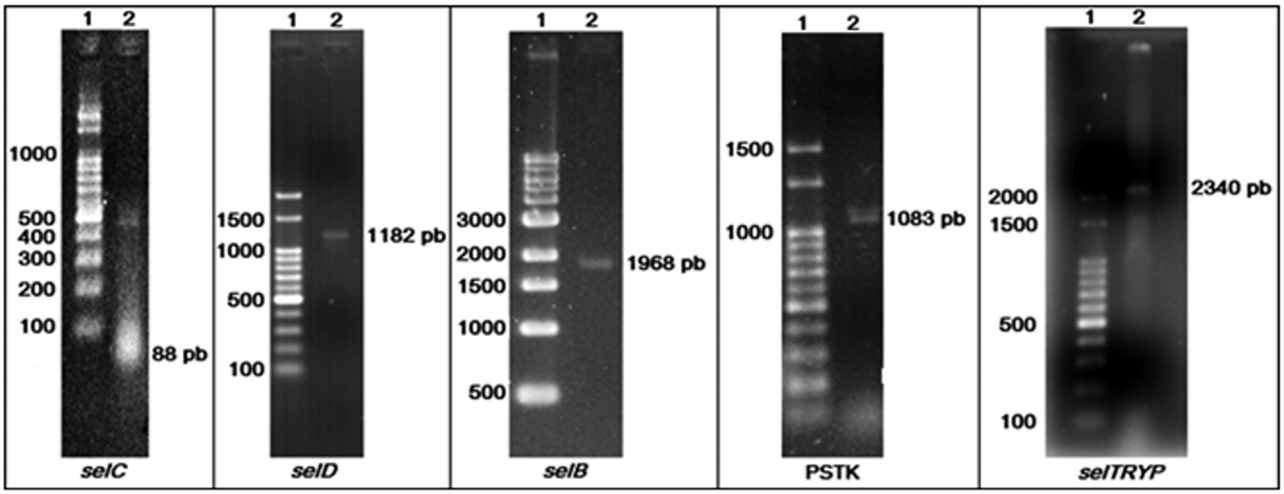

Fig. 1. Detection of gene expression of the selenocysteine pathway in *T. evansi*. Numbers on the sides indicate molecular size in base pairs. Lanes marked as 1: Molecular-weight size marker (100bp lader Ludwig Biotecnologia- Brazil); Lanes marked as 2: PCR product. *selD, selB* and *selTRYP* amplicons ran in 1% agarose gels; *selC* and *PSTK* ran in 2% agarose gels. The gene amplified in each reaction is indicated on the bottom.

Table 1. Oligonucleotide primer sequence used in PCR and RT-PCR assays.

ORF	Primer	Sequence(5'-3')	Amplicon size(bp)	References
selB	selBF	GCC CAT ATG ACA GAA GTT AAT GAT GTT GCC TCT G	1968	Cassago *et al*. 2006
	selBR	GCC CTC TGA GCT ACT GCT GAA GCT GAC TGT G		
selC	selCF	GCC ACG ATG AGC TCA GCT GGT GCT G	88	Cassago *et al*. 2006
	selCR	CAC CAC AAA GGC CGA ATC GAA CGG C		
selD	selDF	ACG TAC GTC ATA TGT CAG AGA AGG AAG GAA AAG TAA TAC	1182	Sculaccio *et al*. 2008
	selDR	ATC TGA GCT ATC AAA TAA TCT ATC ATT TAC CTT CGC TCC CA		
PSTK	PSTKF	CCC ATA TGA CAG TTT GTC TTG TTC TAC TAA	1083	designed by authors
	PSTKR	CCC TCT GAG TTA TCA AAG ATC TAC TAA GGC ATG A		
selTRYP	seltrypF	TAT AAA GGA TCC ATG GTT TCT GAA GC	2340	Lobanov *et al.* 2006
	seltrypR	AAG AGT AAG CTT TTA CCT GGG TCA GC		

All the *T. evansi* proteins (translated from the sequencing results of the *selD*, *PSTK*, *selTRYP* and *selB T. evansi* genes) were compared against proteins from related organisms. The highest identity of the *T. evansi* proteins was with *T. b. gambiense* and the lowest with *T. vivax* (Table 2, Figure 2). All proteins were found in the five species, except selTRYP that is not present in *T. equiperdum* (Table 2). Molecular phylogenetic analysis shows that *T. vivax* proteins are the most different related to other proteins and there are a close relationship among *T. evansi* and *T. b. gambiense* and among *T. brucei* and *T. equiperdum* proteins (Figure 2).

For the SelB (TevSTIB805.4.1890), SelD (TevSTIB805.10.9990) and PSTK (TevSTIB805.10.9870) proteins, domains with the characteristics described in Table 3 were found. In addition, the identities found of each protein with other trypanosomatids are also demonstrated.

Sequence and domain analysis of the predicted proteins for the 3 coding genes *selB*, *selD* and *PSTK* revealed regions with characteristic properties for each expected activity. Domain analysis of *selB* protein (selenocysteine-specific elongation factor, EFSec), revealed GTP binding (PF00009.27) and 50S ribosome-binding GTPase (PF01926.23). *selD* product, SPS, showed conserved regions among protozoans, a non-specific aminoimidazole ribonucleotide synthetase (AIRS) suggested as a putative ATP binding domain. (Miller *et al.* 1999). PSTK, the third selenoprotein-coding gene identified in *T. evansi* presented great similarity with sequences of several species described by Carlson *et al.* (2004), especially at the N-terminal portion. Domain analysis revealed that *T. evansi PSTK* gene product belongs to ATPases associated with diverse cellular activities are a protein family sharing a common conserved module of approximately 230 amino acid residues (Koonin *et al*. 2004). Only a partial sequence was obtained for *selTRYP* gene, which was found to have 99% similarity with the hypothetic protein Tb927.4.3410, described by Lobanov *et al.* (2006) as *T. brucei* selTRYP. Further analysis of *T. brucei selTRYP* coding sequence revealed a rhodanese-like domain, which is found in some dehydrogenases and stress-related proteins (Bordo & Bork 2002), as well as a CXXU motif, which is related to antioxidant activity and present in most selenoproteins (Dikiy *et al.* 2007).

selC nucleotide sequence analysis using ARAGORN tRNA gene prediction tool (http://130.235.46.10/ARAGORN/) revealed the presence of a D-loop, an anticodon loop, a variable loop, a TwC loop and an acceptor stem, suggesting that *selC* codes for a functional tRNA molecule (Figure 3). In addition, sequence alignment with the *selC* gene from *T. brucei, T. cruzi* and *Leishmania major* revealed a high degree of similarity among these species (97% and 90%, respectively).

Demonstration of presence and cellular localization of the selenophosphate synthetase protein in *T. evansi*

To determine the presence and cellular localization of the selenophosphate synthetase protein, encoded by the *selD* gene, western blot and immunocytochemistry staining was performed in fixed cells and cellular extracts of *T. evansi*. SPS was detected as an approximate-

Table 2. BLASTp and Orthovenn results of orthologous genes on selD, selB, PSTK and selTRYP proteins from *T. evansi* (STI805) with *T. brucei* (TREU927), *T. b. gambiense* (DAL927), *T. equiperdum* (TXID5694) *and T. vivax* (Y486)

Protein (gene)	*T. evansi* (STI805)	*T. brucei (TREU927)*			*T. b. gambiense* (DAL927)			*T. equiperdum* (TXID5694)			*T. vivax* (Y486)		
		Protein	E-value	Ident	Protein	E-value	Ident	Protein	E-value	Ident	Protein	E-value	Ident
selD	TevSTIB805.10.9990	Tb927.10.9410	0	99	Tbg972.10.11500	0	100	SCU65447.1	0	99	TvY486_1009330	0	93
selB	TevSTIB805.4.1890	Tb927.4.1820	0	99	Tbg972.4.1730	0	100	SCU68058.1	0	99	TvY486_0401530	0	65
PSTK	TevSTIB805.10.9870	Tb927.10.9290	0	99	Tbg972.10.11380	0	99	SCU72104.1	0	98	TvY486_1009180	8E-82	42
seltryp	TevSTIB805.4.3500	Tb927.4.3410	0	99	Tbg972.4.3420	0	100	–	–	–	TvY486_0403220	0	57

Table 3. BLASTp and domains analysis of selD, selB, and PSTK proteins from *T. evansi* (STI805) with *T. brucei* (TREU927), *T. b. gambiense* (DAL927), *T. equiperdum* (TXID5694) and *T. vivax* (Y486)

Protein	Accession Id	Domain	Start	End	*T. brucei* (TREU927)			*T. b. gambiense* (DAL927)			*T. equiperdum* (TXID5694)			*T. vivax* (Y486)		
					Cover	E-value	Ident	Cover	E-value	Ident	Cover	E-value	Ident	Cover	E-value	Ident
SelD	TevSTIB805.10.9990	AIRS	67	186	100%	1E-91	100.00%	100%	1E-91	100.00%	100%	1E-91	100.00%	100%	6E-77	82.50%
		AIRS_C	198	372	100%	2E-133	99.43%	100%	2E-133	99.43%	100%	2E-133	99.43%	99%	4E-95	69.54%
SelB	TevSTIB805.4.1890	GTP_EFTU	9	256	100%	0.0	99.60%	100%	0.0	100.00%	100%	0.0	99.60%	91%	4E-120	73.59%
		MMR_HSR1	13	140	100%	8E-95	100.00%	100%	8E-95	100.00%	100%	8E-95	100.00%	100%	3E-79	85.94%
PSTK	TevSTIB805.10.9870	AAA_18	6	172	100%	1E-129	99.40%	100%	7E-130	99.40%	100%	3E-129	98.80%	–	–	–

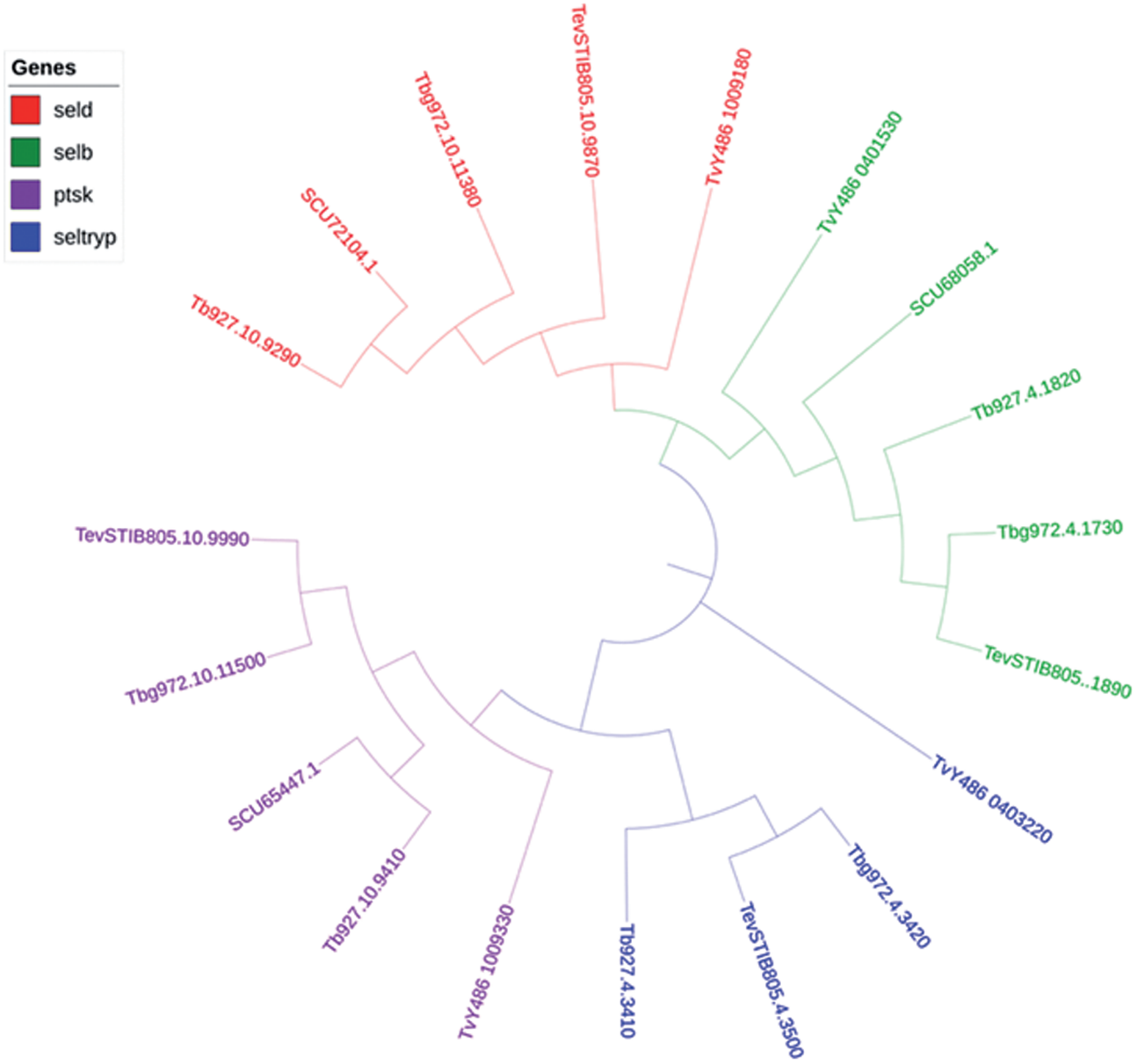

Fig. 2. Molecular phylogenetic analysis by Maximum Likehood method generated in Mega 7 and Plotted in iTOL (Interactive Tree of Life). *selD* (red), *selB* (green), *PSTK* (purple), *selTRYP* (blue). Protein (gene) codes correspond to those described in Table 2.

ly 43 kDa band in the western blot (Figure 4A) with an extranuclear location (Figure 4B). Furthermore, DAPI staining highlighted the lack of kinetoplast DNA in this *T. evansi*, a hallmark used to distinguish it from other closely related protozoans such as *T. brucei*.

DISCUSSION

The selenocysteine inserting system is a metabolic pathway present in the three domains of life, with components demonstrated in the proteomes of eukaryotes, archaea and prokaryotes (Lobanov *et al.* 2006). Trypanosomal orthologues of this pathway have been identified in several members of the kinetoplastid group

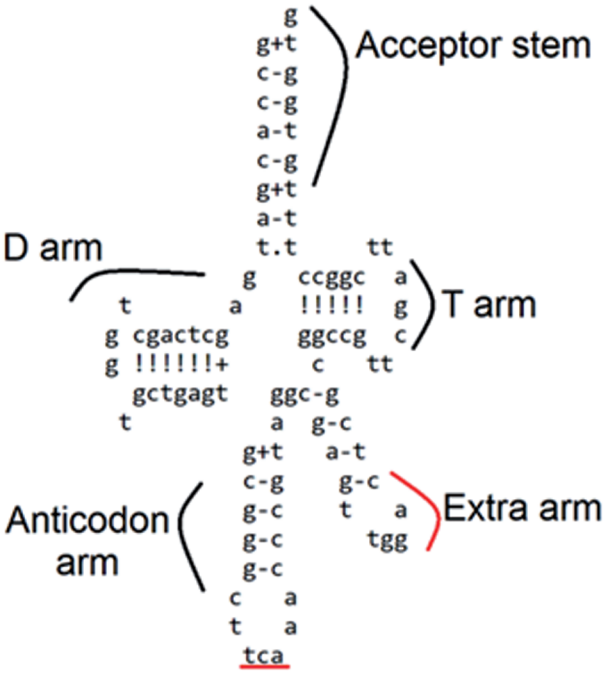

Fig. 3. Secondary structure of *T. evansi* $tRNA^{Sec}$ simulated with ARAGORN. Highlighted in red are the anticodon TCA and the elongated extra arm that are characteristic of the specific tRNA for selenocysteine.

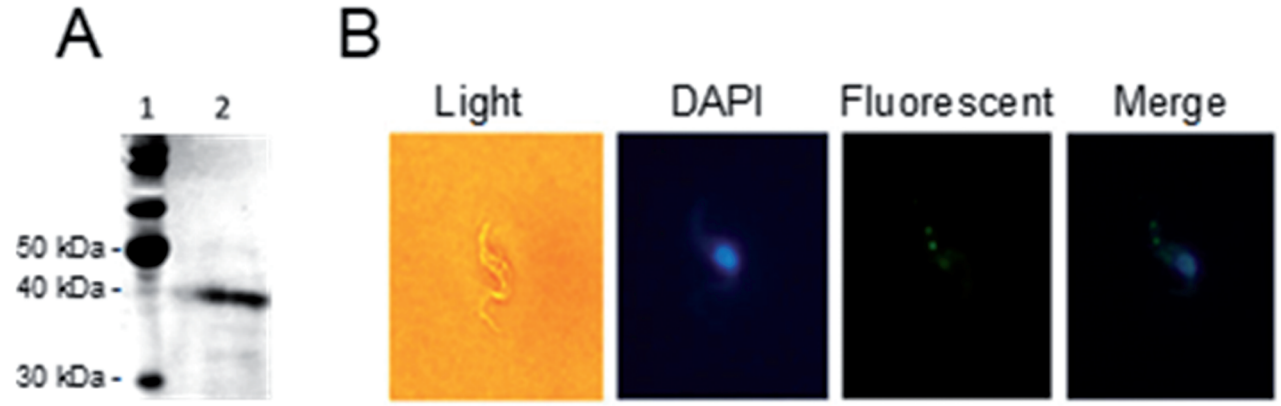

Fig. 4. Detection and cellular localization of the SPS protein, encoded by the *selD* gene, in *T. evansi*. A) Detection of 43kDa SPS by western blot *T. evansi* cellular extracts blotted with an anti-SPS antibody. Lane 1 – Protein Ladder, Lane 2 – *T. evansi* total protein extract. B) Immunocytochemistry analysis on fixed *T. evansi* for the determination of SPS extranuclear cellular localization.

(Cassago *et al.* 2006; Bouzaidi-Tiali *et al.* 2007; Geslain *et al.* 2006; Sculaccio *et al.* 2008), however none of its elements have yet been described in *T. evansi*. Here, we successfully identified key selenocysteine pathway genes in *T. evansi* with evidence of *selB, selC, PSTK, selTRYP* expression at the RNA level and of selenophosphate synthetase (product of *selD* gene) at the protein level. Coding sequence analyses of *T. evansi* genes revealed the presence of conserved domains among protozoans and higher organisms, consistent with the presence of a functional selenocysteine inserting system in this parasite. Taken together, our data demonstrate that *T. evansi* expresses key elements of the selenocysteine inserting system suggesting the parasite's ability to incorporate the micronutrient selenium into selenoproteins.

One of the major functions of selenoproteins is to confer resistance against reactive oxygen species (ROS) and most selenoproteins contain a CxxU motif that predicts this redox role (Papp *et al.* 2007). We

identified the expression of the trypanosomatid exclusive selenoprotein gene *selTRYP* (Lobanov *et al.* 2006) in *T. evansi*, which strongly suggests the utilization of selenoproteins by this parasite. Furthermore, conserved regions and domains are present in key elements of the selenocisteine pathway. *selB* gene sequence codes for regions typical for tRNA elongation factors such as GTP binding and β-ribosomal barrier regions (Bulteau & Chavatte 2015). *selD* product, SPS, presents ATP binding domains (Itoh *et al.* 2009b) and localizes to the cytoplasm. PSTK presents protein kinase domains and *selC* secondary structure prediction shows it shares most features present in eukaryotic tRNASec (Carlson *et al.* 2004; Itoh *et al.* 2009a; Aeby *et al.* 2010).

The absence of kinetoplast DNA (Ventura *et al.* 2002), the extensive philogenetics analysis of *T. evansi* and *T. brucei* (Carnes *et al*. 2015) and the high similarity of the five selenoproteins metabolis genes sequences identified in this study to *T. brucei* sequences corroborates the most accepted hypothesis about the origins of *T. evansi* from a gradual loss of kDNA in *T. brucei* (Jensen *et al.* 2008).

The identification of selenoprotein genes in kinetoplastida is relevant from an evolutionary perspective as they have been identified in the three domains of life but are absent in plants and fungi (Mariotti *et al*. 2019). Thus, the identification and characterization of genes related to the selenocysteine pathway in *T. evansi* can shed further light into the evolution of selenium utilization (Lobanov *et al.* 2006).

Studies have reported that the selenocysteine pathway is not essential for parasite survival under non-stress conditions as knockout cells for O-phosphoseryl-tRNA(Sec) selenium transferase (SepSecS), SPS or PSTK do not show impaired growth or infectivity (Bonilla *et al.* 2016). Aeby *et al.* (2009b) showed that *T. brucei* is highly sensitive to auranofin, a complex that targets selenoproteins, but so are SepSecS knockout cells, indicating that the trypanocidal effect may not be related to selenoproteins. Interestingly, Costa *et al.* (2011) reported impaired growth in SPS knockdown cells exposed to continuous oxidative stress. However, the experiments were carried out in *T. brucei*, not *T. evansi*. Thus, it remains to be determined whether *T. evansi* selenoproteins or specific components of this pathway can be potential drug targets. This is a possibility, since despite very close genetic identity among members of the kinetoplastid group (Carnes *et al.* 2015; Richardson *et al.* 2017), each species presents a myriad of morphophysiological differences, including host specificity, life cycle and disease symptoms upon infection indicating that small genetic changes can bring about significant phenotypical differences.

These facts encourage further investigations into whether the selenocysteine insertion pathway can be a potential novel therapeutic target for *T. evansi*.

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