



Leishmania mexicana recombinant filamentous acid phosphatase as carrier for *Toxoplasma gondii* surface antigen 1 expression in *Leishmania tarentolae*

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Received: 5 March 2021 / Accepted: 7 June 2021 / Published online: 17 June 2021
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Abstract *Leishmania tarentolae* has been used to produce recombinant intracellular and secreted proteins for their easy handling and posttranslational modifications. Filamentous acid phosphatase is a multimeric protein complex composed of many subunits assembled in a linear highly glycosylated filament, which is secreted in vast amounts into the culture supernatant via the flagellar pocket of *Leishmania mexicana* promastigotes. This suggested that the protein could be used as a carrier for the Surface Antigen1 protein of a *Toxoplasma gondii* (SAG1) for easy purification to generate a protein with multiple SAG1 subunits suitable for immunisation. SAG1 has an immunodominant structure that is involved in binding to host cells. Previous studies used this surface protein for vaccination for its immunological importance for triggering a type 1 immune response in the host. This study aims to determine the production of recombinant filamentous protein carried subunits of the surface protein of *Toxoplasma gondii* for vaccination purposes. *Leishmania* codon-optimised SAG1 was cloned as a fusion construct into pLEXSY-ble2.1 plasmid and introduced into *Leishmania tarentolae* to generate recombinant cell lines expressing a filamentous fusion protein called SAP2SAG1. PCR confirmed the correct integration into the small ribosomal subunit RNA gene locus of *Leishmania tarentolae*. Immunofluorescences and Immunoblot analyses were used to detect the fusion protein in the sediment of culture supernatants of recombinant *L. tarentolae* promastigotes after purification by ultracentrifugation. The yield of

purified protein was low that suggested further investigations of other methods for scaling large production of secreted protein.

Keywords SAG1 · *Leishmania tarentolae* · Filamentous recombinant protein · Protein expression

Abbreviations

Amp	Ampicillin
BSA	Bovine serum albumin
bp	Base pairs
ddH ₂ O	Double distilled water
DTT	1,4-Dithiothreitol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
FCS	Foetal calf serum
GPI	Glycosyl phosphatidy linoitol
gDNA	Genomic DNA
HRP	Horse radish peroxidase
kDa	Kilo-Dalton
LB	Luria–Bertani (broth)
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
RPM	Revolutions per minute
SDS	PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSU	Small ribosomal Subunit

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Background

SAP2 Protein of *Leishmania mexicana*

Leishmania mexicana is a protozoan parasite of the genus *Leishmania*, which are the causative agents of a range of diseases called leishmaniasis mostly found in tropical and subtropical regions (Dey et al. 2014). *L. mexicana* insect stage (promastigotes) secrete a filamentous protein of long acid phosphatases (SAP2). SAP2 protein is composed of 383 serine/threonine amino acids rich regions that are enzymatically active and assemble filaments in the flagellar pocket of the promastigotes. Besides, SAP2 protein has a long linear epitope in the COOH-terminal tail that can detect by using monoclonal antibody (mAb) LT8.2.

Leishmania tarentolae

Leishmania tarentolae is a non-pathogenic trypanosomatid protozoan parasite living as promastigotes in the lumen of the intestine of lizards such as the gecko (*Tarentola annularis*) (Wilson and Southern 1979). *L. tarentolae* is distinguished by lacking 250 genes that are expressed in the intracellular amastigote stage. Hence, *L. tarentolae* has been used for heterologous gene expression and recombinant protein production (Raymond et al. 2012). *L. tarentolae* has been used as a protein expression system for both cytoplasmic and secreted proteins. Therefore, this work aims to determine the effectiveness of a novel eukaryotic filamentous protein expression system based on the *L. mexicana* SAP2 in *L. tarentolae* for vaccine development.

Toxoplasma gondii surface antigen 1 (SAG1) protein

Surface antigen 1 (SAG1) is considered the major surface antigen in *T. gondii* and comprises more than 5% of the total amount of protein of the tachyzoite stage of *T. gondii* (Lekutis et al. 2001). SAG1 is a tachyzoite that facilitates the adhesion and invasion of the host cells (Tang et al. 2016). SAG1 exists as a monomer of 250 amino acids with an approximate molecular weight of 30 kDa. It can form homodimer structures, which are attached to the parasite surface by glycosylphosphatidylinositol (GPI) anchors (He et al. 2002). Previous studies have focused on SAG1 for vaccination because it has a critical importance for the immune response in the initial stage of infection involving both the humoral and cellular immune response (Abdizadeh et al. 2015; Lekutis et al. 2001). Vaccination of mice with SAG1 has provided encouraging results with significant protection as measured by a reduction of the number of cysts in vaccinated animals compared with control animals (Lekutis et al. 2001). SAG1 was purified

from tachyzoites and produced as a recombinant protein in *E. coli* or yeast (Wang and Yin 2014).

The aim of this was preparation and expression of recombinant protein of filamentous secreted acid phosphatase 2 fused with SAG1 (SAP2SAG1) in *L. tarentolae* for immunogenic importance in vaccination studies.

Methods

Generation and purification of expression plasmid

A single positive colony was inoculated to 100 ml of LB broth with Carbenicillin (100 µg/ml) and incubated 16–18 h at 37 °C, 220 rpm. The plasmid DNA was purified from the overnight culture by using the Nucleobond® Xtra Midi High copy plasmid protocol (Macherey–Nagel, Germany) until the elution step following the manufacturer's instructions. DNA pellets were resuspended in a total volume of 120 µl ddH₂O and stored at -20 °C for transfection into *L. tarentolae* cells.

Leishmania cell culture

L. mexicana and *L. tarentolae* wild type promastigotes were grown at 27 °C without CO₂ in complete SDM79 media and re-inoculated every 3–4 days. *Leishmania* promastigotes were counted by using a haemocytometer under a light microscope (40 × objective lens), and the averaged of cells were calculated per millilitre (number of cells/ml = number of counted cells × dilution factor (100 × 10⁴).

Transfection of DNA into *Leishmania tarentolae* by using electroporation

Transfection of linearised DNA into *L. tarentolae* cells was performed using a Nucleofector II (Amaxa Biosystems, Lonza, Germany). A maximum volume of 10 µl of purified linearised plasmid DNA was mixed with the electroporation buffer before the cells were resuspended in it. *L. tarentolae* promastigotes were grown until a density of approximately 3 × 10⁷ cells/ml. The cells were harvested by centrifugation for 2 min, at 5600 × g and 4 °C. The supernatant was gently removed from sedimented *L. tarentolae* cells and immediately replaced with 100 µl electroporation buffer by gentle pipetting. The re-suspended cells were transferred to a pre-cooled electroporation cuvette with no air bubbles present. Cells were electroporated using Nucleofector II program V-033 or X-001 and left on ice for 10 min. Electroporated cells were transferred from the cuvette to 10 ml SDM79 medium supplemented with 8% v/v heat-inactivated fetal calf serum, 0.3% v/v hemin

and incubated at 27 °C for 24 h. On the second day, the electroporated cells were diluted 1:40 with supplemented SDM79 medium and the appropriate selective antibiotic was added to the diluted cell cultures. 1:40 dilutions were pipetted into 96 well plates using a multichannel pipette (200 µl per well) and incubated at 27 °C for 10–14 days until turbid wells indicated growth of cloned parasites. Turbid wells were checked using an inverted microscope to confirm the presence of *Leishmania* cells in the well. 200 µl of positive clones identified on the 96 well plates were transferred to 12 well plates containing 2 ml supplemented SDM79 medium with the appropriate selective antibiotic and incubated at 27 °C for 72 h until turbidity was visibly observed in the well. Subsequently, 40 µl from positive clones identified in 12 well plates were used to inoculate 10 ml of supplemented SDM79 medium with the selective antibiotic and cultured at 27 °C for approximately 96 h until the culture reached the logarithmic growth phase.

Isolation of genomic DNA from cloned *L. tarentolae* cells

The “isolate II genomic DNA kit from Bioline, UK” was used to isolate genomic DNA from *L. tarentolae* cells following the manufacturer’s instructions. 3 ml of a stationary phase culture of *Leishmania* promastigotes (approximately 10⁷ parasites) was used. Genomic DNA pellets were resuspended in 100 µl TE buffer, the concentration was determined using a NanoDrop2000® (Thermo Scientific, Wilmington USA), and stored at 4 °C until required.

Polymerase chain reaction (PCR)

PCRs were performed using the Expand High Fidelity PCR System from Roche. 2 Reactions were carried out in a 25 µl PCR tube using a PE thermocycler. The sequences of LeishSSU forward (5′-GATCTGGTTGATTCTGCCAGTAG-3′) and pLexyup1 reverse primers (5′-CCTACGTCAATCGCAGACCT-3′) were used in the first reaction to confirm the correct integration of SAP2SAG1 construct into the rRNA gene locus. While in the second reaction the forward primer was SAP2mod2C (5′-AGC-GACGTCCCTTCCTTCAA-3′) and the reverse was SAG1-2 rev (5′-CCACTACTGCAGCGGCACGA-3′) to approve the fusion of SAP2 and SAG1. The reaction mixture was composed of: template 10–200 ng, forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, 10 × PCR buffer (15 mM MgCl₂) 5 µl, dNTPs (10 mM) 1 µl, HiFidelity DNA Polymerase 0.8 µl, ddH₂O was used to attain a final volume to 25 µl. PCR temperature Sequence: initial denaturation step 94 °C 3 min, then 30 cycles consisting of 94 °C for 45 s, 52 °C for 30 s, 72 °C

for 60 s and a final extension step at 72 °C for 7 min and then 4 °C.

Secreted acid phosphatase assay

An acid phosphatase assay was used for detection of the protein expression in *Leishmania* cell-free culture supernatants, which were prepared from 25 µl culture at the late logarithmic stage by sedimentation at 5600 × g, 4 °C, 2 min. The total volume per well of a 96-well plate was 100 µl composed of a 20 µl supernatant/media sample, 70 µl (50 mM *p*-nitrophenyl phosphate in 100 mM sodium acetate pH 5.0). The plate was incubated at 37 °C for 30 min. The reaction was stopped by addition 10 µl of 2 M sodium hydroxide solution leading to a visible colour change (yellow, positive reaction; pink, no activity). Absorbance readings for the assay were measured at 405 nm using a spectrophotometer (Spectramax M5, Molecular Devices, USA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE consisting of a 10% (v/v) resolving gel and 4% (v/v) stacking gel. Protein samples were diluted in sample buffer with DDT (40 mM), boiled at 95 °C for 10 min. Samples were electrophoresed at 30 mA/gel until the front dye had left the gel (~ 1 h). A protein marker was also loaded into one of the lanes of the gel. Gel running buffer was used in electrophoresis. Gels were placed in Coomassie stain for 20 min. Then the destaining solution was applied on the gel 4–5 times for 20 min until the protein bands were visible.

Immunoblot analysis

The production of SAP2SAG1 protein in the culture supernatant was determined using immunoblot analysis. Proteins were transferred from a 10% SDS-gel to an Immobilon-P Polyvinylidene Difluoride (PVDF) membrane by the electroblotting system (Biometra) at a current of 4 mA/cm² of gel for 90 min. After transfer, the membrane was incubated for one hour at 37 °C in a blocking solution (5% (w/v) milk powder in PBST, 20 mM Tris-HCl pH 7.5). This was then replaced with a blocking solution containing the primary monoclonal antibody (mAb) LT8.2 (1:2000) in the blocking solution and incubated for one hour with gentle agitation at 37 °C. The membrane was then washed four times for 5 min with 1 × PBST at room temperature before goat anti-mouse IgG HRP-linked 1:2000–1:5000 (DAKO, Hamburg, Germany) was added in blocking solution and incubated for one hour at 37 °C. Three times washes for 5 min were

carried out with $1 \times$ PBST and two with $1 \times$ PBS. The blot was incubated and exposed to X-ray films with solutions from the SuperSignal West Pico Chemiluminescent Substrate Kit (Fischer Scientific, Loughborough, UK).

Immunofluorescence microscopy

Leishmania cell culture samples were prepared and fixed on a 10 well-masked slide. Each sample well on the slide was prepared by washing with $50 \mu\text{l}$ of 70% ethanol followed by the application of $20 \mu\text{l}$ of 0.1 mg/ml polylysine in $1 \times$ PBS and incubation at room temperature for 15 min. After the incubation was completed each well on the slide was washed with $50 \mu\text{l}$ per well $1 \times$ PBS solution twice. $20 \mu\text{l}$ of total cell culture or cell culture supernatant in a cell density range of $1\text{--}2 \times 10^7$ cells/ml was fixed to each well with $20 \mu\text{l}$ of 4% *p*-formaldehyde solution by incubation for 15 min at room temperature. After the incubation was completed each well was washed once for 15 min at room temperature with $50 \mu\text{l}$ per well $1 \times$ PBS solution, 50 mM NH_4Cl , 0.1% Saponin. $50 \mu\text{l}$ per well of blocking buffer ($1 \times$ PBS solution, 2% Bovine Serum Albumin (BSA), $1 \times$ PBS, 0.1% Saponin) was applied to the wells and incubated for further 15 min at room temperature. $20 \mu\text{l}$ of the primary antibody mAb LT8.2 in a 1:40 dilution in blocking buffer was applied for 1 h at room temperature followed by two wash steps with $50 \mu\text{l}$ per well $1 \times$ PBS solution. Then the wells were incubated with $20 \mu\text{l}$ per well of a 1:500 dilution of the secondary antibody (goat anti-mouse IgG -conjugated fluorescent tag) in blocking buffer at room temperature for 1 h. The slide was washed three times with $50 \mu\text{l}$ $1 \times$ PBS solution. $10 \mu\text{l}$ Mowiol/DABCO solution was applied to each well before the slide was sealed with a coverslip and left to dry for 15 min. Fluorescence was detected using an Epifluorescence microscope at $60 \times$ magnification.

Results

Generation of pLPhSAP2SAG1 and transfection of *L. tarentolae* promastigotes

A 7984 bp fragment was isolated from pLEXSY-ble2.1 cleaved with *Nco*I + *Nhe*I. pSSUPacSAP2SAG1 was cleaved by *Nco*I + *Xba*I and the 3386 bp and 3164 bp fragments isolated, respectively (Fig. 1). pLPhSAP2SAG1 was produced from ligation of 3386 bp with the 7984 bp fragments. pLPhSAP2SAG1 was transformed into *E. coli* DH5 α , amplified, isolated and cleaved with *Bam*HI, *Not*I and *Hind*III to confirm the identities of the plasmids (Fig. 2A). To generate Linear DNA Fragments for Electroporation, pLPhSAP2SAG1 was cleaved with *Swa*I to

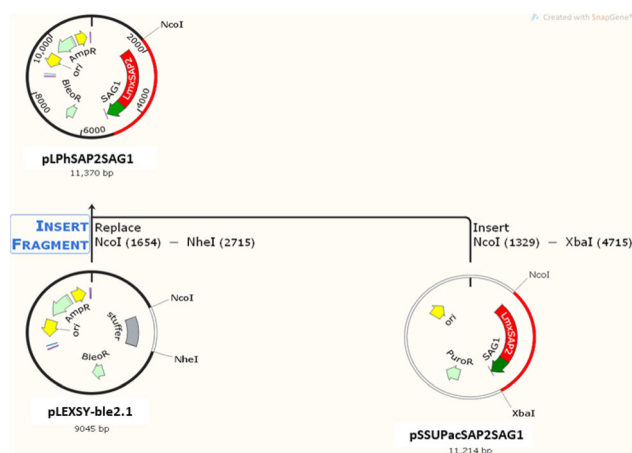


Fig. 1 Generation of pLPhSAP2SAG1 from pLEXSY-ble2.1

isolate the 7207 bp under a sterile condition. Figure 2B show the confirmation of the fragment size. Isolated linear fragments were transfected into *L. tarentolae* promastigotes. Turbid wells were obtained from 1:40 dilution of the electroporated cells were 5 for PhleoSAP2SAG1 on 96 well plates. Four positive cell lines were chosen to scale up in 10 ml cultures for further analysis.

Confirmation of integration of constructs into the 18S rRNA gene locus by polymerase chain reaction (PCR)

To test the correct integration of the construct into the rRNA gene locus specific oligonucleotide primers were chosen for PCR to amplify fragments of distinctive size. Table 1 showed the amplification of an 862 bp DNA fragment proved the correct integration of SAP2SAG1 construct into the rRNA gene locus (Fig. 3A). In a second PCR, a 696 bp amplicon confirmed the fusion of the secreted acid phosphatase gene (SAP2) to the SAG1 coding region (Fig. 3B). *L. tarentolae* wild type, which served as a negative control in the PCR reactions.

Detection of secreted acid phosphatase activity in the culture supernatants of recombinant *L. tarentolae* Promastigotes

Secreted acid phosphatase (SAP2) activity in culture supernatants was used to determine the presence of the recombinant fusion proteins. A quantitative spectrophotometric assay was performed to measure the SAP2 activity from different culture supernatants of recombinant *L. tarentolae* promastigotes grown in SDM79 medium supplemented with 10% iFCS and 7.5 $\mu\text{g}/\text{ml}$ hemin. For comparison purposes, the supernatants of two controls, *L. mexicana* wild type (positive control) and *L. tarentolae*

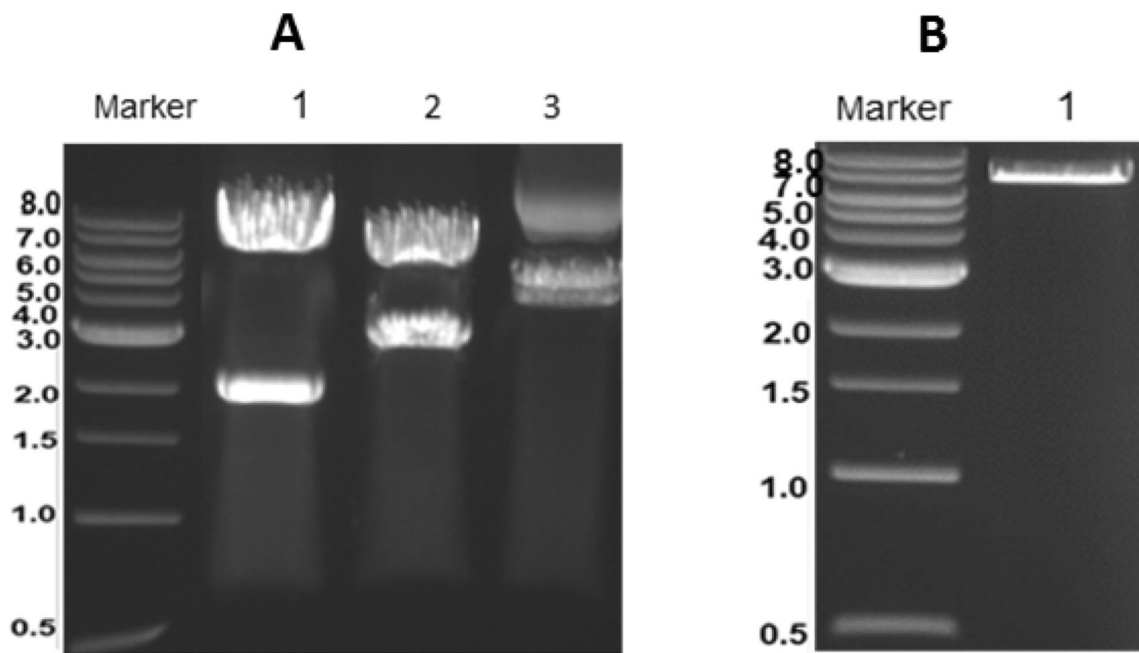


Fig. 2 (A) Restriction analysis of pLPhSAP2SAG1. Lanes 1, BamHI (2223 bp and 9147 bp); lanes 2, NotI (3396 bp and 7974 bp); lanes 3, HindIII, (5094 bp and 6276 bp). DNA size marker in kb.

(B) Generation of linear DNA for electroporation. pLPhSAP2SAG1 cleaved with SwaI resulted in 7207 bp DNA

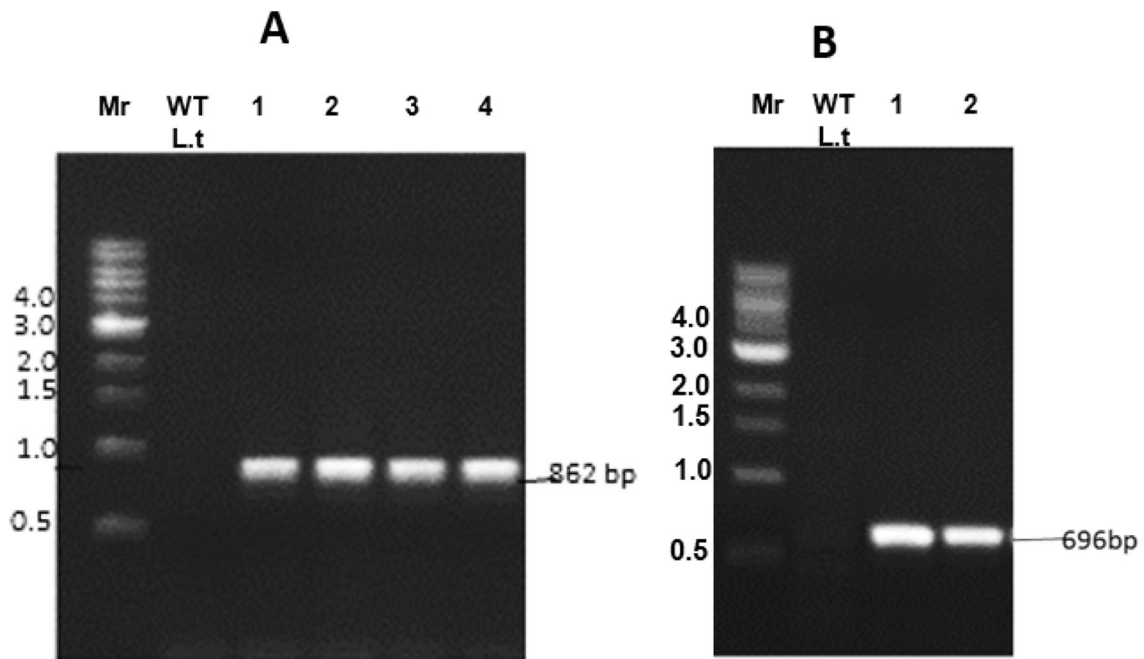


Fig. 3 PCR verification for correct integration of PhleoSAP2SAG1 into the rRNA gene locus of *L. tarentolae*. (A) Integration of SAP2/SAG1 construct into the *L. tarentolae* rRNA gene locus. Lanes 1–4, 862 bp amplicon reflects the correct integration. (B) Fusion of SAG1

to SAP2 for PhleoSAP2SAG1. Lanes 1 and 2, the 696 bp fragments, confirmed the fusion of SAG1 to SAP2. *L. t* WT is *L. tarentolae* wild type (negative control). DNA size marker in kb in A and B

wild type (negative control), were also measured. Four cell lines (A11, E11, F7, F11) of *L. tarentolae* promastigotes transfected with PhleoSAP2SAG1 presented the enzymatic

activity of acid phosphatase activity (Fig. 4). To determine the levels of recombinant protein in supplemented culture supernatants, the cells were grown over three days and the

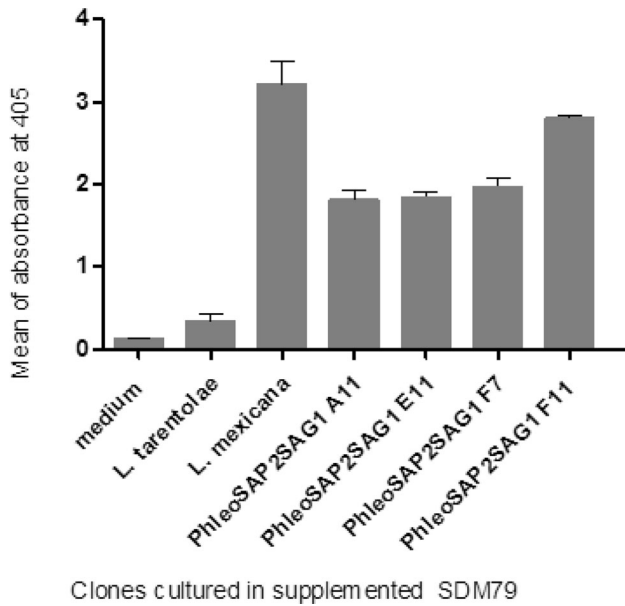


Fig. 4 Secreted acid phosphatase activity in supplemented culture supernatants of *Leishmania* promastigotes. *L. tarentolae* promastigotes transfected with four clones of PhleoSAP2SAG1. *L. mexicana* and *L. tarentolae* were used as positive and negative controls, respectively. Measurements were done in triplicate

cell density and enzymatic activity were measured. On day one a low cell density and enzymatic activity were found. The second and third day presented similar enzymatic activity, but the cell density on day three suggested that the cells were already in the stationary growth phase (Fig. 5A and B). Therefore, for the following experiments, the second day of culture was selected to enrich the fusion protein from the culture supernatants of recombinant cells. To make the purification of the fusion protein from culture supernatants as simple as possible, it was attempted to grow the promastigotes in non-supplemented SDM79 medium. Four *L. tarentolae* cell lines transfected with PhleoSAP2SAG1, *L. tarentolae* and *L. mexicana* wild type were grown in supplemented and a non-supplemented SDM79 medium to determine the enzyme activity in the media. When compared, no activity was observed from promastigotes cells growing in non-supplemented media for two days; in contrast, growing cells in supplemented media showed high enzymatic activity (Fig. 6).

Detection of recombinant protein in culture supernatants by immunofluorescence microscopy

Immunofluorescence analysis of cells and cell culture supernatants were used to detect the SAP2SAG1 fusion protein secreted by *L. tarentolae* promastigotes into their culture supernatants by using the mAb LT8.2. *L. mexicana* wild type promastigotes secrete large amounts of secreted acid phosphatase, served as a positive control. No material

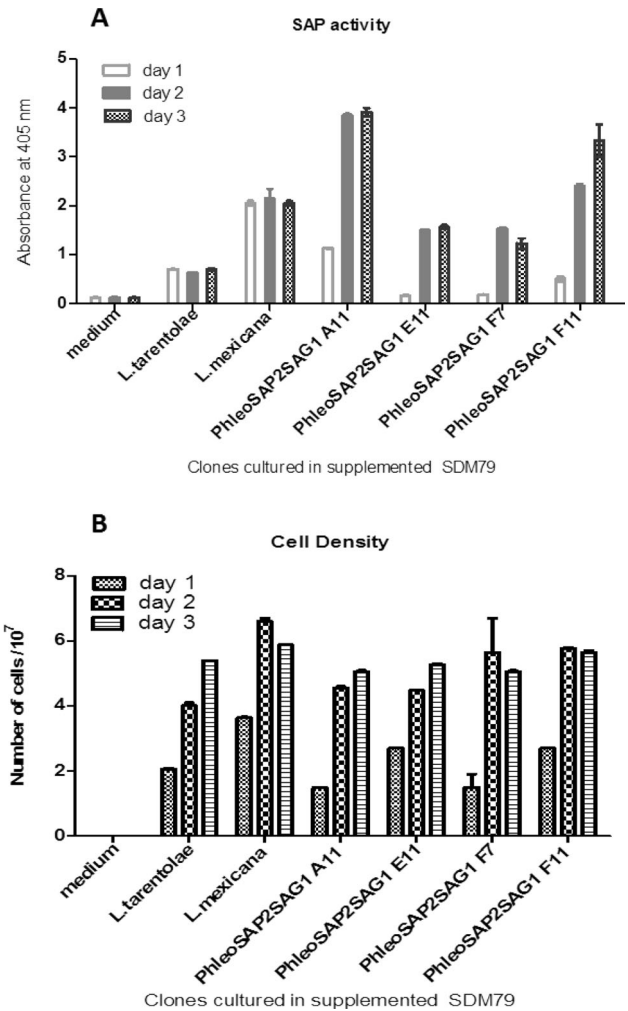


Fig. 5 Time course of secreted acid phosphatase activity in culture supernatants. (A) Enzyme activity of recombinant *L. tarentolae* promastigotes from supplemented culture supernatants followed over three days. Measurements were done in triplicate. (B) Cell density over three days of incubation

detectable with mAb LT8.2 from *L. tarentolae* promastigotes (negative control) in their culture supernatants. Supernatants from *L. tarentolae* promastigotes transfected with PhleoSAP2SAG1 showed green fluorescence less than those from *L. mexicana* in their culture supernatant (Fig. 7).

Detection of recombinant proteins in culture supernatants by immunoblot analysis

Immunoblot analysis was used to detect the recombinant SAP2SAG1 fusion protein in the sediment of culture supernatants of recombinant *L. tarentolae* promastigotes after purified by ultracentrifugation using mAb LT8.2. Strong bands were detected by mAb LT8.2 around 175 kDa in culture supernatants of *L. tarentolae*

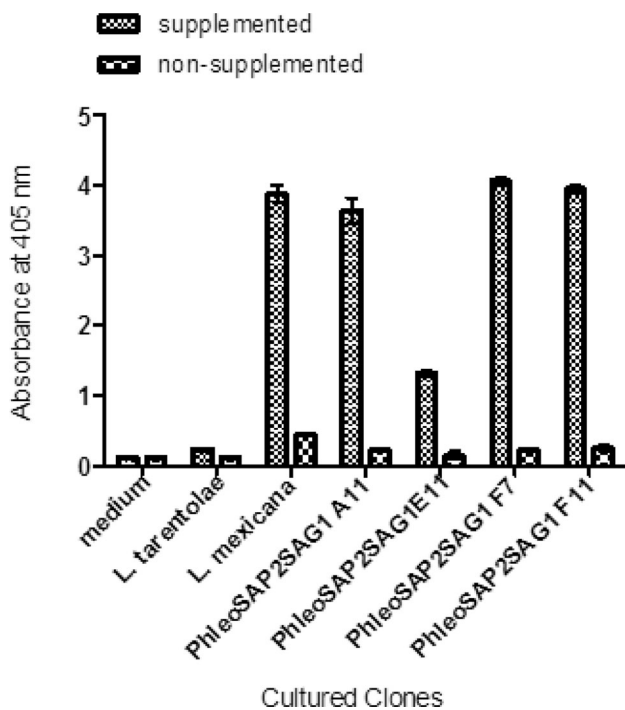


Fig. 6 Comparison of secreted acid phosphatase levels from culture supernatants of *Leishmania* promastigotes grown in supplemented and non-supplemented cultures. WT *L. tarentolae* and *L. mexicana* were used as negative and positive controls, respectively. Measurements were done in triplicate

promastigote transfected with the PhleoSAP2SAG1 construct. *L. mexicana* samples showed a band of 89 kDa corresponding to SAP1 (Martin Wiese et al. 1999), while no band was detectable when using *L. tarentolae* as a negative control (Fig. 8 and 9).

Discussion

DNA constructs generation

Because of its unique features, *L. tarentolae* has previously been used to express secreted proteins like easy growth of parasites, protein purification from culture, and the presence of post-translational modifications (Basile and Peticca 2009; Kovtun et al. 2011). We have constructed a novel filamentous protein expression system using a DNA cassette for integration into the 18S ribosomal RNA gene locus using the pLEXSY-ble2.1 expression vector. Previously, different proteins have been successfully expressed in high levels using pSSU constructs in *Leishmania*, e.g. β -galactosidase and green fluorescent protein (Mißlitz et al. 2000). Human laminin-332 and c-reactive proteins were produced into the culture supernatant of *L. tarentolae* (Dortay et al. 2011; Phan et al. 2009). SAP2 from *L. mexicana* including its N-terminal phosphatase domain, the

serine/threonine-rich repeats and the mAb LT8.2 epitope was used as a carrier for *T. gondii* proteins, SAG1, which were genetically fused to its C-terminus.

Efficiency of electroporation

Trypanosomatids can be efficiently transfected by electroporation of in vitro cultivated promastigotes (Beverley and Clayton 1993). Moreover, the selection of recombinant parasites can easily be performed using antibiotic resistance genes, such as phleomycin, which also used in this investigation to generate permanent and stable transfectants, linear DNA for chromosomal integration (Beverley and Clayton 1993). However, when using higher DNA amounts homologous integration of multiple copies of the transfected DNA could be observed into the *L. tarentolae* genome through homologous recombination (Kianmehr et al. 2016). To confirm integration into the small subunit rRNA gene locus of the *L. tarentolae* genome by homologous recombination diagnostic PCRs were performed. The PCR results proved both the fusion of the SAG1 gene to the SAP2 gene and the integration into the proposed site. It is important to note that transcription in this chromosomal location is under the control of RNA polymerase I, which allows high-level transcription (Teixeira 1998).

Gene expression in *L. tarentolae*

SAP of *L. mexicana* promastigotes was previously expressed in *L. donovani* (Ilg et al. 1991) and *L. major* (Martin Wiese et al. 1999). It is easier to isolate and purify recombinant proteins if they are exported outside the cell. In this study, filaments composed of the SAP2-SAG1 fusion protein was generated and expressed in *L. tarentolae*. To improve protein yield transfection of *L. tarentolae* with multiple copies of the gene of interest integration into different gene loci could have been attempted (Breitling et al. 2002). The recombinant constructs for gene expression were integrated into the 18S small subunit ribosomal RNA gene locus to achieve strong transcription. Interestingly gene regulation, in trypanosomatids, occurs on a post-transcriptional level through intergenic untranslated regions (UTRs) (Clayton 1999). Therefore, the choice of suitable UTRs is crucial for the construction of an efficient expression cassette suitable for large-scale recombinant protein production (Breitling et al. 2002). The commercial plasmid, pLEXSY-ble2.1, contains three different untranslated regions (UTR1, UTR2 and UTR3), which were supposed to increase the expression level of recombinant proteins in *L. tarentolae*. The LEXSY system has been successful previously used to express small secreted proteins, like IFN- γ (Davoudi et al. 2011) and IL-29 (Taromchi et al. 2013).

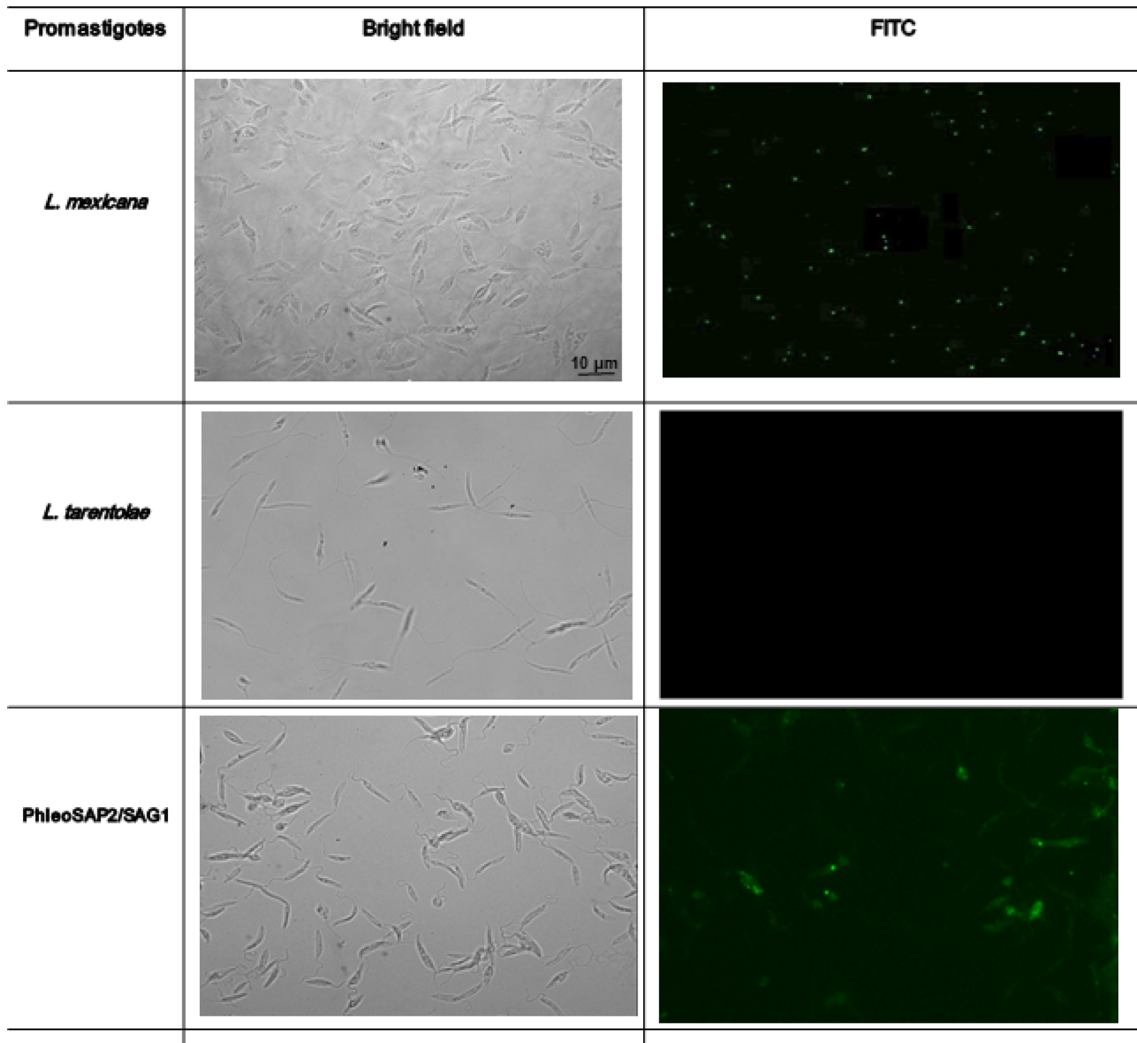


Fig. 7 Immunofluorescence analysis of *Leishmania* promastigotes in medium using mAb LT8.2 to detect secreted acid phosphatase. Bright field microscopy using 40 × magnification on the left and

fluorescence microscopy using 488 nm excitation with FITC filter at the same magnification on the right. Size bar, 10 μm

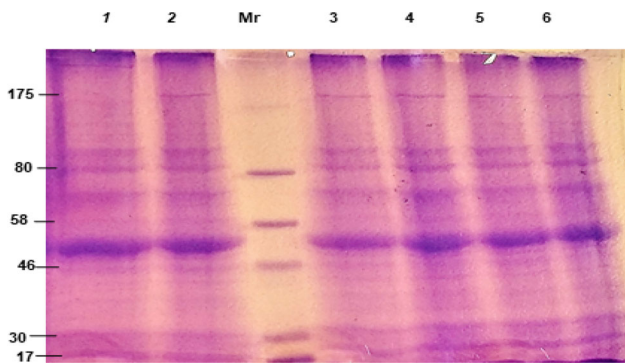


Fig. 8 Coomassie-stained 10% SDS-PAGE of *L. tarentolae* carrying PhleoSAP2SAG1. Lane 1, *L. tarentolae* (negative control); lane 2, *L. mexicana* (positive control); lane 3–6, PhleoSAP2SAG1 (A11, E11, F7, F11). Protein size marker in kDa

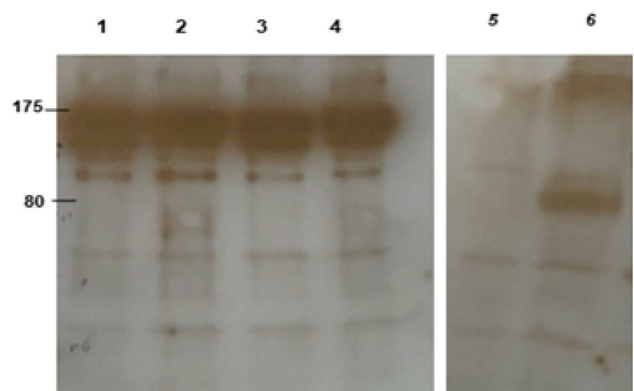


Fig. 9 Immunoblot analysis of *L. tarentolae* carrying PhleoSAP2SAG1. mAb LT8.2 immunoblot of promastigote cells. Lane 1–4, PhleoSAP2SAG1 (A11, E11, F7, F11); lane 5, *L. tarentolae* (negative control); lane 6, *L. mexicana* (positive control). Protein size marker in kDa

Table 1 Oligonucleotide sequences used in PCR and expected fragment sizes for each PCR reaction

Primers	Sequence	Size (bp)
LeishSSU.Fw1	5'-GATCTGGTTGATTCTGCCAGTAG-3'	862
pLexyup1.Rv1	5'-CCTACGTC AATCGCAGACCT-3'	
SAP2MOD2C.Fw2	5'-AGCGACGTCCTTCCCTTCAA-3'	696
SAG1.Rv2	5'-GTGAAGTGGTTCTCCGTCGG-3'	

Secreted acid phosphatase activity

An advantage of the SAP2 fusion protein system is the ease of detecting protein expression by a phosphatase reporter assay directly from cell culture supernatants. The expression level of the SAP2 fusion proteins in *L. tarentolae* was tested by measuring the secreted acid phosphatase activity in the culture supernatant. As long as SAP2 is the only enzyme in the supernatant, we can calibrate the percent protein expressed in the supernatant based on enzyme activity at 405 nm wavelength (Zhang et al. 1992). A 1.041 mol/ml proximate concentration of the SAP2SAG1 fusion proteins from the culture supernatant of *L. tarentolae* growing in 10% iFCS. The secretion signal sequence of *L. mexicana* secreted acid phosphatase was used to produce different proteins (Basile and Peticca 2009). To avoid interference of proteins, present in iFCS (mainly bovine serum albumin) with the purification of the recombinant protein, recombinant parasites were grown in non-supplemented media. No phosphatase activity was detectable in culture supernatants from these cells confirming the importance of iFCS in the culture medium.

Immunofluorescence analyses

The detection of the recombinant proteins by immunofluorescence confirmed the successful secretion of the recombinant proteins into culture supernatants. When comparing the amount of green fluorescence obtained from *L. mexicana* wild type, which served as a positive control, SAP2SAG1 expressed using pLEXSY-ble2.1 revealed filamentous structures along with stronger green fluorescence signals. SAG1 is a prototypic member of a superfamily of surface glycoproteins, which is mostly located at the surface of *T. gondii* (He et al. 2002). This makes it more likely to be secreted normally through the secretory pathway of other protozoan parasites like *L. tarentolae*.

Immunoblot analyses

Immunoblot analyses results of SAP2SAG1 using the mAb LT8.2 showed strong bands around 175 kDa from

supernatants purified by ultracentrifuge. The signal is most likely derived from fusion protein retained in the flagellar pocket or from protein in transit in the secretory pathway. The 175 kDa bands correspond to the full-length recombinant fusion protein. Generally, proteolytic maturation occurs in secretory or transport vesicles carrying proteins from the trans-Golgi network to the cell surface (Lodish et al. 2001). The modifications of SAP2 protein form a filamentous complex with long serine/threonine-rich regions, and N-linked glycans on the N-terminal phosphatase domain which are highly glycosylated might protect the protein against proteolytic degradation (Wiese et al. 1995).

Conclusions

The data gathered by the use of enzyme activity measurement, immunoblot and immunofluorescence analyses in this study provide convincing evidence that the proteins of interest have been expressed successfully. Protein amounts obtained for SAP2SAG1 using pLEXSY-ble2.1 were not determined because of too high serum contaminations in the samples. However, it can be said from the results from the Coomassie-stained gels that the amount of protein is more in the µg than in the mg range. To improve the protein expression levels of the filamentous fusion proteins, further investigation needs to be done to increase the expected efficiency of *L. tarentolae* production of *T. gondii* protein SAG1.

Acknowledgements I would like to thank Prof. Craig W Roberts and Dr. M. Wiese (Strathclyde Glasgow University/UK) for their assistance with protein expression and purification.

Authors' contributions MW analyzed and interpreted the immunoblot and immunofluorescences data. CWR examination was a contributor in writing the manuscript.

Funding This study was funded by the Iraqi ministry of higher education and scientific research/Baghdad University.

Declarations

Conflict of interests No competing interests in this article.

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