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Research Article

RESEARCH ARTICLE

Identification and characterisation of elongation factor Tu, a novel protein involved in *Paracoccidioides brasiliensis*–host interaction

Caroline Maria Marcos[1], Haroldo Cesar de Oliveira[1], Julhiany de Fátima da Silva[1], Patricia Akemi Assato[1], Daniella Sayuri Yamazaki[1], Rosângela Aparecida Moraes da Silva[1], Cláudia Tavares Santos[1], Norival Alves Santos-Filho[2], Deivys Leandro Portuondo[3], Maria José Soares Mendes-Giannini[1] and Ana Marisa Fusco-Almeida[1,*]

[1]Faculdade de Ciências Farmacêuticas, UNESP - Univ Estadual Paulista, Departamento de Análises Clínicas, Laboratório de Micologia Clínica, Araraquara, São Paulo, Brasil, [2]Instituto de Química, UNESP - Univ Estadual Paulista, Departamento de Bioquímica e Tecnologia Química, Unidade de Síntese, Estrutura e Caracterização de Peptídeos e Proteínas, Araraquara, São Paulo, Brasil and [3]Faculdade de Ciências Farmacêuticas, UNESP - Univ Estadual Paulista, Departamento de Análises Clínicas, Laboratório de Imunologia Clínica, Araraquara, São Paulo, Brasil

*Corresponding author: Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas de Araraquara, Rodovia Araraquara-Jaú, km 1, s/n, Campos Ville Araraquara, São Paulo, CEP, 14800-903, Brasil. Tel: +55-(16)-33014638; E-mail: ana.marisa@uol.com.br

One sentence summary: This article will discuss the identification of novel protein of *Paracoccidioides brasiliensis*, EF-Tu, which is important to the fungus-host interaction.

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ABSTRACT

Paracoccidioides spp., which are temperature-dependent dimorphic fungi, are responsible for the most prevalent human systemic mycosis in Latin America, the paracoccidioidomycosis. The aim of this study was to characterise the involvement of elongation factor Tu (EF-Tu) in *Paracoccidioides brasiliensis*–host interaction. Adhesive properties were examined using recombinant PbEF-Tu proteins and the respective polyclonal anti-r*Pb*EF-Tu antibody. Immunogold analysis demonstrated the surface location of EF-Tu in *P. brasiliensis*. Moreover, *Pb*EF-Tu was found to bind to fibronectin and plasminogen by enzyme-linked immunosorbent assay, and it was determined that the binding to plasminogen is at least partly dependent on lysine residues and ionic interactions. To verify the participation of EF-Tu in the interaction of *P. brasiliensis* with pneumocytes, we blocked the respective protein with an anti-r*Pb*EF-Tu antibody and evaluated the consequences on the

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interaction index by flow cytometry. During the interaction, we observed a decrease of 2- and 3-fold at 8 and 24 h, respectively, suggesting the contribution of EF-Tu in fungal adhesion/invasion.

Keywords: *Paracoccidioides brasiliensis*; EF-Tu; fungal–host interaction; fibronectin; plasminogen; moonlighting protein

INTRODUCTION

Paracoccidioidomycosis (PCM), a fungal infection caused by species of the *Paracoccidioides* genus, *Paracoccidioides brasiliensis* and *P. lutzii* (Matute *et al.* 2006; Carrero *et al.* 2008; Teixeira *et al.* 2009), is a neglected disease, and it is estimated that ∼10 million individuals in Latin America are affected (Restrepo *et al.* 2001). *Paracoccidioides brasiliensis* is a thermally dimorphic fungus that at temperatures of ∼37°C is present in the yeast form (the pathogenic form for humans), while at temperatures below 28°C, it develops as a mycelium (the non-pathogenic form) (Shikanai-Yasuda *et al.* 2006). The transition is essential to establish the disease. The initial interaction occurs in the lungs, and it appears to trigger the subsequent activation mechanisms of innate and adaptive immunity, which results in localised infection or overt disease (Pina, Bernardino and Calich 2008).

Over the past decade, there has been recognition of the importance of fungal adhesion to the surface of the host cells. The capacity of a pathogen to colonise and infect the host organism is critically dependent on its ability to attach to the host-cell surface through a wide variety of molecules bound or secreted at the pathogen surface (Tronchin *et al.* 2008; Stones and Krachler 2015). *Paracoccidioides* spp. adhere and invade non-professional phagocytes or epithelial cells, as demonstrated in several studies (Hanna, Monteiro da Silva and Giannini 2000; Mendes-Giannini *et al.* 2000; Mendes-Giannini *et al.* 2004).

Commonly, the initial interaction of a pathogen with the host involves the adhesion to a barrier composed of a thin layer of epithelial or endothelial cells strengthened with extracellular matrix (ECM) that must be impaired for the establishment of the infection (Kozik *et al.* 2015).

Paracoccidioides spp. employ cell wall proteins, called adhesins, to interact with molecules of the ECM present on the host cells, such as laminin, fibronectin, and collagen types I and IV. *Paracoccidioides* spp. adhesins have been extensively characterised, such as gp43 (Vicentini *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (Barbosa *et al.* 2004), 14-3-3 protein (30 kDa) (Andreotti *et al.* 2005; Da Silva *et al.* 2013; Assato *et al.* 2015; Marcos *et al.* 2015) triose phosphate isomerase (Pereira *et al.* 2007), enolase (Donofrio *et al.* 2009; Marcos *et al.* 2012) and malate synthase (Da Silva Neto *et al.* 2009).

The initial binding of the pathogen to host cells is crucial to the delivery of virulence factors and is thus a key determinant of the pathogen's success. Pathogens have a large collection of virulence factors that targets the manipulation of the host-cell machinery to allow infection (Krachler, Ham and Orth 2011).

Other host targets that pathogen microorganisms employ to penetrate and colonise host tissues are coagulation cascade molecules and complement regulators that enable pathogens to evade the host's innate immune response (Wolff *et al.* 2013). Some *Paracoccidioides* spp. proteins bind to plasminogen as fructose 1,6-biphosphate aldolase (Chaves *et al.* 2015) and enolase (Nogueira *et al.* 2010; Marcos *et al.* 2012), which confer the pathogen's ability to spread to deeper tissues once the plasminogen is converted to plasmin, which has fibrinolytic activity to degrade the ECM (Bhattacharya, Ploplis and Castellino 2012).

Proteins that have more than one distinct function are called moonlighting proteins (Jeffery 2015). Examples of such multifaceted proteins in *Paracoccidioides* spp. are the metabolic enzymes aconitase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, isocitrate lyase, malate synthase, triose phosphate isomerase, fumarase and enolase, which act in the virulence of these fungi (Marcos *et al.* 2014).

The elongation factor Tu (EF-Tu) is another moonlighting protein described for several pathogens (Carrasco *et al.* 2015). Its canonical function is in the elongation step of translation, acting as a GTPase on the binding of aminoacyl-tRNA to the ribosome-mRNA complex during protein synthesis (Miller and Weissbach 1977). Other studies reported different functions for this protein: acting as protein disulphide isomerase (Richarme 1998), chaperonin (Caldas *et al.* 1998) and influencing the start of replication by the Qβ replicase (Blumenthal and Hill 1980).

In other pathogens, EF-Tu has been reported to be involved in virulence, contributing to adhesion, invasion and modulation of the immune system (Dallo *et al.* 2002; Crowe *et al.* 2003; Granato *et al.* 2004; Schaumburg *et al.* 2004; Kunert *et al.* 2007; Barel *et al.* 2008; Kesimer *et al.* 2009).

Here, we identified and characterised *P. brasiliensis* EF-Tu as a moonlighting protein that is present on the fungal surface and binds to fibronectin and plasminogen and participates in the fungal interaction (attachment/invasion) with pneumocyte cells. In addition, we demonstrated that lysine residues of *P. brasiliensis* EF-Tu are involved in the interaction with plasminogen. Together, these data contribute towards a better understanding of PCM pathogenesis and how *P. brasiliensis* interacts with the human host.

MATERIALS AND METHODS

Animal experimentation approvals

Animal experiments were performed in strict accordance with Brazilian Federal Law 11 794 establishing procedures for the scientific use of animals and the state law establishing the Animal Protection Code of the State of São Paulo. All efforts were made to minimise suffering, and all animal procedures were approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences of Araraquara—UNESP (Proc. 10/2011/CEUA/FCF).

Paracoccidioides brasiliensis isolate and growth conditions

Paracoccidioides brasiliensis Pb18 yeast cells isolated from a clinical case of PCM were cultured in brain heart infusion (BHI) medium containing 5% sheep blood at 37°C for 2D gel electrophoresis analysis. For the other experiments, the fungus was grown in BHI media supplemented with 1% glucose at 37°C with aeration in a mechanical shaker (150 rpm) and was collected during the early exponential growth phase (72–96 h).

Preparation of *Paracoccidioides brasiliensis* crude extracts

The protein extract was prepared from the yeast form of Pb18. Approximately 300 mg (6×10^4 cells mL^{-1}) of Pb18 was grown for 3–4 days at 37°C on BHI medium with 5% sheep blood, then

scraped off, and mixed with 1 mL of homogenisation buffer (10 mM Tris-HCl, pH 8.8) containing 100 mM phenylmethylsulphonyl fluoride and 1 mg mL^{-1} protease inhibitors (pepstatin, leupeptin, aprotinin, antipain and chymostatin). The mixture containing yeast cells was disrupted with liquid nitrogen and then vortexed for 30 min with glass beads followed by centrifugation at 13 000 *g* for 45 min. The supernatant was used for further protein analysis. The protein concentration was estimated using the Bradford method (Bio-Rad, Hercules, CA, USA), and the samples were analysed by 10% polyacrylamide gel electrophoresis (SDS-PAGE) prior to the 2D electrophoresis according to Laemmli (1970).

Two-dimensional gel electrophoresis and mass spectrometry analysis

The Pb18 proteins cultured in BHI medium with blood were subjected to isoelectric focus using the Ettan IPGPhor 3 (GE Healthcare, Little Chalfont, Bucks, UK) system. The second dimension was performed on a 12.5% gradient polyacrylamide gel as described by Laemmli (1970). The proteins were stained using Comassie Brilliant Blue R350 (GE Healthcare, Little Chalfont, Bucks, UK). The separated protein bands were excised from the gel and washed to eliminate the colouration. The proteins were further reduced with 10 mM dithiothreitol in 100 mM NH_4CO_3 for 35 min at 56°C and then alkylated with 55 mM iodoacetamide in 100 mM NH_4CO_3 for 30 min at room temperature. The proteins were subjected to tryptic digestion using 10 ng mL^{-1} Trypsin Gold (Promega, Madison, WI, USA) and extracted with 50% acetonitrile/5% formic acid.

Tryptic fragments were desalted with C_{18}ZipTips (Millipore, Billerica, MA, USA) and eluted with 1.5 μL of 80% acetonitrile/0.1% trifluoroacetic acid. Tryptic fragments were subjected to Nano-ESI-MS/MS using the Synapt G2-S HDMS mass spectrometer (Waters Corporation, Massachusetts, USA) and then analysed using ProteinLynx and identified by searching the SwissProt database (service of the Laboratório Multiusuários Centralizado de Genômica Funcional Aplicada à Agropecuária e Agroenergia, Departamento de Genética, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo). The sequences were submitted to databases and analysed by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and FASTA3.

Heterologous expression and purification of the Pb*EF-Tu* recombinant (r*Pb*EF-Tu)

Total RNA was obtained from Pb18 yeast cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed with RevertAid H Minus Reverse Transcriptase (Fermentas, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The *P. brasiliensis* EF-Tu gene open reading frame (accession no. PADG˙01949.1, from Broad Institute) was amplified by touch-down polymerase chain reaction (PCR) from cDNA of the Pb18 isolate using the primers F: **CACC**ATGTCTAATATTTCAAGATCGGTCAA/R: CTATTCCTCGAGGACACG (the adaptor nucleotide sequence added for directional cloning is highlighted in bold). The PCR cycling temperatures were as follows: 94°C for 10 min, 40 cycles of 30 s at 94°C, temperature range of 65°C–55°C for 45 s, 30 cycles of 2 min each at 68°C and a final cycle at 68°C for 10 min. The construct PbEF-Tu-pET100/D-TOPO was generated by ligation of the PCR fragment product into a pET100/D-TOPO® expression vector (Invitrogen, Carlsbad, CA, USA). The construct was propagated and maintained in *Escherichia coli* One Shot® TOP 10 cells, while BL21 Star (DE3) cells were used for EF-Tu expression, as indicated by the provider. Briefly, Luria-Bertani medium containing 50 μg mL^{-1} ampicillin was inoculated with an aliquot of transformed BL21 Star (DE3) cells grown overnight and incubated at 37°C with shaking (200 rpm) until the culture reached an $OD_{600nm} = 0.5–0.8$. Isopropyl thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) was added to the cultures, at 0.5 and 1 mM final concentrations, followed by different times of incubation (1, 3, 6 and 24 h) at 37°C with shaking (200 rpm) to determine the best induction conditions. His-tagged r*Pb*EF-Tu was purified using the HisTrap FF Crude column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Eluted fractions were kept at –20°C until analysis by SDS-PAGE and use for other assays. The protein concentrations were measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Generation of polyclonal antibody

Rabbit pre-immune serum was obtained and stored at a –20°C. Male rabbits were injected intradermally with 2 mL of an emulsion consisting of 1 mL of r*Pb*EF-Tu (80 μg mL^{-1}) and 1 mL of complete Freund's adjuvant. Subsequently, booster injections of r*Pb*EF-Tu mixed with incomplete Freund's adjuvant were given weekly for 4 weeks and then monthly for 3 months. The rabbits were bled 7 days after the last dose. The globulin fraction from both pre- and post-immunisation sera was purified by precipitation with 1.56 M ammonium sulphate, pH 5.6, and stored at –70°C.

Western blotting analysis

The recombinant protein crude extract was obtained as described above, and the Pb18 culture filtrate were electrophoresed and transferred to a nitrocellulose membrane as described previously (Singh *et al.* 2005). Following protein transfer, membranes were blocked with 5% non-fat dry milk powder and 1% bovine serum albumin in 0.05 M phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). After three wash steps with 0.05% PBS-T, membranes were probed with a polyclonal anti-r*Pb*EFTu antibody and pre-immune serum (NRS) (both diluted 1:100), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins (diluted 1:1000) (Sigma-Aldrich, St. Louis, MO, USA). Immune complexes were visualised with 3,3′-diaminobenzidine.

Electron microscopy *in vitro* and *in vivo*

C57BL/6 mice were obtained from the Isogenic Breeding Unit (Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil) and used at 8 and 12 weeks of age. Briefly, following anaesthesia of the mice with intraperitoneal injection of a combination containing ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}), the animals were inoculated with 10^6 *P. brasiliensis* yeast cells in 50 μL of PBS by surgical intratracheal inoculation as previously described (Cano *et al.* 1995). After 72 h and 4 weeks post-infection, the lungs were removed and fixed to determine the subcellular localisation of *P. brasiliensis* EF-Tu protein.

The subcellular localisation of the *P. brasiliensis* EF-Tu protein was performed by immunocytochemistry at the ultra-structural level using immunogold labelling. For each experiment, both pneumocytes infected with *P. brasiliensis* (10^8 cells mL^{-1}) for 5 and 8 h and lungs removed from C57BL/6 mice infected with

P. brasiliensis (10^6 cells mL^{-1}) were fixed for 24 h at 4°C (2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) and then processed for ultrathin sectioning by the electron microscopy service of the Institute of Biomedical Sciences (ICB-I) USP-SP. The ultrathin sections were incubated with polyclonal anti-r*Pb*EF-Tu antibody and pre-immune serum (NRS) at a dilution of 1:100 overnight, followed by a goat anti-rabbit antibody labelled with 10-nm colloidal gold particles (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10 at 4°C for 3 h. Sections were finally examined on a Jeol 1010 (Tokyo, Japan) electron microscope at an acceleration voltage of 80 kV (performed in the Laboratório Multiusuário de Microscopia Eletrônica—LMME, FMRP-USP).

Flow cytometry

The inhibition interaction assay of *P. brasiliensis* to A549 cells was determined using the adhesion assay as described by de Oliveira *et al.* (2015). The inoculum of *P. brasiliensis* (5×10^6 cells mL^{-1}) was previously treated with 10 μM [5(6)-carboxyfluorescein diacetate N-succinimidyl ester] for 15 min at 37°C, and then the cells were washed, resuspended in PBS and treated with EF-Tu rabbit antisera (1:100) for 1 h at 37°C. The cells were washed, resuspended in PBS and transferred to 24-well plates (Sigma-Aldrich, St. Louis, MO, USA) coated with the A549 monolayer and incubated for 2, 5, 8 and 24 h at 37°C, 5% CO_2. As positive control, A549 cells were assayed with *P. brasiliensis* without previous antibody treatment, and a negative control was carried out with A549 cells assayed with *P. brasiliensis* treated with pre-immune rabbit serum (NRS) diluted 1:100 in PBS. After each incubation period, the cells were washed with PBS to remove non-adherent cells, detached from plastic with cold PBS and a rubber cell scraper on ice (Bernardino *et al.* 2013), and then fixed in 4% paraformaldehyde. The interaction index was determined by flow cytometry using a FACsCanto (Becton Dickinson, New Jersey, USA) flow cytometer; uninfected cells and unlabelled yeast were used as internal controls to define the gates and for self-fluorescence evaluation. The excitation wavelength was 488 nm, and emitted light was collected with a 530/30-nm bandpass filter. Data were processed and analysed using the FACsDiva software program (Becton Dickinson, New Jersey, USA).

The influence of antisera anti-r*Pb*EF-Tu on the survival of the fungi was evaluated to analyse how this factor interferes with the interaction inhibition assay results. For this, Pb18 was grown in BHI medium supplemented with 1% glucose with or without anti-r*Pb*EF-Tu or rabbit pre-immune antisera (NRS) at a titre of 1:100. Fungus aliquots were obtained at the same times used for the interaction inhibition assay described above, and the viability was verified by measuring metabolic activity using the 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide (Sigma-Aldrich, St. Louis, MO, USA) reduction assay as described by Meshulam *et al.* (1995).

Enzyme-linked immunosorbent assay

ELISA (enzyme-linked immunosorbent assay) was carried out as described by Mendes-Giannini *et al.* (2006). Briefly, 96-well microtitre ELISA plates (Sigma-Aldrich, St. Louis, MO, USA) were coated with ECM proteins (laminin, fibronectin and type IV collagen), and also plasminogen and fibrinogen, all at concentrations of 10 μg mL^{-1}, at 4°C overnight. Following three wash steps with PBS containing 0.05% PBS-T, wells were blocked with 1% BSA w/v for 2 h at RT. Wells were washed three times with PBS-T and incubated with r*Pb*EF-Tu protein (5 μg mL^{-1}) at 37°C for 1 h in triplicate wells. Following incubation, wells were washed thoroughly with PBS-T incubated with a polyclonal anti-r*Pb*EF-Tu antibody (1:100) for 1 h at 37°C. After the wash steps, wells were incubated with HRP-conjugated anti-rabbit immunoglobulins (1:2000) at 37°C for 1 h. The reaction was developed with *o*-phenylenediamine, and the absorbance was measured at 490 nm. Appropriate controls for non-specific binding were performed.

To evaluate the involvement of the lysine-residues and ionic strength in the r*Pb*EF-Tu-plasminogen interaction, binding assays were performed, using the lysine analogue ε-aminocaproic acid (Sigma-Aldrich, St. Louis, MO, USA) (EACA) and sodium chloride (NaCl), respectively, by ELISA. The same protocol described above was followed, except that increased quantities of EACA (0–10 mM) or NaCl (0–200 mM) were added with r*Pb*EF-Tu (5 μg mL^{-1}) to plasminogen-coated wells (10 μg mL^{-1}).

Sequence alignment and molecular modelling of PbEF-Tu

A sequence alignment of *P. brasiliensis* EF-Tu with other validated prokaryotic and eukaryotic EF-Tu from the GenBank/EMLB database was made with CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). GenBank accession numbers of aligned sequences are as follows: *P. brasiliensis* Pb18 (XP˙010757650.1), *P. lutzii* Pb01 (XP_002795445.1), *Emmonsia parva* (gi:824373436), *Blastomyces dermatitidis* (gi:531978878), *E. crescens* (gi:821505882), *Histoplasma capsulatum* (gi:225559613), *Byssochlamys spectabilis* (gi:557726296), *Thermus thermophilus* (gi:541881568) and *Bos taurus* (gi:6137414). These comparisons were used to predict the functional domains in the deduced amino acid sequences. To further characterise the EF-Tu sequence and conformational properties that might influence plasminogen binding, we searched for the best structural homologues of EF-Tu_{Pb} by using variation of protein threading as implemented in the program HHPRED (http://toolkit.tuebingen.mpg.de/hhpred). With the program MODELLER (Sali *et al.* 1995), the sequence of *P. brasiliensis* EF-Tu was aligned to that of *B. taurus* EF-Tu, and then a homology model was built using the X-ray crystallography structure of *B. taurus* EF-Tu (PDB ID 1D2E:A) as the template. The quality and stereochemistry of the model were validated using the program PROCHECK (Laskowski *et al.* 1993). Structural models were visualised by the PyMol Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA, USA).

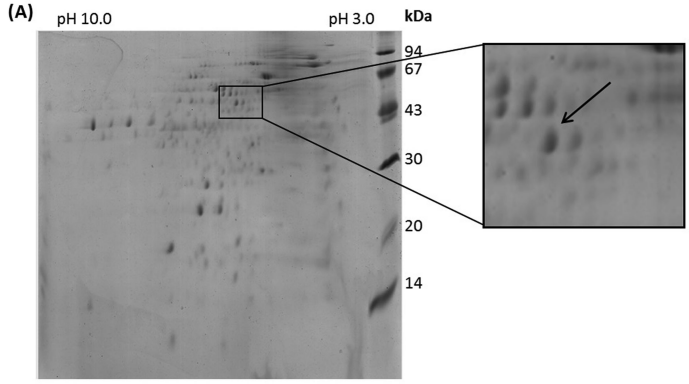
Statistics

One-way ANOVA with Tukey's coefficient post hoc test was used to analyse the results obtained in this study. The results of the statistical analyses were considered significant when the *P*-value was <0.05. The analyses and graph construction were performed using the software Prism 5 (GraphPad Software Inc.).

RESULTS

Identification of EF-Tu protein in *Paracoccidioides brasiliensis* yeast cells

Two-dimensional gel electrophoresis analysis revealed a protein of 48.1 kDa and pI of 5.3 (Fig. 1A). This protein was analysed based on sequences of the internal peptide of *P. brasiliensis*, which spanned five amino acid sequences (Fig. 1B), and these peptides were similar to the *P. brasiliensis* EF-Tu protein. The



(B)

Peptide sequence	Protein Score/ Protein sequence coverage	Mol. Weight (kDa)	Identified protein/Acession number
KDSEVEIIGGGVPTILTK[1]			
GLANFLEYGAIDKAPEER[2]			
QPELGEQKIDELLEAVDT WIPTPQR[3]	25.240.380/(35.6)	48,7	Translation elongation factor Tu *Paracoccidioides brasiliensis* Pb18 (XP_010757650.1)
DTDKPFLMSIEEVFSISGR[4]			
NMITGAANMDGAIVVVA ASDGQMPQTR[5]			

(C)

MSNISRSVKALLQTSRLALQRPRGVNPIHHVLLQ
DRIAFRGMATVFERTKPHVNVGTIGHVDHGKTT
LTAAITKRQAEK**GLANFLEYGAIDKAPEER**[2]KRGI
TISTSHIEYSTDKRHYAHVDCPGHADYIK**NMITG**
AAMDGAIVVVAASDGQMPQTR[5]EHLLLARQV
GVQKIVVFVNKVDAVEDKEMLELVELEMRELLTT
YGFEGEKTPIIFGSALCAMEGR**QPELGEQKIDELL**
EAVDTWIPTPQR[3]**DTDKPFLMSIEEVFSISGR**[4]G
TVASGRVERGILK**KDSEVEIIGGGVPTILTK**[1]VTDI
ETFKKSCDESRAGDNSGLLLRGVKREDIRRGMV
VAVPGSVKAHRFLVSMYVLTEAEGGRRTGFGQN
YRPQMFIRTADEAAELSWPDGDDEAKMVMPG
DNVEMVLKSHRPVAAEAGQRFNIREGGRTVAT
GLVTRVLEE*

Figure 1. Identification of EF-Tu in the yeast cells of *P. brasiliensis*. (**A**) Two-dimensional gel electrophoresis of *P. brasiliensis* proteome from crude protein extract; the highlighted square points to 48.1 kDa protein (pI 5.3). (**B**) Analysis of an *in situ* trypsin digests using Nano-ESI-MS/MS. (C) Hundred percent of identity of amino acid sequences obtained and the EF-Tu sequence regions of *P. brasiliensis*.

amino acid sequences of the peptide showed identity with four regions of the EF-Tu *P. brasiliensis* protein that was deposited in GenBank (XP˙010757650.1): all amino acids sequences shared 100% identity, as shown in Fig. 1C.

Expression, purification and production of polyclonal antibody to r*Pb*EF-Tu

The *P. brasiliensis* EF-Tu recombinant protein (rPbEF-Tu) of 51.1 kDa was detected in bacterial lysates (Fig. 2A). After induction for 6 h with 1 mM IPTG (the optimal tested conditions), the protein was purified from bacterial lysates through a nickel-chelating affinity column and the purity was assessed by SDS-PAGE (Fig. 2B). An aliquot of the purified r*Pb*EF-Tu was used to generate rabbit polyclonal anti-r*Pb*EF-Tu antibody. A western blot confirmed the positive reaction of antibody with the recombinant protein (Fig. 2, lane 1) and crude total extract of Pb18 (Fig. 2, lane 2). We examined whether *P. brasiliensis* EF-Tu is released into the supernatant. As shown in Fig. 2, column 3, this protein was detected by western blot in the culture filtrate of Pb18. No cross-reactivity to the pre-immune rabbit serum (NRS) was confirmed in all samples (Fig. 2, columns 4, 5 and 6).

Cellular localisation of PbEF-Tu by immunoelectron microscopy *in vitro* and *in vivo*

The presence of EF-Tu on the *P. brasiliensis* yeast surface was assessed in different conditions, Fava-Netto culture medium (Fig. 3), A549 infection (for 5 and 8 h of infection) (Fig. 4) and experimental PCM (Fig. 5) in mice to investigate whether the conditions of infection change the location and expression of this protein. A polyclonal antibody that specifically recognises *Pb*EF-Tu was used to examine the location of this protein by immunogold electron microscopy. Immunogold labelling with anti-r*Pb*EF-Tu antibody revealed a random distribution of gold particles on the *P. brasiliensis* cell surface (with all cells showing immunogold labelling at this location) and cytoplasm; additionally, the major yeast cells of *P. brasiliensis* found in the experimental PCM were localised near the nucleus of host cells. Despite being in greater quantity in the cell wall, apparently, its occurrence on this site does not change during the conditions of *in vitro* and *in vivo* infection, but in the experimental PCM in mice, we observed gold particles released to host cells, suggesting that possibly the *P. brasiliensis* EF-Tu is released during mouse infection (Fig. 5). As expected, pre-immune sera were free of gold particles (Figs 3, 4 and 5).

EF-Tu is involved in the *Paracoccidioides brasiliensis* interaction with host cells

Previously, we verified the influence of antisera (anti-r*Pb*EF-Tu and NRS) treatment on the survival of the fungi, and no significant differences were found between the treatments and the untreated control (Fig. S1, Supporting Information); this was done to validate the results of the inhibition interaction assay. To verify the involvement of EF-Tu in the interaction of *P. brasiliensis* with host cells, we blocked this protein with a polyclonal anti-r*Pb*EF-Tu antibody. Antibody treatment was effective in inhibiting the infection (adhesion/invasion), particularly at 8 and 24 h, with reductions of 2-fold and 3-fold, respectively (Fig. 6).

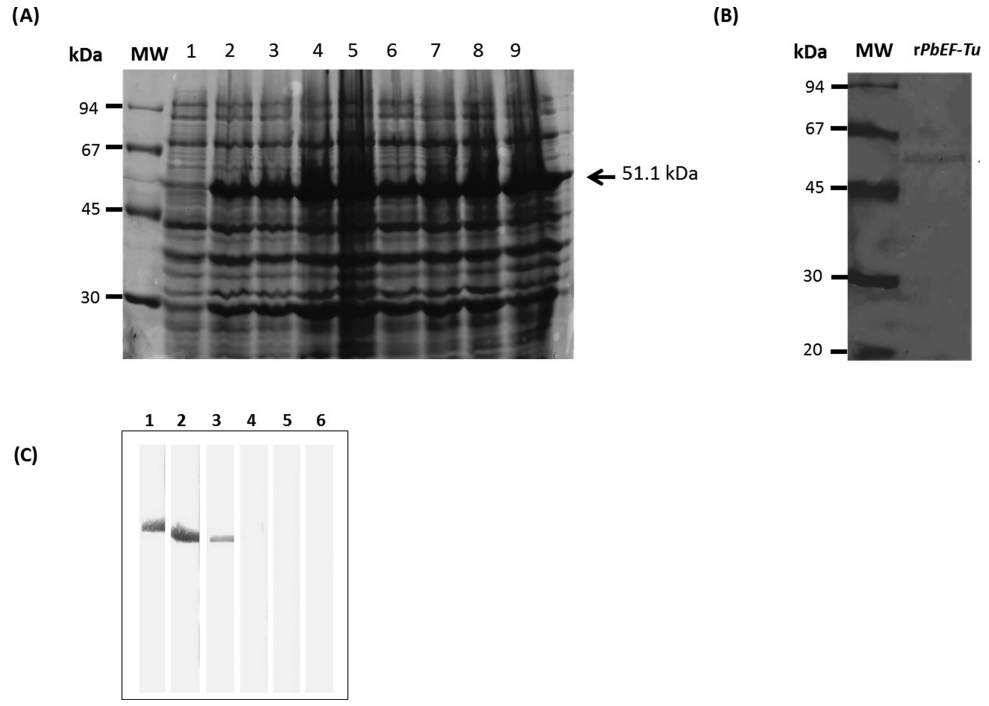


Figure 2. Expression, purification and production of polyclonal antibody to r*Pb*EF-Tu. (**A**) Standardisation of protein-induction conditions; 1: without induction, 2: 1 h of induction, 3: 3 h of induction, 4: 6 h of induction, 5: 24 h of induction, all the last three with 0.5 mM of IPTG, 6: 1 h of induction, 7: 3 h of induction, 8: 6 h of induction, 9: 24 h of induction, all the last three with 1 mM of IPTG; arrow points to the recombinant EF-Tu protein with molecular mass of 51.1 kDa. (**B**) Purified recombinant *Pb*EF-Tu protein. MW: molecular marker. (B) Reactivity of anti-r*Pb*EF-Tu sera by western blotting. 1: reactivity of anti-r*Pb*EF-Tu sera to r*Pb*EF-Tu; 2: reactivity of anti-r*Pb*EF-Tu sera to crude extract protein of Pb18; 3: reactivity of anti-r*Pb*EF-Tu sera to culture filtrate of Pb18; 4: control performed with NRS and r*Pb*EF-Tu; 5: control performed with NRS and crude protein extract of Pb18; 6: control performed with NRS and culture filtrate of Pb18.

r*Pb*EF-Tu interacts with ECM components and coagulation cascade molecules

As EF-Tu is located in the *P. brasiliensis* surface exposed to the extracellular medium, we examined its capacity to bind to host molecules of the ECM and coagulation cascade by ELISA assay. We showed that *Pb*EF-Tu interacts with fibronectin and plasminogen but not with laminin, fibrinogen or type IV collagen (Fig. 7).

Involvement of lysine residues or ionic strength in the r*Pb*EF-Tu-plasminogen interaction

Many pathogen surface proteins interact with plasminogen through lysine residues (Verma *et al.* 2010). The interaction of r*Pb*EF-Tu with plasminogen was analysed more deeply by evaluating the participation of ionic strength and/or lysine residues. Lysine residues are important for the plasminogen–r*Pb*EF-Tu interaction. Figure 8A demonstrates that 0.1 mM EACA is sufficient to significantly reduce (53.3%) the plasminogen binding to r*Pb*EF-Tu. To evaluate other determinant factors of plasminogen binding with proteins, the ionic strength in *Pb*EF-Tu-plasminogen interactions was examined in the presence of increased quantities of NaCl (Salazar *et al.* 2014). With increasing salt concentration, we did not observe a prominent reduction of plasminogen binding to *Pb*EF-Tu, (17%–20%); however, this finding suggests that ionic strength is also involved in this interaction (Fig. 8B).

EF-Tu alignment and lysine residue distribution in PbEF-Tu

The gene for *P. brasiliensis* EF-Tu encodes a polypeptide of 441 amino acids, with a predicted molecular mass of 48.6 kDa. The EF-Tu sequences exhibit various degrees of conservation among particular taxonomic groups, with sequence identity of 61% between prokaryotic sequences and ranging from 60% to 98% identity with other eukaryotic sequences. Surface-exposed lysine residues might potentially be linked to the sites of interaction with plasminogen. Then, the sequence of EF-Tu from *P. brasiliensis* has a total of 23 lysine residues, and 21 of these are conserved among the analysed sequences. The conserved lysine residues identified in EF-Tu of *P. brasiliensis* are highlighted in grey, and two non-conserved lysine residues (K47 and K162) are highlighted in sky blue (Fig. S2, Supporting Information). The lysine residue (a positively charged amino acid) K162 present in EF-Tu of *P. brasiliensis* is not conserved among the species; additionally, in the other microorganisms, it is replaced by a glutamic acid (a negatively charged amino acid) (Fig. S2, Supporting Information).

Based on sequence similarity analysis, *P. brasiliensis* EF-Tu shows 60% amino acid sequence identity with *Bos taurus* EF-Tu (Fig. S2, Supporting Information). This level of sequence identity is suitable enough for the use of *B. taurus* EF-Tu X-ray crystallography structures as a template for PbEF-Tu structure prediction by homology modelling. A *B. taurus* EF-Tu crystal structure PDB:1D2E:A was specifically selected on the basis of the HHpred result (60% identity and 100% probability) and was utilised as a

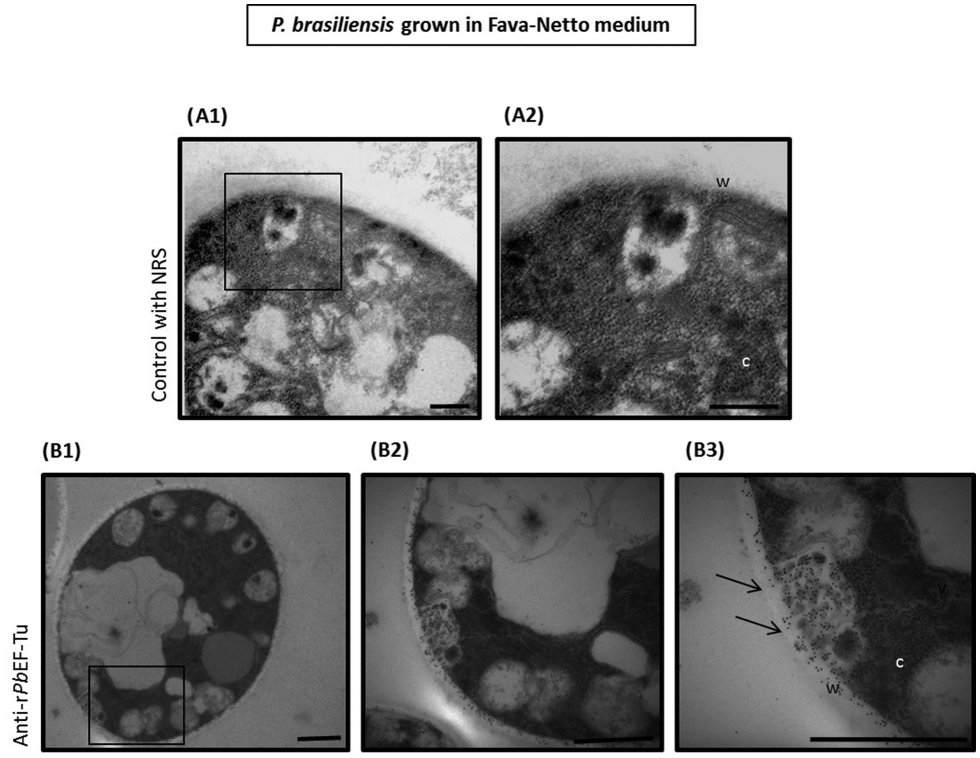


Figure 3. Immunoelectron microscopy detection of EF-Tu in *P. brasiliensis* yeast cells grown in Fava-Netto medium. (**A**1) and (A2) negative control with pre-immune serum (NRS); (**B**1), (B2) and (B3) PbEF-Tu yeast cells localisation. Cell wall (w) and cytoplasm (c) of *P. brasiliensis* yeast cells; gold particles that correspond to *Pb*EF-Tu are observed at the fungus (arrow). Bars = 1 μm.

template for *P. brasiliensis* EF-Tu structure modelling. A structural model for *P. brasiliensis* EF-Tu was built by the MODELLER (Sali *et al.* 1995) program based on atomic coordinates of 1D2E:A and checked using the PROCHECK Program (Laskowski *et al.* 1993). The Ramachandran plot analysis shows that the main-chain conformations for 96.9% are within the most favoured or allowed region (Fig. S3, Supporting Information), which confirmed the quality of the predicted model. The *P. brasiliensis* EF-Tu model demonstrating the lysine residues that are surface-exposed is shown in Fig. 9. From a total of 23 lysine residues found in *P. brasiliensis* EF-Tu, 22 are located at the protein surface, and among these, 21 are conserved residues (highlighted in blue) and two are non-conserved (highlighted in sky blue, K47 and K162) (Fig. 9).

DISCUSSION

Adhesins have a crucial role in the pathogen–host interaction (Govender, Ramsugit and Pillay 2014), and adhesion is the first stage of infection and subsequent colonisation (Jarocki, Padula and Djordjevic 2015). One of the factors involved in the *Paracoccidioides* genus virulence is the ability of yeast cells to adhere to human epithelial cells and ECM components through adhesins, such as enolase and 14-3-3 proteins, as demonstrated by de Oliveira *et al.* (2015).

The ECM provides the anchoring platform for the basement membrane, which is engaged in the building of structural scaffolds and involved in physiological processes, cell signalling and physical barriers (Hynes 2009; Singh *et al.* 2012). The lungs, the first site of *Paracoccidioides brasiliensis* contact with the host, present epithelial cells with various types of ECM components, such as fibronectin that are highly expressed in this organ (Roman 1997) and represent the preferential receptor in the adhesion process of Pb18 isolate of *P. brasiliensis* (De Oliveira *et al.* 2015).

Once adhered to host cells, the pathogens initiate the invasion process to prevent the host immune response using different strategies. To disseminate beyond the site of infection, *P. brasiliensis* and other pathogens require that host physiological barriers such as ECM be degraded as well the complement system regulators to evade the immune system response present on surface plasminogen receptors (Nogueira *et al.* 2010; Marcos *et al.* 2012; Chaves *et al.* 2015); the plasminogen, once bound to and in the presence of host activators (uPA and tPA), is converted to plasmin that has proteolytic activity, leading to invasion of the host cell and dissemination (Lähteenmäki, Edelman and Korhonen 2005; Jarocki, Padula and Djordjevic 2015; Raymond and Djordjevic 2015). The protease activity mediated by plasminogen is in fact a virulence factor used by pathogens (Singh *et al.* 2013, 2015).

It has been shown that EF-Tu can act as a virulence factor in different pathogens, as the surface location mediates binding to fibronectin for *Mycoplasma pneumoniae* (Dallo *et al.* 2002; Balasubramanian, Kannan and Baseman 2008), *Acinetobacter baumannii* (Dallo *et al.* 2012) and *Mycobacterium tuberculosis* (Xolalpa *et al.* 2007); and in other pathogens, as the plasminogen receptor for *Pseudomonas aeruginosa* (Kunert *et al.* 2007), *Listeria monocytogenes* (Schaumburg *et al.* 2004) and *M. tuberculosis* (Xolalpa *et al.* 2007); Factor H for *Pseudo. aeruginosa* (Kunert *et al.* 2007); laminin for *M. avium* (Viale *et al.* 2014) and *Francisella tularensis* facilitate host-cell invasion by interacting with nucleolin (Barel *et al.* 2008). In *P. brasiliensis*, there is a report of

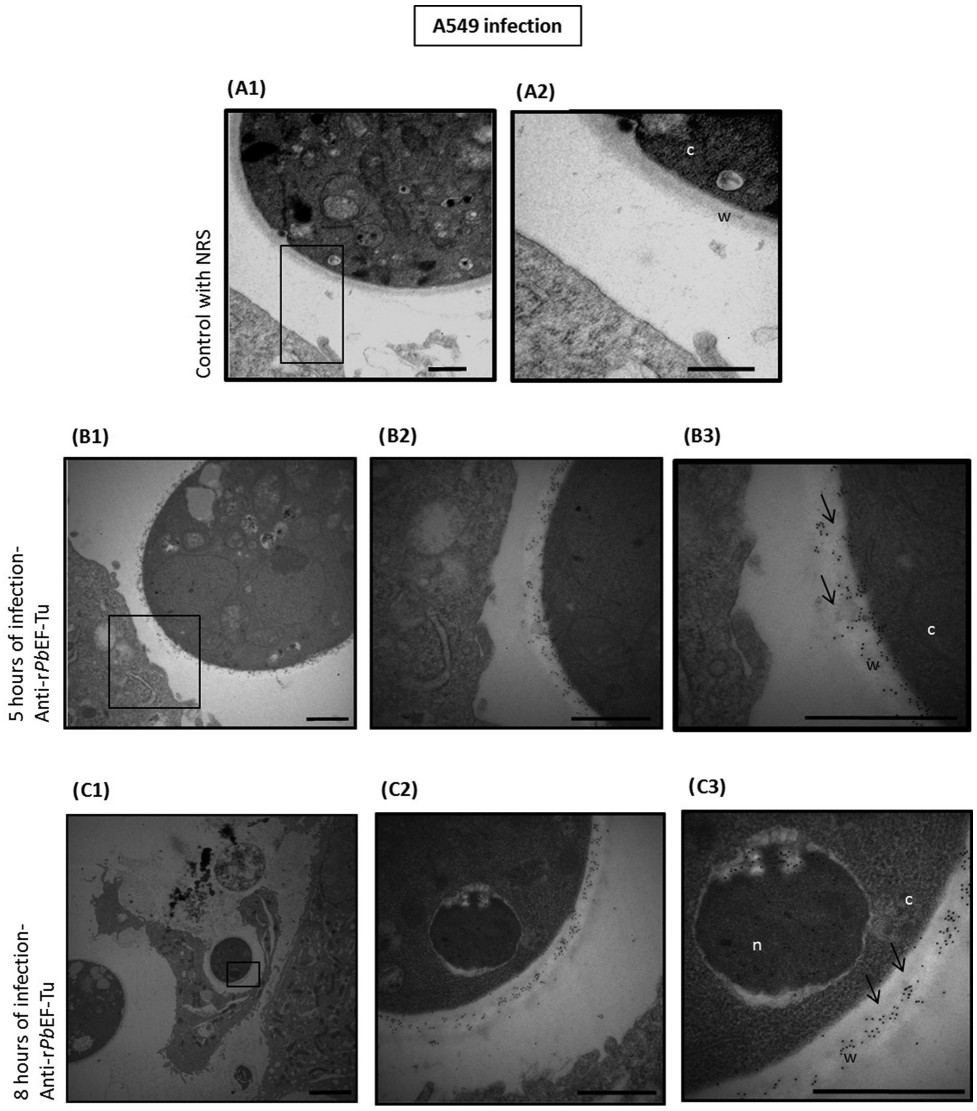


Figure 4. Immunoelectron microscopy detection of EF-Tu_{Pb} in *P. brasiliensis* yeast cells during infection to A549 cells. (**A**1) and (A2) negative control with pre-immune serum (NRS); (**B**1), (B2) and (B3) PbEF-Tu yeast cells localisation within 5 h of infection; (**C**1), (C2) and (C3) PbEF-Tu yeast cells localisation within 8 h of infection. Cell wall (w), cytoplasm (c) and nucleous (n) of *P. brasiliensis* yeast cells; gold particles that correspond to *Pb*EF-Tu are observed at the fungus (arrows). Bars = 1 μm.

EF-Tu overexpression identification through transcriptional analysis of yeast cells when in contact with human plasma (that mimics sites of infection or inflammation) (Bailão *et al.* 2007). The proteomics approach elucidating pathogen growth in complex media, such as the presence of blood or haemoglobin, reveals adhesins that bind to fibronectin expressed under these conditions. The interaction of haemoglobin with the fungal cell surface initiates a signal transduction pathway that ultimately leads to fibronectin receptor expression, but the molecular basis for this signalling is unknown (Pendrak, Krutzsch and Roberts 2000). Donofrio *et al.* (2009) demonstrated that *P. brasiliensis* cultured on blood medium exhibited higher adhesion to pneumocytes, apparently similar to the effect of animal passage.

In addition to its superficial location, EF-Tu has also been found associated with the outer membrane of secreted vesicles in *Burkholderia pseudomallei* (Nieves *et al.* 2010) and *A. baumannii* (Dallo *et al.* 2012), which may represent an export mechanism of this protein from the cytoplasm. We found that *P. brasiliensis* EF-Tu is localised to the cell surface during *in vitro* and *in vivo* infection models. However, the mechanisms of protein exposure to the fungal cell surface remain unclear, and the association of *P. brasiliensis* EF-Tu to the cell surface, confirmed by immunoelectron microscopy and binding experiments in *P. brasiliensis*, is a potential explanation for surface localisation. This protein is released into the culture medium by yeast forms of *P. brasiliensis* and was confirmed by western blotting and immunoelectron microscopy, suggesting that EF-Tu participates in the fungal environment in host tissue.

The next question was whether EF-Tu behaves like a moonlighting protein, contributing to *P. brasiliensis* interaction with the host. We have demonstrated that EF-Tu interacts with ECM and coagulation cascade components, including fibronectin and plasminogen, thus contributing to the fungus–host interaction.

Further investigation of this interaction in the future should be performed, but we speculated, based on literature data and analyses of important motifs in the EF-Tu of *P. brasiliensis*, that

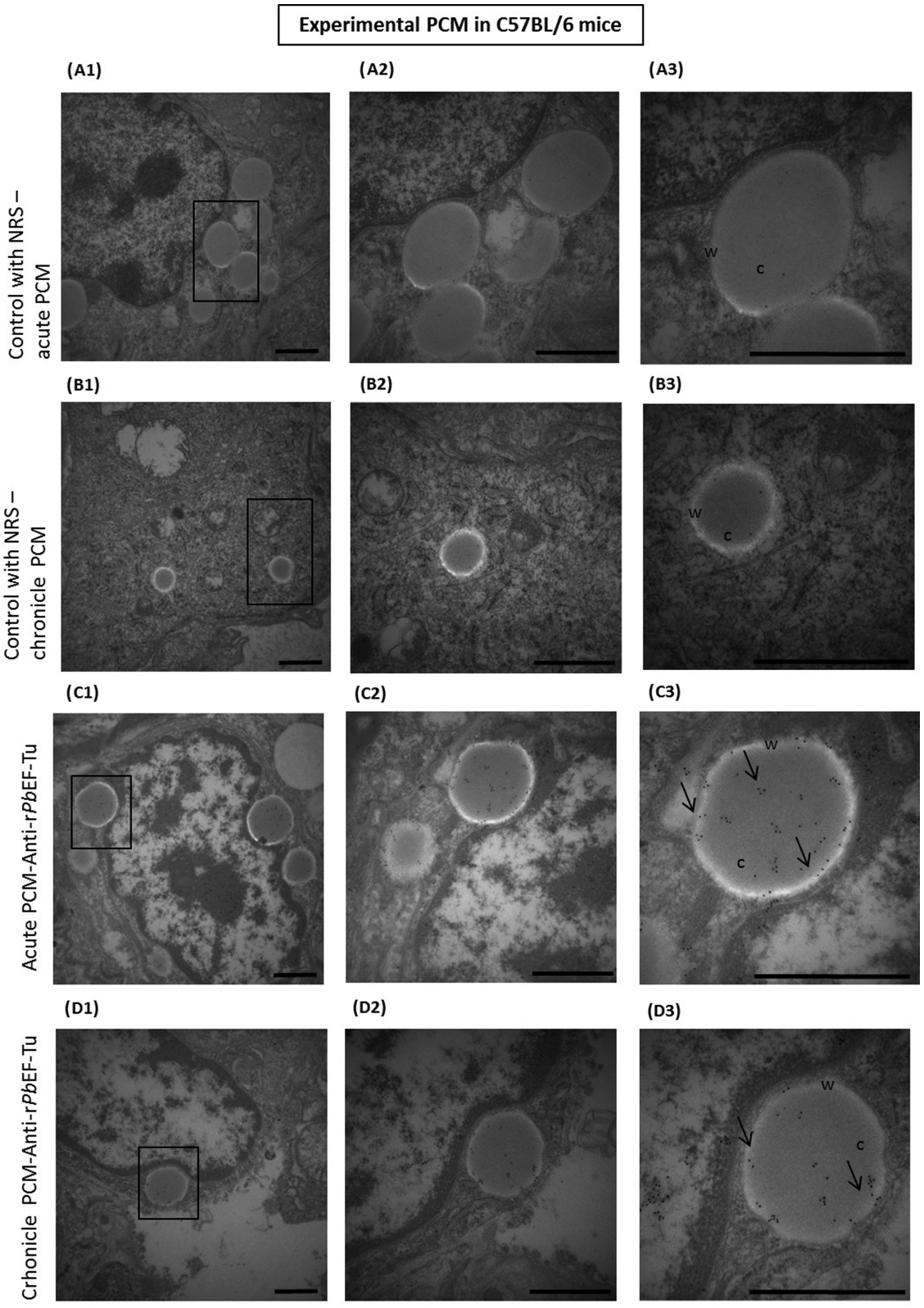


Figure 5. Immunoelectron microscopy detection of EF-Tu_{Pb} in *P. brasiliensis* yeast cells during experimental PCM in C57BL/6 mice. (**A**1), (A2) and (A3) negative control with pre-immune serum (NRS) during acute mouse infection; (**B**1), (B2) and (B3) negative control with pre-immune serum (NRS) during chronicle mouse infection; (**C**1), (C2) and (C3) PbEF-Tu yeast cells localisation during acute mouse infection; (**D**1), (D2) and (D3) PbEF-Tu yeast cells localisation during chronicle mouse infection. Cell wall (w) and cytoplasm (c) of *P. brasiliensis* yeast cells; gold particles that correspond to *Pb*EF-Tu are observed at the fungus (arrows). Bars = 1 μm.

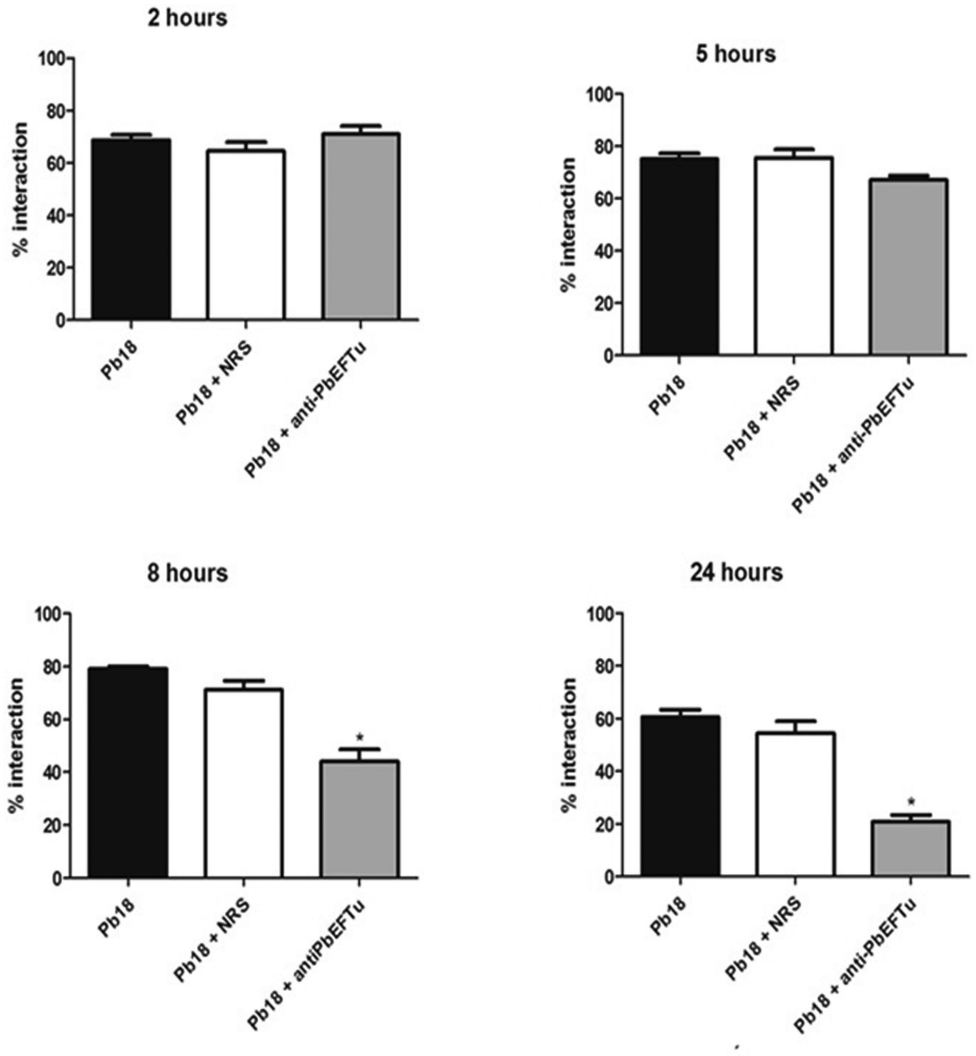


Figure 6. Inhibition of the interaction of *P. brasiliensis* yeast cells with A549 cells using polyclonal anti-r*Pb*EF-Tu antibody. Influence of *Pb*EF-Tu in the interaction of *P. brasiliensis* with A549 cells in different times evaluated by flow cytometry. As controls, we used *P. brasiliensis* treated with rabbit pre-immune serum (NRS) and without any treatment. '*' indicates statistically significant differences between the interaction rates, $P < 0.05$.

it involves binding to plasminogen (Koenigs *et al.* 2013; Koenigs, Zipfel and Kraiczy 2015). The lysine-binding kringle domains of plasminogen mediate the binding to fibrin, components of the ECM, or lysine-containing host and bacterial-binding proteins (Anglés-Cano 1994; Lähteenmäki, Kuusela and Korhonen 2001); this was shown in *A. baumannii* (Koenigs, Zipfel and Kraiczy 2015), *Borrelia burgdorferi* (Koenigs *et al.* 2013) and *Leptospira interrogans* (Wolff *et al.* 2013), where the role of lysine residues in the EF-Tu-plasminogen interaction was examined using the lysine analogue tranexamic acid or EACA and it was found that treatment significantly reduced plasminogen binding to EF-Tu. The *P. brasiliensis* EF-Tu sequence possesses 21 conserved lysine residues and two non-conserved residues of a total of 23, and among these, 22 are surface-exposed. The *P. brasiliensis* EF-Tu structure model and the binding study with plasminogen reveal details of the interaction between the two proteins; this prediction may lead to the establishment of prophylactic and therapeutic approaches.

Furthermore, there is a conserved lysine residue on the EF-Tu proteins that is replaced by arginine on EF-Tu of *P. brasiliensis* (highlighted in green, R298). Despite being a conservative substitution, according to this model, this residue does not appear on protein surface, so this replacement probably does not affect protein interaction. Between the non-conserved lysine residues, comparing mainly with EF-Tu protein from *P. lutzii* model, the non-conserved K162 residue, replaced by a glutamine residue in this specie, although it is apparently on protein surface, appears to not be located in areas of the EF-Tu protein with a net positive charge (data not show) (Koenigs, Zipfel and Kraiczy 2015). Moreover, according to some authors, regardless of the binding of plasminogen by EF-Tu proteins be partially dependent by ionic interactions mediated by lysine residues, other non-ionic interactions could contribute to this binding (Wolff *et al.* 2013; Koenigs, Zipfel and Kraiczy 2015). Therefore, the lysine residue at this position may not interfere with the molecule activity.

In addition to showing that EF-Tu of *P. brasiliensis* interacts with plasminogen, we also demonstrated that this interaction is strongly dependent on lysine residues, as the lysine analogue, EACA, reduced plasminogen binding to r*Pb*EF-Tu; even at 10 mM EACA, binding levels remained almost constant. This effect may be due to the blocking of all lysine binding sites

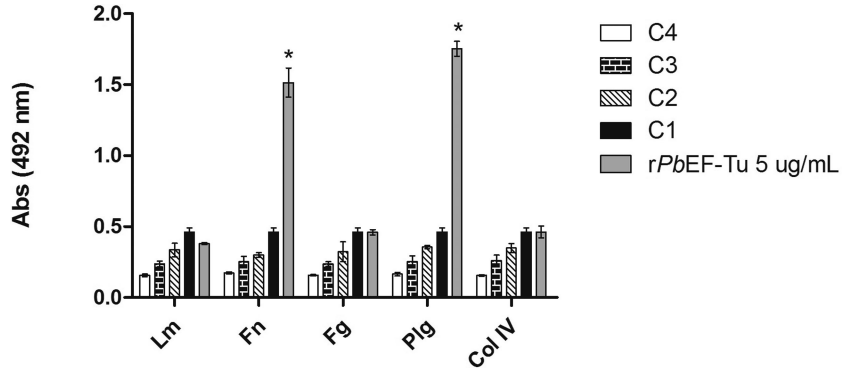


Figure 7. Interaction of *Pb*EF-Tu to ECM and cascade coagulation components. Wells were coated with 10 μg ml^{-1} of each component, Lm: laminin, Fn: fibronectin, Fg: fibrinogen, Plg: plasminogen and Col IV: type IV collagen. Recombinant protein attachment to those components was performed by ELISA. r*Pb*EF-Tu (5 μg ml^{-1}) was added per well. Controls were performed as follows: C1: without ECM or cascade coagulation components; C2: without r*Pb*EF-Tu; C3: ECM or cascade coagulation components and secondary antibody; C4: BSA 0.5%. '*' indicates statistically significant differences between the interaction rates, $P < 0.05$.

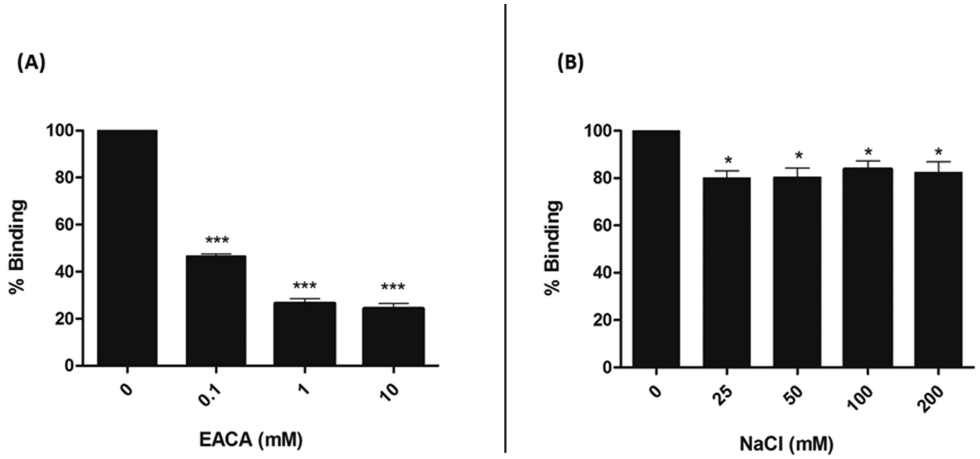


Figure 8. Role of lysine residues and salt in r*Pb*EF-Tu-plasminogen interaction. ELISA of EF-Tu (5 μg ml^{-1}) in the presence (0.1–10 mM) or absence of ε-aminocaproic acid (EACA) (**A**) and in the presence (25–200 mM) or absence of NaCl (**B**). Bound r*Pb*EF-Tu to plasminogen was detected with polyclonal anti-rPbEF-Tu antibody followed by peroxidase-conjugated anti-rabbit IgG. Each point represents the mean absorbance value at 492 nm ± standard deviation of three experiments performed in triplicate. (*** $P < 0.0001$, * $P < 0.05$).

or the participation of other factors that influence the EF-Tu-plasminogen interaction, such as ionic strength demonstrated using NaCl. The chloride anion promotes a closed conformation of plasminogen, which may adversely affect the plasminogen interaction with different proteins. Here, NaCl promoted a low reduction in the plasminogen-EF-Tu interaction, but this is not dependent on the salt concentration. The physiological context of these ionic interactions remains unclear (Salazar *et al.* 2014).

Several studies showed the ability of *P. brasiliensis* for adhesion and/or invasion (Mendes-Giannini *et al.* 2004; Andreotti *et al.* 2005). The adhesion/invasion properties displayed by the cell wall EF-Tu protein of *P. brasiliensis* were investigated by inhibition of the interaction with pneumocytes by the incubation of *P. brasiliensis* with the polyclonal anti-EF-Tu. The results showed that EF-Tu seems to play a role in the later stages (8 and 24 h of infection) of fungal infection.

The virulence factor identification is very important for understanding *P. brasiliensis* pathogenesis and interactions with the host, and these factors may also be used as novel targets in drug and vaccine development. Anti-virulence drugs are a new type of therapeutic drug that target virulence factors, potentially revitalising the drug-development pipeline with new targets. Rather than kill or halt pathogen growth, one emerging strategy is to 'disarm' pathogens by directly targeting virulence using anti-virulence drugs. As anti-virulence drugs are not designed to directly harm their targets, several papers have argued that they will have little effect on the fitness (that is, the net growth rate) of the pathogen in the host and therefore approach the ideal of an 'evolution-proof' drug that does not impose selection for resistance (Allen *et al.* 2014).

We showed for the first time the identification and characterisation of *P. brasiliensis* EF-Tu, a cell surface-localised protein that binds to plasminogen and fibronectin, is involved in the interaction with host cells, and plays an important role in the virulence of the fungi.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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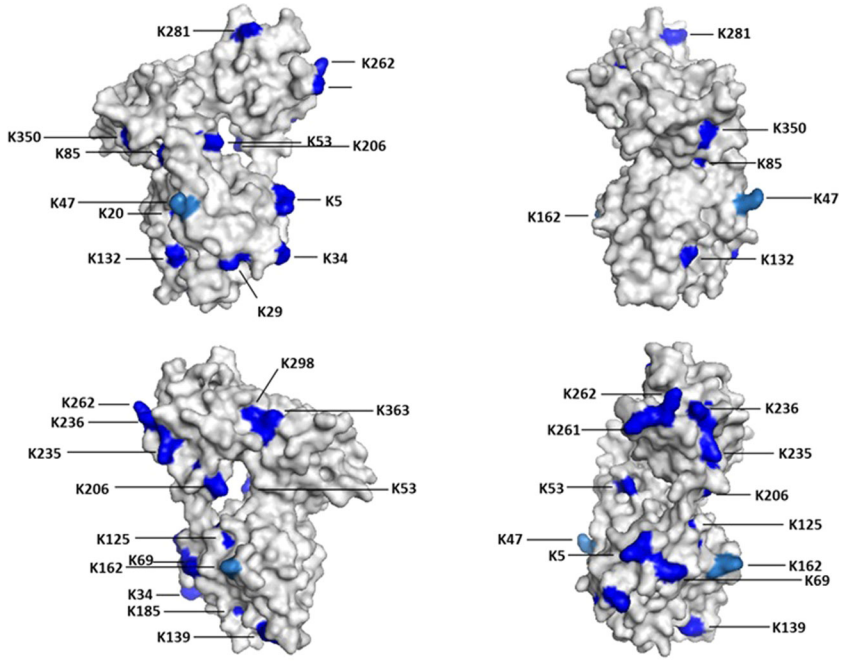

Figure 9. Theoretical model of *P. brasiliensis* EF-Tu in different rotation demonstrating lysine residues surface exposed. Conserved (blue) and non-conserved (sky blue, K47 and K162) lysine residues exposed in the surface. Figure was created using PyMOL, version 1.3 and is based on PDB file 1D2E:A

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Conflict of interest. None declared.

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