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# Molecular cloning, expression and insulin reduction activity of a thioredoxin 1 homologue (TRX1) from the pathogenic fungus *Paracoccidioides lutzii*

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ABSTRACT

The dimorphic fungi *Paracoccidioides* spp. are the etiological agents of paracoccidioidomycosis (PCM), a prevalent systemic mycosis in Latin America. The *Paracoccidioides lutzii* response to oxidative stress is largely unexplored. Thioredoxins (TRX) are involved in the regulation of the redox environment in the cell, responding to oxidative stress in several organisms. In this study, we describe the isolation and characterization of a cDNA encoding a thioredoxin 1 from yeast cells from *P. lutzii*. The cDNA codes for a 12 kDa protein containing the characteristic thioredoxin active site. The thioredoxin 1 gene was expressed in *Escherichia coli* and the isolated thioredoxin 1 recombinant protein as the native *Pl*TRX1 from yeast cells showed insulin reduction activity *in vitro*. We also showed by semi-quantitative RT-PCR analysis that the expression of thioredoxin 1 gene was induced in response to $H_2O_2$ and may exert an antioxidant activity *in vivo*. Our results suggest that the thioredoxin 1 may play an important role in controlling the redox status in *P. lutzii* which may contribute to this organism's virulence.



## 1. Introduction

The fungi of the genus *Paracoccidioides* are important fungal pathogens located predominantly in Central and South America. The complex *Paracoccidioides* spp are composed of two species: *Paracoccidioides lutzii* and *Paracoccidioides brasiliensis*, and are the causative agents of paracoccidioidomycosis (PCM) [1]. At room temperature, *Paracoccidioides* spp takes the form of filamentous mold (mycelia). The main route for infection is by inhalation of airborne fungal propagules. Generation of infectious arthrospores during fungal growth has been well documented [2]. Upon elevation of temperature to that of the mammalian body, the fungus adopts a yeast-like form that is associated with its pathogenic lifestyle [3].

Fungi, like many other organisms, rely on antioxidant defense mechanisms for protection against oxidative damage [4]. A prerequisite for the success of human pathogenic fungus is their ability to defend against reactive oxygen species (ROS) elicited by host cells in the course of an infection. ROS are extremely reactive and cause damage of cellular constituents, including DNA, proteins, lipids, leading to cellular death [5]. Organisms have therefore developed a series of antioxidant defense mechanisms to maintain and protect the cells against oxidative damage. Several genes encoding molecules involved in antioxidant defense have been identified in *Paracoccidioides* spp., such as those encoding for superoxide dismutase, peroxiredoxin, peroxidases, catalases and thioredoxin [6–9].

The thioredoxin system is composed of thioredoxin (TRX), thioredoxin reductase and nicotinamide adenosine dinucleotide phosphate (NADPH) as a proton donor. It is an important conserved system for protection against ROS by reducing peroxides such as $H_2O_2$ to harmless products. TRX is a small (around 12 kDa) ubiquitous protein characterized by a conserved active site sequence –Trp-Cys-Gly-Pro-Cys-Lys–, that function as protein-disulfite reductase. The two amino acid residues of cysteine in the active-site form a dithiol group, which is involved in the thiore-

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doxin reduction of disulfides in a number of proteins. Oxidized thioredoxin (TRX-$S_2$) contains a disulfide bridge that is reduced by NADPH through the catalytic action of the flavoenzyme thioredoxin reductase to its reduced state (TRX-$SH_2$) [10–12].

The antioxidant defense system of microorganisms comprises many different antioxidant molecules. However, little is known about what the specific roles for these molecules in *Paracoccidioides* spp. have, each one may be responsible for the oxidative stress response at a particular ROS or at a particular compartment. Although TRX from several organisms are well described, it had not been characterized in *P. lutzii*.

In this report, we described the molecular cloning and characterization of a cDNA (*Pl*trx1) encoding a thioredoxin 1 (*Pl*TRX1) from yeast *P. lutzii* cDNA library. We have also expressed *Pl*trx1 in *Escherichia coli* cells and demonstrated that recombinant TRX1 (rec*Pl*TRX1) efficiently reduces insulin as the native *Pl*TRX1 from yeast cells. The *Pl*TRX1 activity and the *Pl*trx1 expression from yeast cells in presence of $H_2O_2$ were higher. These results show the participation of *P. lutzii* TRX1 as a response against $H_2O_2$ suggesting that this later could be essential for fungal adaptation to host condition.

## 2. Materials and methods

### 2.1. Strains and growth conditions

*P. lutzii* (ATCC-MYA-826), was used in this study [1]. It was grown as yeast at 36 °C and subcultured every 7 days in semisolid Fava-Neto's medium [13] (1% weight in volume (w/v) peptone; 0.5% w/v yeast extract; 0.3% w/v proteose peptone; 0.5% beef extract; 0.5% w/v NaCl; 4% w/v glucose; and 1.4% w/v agar, pH 7.2) [14]. Mycelium was grown at 22 °C in this same medium, by subculturing every 15 days.

### 2.2. Analysis of yeast cells growth and differentiation of P. lutzii in presence of $H_2O_2$

Exponentially growing yeast cells ($6 \times 10^6$ cells $mL^{-1}$) of *P. lutzii* were tested for the growth after treatment with $H_2O_2$. Yeast cells grown in semi-solid Fava-Neto medium [13] for 7 days at 36 °C were transferred to liquid Yeast Extract-Peptone-Dextrose (YPD) medium (1% w/v Bacto-yeast extract, 2% w/v Bacto-peptone and 2% w/v dextrose). The cultures were treated with 25 mM of $H_2O_2$ for 3 h. After that, the cultures were centrifuged at $3000 \times g$ for 5 min and washed 3 times with $H_2O$ and cells inoculums of $5 \times 10^6$ cells $mL^{-1}$ were incubated in fresh YPD medium at 36 °C with shaking. A control culture without $H_2O_2$ was grown in parallel. Daily, during 7 days of incubation, 10 μL of each culture was collected and transferred to 1 mL of 0.9% NaCl, 2% formaldehyde, 4% Tween 20 solution and the yeast cells growth were estimated by counting in Neubauer chamber. Cell viability was determined using green Janus vital staining.

The conversion from mycelium phase to yeast phase was performed in YPD medium by changing the temperature from 26 to 36 °C. The mycelium culture was initially treated with 25 mM of $H_2O_2$ for 3 h at 26 °C. After this, the culture was centrifuged at $3000 \times g$ for 5 min and washed 3 times with $H_2O$. 100 μg of mycelium was added to fresh YPD liquid medium and incubated at 36 °C, with shaking. The differentiation was visualized by optic microscopy and evaluated daily for 15 days by counting in Neubauer chamber the yeast cells in culture. Both experiments, analysis of the growth and the differentiation were done in triplicate. The yeast cells growth and the mycelium to yeast transition data were also analysed by linear regression, and the results compared by the unpaired Student's *t*-test with significance set when $p \leq 0.05$. The values were expressed as a mean of triplicates.

### 2.3. Obtaining protein extracts and protein analysis

The protein extract was obtained as described previously [15]. Briefly, the treated and no treated yeast cells with 25 mM of $H_2O_2$ (Sigma), and mycelium protein crude extracts were obtained by disruption of frozen cells in the presence of protease inhibitors $N_\alpha$-tosyl-L-lysine chloromethyl ketone (TLCK) (50 μg/ml), 4-chloromercuribenzoic acid (1 mM), leupeptin (20 mM), phenylmethylsulfonyl fluoride (20 mM), and iodoacetamide (5 mM) in homogenization buffer (20 mM Tris–HCl, pH 8.8, 2 mM $CaCl_2$). The mixture was centrifuged at $12.000 \times g$ at 4 °C for 10 min, and the supernatant was used for further analysis of proteins by one-dimensional gel electrophoresis.

Polyacrylamide gel electrophoresis of native proteins and sodium dodecyl sulfate (SDS-PAGE) were performed according to Laemmli [16]. Protein quantification was performed according to Bradford protein assay [17].

### 2.4. Western blot analysis

The cellular extracts and the recombinant protein were submitted to one dimensional gel electrophoresis. The proteins were stained with Coomassie brilliant blue or electrophoretically transferred to nylon membranes. The membranes were stained by Ponceau-S to assess loading of equal amounts of protein and they were incubated in 0.05% (v/v) Tween-20 plus Tris-buffered saline (TBS) containing 1% (w/v) skim milk. The TRX1 protein was detected with a polyclonal antibody raised to the TRX1 of *E. coli* (Sigma) (1:1000 diluted). After reaction with alkaline phosphatase anti-rabbit IgG (Sigma) (1:2000 diluted), the reaction was developed with 5-bromo-4-chloro-3- indolylphosphate/nitroblue-tetrazolium (BCIP/NBT) (Sigma).

### 2.5. Cloning of the cDNA encoding the thioredoxin 1 homologue

For screening of cDNA library from yeast cells of *P. lutzii* a homologue fragment was generated by polymerase chain reaction (PCR). In PCR amplification this yeast cDNA library was used as a template and a partial fragment encoding the thioredoxin 1 was obtained. Based on the conserved sequence of the active site of the TRXs from several organisms and on the other conserved sequence on the carboxi-terminal region sense and antisense degenerated primers were respectively designed. Both primers sense (TRXI) 5′-TGGTGYGGNCCNTGYAAR-3′ and antisense (TRXII) 5′-TCNCGNTACGGNTGNAA*R*-3′ were used in the PCR that was conducted in a total volume of 50 μL. The resulting 140-bp product was subcloned into pGEM-T-Easy (Promega, Madison, WI). The sequence was determined on both strands by automated DNA sequencing, applying the DNA sequencing method of Sanger [18].

A yeast cDNA library was constructed in *Eco*RI and *Xho*I sites of Lambda ZAP II (Stratagene Inc., La Jolla, CA). The screening of this library was carried out with 140-bp probe labeled with [$\alpha^{32}$p]-dCTP. Plating $5 \times 10^6$ plaque forming units (pfu), DNA transfer to membranes and hybridization were performed as described in standard procedure [19]. Six positive clones were isolated and phage particles were released from the plaques. The *in vivo* excision of pBluescript $SK^+$ phagemids in *E.* coli XL1-Blue (MRF') cells was performed using the ExAssist/SOLR protocol from Stratagene. The nucleotide sequence was determined on both strands.

### 2.6. Sequencing of Pltrx1 cDNA and sequence analysis

Nucleotide sequence of the *Pl*trx1 was determined on both strands, using primer walking by the double-strand dideoxy-chain terminator method using a MegaBace 1000 sequencer (Amersham Biosciences, Little Chalfont, UK) for automated sequence analysis. The obtained sequences were translated and compared to all non-redundant polypeptides in the translated National Center for Biotechnology Information (NCBI) database using the BLAST algorithm [20]. Sequence analyses were carried out with the Genetics Computer Group (GCG) package (Madison, Wisconsin, USA) [21] with the PROSITE [22] and Pfam [23] databases. The values of similarity among protein sequences were based on the alignment of amino acid sequences taking into account conserved substitutions of residues [24]. The sequences analyzed were purchased from protein data Bank [25].

### 2.7. Comparison of PlTRX1 sequence and inferred phylogeny

The deduced amino acid sequence was aligned with putative homologous from GenBank database. Phylogenetic relationships of *Pl*TRX1 were carried out with the available TRX1 from the NCBI database. One phylogenetic tree was constructed by multiple sequence alignments using CLUSTAL X [24] and was generated by neighbor-joining method and visualized by TREE-VIEW software. Robustness of branches was estimated using 1000 bootstrapped replicates.

### 2.8. Expression of Pltrx1 in E. coli and recombinant protein purification

The *Pl*trx1 cDNA containing the complete coding region of TRX1 was amplified by PCR using sense (TRXS 5′-CAC**GAATTC**CAATGGTTGTCCAC-3′) and antisense (TRXAT 5′-CCC**CTCGAG**ACTTTCATCTAC-3′) oligonucleotides, which contained engineered *Eco*RI and *Xho*I restriction sites (bold), respectively. The amplification of the *Pl*trx1 was carried out to an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and 30 s and extension at 72 °C for 7 min. The 351-bp PCR product was digested with *Eco*RI and *Xho*I, separated by agarose gel electrophoresis, gel excised and subcloned into the *Eco*RI/*Xho*I sites of pGEX-4T-3 expression vector (Amersham Biosciences) to yield the pGEX-4T-3-trx1 construct. The recombinant plasmid was used to transform the *E. coli* BL21 Star (DE3) competent cells (Invitrogen) using the heat shock method [14]. To confirm that *Pl*trx1 was cloned in correctly orientation in vector, producing an in-frame molecule fused to glutathione *S*-transferase (GST), the complete sequencing of the plasmid was carried out. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM) was used to induce the recombinant protein expression. The recombinant *Pl*trx1 was expressed in the soluble form by the bacteria and the protein was purified by affinity chromatography under nondenaturing conditions, as previously reported [15]. The purity and size of the recombinant protein were evaluated by running the purified molecule on a 12% SDS–PAGE followed by Coomassie blue staining.

### 2.9. Insulin-disulfide reduction assay of recPlTRX1 and of native PlTRX1 from yeast cells

The insulin reduction catalysed by thioredoxin was measured spectrophotometrically at 650 nm at 25 °C as an increase in turbidity, as a result from precipitation of free insulin chain [26]. The assay mixture contained 100 mM potassium phosphate, 2 mM EDTA (pH 7.0), 0.13 mM insulin (0.75 mg $mL^{-1}$, Novo Nordisk) and 0.05 mg $mL^{-1}$ of protein extract of *E. coli* transformed with pGEX-*Pl*trx1 and protein extract of *E. coli* containing pGEX-4T-3 only, as a control. The reaction was initiated by addition of 0.33 mM dithiothreitol (DTT, Sigma) and the absorbance was recorded at 2 min intervals on spectrophotometer (Pharmacia Biotech, Utrospec 2000). Negative control was performed using DTT without rec*Pl*TRX1. The blank contained all components minus the DTT. Total volume reaction was 600 μL. The assay of TRX activity was also performed to the yeast cells protein extract of *P. lutzii* (0.05 mg $mL^{-1}$) cultivated in a medium absence and presence 25 mM of $H_2O_2$. These assays were performed in triplicate. The results were compared by applying statistical analyses (Student's *t* test). Significant differences were obtained when $p \leq 0.05$.

### 2.10. RNA isolation and semi-quantitative RT-PCR analysis

Total RNA from yeast cells after treatment with 25 mM $H_2O_2$ during 30 min and 1 h and from no treated yeast cells was isolated by Trizol method, according to manufactures instructions (Invitrogen, Carlsbard, CA, USA). To remove genomic contamination, the RNA samples were treated with RNase-free DNase-I (Invitrogen, Carlsbard, CA, USA) at 37 °C for 15 min, following standard procedures. RNA concentration and purity were determined spectrophotometrically and RNA integrity was visualized by electrophoresis.

Semi-quantitative reverse transcription polymerase chain reactions (sqRT-PCR) were performed for *Pl*trx1 gene to show the pattern of expression of this gene in presence of $H_2O_2$. Total RNA was extracted from *P. lutzii* yeast cells after treatment with 25 mM $H_2O_2$ for 30 min and 1 h and from *P. lutzii* no treated yeast cells, as described. First-strand cDNA was synthesized with 1 μg total RNA by reverse transcription using the SuperScript II reverse transcriptase (Invitrogen) in the presence of the synthetic oligonucleotide dT (Invitrogen). cDNA synthesis reaction was performed at 45 °C for 60 min cDNA was used for PCR in 50 μL reaction mixture containing trx1 sense (TRXS) and antisense (TRXAT) specific primers. Specific primers to ribosomal 28S of *P. lutzii* were used in PCR as internal control, sense and antisense, respectively: 5′-CGAAGACGGGATTCTCACC-3′ and 5′-CGGATCAGGTAGGGATACC-3′. PCR conditions were: 94 °C for 2 min; annealing at 50–65 °C for 2 min; 25–35 cycles at 72 °C for 1 min and 30 s, extension at 72 °C for 7 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure linear phase of amplification.

Amplicons were analyzed by agarose gels electrophoresis (1%). The analyses of relative differences were performed by using Scion Image Beta 4.03 program. Control reactions without RT and RNA were performed (data not shown). The RT-PCR product of *Pl*trx1 (351-bp) was gel-purified, subcloned into plasmid pGEM-T-Easy (Promega, Madison, WI, USA) and sequenced as described previously.

## 3. Results and discussion

### 3.1. Effect of $H_2O_2$ on yeast cell growth and on dimorphism of P. lutzii

*P. lutzii* presented a prominent activation of antioxidant enzymes and a rearrangement of the metabolism during oxidative stress [27]. The effect of $H_2O_2$ in yeast cells growth and in the mycelium to yeast transition was analyzed. Fig. 1A shows the results of yeast cell growth curve in presence of 25 mM $H_2O_2$ was affected and showed significant difference as compared to the control ($p \leq 0.05$). A protein band of 12 kDa corresponding to TRX1 was detected using polyclonal antibody against TRX1 from *E. coli* (Sigma) in both protein extracts blotted, yeast cells in absence and

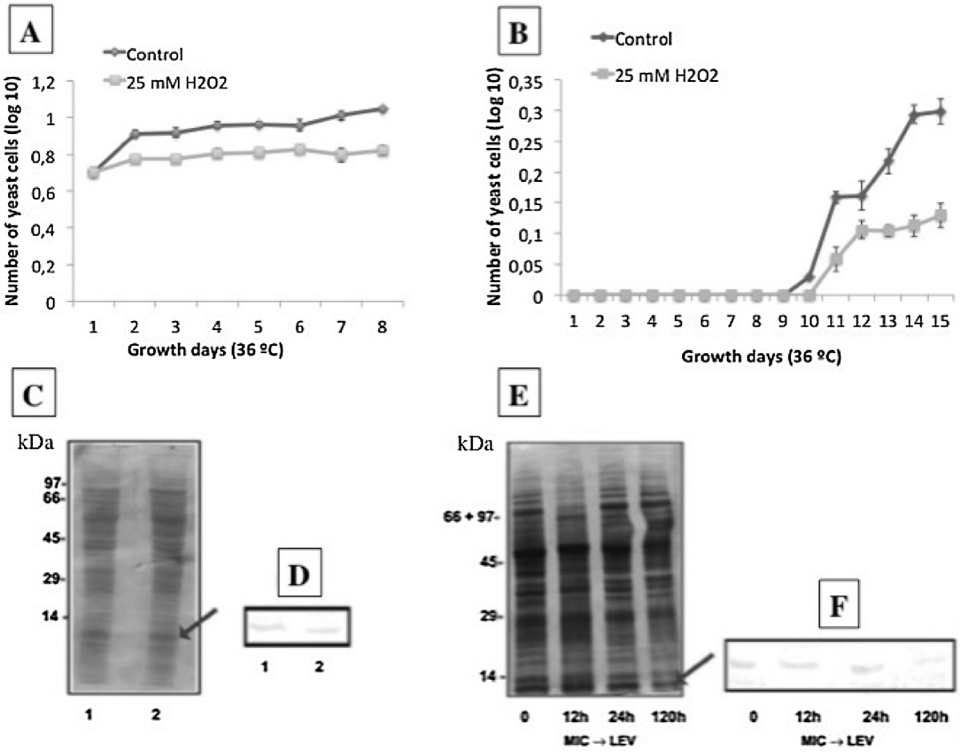


**Fig. 1.** Effect of $H_2O_2$ on yeast cells growth, on mycelium to yeast transition and on the protein patterns during the yeast cells growth and during the transition from mycelium to yeast of *P. lutzii*. (A) Yeast cells from logarithmic phase were treated with 25 mM $H_2O_2$ for 3 h at 36 °C. After this, they were washed and an inoculum's of $5 \times 10^6$ cells $mL^{-1}$ was resuspended in fresh YPD medium at 36 °C, with shaking. (B) Mycelium was incubated at 26 °C in presence 25 mM $H_2O_2$ for 3 h. After this treatment, the mycelium was washed and 100 μg of mycelium was transferred to fresh YPD medium and incubated at 36 °C, with shaking. Aliquots were taken (A and B) daily and yeast cells were counted in Neubauer chamber. The values were expressed as a mean of triplicate. (C) and (E) The proteins (20 μg) were fractionated by uni-dimensional gel electrophoresis (15% SDS-PAGE) and stained by Coomassie blue. (D) and (F) Western blot of total cell extract from yeast cells control (lane 1) and yeast cells treated with $H_2O_2$ for 3 h (lane 2) and from mycelium treated with $H_2O_2$ for 3 h and incubated at 36 °C at 0, 12, 24 and 120 h, respectively. The protein extracts (20 μg for each lane) were probed with the polyclonal antibody to TRX 1 from *E. coli*.

in the presence of 25 mM $H_2O_2$, though it wasn't possible to observe quantitative differences between these two conditions by western blot analysis (Fig. 1C and D). In addition, proteomic analysis of *Paracoccidioides* during oxidative stress induced by $H_2O_2$ revealed an activation of ROS detoxifying enzymes such as thioredoxin [7].

The cellular transition from mycelium to yeast was determined in 25 mM of $H_2O_2$ (Fig. 1B). The graphic shows that $H_2O_2$ affects morphological transition *in P. lutzii*. The culture treated with $H_2O_2$ showed a reduction of approximately 44%, by the 15th day of transition, in the percentage of yeast cells (relative to the control without treatment), and the result is statistically significant ($p \leq 0.05$). We also identified bands in western blot performed with protein extract obtained during differentiation, after 3 h of mycelium treatment with $H_2O_2$, that correspond to native *Pl*TRX1 (Fig. 1E and F). These results related to the expression of TRX1 during all differentiation process bringing insights about the role of this protein in dimorphism of *P. lutzii* during the oxidative stress caused by $H_2O_2$-treatment.

It is worth mentioning that this fungal has a house-keeping expression of anti-oxidant genes that maintain the level of ROS inside the tolerable pattern by the fungi [8]. To some pathogenic organisms the ROS may function as a stimulating factor to morphogenesis. Furthermore, it is known that cells in exponential phase growth are more sensitive to $H_2O_2$ than cells in the stationary phase growth [28] and when cells enter in the stationary phase there is an increase in the expression of the proteins in response to oxidative stress. Analyzes of the yeast cells growth from *P. lutzii* were performed from cultures in exponential phase, justifying the greater sensibility to the $H_2O_2$ treatment.

### 3.2. Isolation, sequence analysis of the Pl*trx1* cDNA and characterization of Pl*TRX1* deduced protein

To isolate the complete cDNA enconding the 12 kDa TRX1 of *P. lutzii*, we obtained a 140-bp PCR product using the sense (TRXI) and antisense primers (TRXII) (Fig. 2A). This PCR product showed homology with sequences of TRX from several microorganisms present in the database, besides showing 100% identity with the *P. lutzii* thioredoxin identified in the comparative genome project the *Paracoccidioides* genus (GenBank accession number XM_002795612) (data not shown). The entire cDNA (*Pl*trx1) was isolated by screening the yeast cDNA library of *P. lutzii*. The *Pl*trx1 sequence was 811-bp nucleotides in length, contained an open reading frame (ORF) of 351-bp, and the deduced amino acid sequence presented 116 residues with predict molecular mass and p*I* of 12 kDa and 5.2, respectively, which is similar in size to previously reported TRX. The ORF starts at an ATG codon and the first methionine at position 296 in the nucleotide sequence is in the context of an initiation codon [29]. The *Pl*trx1 presented 295 and 165 bases at the 5′ and 3′ untranslated regions, respectively. A polyadenyation signal ATAAAA was identified at 770 nucleotides in the downstream sequence of the cDNA [30] and it is followed by a t/gt-rich element [31]. The deduced *Pl*TRX1 presented one highly conserved motif –Trp-Cys-Gly-Pro-Cys- in the C-terminal region, described as a characteristic TRX active site at amino acids 41–45. The major feature of TRXs is the presence of two cysteine residues involved in this conserved catalytic site, which are critic for TRX activity. Two other cysteine residues out side of the catalytic site at positions 37 and 72 were identified in *Pl*TRX1 and they have been described as important in TRX function [32].

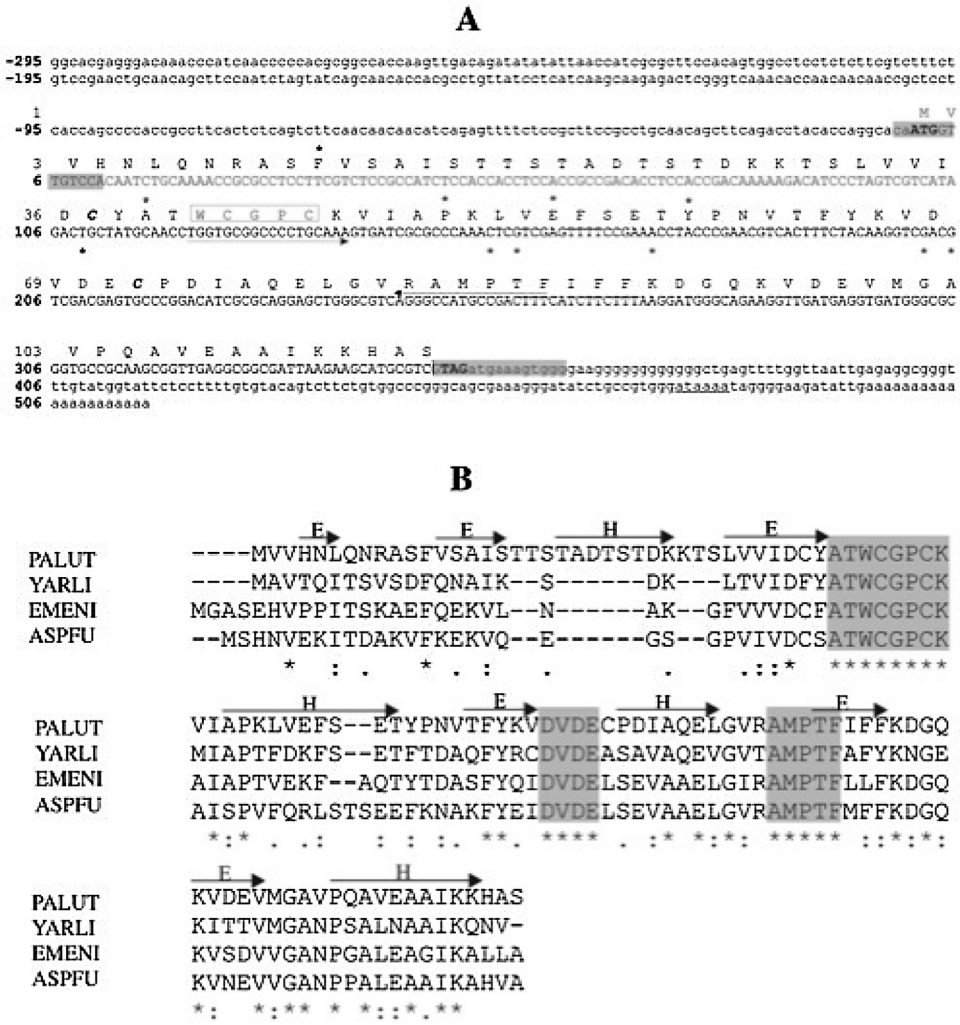


**Fig. 2.** (A) Nucleotide sequence and deduced amino acid sequence of the *P. lutzii* cDNA encoding the TRX1 homologue. Nucleotides are numbered in bold (left margin). Nucleotides 5′ to the initiation codon (bold) are indicated by negative numbers. The presumptive start and stop codons are in bold. The putative polyadenilation signal ATAAAA is underlined. Numbering of the amino acid sequence begins at the methionine encoded by the first initiation codon and ends at the first termination codon, both in bold. The deduced amino acid sequence is shown above the nucleotide sequence (single letter code). The amino acids potentially associated to catalysis are boxed. The structural important residues present in majority of TRXs1 sequences are indicated by asterisks. The two extra cysteine residues outside catalytic site are in italic and bold. Arrows mark TRXI and TRXII oligonucleotides, and gray boxes mark TRXS and TRXAT oligonucleotides. **(B)** Alignment of deduced *Pl*TRX1 and related sequences of fungi TRXs to produce the secondary structure alignment. The alignment was performed by using the CLUSTAL X program. *Pl*TRX1 (PARBR) was aligned with those from YARL1, *S. cerevisiae* (GenBank accession number Q6C399); EMEN1, *Emericella nidulans* (Genbank accession number Q5BH10); ASPFU, *Aspergillus fumigatus* (GenBank accession number Q4WV97). The letters H and E above the TRX sequence correspond to regions of α-helix and β-strand, respectively. Positions in which a gap was introduced to optimize homology are shown as dashes. The gray boxes show the highly conserved residues amino acids among these fungi TRXs1. The important amino acid residues to the secondary structure pattern are in italics. The amino acids potentially associated to catalysis are in bold. Asterisks (*) define identity, two points (:) define conservative substitutions and one point (.) define semi conservative substitutions.

From the sequence analysis we also observed that *Pl*TRX1 has all structurally important amino acids (Fig. 2A). These include, besides the active center consensus sequence, structural important residues such as Phe12, Ala39, Pro50, Glu54, Tyr59, Asp70, Pro85, Thr86, Lys91, Gly101 and Ala102 corresponding to Phe27, Ala29, Pro40, Glu44, Tyr49, Asp61, Pro76, Thr77, Lys82, Gly92 and Ala93 in the *E. coli* TRX sequence [33]. Furthermore, *Pl*TRX1 sequence is not preceded by transit peptides as has been described for some TRXs [34].

### 3.3. Comparison of *Pl*TRX1 deduced amino acid sequence and phylogenetic analysis

A search performed in FASTA on the GenBank database showed a high number of identical and conserved amino acids among TRX sequences from both prokaryotic and eukaryotic microorganisms. By comparison to fungi TRX sequences, the secondary structural elements of *Pl*TRX1 were mapped by using the Jpred 4 program (http://www.compbio.dundee.ac.uk/jpred/) [35]. The alignment of the *Pl*TRX1 with these sequences was performed using the Clustal X program [24] (Fig. 2B). The analysis showed that *Pl*TRX1 shared with other microbial TRXs a secondary structure characteristic of the open twisted alpha/beta (5 beta-sheets associated in a parallel and anti parallel manner and surrounded by 4 helices). Five proline and three glycine residues out site catalytic region are common residues in or close to bends. They are highly conserved in TRXs and fulfill this function [36] and these same residues were observed in *Pl*TRX1 sequence. The region corresponding to TRX catalytic site presented 100% of identity in all sequences aligned. Besides this conserved site, two other regions, –Asp-Val-Asp-Glu- and Ala-Met-Pro-Thr-Phe- showed high degree of identity.

The interaction between TRX1 and its substrate proteins has been suggested the involvement of the Cys-Gly-Pro-Cys active site and several residues, including *cis*-Pro76 and Gly92, which form a

moderately hydrophobic surface around the active site facilitating interactions with other enzymes [37]. The corresponding residues in *Pl*TRX1 deduced sequence are Pro85 and Gly102, which maybe could present the same functions in the *Pl*TRX1 interactions with its substrate. Among the several conserved amino acid residues in *E. coli* TRX1, Asp59 and Gly84 are considered as contributing to the right folding of the protein, whereas the *cis*-Pro76 was described to interact with the active site in the 3-D structure of TRX [36]. We could observe these same amino acid residues in *Pl*TRX1, Asp68 and Gly93 and *cis*-Pro85. The aspartic amino acid residue (Asp26) in *E. coli* TRX1 might participate in the *E. coli* TRX interaction with its substrate [36]. *Pl*TRX1 also has an Asp amino acid residue at position 36 which corresponds to the only negatively charged residue in the interior of the molecule as occurs in *E. coli* TRX1 [12].

The NCBI database was accessed to search complete protein sequences of thirty-one TRXs1 from several organisms. The TRXs1 sequences were downloaded and aligned using the multiple-alignment, a neighbor-joining tree was constructed to estimate the distances among these TRX sequences. To visualize the relationship between *Pl*TRX1 and other TRXs1 in terms of amino acid sequence similarity, a phylogenetic tree was constructed (Fig. 3). In the drawen phylogenetic tree *P. lutzii* was enclosed in the fungi clade together with *Neurospora crassa*.

### 3.4. Expression and purification of the recombinant Pl*TRX1*

The cDNA encoding the *P. lutzii* TRX1 was subcloned into the expression vector pGEX-4T-3 to obtain the recombinant fusion protein. After induction with IPTG, a 38-kDa recombinant protein was detected in bacterial lysates (Fig. 4A, lane 4). The protein was not present in extracts from control cells. *E. coli* BL21 cells harboring the expression vector empty harvested before and after induction (Fig. 4A, lane 1 and 2) or in extracts from not induced *E. coli* cells carrying the expression vector pGEX-4T-3-trx1 (Fig. 4A, lane 3). The fusion protein bound to glutathione-sepharose (Lane 5) was blotted and reacted with TRX1 polyclonal antibody of *E. coli* (Lane 6). The predicted molecular size of the recombinant protein was 38 kDa, which included the vector-encoded fusion peptide of 26 kDa at its N-terminus.

### 3.5. Reductive activity of recombinant Pl*TRX1* and native Pl*TRX1* from yeast cells extract

The biological activity of rec*Pl*TRX1 protein was evaluated by insulin reduction assay, where the precipitation of $\beta$-chain of insulin was dependent on the time of incubation [26]. All TRXs reduce disulfide bonds, in this way exerting their major anti-oxidant function. To show that *Pl*TRX1 functions as an oxidoreductase, we analyzed the redox activity of the rec*Pl*TRX1 and of the native *Pl*TRX1 from yeast cells protein extract by the insulin reduction assay. In the absence of rec*Pl*TRX1 (DTT), increase in the turbidity wasn't observed during the first 50 min of incubation (Fig. 5A). In contrast, the presence of purified recombinant *Pl*TRX1 increased the rates of insulin reduction (Fig. 5A), which was detectable in the first minute of incubation. It exhibited approximately 3-fold higher activity than the negative control (DTT). The results indicate that the GSH fused to TRX didn't affect rec*Pl*TRX1 activity. We also observed that the insulin reduction for yeast cells increased when the cells were treated with $H_2O_2$ during 3 h (Fig. 5B). In fact, when was compared the treated and no treated cells, these latter showed a low activity (control). Additional experiment was performed to prove that this reduction activity was caused by *Pl*TRX1 and not by other *P. lutzii* reductases present in yeast protein extract. Thus, the yeast protein extracts were incubated with the antibody against *E. coli* TRX1 (Sigma) and the insulin reduction was measured. The treated ($H_2O_2$) extract that before

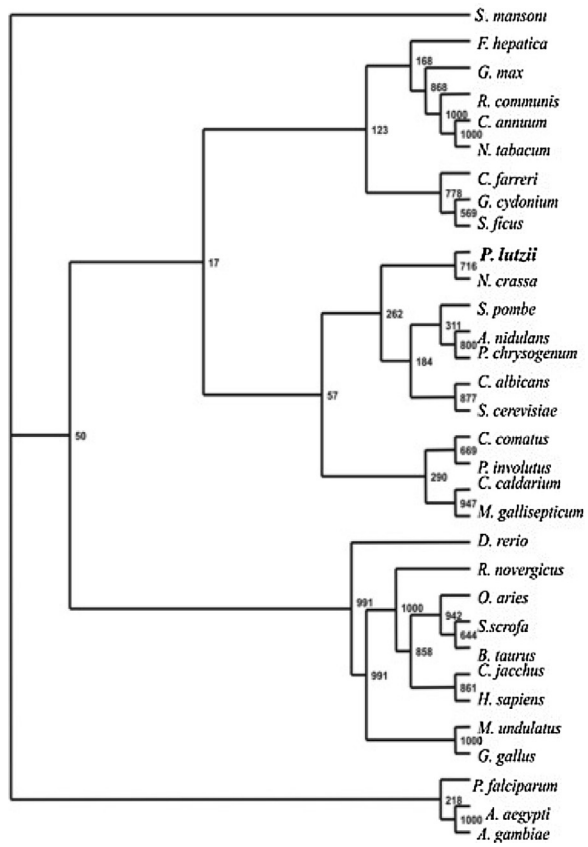


**Fig. 3.** The phylogenetic analysis was based on amino acid sequences of several TRXs1. The tree was calculated by neighbor-joining method implement in the program Clustal X and drawn by using the program Tree View software. The numbers of the branches are bootstrap values obtained with 1000 replications and indicate the percentage of times all species to the right appear as a monophyletic cluster. The TRX1 amino acids sequences described at database were used for construct tree: *Schistosoma mansoni* (GenBank accession number AAL79841); *Fasciola hepatica* (GenBank accession number AAF14217); *Glycine max* (GenBank accession number AAS88427); *Ricinus communis* (GenBank accession number CAA94534); *Capsicum annuum* (GenBank accession number AAR83852); *Nicotiana tabacum* (GenBank accession number CAA41415); *Chlamys farreri* (GenBank accession number AAV73827); *Geodia cydonium* (GenBank accession number CAA76654); *Suberites ficus* (GenBank accession number CAG25528); *Neurospora crassa* (GenBank accession number AAK07845); *Schizosaccharomyces pombe* (GenBank accession number AAF05765); *Aspergillus nidulans* (GenBank accession number AAB24444); *Penicillium chrysogenum* (GenBank accession number CAA53726); *Candida albicans* (GenBank accession number XP_719372); *Saccharomyces cerevisiae* (GenBank accession number CAA97572); *Coprinus comatus* (GenBank accession number CAB52130); *Paxillus involutus* (GenBank accession number AAS19462); *Cyanidium caldarium* (GenBank accession number CAA79820); *Mycoplasma gallisepticum* (GenBank accession number AAF19044); *Danio rerio* (GenBank accession number AAH49031); *Rattus novergicus* (GenBank accession number AAH58454); *Ovis aries* (GenBank accession number NP_001009421); *Sus scrofa* (GenBank accession number NP_999478); *Bos taurus* (GenBank accession number AAC83380); *Callithrix jacchus* (GenBank accession number AAK30295); *Homo sapiens* (GenBank accession number AAF87085); *Melopsittacus undulatus* (GenBank accession number AAO72714); *Gallus gallus* (GenBank accession number NP_990784); *Plasmodium falciparum* (GenBank accession number NP_702434); *Aedes aegypti* (GenBank accession number AAK70900); *Anopheles gambiae* (GenBank accession number AAF68382).

of the incubation with antibody showed higher activity than the reaction with only DTT, it had lower activity after antibody incubation (Fig. 5B). The effect of the antibody in the enzymatic assays of *P. lutzii* showed that the greater activity in yeast extract was due to *Pl*TRX1. So, these assays demonstrated the functionality of this system in *P. lutzii* oxidative stress response and that purified recombinant protein is a TRX and the most important it is catalytically active. Our results suggested that this protein has the TRX-like

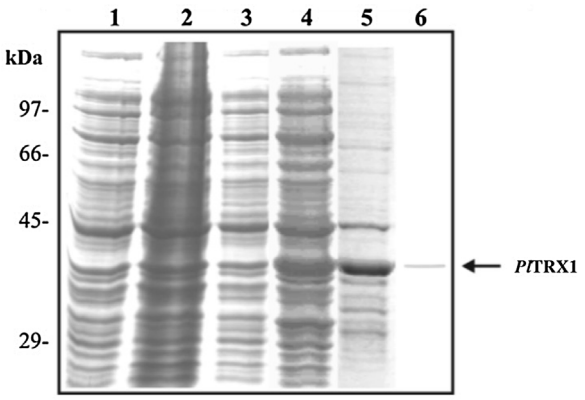


**Fig. 4.** SDS-PAGE and western blotting analysis of the 38 kDa recombinant protein expressed by *E. coli* transformed with *Pl*trx1 construct. *E. coli* BL21 cells harboring pGEX-4T-3 (lane 1 and 2) and pGEX-4T-3-trx1 (lane 3 and 4) were grown at 30 °C to an $A_{260}$ of 0.7 and harvested before (lane 1 and 3) and after (lane 2 and 4) a 3 h of incubation with 0.1 mM IPTG. Lane 5, the affinity-isolated recombinant GST-TRX1. Electrophoresis was carried out on 15% SDS-PAGE and the proteins were stained by Coomassie blue R-250. Lane 6, western blotting of the recombinant GST-TRX1 protein. The recombinant GST-TRX1 protein were run in parallel, blotted onto nitrocellulose membrane, and was detected by using polyclonal antibody to TRX 1 from *E.* coli (Sigma). Arrow indicates the recombinant GST-TRX1 protein. Molecular Markers are indicated on the left (Amersham Biosciences).

activity and this redox feature is reported for playing an important role in the cellular defense against oxidative stress [38].

### 3.6. Analysis of the expression of Pl*trx1*

The expression of the *Pl*trx1 gene was investigated in yeast cells in $H_2O_2$ presence. The transcription rates of many yeast genes are increased in response to diverse environmental and physiological insults, such as nitrogen starvation, osmotic shock, heat shock, metals and oxidative stress [39–41]. The transcription of *Pl*trx1 gene was analyzed by sq-RT-PCR. The sq-RT-PCR was carried out using total RNA from treated (25 mM $H_2O_2$ for 30 min and 1 h) and no treated (control) yeast cells. To evaluate that the obtained results are representative of the real condition which the yeast cells were submitted and not due to the differences in RNA contents, the same procedure was performed using an internal control, ribosomal RNA 28S from *P. lutzii*. Fig. 6 shows the amplification of 351-bp fragments from RT-PCR using TRX1 specific primers and of 223-bp from RT-PCR using 28S ribosomal primers. As shown in Fig. 6, the yeast cells treated with $H_2O_2$ showed a level of *Pl*trx1 expression higher than the no treated yeast cells and increase wasn't observed in 28S ribosomal transcript by analyses using Scion Image Beta 4.03 program. We also sequenced the 351-bp products and compared the sequences obtained with *Pl*trx1 (data not shown), in fact these products presented 100% of homology with *Pl*trx1 sequence.

Several studies have emphasized that the maintenance of redox homeostasis is crucial not only for many biological processes such as differentiation, regulation of specific genes, signaling pathways, but also for survival of pathogens in mammalian hosts [42–44]. Thioredoxins were more important than glutaredoxins in mediating resistance to oxidative stress caused by hydroperoxides [28]. They reported that the overexpression of TRX1 and TRX2 in *Saccharomyces cerevisiae* was found to increase the resistance of wild-type strain to $H_2O_2$. Interestingly, *P. lutzii* showed an increase in *Pl*trx1 expression when treated with $H_2O_2$, suggesting that in this fungus thioredoxins may play a key role in protection against oxidative stress, as in *S. cerevisiae*. In order to analyze if the level of *Pl*trx1 transcripts was changed by yeast cells treatment with $H_2O_2$, processed RNA from exponential yeast cells of *P. lutzii* treated with $H_2O_2$ was used in the sqRT-PCR reaction. The occurrence of a higher amplification of *Pl*trx1 in cells submitted to $H_2O_2$ treatment shows that this transcript could be very important to the *P. lutzii* in response to oxidative stress. Studies performed with *S. cerevisiae* about the expression of genes encoding the components of the thioredoxin system, thiol-specific antioxidant (TSA1), TRX2 and thioredoxin reductase 1, showed that these genes were induced in response to $H_2O_2$ [45–48]. It showed a rise in the level of thioredoxin mRNA when compared to the mRNA involved in glutathione biosynthesis, suggesting that the induction of the thioredoxin system occurs before other systems related to oxidative stress response. It is interesting to mark that the yeast AP-1-like transcription factor (Yap1p) the major regulator of *S. cerevisiae* response to $H_2O_2$ [46,49] was also described in *Paracoccidioides* spp. transcriptome [8], suggesting that this fungus has similar mechanism of response to $H_2O_2$. Our results demonstrated another existence of similarity between the antioxidant systems from *P. lutzii* and *S. cerevisiae* related to the conserved *Pb*TRX1 secondary structure pattern.

## 4. Conclusions

This is the first study to report gene cloning and expression of thioredoxin 1 from the *Paracoccidioides lutzii*. The thioredoxin system plays a central role in redox control by regulating the activity of transcription factors and enzymes and protecting cells against oxidative stress. The trx1 gene from *P. lutzii* was cloned, expressed and the purified protein showed insulin reduction activity *in vitro*. We also showed that the expression of thioredoxin 1 gene was induced in response to $H_2O_2$ and may exert an antioxidant activity *in vivo*. Our results describe the importance of TRX1 for the main-

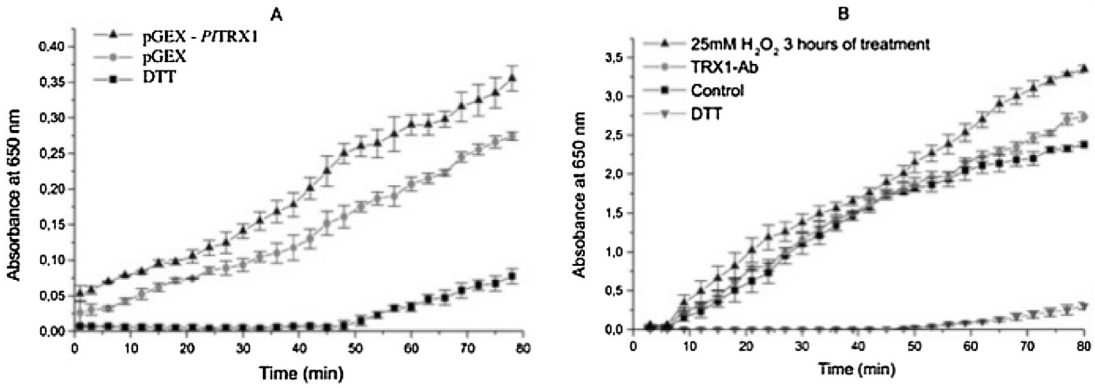


**Fig. 5.** rec*Pl*TRX1 and native *Pl*TRX1 catalyzes the reduction of insulin. The increase in turbidity measured at 650 nm was plotted against reaction time. The conditions under which the catalytic activity of TRX1 was measured are presented in the text. DTT was used as a negative control. (A) – Insulin reduction by rec*Pl*TRX1. (B) – Insulin reduction by native *Pl*TRX1 from yeast cells protein extract. The values represent the media and standard deviations of three independent experiments.

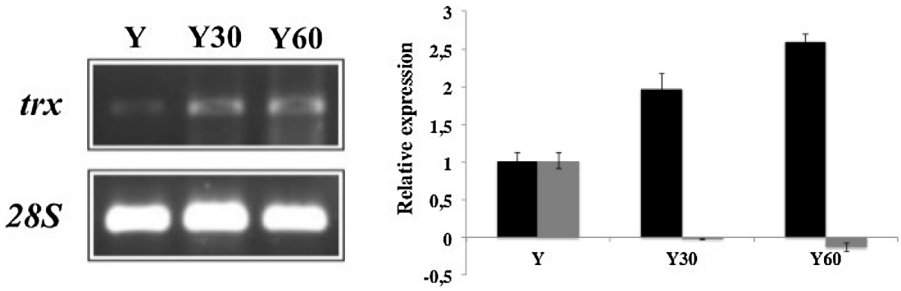


**Fig. 6.** Semi-quantitative RT-PCR analyses for *Pl*trx1 of yeast cells exposure to $H_2O_2$. Semi-quantitative RT-PCR analysis was carried out with specific oligonucleotides sense and antisense to *Pl*trx1 and r28S, as described. Numbers associated with the bars indicate fold differences relative to the data for the reference *in vitro* cultured yeast cells, which were established by densitometry analysis. Using varied cycle numbers, the exponential phase of primer was determined and used to allow semi-quantitative analysis of the respective reactions. The same amounts of cDNAs were used for all PCRs. The RNAs used for RT-PCR were obtained from an independent sample of *in vitro* cultured yeast cells and from sample *in vitro* cultured yeast cells incubated with 25 mM $H_2O$ for 30 min and 1 h. The sqRT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The sizes of the amplified DNA fragments from *Pl*trx1 and from r28S are written on the right side of the figure. The RNAs samples were obtained from: yeast cells, *in vitro* cultured (Y); yeast cells incubated with 25 mM $H_2O_2$ for 30 min (Y 30); yeast cells incubated with 25 mM $H_2O_2$ for 1 h (Y60). Ribosomal 28S was used as an internal control.

tenance of the redox homeostasis in *P. lutzii* controlling the redox status during yeast growth and oxidative insults. Further studies are needed in order to elucidate the possible implications of TRX1 in oxidative stress response in *P. lutzii* and the molecular mechanism of action of TRX1.

## Competing interests

The authors have declared that no competing interests exist.

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