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The catalases of *Paracoccidioides brasiliensis* are differentially regulated: Protein activity and transcript analysis

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ABSTRACT

Paracoccidioides brasiliensis is a fungal pathogen of humans. The *P. brasiliensis* response to oxidative stress is largely unexplored. We report the analysis of three catalases, *Pb*CatA, *Pb*CatP and *Pb*CatC. The former are monofunctional catalases and the latter is a catalase-peroxidase. Differential expression of catalases as measured by activity and by quantitative analysis of transcripts was observed in the morphological conversion and in response to different stress conditions. *Pb*CatA manifested higher activity in the mycelial phase, showed increased activity during transition from mycelium to yeast and during conditions of endogenous oxidative stress. Consistent with our previous studies, *Pb*CatP manifested higher activity in yeast cells since it is putatively involved in the control of exogenous reactive oxygen species. *P. brasiliensis* displays an oxidative stress response following phagocytosis by macrophages, inducing the expression of catalase A and P transcripts. *Pb*CatC displayed a relatively constant pattern of expression, being modestly induced in cells exposed to osmotic and heat stress.

1. Introduction

Paracoccidioides brasiliensis is an important fungal pathogen located predominantly in Central and South America. The fungus is the causative agent of paracoccidioidomycosis (PCM). At room temperature, *P. brasiliensis* 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 (Bustamonte-Simon et al., 1985). Upon elevation of temperature to that of the mammalian body, the fungus adopts a yeast-like form that is associated with its pathogenic lifestyle (Restrepo et al., 2001).

Fungi, like many other organisms, rely on antioxidant defense mechanisms for protection against oxidative damage (Giles et al., 2006). 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 (Halliwell and Gutteridge, 1999). 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 *P. brasiliensis*, such as those encoding for superoxide dismutase, peroxiredoxin, peroxidases, thioredoxin and catalases (Moreira et al., 2004; Campos et al., 2005).

Several families evolved in the ancestral genomes capable of H_2O_2 dismutation. The most abundant are heme-containing enzymes that are divided in two main groups, typical or monofunctional catalases (E.C. 1.11.1.6) and catalase-peroxidases; both types of heme enzymes exhibit high catalase activities (Zamocky et al., 2007). The proteins contribute to the pathogenesis of several microorganisms, including fungi as the oxidative stress response in the virulence of fungi has been emphasized by the observation that mutations that inactivate such responses attenuate the virulence of the pathogens (Wysong et al., 1998; Paris et al., 2003).

Our laboratory had previously characterized a small-subunit monofuctional catalase in *P. brasiliensis*, named catalase P (*Pb*CatP). The protein was characterized as an immunodominant antigen of *P. brasiliensis* reacting with sera from PCM patients (Fonseca et al., 2001). *Pb*CatP is expressed at high levels in the fungus yeast phase and is positively regulated in the transition from mycelium to yeast and in contact of the cells with H_2O_2 (Moreira et al., 2004). Additionally, some oxidative stress functions are induced when *P. brasiliensis* is induced to differentiate to yeast cells (Bastos et al., 2007), when yeast cells are exposed to human plasma (Bailão et al., 2007), or when yeast cells infect macrophages (Silva et al., 2007). This observation is consistent with the idea that *P. brasiliensis*

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must cope with reactive oxygen species in the course of development and infection.

In the present study we sought to identify novel classes of *P. brasiliensis* catalases and to elucidate the contribution of such molecules to the *P. brasiliensis* antioxidant defense system. We characterized three members of the *P. brasiliensis* catalase family that are differentially expressed on dependence of the stress condition. Our results are consistent with the hypothesis that *Pb*CatA can mainly play a role in the protection of *P. brasiliensis* against endogenous stress, while *Pb*CatP could preferentially protect fungal cells against exogenous stress. The complete cDNA sequence of the *PbCATA* was obtained.

2. Materials and methods

2.1. Cloning of the cDNA encoding catalase A and sequence analysis

A *P. brasiliensis* yeast cells cDNA library was constructed in EcoRI and XhoI sites of λ ZapII (Stratagene, La Jolla, CA, USA), as described (Moreira et al., 2004). The screening of the library was performed using a partial cDNA encoding catalase A obtained from a cDNA library of yeast cells treated with human plasma (Bailão et al., 2007). Plating 5×10^6 plaque forming units (p.f.u), DNA transfer to membranes and hybridization were performed as described in standard procedures (Sambrook and Russel, 2001). Two positive clones were obtained and phage particles were released from the plaques. DNA sequencing was performed by the double-strand dideoxy-chain termination method (Sanger et al., 1977) by using a Mega BACE 1000 sequencer (GE Healthcare, Chalfont St Giles, UK) for automated sequence analysis. The cDNA was translated and compared to all non-redundant polypeptides in the translated NCBI (http://www.ncbi.nlm.nih.gov) database. The alignment of *PbCATA* with other catalases from fungi was generated with Clustal X software (http://www.ebi.ac.uk/clustalx/). The PROSITE (http://www.us.expasy.org/prosite), Pfam (http://www.sanger.ac.uk/sftwer/pfam/index.shtml) and Sprint (http://umber.sbs.man.ac.uk/dbbrowser/sprint/) algorithms were used for the analysis of the deduced protein.

The complete cDNA (*PbCATA*) presents 2619 nucleotides (Fig. 1, Supplementary material). The open reading frame is 2277 bp in length and codes for a 759 amino acids protein (GenBank Accession No. AAR87484).

Appreciable sequence similarities were found between the predicted ORF product and known catalases A, as shown in Supplementary Fig. 1. The translated amino acid sequence predicted a protein with a molecular mass of 84 kDa and pI of 6.12. Sequence analysis of the deduced *P. brasiliensis* *Pb*CatA, revealed the seven-element fingerprint of the catalase protein family. Foremost, both catalase consensus patterns are present: the active site at position 82–98 and at position 390–398 the proximal heme binding signature. Canonical motifs of catalases are depicted in Fig. 1, Supplementary material.

2.2. Paracoccidioides brasiliensis isolate and growth conditions and cellular extracts preparation

The *P. brasiliensis* isolate *Pb*01 (ATCC MYA-826) has been investigated by our laboratory and was cultivated in Fava-Neto's medium (1% w/v peptone, 0.5% w/v yeast extract, 0.3% w/v proteose peptone, 0.5% w/v beef extract, 0.5% w/v NaCl, 1% w/v agar, pH 7.2) at 36 °C for the yeast form and at 22 °C for the mycelium phase. For some experiments the fungal cells were transferred to the medium of McVeigh and Morton (MMcM) with modifications (15.0 mM glucose, 10.0 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13.0 mM glycine, 3.0 μM vitamin B1, pH 6.3) (Restrepo and Jiménez, 1980) or YPD medium (0.5% w/v yeast extract, 0.5% w/v casein peptone and 1.5% w/v glucose, pH 6.3). The cellular extracts were prepared as described (Fonseca et al., 2001). Yeast and mycelium protein crude extracts were obtained by disruption of frozen cells in the presence of proteases inhibitors *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (50 μg/mL), 4-chloromercuribenzoic acid (1 mM), leupeptin (20 mM), phenylmethylsulfonyl fluoride (20 mM) and

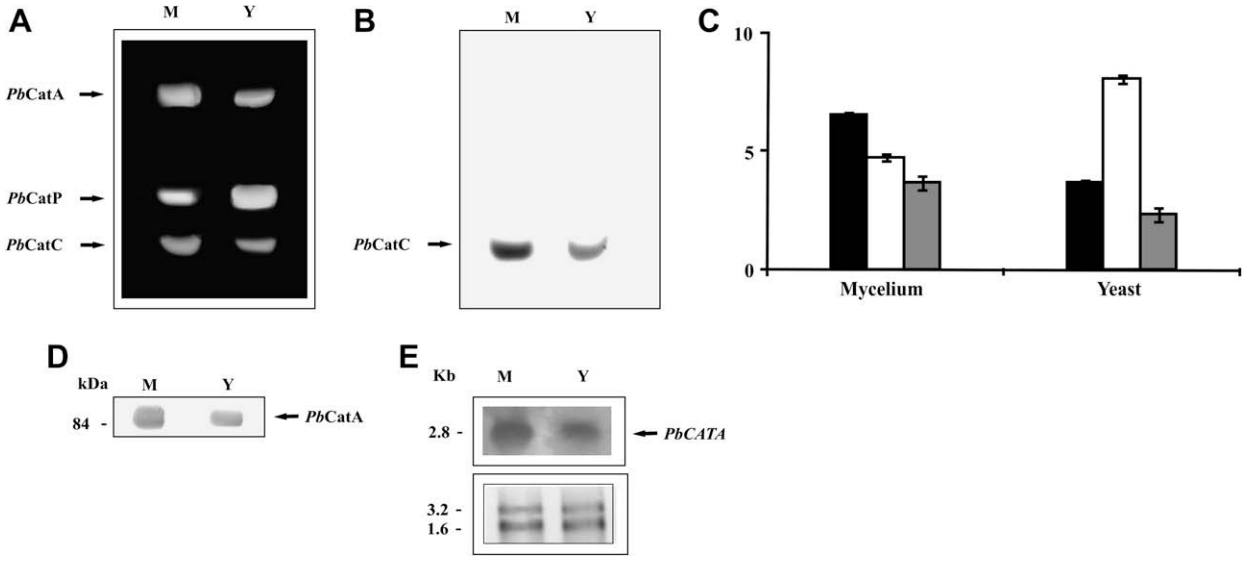

Fig. 1. Expression analysis of catalases in *P. brasiliensis*. (A) Activity of catalases in *P. brasiliensis* mycelium and yeast cells. Protein extracts from mycelium (M) and yeast (Y), 30 μg, were separated on a 10% acrylamide gel under nondenaturing conditions. Catalase activity was visualized by potassium ferricyanide-negative staining. (B) Catalase-peroxidase expression was visualized by reaction to *o*-dianisidine substrate. (C) Catalase expression was quantified. Three catalase species were detected, *Pb*CatA (black), *Pb*CatP (white) and *Pb*CatC (gray). Bars represent the standard deviation of three independent experiments. (D)Western-blot analysis of *P. brasiliensis* protein extracts. Protein extracts from mycelium (M) and yeast (Y) were obtained and equal amounts (30 μg) were fractionated on a 10% SDS–PAGE, blotted to a membrane and detected by using the antibody anti-Cat 1 of *N. crassa* (1:6000 diluted). (E) Northern blot analysis of *PbCATA* transcript. Ten micrograms of total RNA from mycelium (M) and yeast (Y) was fractionated and hybridized to the complete cDNA of catalase A. The rRNAs from the ethidum-bromide stained agarose gel were used for the loading control.