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Differential *PbP27* expression in the yeast and mycelial forms of the *Paracoccidioides brasiliensis* species complex

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ABSTRACT

p27 is an antigenic protein produced by *Paracoccidioides brasiliensis*, the etiologic agent of paracoccidioidomycosis (PCM). Despite its unknown function, it has been suggested as a putative virulence factor, proposed as a suitable target for the design of diagnostic tools and vaccines, and considered as an enhancer in antifungal treatment of PCM. We evaluated sequence polymorphisms of *PbP27* gene sequence among isolates, finding some polymorphisms associated with the isolates' phylogenetic origin. In order to determine if there was a differential expression pattern between morphological states and among isolates, we also evaluated *PbP27* expression, at transcriptional and translational levels, in mycelia and yeast cultures in 14 isolates belonging to the *P. brasiliensis* species complex (S1, PS2, PS3, and "*Pb01-like*", proposed to be named *Paracoccidioides lutzii*) by two techniques, real time RT-PCR (RT-qPCR) and protein dot blot. For the latter, four protein extracts from different cell localizations (SDS or β-mercaptoethanol, cytoplasmic and extracellular proteins) were analyzed for each isolate. p27 was present in the four extracts evaluated, mainly in the SDS extract, corresponding to an extract containing proteins loosely attached to the cell wall. This information correlates with immunohistochemical analysis, where positive staining of the yeasts' cell wall was observed. We found that p27 was present in all isolates, mainly in the yeast form. This pattern was corroborated by RT-qPCR results, with higher expression levels found in the yeast form for most of the isolates. The results provide new insights into the expression patterns of this protein, and further characterize it in view of potential uses as a diagnostic and/or therapeutic tool.

1. Introduction

Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America, with a restricted geographic distribution ranging from Mexico to Argentina, excluding some countries as Nicaragua, Chile, Guyana and the Caribbean islands (Brummer et al., 1993; Restrepo et al., 2001). Usually this infection is asymptomatic, affecting males and females at the same rate (Brummer et al., 1993). However, the clinical form is in 90% of the cases a chronic disease with higher incidence in males, characterized by primary lung compromise that can disseminate to mucosae, skin and other organs. The remaining 10% of the cases, usually described in children and young persons, are characterized by an acute/subacute form with compromise of the reticuloendothelial system (Borges-Walmsley et al., 2002). In absence of treatment, this disease is mostly fatal (Borges-Walmsley et al., 2002; Brummer et al., 1993).

The etiologic agent of this mycosis is *Paracoccidioides brasiliensis* sensu lato (s.l.), a dimorphic fungal pathogen that grows as a mold at temperatures below 26 °C, whereas at temperatures between 35 °C and 37 °C it grows as multibudding yeasts, the pathogenic form of this fungus (Borges-Walmsley et al., 2002). High phenotypic diversity among *P. brasiliensis* s.l. isolates has been reported in morphology, rate of growth, and virulence (Borba Cde et al., 2008; Burger et al., 1996; Carvalho et al., 2005; Molinari-Madlum et al., 1999), and recent phylogenetic studies have revealed that this species actually contains at least four cryptic phylogenetic species: two paraphyletic species (S1 [Species 1] and PS2 [Phylogenetic Species 2]), and two monophyletic species (PS3 [Phylogenetic Species 3] and the "*Pb01-like*" species for which the name *Paracoccidioides lutzii* sp. nov. has been proposed) (Matute et al., 2006; Teixeira et al., 2009).

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A number of studies in *P. brasiliensis* s.l. have focused on the identification of specific antigenic proteins that can be used for the serological diagnosis of PCM, for follow up of patients in response to therapy, and for possible development of immunization alternatives. In this way, the immunodominant antigen gp43, the first one described (Puccia et al., 1986), has been found useful for diagnosis (Puccia and Travassos, 1991) and to protect against experimental infection in mice (Marques et al., 2006; Pinto et al., 2000; Taborda et al., 1998). Other antigens also assayed for diagnosis, follow up of patients and/or immunization include gp70 antigen (da Silva et al., 2004; de Mattos Grosso et al., 2003) and the heat shock protein 87-kDa antigen (Díez, 2004; Gomez et al., 1998, 1997).

Another such antigen is a 27-kDa protein (p27), obtained from a *P. brasiliensis* cDNA library (McEwen et al., 1996), with potential use for diagnosis due to a high sensitivity and specificity shown (Diez et al., 2003; Fernandes et al., 2011a; Ortiz et al., 1998, 1996). In addition, it has been shown to promote protective immunity in BALB/c mice against infection with *P. brasiliensis* yeast forms (Fernandes et al., 2011b; Reis et al., 2008), enhancing the PCM antifungal therapy (Fernandes et al., 2011b), and has been suggested as a putative virulence factor (Matute et al., 2008; Ortiz et al., 1996; Reis et al., 2008). Nonetheless, the function of this protein remains unknown.

A common approach for recognizing putative virulence factors in dimorphic fungi is to establish a correlation between increased protein expression and the pathogenic form of the fungus (Rappleye and Goldman, 2006), since it has been shown that the dimorphic ability of these fungi is crucial for their pathogenicity (Nunes et al., 2005; Rappleye and Goldman, 2006), and that those isolates unable to transform into yeast are not virulent (Borges-Walmsley et al., 2002). In this study, *PbP27* expression was examined at the transcriptional and translational levels in the mycelia and yeast forms of different isolates of the species complex of *P. brasiliensis* in order to detect potential differential expression patterns. We also characterized the nucleotide polymorphisms among different isolates.

2. Materials and methods

2.1. Strains and culture conditions

Fourteen isolates belonging to the four cryptic phylogenetic species previously described (S1, PS2, PS3, and "*Pb01-like*" cluster) (Matute et al., 2006; Teixeira et al., 2009) were included in this study (Table 1). For maintenance, the mycelial form was grown in the modified synthetic McVeigh and Morton medium (Restrepo and Jimenez, 1980) at room temperature (18–23 °C). To obtain the yeast form, mycelia was grown in anaerobic conditions in Sabouraud agar supplemented with 0.2% asparagine and 1% thiamine at 37 °C and subcultured every 4–5 days until a complete reversion was observed. For experiments, all isolates were cultured in Brain Heart Infusion broth supplemented with 0.2% asparagine and 1% glucose, and incubated in constant agitation at 20 °C for 8–10 days and at 37 °C for 4–6 days, for mycelial and yeast forms respectively. Two independent cultures were grown for each form of each isolate in study. Aliquots of each culture were used for RNA, DNA and protein isolation.

Table 1
Isolates evaluated in this study.

Isolate[a]	Country	Source	Species
A1 (A1)	Argentina	Acute PCM	S1
Pb339 (B18)	Brazil	Chronic PCM	S1
T4B14 (B3)	Brazil	Armadillo	S1
Pb18 (B17)	Brazil	Chronic PCM	S1
T15LN1 (B10)	Brazil	Armadillo	S1
BT84 (B15)	Brazil	PCM	PS2
T10B1 (B7)	Brazil	Armadillo	PS2
Uberlandia (B13)	Brazil	Dog Food	PS2
Pb2 (V2)	Venezuela	Chronic PCM	PS2
ATCC 76533 (C13)	Colombia	Chronic PCM	PS3
ATCC 60855 (C4)	Colombia	Chronic PCM	PS3
P204 (C7)	Colombia	Chronic PCM	PS3
P206 (C17)	Colombia	Chronic PCM	PS3
Pb01 (–)	Brazil	Acute PCM	*"Pb01-like"*

[a] Isolates were provided by the Corporación para Investigaciones Biológicas – CIB collection. Name in brackets is according to the nomenclature given by Matute et al. (2006).

2.2. Nucleic acids isolation

RNA isolation was performed as previously described (Garcia et al., 2010). Briefly, cells were harvested, disrupted by grinding in liquid nitrogen and mixed with Qiazol reagent (Qiagen, Duesseldorf, Germany) for RNA extraction according to the supplier's instructions. In order to verify the RNA integrity, samples were electrophoresed in 1.2% denaturing formaldehyde agarose gels and visualized with UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion to verify that there was no significant degradation.

DNA was removed by Amplification Grade DNase I treatment (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's procedure. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the β-tubulin gene using oligonucleotides BTubE2F: 5′-AGATGTTC GATCCTAAGAACATGA-3′ and BTubE2R: 5′-ATAGCTGAGCAGGTAA GGTAACG-3′.

For DNA preparation, cells were mixed with lysis buffer (1 mM EDTA, 10 mM TrisHCl [pH 8.0], 1% Sodium Dodecyl Sulfate (SDS), 2% Triton X100, 100 mM NaCl) and disrupted using glass beads or maceration of frozen cells for yeast and mycelia respectively (Morais et al., 2000; van Burik et al., 1998). Extraction was carried out with phenol chloroform-isoamyl alcohol (25:24:1) as previously described (Sambrook and Russell, 2001). RNA was removed by RNase I treatment at 37 °C for 30 min. DNA integrity was examined by electrophoresis on 1% agarose gels.

RNA and DNA quantification and additional quality evaluation were carried out using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA).

2.3. PCR conditions and sequencing

The sequence of the *PbP27* gene from the Pb18 isolate, obtained from the Broad Institute *P. brasiliensis* Database (Locus PABG_07332.1), was used to design the following primers for DNA sequencing: P27F, 5′-GACGAGCTGAAAACTGTTGT-3′ and P27R, 5′-CTAGTTGTGGAAGACAGCG-3′. Amplification reactions were composed of 1x Taq Buffer (100 mM Tris–HCl pH 8.5; 500 mM KCl and 1% (v/v) Triton X-100) (Tucantaq, Corpogen, Bogotá, Colombia), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, 1 U of Taq Polymerase (TucanTaq DNA Polimerasa, Corpogen, Bogotá, Colombia) and 0.4 μg of DNA. A reaction without DNA was included as a negative control to rule out possible contamination. PCR amplifications were performed on a MyCycler IQ (Bio-Rad, Hercules, CA, USA) with an initial denaturing cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C (30 s), 58 °C (30 s) and 72 °C (30 s), with a final cycle at 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gels visualized by staining with ethidium bromide and sequenced using the BigDye™ Terminator sequencing chemistry on an ABI 3730Xl DNA sequencer (Applied Biosystems). Sequence assembly and editing was performed manually on CLC Genomics Workbench (http://www.clcbio.com).