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# Involvement of the 90 kDa heat shock protein during adaptation of *Paracoccidioides brasiliensis* to different environmental conditions

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ABSTRACT

*HSP90* is a molecular chaperone that participates in folding, stabilization, activation, and assembly of several proteins, all of which are key regulators in cell signaling. In dimorphic pathogenic fungi such as *Paracoccidioides brasiliensis*, the adaptation to a higher temperature, acid pH and oxidative stress, is an essential event for fungal survival and also for the establishing of the infectious process. To further understand the role of this protein, we used antisense RNA technology to generate a *P. brasiliensis* isolate with reduced *PbHSP90* gene expression (*PbHSP90*-aRNA). Reduced expression of *HSP90* decreased yeast cell viability during batch culture growth and increased susceptibility to acid pH environments and imposed oxidative stress. Also, *PbHSP90*-aRNA yeast cells presented reduced viability upon interaction with macrophages. The findings presented here suggest a protective role for *HSP90* during adaptation to hostile environments, one that promotes survival of the fungus during host–pathogen interactions.


## 1. Introduction

The success of pathogenic microorganisms depends greatly on their ability to sense alterations in the environment, followed by induction of proteins that are involved in varied protective responses (Goldani et al., 1994; Rokhlenko et al., 2007). Among them are the heat shock proteins (HSPs) (Shapiro and Cowen, 2010), important for the adaptation of microbial cells to changing environmental conditions, such as thermal adaptation (Goldani et al., 1994). Adaptation of human pathogens to a higher temperature during host infection is an event frequently concomitant with cellular differentiation, a common process observed in dimorphic pathogenic fungi (da Silva et al., 1999).

Dimorphic fungi are a group of pathogens, distributed worldwide, that can infect both immunocompromised and immunocompetent individuals. These include *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Coccidioides posadasii* (Krajaejun et al., 2007; Colombo et al., 2011). The dimorphic fungus *P. brasiliensis*, the etiological agent of paracoccidioidomycosis (PCM), has the ability to grow either in the yeast or the mycelial form in response to specific environmental conditions (Maresca and Kobayashi, 2000; Colombo et al., 2011). The disease is acquired after inhalation of conidia or small hyphal fragments, which convert to yeast cells in the lungs (Brummer et al., 1993). The morphological transition and the capacity to grow as yeast cells are essential steps for the progression of the disease and dissemination to other tissues (Nemecek et al., 2006).

To respond to environmental changes during the morphological conversion, *P. brasiliensis* expresses, among other molecules, different HSPs (Goldani et al., 1994; Shapiro and Cowen, 2010). These proteins are a family of evolutionarily conserved molecules detected in both prokaryotic and eukaryotic cells (Lindquist and Craig, 1988; Bonnefoy et al., 1994; Kiang and Tsokos, 1998; Burnie et al., 2006). Most HSPs are expressed constitutively and are thought to have a protective role during temperature changes or against harmful insults such as certain environmental stress conditions (e.g., oxidative injury, acidic environments and when cells are challenged with cytotoxic drugs) (Kiang and Tsokos, 1998; Nicola et al., 2005; Nicola et al., 2008). HSPs are categorized into families

*Abbreviations:* HSP90, heat shock protein 90 kDa; aRNA, antisense RNA; ROS, reactive oxygen species; ATMT, *Agrobacterium tumefaciens*-mediated transformation; PCM, paracoccidioidomycosis.

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on the basis of their molecular weights such as HSP27, HSP60, HSP70, HSP110/104, and HSP90 (Kregel, 2002).

HSP90 is a molecular chaperone that participates in the folding, stabilization, activation, and assembly of a diverse but select set of client proteins, which are key regulators of cell signaling, thus important under both normal and stressful conditions (Csermely et al., 1998; Sreedhar et al., 2003; Cowen and Lindquist, 2005; Nicola et al., 2008). In eukaryotic cells, *HSP90* is involved in cytoprotection (Sreedhar et al., 2003) and its inhibition causes a large number of phenotypic alterations compromising cellular integrity (Sreedhar et al., 2003; Cowen and Lindquist, 2005). Moreover, *HSP90* has been proven to be essential for cellular viability in fungi such as *Saccharomyces cerevisiae* and *Candida albicans* (Swoboda et al., 1995; Shapiro and Cowen, 2010). Previous studies have also suggested that *HSP90* may assist *P. brasiliensis* yeast cells to cope with a variety of physiological stresses including temperature, host interaction (e.g., acidic environments in the phagolysosome) and oxidative injury *inter alia* (Leung and Gershwin, 1991; Nicola et al., 2008; Hernandez et al., 2011).

The main goal of this work was to evaluate the relevance of *PbHSP90* in *P. brasiliensis* yeast cells placed under different stress conditions, namely acidic and oxidative environments. To achieve this we down-regulated *PbHSP90* expression with antisense RNA (aRNA) technology (Almeida et al., 2007; Almeida et al., 2009; Hernandez et al., 2010; Ruiz et al., 2011) and analyzed yeast cell behavior under different conditions. In addition, we performed a sequence analysis in order to determine the protein domain conservation and build the phylogenetic tree that shows the relationships of the selected species.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

*P. brasiliensis* yeast cells (strain ATCC 60855) were used and maintained at 36 °C by subculturing in brain heart infusion (BHI) medium supplemented with 1% glucose (Beckton Dickinson and Company, Sparks, MD). Unless indicated otherwise, yeast cells were grown in BHI liquid medium at 36 °C with aeration on a mechanical shaker and were routinely collected during their exponential phase of growth (72–96 h). Samples were collected for RNA extraction and quantification of gene expression analysis (Garcia et al., 2009).

*A. tumefaciens* strain LBA1100 (Beijersbergen et al., 1992) was used as the recipient for the binary vectors constructed in this study. Bacterial cells were maintained at 28 °C in Luria–Bertani (LB) medium containing kanamycin (100 mg/ml). *Escherichia coli* DH5α was grown at 37 °C in LB medium supplemented with appropriate antibiotics and was used as the host for plasmid amplification and cloning (Sambrook et al., 1998).

### 2.2. Protein sequence and phylogenetic analysis

Phylogenetic analysis and conservation domain analysis were done for the following taxa, including nine in the order Onygenales, two representative pathogenic fungi from the order Eurotiales, one model fungus from the order Sordariales, and the fungus with the best characterized Hsp90 protein, respectively as follows: *P. brasiliensis*, *Paracoccidioides lutzii*, *H. capsulatum*, *B. dermatitidis*, *C. posadasii*, *C. immitis*, two dermatophytes: *Microsporum canis*, *Microsporum gypseum*, *Uncinocarpus reesii*, *Penicillium marneffei*, *Aspergillus fumigatus*, *Neurospora crassa*, and *S. cerevisiae*. BLAST was done to search the single-copy similarity for PbHsp90p with BLASTP at the Broad Institute platform (http://www.broadinstitute.org). InterProScan was used to identify the protein domains (Zdobnov and Apweiler, 2001), by sequence comparison with InterPro collection of protein signature databases in the EMBL-EBI (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

Multiple sequence alignments were constructed using Muscle (Edgar, 2004) and the alignments were pruned with Gblocks (Castresana, 2000). Phylogenetic trees were constructed employing three methods, performed by distance computation, maximum likelihood and Bayesian inference (Huelsenbeck and Ronquist, 2001; Guindon and Gascuel, 2003; Felsenstein, 2005).

Finally, prediction gene analyses were implemented to manually curate the *P. brasiliensis* (Pb03/Pb18) and *P. lutzii* (Pb01) Hsp90 gene-protein annotations, handling the genomic sequences available in the Broad Institute Platform, using for this Augustus 2.3 (Stanke et al., 2004) and Fgenesh (not versioned – Softberry) gene predictors, and BLAST to check out for the best prediction.

### 2.3. Generating P. brasiliensis PbHSP90-aRNA isolates

DNA from *P. brasiliensis* wild-type (PbWT) strain (ATCC 60855) was extracted from yeast cells cultures during exponential growth using the TRIzol reagent (Invitrogen, Carlsbad, CA). We employed a Platinum high-fidelity Taq DNA polymerase (Invitrogen®) to amplify aRNA oligonucleotides, designed on the sequence PADG_07715 (www.broadinstitute.org) of *PbHSP90* gene from PbWT DNA: AS1 (116 bp), AS2 (122 bp), and AS3 (157 bp).

*P. brasiliensis* plasmid construction for aRNA and *Agrobacterium tumefaciens*-mediated transformation (ATMT) were performed as previously described (Almeida et al., 2007; Almeida et al., 2009; Hernandez et al., 2010; Hernandez et al., 2011; Ruiz et al., 2011). The amplified *PbHSP90* aRNA oligonucleotides were inserted into the pCR35 plasmid under the control of the calcium binding protein 1 (CBP-1) promoter region from *H. capsulatum* (Rappleye et al., 2004). The pUR5750 plasmid was used as a parental binary vector to harbor this aRNA cassette within the transfer DNA (T-DNA). The constructed binary vectors were introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation as described previously (den Dulk-Ras and Hooykaas, 1995), and isolated by kanamycin selection (100 mg/ml).

In *P. brasiliensis* yeast cells, ATMT was done using *A. tumefaciens* cells harboring the desired binary vector, as described previously by Almeida et al. (2007). A 1:10 *P. brasiliensis*/*A. tumefaciens* ratio was employed during the 3 days period of co-culture at 28 °C. Selection of *P. brasiliensis* transformants was performed in BHI solid media containing hygromycin B (Hyg; 100 mg/ml) over a 15 days incubation period at 36 °C. Randomly selected Hyg resistant transformants were tested for mitotic stability. During the assays carried out in this study and as controls, *P. brasiliensis* yeast cells were also transformed with the empty parental vector pUR5750 (PbEV).

### 2.4. Gene expression analysis

Total RNA was obtained from PbWT, PbEV and *P. brasiliensis PbHSP90*-aRNA yeast cells using the TRIzol reagent (Invitrogen®). Total RNA was treated with DNase I (Thermo Scientific®) and tested using a conventional PCR with β-tubulin primers to confirm the absence of chromosomal DNA contamination (Goldman et al., 2003). cDNA was synthesized using 2 μg of total RNA with Maxima® First Strand cDNA synthesis kit for RT-qPCR, according to the manufacturer's instructions (Fermentas®).

Real-time PCR was done using a Maxima® SYBR Green/Fluorescein qPCR Master Mix (2X) (qRT-PCR) kit with SYBR green, according to the manufacturer's instructions (Fermentas®). The CFX96 real time PCR detection system (Bio-Rad, Hercules, CA) was used to measure gene expression levels. *PbHSP90* expression was evaluated in PbWT, PbEV and Pb*HSP90*-aRNA yeast cells at different time

points. Melting curve analysis was performed after the amplification phase to eliminate the possibility of nonspecific amplification or primer-dimer formation. Fold changes in mRNA expression were calculated using the $2^{\Delta\Delta CT}$ formula, where ΔΔCT is the difference in the threshold cycle (CT) between the target gene and the β-tubulin gene (housekeeping gene) (Livak and Schmittgen, 2001). Each experiment was done in triplicate, and the expression level was measured in triplicate.

### 2.5. Viability and growth curve in P. brasiliensis yeast cells

PbWT, PbEV and Pb*HSP90*-aRNA yeast cells were grown in BHI liquid medium at 36 °C and samples were collected at specific time points to determine growth curves by spectrophotometric analysis [$OD_{600\ nm}$; SmartSpec Plus (Bio-Rad, Hercules, CA)].

The viability of yeast cells was evaluated using ethidium bromide-fluorescence staining (Calich et al., 1979) and standard cell count techniques in the fluorescence microscope Laborlux S (Leica, Germany). Briefly, *P. brasiliensis* multi-budding yeast cells were passed through a syringe with a 0.8 mm needle before starting any of the experimental conditions evaluated here, in order to obtain more separate cells and facilitate the subsequent cell count. Finally, we collected the samples of each experiment and determined the viability percentage as was described by Calich et al. (1979).

### 2.6. Exposure of yeast cells to acid environments and induction of oxidative stress

PbWT, PbEV and Pb*HSP90*-aRNA were grown in BHI liquid supplemented with 1% glucose at different pHs [8.0, 7.3 (pH of BHI medium), 6.5 and 6] during 12, 24, 48, 72, and 96 h. *PbHSP90* expression analysis (Hernandez et al., 2011) and determination of the yeast cell viability was performed as described above (Calich et al., 1979).

For induction of exogenous oxidative stress, we used Thimerosal – Ethylmercurithiosalicylic acid sodium salt – (Sigma–Aldrich® Ref T5125) and hydrogen peroxide ($H_2O_2$). Thimerosal assays were done incubating $2.5 \times 10^6$ *P. brasiliensis* yeast cells in BHI liquid at different concentrations (0.01%, 0.02% and 0.04%), during 12 and 24 h. Afterwards, cell viability was determined as previously described (Calich et al., 1979). For $H_2O_2$ assays, $3.5 \times 10^6$ *P. brasiliensis* yeast cells were added in 2 ml of PBS containing 0.125 M $H_2O_2$, yeast cells were incubated during 1, 3, and 6 h at 37 °C; finally, we determined viability at the end of each incubation time as previously described (Calich et al., 1979).

### 2.7. Interaction of P. brasiliensis yeast cells with alveolar macrophages

The MH-S cell line, which corresponds to mouse alveolar macrophages transformed with SV40, was obtained from the European Cell Cultures Collection (ECACC No. 95090612). IFN-γ-activated alveolar macrophages were grown in RPMI 1640 medium + 2 mM Glutamine (Invitrogen®) + 0.05 mM 2-Mercaptoethanol (Sigma Aldrich, USA) + 10% fetal bovine serum (Invitrogen®). For the assays, we used confluent monolayers obtained by adding $4 \times 10^5$ cells per well to 24-well tissue culture plates (Nunc, Kamstrup, Denmark) incubated for 24 h at 36 °C with 5% $CO_2$ prior to evaluating interaction with PbWT and Pb*HSP90*-aRNA yeast cells. Alveolar macrophages were activated for 18 h by adding recombinant Mouse IFN-γ (BD Pharmigen. Ref 554587) at 100 ng/ml. MH-S cells monolayer (activated and non-activated) were washed once with RPMI 1640 culture medium and co-cultured with *P. brasiliensis* yeast cells at a concentration of $8 \times 10^4$ yeast cells per well (corresponding to a ratio 1:5 for *P. brasiliensis* yeasts: IFN-γ activated alveolar macrophages), and incubated during 1, 3, 6, 12, and 24 h at 36 °C with 5% of $CO_2$. After this interaction, *PbHSP90* gene expression (Ruiz et al., 2011) and colony forming units were determined to establish the viability percentage (Kurita et al., 1993; Ruiz et al., 2011).

### 2.8. Statistics analysis

Data are presented as means ± S.E. (standard error), averaged for minimum three replicates of the experiments. Error bars are standard deviations. Data were analyzed with the Student's *t* test. A $p$ value <0.05 was accepted as indicating a statistically significant difference compared with controls.

## 3. Results

### 3.1. Protein sequence analysis reveals three conserved domains in the predicted PbHsp90p and relatedness to order Onygenales

Using BLASTP to find the orthologous sequence of PbHsp90p, we found the single-copy of the gene/protein in all fungi of the order Onygenales, but not in all isolates available in the Broad Institute Platform. Also, for Pb03/Pb18 and Pb01 we found genome annotation inconsistencies, regarding the expected length of *HSP90*. Sequence prediction and gene analysis was performed using Augustus and Fgenesh, completing the protein sequence to allow the identification of the three main domains with high quality and reliability. These sequences were used for conserved domain and phylogenetic analysis.

The *HSP90* of all studied fungi presented the three predominant domains of the eukaryotic Hsp90, including the N-terminal domain, the middle-domain and C-terminal domain. The alignment of the N-terminal domain of Onygenales provided the highest rate of conservation, including the Hsp90 high conservation site. This domain is important, owing to the presence of the catalytic region and ATP-ase activity, which is broadly used to inhibit the protein activity (Howes et al., 2006; Nicola et al., 2008) (Fig. 1a and b).

On the other hand, the phylogenetic trees displayed the same topology in each analysis showing the relationships of Hsp90 in the taxa and exhibited evolutionary relationships between the different species used in this work and, as expected, divided them into different groups (Sharpton et al., 2009). The Hsp90 of the dimorphic pathogenic fungi, the ones from the non-pathogenic *U. reesii* and that from the two dermatophytes, were phylogenetically related to each other in the order Onygenales clade.

### 3.2. Decreasing PbHSP90 gene expression affects growth rate and cell viability in P. brasiliensis yeast cells

Three different regions of *HSP90* were selected to design aRNA oligonucleotides and generate knockdown isolates; two directed to exon 1 (116-bp and 122-bp) and 1 directed to exon 2 (157-bp) (Fig. 2a). To pursue our main goals in this work, we selected a mitotically stable isolate with the highest decrease in *PbHSP90* expression (±56%) (Fig. 2b). We initially inquired on the effect of the down regulation of *PbHSP90* during batch culture growth and viability of yeast cells. A reduction was observed in the *PbHSP90*-aRNA isolate when compared to either PbWT or PbEV (Fig. 3a), more pronounced after 24 h of incubation, with statistically significant differences between the control isolates and the *PbHSP90*-aRNA isolate. We also observed a general decrease in viability of *PbHSP90*-aRNA yeast cells throughout all evaluated time points (Fig. 3b). Statistically significant differences were detected as early as 12 h, becoming more evident at 48 and 72 h.

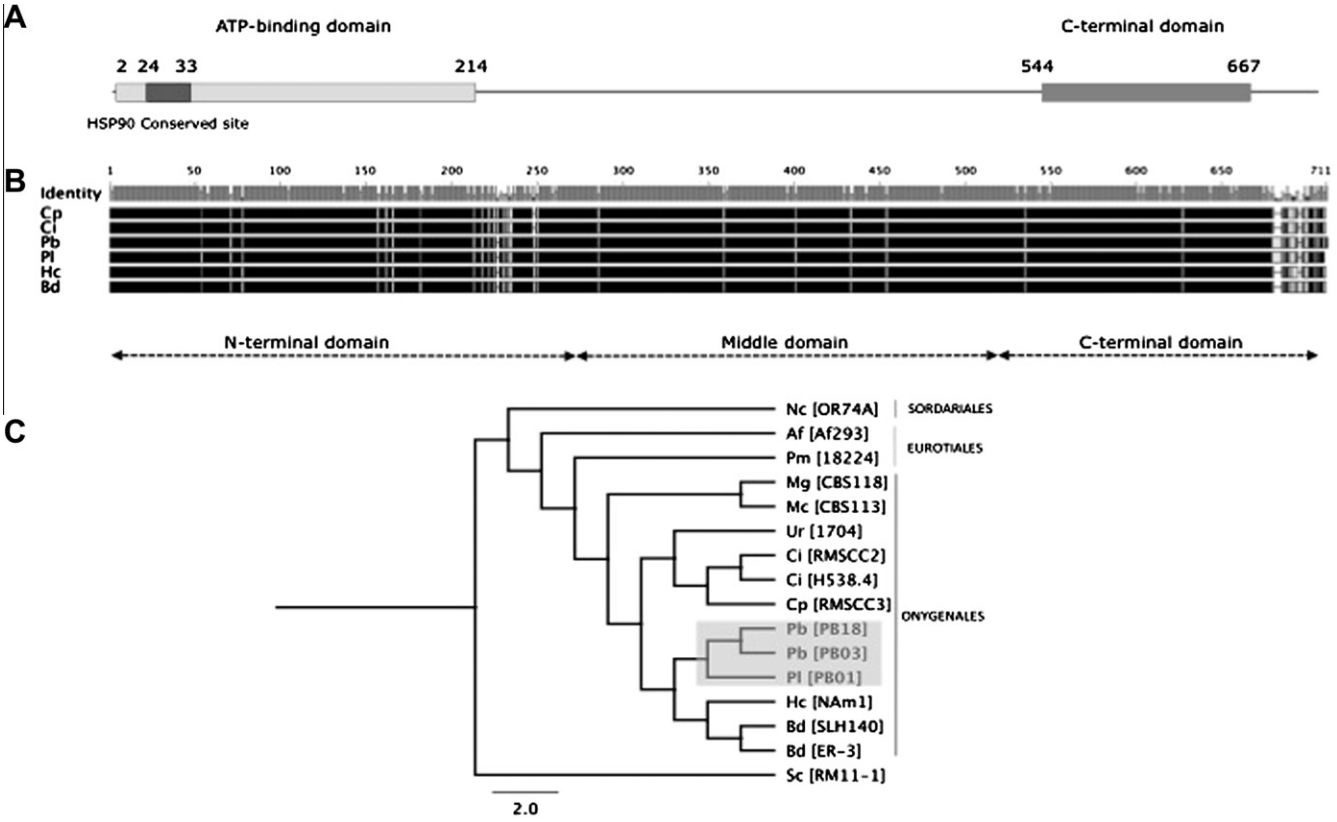


**Fig. 1.** Multiple alignments of amino acid (aa) sequences deduced from the Hsp90 gene corresponding to the pathogenic Onygenales clade. (A) Conserved domains: ATP-binding domain [2–214 aa]; Hsp90 conserved site [24–33 aa]; and C-terminal domain [544–667 aa]. (B) Black boxes represent the regions with high conservation (100%), gray and white regions are different in the clade (*Paracoccidioides*, *Histoplasma*, *Blastomyces and Coccidioides*). In all domains, high quantities of amino acid sequences have conserved values (100%). This alignment was generated using Muscle and visualized in Geneious Pro. (C) Hsp90 phylogeny shows its relatedness in members of the order Onygenales. Phylogenetic tree of relatedness between the orthologous Hsp90 constructed by three methods (computation of distance, maximum likelihood and Bayesian inference) resulting in the same topology showed in the tree. The phylogenetic tree is consistent with the expected phylogeny and groups the Hsp90 of different fungi in their respective order, the vast majority pathogens (Sharpton et al., 2009). In the gray box corresponding to the *Paracoccidioides* clade, the two divergent species for the genera, *P. brasiliensis* and *P. lutzii*, is depicted (Teixeira et al., 2009). Dendogram was generated using Figtree version 1.3.1. Af: *Aspergillus fumigatus;* Pm: *Penicillium marneffei;* Mg: *Microsporum gypseum;* Mc: *Microsporum canis;* Ur: *Uncinocarpus reesii;* Ci: *Coccidioides inmitis;* Cp: *Coccidioides posadasii;* Pl: *Paracoccidioides lutzii;* Pb: *Paracoccidioides brasiliensis;* Hc: *Histoplasma capsulatum;* Bd: *Blastomyces dermatitidis;* Nc: *Neurospora crassa;* and Sc: *Saccharomyces cerevisiae.*

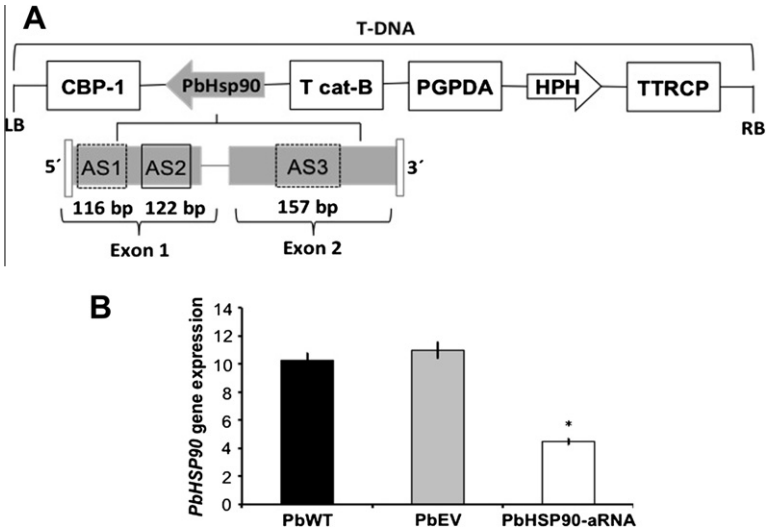


**Fig. 2.** Silencing *HSP90* in *P. brasiliensis*. (A) Transfer DNA (T-DNA) inserted into *P. brasiliensis* yeast cells via ATMT in order to silence *PbHSP90*. AS1, AS2 and AS3 oligonucleotides were placed under control of the calcium binding protein (CBP-1), the terminator (Cat-B) and harboring hygromycin B phosphotransferase (HPH) under control of glyceraldehyde 3-phosphate of *Aspergillus nidulans* (PGPDA) and with the terminator (TTRCP). Modified by Hernandez et al. (2011). (B) Gene expression levels of *PbHSP90* obtained by RT-qPCR assay, normalized with housekeeping gene β-tubulin in PbWT, PbEV and Pb*HSP90*-aRNA yeast cells grown at exponential phase. The low expression levels in *PbHSP90*-aRNA were maintained after successive sub-cultures.

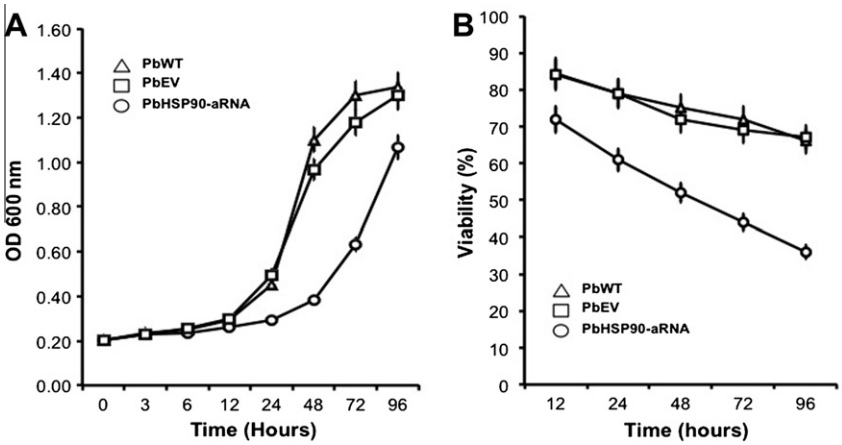


**Fig. 3.** Viability and growth curve in *P. brasiliensis* yeast cells. (A) Growth curve in PbWT, PbEV and *PbHSP90*-aRNA. Yeast cells were grown in BHI liquid medium at 36 °C, $OD_{600\ nm}$ was determined through each time point. (B) Viability of PbWT, PbEV and *PbHSP90*-aRNA was determined using fluorescein diacetate and ethidium bromide (Calich et al., 1979).

### 3.3. PbHSP90 is important for adaptation of yeast cells to acid environments

In order to study the role of *PbHSP90* in response to acid environments, we cultured yeast cells (PbWT, PbEV and Pb*HSP90*-aRNA) at different pHs (6.0, 6.5, 7.3 and 8.0) and evaluated *PbHSP90* gene expression and yeast cell viability at different time points. *PbHSP90*-aRNA yeast cells consistently presented lower *PbHSP90* expression levels throughout all time points and evaluated pHs (Fig. 4a). During exposure to acid environments (pH 6.0 and 6.5) a higher expression of *PbHSP90* was detected in PbWT compared to when exposed to higher pHs (7.3 and 8.0) (Fig. 4a).

*PbHSP90*-aRNA yeast cells presented decreased viability when compared to PbWT (Fig. 4b). On the other hand, in PbWT no alterations in cell viability were observed independent of the culture media's pH. In addition, both PbWT and *PbHSP90*-aRNA yeast cell viability decreased throughout the growth curve in all studied pHs (Fig. 4b).

### 3.4. Reduced expression of PbHSP90 leads to increased susceptibility to oxidative stress

Oxidative injury has been previously reported to induce expression of *HSP90* in *P.* brasiliensis (Kiang and Tsokos, 1998; Nicola et al., 2005; Nicola et al., 2008). Thus, we also attempted to find out how the absence of wild-type *PbHSP90* levels would affect the response of yeast cells to oxidative stress. We exposed *P. brasiliensis* yeast cells (PbWT, PbEV and *PbHSP90*-aRNA) to both thimerosal, which induces cellular damage through indirect oxidative stress (Crespo-Lopez et al., 2009), and $H_2O_2$ as a direct source of oxidative

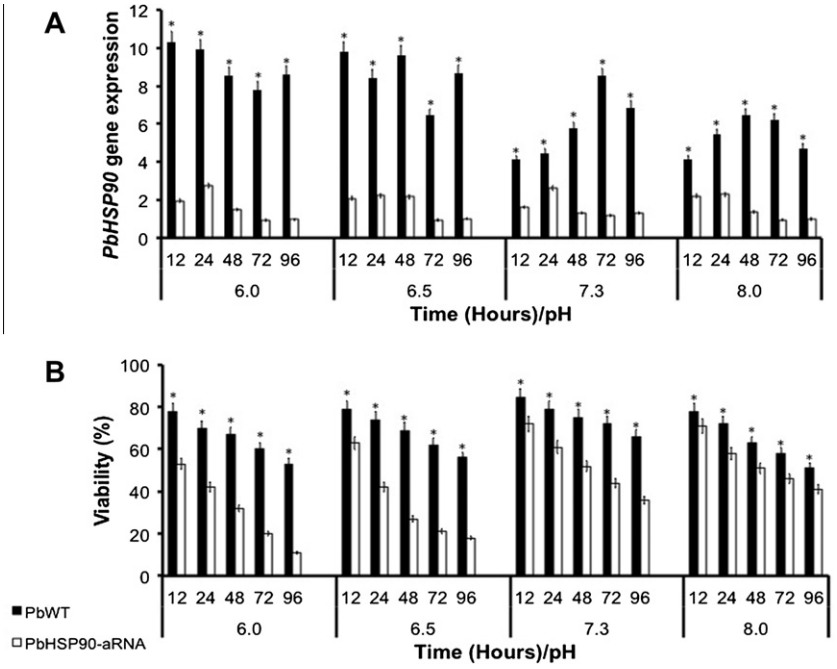


**Fig. 4.** Viability and *PbHSP90* gene expression in *P. brasiliensis* yeast cells exposed to acid environments. Yeast cells were cultured in BHI at different pHs, ranging from 6.0 to 8.0. (A) *PbHSP90* gene expression levels obtained by RT-qPCR assay, normalized with housekeeping gene β-tubulin in *P. brasiliensis* yeast cells grown at different pH values. (B) Viability of PbWT and Pb*HSP90*-aRNA was determined at each time point and pH values. *PbHSP90*-aRNA had the lowest values of gene expression and also the lowest viability percentages.

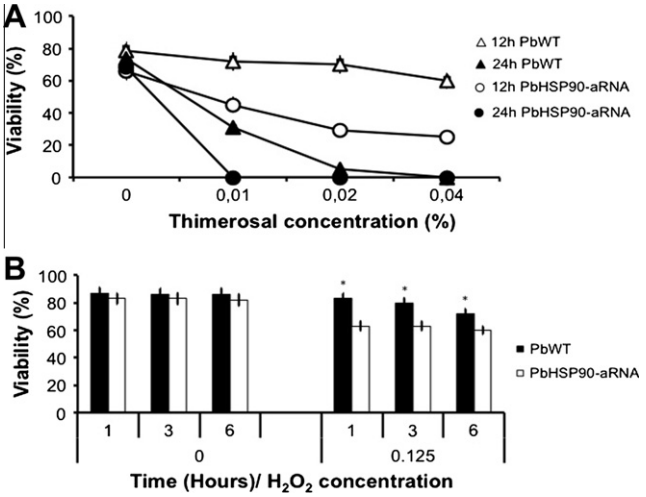


**Fig. 5.** Viability in *P. brasiliensis* yeast cells exposed to oxidant agents. *P. brasiliensis* yeast cells were incubated in the absence and in the presence of thimerosal (indirect oxidative stress induction) and $H_2O_2$ (direct oxidative stress induction). (A) Viability in *P. brasiliensis* yeast cells exposed to thimerosal. *P. brasiliensis* yeast cells incubated without thimerosal (0%) and at different concentrations of this compound (0.01%, 0.02% and 0.04%), during 12 and 24 h. After that incubation for the specified times, viability percentages via ethidium bromide-fluorescein diacetate stain was verified (Calich et al., 1979). White forms represent data obtained after 12 h of incubation and black forms data obtained after 24 h of incubation. (B) Viability in *P. brasiliensis* yeast cells exposed to $H_2O_2$. Yeast cells were incubated without $H_2O_2$ (0) and in the presence of this compound (0.125 M). Viability was determined after 1, 3 and 6 h of incubation (Calich et al., 1979).

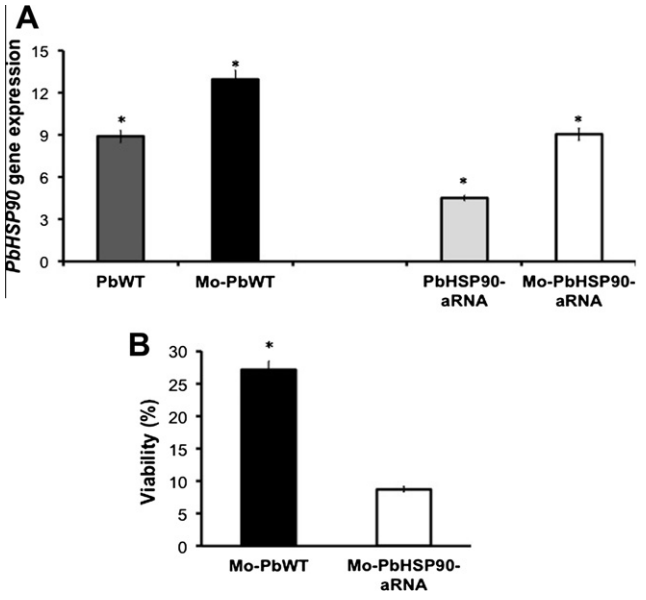


**Fig. 6.** *PbHSP90* gene expression and viability in *P. brasiliensis* yeast cells after interaction with macrophages. (A) *PbHSP90* gene expression in PbWT and *PbHSP90*-aRNA yeast cells isolates, co-cultivated in the presence and in the absence of IFN-γ activated macrophages. Gene expression levels were obtained by RT-qPCR assay and normalized with housekeeping gene β-tubulin. After interaction, we observed an increase in *PbHSP90* gene expression in both, PbWT and *PbHSP90*-aRNA isolates. (B) Viability percentage in *P. brasiliensis* yeast cells after incubation in the presence of IFN-γ activated macrophages (Mo). Silencing of *PbHSP90* gene affects *P. brasiliensiśs* capacity to survive the interaction with macrophages. Mo-PbWT: Activated macrophages-PbWT interaction; Mo-*PbHSP90*-aRNA: Activated macrophages-*PbHSP90*-aRNA interaction.

stress. After 12 h of incubation with thimerosal, we observed a significant decrease in *PbHSP90*-aRNA yeast cells viability when compared to PbWT. In fact, after 24 h of treatment with this compound, no viable *PbHSP90*-aRNA yeast cells were detected at any of the tested concentrations (Fig. 5a), while PbWT viable yeast cells were still detected at 0.01% and 0.02%. Following 24 h of treatment with 0.04% thimerosal, no viable cells were detected for either isolate.

Regarding exposure to $H_2O_2$, *PbHSP90*-aRNA yeast cells were significantly more susceptible than PbWT yeast cells (Fig. 5b).

### *3.5. PbHSP90 gene is important for interaction of P. brasiliensis yeast cells with macrophages*

We also evaluated *PbHSP90* expression and viability in yeast cells after interacting with IFN-γ activated macrophages for 3 h. Higher expression levels of *PbHSP90* gene were detected in PbWT yeast cells during the interaction with activated MH-S cells in comparison to Pb*HSP90*-aRNA yeast cells (Fig. 6a). Moreover, Pb*HSP90*-aRNA yeast cell viability was severely reduced when compared to PbWT cells 3 h post-interaction with activated macrophages (Fig. 6b).

## 4. Discussion

*HSP90* has been previously associated to physiologic responses of pathogenic fungi to diverse stressful events, namely during host–pathogen interaction or temperature-induced morphological changes (Leung and Gershwin, 1991; da Silva et al., 1999; Nicola et al., 2008). The main goal of this work was to increase knowledge on the role of this chaperone in *P. brasiliensis* yeast cells. Although previous studies have employed *HSP90* inhibitors to better understand its biological function, the use of chemicals as geldanamycin has been shown to also induce superoxide production, altering cellular structures and causing cell lysis, ultimately creating difficulties to evaluate the role of *HSP90* in specific cellular processes (Hodgetts et al., 1996; Sreedhar et al., 2003; Nicola et al., 2008). Therefore, we generated *P. brasiliensis PbHSP90*-aRNA isolates to evaluate the effect of reduced *PbHSP90* expression in yeast cells.

Initial results in our study show that absence of wild-type levels of *PbHSP90* reduced *P. brasiliensis* yeast cell viability and growth rate (Fig. 3). Pharmacological knock out with specific Hsp90p inhibitors (e.g., radicicol and geldanamycin) were also shown to alter yeast cell viability in *P. brasiliensis* (Nicola et al., 2008). Moreover, previous studies in *S. cerevisiae* demonstrated that inhibition of Hsp90p increased cell lysis while mutation in the *HSP90* gene reduced growth rate (Borkovich et al., 1989; Sreedhar et al., 2003). Sreedhar and co-workers also demonstrated that *HSP90*-inhibiting ribozymes induced loss of cellular integrity in different eukaryotic cell types (Sreedhar et al., 2003). Additionally, in the filamentous fungus *Podospora anserina*, a mutation in *HSP90* has been shown to alter cell cycle regulation and sexual development (Loubradou et al., 1997).

Our data also shows that *HSP90* gene expression increases when *P. brasiliensis* yeast cells are exposed to varied exogenous stress. Batch culture growth in more acidic environments induced a higher expression of *PbHSP90* in PbWT cells, together with significantly increased yeast cell viability when compared to the *P. brasiliensis PbHSP90*-aRNA isolate (Fig. 4). In fact, lower medium pH was generally associated to either higher *HSP90* expression or lower yeast cell viability exacerbated in the *P. brasiliensis PbHSP90*-aRNA isolate. Interestingly, work in our laboratory has shown that *P. brasiliensis* yeast cells acidify the culture medium throughout batch culture growth (manuscript under preparation, Hernandez, O. et al.). This could also explain the gene expression profile observed at higher initial culture medium pHs (8.0 and 7.3) as a boost in *HSP90* expression occurred after 72 h when

the culture medium became more acidic (manuscript under preparation, Hernandez, O. et al.). Taking our results into account, one could hypothesize that induction of *PbHSP90* gene expression is important for *P. brasiliensis* to cope when exposed to acid pH values.

Challenge of *P. brasiliensis* with oxidative agents has been shown to induce higher gene expression levels of *PbHSP90* (Goldani et al., 1994; Nicola et al., 2008). During this study we also inquired on the role of *PbHSP90* upon oxidative injury with either thimerosal or $H_2O_2$. Both agents induced a more severe loss in viability of Pb*HSP90*-aRNA yeast cells (Fig. 5), although to a greater extent when exposed to thimerosal. This could be explained by the fact that thimerosal has been shown to act in two ways: (i) inducing cellular damage through an increase in ROS levels and (ii) provoking the inactivation of enzymes involved in the detoxification of ROS (catalase, glutathione peroxidase and superoxide dismutase) (Crespo-Lopez et al., 2009). On the other hand, $H_2O_2$ only affects the redox state of the cell and the enzymes still work to detoxify the cell and prevent the cellular damage induced by ROS. These data seem to suggest that *PbHSP90* may represent an important mechanism in the activation of oxidative stress defense.

The above-mentioned conditions (acid environments and oxidative stress) are present in the host, specifically within the macrophageśs phagolysosome, where *P. brasiliensiś* capacity to endure such environmental stresses proves crucial for pathogenicity and disease progression. We show that *PbHSP90* gene expression increases during the initial interaction with macrophages in both PbWT and *PbHSP90*-aRNA yeast cells (Fig. 6). In addition, *PbHSP90*-aRNA yeast cell viability was significantly lower than PbWT. Altogether these data seem to suggest that *HSP90* plays an important role at the beginning of interaction of the fungal pathogen with host cells, possibly through the induction of important molecular events that increases the fungus' capability to defend itself against exogenous oxidative and acidic stress. Previous studies have shown that over-expression of Hsp90p increases the virulence of *S. cerevisiae* in a mouse model of infection (Hodgetts et al., 1996; Burnie et al., 2006). Moreover, Hsp90p has been associated to the immunopathogenicity of infectious diseases as an immunodominant antigen (Burnie and Matthews, 2004; Matthews and Burnie, 2004; Nicola et al., 2005; Burnie et al., 2006), and the induction of specific antibodies against this protein protects against different types of infections (Kaufmann, 1990; Bonnefoy et al., 1994).

Phylogenetic analysis performed in this work confirmed the predicted similarity of Hsp90p among the Onygenales (Fig. 1). This is a highly conserved protein and its evolution rate is linked and proportional to the evolution rate of the selected taxa, presenting the same phylogeny reported for other genes under neutral selection for both the *Paracoccidioides* clade and the two divergent groups for the genera *P. brasiliensis* and *P. lutzii* (Fig. 1c) (Teixeira et al., 2009). Adding this to our data that suggests a protective role for *PbHSP90* in *P. brasiliensis* and to the fact that altering the function of Hsp90p can render pathogens more responsive to treatment and can thwart the evolution of fungal drug resistance (Cowen, 2009), seems to suggest that compromising *HSP90* function can transform antifungals from ineffective to highly efficacious agents to the management of infections caused by these human fungal pathogens (Diezmann et al., 2012).

Altogether, our data show that *HSP90* is required for *P. brasiliensis* to cope with different *in vitro* environmental stresses such as oxidative injury, low pH conditions and interaction with host effector cells as macrophages. Future studies will be directed to understand what are the implications of reduced *PbHSP90* levels in a mouse model of infection and which is the role of *HSP90* in the thermodimorphism of *P. brasiliensis*.

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## References

Almeida, A.J., Carmona, J.A., et al., 2007. Towards a molecular genetic system for the pathogenic fungus *Paracoccidioides brasiliensis*. Fungal Genet. Biol. 44 (12), 1387–1398.
Almeida, A.J., Cunha, C., et al., 2009. *Cdc42p* controls yeast-cell shape and virulence of *Paracoccidioides brasiliensis*. Fungal Genet. Biol. 46 (12), 919–926.
Beijersbergen, A., Dulk-Ras, A.D., et al., 1992. Conjugative transfer by the virulence system of Agrobacterium tumefaciens. Science 256 (5061), 1324–1327.
Bonnefoy, S., Attal, G., et al., 1994. Molecular characterization of the heat shock protein 90 gene of the human malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. 67 (1), 157–170.
Borkovich, K.A., Farrelly, F.W., et al., 1989. Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. 9 (9), 3919–3930.
Brummer, E., Castaneda, E., et al., 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. 6 (2), 89–117.
Burnie, J., Matthews, R., 2004. Genetically recombinant antibodies: new therapeutics against candidiasis. Expert Opin. Biol. Ther. 4 (2), 233–241.
Burnie, J.P., Carter, T.L., et al., 2006. Fungal heat-shock proteins in human disease. FEMS Microbiol. Rev. 30 (1), 53–88.
Calich, V.L., Purchio, A., et al., 1979. A new fluorescent viability test for fungi cells. Mycopathologia 66 (3), 175–177.
Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17 (4), 540–552.
Colombo, A.L., Tobon, A., et al., 2011. Epidemiology of endemic systemic fungal infections in Latin America. Med. Mycol. 49 (8), 785–798.
Cowen, L.E., 2009. Hsp90 orchestrates stress response signaling governing fungal drug resistance. PLoS Pathog. 5 (8), e1000471.
Cowen, L.E., Lindquist, S., 2005. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science 309 (5744), 2185–2189.
Crespo-Lopez, M.E., Macedo, G.L., et al., 2009. Mercury and human genotoxicity: critical considerations and possible molecular mechanisms. Pharmacol. Res. 60 (4), 212–220.
Csermely, P., Schnaider, T., et al., 1998. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol. Ther. 79 (2), 129–168.
da Silva, S.P., Borges-Walmsley, M.I., et al., 1999. Differential expression of an hsp70 gene during transition from the mycelial to the infective yeast form of the human pathogenic fungus *Paracoccidioides brasiliensis*. Mol. Microbiol. 31 (4), 1039–1050.
den Dulk-Ras, A., Hooykaas, P.J., 1995. Electroporation of Agrobacterium tumefaciens. Methods Mol. Biol. 55, 63–72.
Diezmann, S., Michaut, M., et al., 2012. Mapping the Hsp90 genetic interaction network in Candida albicans reveals environmental contingency and rewired circuitry. PLoS Genet. 8 (3), e1002562.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.
Felsenstein, J., 2005. Using the quantitative genetic threshold model for inferences between and within species. Philos. Trans. R. Soc. London B: Biol. Sci. 360 (1459), 1427–1434.
Garcia, A.M., Hernandez, O., et al., 2009. Identification of genes associated with germination of conidia to form mycelia in the fungus *Paracoccidioides brasiliensis*. Biomedica 29 (3), 403–412.
Goldani, L.Z., Picard, M., et al., 1994. Synthesis of heat-shock proteins in mycelia and yeast forms of *Paracoccidioides brasiliensis*. J. Med. Microbiol. 40 (2), 124–128.
Goldman, G.H., dos Reis Marques, E., et al., 2003. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of Candida albicans virulence and pathogenicity genes. Eukaryot Cell 2 (1), 34–48.
Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.
Hernandez, O., Almeida, A.J., et al., 2011. The hydrolase PbHAD32 participates in the adherence of *Paracoccidioides brasiliensis* conidia to epithelial lung cells. Med. Mycol..
Hernandez, O., Almeida, A.J., et al., 2010. A 32-kilodalton hydrolase plays an important role in *Paracoccidioides brasiliensis* adherence to host cells and influences pathogenicity. Infect. Immun. 78 (12), 5280–5286.
Hernandez, O., Garcia, A.M., et al., 2011. Gene expression during activation of *Paracoccidioides brasiliensis* conidia. Yeast 28 (11), 771–781.
Hodgetts, S., Matthews, R., et al., 1996. Over-expression of *Saccharomyces cerevisiae* hsp90 enhances the virulence of this yeast in mice. FEMS Immunol. Med. Microbiol. 16 (3–4), 229–234.
Howes, R., Barril, X., et al., 2006. A fluorescence polarization assay for inhibitors of Hsp90. Anal. Biochem. 350 (2), 202–213.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17 (8), 754–755.
Kaufmann, S.H., 1990. Heat shock proteins and the immune response. Immunol. Today 11 (4), 129–136.
Kiang, J.G., Tsokos, G.C., 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol. Ther. 80 (2), 183–201.
Krajaejun, T., Gauthier, G.M., et al., 2007. Development and application of a green fluorescent protein sentinel system for identification of RNA interference in Blastomyces dermatitidis illuminates the role of septin in morphogenesis and sporulation. Eukaryot Cell 6 (8), 1299–1309.
Kregel, K.C., 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol. 92 (5), 2177–2186.
Kurita, N., Sano, A., et al., 1993. An improved culture medium for detecting live yeast phase cells of *Paracoccidioides brasiliensis*. J. Med. Vet. Mycol. 31 (3), 201–205.
Leung, P.S., Gershwin, M.E., 1991. The immunobiology of heat shock proteins. J. Investig. Allergol. Clin. Immunol. 1 (1), 23–30.
Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25 (4), 402–408.
Loubradou, G., Begueret, J., et al., 1997. A mutation in an HSP90 gene affects the sexual cycle and suppresses vegetative incompatibility in the fungus *Podospora anserina*. Genetics 147 (2), 581–588.
Maresca, B., Kobayashi, G.S., 2000. Dimorphism in Histoplasma capsulatum and Blastomyces dermatitidis. Contrib. Microbiol. 5, 201–216.
Matthews, R.C., Burnie, J.P., 2004. Recombinant antibodies: a natural partner in combinatorial antifungal therapy. Vaccine 22 (7), 865–871.
Nemecek, J.C., Wuthrich, M., et al., 2006. Global control of dimorphism and virulence in fungi. Science 312 (5773), 583–588.
Nicola, A.M., Andrade, R.V., et al., 2005. Molecular chaperones in the *Paracoccidioides brasiliensis* transcriptome. Genet. Mol. Res. 4 (2), 346–357.
Nicola, A.M., Andrade, R.V., et al., 2008. The stress responsive and morphologically regulated hsp90 gene from *Paracoccidioides brasiliensis* is essential to cell viability. BMC Microbiol. 8, 158.
Rappleye, C.A., Engle, J.T., et al., 2004. RNA interference in Histoplasma capsulatum demonstrates a role for alpha-(1,3)-glucan in virulence. Mol. Microbiol. 53 (1), 153–165.
Rokhlenko, O., Wexler, Y., et al., 2007. Similarities and differences of gene expression in yeast stress conditions. Bioinformatics 23 (2), e184–e190.
Ruiz, O.H., Gonzalez, A., et al., 2011. Alternative oxidase mediates pathogen resistance in *Paracoccidioides brasiliensis* infection. PLoS Negl. Trop. Dis. 5 (10), e1353.
Sambrook, J., Fritsch, F., et al., 1998. Molecular Cloning: A Laboratory Manual. C.S.H. Laboratory, Cold Spring Harbor, NY.
Shapiro, R.S., Cowen, L., 2010. Coupling temperature sensing and development: Hsp90 regulates morphogenetic signalling in Candida albicans. Virulence 1 (1), 45–48.
Sharpton, T.J., Stajich, J.E., et al., 2009. Comparative genomic analyses of the human fungal pathogens Coccidioides and their relatives. Genome Res. 19 (10), 1722–1731.
Sreedhar, A.S., Mihaly, K., et al., 2003. Hsp90 inhibition accelerates cell lysis. Anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90 inhibitors involving both superoxide- and Hsp90-dependent events. J. Biol. Chem. 278 (37), 35231–35240.
Stanke, M., Steinkamp, R., et al., 2004. AUGUSTUS: a web server for gene finding in eukaryotes. Nucleic Acids Res. 32, W309–W312 (Web Server issue).
Swoboda, R.K., Bertram, G., et al., 1995. Structure and regulation of the HSP90 gene from the pathogenic fungus Candida albicans. Infect. Immun. 63 (11), 4506–4514.
Teixeira, M.M., Theodoro, R.C., et al., 2009. Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. Mol. Phylogenet. Evol. 52 (2), 273–283.
Zdobnov, E.M., Apweiler, R., 2001. InterProScan – an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17 (9), 847–848.