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RESEARCH ARTICLE

A conserved dimorphism-regulating histidine kinase controls the dimorphic switching in *Paracoccidioides brasiliensis*

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One sentence summary: PbDRK1, a conserved histidine kinase, acts as a regulator of dimorphism in *Paracoccidioides brasiliensis*, participates in the response to osmotic stress and its virulence.

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ABSTRACT

Paracoccidioides brasiliensis and *P. lutzii*, thermally dimorphic fungi, are the causative agents of paracoccidioidomycosis (PCM). *Paracoccidioides* infection occurs when conidia or mycelium fragments are inhaled by the host, which causes the *Paracoccidioides* cells to transition to the yeast form. The development of disease requires conidia inside the host alveoli to differentiate into yeast cells in a temperature-dependent manner. We describe the presence of a two-component signal transduction system in *P. brasiliensis*, which we investigated by expression analysis of a hypothetical protein gene (PADG_07579) that showed high similarity with the dimorphism-regulating histidine kinase (*DRK1*) gene of *Blastomyces dermatitidis* and *Histoplasma capsulatum*. This gene was sensitive to environmental redox changes, which was demonstrated by a dose-dependent decrease in transcript levels after peroxide stimulation and a subtler decrease in transcript levels after NO stimulation. Furthermore, the higher *PbDRK1* levels after treatment with increasing NaCl concentrations suggest that this histidine kinase can play a role as osmosensing. In the mycelium-yeast (M→Y) transition, *PbDRK1* mRNA expression increased 14-fold after 24 h incubation at 37°C, consistent with similar observations in other virulent fungi. These results demonstrate that the *PbDRK1* gene is differentially expressed during the dimorphic M→Y transition. Finally, when *P. brasiliensis* mycelium cells were exposed to a histidine kinase inhibitor and incubated at 37°C, there was a delay in the dimorphic M→Y transition, suggesting that histidine kinases could be targets of interest for PCM therapy.

Keywords: dimorphism; histidine kinase; phosphorelay; *Paracoccidioides*

INTRODUCTION

Paracoccidioides brasiliensis and *P. lutzii* are thermally dimorphic fungi and the causative agents of paracoccidioidomycosis (PCM), an important human disease in Latin America (Desjardins *et al.* 2011; Martinez 2015). PCM has a multitude of clinical presentations, from cutaneous to systemic forms, and can spread to various tissues, especially from the lungs. The ability of the fungus to cause disease relies on its capacity to switch from the

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mycelium to yeast form (M→Y) at the site of infection, along with an ineffective immune response from the host (Borges-Walmsley *et al.* 2002; de Oliveira *et al.* 2015). Nemecek, Wüthrich and Klein (2006) reported one of the largest comprehensive mechanistic descriptions of the switch to the pathogenic form among dimorphic fungi. They characterised the open reading frame of *DRK1* (dimorphism-regulating histidine kinase 1), which is responsible for controlling the M→Y transition in *Histoplasma capsulatum* and *Blastomyces dermatitidis* fungi. When the *DRK1* gene was knocked down and the mycelium cells were incubated at 37°C, the fungus was only able to form pseudohyphae, which expressively reduced their virulence. The expression of the *DRK1* gene in dimorphic fungi is phase specific for the yeast form of *Sporothrix schenckii* (Hou *et al.* 2013) and is fundamental to the M→Y transition in *H. capsulatum*, *B. dermatitidis* (Nemecek, Wüthrich and Klein 2006) and *Penicillium marneffei* (Boyce *et al.* 2011).

Histidine kinases are highly conserved proteins belonging to two-component signal transduction (TCST) systems, which are based on histidine and aspartate residue phosphorylation (Wuichet, Cantwell and Zhulin 2010). These systems are activated by a trans-autophosphorylation mechanism performed by the catalytic domain of the histidine kinase (Stock, Robinson and Goudreau 2000). After the catalytic domain phosphorylates a conserved histidine residue, the protein transfers the phosphorylation to a conserved aspartate residue in another protein containing a receiver domain (Capra and Laub 2012). Drk is a histidine kinase belonging to a variant of this system known as the phosphorelay. In this variant, a unique, hybrid histidine kinase contains the catalytic and the receiver domains (Capra and Laub 2012; Boyce and Andrianopoulos 2015).

TCST and phosphorelay systems have a well-documented role as determinants of pathogenesis in fungi, such as *Candida albicans*, *Cryptococcus neoformans*, *B. dermatitidis* and *H. capsulatum*, and in bacteria (Calera and Calderone 1999; Calera, Zhao and Calderone 2000; Hoch 2000; Chauhan *et al.* 2003; Bahn *et al.* 2006; Nemecek, Wüthrich and Klein 2006; Mitrophanov and Groisman 2008). Furthermore, these systems are absent in mammalian cells. In fungi, TCST/phosphorelay systems have been implicated mainly in the oxidative and osmotic stress responses and in virulence switches from non-pathogenic to pathogenic states. Because TCST/phosphorelay systems are absent in mammalian cells, they are attractive targets for the development of new antifungals with the potential for minimal side effects in humans (Chauhan and Calderone 2008; Shor and Chauhan 2015). Indeed, *Saccharomyces cerevisiae* expressing the heterologous histidine kinase CaNiK1p from *C. albicans* becomes susceptible to antibiotics targeting this protein (El-Mowafy, Bahgat and Bilitewski 2013).

Recently, Boyce and Andrianopoulos (2015) noted several genes with some role in pathogenesis among the dimorphic fungi. Although we now know some important genes that contribute towards pathogenesis in the yeast form of *P. brasiliensis*, notably *CDC42p*, *AOX*, *RBT5*, *GP43*, *HSP90* and *P27* (Almeida *et al.* 2009; Ruiz *et al.* 2011; Tamayo *et al.* 2013; Torres *et al.* 2013, 2014; Bailão *et al.* 2014), the signalling pathways that control the morphological changes in *P. brasiliensis* are poorly understood.

Almeida *et al.* (2009) reported that *P. brasiliensis* yeast cells deficient in Rho-GTPase Cdc42p expression have a reduction in the size of cells and buds, as well in virulence. Rho-GTPase Cdc42p is a mitogen-activated protein kinase (MAPK) downstream of Ras, and its activity was demonstrated to be important to the M→Y transition in *P. brasiliensis* (Fernandes *et al.* 2008). By using a specific inhibitor of farnesylation, Fernandes *et al.* (2008) prevented Ras from anchoring on the membrane of *P. brasiliensis*, causing a delay in the M→Y transition. In the present study, we identified in the genome of the *P. brasiliensis* histidine kinase-like *DRK1* gene. We investigated its expression during the mycelium to yeast transition, under different stress conditions and in virulent and attenuated isolates. Our results show that *PbDRK1* is involved in cell morphogenesis and differentiation, osmotic stress response and it also may be a possible virulence regulator. These findings suggest that Drk1 plays an important role in controlling the switch to the pathogenic state in *P. brasiliensis*.

MATERIALS AND METHODS

Fungal isolate and growth conditions

Paracoccidioides brasiliensis (isolate Pb18) was used for the experiments. Yeast extract-peptone-dextrose modified medium (mYPD) (0.5% yeast extract, 1% casein peptone and 0.5% glucose, pH 6.7) was used to cultivate yeast or mycelium cells, which were cultured at 37°C or 25°C, respectively. After three passages on solid medium, the virulent Pb18 (vPb18) isolate was used to infect mice (B10.A) and then reisolated. The attenuated isolate (aPb18) was maintained in culture for at least 5 years. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

Paracoccidioides RNA isolation

Paracoccidioides brasiliensis yeast cells were grown in mYPD broth for 5 days at 37°C with continuous shaking at 150 rpm, then incubated in RPMI 1640 medium for 24 h. Cells were washed with phosphate-buffered saline (PBS) and resuspended in medium containing 0.1, 1 or 10 mM H_2O_2. For nitrosative stress assays, the yeasts cells were incubated with 0.25, 1 or 10 μM $NaNO_2$ (in mildly acidic culture medium, pH 5.6; in this condition $NaNO_2$ releases NO) for 5 h at 37°C with continuous shaking at 150 rpm (Nathan and Shiloh 2000; Schnappinger *et al.* 2003; Rhee *et al.* 2005; Haniu *et al.* 2013). For evaluation of gene expression in *P. brasiliensis* undergoing heat shock stress, yeast cells growing in mYPD at 37°C with continuous shaking at 150 rpm were transferred to fresh medium at 42°C, and aliquots were collected after 0, 30 and 60 min. For evaluation of osmotic stress response, yeast cells growing in mYPD supplemented with 50, 150 or 300 mM NaCl at 37°C were collected after 1 h. Fully formed yeast cells were mechanically disrupted by vortexing with glass beads for 10 min in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Medium without stimulus was used as a control for all the experiments, and fungal viability was evaluated by trypan blue staining.

RNA purification during the M→Y transition from 25°C to 37°C was obtained using the protocol described by Batista *et al.* (2007). Briefly, cell pellets of *P. brasiliensis* mycelium or mycelium undergoing the phase transition were packed in aluminium foil, frozen in liquid N_2 and ground. The resulting powder was thawed in TRIzol. Ground cells in TRIzol were processed as suggested by the manufacturer for the purification of RNA, which was then quantified with a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientifics, Wilmington, DE, USA). The quality of the extracted RNA was verified using 1% agarose gel electrophoresis, and the RNA was stored at –80°C in H_2O.

Real-Time quantitative RT-qPCR

The abundance of *P. brasiliensis DRK1* transcripts for different experimental conditions was quantified by RT-qPCR. After the

assessment of the purity of the extracted RNA, samples were processed to obtain cDNA with the ProtoScript First Strand cDNA Synthesis kit (New England BioLabs, Ipswich, MA, USA). The 20-μL reaction contained 0.2 μL of cDNA, 0.1 μM of forward and reverse primers and SYBR® Green Master Mix (Applied Biosystems, USA). RT-qPCR runs were performed using the ABI StepOne Plus Real-Time PCR System (Applied Biosystems) with the following reaction conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The dissociation curve included an additional cycle of 15 s at 95°C, 20 s at 60°C and 15 s at 95°C. DNA contamination was evaluated via PCR amplification of the *GP43* gene (accession no. U26160). Negative controls contained neither DNA nor RNA. The relative expression ratio (experimental/control) was determined based on the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) after normalisation to the transcript levels of α-tubulin (*α-TUB*) and 60S ribosomal protein L34 (*L34r*). The data are presented as the mean values of triplicate RT-qPCR runs. The gene-specific primer pairs were as follows: *DRK1* forward: 5′ GGAGGGTAAATGGAAGGATCTG 3′, reverse: 5′ AAAGTTCTCCCTTGGCGATAG 3′; *α-TUB* forward: 5′ CGGCATATGGAAAATACATGGC 3′, reverse: 5′ GTCTTGGCCTTGAGAGATGCA 3′; and *L34r* forward: 5′ CAAGACTCCAGGCGGCAAC 3′, reverse: 5′ GCACCGCCATGACTGACG 3′.

Histidine kinase inhibition assay

Paracoccidioides brasiliensis yeast cells were grown in mYPD broth for 7 days at 25°C with continuous shaking at 150 rpm to induce the Y→M transition. Cultures were examined by microscopy to confirm the completion of the Y→M transition. Media containing fully formed mycelium cells were supplemented with iprodione (Pestanal, Sigma-Aldrich) to a final concentration of 20 μM, the temperature was increased to 37°C to induce the M→Y transition, and the transition was monitored in iprodione-supplemented and non-supplemented cultures by bright field microscopy at 5, 10, 24, 48, 72 and 96 h. Because iprodione is insoluble in water, dimethyl sulphoxide (DMSO) was used to solubilise the drug. Because the drug undergoes photodegradation in solution (Lassalle *et al.* 2014), it was always freshly prepared and stored in the dark.

Fungi susceptibility to iprodione

To test the susceptibility of *P. brasiliensis* yeast cells to iprodione, a Drk1 inhibitor, the cells (1×10^5) were seeded in 6-well culture plates. Medium supplemented with 20 μM of iprodione in DMSO was added every 24 h, and cells were counted using a Neubauer chamber. Wells containing only medium were used as a negative control, and DMSO was used as a vehicle control. The experiment was performed in quadruplicate. After 5 days of incubation with iprodione, a suspension of 1×10^6 cells was washed three times and incubated with 3 μM propidium iodide in filtered PBS for 30 min at room temperature and analysed by flow cytometry (BD FACSCalibur).

In Silico analysis

Protein domains were found by inputting sequences into the InterPro protein families database (Mitchell *et al.* 2015). Phobius predictor, an algorithm based on a hidden Markov model, was used to search for signal peptide and transmembrane protein topology (Käll, Krogh and Sonnhammer 2004). Additional searches were performed using the Basic Local Alignment Search Tool.

Spot test

The osmotic sensitivity of the yeast strains was determined by the drop test standard technique. Briefly, yeast cultures in an early stationary phase of growth (2×10^6 cells/mL) were obtained after 4–5 days of growth in mYPD media at 37°C. The yeast cultures were serially diluted (1:10 at each step) in a sterile saline solution (0.9% NaCl), and 30 μL of each suspension was plated on mYPD with different NaCl concentrations (50, 100, 150, 200 or 300 mM) with and without 20 μM iprodione supplementation. Negative controls were plated on media without NaCl. Plates were photographed after 7 days of growth at 37°C.

Statistical analysis

Data are expressed as the means ± SD. Significance was assessed by one-way analysis of variance with Student's *t*-test used for comparison. The results with $P < 0.05$ were considered statistically significant.

RESULTS

Histidine kinases are highly conserved between dimorphic and other relevant fungi

The *PbDRK1* gene is located at locus PADG_07579 (Broad Institute accession number) within the negative strand of the *Paracoccidioides brasiliensis* genome and contains a coding sequence of 4054 nucleotides with only one 3′ intron. The putative Drk1 protein of *P. brasiliensis* contains 1292 amino acids and has an estimated molecular mass of ~142 kDa and a basic isoelectric point of 5.34. The N-terminus contains six HAMP domains (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases), a histidine phosphotransfer and a dimerisation domain, which harbours the conserved histidine that is the site of both the autophosphorylation and phosphotransfer reactions (Capra and Laub 2012). We observed an extremely conserved protein organisation of Drk1 among members of the Eurotiomycetes family (e.g. *P. brasiliensis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Aspergillus nidulans*) (Fig. 1A). The number and position of the HAMP domains are conserved. The first PbDrk1 HAMP domain contains 56 amino acid residues, whereas all others have 53 amino acids. Drk1 from *Cryptococcus neoformans* and *Candida albicans* contain seven and five HAMP domains, respectively. Furthermore, PbDrk1 also presents a conserved histidine kinase (226 aa) and histidine kinase-like ATPase, C-terminal (HATPase c, 119 aa) domains, which correspond to the catalytic and ATP-binding domain (CA). These domains can either relay signals from the periplasmic sensory domains to the dimerisation and histidine-phosphotransfer domain and CA domains or, in some cases, directly recognise cytoplasmic signals (Möglich, Ayers and Moffat 2009; Parkinson 2010). Finally, all fungal Drk1 analysed contain in the C-terminus a response regulator receiver domain, which has an aspartate that receives the phosphate group from its cognate histidine kinase (Fig. 1A). These features indicate that this gene encodes a putative hybrid histidine kinase protein. By using the Gene Ontology Annotation database, we found that PbDrk1 is predicted to be an integral transmembrane protein. The computer program TargetP (Nielsen *et al.* 1997) (http://www.cbs.dtu.dk/services/TargetP/) and Phobius (Käll, Krogh and Sonnhammer 2004) (http://phobius.sbc.su.se/) predicted a putative signal peptide within the 19 first amino acid residues in *P. brasiliensis*, *B. dermatitidis*, *H. capsulatum*, *C. immitis* and *Neurospora crassa* (Fig. 1A).

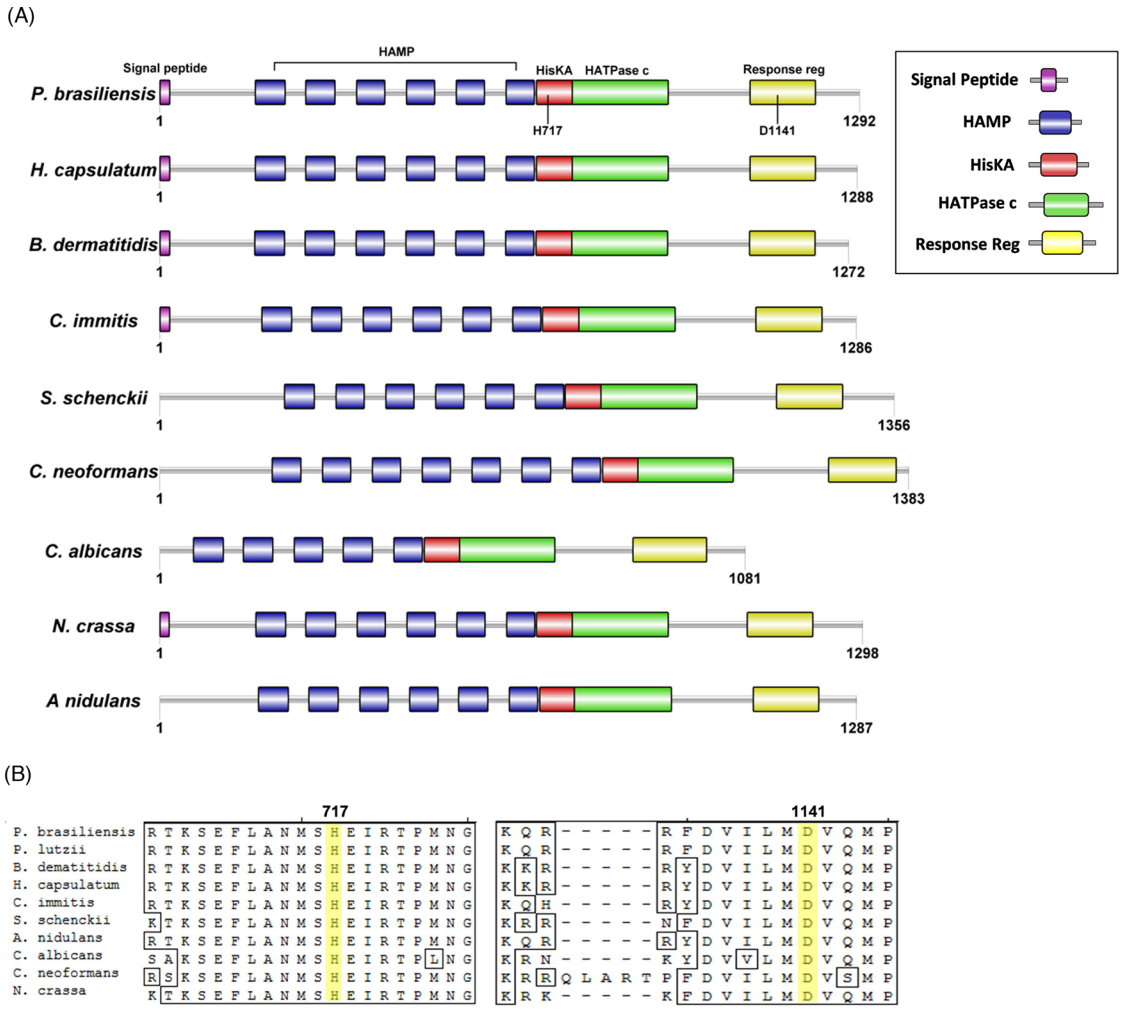

Figure 1. *In silico* analyses of histidine kinases from group III. (**A**) Representation of the Drk1 protein from dimorphic and other fungi showing the highly conserved domains HAMP (blue), HisKA (red), HATPase c (green), Response Reg (yellow) (*P. brasiliensis* PADG_07579, *H. capsulatum* HCBG_00429, *B. dermatitidis* BDFG_06743, *C. immitis* CIMG_04512, *S. schenckii* AFS50004, *C. neoformans* CNAG_01850, *C. albicans* AF029092, *N. crassa* NCU02815, *A. nidulans* FGSCA4). (**B**) Multiple alignment of Drk1 proteins from *P. brasiliensis* and several other fungi showing conservation of histidine (H717) and aspartate (D1141) residues predicted to be the sites of phosphorylation. Both residues are highlighted in yellow.

A comparison of fungal histidine kinase homologues, such as Drk1 of *H. capsulatum*, *B. dermatitidis*, *C. immitis*, *Sporothrix schenckii*, *C. neoformans*, *A. nidulans*, *N. crassa* and *C. albicans*, revealed a high percentage of shared identity, especially among the dimorphic fungi (over the 80%). In these species, Drk1 is highly conserved and structurally related to the histidine kinase from group III, as described by Catlett, Yoder and Turgeon (2003) (Fig. 1B). Excluding the *PbDRK1* gene (PADG_07579), the *P. brasiliensis* (isolate Pb18) genome contains four other genes for hypothetical histidine kinases (PADG_00903, PADG_00905, PADG_04063 and PADG_07139), but only *PbDRK1* belongs to group III histidine kinases. The presumptive autophosphorylated histidine residue was detected (His-717) while the canonical receiver domain (865–986 aa) containing a phosphorylatable aspartate residue (Asp-1141) was observed in the C-terminal region of PbDrk1 (Fig. 1B). After this *in silico* characterisation, we investigated the role of the *PbDRK1* gene in the responses of *P. brasiliensis* to different types of stresses and dimorphic switching.

PbDRK1 is a phase-specific gene preferentially expressed in the yeast form of virulent *Paracoccidioides brasiliensis*

To verify whether the *PbDRK1* gene generates a functional product or not, we conducted a RT-qPCR assay, which showed that the gene is expressed in *P. brasiliensis* yeast cells. Because the *DRK1* gene has been associated with dimorphic switching in *H. capsulatum* and *B. dermatitidis* (Nemecek, Wüthrich and Klein 2006), we evaluated *PbDRK1* expression during dimorphism in *P. brasiliensis*. We measured *PbDRK1* transcript levels at the M→Y transition, which showed *PbDRK1* transcription to be phase specific to the yeast form (Fig. 2A). Under dimorphic transition, the *PbDRK* expression appeared to be biphasic. There appear to be two expression peaks, one in 24 h (after M→Y transition) and other in 96 h. The expression of this gene increases as the mycelium reverts to yeast form until the transcript reaches stable levels (96 h). In other models, some genes involved in differentiation exhibit its biphasic expression profile, such as the *rnrB* gene of the *Dictyostelium discoideum* (MacWilliams *et al.* 2000). In

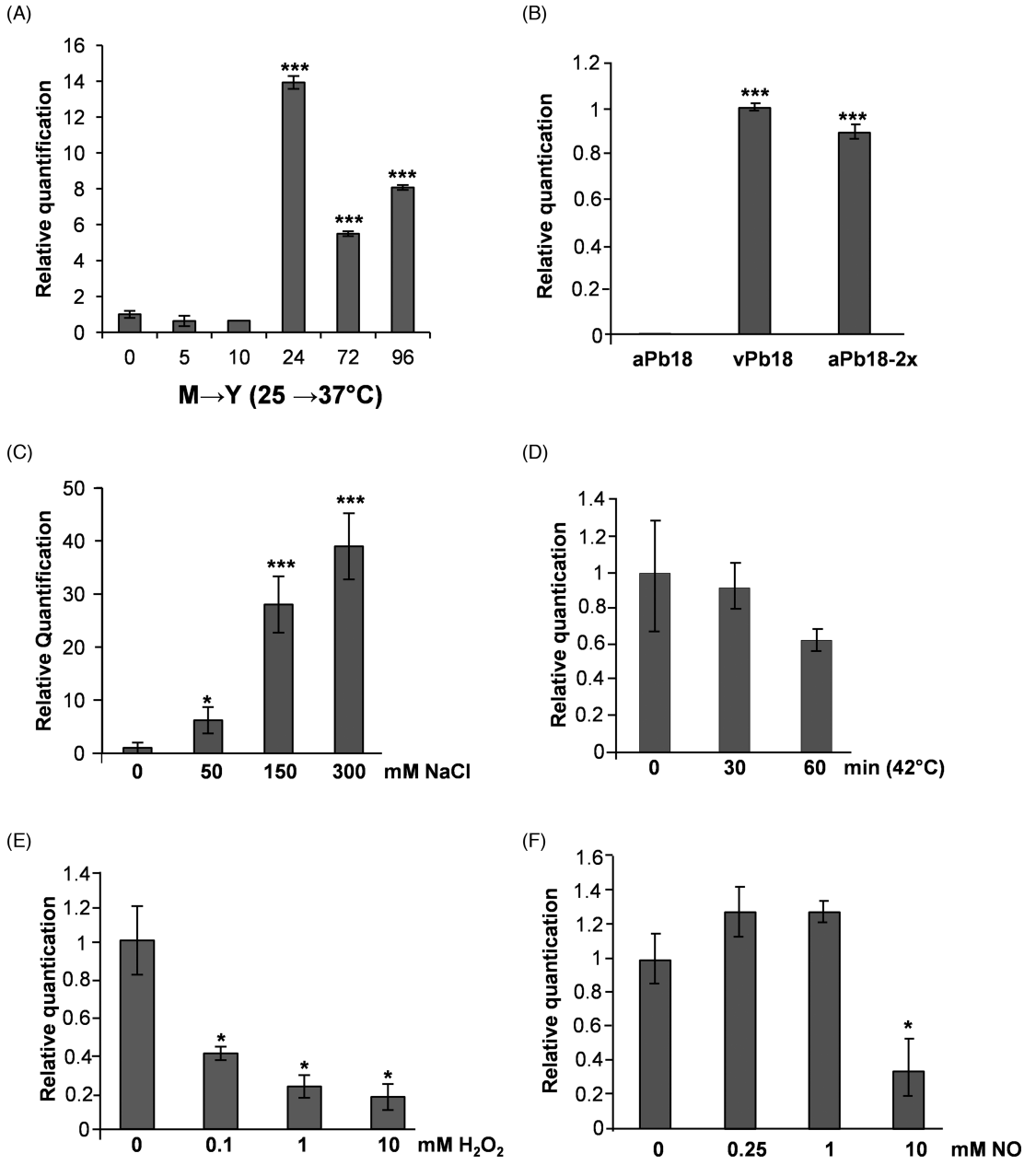

Figure 2. Reverse transcriptase qPCR of *PbDRK1* from *P. brasiliensis* under multiple conditions. (**A**) *PbDRK1* (PADG_07579) transcript levels were measured throughout the *P. brasiliensis* mycelium to yeast transition at 37°C for up to 96 h. (**B**) *PbDRK1* expression in *P. brasiliensis* attenuated (aPb18), virulent (vPb18) and aPb18 after two passages through animals (aPb18-2x) to restore virulence. (**C**) *PbDRK1* expression after treatment with different NaCl concentrations. (**D**) *PbDRK1* expression after heat shock at 42°C for 30 or 60 min. (**E**) *PbDRK1* expression after treatment with different peroxide concentrations. (**F**) *PbDRK1* expression after treatment with different NO concentrations. The change in transcriptional levels was calculated via the ΔΔCt method, with two housekeepers (*α-TUB* and *L34r*). All of the data shown in this figure were analysed using Student's *t*-test. Error bars correspond to the standard deviation of measurements performed in triplicate, and asterisks indicate statistically significant differences in expression (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). The results shown are representative of three independent experiments.

yeast cells, the *PbDRK* expression was always higher than from the mycelium levels. Indeed, we examined mycelium cells cultured at 25°C for several days and found that the expression of *PbDRK1* was always lower than that from yeast cells cultured at 37°C (Fig. 1S, Supporting Information). This showed that *PbDRK1* transcripts are yeast-phase enriched in *P. brasiliensis*. To assess this gene as a possible virulence factor, we compared its transcript levels in the virulent fungus (vPb18), in its attenuated form (aPb18), and in its attenuated form that had regained virulence (aPb18-2x) after two passages of aPb18 in mice, according to Castilho *et al.* (2014). Expression analysis revealed that *PbDRK1* was upregulated in both the virulent strain and the attenuated strain that regained virulence and was isolated after two passages in mice (aPb18-2x) (Fig. 2B). *PbDRK1* expression was enriched more than 10-fold in vPb18 and aPb18-2x compared to aPb18. These results demonstrate that the expression of *PbDRK1* is a possible virulence factor and can be modulated by the host response.

In other fungi, the group III histidine kinases have been found to function as osmosensors that are essential for fungal survival under high osmolarity stress (Alex, Borkovich and Simon 1996; Schumacher, Enderlin and Selitrennikoff 1997; Alex *et al.* 1998;

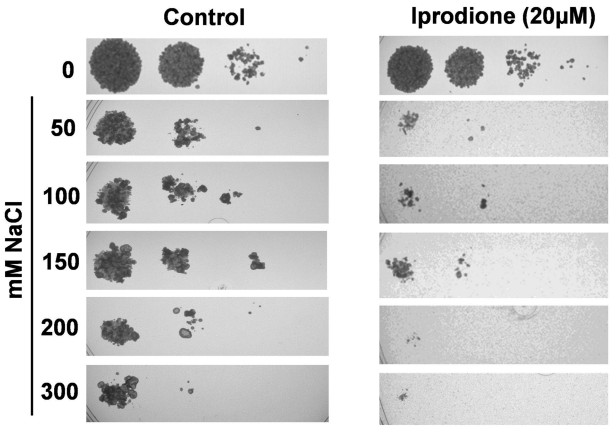

Figure 3. Spot assay under osmotic stress before and after treatment with iprodione. A total of 2×10^6 *P. brasiliensis* yeast cells were spotted on mYPD supplemented with different NaCl concentrations and allowed to grow for 7 days. The left panel shows cells with no treatment, the right panel shows cells from medium supplemented with iprodione at 20 μM. The results shown are representative of three independent experiments.

Nagahashi *et al.* 1998; Srikantha *et al.* 1998; Cui *et al.* 2002; Motoyama *et al.* 2005). To evaluate *PbDRK1* expression in different salt concentrations, we analysed its mRNA levels after osmotic stress. When *P. brasiliensis* yeast cells were treated with different concentrations of NaCl, *PbDRK1* levels increased in a dose-dependent manner (Fig. 2C). After treatment with 300 mM NaCl, the transcription of *PbDRK1* increased 40-fold, revealing that this gene is also responsive to osmotic stress in *P. brasiliensis*.

Other stress conditions were evaluated. Given that temperature is a critical factor in fungus dimorphism, we investigated if the *PbDRK1* gene was regulated by heat shock. The differences in expression of this gene were not significant at either 30 or 60 min after heat shock treatment (42°C) (Fig. 2D). Furthermore, we tested *PbDRK1* expression following reactive oxygen species (ROS) or reactive nitrogen species (RNS) treatment. The gene showed redox sensitivity as evidenced by a 2.5-fold decrease in levels of the transcript after 0.1 mM peroxide stimulation and a 4.4- and 5.9-fold decrease in levels of the transcript after 1 mM and 10 mM H_2O_2 stimulation, respectively (Fig. 2E). The response of the gene to the nitrosative stress was not the same. Indeed, *DRK1* expression decreased only at higher NO concentrations (Fig. 2F).

To investigate the role of *PbDRK1* under osmotic stress, the Pb18 strain was exposed to different concentrations of NaCl and the presence or absence of iprodione, a pharmacological inhibitor used in other fungi (Boyce *et al.* 2011; Fillinger *et al.* 2012; El-Mowafy, Bahgat and Bilitewski 2013). In the controls we observed that Pb18 is sensitive to concentrations higher than 200 mM NaCl (Fig. 3, left panel). Furthermore, we found that when the Pb18 strain was incubated in mYPD supplemented with 20 μM iprodione, it was more strongly influenced by osmotic stress compared to control (Fig. 3, right panel). These results indicate that PbDrk1 plays an important role in the osmotic stress response in *P. brasiliensis*.

Inhibition of PbDrk1 protein causes a delay in the mycelium to yeast transition

Once the *PbDRK1* expression profile was assessed under diverse conditions, we examined the effects of PbDrk1 inhibition with iprodione. When yeast cells were treated with concentrations of iprodione up to 40 μM the fungus viability was maintained (data not shown). Then, we chose the concentration of 20 μM of iprodione to the inhibition assays. Moreover, 1×10^6 viable yeast cells of *P. brasiliensis* were cultured for 4 days in YPD medium with or without treatment with iprodione. The viable cell counts were performed at specific points during cultivation. No changes in the growth curve, for both samples (in the presence or absence of 20 μM iprodione), were observed (data not shown). Thus, we can say that 20 μM iprodione was not toxic to the cell and did not inhibit fungal growth.

Yeast cells were reverted to mycelium by incubating cells at 25°C and the complete transition was followed by microscopy. *Paracoccidioides brasiliensis* mycelium cells were incubated at 37°C with 20 μM iprodione, 1 μL DMSO, or only culture medium and examined by microscopy up to 96 h. At normal conditions, *P. brasiliensis* mycelium cells start to revert into yeast form after 24 h incubation at 37°C and complete the transition after 96–120 h (Fig. 4A, left panel). The mycelium cells treated with 1 μL DMSO showed a similar transition profile (Fig. 4A, centre panel) when compared to the control. Interestingly, when mycelium cells were treated with 20 μM iprodione and incubated at 37°C, there was a delay in the transition to yeast form, indicating that PbDrk1, in addition to being phase specific, is an important regulator of dimorphic switching in *P. brasiliensis* (Fig. 4A, right panel). The delay effect in switching to the pathogenic phase was observed in both virulent (vPb18) and attenuated (aPb18) fungus (data not shown). Then, we compared the *PbDRK1* transcript levels between the yeast cells from two attenuated strains: one always growing as yeast cells (aPb18) and another that was induced to switch to mycelium and again reverted to yeast (aPb18Rev). Strikingly, we found that when attenuated fungus is forced to perform the M→Y transition it increases the *PbDRK1* levels compared to that attenuated fungus that stayed a long time without transition (Fig. 4B).

DISCUSSION

The *PbDRK1* gene encodes a hybrid histidine kinase, which is highly conserved among dimorphic and other fungi such as *Aspergillus nidulans*, *Neurospora crassa* and *Candida albicans* (Fig. 1). After the mutations in group III histidine kinases were associated with resistance to iprodione or fludioxonil treatment in *N. crassa* (Ochiai *et al.* 2001), the drug was considered an inhibitor specific for group III histidine kinases. According to the proposed classification (Catlett, Yoder and Turgeon 2003), the *PbDRK1* protein is structurally related to group III histidine kinases, which explains why we could use iprodione as an inhibitor of the *PbDRK1* pathway. While the *P. brasiliensis* (isolate Pb18) genome contains five genes for hypothetical histidine kinases (PADG_07579, PADG_00903, PADG_00905, PADG_04063 and PADG_07139), only *PbDRK1* belongs to group III, as demonstrated by the presence of repeated HAMP domains, a characteristic feature of this group of proteins. According to the Catlett, Yoder and Turgeon (2003) classification, there are 11 histidine kinase groups as determined by phylogeny analyses. *Paracoccidioides brasiliensis* has five histidine kinases-representing groups II, III, IV, V and VII (Fig. 2, Supporting Information).

When we examined the effects of PbDrk1 protein pharmacological inhibition, we found a significant delay in the transition to yeast form, independently whether the fungus was virulent or not, indicating that *PbDRK1* is an important regulator of

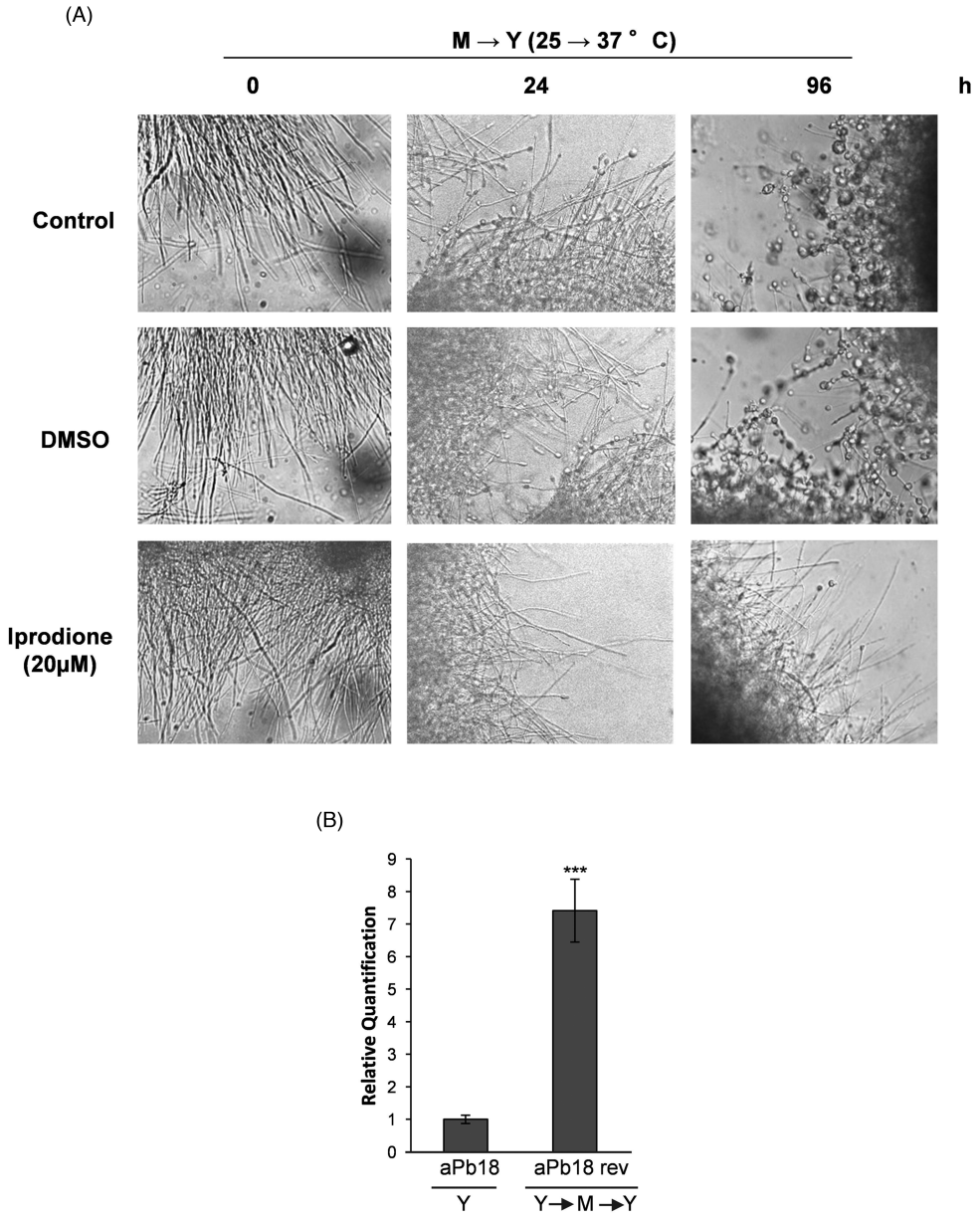

Figure 4. *Paracoccidioides brasiliensis* mycelium to yeast transition followed by bright field microscopy through 4 days. (**A**) Mycelial cells growing in the early exponential phase were induced to undergo morphological transformation by changing the temperature of incubation. *Paracoccidioides brasiliensis* mycelium cells were treated with DMSO or 20 μM iprodione and incubated at 37°C for 4 days. After 48-h incubation, the mycelium cells started to differentiate into yeast in untreated (control) and DMSO-treated cells, but not in cells treated with 20 μM iprodione. (**B**) *PbDRK1* (PADG_07579) transcript levels were measured from the attenuated yeast cells (aPb18) and after aPb18 yeast cells reverted to mycelium form (Y→M at 25°C) and again reverted to yeast form (after 5 days of M→Y transition at 37°C). This isolate was named as aPb18Rev (Y→M→Y). The difference was significant with a $P < 0.001$. The results shown are representative of three independent experiments.

dimorphic switching to the pathogenic state in *P. brasiliensis* (Fig. 4). These findings are consistent with the control of dimorphism in *Blastomyces dermatitidis* and *Histoplasma capsulatum*, as reported by Nemecek, Wüthrich and Klein (2006). In that report, Nemecek, Wüthrich and Klein (2006) presented evidence that in addition to regulating expression of virulence genes, *DRK1* is essential to the maintenance of suitable sporulation and cell-wall integrity in dimorphic fungi. Consistent with these findings, we reported that the expression of *PbDRK1* is abundant in the yeast phase and lower in the mycelium phase. This expression profile is also consistent with the results reported by Hou *et al.* (2013) while investigating the control of dimorphism in *Sporothrix schenckii*. Indeed, *PbDRK1* transcript levels greatly increased in the highly virulent *P. brasiliensis* isolate compared to the attenuated yeast (Fig. 2B).

Surprisingly, attenuated yeast cells are able to recover the high levels of *PbDRK1* when forced to perform the Y→M→Y transition (Fig. 4B). Probably, this effect is a consequence of the cellular machinery reorganisation or expression/activation of transcription factors occurred due to switching process. An analysis of *P. brasiliensis* transcriptome obtained from M→Y transition revealed a modulation of 2583 genes (Nunes *et al.* 2005), which could aid to explain the reactivation of *PbDRK1* at the attenuated fungus subjected to Y→M→Y transition. Monteiro *et al.* (2009) reported that *P. lutzii* yeast cells culture differ significantly in gene expression when allowed to grow for 5 or 14 days, while

mycelium cells maintain a clustered pattern of gene expression independently if cultured for 5 or 14 days. Probably, the yeast form has a more varied repertoire of responses to stresses than mycelium, the environmental form.

The group III histidine kinases are directly linked to the MAPK cascade and regulate its activation in response to osmotic conditions in the extracellular environment, resulting in the activation of the Hog1 pathway (Maeda, Wurgler-Murphy and Saito 1994; Posas *et al.* 1996). This link to the MAPK cascade has been observed in several fungi, including human pathogens (Bahn *et al.* 2006). El-Mowafy, Bahgat and Bilitewski (2013) reported that *Saccharomyces cerevisiae* expressing heterologous histidine kinase *CaNIK1p* from *C. albicans* becomes susceptible to treatment with iprodione and that this effect was due to the activation of MAPK Hog1p. Hagiwara *et al.* (2007) reported similar results while examining the treatment of *A. nidulans* with iprodione, in which the fungus' rate of growth was severely inhibited by treatment. We found that *P. brasiliensis* treated with Drk1 inhibitor showed growth reduction on the osmotic stress plates tested in our experiments, which suggests a relationship between the Hog1 pathway and Drk1 in *P. brasiliensis*.

Altogether, these findings point to a potential new target for antifungal therapy. Notably, protein kinases have been considered promising targets for diverse therapies (Sebolt-Leopold 2000; Doerig *et al.* 2005; Naula, Parsons and Mottram 2005). If the histidine kinases or any other component of the phosphorelay system are found to be absent in human cells, the specific inhibition of these proteins could provide beneficial effects as an adjuvant in antifungal therapy with minimal side effects (Shor and Chauhan 2015). Although iprodione has shown interesting results against some medically important fungi (Hagiwara *et al.* 2007; Chauhan and Calderone 2008; El-Mowafy, Bahgat and Bilitewski 2013), this drug must be only considered for *in vitro* assays. A computational study by Galli *et al.* (2014) reported a lower docking score value for human androgen receptor interacting with iprodione compared to its endogenous ligand, testosterone. This means that iprodione could compete with testosterone for the receptor and potentially cause endocrine side effects. The only similarity the *PbDRK1* gene has with any human endocrine receptor is a peptide of 15 amino acids in length (KRVARDVGVDGKMGG) that is similar to the human prolactin receptor (RefNG_029042.2). Although there is no important similarity between the *PbDRK1* gene and any human gene, a previous study by Blystone *et al.* (2007) reported a delay in pubertal development and reduced serum testosterone levels in male rats when treated with iprodione. They suggested that the mechanism by which iprodione exerts this endocrine effect was through the inhibition of steroidogenesis within the testis of pubertal rats, rather than by antagonising the androgen receptor.

Finally, we investigated the role of the *PbDRK1* gene in the responses of *P. brasiliensis* to different types of stress. Our data do not show important differences in *DRK1* expression after heat shock treatment. Furthermore, *PbDRK1* expression showed redox sensitivity, as evidenced by a decrease in transcript levels in a dose-dependent manner after peroxide stimulation and a similar, but less pronounced, decrease after NO stimulation. Whether these differences are important remains to be investigated.

CONCLUSIONS

We have described a gene that regulates dimorphism in *P. brasiliensis* and identified a drug which delayed dimorphic switching to the fungus' pathogenic phase *in vitro*. In addition to being responsive to osmotic adversities, the *PbDRK1* gene is redox sensitive and highly expressed in the virulent fungus compared to the attenuated fungus. Furthermore, we shown that *PbDRK1* gene is fundamental in the M→Y transition once this switching process is enough to reactivate the gene at the attenuated fungus. These findings indicate an association between the gene and virulence and suggest a new target for adjuvant therapy against paracoccidioidomycosis. Finally, for the first time, we present evidence of a TCST system functioning in *P. brasiliensis*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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