

Decreased expression of 14-3-3 in *Paracoccidioides brasiliensis* confirms its involvement in fungal pathogenesis

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The interaction between the fungal pathogen *Paracoccidioides brasiliensis* and host cells is usually mediated by specific binding events between adhesins on the fungal surface and receptors on the host extracellular matrix or cell surface. One molecule implicated in the *P. brasiliensis*-host interaction is the 14-3-3 protein. The 14-3-3 protein belongs to a family of conserved regulatory molecules that are expressed in all eukaryotic cells and are involved in diverse cellular functions. Here, we investigated the relevance of the 14-3-3 protein to the virulence of *P. brasiliensis*. Using antisense RNA technology and *Agrobacterium tumefaciens*-mediated transformation, we generated a 14-3-3-silenced strain (expression reduced by 55%). This strain allowed us to investigate the interaction between 14-3-3 and the host and to correlate the functions of *P. brasiliensis* 14-3-3 with cellular features, such as morphological characteristics and virulence, that are important for pathogenesis.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by fungi of the *Paracoccidioides* species complex (*P. brasiliensis* and *P. lutzii*). PCM is important due to the frequency and severity of the cases.¹ It is geographically confined to Latin America, with more than 10 million people possibly infected in this continent,² and the highest incidence occurs in Brazil (80%).^{3,4}

Fungi from the *Paracoccidioides* genus are thermal dimorphic fungi that grow both in a yeast form (at approximately 37°C) and as mycelia (below 25°C). Infection occurs mainly by inhalation of airborne propagules released by the mycelial form; once in the lungs and in contact with the host body temperature, it transforms to its yeast form, which is responsible for the symptoms and clinical manifestations during disease.^{5,6}

The development and the course of the microbial infection can be considered a multifactorial process that results from the interaction between the virulence factors of pathogen, leading to the pathogen's establishment in the host and causing disease, and the host's ability to prevent and resist microbial colonization or invasion.⁷⁻¹⁰

The pathogen uses a large repertoire of surface molecules, specifically adhesins, that can bind to the extracellular matrix (ECM) of various cell types in the host, inducing endocytosis in

normally non-phagocytic cells (epithelial and endothelial cells) during the invasion process.^{11,12} This interaction with the ECM has been correlated with the processes of adhesion and invasion. The ECM is composed of components such as collagen, laminin, fibronectin, vitronectin and proteoglycans, which participate in the regulation of physiological processes including cell migration, signaling and the transport of solutes across cellular barriers. The respiratory tract, the primary focus of PCM, is composed of tissues rich in ECM, such as laminin and various types of collagen. Moreover, alveolar fibroblasts in the lungs secrete ECM components.¹²⁻¹⁴

A required step for colonization and ultimately the development of disease is the ability of pathogens to adhere to host surfaces. The ability to adhere is a biological phenomenon that is widely distributed and shared by many pathogens, enabling the colonization of their respective habitats.^{15,16} Some adhesins have been described for *P. brasiliensis*, such as gp43,^{17,18} glyceraldehyde-3-phosphate dehydrogenase,¹⁹ triose phosphate isomerase,²⁰ enolase,^{21,22} a 32-Kda hydrolase,^{23,24} malate synthase,²⁵ isocitrate lyase²⁶ and the 14-3-3 protein (30 KDa)^{27,28}; this last adhesin is the focus of the present study.

Strong evidence has resulted in the classification of the 14-3-3 protein of *P. brasiliensis* as an adhesin, including its ability to bind laminin and its association with virulent isolates of the

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fungus.²⁷ Additionally, immunocytochemical studies using both *in vitro* and *in vivo* models have demonstrated the ubiquitous distribution (cytosol and cell membrane) of the 14-3-3 protein in the yeast form of *P. brasiliensis*. A significant increase in the amount of 14-3-3 was observed in the fungal cell wall during interaction with pneumocytes and in an experimental acute infection in mice. Inhibition assays using the recombinant 14-3-3 protein and anti-14-3-3 antibody to study the interaction of *P. brasiliensis* with pneumocytes reported a variation in the rate of adherence at different stages during infection, suggesting an important role for 14-3-3 in fungus-pathogen interactions.²⁸

The aim of this study was to develop an isolate of *P. brasiliensis* with low expression of the 14-3-3 protein using antisense RNA technology. We used transformation mediated by *Agrobacterium tumefaciens* to elucidate the role of this protein in *P. brasiliensis* pathogenesis by investigating its biological function and

involvement in virulence. These factors have not previously been described for this fungus.

Results

Knockdown of Pb14-3-3 expression

Using antisense (aRNA) technology and *A. tumefaciens*-mediated transformation (ATMT), we constructed an antisense RNA targeting the exon-coding sequence of the Pb14-3-3 gene to study the role of 14-3-3 in *P. brasiliensis* (Fig. 1A). We confirmed the integration of the hygromycin cassette into the genomic DNA of one hygromycin-resistant transformant (Fig. 1B). To pursue our main goals in this work, we selected a mitotically stable isolate with the highest decrease in Pb14-3-3 expression (55%) confirmed by RT-qPCR. The reduction in the transcript

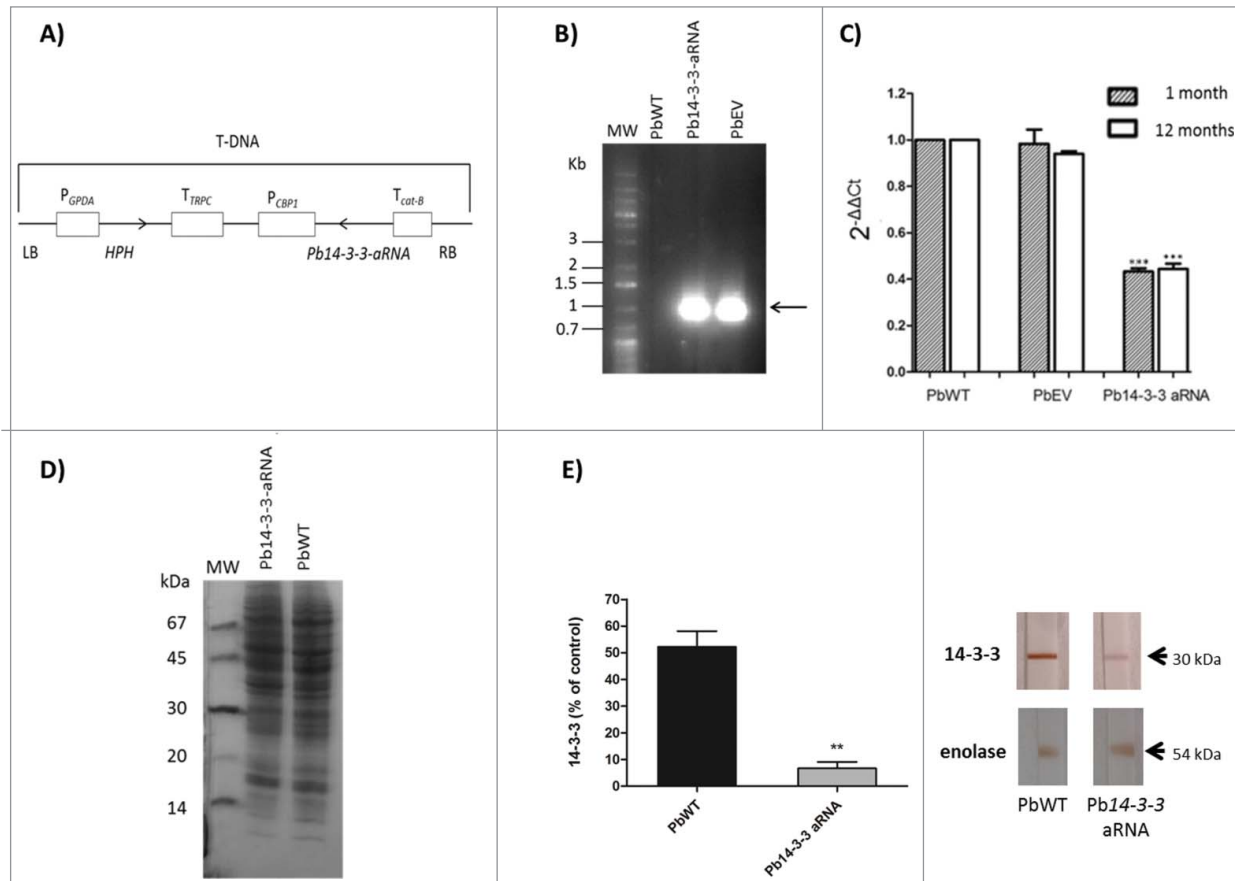


Figure 1. Inhibition of Pb14-3-3 expression using aRNA and *A. tumefaciens*-mediated transformation. (A) tDNA (T-DNA) inserted into *P. brasiliensis* by ATMT to silence Pb14-3-3. The anti-sense oligonucleotides were produced based on the Pb18 (PbWT) genomic sequence, as detailed in the Materials and Methods section. The sequence was cloned under the control of CBP-1 promoter (calcium-binding protein). The construct was sub-cloned into T-DNA region of the binary vector pUR5750 harboring hygromycin B phosphotransferase (HPH) driven by the glyceraldehyde-3-phosphate of *Aspergillus nidulans* (PGPDA) and with terminator (TTRPC) as described previously.³¹ (B) PCR fragments amplified with an HPH-specific primer yielded a 1,000 bp internal fragment. Pb14-3-3 aRNA, PbWT and PbEV were used as the templates. MW, DNA molecular marker. (C) Pb14-3-3 expression levels in PbWT, PbEV and Pb14-3-3 aRNA after 1 month and over 12 months of subculture. Gene expression levels obtained by RT-qPCR were normalized to the internal control TUB2; *** $p < 0.0001$ when compared with PbWT and PbEV; (D) SDS-PAGE gel of total proteins of PbWT and Pb14-3-3 aRNA; (E) Immunoblots of the total proteins tested with the anti-14-3-3 monoclonal antibody and anti-enolase (used as an internal control) and quantified using ImageJ software; $p = 0.0021$.

Table 1. Percentage of budding cells and number of buds by cell exhibited by the isolates.

Isolates	% of budding cells	mean buds/cell (± SD)
PbWT	83	2.23 (1.6)
PbEV	81	1.82 (1.5)
Pb14-3-3 aRNA	67	0.95 (1.0)

SD: standard deviation.

levels of Pb14-3-3 (band size of 30 kDa) was confirmed even after 12 months of successive subcultures of Pb14-3-3 aRNA (Fig. 1C). Next, SDS-PAGE under reducing conditions and immunoblotting (Fig. 1D) was performed with monoclonal anti-14-3-3 antiserum and polyclonal anti-enolase (as an internal control, band of 54 kDa) (Fig. 1E). The Pb14-3-3 aRNA strain expressed noticeably reduced levels of the 14-3-3 protein.

Pb14-3-3 silencing affects yeast cell morphology and morphological transition without affecting cell viability or fungal growth

The variability of budding of the yeast cells is shown in Table 1. PbWT and PbEV were not significantly different, with approximately 83% and 81% of yeast cells showing buds, respectively. The observed mean number of buds per yeast cell was 2.23 buds/yeast cell for PbWT and 1.82 buds/yeast cell for PbEV. In contrast, after knockdown with Pb14-3-3 aRNA, 67% of yeast cells possessed buds, with 0.95 buds/yeast cell, a significant reduction compared to the other isolates (Table 1).

The estimated diameters are depicted in Figure 2. Pb14-3-3 aRNA yeast cells were approximately 5 μm, significantly smaller than PbWT and PbEV, which averaged 9.19 μm and 8.67 μm in diameter, respectively. Microscopic observations indicated

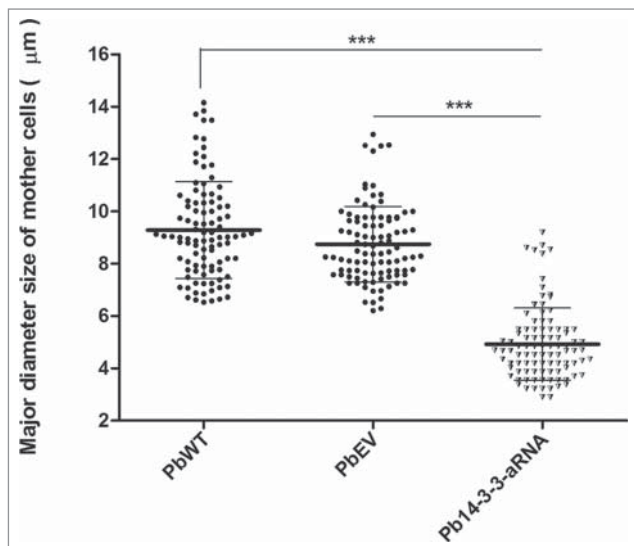


Figure 2. Knocking-down Pb14-3-3 in *P. brasiliensis* alters yeast cell size. Analysis of major diameter size of mother cells of PbWT, PbEV and Pb14-3-3 aRNA isolates. (***) $p < 0.0001$; $n = 100$ mother cells.

that some yeast cells (12%) of Pb14-3-3 aRNA were more elongated and filamentous than the wild-type cells and cells harboring the empty vector, but this was not the predominant phenotype (Fig. 3).

Coupled with the previous evidence, Pb14-3-3 silencing affected the fungal transition. To test the effect of Pb14-3-3 on fungal dimorphism, cells in each phase, yeast and mycelium, were incubated at 25°C and 37°C, respectively, until 100% conversion was observed.

At 25°C, after 24 h, PbWT, PbEV and Pb14-3-3 aRNA did not show differences, and we observed that approximately 15–20% of cells showed budding with neck elongation (transforming yeast). By 48 h, the percentage of transforming yeasts increased (to approximately 34.6–48.1%), and chlamyospore-like cells (differentiating hyphae) were present in all isolates PbWT (13.3%), PbEV (10%) and Pb14-3-3 aRNA (7.1%); however, at this time, hyphae were found only in PbWT (5%) and PbEV (2%). At 72 h, the majority of cells of PbWT (68%) and PbEV (58%) were hyphae, while those with Pb14-3-3 aRNA were yeast. Incubation for 120 h was sufficient to obtain 100% transition to mycelium for PbWT and PbEV. For the Pb14-3-3 aRNA isolate, this transition occurred gradually and slowly, requiring 10 days for completing the yeast-mycelium transition (Fig. 4E).

At 37°C, the same test was performed to verify the mycelium-yeast transition. After 24 h, most of the hyphae (58–82%)

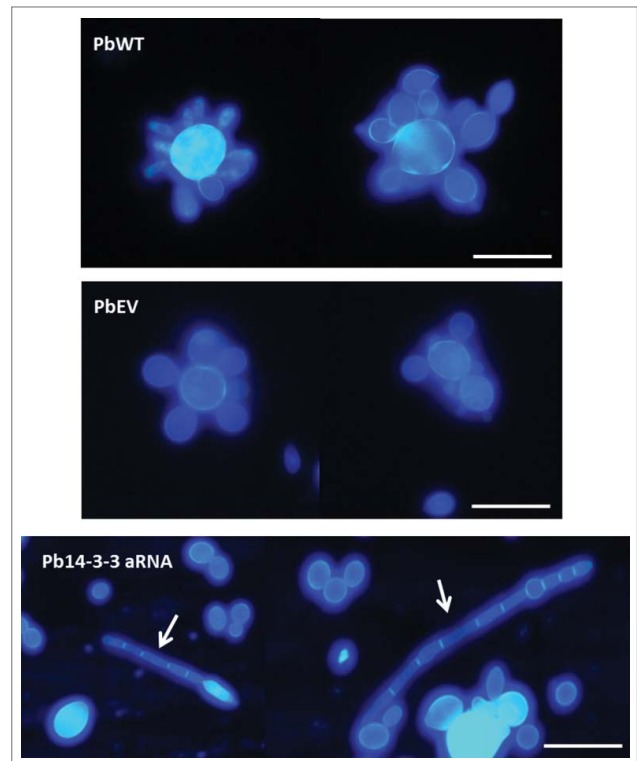


Figure 3. Silencing of Pb14-3-3 leads to distinct *P. brasiliensis* yeast cell morphology. Microscopic evaluation of PbWT and PbEV yeast cells and yeast cells generated from the Pb14-3-3 aRNA strain using CalcoFluor; magnification 40X. White bars correspond to 20 μm. White arrows indicate yeast cells showing elongation and the presence of filamentation.

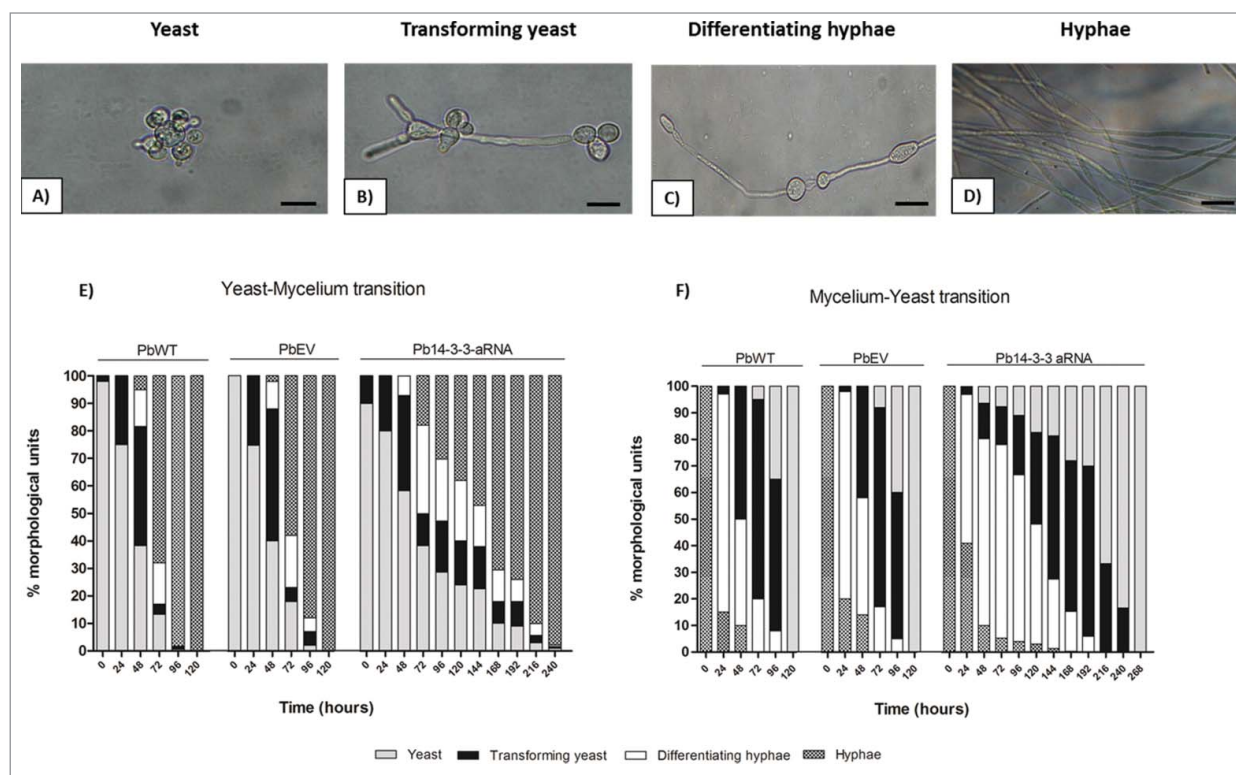


Figure 4. Influence of Pb14-3-3 in the temperature morphological transition. Mycelia or yeast were induced to transform by changing the incubation temperature from 25°C to 37°C or from 37°C to 25°C. Morphological units were classified as described by Nunes et al., 2005⁸³: (A) Yeast (multiple budding), (B) Transforming yeast (yeast with neck elongation), (C) Differentiating hyphae (chlamyospore-like cells) and (D) hyphae. (E) Yeast-Mycelium transition at 25°C. (F) Mycelium-Yeast transition at 37°C. A total of 300 cells were counted at each time, and the results are shown as the mean.

became enlarged, indicating the start of differentiation (differentiating hyphae) for all isolates. However, by 48 h of incubation, the Pb14-3-3 aRNA isolate showed impairment of the mycelium-yeast transition, as determined by the slight growth of transforming yeasts (13.3%) when compared to PbWT (50%) and PbEV (42%). While PbWT and PbEV advanced in the mycelium-yeast transition, achieving complete mycelium-yeast transition within 5 days, the silenced isolate with Pb14-3-3 aRNA required 11 days to complete the same process (Fig. 4F).

The decrease in expression of Pb14-3-3 did not alter the yeast cell vitality or viability. Moreover, no significant differences between the Pb14-3-3 aRNA strain and the control were detected during batch culture growth of yeast cells (Fig. 5A). We also studied the viability of Pb14-3-3 aRNA, PbWT and PbEV yeast cells at different time points during batch culture using XTT and Trypan blue exclusion assays, calculating the percentage of live cells in each test strain based on the OD measured from the heat-killed *P. brasiliensis*, used as dead control cells, and no major differences were found in either viability test (Fig. 5B and 5C).

Differential interaction kinetics of Pb14-3-3 aRNA with pneumocytes

To elucidate the role of Pb14-3-3 in the interaction (adhesion/internalization) of *P. brasiliensis* with host cells, we infected the A549 epithelial human lung cell line with PbWT, PbEV and Pb14-3-3 aRNA strain yeast cells. After 2 h of interaction, the

interaction capacity of Pb14-3-3 aRNA yeast cells was 10-fold decreased compared with PbWT. It was also possible to observe 2-fold decrease in the Pb14-3-3 aRNA interaction rate after 5 h of incubation when compared with PbWT, suggesting the involvement of Pb14-3-3 in interactions between *P. brasiliensis* and host cells. Yeast cells of the PbWT and PbEV strains showed no changes in interaction rates at the evaluated times (Fig. 6).

14-3-3 in *P. brasiliensis* is important for binding to ECM proteins

The influence of Pb14-3-3 on binding to ECM proteins was evaluated using an ELISA with an anti-cell free antibody that recognized the isolates equally (data not shown). Pb14-3-3 aRNA cells showed a significant reduction in binding to laminin (28%) and fibronectin (14%) when compared to PbWT. No differences were observed regarding the adhesion of the strains to type I and type IV collagen (Fig. 7).

14-3-3 is an important virulence factor for *P. brasiliensis*

The relevance of Pb14-3-3 during the host-pathogen interaction was further evaluated using a *G. mellonella* infection model. To evaluate this hypothesis, the isolates PbWT, PbEV and Pb14-3-3 aRNA (5×10^6 cells/larva) were used to infect *G. mellonella* larvae that were maintained at 37°C, and the virulence of these fungi was evaluated for 7 days. Comparing the percentage of survival at the end of the experimental for all strains tested

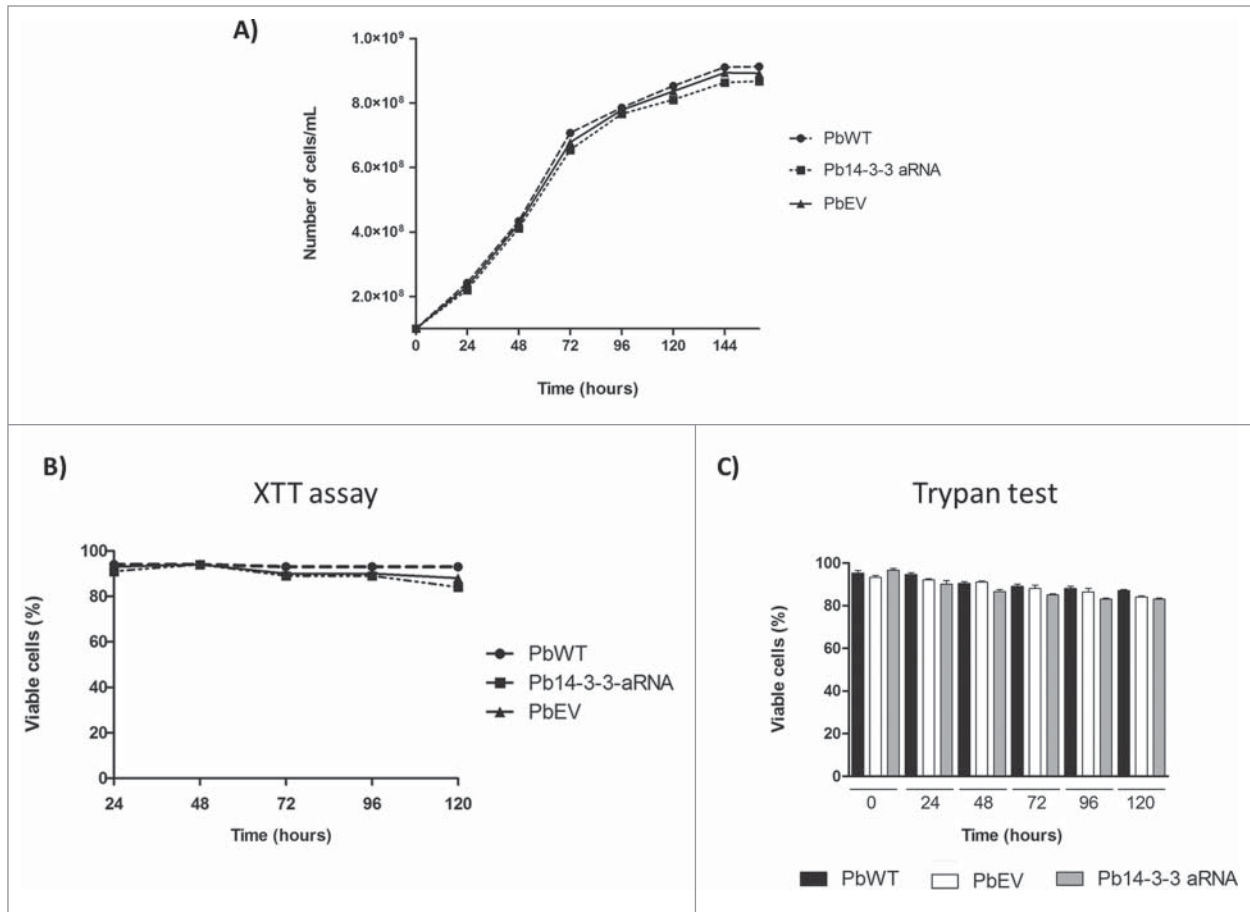


Figure 5. Down-regulation of *Pb14-3-3* does not affect cell viability or vitality of *P. brasiliensis* yeast cell. *P. brasiliensis* isolates PbWT, PbEV and *Pb14-3-3* aRNA were evaluated by (A) cell counts in a Neubauer chamber, (B) a colorimetric XTT reduction assay and (C) Trypan blue exclusion test at different time points.

demonstrated that larvae infected with the Pb18 strain (PbWT) began to die at day 3, with no surviving larvae at the end of the experimental period. In contrast, larvae infected with the *Pb14-3-3* aRNA strain survived longer: the larvae began to die at day 3, but the survival percentage of larvae infected with *Pb14-3-3* aRNA was significantly ($p < 0.0001$) higher (35% survival) than for PbWT and PbEV at the end of the 7-day experiment (Fig. 8A). No significant difference was observed between PbEV and PbWT, and no mortality was observed in the PBS-injected *G. mellonella* larvae (Fig. 8A). Additionally, a control group inoculated with heat-killed fungal cells showed 100% survival, indicating that the larvae did not die from osmotic stress.

To compare the fungal replication kinetics over the course of the infection of wild-type (PbWT) and the silenced strain (*Pb14-3-3* aRNA), the numbers of colony-forming units (CFUs) per larva were evaluated. At time zero, the isolates showed no differences based on CFU counting (Fig. 8B). After 24 h of infection, PbWT and PbEV showed no differences in CFUs compared with the initial time, but for *Pb14-3-3* aRNA, the number of CFUs was significantly lower than that for PbWT (4.4-fold lower) and PbEV (4.5-fold lower) (Fig. 8B). By 48 h of infection, PbWT, PbEV and *Pb14-3-3* aRNA underwent replication,

evidenced by the increase in the fungal burden. Nevertheless the silenced isolate, *Pb14-3-3* aRNA, was also observed to have significantly fewer CFUs than PbWT (2.8-fold lower) and PbEV (2.7-fold lower) despite showing an increase in CFUs compared to the 24 h time point (Fig. 8B), supporting the results of the survival curve.

Discussion

Antisense RNA silencing has proven to be a strong molecular biological tool for studying the functions of cellular genes and identifying the genetic factors involved in the virulence and the dimorphic nature of *P. brasiliensis*.²⁹⁻³¹

Previous studies focused on elucidating the function of the 14-3-3 protein in the pathogenesis of *P. brasiliensis*.^{27,28} Those studies were limited to the use of the recombinant 14-3-3 protein and the anti-14-3-3 antibody, and they therefore indirectly explored the role of 14-3-3 without manipulating gene expression. This is the first report to use a strain of *P. brasiliensis* with reduced transcript and protein levels of 14-3-3 to elucidate the

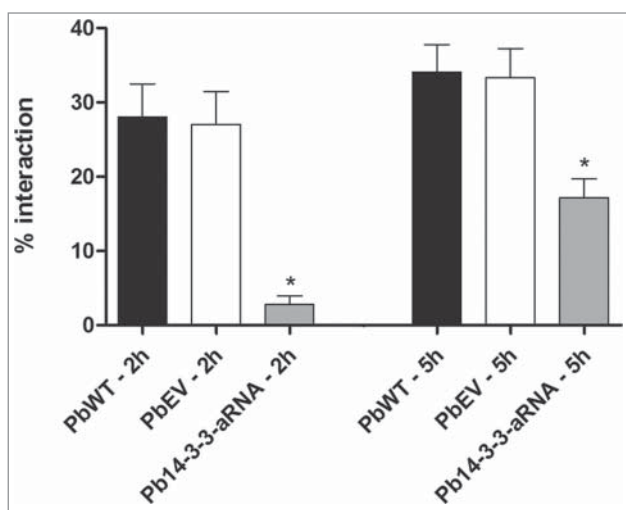


Figure 6. Interaction of *Pb14-3-3* aRNA yeast cells with pneumocytes is affected during the early stage of infection. The interaction was assessed by indirect immunofluorescence and analyzed by flow cytometry at different time points. * $p < 0.05$ when comparing PbWT and PbEV with *Pb14-3-3* aRNA at 2 and 5 hours after infection.

role of this protein in host-pathogen interactions and in the biology and virulence of *P. brasiliensis*.

The *Pb14-3-3* aRNA-silenced strain of *P. brasiliensis* was used in all of the experiments, with PbWT and PbEV serving as controls. The reductions in both gene and protein expression levels in the *Pb14-3-3* aRNA strain were confirmed prior to its experimental use. The silenced strain demonstrated a yeast cell viability and growth rate similar to the controls. Based on these results, we can hypothesize that this protein is not important for fungal metabolism. In contrast, our results suggest a role in fungal adherence with an effect on virulence.

Interestingly, the decreased expression of the *Pb14-3-3* gene affected the morphology of yeast cells maintained at 37°C, resulting in smaller yeast cells, fewer buds per yeast cell and the presence of a few yeast cells with a more elongated morphology, including the presence of filamentation. The report by Peraçoli et al. in 1999³² demonstrated that the isolate Pb-T, recently isolated from armadillos and more virulent than Pb18, showed a significantly higher number of buds per mother cell than did Pb18. Likewise, the silenced isolate *Pb14-3-3* aRNA, with fewer budding yeast cells, proved to be less virulent than Pb18 (PbWT). These findings support the previous hypothesis, that the most virulent isolate has more buds, but evaluating the contribution of morphological differences to disease progression requires further investigation of the polymorphisms present in *P. brasiliensis*.³³

Other evidence pointing to the possible involvement of the 14-3-3 protein in *P. brasiliensis* morphology includes the findings that both the yeast-to-mycelium transition and the reverse transition were hampered in the isolate with reduced expression of this protein. This suggests that *Pb14-3-3* is important for pathogenicity that its closely associated with dimorphic transition: *P. brasiliensis* mycelium of strains that are unable to transform into yeast

are not virulent^{34,35} because the yeast form is more resistant to the killing mechanisms of various host cells, thereby contributing to the survival of the pathogen in this environment.³⁶

Previous studies with dimorphic fungi such as *P. brasiliensis* have demonstrated that these fungi require their morphological transitions for efficient adherence and invasion of the host to establish infection.³⁷ Morphological transitions in dimorphic fungi are highly complex events triggered by a wide range of environmental factors and consisting of a reversible change in the growth pattern, alternating between the unicellular yeast form and the filamentous form (hyphae and pseudohyphae).³⁸ Understanding the mechanisms that regulate these events is of extreme interest due to implications for pathogenesis and cell differentiation, as observed with the inhibition of the transition to the parasitic form by the female hormone estrogen.³⁹ The proteins Bmph1p and Bmph2p in *Saccharomyces cerevisiae* are homologous to *Pb14-3-3* and regulate pseudohyphal growth through the MAPK (mitogen-activated protein kinase) signaling pathway. This interaction has been shown to regulate fungal morphogenesis.⁴⁰ Further, Kraus et al.⁴¹ demonstrated that the ArtA protein of *Aspergillus nidulans*, which also has high homology to the *Pb14-3-3* protein, is involved in preventing septum formation.

Additionally, 14-3-3 has been shown to be involved in the filamentation of other dimorphic fungi such as *Candida albicans*⁴² and *Yarrowia lipolytica*. In these microorganisms, the filamentous growth is proportional to the level of expression of the 14-3-3 protein.³⁸

Studies have shown that the variability of cell size within a fungal cell population alters pathogenicity via altering phagocytosis.^{43,44} Phagocytosis, along with the resultant killing by macrophages and monocytes in the lungs, is one of the first lines of defense by the host immune system,⁴⁵ thus demonstrating that in *P. brasiliensis*, a decrease in cell size leads to increased phagocytosis and decreased virulence⁴⁴.

The evidence presented in this study demonstrated that reduced *Pb14-3-3* expression induced morphological alterations, as change in size, which may affect the ability of host cells to phagocytose the fungus, suggesting that this protein plays an important role in the yeast form in *P. brasiliensis*, thereby contributing to its virulence. However, the specific mechanism by which this gene silencing was able to generate morphological changes was not elucidated in the present study and requires further investigation.

Many molecular interaction events and the subsequent establishment of infection that occurs between the pathogenic fungi and host are mediated by adhesion proteins on the fungal surface that are capable of binding to the host cell's ECM.⁴⁶ Mendes-Giannini et al.⁴⁷ observed a relationship between virulence and adherence to the ECM when comparing several *P. brasiliensis* isolates that showed different levels of virulence *in vivo*; the authors demonstrated that the isolate Pb18, which was recently isolated from an animal, had a greater ability to adhere to ECM components. Confirming the results of Andreotti et al., 2005,²⁷ we demonstrated that low expression of *Pb14-3-3* resulted in a reduction of binding to laminin; this interaction was assessed by ELISA, which has greater sensitivity and specificity than the

immunoblotting that was performed in the study cited above. Additionally, the Pb14-3-3 protein has been reported to interact with other ECM components, including fibronectin, suggesting that Pb14-3-3 is likely involved in the virulence of *P. brasiliensis* through interaction with ECM components. This interaction represents a critical step in the adherence and invasion of host tissues. For collagen type I and IV, we found no differences in the binding of isolates, suggesting that this component of the ECM is not relevant to the interaction between Pb14-3-3 and host cells.

P. brasiliensis uses a sequence of different mechanisms to establish successful infections, beginning with the first contact with host cells and extending through the later stages of disease.^{48,49}

The reduced initial adhesion observed in this study suggests that the Pb14-3-3 gene products strongly influence the initial adhesion, as reported by Andreotti et al., 2005,²⁷ Da Silva et al., 2013²⁸ and De Oliveira et al., 2015,⁵⁰ but the requirement for these proteins was bypassed following extended contact with the host cell.

Together, these data suggest that the adhesion of *P. brasiliensis* to pneumocytes relies on a combination of factors, including the expression of different adhesins, and that the defects elicited by the silencing of a single gene, as shown herein for Pb14-3-3, are offset by prolonged contact with host cells.

The huge armamentarium of virulence factors is controlled by an extremely complicated regulatory network that coordinates and synchronizes all of the elements involved.⁵¹ This regulation is important not only from the perspective of guaranteeing the expression of individual virulence elements but also to confer cross-talk between these determinants to ensure the appropriate response of the fungus in which all of the stages are activated following a temporal hierarchy.

To verify the importance of the Pb14-3-3 protein to the virulence of *P. brasiliensis*, we used the invertebrate infection model *Galleria mellonella*. Several studies have established *G. mellonella* as a useful model for evaluating fungal virulence,⁵²⁻⁵⁶ including for *P. lutzii*, another species belonging to the *Paracoccidioides* species complex,⁵⁷ with the advantage that the innate immune response is highly similar between insects and mammals,^{58,59} 60

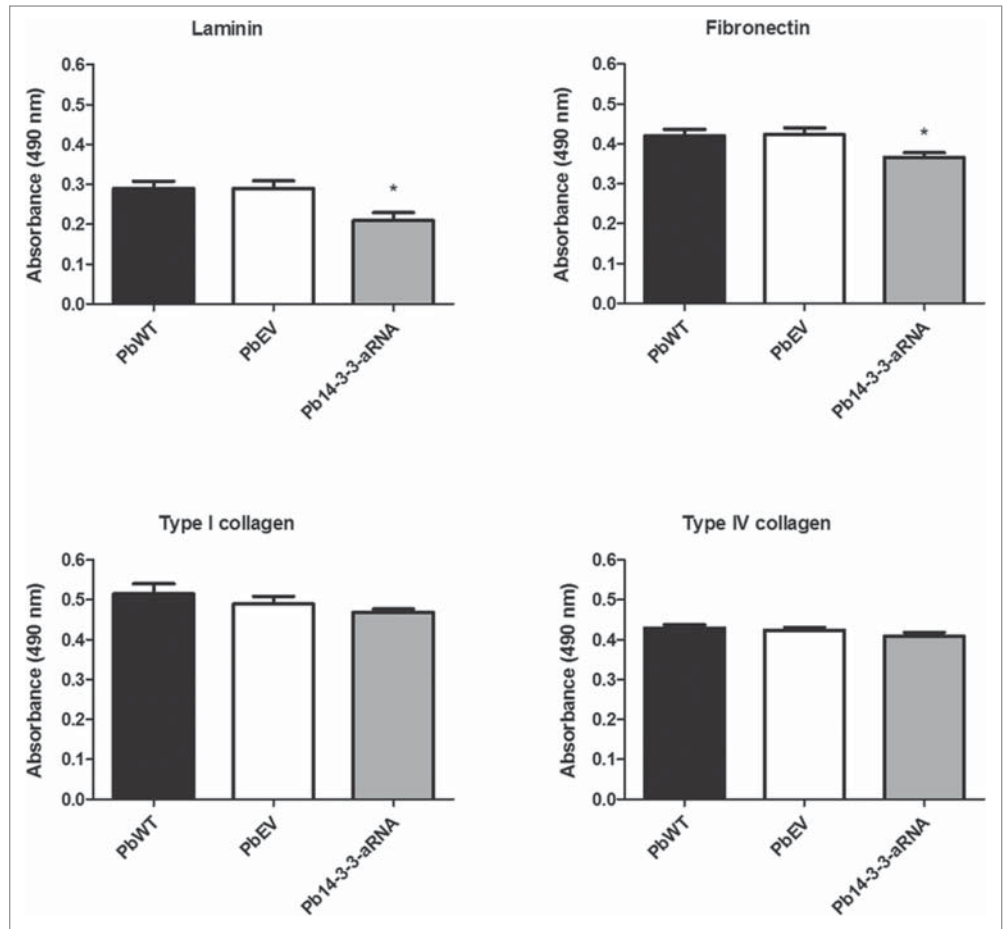


Figure 7. Binding of the *Pb14-3-3* mutant to ECM is reduced compared to the PbWT. Binding yeast cells of the strains PbWT, PbEV and *Pb14-3-3* aRNA to the ECM components laminin, fibronectin, and type I and type IV collagen ($10 \mu\text{g mL}^{-1}$). The interaction was determined by ELISA with peroxidase. * $p < 0.05$ when *Pb14-3-3* aRNA was compared to PbWT and PbEV for laminin and fibronectin.

including antimicrobial peptides and cells with phagocytic activity in hemolymph that function in a manner similar to human phagocytes.⁶¹⁻⁶³

Decreased expression of Pb14-3-3 resulted in a significant reduction of the virulence phenotype as reflected in the survival curve and CFU experiment with *G. mellonella* model. There was a reduction in the fungal burden in the larvae infected with the silenced strain, probably due to difficulty in the establishment in the host, leading to a lower resistance to killing by the host's immune system. Therefore, the silenced isolate was considered less virulent than Pb18 (PbWT), supporting the hypothesis that Pb14-3-3 is involved in the pathogenicity of *P. brasiliensis*.

A panel of mutants of the 14-3-3 protein (Bmh1p) of *C. albicans* was used to verify that specific mutations affect different pathways associated with virulence, including those involved with the formation of filaments and interactions with cells of the host immune system.⁶⁴ The involvement of the 14-3-3 protein has recently been reported to modulate the TLR (toll-like receptor) signaling pathway. It was demonstrated that 14-3-3 impairs TLR2, TLR3, TLR4, TLR7/8, and TLR9 ligand-induced IL-6, TNF α , and IFN- β production, thereby playing a major

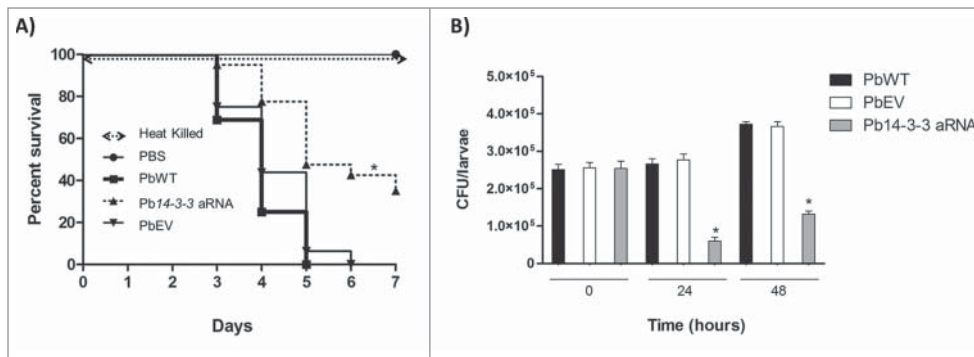


Figure 8. Silencing of *Pb14-3-3* decreases the virulence of *P. brasiliensis* in a *Galleria mellonella* infection model. (A) Representative survival curves of an experimental infection performed in *G. mellonella* with PBS; 5×10^6 PbWT, PbEV, *Pb14-3-3* aRNA and heat-killed yeast cells; $p < 0.0001$ when *Pb14-3-3* aRNA were compared with PbWT and PbEV and B) Number of CFUs in larvae sacrificed and homogenized at 0, 24 and 48 h after infection (* $p < 0.05$ when compared *Pb14-3-3* aRNA with PbWT and PbEV).

regulatory role in balancing the host inflammatory response to viral and bacterial infections by modulating the TLR signaling pathway.⁶⁵ Toll-like receptors are a group of pattern-recognition receptors that play a crucial role in the induction of innate immunity, the first line of defense against bacteria, viruses and fungi.⁶⁶ Roles for TLR2 and TLR4 in the recognition and internalization of *P. brasiliensis* have been reported in human monocytes and neutrophils.⁶⁷ Considering that the 14-3-3 protein of *P. brasiliensis* is more abundant at the cell surface when in contact with the host (da Silva et al., 2013), we suggest that decreased levels of this protein results in the reduced interaction of the fungus with host structures and lead to a decreased ability to evade host responses, possibly due to the modulation of the initial immune response, thereby resulting in the reduced virulence of *Pb14-3-3* aRNA.

The characterization of virulence factors is essential for improving our understanding of fungal pathogenesis and of the interactions between fungal pathogens and the host. These studies may also serve as a baseline for exploring the development of prophylactics or new therapeutic strategies. This study established that *Pb14-3-3* silencing differentially regulated important of the interaction with the host cells, such as morphology, the attachment index for ECM/pneumocytes and virulence. Therefore, 14-3-3 is an interesting therapeutic target for combating infection and modulating inflammatory conditions associated with PCM due to *P. brasiliensis*.

Materials and Methods

Microorganisms and culture media

For this study we used yeast cells of *P. brasiliensis*, strain Pb18 (referred to herein as PbWT, wild-type). The fungus was grown in BHI medium supplemented with 1% glucose (Beckton Dickinson and Company, Sparks, MD) at 37°C with aeration in a mechanical shaker and was collected during the early exponential phase (72–96 h). The isolate Pb18 was used in the genetic

manipulation; this study is the first to address the transformation of the isolate Pb18.

The *Agrobacterium tumefaciens* strain LBA1100⁶⁸ was used to carry the binary vector used in this study; recombinant *A. tumefaciens* containing the binary vector pUR5750 (conferring kanamycin resistance in *A. tumefaciens* and *Escherichia coli*) was maintained at 28°C in LB medium supplemented with 100 $\mu\text{g mL}^{-1}$ kanamycin.

E. coli DH5 α grown at 37°C on LB medium supplemented with 0 $\mu\text{g mL}^{-1}$ kanamycin was used as host for plasmid propagation and cloning.⁶⁹

Obtaining *P. brasiliensis* with 14-3-3 silencing

We followed the anti-sense strategy and *A. tumefaciens*-mediated transformation protocol described previously by Almeida et al.⁷⁰ and Ruiz et al.⁷¹ to obtain *P. brasiliensis* mutants with silencing of *Pb14-3-3*. DNA from Pb18 was extracted from yeast cultures during exponential growth, and a high-fidelity proof-reading DNA polymerase (Taq DNA Polymerase High-Fidelity, Invitrogen, USA) was employed to amplify an aRNA oligonucleotide sequence targeting the exon coding sequence of *Pb14-3-3* (GenBank accession number AY462124); the oligonucleotide primers used are *Pb14-3-3* Forward (5'-CCGCTCGAGCG-GAAGGGGGACTACCACCGCT-3') and *Pb14-3-3* Reverse (5'-GGCGCGCCCTGAGCAACCTCAGTTGCAT-3').

The *Pb14-3-3* fragment was inserted into the pCR35 plasmid under the control of the promoter region of the calcium-binding protein gene (CBP-1) from *Histoplasma capsulatum*⁷² and propagated in *E. coli* DH5 α .⁶⁹ The CBP-1 promoter-AS cassette was subcloned into the pUR5750 plasmid (harboring a hygromycin B phosphotransferase gene), allowing the parental binary vector to harbor the transferred DNA (T-DNA) with the antisense molecule. Pb18 yeasts transformed with an empty vector (PbEV) were used as a *Pb18* recombination control as previously described.^{24,44} After transformation, the cells were spread onto selective BHI media supplemented with 200 $\mu\text{g mL}^{-1}$ hygromycin B (Sigma-Aldrich). Prior to incubation, the plates were air-dried in a safety cabinet for 30 min. The selection plates were monitored for colony transformation and for colony-forming ability at 36°C for 15–20 days.⁴⁴ Randomly selected hygromycin-resistant transformants were tested for mitotic stability.

Molecular detection of the hygromycin resistance gene (HPH)

Genomic DNA from PbWT, PbEV and *Pb14-3-3* aRNA yeast cells were isolated according to the glass beads protocol described by van Burik et al.,⁷³ To confirm the presence of the hygromycin B resistance cassette, PCR (polymerase chain reaction) amplification was performed to detect the HPH 1000-bp

amplification product using oligonucleotides primers *hphF* (5'-AACTCACCGCGACGTCTGTCGA-3') and *hphR* (5'-CTACACAGCCATCGGTCCAGA-3'). The PCR cycling temperatures were as follows: 94°C for 10 min, 40 cycles of 15 s at 94°C, 1 min each at 56°C and 65°C and finally one cycle at 65°C for 10 min. The reaction products were analyzed using 1% agarose gels and visualized with GelRed under UV light.

Gene expression analysis

Total RNA was obtained from PbWT, PbEV and *Pb14-3-3* aRNA yeast cells using the TRIzol[®] reagent (Invitrogen). cDNA synthesis was performed with RevertAid[™] H Minus Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. cDNAs were subjected to a qRT-PCR reaction to measure *Pb14-3-3* expression levels; this reaction was performed using Maxima SYBR Green/ROX qPCR Master Mix 2X according to the manufacturer's instructions (Fermentas) in a 7500 Cyclo Real-Time System (Applied Biosystems[®]). *Pb14-3-3* expression was evaluated in PbWT, PbEV and *Pb14-3-3* aRNA yeast cells; β -tubulin was selected as the endogenous control.⁷⁴ The sequences of the oligonucleotide primers used for the amplification of the target gene were *Pb14-3-3RT-forward* (5'- AATCTGCTTTCCGTTGCCTA-3') and *Pb14-3-3RT-reverse* (5' GTTTTGGCGGTACTCCTTGA-3'), the qRT-PCR product span any of the region targeted by antisense. For the β -tubulin gene, the primer sequences were β -tubulin RT-*forward* (5'- GTGGACCAGGTGATCGATGT-3') and β -tubulin RT-*reverse* (5'- ACCCTGGAGGCAGTCACA-3'). 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 $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T$ is the difference between experimental conditions (silenced vs. non-silenced strains) after normalization to β -tubulin.⁷⁵ Each experiment was performed in triplicate, and the expression levels were measured 3 times.

Western blotting

To obtain the total protein extracts, log-phase cells of PbWT and *Pb14-3-3* aRNA were concentrated and subsequently washed 3 times with ultrapure water via centrifugation 10,000 \times g. The obtained pellets were resuspended in 10 mM Tris-HCl (pH 8.8) added with 1 mL PBS containing 100 mM phenylmethylsulfonyl fluoride and 1 mg mL⁻¹ protease inhibitors (pepstatin, leupeptin, aprotinin, antipain, and chymostatin). The cells were frozen in liquid nitrogen and disrupted by maceration with glass beads, the mixture was vortexed twice 15 min and centrifuged at 5,000 \times g for 15 min, and the supernatant was analyzed. The protein concentration was estimated by the Bradford method. SDS-PAGE was performed with equivalent amounts of proteins (0.025 mg) isolated from PbWT and *Pb14-3-3* aRNA according to Laemmli.⁷⁶ For Western blotting experiments, the proteins were transferred onto nitrocellulose membranes (Hybond-C extra, Amersham Biosciences[®]) as described by Sambrook et al.⁶⁹. The membranes were blocked with a solution containing PBS, 5% skim milk and 1% bovine serum albumin (BSA) for

4 h at room temperature. After three washes with PBS, the membranes were incubated with the monoclonal anti-14-3-3 (Imuny[®], diluted 1:500) antibody overnight, and this assay was also performed with polyclonal anti-enolase²¹ as an internal control. The anti-IgG secondary antibody conjugated with peroxidase (Sigma[®]) diluted 1:1000 was added after further washing, and the 14-3-3 and enolase proteins were visualized by adding 3,3' diaminobenzidine and hydrogen peroxide as a substrate. Quantification of bands was performed using ImageJ software (NIH) for all blots. Values were calculated as the ratio of the protein of interest (14-3-3) to enolase.

Growth curve and viability assays

For the growth curve, the yeast cells of all isolates were grown in BHI broth at 37°C with shaking, and samples were collected to determine cell number using a standard Neubauer system⁷⁷ at different times until the stationary phase was reached. All cultures were begun with an initial concentration of 1×10^8 cells mL⁻¹.

Viability was assessed by measuring the metabolic activity of 2,3-bis(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) (Sigma) in a reduction assay as indicated by the manufacturer. Individual inoculums (200 μ L) of PbWT, PbEV and *Pb14-3-3* aRNA with 10^6 cells mL⁻¹, 50 μ L of XTT salt solution (1 mg mL⁻¹ in PBS) and 4 μ L of menadione solution (1 mM in acetone; Sigma) were added to develop the reaction. Microtiter plates were incubated at 37°C for 5 h.^{78,79} Fungal mitochondrial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change that correlates with cell viability. The colorimetric change was measured using a microtiter plate reader (Kasvi[®]) at 492 nm. In all experiments, microtiter wells containing heat-killed *P. brasiliensis* and minimal medium alone were included as negative controls.

To confirm the viability results obtained from the XTT assay, a trypan blue dye-exclusion test was performed based on the principle that intact cellular membranes of live cells exclude the dye.^{80,81} For counting yeast cells, the samples were collected at various times, and 10 μ L of the cell solution was added to 190 μ L trypan blue solution and diluted to a final volume of 1 mL as described by Do Carmo Silva et al., 2015.⁸² Yeast cells were analyzed under a light microscope with a 40X lens.

Microscopy

To determine morphometric alterations of yeast cell populations, samples of PbWT, PbEV and *Pb14-3-3* aRNA were evaluated. The yeast cells were grown under standard conditions to the exponential phase as described above. Then, they were collected and washed with PBS, followed by the preparation of suspensions with 10^6 cells mL⁻¹ (in PBS). Then, 50 μ L samples of these suspensions were stained using a 25 μ M solution of Calcofluor White M2R[®] (Life Technologies) and observed under a Leica DMLB fluorescence microscope using the 40X objective.

A DFC350FX Leica camera was used for image capture. Under microscopic observation, we recorded the following parameters: the percentage of budding cells, the number of buds exhibited by each cell and the diameter (the major transverse or

longitudinal axes) for 100 yeasts from each preparation. Pictures were processed using ImageJ (<http://rsb.info.nih.gov/ij>).

Additionally, the morphological temperature shift from 25°C to 37°C and vice versa was examined to investigate the influence of Pb14-3-3 in *P. brasiliensis* dimorphism. For this, fungal cells of PbWT, PbEV and Pb14-3-3 aRNA were cultivated in modified YPD liquid medium (0.5% yeast extract, 0.5% casein peptone, 1.5% glucose, pH 6.5) at either 37°C (yeast phase) or 25°C (mycelial phase) at 150 rpm for several days until the conversion was complete. Cultures were collected every 24 h and evaluated under brightfield microscopy, and a total of 300 cells from random fields were counted at 40X magnification. The transition was classified in 4 different morphological states as described by Nunes et al., 2005⁸³: hyphae, differentiating hyphae, transforming yeast and yeast.

Interaction of *P. brasiliensis* to pneumocytes

Pneumocytes monolayers (A549 epithelial lineage) were cultured for approximately 24 h in HAM-F12 medium (Cultilab) supplemented with 10% fetal bovine serum. Then, these monolayers were washed and infected with 10^5 cells mL⁻¹ of PbWT, PbEV and Pb14-3-3 aRNA yeast cells previously stained for 30 min at 37°C with 10 µM of CFSE (5,6 – carboxyfluorescein diacetate N-succinimidyl ester) (BioChemika), which stains the fungal cell wall, washed and resuspended in HAM-F12 medium. The infected cells were incubated for 2 h and 5 h (early times post-inoculation) at 37°C, 5% CO₂. Subsequently, after each incubation period, the cells were washed with PBS to remove non-adherent cells, detached from plastic with cold PBS and a rubber cell scraper on ice,⁸⁴ and then fixed in 4% paraformaldehyde.

The interaction rate was determined by flow cytometry using a FACsCanto™ (Becton Dickinson); uninfected cells and unlabeled yeast were used as internal controls to define the gates and for evaluating autofluorescence. The excitation wavelength was 488 nm, and emitted light was collected via the 530/30 nm band pass-filter. Data were processed and analyzed using FACs-Diva software. The assays were performed in triplicate, and the mean results are expressed as the percentage of interaction (fluorescent cells) detected in the samples.

Ligand affinity of *P. brasiliensis* to ECM components

Next, 96-well polypropylene ELISA microtiter plates (Corning®) were precoated with ECM proteins (10 µg mL⁻¹) in carbonate buffer (pH 9.6) for 1 h at room temperature. After blocking with 1% BSA (w/v) and 0.05% PBS-Tween, the plates were incubated individually with 5×10^6 cells mL⁻¹ of PbWT, PbEV and Pb14-3-3 aRNA for 15 h at 37°C in triplicate wells. The cells were washed, and then anti-cell free antibody (1:100) (a polyclonal antibody against the *P. brasiliensis* cell wall-associated antigens) in 0.5% BSA and 0.05% PBS-Tween was added for 1 h at 37°C. Peroxidase-conjugated anti-rabbit IgG (1:2000; Sigma®) in 0.05% BSA and 0.05% PBS-Tween was added for 1 h at 37°C. The reaction was developed using citrate (pH 4.9) conjugated with o-phenylenediamine as a chromogenic substrate. Negative controls were performed by incubating the matrix with the primary and secondary antibodies alone and with 0.05% BSA. The absorbance was measured at 490 nm. The capacity

of anti-cell free antibody to recognize the isolates equally was verified by Western blotting analysis (data not shown).

Evaluation of the virulence of *P. brasiliensis* using the *Galleria mellonella* model

Galleria mellonella larvae (number of larvae per group=16) weighing from 0.1 to 0.2 g were selected and used for the virulence assay. PbWT, PbEV and Pb14-3-3 aRNA yeast cells were harvested at the exponential phase of growth in BHI liquid medium at 37°C. Prior to the infection, the cells were washed thrice with PBS, passed through a syringe to eliminate cells clumps, and a total of 5×10^6 cells prepared in PBS were injected into each larva through the last left pro-leg using a 10 µL Hamilton syringe (Hamilton, USA) after cleaning the pro-leg with 70% ethanol. Each experiment was repeated 3 times independently. One additional group of larvae was inoculated with PBS to monitor the effects on survival due to physical injury, and another control group was inoculated with heat-killed fungal cells to test whether larvae were dying from osmotic stress because of the inoculum size. After the infection, the larvae were incubated in Petri dishes at 37°C for 7 days and checked daily for mortality (based on lack of movement after manipulation with forceps).⁸⁵

For evaluating the fungal burden of the different isolates, 5 larvae per group were infected as detailed above and sacrificed at each time point (0, 24 and 48 h). The larvae were homogenized in 1 mL sterile PBS, and the homogenates were plated on BHI agar containing chloramphenicol (30 mg mL⁻¹) as described by Singulani et al. (unpublished data). Plates were incubated at 37°C, and colonies were counted daily until no increase in counts was observed.

Statistical analysis

One-way ANOVA with Tukey's coefficient was used to analyze the results obtained in this study. The results of the statistical analyses were considered significant when the p value was < 0.05. Prism 5 software (GraphPad Software Inc.) was used for the analyses and for graph construction. The Western blotting analyses were performed using Student's *t*-test with the p-value set at $p < 0.05$.

For the survival curves, statistical analyses from the results of 3 independent experiments were performed using the Log-rank (Mantel-Cox) test in GraphPad Prism software with the p-value set at $p < 0.05$ and $p < 0.0001$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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