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Original Article

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Alternative oxidase plays an important role in *Paracoccidioides brasiliensis* cellular homeostasis and morphological transition

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Abstract

Paracoccidioides brasiliensis is the etiologic agent of one of the most common systemic mycoses in Latin America. As a dimorphic fungus, it must adapt to different environments during its life cycle, either in nature or within the host, enduring external stresses such as temperature or host-induced oxidative stress. In this study we addressed the role of alternative oxidase (*PbAOX*) in cellular homeostasis during batch culture growth and the morphological transition of *P. brasiliensis*. Using a *PbAOX*-antisense-RNA (*PbAOX*-aRNA) strain with a 70% reduction in gene expression, we show that *PbAOX* is crucial for maintaining cell viability and vitality during batch culture growth of yeast cells, in what appears to be a pH-dependent manner. We also show that silencing of *PbAOX* drastically reduced expression levels of other detoxifying enzymes (*PbY20* and *PbMSOD*). In addition, our data indicate that *PbAOX* plays a role during the morphological transition, namely, during the yeast-to-mycelia germination and mycelia/conidia-to-yeast transition, essential events during the establishment of infection by dimorphic fungal pathogens. Altogether, our findings support the hypothesis that *PbAOX* is important for the maintenance of cellular homeostasis, possibly by assisting redox balancing during cell growth and the morphological switch of *P. brasiliensis*.

Key words: alternative oxidase, homeostasis, oxidative stress, *P. brasiliensis*, transition.

Introduction

Many biological processes in organisms, ranging from fungi and plants to animals, are dependent on molecular oxygen (O_2). The respiratory chain, coupling oxidative phosphorylation and the electron transport chain at the inner mitochondrial membrane, is an essential process for the production of ATP [1]. The dominant branch of the respiratory chain in most organisms, including mammalian cells, plants, and fungi, is associated to cytochrome c oxidase [2–4]. The use of O_2 as the final electron receptor of the respiratory chain can lead to the production of reactive oxygen species [5], such as superoxide (O_2^-) and hydroxyl (OH^*) radicals or unstable molecules as hydrogen peroxide (H_2O_2), all of which may ultimately alter the bioenergetic status of the cell or affect essential metabolic pathways [6,7]. Nonetheless, ROS production has also been associated to important cellular functions such as intracellular signaling, mainly regarding mitochondrial-linked metabolism [8]. In addition, ROS produced by immune cells (e.g., macrophages) during host-pathogen interaction is essential to prevent invading microorganisms, in particular human pathogenic fungi such as *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Candida albicans*, and *Aspergillus fumigatus* [9,10].

A non-protonmotive non-energy-conserving pathway, referred to as alternative oxidase (AOX) respiration, has been previously described, mostly in plants and fungi, but also in prokaryotes and some animal species [8]. This pathway branches from the respiratory chain at the level of the quinine pool and couples oxidation of ubiquinone to the reduction of O_2 to H_2O [11]. AOXs have been proven to be involved in cellular response to temperature, ROS-induced stress, and infection (e.g., in phytopathogens) [12].

P. brasiliensis is the etiologic agent of Paracoccidioidomycosis (PCM), one of the most common systemic mycosis in Latin American countries [13]. At environmental temperature this fungus grows as a mold producing infectious arthroconidia that are inhaled by the host, reaching the lungs' alveoli were they interact with epithelial cells and are phagocytosed by macrophages [14]. At 37°C, *P. brasiliensis* shifts to the parasitic yeast form, and the onset of disease may or may not occur depending on the virulence of the strain and host-related factors [15]. *P.* brasiliensis must therefore adapt to different environments during its life cycle, either in nature or within the host, enduring distinct external stresses such as temperature, different nutritional requirements or host-induced oxidative stress, or extensive cellular changes during the morphological transformation [16–18].

P. brasiliensis has been shown to posses a powerful antioxidant defense system enabling it to survive ROS-mediated oxidative stress [19,20]. The analysis of the mitochondrial function of *P. brasiliensis* yeast cells revealed the existence of the alternative respiratory chain previously demonstrated to play an important role in the reduction of formation of ROS and other oxidative-inducing agents [11,21–24]. More recently, Martins and coworkers showed that AOX is an important player in intracellular redox balancing and that gene expression is regulated throughout the first stages of the mycelium to yeast transition [23].

The main goal of this work was to elucidate the role of *PbAOX* in *P. brasiliensis* regarding cellular homeostasis during batch culture growth and the morphological transition. We show that *PbAOX* is important to sustain cellular vitality and viability during batch culture growth by maintaining extracellular pH. In addition, our data indicate that *PbAOX* plays a role during the morphological transition, namely, during the yeast-to-mycelia (YM) germination and mycelia/conidia-to-yeast (M/C-Y) transition.

Materials and methods

Microorganisms

The *P. brasiliensis* strains used during this work were the wild-type strain ATCC 60855 (PbWt) and a strain previously generated in our laboratory by antisense RNA (aRNA) technology with a 70% reduced gene expression of *PbAOX* (*PbAOX*-aRNA) [25]. As a control, we employed *P. brasiliensis* strain with down regulation in a gene involved in the adherence to host cells (*PbHAD32*) and a *P. brasiliensis* strain harboring the empty vector (PbEV), in other words, without the aRNA sequence. Yeast cells were maintained at 36°C by subculturing in brain heart infusion solid media supplemented with 1% glucose (BHI) (Becton Dickinson and Company Sparks, MD 21152 USA). For specific assays, batch culture growth was performed in BHI with different initial pH: 7.3 (normal pH), 7.8 and 8.3 and at pH 7.3 with MES buffer 50 mM. *P. brasiliensis* mycelia was grown on Synthetic McVeigh Morton (SMVM) medium at 20°C [26]. *P. brasiliensis* conidia were produced as previously described, using the glass-wool filtration protocol [27]. Cell quantification and viability were determined using Neubauer chamber counting and ethidium bromide-fluorescence staining procedures, respectively [28].

Gene expression analysis

Total RNA was obtained from PbWt and *PbAOX*-aRNA yeast, mycelia, and conidia cells by treatment with TRIzol® (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and tested using a conventional polymerase chain reaction (PCR)

with β-tubulin primers (left gtggaccaggtgatcgatgt and right accctggaggcagtcac) to confirm absence of chromosomal DNA contamination. The cDNA was synthesized using 2 μg of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Real-time PCR was performed using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green, according to the manufacturer's instructions (Invitrogen). The CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) was used to measure *PbAOX*, *PbY20*, *PbMSOD,* and *PbHAD32* gene expression levels. 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 $2^{-\Delta CT}$ formula, where $-\Delta CT$ is the difference between the target gene and β -tubulin, a housekeeping gene [29]. Each experiment was done in triplicate and the expression level was measured three times.

Viability and vitality of *P. brasiliensis* cells

The viability and vitality of *PbWt* and *PbAOX*-aRNA yeast cells were analyzed during batch culture growth in BHI liquid medium at different initial pH (7.3, 7.8, and 8.3). Viability was evaluated using ethidium bromide-fluorescence staining procedures [28] and by determining colony-forming units (CFU) from serial dilutions of the selected growth/treatment condition plated on BHI supplemented with 0.5% glucose, 4% horse serum, and EDTA 300 mM [30] CFUs were counted after 7 days of incubation at 36°C.

Vitality was evaluated as the ability to absorb glucose with later activation of a cell membrane proton pump [31] and subsequent acidification of the media due to released H^+. PbWt and *PbAOX*-aRNA yeast cells were grown in BHI liquid medium, and measurement of the vitality was made at different time points during batch culture growth. Cell samples were collected, washed twice with sterile water (pH 7.0), and suspended in a final volume of 8 ml of water (pH 7.0). Two ml of this suspension were add to a beaker with 38 ml of water, and when pH became stable (between 5.5–6), 10 ml of 20% glucose were added. The pH of the experimental media was evaluated each 3 min up to 60 min to evaluate the increase of H+ in the media. In addition, PbWt, PbEV, and *PbAOX*-aRNA were cultured in a BHI media with MES buffer 50 mM at pH 7.3.

Temperature-induced morphological transition

The morphological transition was evaluated in both PbWt and *PbAOX*-aRNA strains. The Y-M and C-M germination processes were performed in BHI liquid medium at 20°C. The M-Y and C-Y transition was carried out by incubating mycelia/conidia at 36°C in BHI liquid medium. All assays were carried out in 500 ml Erlenmeyer flasks with 250 ml of medium. The 20 ml culture samples were collected during the transition process at defined time points for RNA extraction and quantification of *PbAOX* gene expression [32,33]. Furthermore, vitality and viability assays were performed on the same cell samples as described above.

Microscopic evaluation of cellular morphology was carried out with an AxiosterPlus (Zeiss) microscope, and images were acquired with a Power shot G5 camera (Canon).

Statistical analysis and modeling

Data are reported as mean ± standard error of the mean, and all assays were repeated at least three times. All statistical analysis was performed using SPSS statistics 17.0 program with ANOVA. A P value less than or equal to 0.05 was considered statistically significant. To correlate the variables we use a comparison of alternative models and multiple regressions, including also non-linear models. A P value less than or equal to 0.05 was considered statistically significant.

For the comparison of PbWt and *PbAOX*-aRNA strains, we determined trendlines, equations, and correlation coefficients for extracellular pH and viability during batch culture growth (Fig. 2). For viability and culture time, we modeled time-response and determined the cell mortality:

$$\text{Final viability} = \text{Initial viability} - \text{mortality} \times \text{time}^2$$

Thus,

$$\text{Final viability} - \text{Initial viability} = \text{Viability variation} = -\text{mortality} \times \text{time}^2$$

in other words,

$$\text{Viability variation/time}^2 = -\text{mortality}.$$

Here, mortality is defined as viability variation/time^2 and the negative sign indicates a decrease in mortality. R-square was used to explain the variability. The correlation coefficient, quantifying the relation between variables, was between 0.95 and 1. Calculations were done using STATGRAPHICS Centurion XV, version 15.1.02 software.

Results

PbAOX helps maintain cellular viability, vitality, and extracellular pH during batch culture growth

We first investigated the possible role(s) of *PbAOX* during batch culture growth of *P. brasiliensis* yeast cells in BHI liquid medium. Significant differences were detected between PbWt and *PbAOX*-aRNA yeast cells during batch culture growth and in cellular viability (Fig. 1A and 1B,

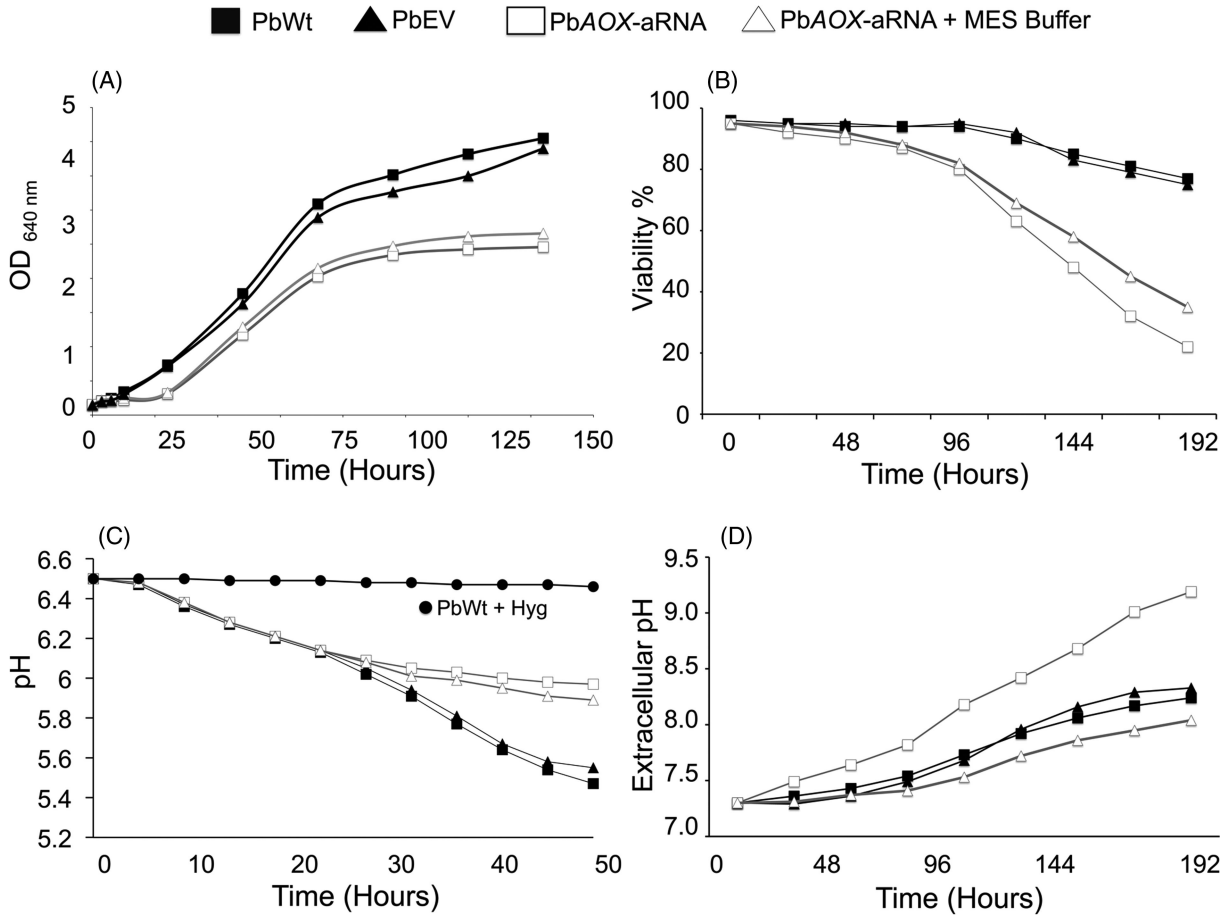

Figure 1. Gene silencing of *PbAOX* affects cell growth, viability/vitality and extracellular pH. Batch culture growth of PbWt and *PbAOX*-aRNA yeast cells; and PbWt treated with Hygromicin at 150 μg/ml: (A) fungal growth curve (OD_{640nm}), (B) cell viability, (C) vitality and (D) extracellular pH.

respectively). Viability of *PbAOX*-aRNA yeast cells was reduced at as early as 48 h of batch culture, decreasing drastically after 72 h. Using the model proposed in Materials and Methods, we determined the mortality of both strains. Our results indicate that the mortality of *PbAOX*-aRNA yeast cells during batch culture growth is 4 times higher than in PbWt yeast cells (Fig. 1B). Furthermore, the vitality of *PbAOX*-aRNA yeast cells was significantly decreased in comparison to PbWt yeast cells. Similar results were observed in *PbAOX*-aRNA yeast cells when they were cultured in a media with MES buffer (Fig. 1C). PbWt yeast cells treated with Hygromycin (150 μg/ml) were used as a metabolically inactive control.

Variation in extracellular pH was also measured during batch culture growth (Fig. 1D). We observed higher levels of extracellular pH during growth of *PbAOX*-aRNA yeast cells, particularly after 72 h of batch culture. An increase in extracellular pH was detected throughout batch culture growth of *PbAOX*-aRNA yeast cells, with twice the slope of *PbWt* yeast cells, indicating a pronounced decrease in cell vitality. A low variation in the extracellular pH was observed in the culture media with MES buffer during growth of *PbAOX*-aRNA yeast cells (Fig. 1D).

Maintenance of extracellular pH during growth is crucial for cellular viability

AOX plays an important role in cellular detoxification of ROS and homeostasis [6,7]. Taking into account our initial results, we questioned whether or not knock-down of the *PbAOX* gene and the corresponding decrease in cellular viability (Fig. 1B) could be associated with alterations in extracellular pH during batch culture and possible consequences on cellular homeostasis. Therefore, we evaluated yeast cell viability during batch culture growth but at different initial pHs: 7.3 (the normal pH for BHI medium, not shown), 7.8, and 8.3. A pH-dependent decrease in viability was observed in *PbAOX*-aRNA yeast cells; meanwhile no major alterations were identified in yeast cell viability of the *PbWt* strain at any of the studied pHs. (Fig. 2) Similar results were observed regarding the PbEV (data no shown).

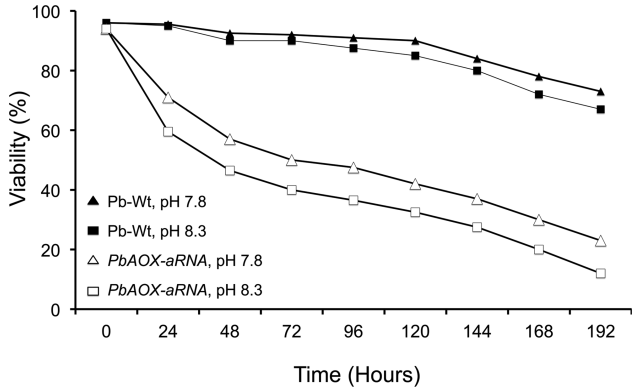

Figure 2. *PbAOX*-aRNA strain has decreased cell viability during batch culture growth at initial pHs above 7.3. Cell viability of PbWt and *PbAOX*-aRNA yeast cells is shown during batch culture growth in media with an initial pH of 7.8 or 8.3.

We also determined gene expression of *PbAOX* and two genes (*PbSOD* and *PbY20*) involved in the oxidative-stress response in *P. brasiliensis* yeast cells [34,35]. As control for cell viability we also evaluated *PbHAD32* gene expression in PbWt and PbAOX-aRNA, which expression profile was previously studied in our laboratory [36,37] (Fig. 3D). In *PbWt* yeast cells, *PbAOX*, *PbSOD*, and *PbY20* gene expression varied depending on the initial pH medium (Fig. 3A-C, left panel): at pH 7.3, a continuous increase in expression of these three genes was detected along batch culture (except for *PbAOX* at the last time point, 120 h); at pH 7.8, a peak in expression of these three genes was detected at 24 h, decreasing throughout time; at pH 8.3, a similar behavior to pH 7.8 was detected, although generally with lower gene expression levels. *PbHAD32* gene expression levels were similar at the evaluated pHs. (Fig. 3D left panel). In *PbAOX*-aRNA strains, gene expression of *PbAOX* was kept at low levels under all studied conditions (Fig. 3A, right panel). However, knock-down of *PbAOX* severely decreased expression of also *PbSOD* and *PbY20* in the *PbAOX*-aRNA yeast cells (Fig. 3B and C, right panel). Nevertheless, *PbHAD32* gene expression was at similar between *PbAOX*-aRNA and PbWt strains at the evaluated pHs (Fig. 3D, right panel).

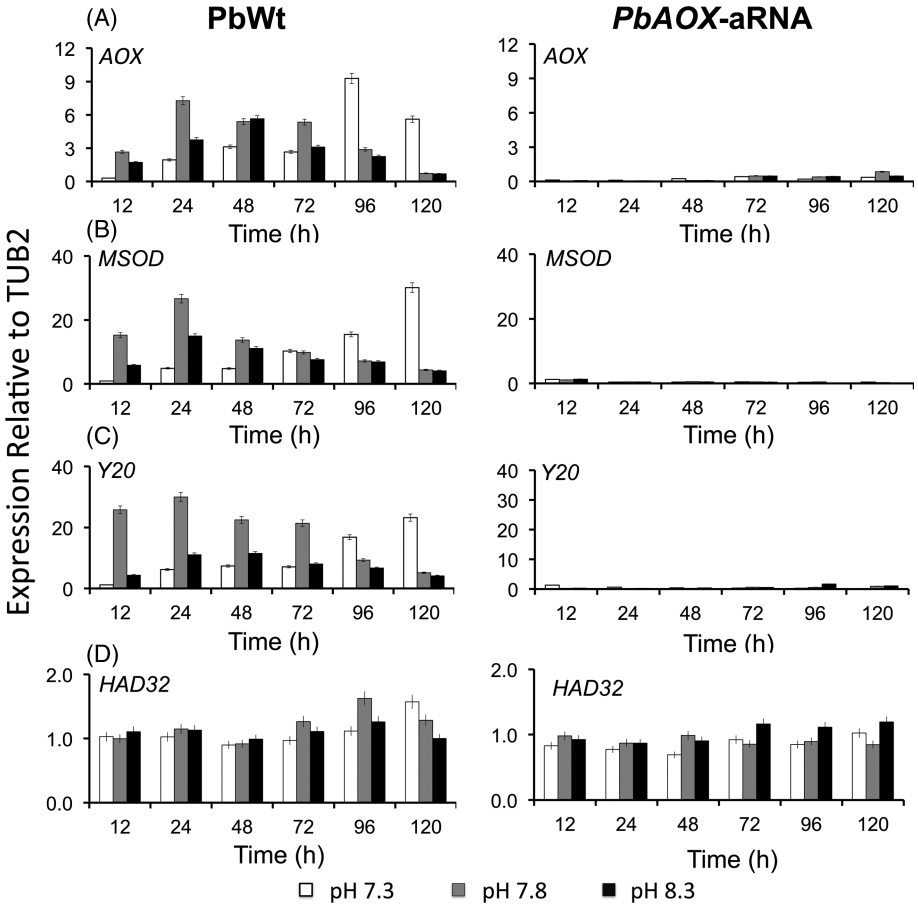

Figure 3. *PbAOX* silencing affects gene expression of detoxifying proteins. Gene expression levels of (A) *PbAOX*, (B) *PbMSOD*, (C) *PbY20,* and (D) *PbHAD32* in PbWt and *PbAOX*-aRNA yeast cells after culture in BHI medium at different pH (7.3, 7.8, and 8.3).

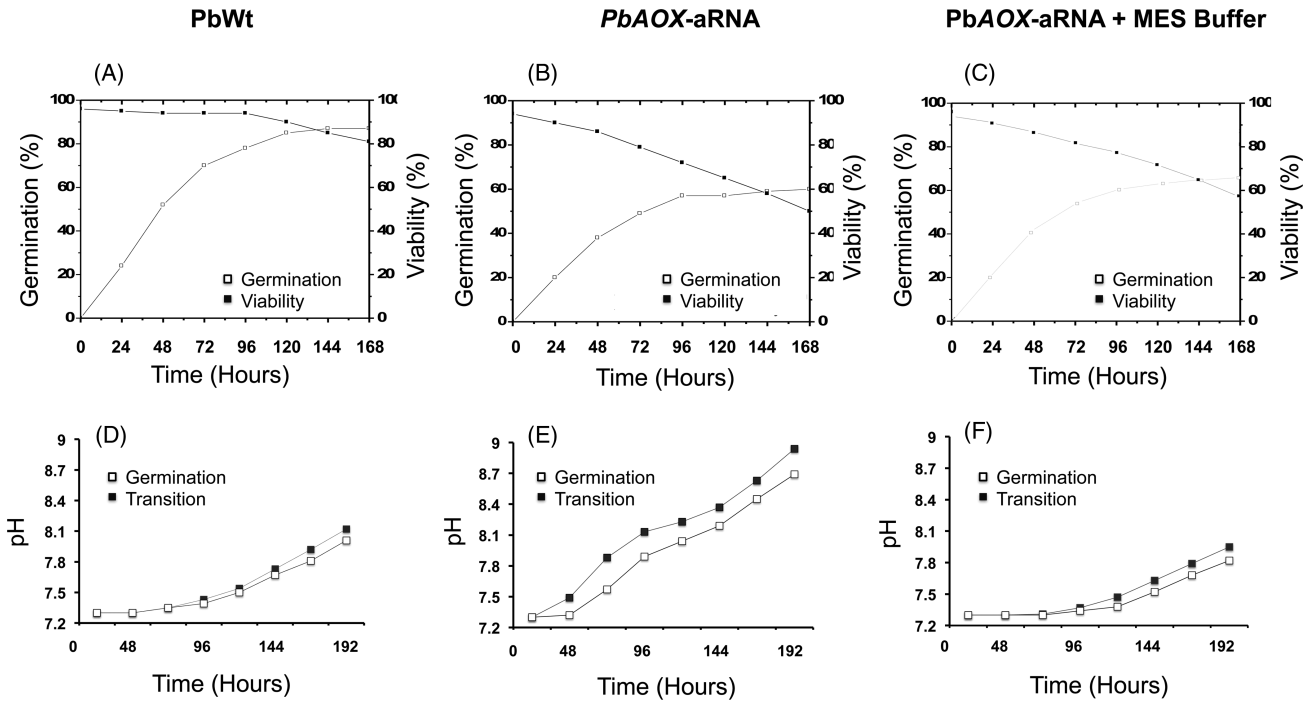

Figure 4. *PbAOX* plays an important role during the morphological shifts, Y-M germination and M-Y transition. Germination (% of yeast cells with filamentous branching) and cell viability of (A) PbWt, (B) *PbAOX*-aRNA, and (C) *PbAOX*-aRNA + MES buffer strains. Extracellular pH during the Y-M germination and M-Y transition of strains (D) PbWt, (E) *PbAOX*-aRNA, and (F) *PbAOX*-aRNA + MES buffer strains.

PbAOX plays an important role during the morphological transformation

As *PbAOX* has been previously described as an important molecule during the response to changes in environmental temperature [23], we also analyzed its role in the thermal dimorphism of *P. brasiliensis*. We initially evaluated several biological parameters during the yeast-to-mycelia (Y–M) germination. The *PbAOX*-aRNA strain presented both impaired Y-M morphological germination and significantly lower viability when compared to PbWt. The decrease in the morphological germination and viability was also observed in a *PbAOX*-aRNA strain cultured with MES buffer (Fig. 4A, B, C). Moreover, the extracellular pH was significantly higher for *PbAOX*-aRNA yeast cells than for PbWt during both the Y-M and M-Y processes (Fig. 4C–D).

The C-Y transition and C-M germination were also analyzed. No differences were detected in the capacity to produce conidia from mycelia in the PbWt, PbWt+EV or *PbAOX*-aRNA strains (data not shown). We also determined cellular viability of both the PbWt and *PbAOX*-aRNA strains placed at temperatures inducing either the C-Y or the C-M morphological switch. PbWt cells presented ≈90% viability at both temperatures, whereas *PbAOX*-aRNA cellular viability was decreased during the C-M germination (≈80%) and severely impaired during the C-Y transition (≈5%). The quantification of the morphological switches, both C-Y and C-M, revealed concurring results. While the C-Y transition was severely impaired in *PbAOX*-aRNA strains, only a slight delay was detected during the C-M germination when compared to *PbWt* cells (Fig. 5).

Discussion

Reactive oxygen species or ROS (H_2O_2, O_2^- and OH^-) are produced as an outcome of fungal metabolic activity and are implicated in cell differentiation and development but can also damage biomolecules, alter cellular processes and diminish cell survival [38]. ROS, primarily produced in the mitochondria, are generally counteracted in fungi via different strategies, which include detoxification by antioxidants and repair of macromolecular damage [5]. Enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (e.g., glutathione peroxidase, GPX) have been described as important antioxidants in fungi [39–41]. In addition, fungi use alternative branches of the mitochondrial respiratory chain to prevent ROS generation. Alternative oxidase (AOX) is an important player of this pathway, oxidizing ubiquinone and decreasing the potential for production of O_2^- by providing an alternative to eliminate electrons [8–11]. Its expression can be triggered by a number of stress signals including temperature and ROS, and it has previously been shown to play an important role in

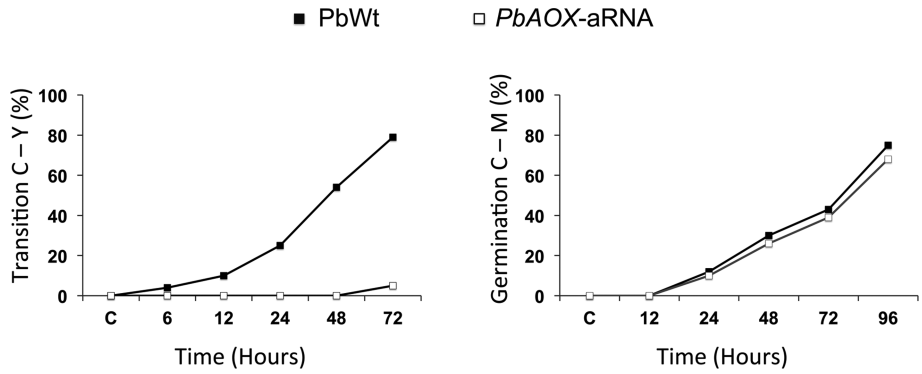

Figure 5. *PbAOX* is important during the C-Y transition and C-M germination in *P. brasiliensis*. Quantification of (A) the C-Y transition process in PbWt and in PbAOX-aRNA and (B) the C-M germination process in PbWt and in PbAOX-aRNA.

cellular detoxification of ROS and homeostasis [6,7]. In other eukaryotes, AOXs have been described to indirectly shift the energy status of cells owing to the non-phosphorylating nature of the alternative respiratory pathway. This is possible achieved in combination with a variety of alternative NAD(P)H dehydrogenases that are co-regulated with AOX, thus contributing to the maintenance of metabolic homeostasis [8].

As a thermal pathogenic dimorphic fungus, *P. brasiliensis* must adapt to different environments during its life cycle. The survival under distinct conditions and as different morphological forms necessarily embodies the capacity to endure a diversified set of external stresses, ranging from temperature changes to host-imposed oxidative stress. In this study, we intended to analyze the relevance of *PbAOX* under different physiological conditions and external stimuli. Our data indicate that *PbAOX* is crucial for maintaining cell viability and vitality during batch culture growth of yeast cells. The decrease in cell viability and vitality seen in the *PbAOX* knock-down strain was accompanied by an increase in the pH of the culture media. Previous work in our laboratory demonstrated that the same *PbAOX*-aRNA employed in this study was more susceptible to exogenous-induced oxidative stress [25]. The decrease in the viability of *PbAOX* knock-down strain could be correlated mainly due to a low capacity to counteract ROS and not to pH increase. Accordingly, similar results were observed in *PbAOX*-aRNA grown with MES buffer in which the pH values were kept stable during the assays.

The production of OH^- may increase intracellular and extracellular pH and can be eliminated as H_2O molecules due to the action of antioxidant enzymes [42,43]. The extracellular pH increase in the *PbAOX*-aRNA strain could suggest that *PbAOX* may play an important role in the maintenance of intracellular redox levels affecting the metabolism of the cell and the production of metabolites possibly affecting the extracellular pH, thus contributing to the maintenance of viability/vitality of *P. brasiliensis* yeast cells during batch culture growth. Nonetheless, future studies are required to understand how *PbAOX* directly or indirectly alters OH^* concentration.

The significant increase in extracellular pH during batch culture of *PbAOX*-aRNA yeast cells also led us to question whether *PbAOX*, as well as other detoxifying enzymes (*PbY20* and *PbMSOD*), could also play a role in cellular response to different environmental redox states. *PbY20* encodes an enzyme that participates in various electron-transport systems by replacing ferredoxin under iron-limiting conditions in a number of pathogenic microorganisms [34,44]. *PbMSOD* belongs to a large family of enzymes that may use manganese or iron as co-factors to scavenge superoxide anion in mitochondria [35]. Cell viability of PbWt yeast slightly decreased in the presence of a higher initial pH of the culture media (7.8 and 8.3). Concurrently, in standard media (pH 7.3) the expression of *PbAOX*, *PbMSOD*, and *PbY20* peaked only at the end of batch culture growth when extracellular pH was at higher levels. Interestingly, an increase in the initial pH induced a peak in gene expression as early as 24 h, suggesting that oxidative stress machinery may be important to maintain cell viability at basic pH. The absence of wild-type *PbAOX* mRNA levels severely reduced cell viability during batch culture growth in what appears to be a pH-dependent manner. Fabrizio *et al.* demonstrated increased expression of MSOD under oxidative stress in *S. cerevisiae* [45]. In *Cryptococcus neoformans* it has been suggested that AOX makes a significant contribution to metabolism, plays an important role in the yeast's defense against exogenous oxidative stress, and contributes to the virulence of this fungus [46]. Other studies in this fungus suggested that MSOD plays a role in its adaptation to host temperature by regulating steady-state concentrations of oxygen radicals in mitochondria [2,47]. Importantly, silencing of *PbAOX* drastically reduced *PbMSOD* and *PbY20* levels throughout batch

culture growth. To further corroborate that differences in gene expression amongst both strains were not due to reduced viability or altered gene expression of the housekeeping gene *TUB*, we also evaluated *PbHAD32* a gene involved in the *P. brasiliensis* adherence to host cells, [36,37] which is not correlated to oxidative stress response [36,37]. Also, previous work in our laboratory showed that *PbAOX*-aRNA yeast cells present decreased resistance to exogenous H_2O_2 [25]. Altogether, our data suggests that the *PbAOX* may be involved in some manner in the prevention against damage by oxygen-mediated free radicals produced during cell metabolism via interaction with mitochondrial enzymes [34,35,48].

The temperature-dependent morphological switch from conidia/mycelia to yeast is an essential event in establishing infection by dimorphic human fungal pathogens. The adaptation to host conditions embodies diverse ATP-dependent metabolic processes that underlie the complex structural and biochemical shift that must occur [1,7]. Under normal conditions, there is an increase of ROS production in the wake of these metabolic processes that are eliminated by intracellular detoxifying systems [6]. In *P. brasiliensis*, several studies have addressed the metabolic alterations that occur during the Y-M germination and M-Y transition processes. While initial reports suggested that mycelia favor an aerobic metabolism and yeast cells favor a fermentative metabolism, recent evidence seem to suggest that either form is prepared to perform aerobic or anaerobic respiration [19,23,24]. In fact, genes encoding proteins from the mitochondrial electron transport chain have been shown to be up-regulated in *P. brasiliensis* yeast cells and are involved with defense against oxidative stress [21,24]. In this study, we showed that reducing *PbAOX* expression significantly decreases the capacity to shift from Y-M and is correlated with an increase of extracellular pH. Even though conidia production is not impaired in *PbAOX*-aRNA mycelia, several differences were detected during the C-Y transition and the C-M germination. While the C-M germination was only slightly affected, the C-Y transition was greatly impaired due to the severe decrease in *PbAOX-aRNA* conidia viability when placed at 36°C. However, a direct connection to AOX with a decrease during the C-Y transition cannot be conclusively made due to alterations in the pH of the culture media of the mutant, which could reduce fungal viability during the transition process. Work in our laboratory previously showed that *PbAOX* is highly expressed during the first 24 h of C-Y while it is maintained at steady levels during the C-M germination [49]. These data further support the relevance of *PbAOX* in the fungus' adaptation to alterations in the intracellular redox balance. Nonetheless, further studies are required to elucidate how this enzyme assists the mitochondrial respiratory chain. We suggest that the observed peak in *PbAOX* gene expression (Fig. 3A) is a result of cytochrome saturation at the mitochondrial respiratory chain. This signal to increase production of this enzyme may assist the cells to balance intracellular redox state during batch culture growth. Conversely, in *PbAOX*-aRNA yeast cells, even though cytochrome reaches saturation quickly the severely reduced protein levels cannot cope with the redox imbalance, thus leading to a decrease in viability and vitality.

Our findings support the hypothesis that *PbAOX* is an essential enzyme in maintaining cellular homeostasis, possibly by assisting redox balancing during cell growth and the morphological switch of *P. brasiliensis*. Future studies are required to better understand how this alternative pathway helps regulate changes in the flow of electrons in the mitochondrial electron chain, ROS generation, fungal development, and its overall relevance during host infection, not only in *P. brasiliensis*, but also in other human pathogenic dimorphic fungi. Particularly, assays with defined-buffered medium will be conducted to further elucidate the connection between *PbAOX's* function, metabolic pathways and adaptation to different environmental conditions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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