Involvement of an Alternative Oxidase in Oxidative Stress and Mycelium-to-Yeast Differentiation in *Paracoccidioides brasiliensis*""

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*Paracoccidioides brasiliensis* is a thermomorphic human pathogenic fungus that causes paracoccidioidomycosis (PCM), which is the most prevalent systemic mycosis in Latin America. Differentiation from the mycelial to the yeast form (M-to-Y) is an essential step for the establishment of PCM. We evaluated the involvement of mitochondria and intracellular oxidative stress in M-to-Y differentiation. M-to-Y transition was delayed by the inhibition of mitochondrial complexes III and IV or alternative oxidase (AOX) and was blocked by the association of AOX with complex III or IV inhibitors. The expression of *P. brasiliensis aox* (Phaox) was developmentally regulated through M-to-Y differentiation, wherein the highest levels were achieved in the first 24 h and during the yeast exponential growth phase; Phaox was upregulated by oxidative stress. Phaox was cloned, and its heterologous expression conferred cyanide-resistant respiration in *Saccharomyces cerevisiae* and *Escherichia coli* and reduced oxidative stress in *S. cerevisiae* cells. These results reinforce the role of PhAOX in intracellular redox balancing and demonstrate its involvement, as well as that of other components of the mitochondrial respiratory chain complexes, in the early stages of the M-to-Y differentiation of *P. brasiliensis*.

*Paracoccidioides brasiliensis*, which is a thermally dimorphic fungus, is the etiological agent of paracoccidioidomycosis (PCM), which is the most prevalent human systemic mycosis in Latin America (5, 52) and affects almost 10 million individuals in Latin America (54). It is acquired by the inhalation of airborne microconidia, which reach the pulmonary alveolar epithelium and transform into the pathogenic yeast form (52, 54, 59). The human form of PCM that is caused by this fungus is characterized by a range of clinical manifestations, ranging from asymptomatic forms to severe, disseminated, and often fatal disease. Usually, fibrosis sequelae in the affected organs may interfere with the well-being of the patient (50).

During infection, thermomorphic fungi, such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *P. brasiliensis*, differentiate from a mycelial into a pathogenic yeast form, a transition that can also be induced (*in vitro*) by temperature changes from between 23 and 26°C to between 35 and 37°C. In addition, these pathogens are often subjected to significant environmental stresses, including exposure to the reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are produced by the host cells (6, 27, 45).

Elevated sublethal temperatures (thermal stress or heat shock) increase ROS generation and oxidative damage in a variety of eukaryotic cells (16, 49, 60, 73). Mitochondria are the primary intracellular sources of ROS, which are generated in respiratory chain complexes I and III and can damage biomolecules, such as nucleic acids, lipids, and proteins (20–22). In order to control ROS levels, cells employ diverse detoxification mechanisms, including superoxide dismutase, catalase, the glutathione/thioredoxin system (15), and uncoupling proteins (26).

In addition to proton-pumping complexes, the mitochondrial respiratory chains of plants and fungi possess alternative pathways, which are primarily alternative NADH-ubiquinone oxidoreductases and alternative ubiquinol oxidases (cyanide-resistant oxidases) that reportedly prevent ROS generation (28, 35). We have previously demonstrated the presence of both activities in the mitochondrial respiratory chain of *P. brasiliensis* yeast cells, as well as the presence of whole (complex I to V) and functional respiratory chains (40). Here, we addressed respiratory chain complexes and alternative pathways, as well as oxidative stress, in the mycelium-to-yeast (M-to-Y) differentiation of the fungus. Moreover, we cloned alternative oxidase (AOX) and heterologously expressed *P. brasiliensis aox* (Phaox) in *Escherichia coli* and *Saccharomyces cerevisiae*. We found that AOX and other respiratory chain complexes are closely involved in the early stages of the M-to-Y differentiation of *P. brasiliensis*.

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MATERIALS AND METHODS

Chemicals. Antimycin A (Ant A), benzohydroxamic acid (BHAM), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), EGTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), flavone, potassium cyanide (KCN), rotenone, salicylhydroxamic acid (SHAM), and menadione were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (H₂O₂), sodium nitropusrite (SNP), and absolute ethanol were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. All aqueous stock solutions were prepared using glass-distilled deionized water, except for water-insoluble compounds, which were prepared in DMSO or absolute ethanol.

Strains and culture conditions. P. brasiliensis isolate 18 was maintained in the mycelial form in a solid Sabouraud medium (BD, NJ) at room temperature and in the yeast form in a solid PGY medium (0.5% [wt/vol] peptone, 1% [wt/vol] glucose, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] agar) at 35.5°C. The routine cultivation of P. brasiliensis yeast cells was performed in liquid PGY medium (complete medium) under rotary shaker agitation at 35.5°C (58). In some experiments of M-to-Y differentiation, the fungus was grown in a liquid minimal medium (55) that had been supplemented with 0.2 g/liter cysteine, 0.1 g/liter methionine, and 0.1 g/liter tyrosine.

S. cerevisiae yeast strain INVSc1 (Invitrogen) was grown in a Sc-URA medium (0.067% [wt/vol] yeast nitrogen base without amino acids, 2% [wt/vol] glucose or galactose [fermentable medium], or 2% [wt/vol] glycerol/ethanol [nonfermentable medium]) with amino acids or nitrogen bases as required.

P. brasiliensis spheroplast preparation. The spheroplasts of P. brasiliensis were produced from yeast cells in the exponential (72- to 96-h) growth stage. Cells were harvested by the centrifugation of 150 ml of culture medium, washed with a cold phosphate buffer solution (PBS), and pelleted by resuspending yeast cells at 100 rpm for 3 min. The pellet was resuspended in a mitochondrial buffer. The protein concentration of the mitochondrial pellet was resuspended in a mitochondrial buffer. The protein concentration of the mitochondrial pellet was determined by the Biuret assay using BSA as standard (18).

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M-to-Y differentiation of P. brasiliensis in complete and minimum media. P. brasiliensis yeast cells were initially grown to the mid-exponential growth phase in a liquid PGY medium at 35.5°C. Cells were harvested by centrifugation, washed twice using fresh medium, and then incubated for 10 days at 26°C in order to obtain P. brasiliensis mycelia. Mycelial cells were harvested, washed twice in sterile water, inoculated in liquid PGY or minimum medium, and incubated at 35.5°C for 10 days (12). Aliquots were collected at 0, 10, 24, 48, 72, 120, and 240 h after the temperature was shifted (26°C to 35.5°C) for visual analyses or were processed for RNA isolation and real-time PCR. Dimorphic transition was quantified by counting the different morphotypes that are produced during this process in accordance with parameters previously described (12, 48). Fungal clumps were broken by vortexing and up/down pipetting and then analyzed under the microscope.

Mitochondrial respiratory chain inhibitors were added to the cultures at time zero if the following concentrations: 2 mM SHAM, 1 mM KCN, 1.8 μM antimycin A, 2 mM BHA, 300 μM flavone, and 20 μM rotenone. In some flasks, compounds were added in combinations, such as KCN-SHAM, antimycin A-SHAM, and rotenone-SHAM. DMSO and ethanol were also tested in order to determine the possible effects of these solvents on fungal differentiation. The addition of compounds that showed no effect on fungal differentiation was repeated after 24 or 72 h to rule out the possibility of drug degradation. Aliquots were collected at 24, 48, 72, 120, and 240 h, and images were captured using a 40× objective lens, resulting in a final magnification of X400, using an Olympus BX51 model U-LH100-3 microscope (Tokyo, Japan) coupled to an Olympus model C-5060 wide-zoom digital camera (Tokyo, Japan).

Determination of P. brasiliensis cellular viability. The MTT metabolic assay was performed according to the method of Malavazi et al. (36), with some modifications. Cells were washed three times with PBS, and the amount equivalent to 400 micromolars of total protein was added to each well with 10 μl of MTT solution (5 mg/ml in PBS) in a final volume of 160 μl. The mixture was incubated in the dark at 2 h at 37°C, and after that, the 96-well plate was clamped at 3,600 × g for 10 min. The content of each well was resuspended in 100 μl of isopropanol containing 5% (vol/vol) 1 M HCl was added to dissolve the formazan crystals. After 12 h of incubation in the dark at room temperature, the optical density was measured spectrophotometrically with a microtiter plate reader at 570 nm. All the MTT determinations were done in quadruplicate; the results are expressed as percentages of the values (mean) compared to the values observed at time zero for the control culture (100%). Aliquots from the control group were fixed with 4% paraformaldehyde (in PBS) or heat killed by autoclaving in order to obtain the values for nonviable cells.

Growth of the P. brasiliensis yeast form in the presence of mitochondrial respiratory chain inhibitors and inducers of oxidative stress. P. brasiliensis yeast cells were initially grown to the mid-exponential growth phase in a liquid PGY medium at 35.5°C. Cells were harvested by centrifugation, washed twice using fresh medium, resuspended in the same medium that contained the inhibitors or inducers to be tested, and then incubated for 12 h at 35.5°C. After this first incubation, the compounds were readded to the cultures and then incubated under the same conditions for 1 h. The cells were harvested by centrifugation, washed twice in sterile water, and processed for RNA isolation and real-time PCR assays. The concentrations of the compounds after single additions were as follows: 30 mM H₂O₂, 0.5 mM menadione, 1 mM SNP, 2 μM rotenone, 500 μM flavone, 10 μM antimycin A, and 1.8 μM rotenone-SHAM. DMSO and ethanol were also tested in order to determine their possible impact on gene expression levels.

Determination of carbonylated protein levels during the M-to-Y differentiation of P. brasiliensis. Oxidative modification of proteins by oxygen free radicals was monitored by Western blot analysis of carbonyl groups by use of an OxyBlot protein oxidation detection kit (Chemicon International, Inc.) as previously described (10, 68). Both treated and control cultures had the total proteins extracted by homogenization liquid nitrogen in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 0.2% Triton X-100, 1 mM benzanilide, and 10 mg/ml each of leupeptin, pepstatin, and antiprotease. The homogenates were centrifuged at 20,800 × g for 60 min at 4°C. Total protein concentrations were determined by the Bradford method, and 20 μg was subjected to derivatization with dinitrophenylhydrazine (DNPH). About 10 μg of proteins was treated in a 12% (wt/vol) SDS-PAGE gel and electrophoebolled to a nitrocellulose membrane. The membrane was incubated with the antibody anti-DNPH moiety of the proteins, and the immunoblot was detected by a chemiluminescent reaction. Densitometric analysis was performed by using the Image J program (available at http://rsweb.nih.gov/ij/index.html).

Measurement of intracellular ROS in P. brasiliensis yeast form and S. cerevisiae cells. Intracellular ROS levels were determined as previously described by Ruy et al. (56) and Magnani et al. (34), with some modifications. Changes in the fluorescence of the probe 5-((and 6)-chloromethyl)-2′,7′-dichlorodihydrofluorescein diacetate, acetylated (CM-H₂DCFDA) (Invitrogen) due to oxidation by...
ROS were monitored using a Hitachi F-4500 fluorescence spectrophotometer at 30°C under agitation. The excitation and emission wavelengths were 503 and 529 nm, respectively. Assays were performed using 5 × 10^4 cells/ml of spore yeast cells from both P. brasiliensis and S. cerevisiae in a 1.8 ml of a standard incubation medium that contained 0.6 mM succinate, 10 mM HEPES-KOH at a pH of 7.2, 5 mM MgCl2, 2 mM KCl, and 0.5 mM EGTA at 30°C. The oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) was used in 1.8 ml of a standard incubation medium that contained 1% (wt/vol) sodium dodecyl sulfate into S. cerevisiae mitochondria were obtained by sonication in 1% (wt/vol) sodium dodecyl sulfate at a pH of 7.2, 5 mM MgCl2, 2 mM KCl, and 0.5 mM EGTA. The PCR products were then ligated into the plasmid PCR2.1-TOPO (Invitrogen). Several clones were sequenced so to exclude any possible nucleotide mutation during PCR amplification. Inserts that were amplified from cDNA were transferred from PCR2.1-TOPO to pET28a (+) (Novagen) and pYES2-NCT (Invitrogen) expression vectors. pET28a-Pbaox and the empty vector pET28a (+) were transformed into E. coli strain Rosetta(DE3)pLyS8 according to the method of Sambrook et al. (57a). pYES-Pbaox and the empty vector pYES2-NCT were transformed into S. cerevisiae by use of the lithium acetate method (61).

Analysis of nucleotide and amino acid sequences. Pbaox cDNA and genomic DNA sequences as well as the deduced amino acid sequences were analyzed to determine the presence of possible intron regions, trans-membrane domains, and mitochondrial signal peptides by use of previously described methods (67). The bioinformatics tools that were used in these analyses are available at http://www.ncbi.nlm.nih.gov/ (tools using, e.g., the method of Kyte and Doolittle [30], the ClustalW algorithms) and at http://www.ncbi.nlm.nih.gov/ (tools using, e.g., the method of Kyte and Doolittle [30], the ClustalW algorithms). The mitochondrial localization of the P. brasiliensis mitochondrial network, the identity of the protein, and the isoelectric point determination, and the ClustalW algorithms) and at http://www.ncbi.nlm.nih.gov/ (tools using, e.g., the SIGNALP 3.0 server [3]).

Expression of Pbaox in S. cerevisiae and E. coli. The expression of Pbaox in the E. coli strain Rosetta(DE3)pLyS8 was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium and incubation at 37°C for 12 h in an LB medium that had been supplemented with chloramphenicol (35 μg/ml) and kanamycin (35 μg/ml).

In S. cerevisiae cells, the expression of Pbaox was achieved by growing cells in a SC-Ura− liquid medium that had been supplemented with 2% (wt/vol) galactose at 30°C for 24 h.

Immunodetection of Pbaox. Protein samples from E. coli and S. cerevisiae mitochondria were obtained by sonication in 1% (wt/vol) sodium dodecyl sulfate (SDS). Proteins (50 μg) were mixed with a sample buffer that contained 125 mM Tris-HCl at a pH of 6.8, 20% (vol/vol) glycerol, 5% (wt/vol) SDS, 0.004% (wt/vol) bromophenol blue, and 100 mM DTT, which were then loaded and separated by SDS-PAGE (31), transferred to nitrocellulose membranes (Bio-Rad), and immunoblotted with a 1:300 dilution of mouse monoclonal antibodies that had been raised against the His6 tag. The membranes were subsequently incubated with goat anti-mouse IgG that had been conjugated with horseradish peroxidase (ECL Western blotting detection reagents (GE Healthcare).

Oxygen uptake measurements with S. cerevisiae and E. coli. Oxygen uptake was measured with a Clark-type electrode (72) that had been fitted to a Gilson oxygen chamber (Gilson Medical Electronics, Inc., Middleton, WI) in 1.8 ml of a standard incubation medium that contained 0.6 mM succinate, 10 mM HEPES-KOH at a pH of 7.2, 5 mM MgCl2, 2 mM KC1, and 0.5 mM EGTA at 30°C. The initial solubility of oxygen in the reaction buffer was considered to be 445 ng of atoms of O2/ml (23). Further additions are indicated in the figure legends. DMSO and ethanol were also tested in order to determine any possible impact of these solvents on oxygen uptake. Respiratory parameters were determined as previously described (8).

RNA isolation, real-time PCRs, and reverse transcription-PCRs (RT-PCRs). Yeast cells were disrupted with glass beads and by grinding in liquid nitrogen (17) and immediately mixed with Trizol (Invitrogen) for RNA extraction in accordance with the supplier’s recommendations. The presence of RNA contamination after RNase-free DNase treatment was verified by real-time PCR quantification of the P. brasiliensis α-tubulin gene Ptaulb (http://143.107.203.68/pbever2/default.html) using oligonucleotides 5′-ACCAACGCTTGGAGAACAGTCTG-3′ (forward) and 5′-CAGGACCATCGCACCCTAACC-6-carboxyfluorescein (FAM)–G–3′ (reverse).

All real-time PCRs were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems) as described by Semighini et al. (64). All P. brasiliensis real-time PCR products yielded a single band with the expected sizes according to visualizations on agarose gels (data not shown). Because there is no ideal control for gene expression, we first compared the α-tubulin and hexokinase gene probes as normalizers for the expressions of these genes. No difference was observed between the two normalizers. Accordingly, the α-tubulin gene was used to normalize all of the expression results (data not shown). Real-time PCR quantification of ndh2 (alternative NADH dehydrogenase gene; GenBank accession number CN240857), ndh complex I subunit 6 gene; GenBank accession number AY268589), and AOX (GenBank accession number EUH19342) was achieved using, respectively, the following LUX primers (Invitrogen): 5′-CTGGCGGGAATGCTTCTGGAT-3′ (forward) and 5′-CGGTTGTCCCGGTGTGTCACAC-FAM-G–3′ (reverse), 5′-CGACGGAGCACTCTGGTATG-3′ (forward) and 5′-GGTTAGCTCTTCCTGAGAACAGAC-FAM-G–3′ (reverse), and 5′-GTGGTGTCCCTGGATATTG-3′ (forward) and 5′-CGGCTGATCCCTCATTACCC-FAM-G–3′ (reverse).

Statistical analysis. Statistical analysis for comparing the values that were obtained in the gene expression level experiments was performed with one-way analysis of variance (one-way ANOVA) followed by the Newman-Keuls post hoc test. The values that were obtained in the intracellular ROS generation experiments were compared using two-way ANOVA followed by Bonferroni’s post hoc test. In both analyses, P values of <0.05 indicated significance. The software used for the calculations was GraphPad Prism, version 4.0, for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effects of mitochondrial respiratory chain inhibitors on P. brasiliensis M-to-Y differentiation. After the characterization of oxidative phosphorylation in the yeast form of P. brasiliensis (40), we assessed the involvement of mitochondria in its M-to-Y differentiation using classical inhibitors of the mitochondrial respiratory chain and alternative pathways. Twenty-four hours after the temperature was shifted from 26°C to 35.5°C, chlamydospore-like structures were observed (48), which de note the start of the morphological changes from mycelia to yeast (Fig. 1) that were delayed by the presence of complex IV inhibitor KCN (the first chlamydospore-like structure observed after 48 h) (Fig. 1). In order to rule out the possibility that the compound was degraded during the course of the experiment, KCN was also added to the culture after 24 and 72 h, and no change was observed in relation to the inhibition pattern that corresponded with a single KCN addition (data not shown).

Inhibitors of complex III (antimycin A) and AOXs (SHAM) delayed M-to-Y differentiation for up to 120 h (Fig. 1). Similarly to the assays with KCN, multiple additions of antimycin A did not increase the inhibition of M-to-Y differentiation; however, the inhibition of complex III or IV with the simultaneous inhibition of AOX (Ant A-SHAM or KCN-SHAM) completely quenched M-to-Y differentiation (Fig. 1). The M-to-Y transition was quantified by counting the different morphotypes that are observed during this process. Morphological units were arbitrarily classified as hyphae, differentiating hyphae (characterized by the development of chlamydospore-like structures), transforming yeast (characterized by the production of multiple buds by the chlamydospore), and yeast. One day later, 75% of control cells had started the M-to-Y transition (Fig. 2A), but it took 3 days to obtain 75% of completely differentiated yeast cells (Fig. 2B). As shown in Fig. 1, the initiation of M-to-Y transition was delayed for up to 2 days by KCN and for up to 4 days by antimycin A or SHAM (Fig. 2). Ten days later, 75% of antimycin A-treated cells had completed the M-to-Y transition, but only 25% of the SHAM-treated cells had completed this process (Fig. 2B). On the other hand, the associations KCN-SHAM or antimycin A-SHAM completely blocked the M-to-Y transition (Fig. 2).

After 240 h and under the condition of simultaneous inhi-
FIG. 1. Images of *P. brasiliensis* during M-to-Y differentiation in the presence of mitochondrial respiratory chain inhibitors. M-to-Y differentiation was induced as described in Materials and Methods. Mitochondrial respiratory chain inhibitors were added just before the temperature was shifted from 26°C to 35.5°C. Their concentrations per addition, even when combined, were as follows: for SHAM, 2 mM; for KCN, 1 mM; and for antimycin A, 1.8 μM. In some of the experiments, drugs were added once more after 24 or 72 h, but there were no noticeable differences between the results obtained with single additions and those obtained with multiple additions (data not shown). Images were selected from a single experiment that was representative of at least three independent experiments. Images were captured using a 40× objective lens, resulting in a final magnification of ×400, with an Olympus BX51 model U-LH100-3 microscope that was coupled to an Olympus model C-5060 wide-zoom digital camera. Black arrows indicate chlamydomes, which are characteristic structures during M-to-Y differentiation.
tion, mycelia that did not differentiate to yeast were harvested, washed twice with a fresh PGY medium, and incubated without inhibitors for an additional 240 h, and no culture was able to differentiate to yeast. Other respiratory chain inhibitors, such as rotenone (complex I) and flavone (alternative NADH dehydrogenases), or solvents, such as DMSO and ethanol, did not affect \textit{P. brasiliensis} differentiation (data not shown). Moreover, none of the inhibitors that were used in the differentiation assays affected the vegetative growth of the \textit{P. brasiliensis} yeast form (not shown). To verify whether the undifferentiated mycelia have been killed or just blocked by the inhibitors, we determined the viability of the cells during M-to-Y differentiation using the MTT assay. The viability of untreated cells was unaffected for the first 2 days of culture and then decreased until day 10, when the viability was 75% compared to the level observed at time zero (Fig. 3). Cells treated with SHAM had decreased viability on days 1, 3, and 4, when 60% of the cells were still viable, remaining stable after 10 days of culture (Fig. 3). KCN-SHAM- and antimycin A-SHAM-treated cells showed pronounceable decreases in viability compared to the levels for control and SHAM-treated cells throughout the 10 days of culture (Fig. 3). However, 7 days after 40% of the cells were still viable, which was lower than the level of viability for the control cells but considerably higher than the level of viability for heat-killed or fixed cells (values comparable to those observed for the background with the MTT method) (Fig. 3).

Expression of \textit{Pbaox} during M-to-Y differentiation and the vegetative growth of the yeast form. Because inhibitors of mitochondrial AOX interfered with the \textit{P. brasiliensis} differentiation process, we assessed the expression levels of \textit{Pbaox} during M-to-Y differentiation and the vegetative growth of the yeast form. The \textit{Pbaox} transcript levels increased by 345% after 10 h of incubation at 35.5°C in cells that were differentiating in a complete medium and decreased to their lowest levels (25% of the expression levels observed at time zero) at 48 h (Fig. 4A). A second increase in \textit{Pbaox} transcript levels was observed after 72 h, and higher levels, which were comparable to those achieved at 10 h, were reached after 240 h. In cells that were differentiating in a minimal medium, the first peak of \textit{Pbaox} expression was delayed (48 h) in comparison to the level for cells that were grown in a complete medium (Fig. 4A). Additionally, in a minimal medium, the second peak of expression was not observed for up to 240 h (Fig. 4A).

We observed that the expression levels of \textit{Pbaox} were modulated during the growth curve of the \textit{P. brasiliensis} yeast form. The expression of \textit{Pbaox} was lower in the first 24 h as well as during the stationary growth phase (120 and 240 h) (Fig. 4B). On the other hand, the \textit{Pbaox} transcript levels exhibited a gradual increase from 48 to 96 h to levels that were nearly 3-fold higher than those at zero time and 24 h and those in the stationary phase (Fig. 4B). This period of higher levels of \textit{Pbaox} expression corresponds to the early- and mid-exponential phases of growth for the yeast form.

Oxidative stress during M-to-Y differentiation of \textit{P. brasiliensis}. It has been proposed that oxidative stress conditions upregulate the expression of AOX genes. In order to evaluate this hypothesis, we measured oxidative stress during M-to-Y differentiation by determining the levels of protein carbonylation. Proteins were isolated from control and SHAM-treated cells throughout 10 days of culture and then processed. There was an increase of carbonylated proteins during M-to-Y differentiation as shown by the intensity of the bands at days 1, 2, and 3 (around 50% increased), day 4 (150%), and day 10...
(50%), in comparison to the level observed for the band at time zero (Fig. 5, left panels). The increase of carbonylated proteins was even higher when the M-to-Y differentiation occurred under the inhibition of AOX by SHAM (Fig. 5, right panels). All time points analyzed under this condition showed increased levels of carbonylated proteins, with the values ranging from 1.5 to 3.5 times higher than those observed at time zero (Fig. 5B; see also Fig. S1B in the supplemental material).

**Effects of oxidative stress on the expression of mitochondrial respiratory chain genes in the yeast form.** We challenged *P. brasiliensis* yeast cells with different compounds that are known to generate ROS and RNS and measured the corresponding *Pbaox* expression levels. In comparison to untreated control cells, SNP- and KCN-treated cells exhibited nonsignificant increases in *Pbaox* expression (Fig. 6A). *H₂O₂*, rotenone, rotenone-KCN, and antimycin A treatments caused 2-fold increases in *Pbaox* expression, whereas menadione and menadione-SNP increased the levels of *Pbaox* transcripts by almost 3- and 4-fold, respectively (Fig. 6A); however, SHAM, which is a specific inhibitor of AOXs, did not significantly impact the expression of *Pbaox* (demonstrated in Fig. 6A). It was not possible to test AOX inhibitors, because they interfere with this ROS assay (56).

**Cloning, sequence analysis, and heterologous expression of *Pbaox*.** We cloned and heterologously expressed *Pbaox* in order to investigate its function during the differentiation process and pathogenesis of the fungus.

Analyses of *Pbaox* demonstrated a coding sequence of 1,136 bp, which carries an open reading frame of 1,059 bp that encodes 352 amino acids with a predicted molecular mass of 40.3 kDa and a theoretical isoelectric point of 9.25. The alignment of cDNA and genomic DNA sequences revealed the presence of a 77-bp intron region from nucleotides 262 to 339 (see Fig. S2 in the supplemental material). Alignments of the deduced *Pbaox* amino acid sequence with AOXs from *H. capsulatum*, *Neurospora crassa*, *Candida albicans*, and *Trypanosoma brucei brucei* showed 72%, 46%, 40%, and 29% similarities, respectively (Fig. S3), including two hydrophobic regions (W147 to R169 and W209 to L231) (Fig. S2) and the four primary conserved regions among the AOXs (LET, NERMHL, LEEA, and RADE-H) (Fig. S3) that contain the metal-binding amino acids E155, E194, H197, E245, E300, and H303 and the ubiquinone-binding amino acids Y230 and Y252 (44, 66).

**Phaox** was subcloned into the expression vector pET28a(+) for expression in *E. coli* cells and into pYES2-NTC for expression in *S. cerevisiae*. The final constructs were named pET28-Phaox and pYES-Phaox. The expected protein sizes were 42 kDa in *E. coli* (PhAOX plus an N-terminal His tag) and 48 kDa (PhAOX plus an N-terminal and C-terminal His tags) in *S. cerevisiae*. The expression of PhAOX was confirmed by Western blotting via an anti-His tag antibody (Fig. 7D and 8D). In isolated *S. cerevisiae* mitochondria, a band was detected at the expected size (48 kDa) in addition to a smaller band at around 42 kDa that corresponded to the expected size without the mitochondrial signal peptide (Fig. 7D, lane 3). In *E. coli* pET28-Phaox samples, a single band at the expected size (42 kDa) was detected (Fig. 8D, lane 3).
Heterologous expression of Pbaox confers cyanide-resistant respiration to S. cerevisiae and E. coli cells. The polarographic measurement of oxygen uptake by E. coli and S. cerevisiae in both PbAOX-expressing and control cells showed that KCN completely inhibited oxygen uptake in S. cerevisiae/pYES (Fig. 7C) and E. coli/pET28 (Fig. 8C) cells that did not overexpress PbAOX; however, KCN only partially inhibited oxygen uptake in PbAOX-overexpressing S. cerevisiae/pYES2-Pbaox (Fig. 7A) and E. coli/pET28-Pbaox (Fig. 8A) cells. Moreover, the addition of SHAM or BHAM (specific inhibitors of AOXs) partially inhibited oxygen uptake in PbAOX-overexpressing S. cerevisiae/pYES2-Pbaox (Fig. 7A) and E. coli/pET28-Pbaox (Fig. 8A) cells. These agents also inhibited KCN-resistant respiration and quenched oxygen uptake in PbAOX-overexpressing S. cerevisiae/pYES2-Pbaox (Fig. 7A) and E. coli/pET28-Pbaox (Fig. 8A) cells.

S. cerevisiae Pbaox-expressing cells exhibited compromised cell growth and a decreased intracellular ROS generation. Pbaox overexpression and activity were confirmed for both S. cerevisiae and E. coli cells. In order to determine the influence of PbAOX activity in S. cerevisiae cells, we evaluated their growth curves and intracellular ROS generation. PbAOX-overexpressing cells exhibited a lower growth rate and lower cell numbers during both exponential and stationary stages in both fermentable (see Fig. S5A in the supplemental material) and nonfermentable (Fig. S4B) media.

The intracellular oxidative status of S. cerevisiae cells was determined via a CM-H$_2$DCFDA fluorescent probe. As can be observed in Fig. 9, the fluorescence measured in S. cerevisiae/pYES cells after 10, 30, and 50 min increased by approximately 25%, 50%, and 75%, respectively. An increase in fluorescence...
in *S. cerevisiae/pYES-Pbaox* cells was also observed, although this increase was significantly lower than those that were observed in cells that were not expressing *PbAOX* at 30 and 50 min. Similarly to the data that were obtained from *P. brasiliensis* yeast cells (Fig. 6B), the exposure of *S. cerevisiae* to KCN did not increase ROS generation in comparison to that in control cells, even though the level of ROS after 50 min was significantly lower in *PbAOX* expressing-cells that were exposed to KCN than in control cells. Antimycin A increased ROS generation by 50%, 75%, and 150% after 10, 30, and 50 min.
FIG. 8. Cyanide-resistant respiration in Phaox-expressing E. coli. (A and B) E. coli/pET28-Phaox-induced cells. (B) E. coli/pET28. Cells were incubated at 30°C in 1.8 ml of a respiration medium. At the indicated time points, KCN (1 mM) and SHAM (2 mM) were added. The rate of oxygen uptake was expressed as ng atoms of oxygen/min. All the assays were performed with 0.2 mg/ml of proteins. The depicted graphs are representative of at least three independent assays. (D) Reverse image of the immunodetection of Phaox in Phaox-expressing E. coli. Fifty micrograms of protein was loaded per lane. Samples were prepared, as described in Materials and Methods, from induced E. coli cells that had been transformed with the empty vector (lane 1), induced E. coli cells that had been transformed with the empty vector (lane 2), induced E. coli cells that had been transformed with pET28-Phaox (lane 3), and uninduced E. coli cells that had been transformed with pET28-Phaox (lane 4). SDS-PAGE and Western blot techniques were performed as described in Materials and Methods. His-tagged Phaox was detected using a monoclonal antibody that was raised against the His tag. The expected molecular mass for the final protein is about 42 kDa.

FIG. 9. The heterologous expression of the Phaox gene decreases the generation of intracellular ROS in S. cerevisiae cells. Cell culture, induction of Phaox expression, spheroplast generation, and ROS measurement procedures were performed as described in Materials and Methods. The spheroplasts of S. cerevisiae/pYES-Phaox and S. cerevi-
siae/pYES cells (5 × 10^6 cells/ml) were loaded with the CM-
H2DCFDA (5 μM) fluorescent probe for 30 min at 37°C. The oxida-
tion of the probe by ROS was monitored using a Hitachi F-4500 fluorescence spectrophotometer at 30°C under agitation. The excita-
tion and emission wavelengths were 503 and 529 nm, respectively. The concentrations of the inhibitors in each addition were as follows: for KCN, 1 mM, and for antimycin A, 1.8 μM. The values represent increases in fluorescence at 10, 30, and 50 min in comparison to that at time zero (before the addition of inhibitors), and these values are shown as the averages ± SEMs of results from three independent assays. Asterisks indicate P values of <0.05 between S. cerevisiae/pYES-Phaox and S. cerevisiae/pYES cells. The statistical analyses are described in Materials and Methods.

involved in the sensing of different stresses in dimorphic fungi, including temperature, osmotic or oxidative stress, nutrient deprivation, redox potential, and host-derived factors (46). Therefore, it is important to assess the possible correlations that exist between mitochondrial function, ROS generation, and fungal development processes in P. brasiliensis.

Mitochondria are the primary intracellular sources of ROS, which have the potential to damage biomolecules and interfere with cellular processes (20–22). In order to protect from these deleterious effects of ROS, fungal cells have developed a series of mechanisms to reduce ROS levels (15). In some plants and fungi, the mitochondrial respiratory chain possesses alternative pathways in addition to proton-pumping complexes in order to prevent ROS generation, specifically, alternative NADH-ubiquinone oxidoreductases and alternative ubiquinol oxidases (AOXs) (28). Alternative NADH-ubiquinone oxidoreductases are located in the mitochondrial matrix, wherein they oxidize internal NADH, or are alternatively located in the intermembrane space, wherein they could oxidize cytosolic NADH and/or NADPH (29). Alternative ubiquinol oxidases directly transfer electrons from ubiquinol to oxygen. AOXs have been documented to occur in plants (4, 65, 74), algae (71), yeasts (75), free-living amoebae (25), and protozoa (69) as well as in pathogenic fungi, such as Aspergillus fumigatus (72), H. capsulatum (27, 38), and P. brasiliensis (17, 39, 40, 42).

The inhibition of complex III and/or AOX, which interferes with ROS homeostasis, was observed to affect the M-to-Y differentiation of P. brasiliensis. The inhibition of AOX has
been reported to affect the development and/or differentiation of several pathogens, such as *C. albicans* (56), *Cryptosporidium parvum*, *T. gondii*, *Plasmodium falciparum* (55), and *T. brucei brucei* (47); however, it is still unclear whether the killing of these microorganisms due to mitochondrial respiratory chain inhibition occurs by energy deficiency, ROS generation/cell damage, or both.

It has been reported that in *P. brasiliensis*, several genes that relate to bioenergetic processes (mitochondrial and cytosolic) are modulated in a pattern that suggests a more favorable aerobic metabolism in the mycelial phase and fermentative metabolism in the yeast phase (11, 12, 48). Conversely, the upregulation of genes related to mitochondrial functions during the yeast phase of *P. brasiliensis*, such as the cytochrome *c* oxidase complex (1), ATP synthase (7), NADH-ubiquinone oxidoreductase (complex I), and alternative NADH-ubiquinone oxidoreductase (40), as well as the electron transfer flavoprotein-ubiquinone oxidoreductase, which is a component of complex III (14), has been reported.

In a previous work, we characterized, in situ, the oxidative phosphorylation processes of *P. brasiliensis* mitochondria during the yeast phase (40). We demonstrated the presence and activity of a complete mitochondrial respiratory chain (complexes I to V) as well as an alternative NADH-ubiquinone oxidoreductase and an AOX. Our previous and present results in combination with previous literature (1) suggest that *P. brasiliensis* can perform aerobic and anaerobic respiration in both morphological forms. In this regard, it has been reported that, upon phagocytosis, there is a shift from fermentative to nonfermentative metabolism in intracellular pathogens, which may use two-carbon compounds from fatty acid degradation for energy production via the glyoxylate cycle (2, 13, 19, 32, 33, 62). Indeed, the upregulation of genes from the glyoxylate cycle has been reported to occur during the M-to-Y differentiation of *P. brasiliensis* in culture medium (11, 12, 17, 48) and upon the internalization of *P. brasiliensis* by murine macrophages (9, 70). Additionally, nutritional stress conditions have been observed to influence the expression levels of the glyoxylate cycle-related genes of cultured yeast cells (9). In this regard, the upregulation of *Pbaox* during the first hours of M-to-Y differentiation and during the exponential growth of the yeast form could be associated with fatty acid degradation. This might occur by a decreased overflow of electrons in the mitochondrial respiratory chain and/or in association with the external alternative NADH-ubiquinone oxidoreductase involving the oxidation of cytosolic NADH that is generated in the glyoxylate cycle or in fermentative pathways, which subsequently balance the cytosolic redox potential, as we previously suggested (40). It had been reported that *H. capsulatum*, * Blastomyces dermatitidis*, and *P. brasiliensis* possess three different respiratory phases during morphogenesis (37, 42). After the temperature shift, there is an uncoupling of oxidative phosphorylation and a progressive decrease of respiration (stage 1). The dormant period (stage 2) occurs from between 24 and 40 h to 6 days, which is characterized by decrease in the concentrations of mitochondrial electron transport components. Finally, stage 3 is characterized by restoration of cytochrome components, normal respiration, induction of a cytosolic cysteine oxidase, and completion of the transition to yeast morphology (43, 57). During stage 2, cysteine and other sulphydryl compounds are able to induce the shunt pathways utilizing cytochrome oxidase and alternative oxidase, which is inhibited by cyanide and SHAM but is resistant to antimycin A inhibition (57). It is speculated that the inactivation of mitochondrial respiration during stage 2 results from a temperature-induced increase in oxidation-reduction potential; under this condition, the sulphydryl compounds would act as reducing agents, which may be required for maintenance of the activity of the remaining mitochondrial components (37). In accordance with these previous reports, our findings show that there is an increase in the carbonylated proteins (oxidative stress) during M-to-Y transition, which is correlated with the upregulation of *Pbaox*. It has been reported that AOXs are upregulated in fungi that are under mitochondrial respiratory chain inhibition and/or oxidative stress conditions (24, 27, 28, 35). In agreement, our data show that *Pbaox* expression is also upregulated by ROS/RNS-generating compounds and mitochondrial respiratory chain inhibitors. The higher level of induction of *Pbaox* expression after the inhibition of complex I by rotenone and complex III by antimycin A may relate to the increased ROS generation upon inhibition of these complexes. Indeed, rotenone and antimycin A were observed to cause intracellular oxidative stress in the yeast cells of *P. brasiliensis*. Apparently, the regulation of *Pbaox* expression relates more to intracellular oxidative status than to electron flow (solely) in the respiratory chain. The AOX-specific inhibitor SHAM did not affect *Pbaox* expression, suggesting that *PbAOX* activity does not control its own expression. Nevertheless, the inhibition of complex I by rotenone increased the expression of its own subunit 6, which is similar to what has been observed for other oxidative-stress-generating compounds (see Fig. S2 in the supplemental material).

We demonstrated that SHAM and antimycin A delayed the M-to-Y transition and that SHAM-antimycin A and SHAM-KCN associations promoted a complete and irreversible inhibition of the M-to-Y conversion. These effects may be associated with the increase of oxidative stress inside the cells, as suggested by the higher levels of carbonylated proteins in SHAM-treated cells during M-to-Y conversion. It was previously demonstrated that *p*-chloromercuriphenylsulfonic acid (PCMS), a sulfhydryl inhibitor, permanently and irreversibly blocked the M-to-Y transition in *H. capsulatum* (43). Thus, it is reasonable to assume that extreme alterations of the intracellular redox balance at early stages of the M-to-Y transition may affect crucial steps for the differentiation of dimorphic fungi.

*Pbaox* and its expression (17) and AOX activity (40, 42) have previously been described for *P. brasiliensis*; however, the characterization of the *Pbaox* gene and protein sequences, as well as its heterologous activity in eukaryotic and prokaryotic systems, is described here for the first time. As demonstrated, *Pbaox* is very similar to other fungal AOXs in terms of its nucleotide and amino acid sequences, catalytic domains, and trans-membrane regions.

The heterologous expression of *Pbaox* in *S. cerevisiae* demonstrates that the protein was correctly processed and targeted to the mitochondria, as demonstrated by Western blot analysis. The presence of a second band with a molecular size that corresponds to a combination of *PbAOX* before peptide signal cleavage and two histidine tags (N and C terminals) is understandable, because most mitochondrial preparations are en-
riched fractions; therefore, the presence of vesicles and other cytosolic structures is not insignificant. Moreover, the presence of KCN-resistant respiration in PhaOx-expressing cells confirms that the heterologous protein was functional. In bacteria, we were also able to express PhaOX as a functional enzyme, as demonstrated by Western blotting (Fig. 8) and the presence of KCN-resistant respiration in PhaOx-expressing bacteria (Fig. 8). Interestingly, this KCN-resistant respiration arrested growth in S. cerevisiae cells under culturing in both fermentable and nonfermentable media (see Fig. S5 in the supplemental material). A similar effect was previously described for S. cerevisiae via the overexpression of a heterologous AOX from the yeast Hansenula anomala (41). In that study, the increased levels of Krebs cycle enzymes in response to AOX energy-dissipating activity confirmed the close link between AOX and the cycle. The authors proposed that AOX has a direct effect in reducing substrate availability and a regulatory role in energy balance.

The present study demonstrates, for the first time, that heterologously expressed AOX in S. cerevisiae decreases intracellular ROS generation compared to the level for cells that bear only the empty vector. This finding supports the hypothesis that AOXs play an important role in intracellular redox balancing. PhaOx is a unique enzyme in P. brasiliensis that is not present in its mammalian hosts. In this context, we have demonstrated that PhaOAX and other components of the mitochondrial respiratory chain are critical during the early stages of M-to-Y differentiation, which is an essential step for the pathogenesis and establishment of PCM. Therefore, PhaOAX is a potential chemotherapeutic target for PCM.

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