Transcriptome Analysis of *Paracoccidioides brasiliensis* Cells Undergoing Mycelium-to-Yeast Transition

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*Paracoccidioides brasiliensis* is a thermomorphogenic fungus associated with paracoccidioidomycosis (PCM), a systemic mycosis prevalent in South America. In humans, infection starts by inhalation of fungal propagules, which reach the pulmonary epithelium and transform into the yeast parasitic form. Thus, the mycelium-to-yeast transition is of particular interest because conversion to yeast is essential for infection. We have used a *P. brasiliensis* biochip carrying sequences of 4,692 genes from this fungus to monitor gene expression at several time points of the mycelium-to-yeast morphological shift (from 5 to 120 h). The results revealed a total of 2,583 genes that displayed statistically significant modulation in at least one experimental time point. Among the identified gene homologues, some encoded enzymes involved in amino acid catabolism, signal transduction, protein synthesis, cell wall metabolism, genome structure, oxidative stress response, growth control, and development. The expression pattern of 20 genes was independently verified by real-time reverse transcription-PCR, revealing a high degree of correlation between the data obtained with the two methodologies. One gene, encoding 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), was highly overexpressed during the mycelium-to-yeast differentiation, and the use of NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione], a specific inhibitor of 4-HPPD activity, as well as that of NTBC derivatives, was able to inhibit growth and differentiation of the pathogenic yeast phase of the fungus in vitro. These data set the stage for further studies involving NTBC and its derivatives as new chemotherapeutic agents against PCM and confirm the potential of array-based approaches to identify new targets for the development of alternative treatments against pathogenic microorganisms.

Most systemic infections of immunocompetent humans are caused by dimorphic fungi. This group of pathogens includes Blastomyces dermatitidis, Histoplasma capsulatum, *Paracoccidioides brasiliensis*, and *Coccidioides* spp., and thermally regulated dimorphism is their main defined genetic trait (34). *P. brasiliensis* is the causative agent of paracoccidioidomycosis (PCM), a systemic mycosis prevalent in Latin America and with a broad geographic distribution that spans from Mexico to Argentina (47). It is estimated that as many as 10 million individuals are infected with *P. brasiliensis*, acquired by inhalation of airborne microconidia, which reach the pulmonary alveolar epithelium and transform into the parasitic yeast form (43). Pathogenicity is intimately linked to the dimorphic transition, since strains of *P. brasiliensis* (and also of *H. capsulatum* and *B. dermatitidis*) that are unable to transform into yeasts are not virulent (3, 38, 44, 47). Signaling pathways that control the morphological changes in *P. brasiliensis* are poorly understood, but the involvement of both cyclic AMP and mitogen-activated protein kinase signal transduction pathways has been reported for other dimorphic fungi (26). The dimorphic transition also involves changes in cell wall composition, such as migration and reorganization of membrane lipids, especially glycosphin-golipids (27, 50, 52), and structural alterations in the carbohydrate polymers. As the fungus adopts the yeast form, an increase in chitin content is observed in the cell wall, followed by a change in the glucan anomic structure from a β-1,3-linked polymer to an α-1,3-glucan (47). The surface α-glucan may play a role against the host defense mechanisms, due to the incapacity of phagocytic cells to digest α-1,3-glucan (46). The identification of genes specifically involved in the mycelium-to-yeast dimorphic shift has been the subject of great interest, especially after the genome of *P. brasiliensis* was submitted to extensive expressed sequence tag (EST) sequencing, which allows the identification of several *Candida albicans* virulence and pathogenicity homologues (12, 13, 15). In a pre-
liminary attempt to isolate phase-specific transcripts, Venâncio et al. (51) have employed the technique of differential display, which led to the identification of many differentially transcribed cDNA fragments specific for each phase. With the aid of genomics-based approaches, such as electronic subtraction, suppressive subtraction hybridization, real-time reverse transcription-PCR (RT-PCR), and low-density array hybridizations, further information regarding phase-specific gene expression has been obtained from both mycelium and yeast cells (12, 13, 15, 35). Nonetheless, very few genetic determinants identified in this fungus are known to be directly involved in either phase transition and/or pathogenicity, although the experiments mentioned above identified the up-regulation of genes that may play a role in the structural or metabolic changes that take place during morphogenesis or may be necessary for colonization and survival in the host (44).

For a more comprehensive evaluation of genes that have their expression modulated during the mycelium-to-yeast transition, we performed a large-scale analysis of gene expression in P. brasiliensis using a microarray hybridization approach. Since the complete genome of P. brasiliensis has not yet been sequenced, we constructed a 4,692-element array containing amplified EST sequences that have been functionally classified by similarity to known genes (15). Using this array, we identified 2,583 genes whose expression displayed statistically significant modulation during the mycelium-to-yeast transition, and analysis of these data allowed us to identify specific genes and gene sets whose induction seem to be related to biochemical and morphological changes associated with transformation into the pathogenic yeast form. These include genes involved in amino acid catabolism, signal transduction, protein synthesis, cell wall metabolism, genome structure, oxidative stress response, growth control, and development. One of these genes, encoding the enzyme 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), has been evaluated as a new potential drug target through the use of NTBC [2-(2-nitro-4-trifluoromethyl-benzoyl)-cyclohexane-1,3-dione], a specific 4-HPPD inhibitor (53) that may provide an alternative chemotherapeutic strategy against PCM and other fungus-related diseases.

**MATERIALS AND METHODS**

**Strains, culture medium, and disk diffusion assay.** We used P. brasiliensis isolate 18, named Pb18 (provided by Z. P. Camargo, UNIFESP, São Paulo, Brazil) throughout the work. Fungal cells were cultivated in modified YPD medium (Bacto peptone at 5 g/liter, glucose at 10 g/liter, yeast extract at 5 g/liter, 25.0, 50.0, 100.0, or 200.0 μg/ml of NTBC and all derivatives. After drying, they were placed onto inoculated agar plates. The plates were incubated at 37°C, and the diameters of the inhibition zones were evaluated after 7 days. Each disk diffusion assay was performed twice.

**RNA isolation.** Yeast cells and mycelium were disrupted with glass beads and by grinding in liquid nitrogen, respectively (15), and immediately mixed with Trizol (Gibco-BRL) for RNA extraction following the supplier’s recommendations. To minimize the RNA contamination, 20 μg of RNA from each treatment was fractionated in 2.2 M formaldehyde, placed on a 1.2% agarose gel, stained with ethidium bromide, and visualized with UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion to verify that the RNA was intact. RNase-free DNase treatment was done in a final volume of 100 μl containing 40 mM Tris-HCl (pH 7.5) and 6 mM MgCl₂, 1 μl of RNasin (40 U/μl; Promega), 10 μl of RNase-free DNase (1 U/μl; Promega or Life Technologies), 2.5 μl of 200 mM dithiothreitol, and 10 μl of total RNA. The reaction was incubated at 37°C for 60 min and stopped by incubating at 70°C for 30 min. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the FP3 gene using oligonucleotides GP43-2030 (5′-AACAAGGTCACCTGCACTA-3′) and GP4-2031 (5′-GGGGCAGAG AAGCATCCG-3′) (26).

**Microarray hybridizations and data analysis.** The array consists of cDNA inserts from representative ESTs from 4,692 expressed genes that were functionally classified by similarity to known genes (15). These inserts were PCR amplified with the M13 forward and reverse primers, and the amplified products were purified with a QIAquick 96 PCR purification system (QIAGEN), dried in a Savant Speed Vac system, resuspended in 50% dimethyl sulfoxide at a final concentration of 100 to 200 μg/ml and spotted onto 25- by 75-mm CMT-GAPS coated slides (Corning) using an Affymetrix 427 arrayer, according to the manufacturer’s instructions. Following spotting in the Pb. brasiliensis microarrays, please contact the authors for further details.

The RNA samples extracted with Trizol as described above were further purified with aid of an RNeasy kit (QIAGEN) and labeled by incorporation of Cy3- or Cy5-dCTP, as described by Bowtell and Sambrook (4). Briefly, 30 μg of total P. brasiliensis RNA was mixed with 2 μg of oligo(dt)12-18 in 20 μl of water and heated to 65°C for 10 min. The tube was briefly chilled on ice and mixed with 4 μl of 5× first-strand buffer, 8 μl of 5× second-strand buffer (Invitrogen), and 1.5 μl of Superscript II (200 units/μl) (Invitrogen). The reaction was then incubated at 42°C for 1.5 h and terminated by a 15-min incubation at 65°C. The RNA was degraded by the addition of 1 μl of 10 μg/ml RNase A and incubated at 37°C for 1 h. The resulting first-strand cDNA was purified and concentrated with the aid of a Microcon YM-30 cartridge, and the final volume was adjusted to 29 μl. One μl of 4-μg/ml random primer solution (9-mers) and 4 μl of 10× labeling solution (Promega or Life Technologies) were added to each sample, and the cDNA was denatured by mixing with 4 μl of 5× first-strand buffer, 8 μl of 5× second-strand buffer (Invitrogen), and 1.5 μl of Cy3- or Cy5-labeled dCTP (1 μM) (Amersham Biosciences), and 1 μl of the Klenow fragment (50 units/μl) (Invitrogen). The reaction was then incubated at 37°C for 2 h, and the final labeled cDNA was purified in a Microcon YM-30 cartridge (three washes).

Labeled cDNAs were mixed, dried in a Savant speed vacuum, and resuspended in 100 μl of 1× hybridization buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, 0.25 mg/ml salmon sperm DNA, 0.01% sodium dodecyl sulfate (SDS), 50% formamide, and 92.5 ng/ml poly(dT)25 U (Amersham). Arrays were hybridized overnight (42°C) in a Gene-Tac hybridization station (Genomic Solutions, Inc., Ann Arbor, MI) and washed twice (42°C) in 0.5X SSC and 0.1% SDS, which was followed by two washes in 0.06× SSC and 0.01% SDS and two final washes in 0.006× SSC. All washing steps consisted of 1 min of flow followed by 5 min of incubation. Slides were then dried and subjected to fluorescence detection with a GMS 418 array scanner (Affymetrix Inc., Santa Clara, CA). Images were analyzed with the TIGR Spotfinder program (v.2.2.4). All spots with median values lower than the median local background plus 2 standard deviations were flagged and excluded from further analyses. Each time point was analyzed with four independent hybridizations (two pairs of hybridizations with dye swaps within each pair). Since each biochip carried two replicas of the arrayed genes, a total of eight intensity readings were generated for each element in the microarray. The results from each hybridization were submitted to a series of mathematical transformations with the aid of the software TIGR MIDAS v.2.19. These included filtering out all spots that were integrated intensity values below 10,000 analog-to-digital units, normalizing between the two channels with the aid of the Lowess algorithm, and regularizing standard deviations of the Cy5/Cy3 ratios across all sectors (blocks) of the array. Finally, replicated slides were submitted to a dye swap consistency check, and the results from each individual experiment were loaded into the
software TIGR Multi-Experiment Viewer, v.3.01. Experiments were then normalized, and genes that displayed statistically significant modulation were identified by a one-way analysis of variance, considering a P value of <0.01 as a cutoff. Further clustering analyses of the modulated genes have also been performed with the aid of TIGR Multi-Experiment Viewer. For details regarding the use of the TIGR microarray software suite (TM4), see the work of Saeed et al. (45).

Raw and normalized data from all microarray hybridizations, as well as the microarray complete annotation file, have been submitted in MIAME (Minimum Information About a Microarray Experiment)-compliant format to NCBI’s Gene Expression Omnibus and can be assessed through series number GSE 3238. A tab-delimited file containing the list of significant genes and the averaged expression profile of such genes can be downloaded from http://143.107.203.68/pbm2ytrans/index.html.

Real-time PCRs and RT-PCRs. All the real-time PCRs and RT-PCRs were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). TaqMan EZ RT-PCR kits (Applied Biosystems) were used for RT-PCRs. The thermocycling conditions comprised an initial step at 50°C for 2 min followed by 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min. TaqMan PCR reagent kits were used for PCRs. The thermocycling conditions comprised an initial step at 50°C for 2 min followed by 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min. Since there is no ideal control for gene expression, we first compared several genes, such as those encoding γ-tubulin, hexokinase, and a translation factor, as normalizers for the expression experiments. We saw no difference by using any of these normalizers. Accordingly, the calibrator gene used along the expression experiments was the γ-tubulin gene (data not shown). The reactions and calculations were performed according to the work of Semighini et al. (48). Primer and probe sequences are described in Table S1 in the supplementary materials at http://143.107.203.68/pbm2ytrans/index.html. Correlation coefficients comparing the log2 ratios for RT-PCR and array data were calculated with the aid of the software Biostat v2.0.

RESULTS

Morphological evaluation of the mycelium-to-yeast transition in P. brasiliensis. The temperature shift from 26° to 37°C is the single condition known to trigger the mycelium-to-yeast transition in P. brasiliensis. We followed the different steps of the dimorphic transition and established parameters to quantify the different morphotypes that are produced during this process (Fig. 1). We arbitrarily classified the transition in four different morphological states: (i) hyphae; (ii) differentiating hyphae, characterized by the development of chlamydospore-like cells, produced by intercalary or lateral swellings in the fertile hyphae; (iii) transforming yeast, characterized by the production of multiple buds by the chlamydospore; and (iv) mature, multibudding yeast. This classification helped us to establish quantitative parameters to assess the morphological transition at each time point. It is clear that a 120-h incubation period is enough to obtain differentiation of the mycelium into yeast forms (over 85% of the morphotypes in the culture are mature yeasts, while those remaining are transforming yeasts). Since gene expression modulations at the earlier stages of the differentiation process might be important in triggering the mycelium-to-yeast transition, we decided to evaluate gene expression variations at each of the time points, which were 5, 10, 24, 48, 72, and 120 h after the temperature shift. Thus, total RNA extracted from these cultures was used to synthesize fluorescence-labeled cDNAs for competitive microarray hybridizations.

Identification of genes that have their expression modulated during the mycelium-to-yeast transition. To assess P. brasiliensis gene expression on a large scale, we constructed a microarray carrying PCR-amplified cDNA representatives of 4,692 nonredundant EST clusters obtained from a P. brasiliensis
TABLE 1. Comparison of gene expression values obtained with microarray hybridization and real-time RT-PCR

| Gene product (gene) | Value from array/RT-PCR at*: | Correlation coefficient (R<sup>A</sup>/R<sup>B</sup>)<sup>a</sup> |
|---------------------|-------------------------------|---------------------------------|
| Branched-chain alpha-keto acid dehydrogenase E2 subunit (MAS1216) | 0.02/1.57 | 0.89/0.94 |
| Sulphur transcription regulator (MAS1261) | 0.72/1.80 | 0.88/0.94 |
| Alkaline serine protease (MAC0354) | 2.92/1.5 | 0.66/0.77 |
| Peptide methionine sulfoxide reductase (MAC0725) | 0.28/1.08 | 0.76/0.60 |
| Low-affinity hexose transporter (MAS1077) | -1.27/2.33 | 0.74/0.71 |
| Homogentisate dioxygenase (MAC0559) | -0.65/2.04 | 0.74/0.60 |
| Membrane protein YDL237w (MAS1188) | 2.23/1.55 | 0.96/0.50 |
| GPR/FUN34 family protein (MAS2134) | -0.57/1.51 | 0.71/0.50 |
| Alcohol dehydrogenase (MAC0660) | 1.5/2.0 | 0.70/0.60 |
| Thioredoxin (MAS1661) | 1.58/1.43 | 0.68/0.46 |
| Immunoreactive protein precursor (MAS1924) | 2.35/0.85 | 0.66/0.03 |
| Unknown protein (MAS1735) | -0.63/0.97 | 0.60/0.55 |
| Unknown protein (MAC0195) | 3.02/1.03 | 0.41/0.60 |
| 4-Hydroxyphenylpyruvate dioxygenase (MAC0550) | 1.94/1.39 | 0.58/0.37 |
| ADP ribosylation factor-like protein 1 (MAC0849) | -0.35/1.6 | 0.56/0.54 |
| Unknown protein (MAC0867) | 0.03/1.46 | -0.17/0.54 |
| Branched-chain alpha keto acid dehydrogenase E1 (MAS1260) | 0.58/1.13 | -0.17/0.54 |
| Stress-induced protein STII (MAS0665) | 2.78/0.69 | 0.37/0.50 |
| Unknown protein (MAS2305) | -1.05/1.5 | -0.002/0.08 |
| Peroxiredoxin V protein (MAC1221) | 2.05/1.37 | -0.51/0.31 |

<sup>a</sup> The values are shown as the log<sub>2</sub> ratios between the values obtained at the indicated experimental time points and the reference value (0 h).

<sup>b</sup> We calculated both Pearson’s (R<sub>P</sub>) and Spearman’s (R<sub>S</sub>) correlation coefficients for each pair of curves. The former is more adequate for comparisons in which the data assume a normal distribution, while the latter is more appropriate for data sets which do not follow a normal distribution. The higher R value is highlighted in bold. Correlation coefficients were calculated after comparison of the array and RT-PCR data for each gene.

We also compared the gene expression variation estimated by these two methodologies by calculating both Pearson’s (R<sub>P</sub>) and Spearman’s (R<sub>S</sub>) correlation coefficients for the log<sub>2</sub> ratios obtained by the two approaches. As shown in Table 1, positive correlation was observed for both R<sub>P</sub> and R<sub>S</sub> in 16 out of 20 genes (80% of the cases). Furthermore, the value of either R<sub>P</sub> or R<sub>S</sub> was above 0.50 (indicating moderate to strong correlation) in 17 out of 20 genes (85% of the cases). Thus, although we were able to detect some discrepancies between the two methodologies, it seems that our microarray hybridization approach is capable of providing information about <i>P. brasiliensis</i> gene expression modulation with a considerably high level of confidence.

Most relevant metabolic aspects of the mycelium-to-yeast transition. A direct analysis of the modulated genes identified through our microarray hybridization experiments allowed us to detect up- and down-regulation of several genes involved in a variety of cellular processes. Their specific modulation is likely to be implicated with the <i>P. brasiliensis</i> dimorphic shift, as is their response to heat shock and other environmental stimuli to which the culture has been submitted during the dimorphic transition. Some of these genes have been manually selected from our list of significant genes (available at
http://143.107.203.68/pbm2ytrans/index.html) and organized in hierarchical clusters, which are shown in Fig. 2 and 3.

The first thing to attract our attention was the behavior of genes that encode ribosomal proteins, since coordinated overexpression of approximately 60 of these genes was observed after 24 h of temperature shift (Fig. 2). The same overexpression was observed with the gene encoding an RNA polymerase I transcription factor (MAS0157; see Fig. 5B), indicating that the dimorphic transition is likely to involve intense synthesis of new ribosome particles, affecting the rate of protein synthesis as the fungus transforms into the yeast pathogenic form.

Furthermore, it has been well documented that *P. brasiliensis* yeast cells undergo several changes at the surface level, such as an increase in cell wall chitin content, in contrast to hyphae (for a review, see reference 3). In fact, our microarray hybridizations identified up-regulation of a series of genes encoding chitin synthases upon temperature shift, whereas chitinases and endochitinases were simultaneously down-regulated (Fig. 3A). Interestingly, our biochip carried three other chitin synthase genes (MAS2882, MAS1009, and MAC1249) that did not show statistically significant variation in expression during the experiment, suggesting that chitin biosynthesis might also occur through the activation of alternative genes that do not respond to heat shock stimuli and/or do not belong to the dimorphic shift regulon. Kraus and coworkers (24) have also shown increased expression of a *Cryptococcus neoformans*
chitin synthase gene during growth at 37°C. Niño-Vega et al. (41) have shown that despite the fact that yeast cells contain more chitin than hyphal cells do, the levels of mRNA for chitin synthase genes CHS1, CHS2, CHS4, and CHS5 were higher in hyphal cells than in yeast cells. The ESTs that correspond to CHS1 and CHS2 are not present in our microarray, and those corresponding to CHS4 and CHS5 (MAS1009 and MAC1249) did not display statistically significant modulation upon analysis, as more expressed during the mycelium-to-yeast transition. It is possible that these four chitin synthase genes are specifically involved in chitin biosynthesis during the saprophytic phase.

Since the dimorphic transition in *P. brasiliensis* is triggered by an increase in temperature, we decided to evaluate the expression of genes encoding heat shock proteins during the mycelium-to-yeast transition. The biochip carried a total of 25 heat shock proteins (including putative heat shock proteins, as predicted by BLAST analyses). Only three of these Hsp genes did not show statistically significant variation throughout our experiment (MAC1157, MAS2388, and MAS0578). The remaining 22 Hsp genes have their expression profiles displayed in Fig. 3B, which shows that about 70% of them are clearly induced early after temperature shift (5 h). However, at time point 10 h, these genes exhibit rather distinct expression patterns. Only two of them, *HSP10* (MAC0138) and *HSP60* (MAS1469), seem to be up-regulated throughout all analyzed time points. A second group of Hsp genes is characterized by severe down-regulation after 10 h, which occurs during the first 72 h of the differentiation process. Interestingly, this repression pattern seems to cease after 120 h, when their expression mostly returns to constitutive levels.

Alterations in chromatin structure are also likely to be induced by the temperature shift, since transcriptional repression of all histone genes is verified at time point 5 h (Fig. 3C). Interestingly, all histone genes display continued down-regulation until time point 72 h at least, with the exception of histone H4.2, which, coincidentally, has been shown to be under the control of a distinct regulatory mechanism in *Penicillium funiculosum* (2). Down-regulation of histone genes as a function of dimorphism has already been observed in the

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**FIG. 3.** Hierarchical clustering showing the pattern of expression of *P. brasiliensis* genes encoding proteins involved in chitin synthesis and degradation (A), heat shock proteins (B), and histones (C) during the mycelium-to-yeast transition. The color code displays the log₂(Cy5/Cy3) ratio for each time point and has Cy3 as the reference value (hyphae at time point 0).
pathogenic fungus *Cryptococcus neoformans* (49) and may result in significant alterations in the cellular transcriptome, since depletion of histone H4 in *Saccharomyces cerevisiae* induced changes in the expression of approximately 25% of its genes (55). This information is consistent with the observations reported herein, where more than 2,500 genes had their expression modulated during the dimorphic transition. Moreover, down-regulation of histone mRNA levels is known to occur in response to perturbations in cell cycle progression, which might be triggered by the heat shock stimulus and the consequent mycelium-to-yeast dimorphic shift (57).

Detailed analysis of the entire data set of modulated genes (see supplementary materials at http://143.107.203.68/pbm2ytrans/index.html) also allowed the identification of up-regulated genes whose products, such as two G-proteins (an α-subunit encoded by MAS0201 and a β-like subunit encoded by MAC0335), two Ser/Thr protein kinases (MAS0755, MAC0845), a cyclic AMP-dependent protein kinase (MAS1886), a 14-3-3-like protein (MAS0366), a Pho-85 cyclin-dependent kinase (MAS0866), a calmodulin-like protein (MAC1225), and the calmodulin-dependent phosphatase calcineurin (MAS2387), might be directly involved in signal transduction, intracellular signaling, and cell cycle progression.

Several genes identified as up-regulated in our experiments are directly involved with pathways that have already been implicated in the mycelium-to-yeast differentiation of dimorphic fungi. For instance, the consistent up-regulation of both the calmodulin gene (MAC1225) and the gene for the regulatory subunit of calcineurin (MAS2387) is likely to influence important morphogenesis-associated signal transduction pathways. In many fungi, Ca²⁺/calmodulin seems to be involved in various aspects of fungal development, including conidium and appressorium formation, hyphal extension and branching, mycelial dimorphism, photomorphogenesis, and fungal pathogenicity. Calcineurin, a Ca²⁺/calmodulin-dependent protein phosphatase, is essential for survival of *C. neoformans* and *C. albicans* at 37°C (23). Recently, de Carvalho et al. (9) have shown that several inhibitory drugs of the Ca²⁺/calmodulin signaling pathway, such as calmidazolium (R24571), trifluoperazine, and W7, were able to inhibit the *P. brasiliensis* mycelium-to-yeast transition.

A series of genes involved in maintenance of the intracellular redox potential and protection against oxidative stress have also been shown to be overexpressed at several time points of the dimorphic shift. These include two independent glutaredoxin genes (MAS2032 and MAS0292), three thioredoxin genes (MAS1661, MAS0752, and MAC0975), one peroxiredoxin gene (MAC1221), a thiol-specific antioxidant gene (MAC1412), a methionine sulfoxide reductase gene (MAS0725) and the gene for yeast-specific protein Y20 (MAC1168), which was recently characterized as a flavodoxin-like protein (8).

**Cluster analysis of modulated genes.** The 2,583 modulated genes have also been analyzed with the aid of a K-means algorithm in an attempt to cluster genes according to the similarities in their expression profiles. Their distribution into 56 distinct clusters shows a large number of genes with minor alterations in their expression levels, while others were more severely up- or down-regulated at one or more steps of the differentiation process (Fig. 4). We focused our attention on the two clusters that seemed to contain genes with the most intense and consistent up-regulation profiles (Fig. 4, clusters 13 and 55 [as counted from left to right, beginning at the top left panel; indicated with bold lines]). In cluster 13, genes were highly expressed after 48 h (Fig. 5A), while cluster 55 contained genes that were overexpressed at all transition time points (Fig. 5B).

In cluster 13, we have observed a series of genes associated with membrane structure, such as *HXT3*, which encodes a low-affinity hexose transporter (MAS2375), as well as the gene encoding the major *P. brasiliensis* immunodominant antigen, *GP43* (2). A member of the GPR/FUN34 family (MAS2134) is also in this cluster. While the exact function of proteins that belong to this family is yet to be established, most of them are predicted to be plasma membrane proteins (1). We also observed up-regulation of the transcription factor gene *METR*, which encodes a zinc finger transcription activator that is essential for the induction of genes involved in the sulfate assimilation pathway (37) and of the gene encoding the flavodoxin-like protein Y20. Finally, cluster 13 shows the up-regulation of the genes coding for subunit E2 of branched-chain α-keto acid dehydrogenase (MAS1216 and MAS0435), involved in oxidative degradation of branched-chain amino acids (Fig. 5A). Interestingly, subunit E1 of this enzyme displays a similar expression profile (see MAC1260 in Table 1 and in the supplementary materials provided at http://143.107.203.68/pbm2ytrans/index.html). A direct comparison of the expression profiles from these two genes yielded a correlation coefficient (Rₚ) of 0.91, as measured by real time RT-PCR.

Cluster 55, on the other hand, contains a series of genes that display consistent overexpression from the very beginning of the dimorphic shift (Fig. 5B). Among the genes found in this cluster, we observed a homologue of an immunoreactive protein precursor from *Coccidioides immitis* (MAS1924), which has been shown to encode a factor that stimulates T-cell response in humans and has been used as a protective vaccine in mice (11). Gene homologues encoding an alcohol dehydrogenase III (MAC1062) and a formamidase (MAS0378) and seven genes encoding unknown proteins (MAC1307, MAC1404, MAS0549, MAS1359, MAS1371, MAS1593, and MAS2393) have also been found within this group of genes. However, the most interesting member of cluster 55 was a gene encoding a homologue of a T-cell-reactive protein from *C. immitis*, which has been shown to encode 4-hydrophenyl-pyruvate dehydrogenase (4-HPPD; EC 1.1.3.27) (54, 56), the enzyme involved in the second step of aromatic amino acid catabolism, i.e., the conversion of 4-hydroxyphenyl-pyruvate to homogentisate and carbon dioxide in the presence of oxygen and ferrous ion. This gene was recognized by three independent probes spotted in our array (MAC1409, MAC0550, and MAS0502). All of them indicate that the expression of 4-HPPD starts early after temperature shift and might be one of the most intense up-regulations observed during the mycelium-to-yeast differentiation process, reaching an increase in expression of up to 15-fold, as measured by real-time RT-PCR.

**NTBC can block *P. brasiliensis* mycelium-to-yeast transition.** Clusters 13 and 55 showed that genes encoding enzymes involved in amino acid catabolism, such as branched-chain α-keto acid dehydrogenase (catabolism of branched amino acids) and 4-HPPD (catabolism of phenylalanine and ty-
rosine), were highly overexpressed during the mycelium-to-yeast transition (Fig. 5A and B). In addition, we have also observed an increase in expression of two other genes encoding proteins that participate in the catabolism of aromatic amino acids, i.e., homogentisate dioxygenase (MAC0559) and fumarlyacetacetate hydrolase (MAC0670) (see supplementary materials at http://143.107.203.68/pbm2ytrans/index.html). Taken together, these results suggested that the activation of some amino acid catabolic pathways could be relevant to \textit{P. brasilienis} mycelium-to-yeast transition.

Thus, in a preliminary attempt to verify if amino acid catabolism is essential for the mycelium-to-yeast transition (Fig. 5A and B). In addition, we have also observed an increase in expression of two other genes encoding proteins that participate in the catabolism of aromatic amino acids, i.e., homogentisate dioxygenase (MAC0559) and fumarlyacetacetate hydrolase (MAC0670) (see supplementary materials at http://143.107.203.68/pbm2ytrans/index.html). Taken together, these results suggested that the activation of some amino acid catabolic pathways could be relevant to \textit{P. brasilienis} mycelium-to-yeast transition.

Thus, in a preliminary attempt to verify if amino acid catabolism is essential for the mycelium-to-yeast transition, we decided to test the capacity of \textit{P. brasilienis} hyphae to differentiate in the presence of 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione, also known as NTBC or nitisinone (18, 22). NTBC is a potent specific inhibitor of 4-HPPD and has been used to control the activity of this enzyme in a broad variety of organisms, including bacteria, plants, and humans (5, 30). Adding increasing concentrations of NTBC to the media and submitting \textit{P. brasilienis} hyphae to growth at 37°C, we verified that NTBC was able to inhibit the dimorphic transition in a dose-dependent manner (Fig. 6). When exposed to low concentrations of the drug (i.e., 5 to 50 \(\mu\)g/ml), \textit{P. brasilienis} mycelium was unable to transform into yeast cells, assuming typical pseudohyphal morphology even after 10 days at 37°C (Fig. 6B to D). However, hyphae could not go through any kind of morphological transition and remained as mycelium when exposed to 100 \(\mu\)g/ml of NTBC (Fig. 6E). Interestingly, we did not observe any effect of 5 to 50 \(\mu\)g/ml of NTBC either on the yeast-to-mycelium transition or on mycelium growth (data not shown), which is consistent with the preferential expression of 4-HPPD in yeast cells.

To verify the direct impact of NTBC on growth of the pathogenic yeast form of \textit{P. brasilienis}, disk diffusion assays were performed with increasing concentrations of the drug. As observed in Fig. 7A, concentrations above 100 \(\mu\)g/ml of NTBC could clearly inhibit the growth of \textit{P. brasilienis} yeasts, indicating that this drug may be a good lead for the development
of new drugs against this pathogenic fungus. Thus, we ex-
tended this approach to evaluate the effect of a series of NTBC
derivatives, whose chemical structures are shown in Fig. 7
(compounds 1, 4, and 5 were prepared according to the meth-
ods of Wu et al. [53] and Lin et al. [28], while the chemical
synthesis of compound 8 will be described elsewhere). The
results obtained from the disk diffusion assays showed that the
chemical modifications introduced into compounds 1, 4, and 5
had minor impact on \textit{P. brasiliensis} growth compared to the
impact of NTBC. Nonetheless, compound 8 was about 40 times
more potent than NTBC in its capacity to inhibit the growth of
\textit{P. brasiliensis} pathogenic yeast cells, since the effect of this drug
could be clearly observed even at concentrations as low as
$5 \mu g/ml$ (Fig. 7E, right plate). Compound 8 can also completely
block the mycelium-to-yeast transition at the concentration of
$10 \mu g/ml$ (data not shown).

**DISCUSSION**

The results of the present work demonstrate the use of high-throughput microarray hybridization analysis to examine
gene expression during the mycelium-to-yeast transition in the
pathogenic fungus \textit{P. brasiliensis}, a process that is intimately
related to the pathogenicity of dimorphic fungi. However, the
analysis of gene expression during this process involves a tem-
perature shift used to promote the dimorphic transition, mak-
ing the mycelium-to-yeast differentiation inherently associated
with the activation of genes that respond to adaptation to heat
shock and other environmental stimuli as well. So far, no
attempts have yet been made to obtain \textit{P. brasiliensis} yeast cells
at room temperature and thereafter follow the expression of
specific genes in cultures that have not been submitted to a
heat stress. Nonetheless, several genes identified in this study
corroborate previous analyses regarding the pathobiology of
dimorphic fungi and provide new insights into our understand-
ing of \textit{P. brasiliensis} and its adaptation to the environment
within the human host.

Our approach differs from those employed in other studies
aimed at analyzing the transcriptome of dimorphic fungi, such
as the experiments described by Hwang et al. (21) and Kraus et
al. (24), who used genomic DNA microarrays to identify dif-
ferentially expressed genes in the human-pathogenic fungi \textit{H.}
\textit{capsulatum} and \textit{C. neoformans}, respectively. While these au-
thors used whole-genome microarrays, our cDNA microarray
has been constructed with EST sequences derived from the
yeast pathogenic stage of this fungus, which had previously
been clustered and annotated (15), allowing a more precise
and direct identification of modulated genes. Moreover, those studies attempted only to compare gene expression between two specific morphological stages of the fungi (yeast versus mycelium), while we chose to analyze transcription modulation from 5 to 120 h into the mycelium-to-yeast dimorphic transition. This allowed us to identify genes that are specifically up- or down-regulated not only at the endpoints of differentiation but also at different time points of the transition process.

This choice of approach also distinguishes the present work from recently published papers that employed in silico electronic subtraction, suppressive subtraction hybridization, and low-density array hybridization to assess transcriptome differences between fully differentiated mycelium and yeast forms of *P. brasiliensis* grown in either complete or minimal medium (12, 13, 15, 35). Although such papers identified a relatively small number of differently expressed genes compared with the 2,583 genes reported as modulated in the present work, some similarities have been found, reinforcing the involvement of certain metabolic pathways in differentiation of the yeast pathogenic phase of the fungus. For instance, Felipe et al. (13) suggested that the metabolism of mycelium cells tends to be more aerobic than that of yeast cells, considering that, in the saprophytic phase, genes encoding isocitrate dehydrogenase and succinyl-coenzyme A synthase are overexpressed. In contrast, the gene encoding alcohol dehydrogenase I is up-regulated in yeast cells, suggesting that yeast metabolism favors fermentation and consequent production of ethanol. In this study, we have also observed intense overexpression (up to eightfold) of alcohol dehydrogenase I (MAC0060) during the mycelium-to-yeast transition. Moreover, pyruvate decarboxylase, the other enzyme involved in ethanol production from pyruvate also displays an ~2-fold increase at time point 120 h of the differentiation process (see MAS0981 in the supplementary materials at http://143.107.203.68/pbm2ytrans/index.html). This information reinforces the idea that yeast cells possess an energy metabolism biased towards ethanol production through fermentation. Surprisingly, however, our microarray data analysis was able to detect only minor alterations in the expression of isocitrate dehydrogenase and succinyl-coenzyme A synthase. A similar situation has been observed with genes of the glyoxylate pathway, whose activation might be related to pathogenicity in dimorphic fungi (31). Our microarray hybridization experiments show no alteration in the expression of isocitrate lyase (MAC1422) at time point 120 h compared to that seen in mycelial cultures, contrary to what has been reported by Felipe and coworkers (13). However, our results show that yeast cells harvested at time point 120 h display a ~2.5-fold increase in the expression of malate synthase (MAC1234), reinforcing the idea that the glyoxylate pathway is more active in the yeast form of *P. brasiliensis*.

These and other discrepancies might be a result of biological variation associated with the different *P. brasiliensis* strains under consideration, but the use of different culture media and conditions is also likely to exert a great influence on the differential expression of many genes and pathways, as recently demonstrated by Marques and coworkers in their studies involving suppressive subtraction hybridization and macroarray hybridization of *P. brasiliensis* (35). Many results obtained in that work are also consistent with the experiments described herein, such as the yeast-specific overexpression of several Hsp genes and factors involved with oxidative stress response, such
as TSA (thiol-specific antioxidant) and GST1 genes, encoding thioredoxin peroxidase and a glutathione-S-transferase, respectively. We have also identified the Y20 gene as 12 times more expressed at the end of the mycelium-to-yeast transition. The protein encoded by this gene has previously been characterized by Cunha et al. (7) by comparing proteome patterns from both forms of *P. brasiliensis* with two-dimensional gel electrophoresis. More recently, PbY20 was recognized as a member of the flavin mononucleotide flavodoxin-like WrbA family, which is involved in heat shock and oxidative stress in biological systems (8). Felipe et al. (13) have also shown that Y20 was preferentially expressed in the yeast phase. These proteins are likely to be used as antioxidants to reduce the reactive oxygen species and their effects during yeast growth. Kraus et al. (24) have shown increased expression of *C. neoformans* genes involved in promoting resistance to reactive oxygen species (catalases and oxidases) during growth at 37°C. Moreover, it remains to be investigated whether *P. brasiliensis* genes involved in promoting resistance to reactive oxygen species are also induced by heat shock and other types of cellular stress as part of a general stress response, as observed in *S. cerevisiae* (14). These observations suggest that antioxidant systems in *P. brasiliensis* and other dimorphic fungi could be extremely important not only for survival in macrophages but also for detoxification of oxygen free radicals that will be much more detrimental at 37°C.

Another interesting set of results includes the observation of increased expression of genes that encode proteins involved in amino acid catabolism, particularly in the case of aromatic and branched-chain amino acids (BCAAs). Felipe et al. (13) reported that branched-chain aminotransferase, the first enzyme involved in degradation of BCAAs, is preferentially expressed in yeast cells, while we observed intense overexpression of the branched-chain α-keto acid dehydrogenase complex (7.5-fold in the case of MAS0435) responsible for the second (and committed) step of BCAA degradation after 48 h of the mycelium-to-yeast transition. Although an eventual increase in both BCAA and aromatic amino acid degradation might reflect a simple adaptation to the use of these biomolecules as an energy source, it must be noted that such pathways are implicated in other metabolic functions. For instance, BCAAs are required for branched-chain fatty acid synthesis (19, 42), which might be implicated in the phenomena of migration and reorganization of membrane lipids during the dimorphic transition of *P. brasiliensis* (27, 50, 52). More importantly, BCAAs can serve as precursors of fusel alcohols, such as isoamylalcohol, which has been shown to be an important player in a morphogenesis checkpoint that triggers pseudohyphal formation in *S. cerevisiae* (36).

The degradation of aromatic amino acids has also been found to be associated with other metabolic pathways. For example, phenylalanine and its oxidation product, tyrosine, are
precursors for the synthesis of melanins, which are thought to play a protective role in the establishment of pathogenicity by several fungi (25). In most fungal species, a specific type of melanin, called eumelanin, is formed by oxidation of diphenolic compounds, such as L-3,4-dihydroxyphenylalanine (L-DOPA), which are polymerized by a copper-dependent diphenol oxidase from the laccase family. *P. brasiliensis* conidia, as well as yeast cells, are capable of synthesizing melanin-like compounds in vitro and in vivo, but the exact type of melanin produced by these cells remains to be fully characterized (16).

Although a laccase-like activity has been observed in *P. brasiliensis* cellular extracts, *P. brasiliensis* conidia are able to produce the pigment even in the absence of L-DOPA (16), and a homologue for the highly conserved laccase gene has not been identified, even after extensive EST sequencing of this fungus (12, 13, 15). Thus, the 15-fold up-regulation of 4-HPPD during mycelium-to-yeast transition must be noted, since this enzyme has already been described as the product of *melA*, which is involved with the synthesis of pyomelanin, a melanin-like pigment typically found in bacteria which is formed through the oxidative-dependent polymerization of homogentisic acid, the end product of 4-HPPD (39).

It has not escaped our attention that the increased expression of 4-HPPD could also be associated with resistance to oxidative attacks, since homogentisic acid has been shown to act as a scavenger of reactive oxygen species or nitric oxide-derived compounds, which might help to block the oxidative burst mediated by macrophages (20). Since nitric oxide seems to be decisive to the success of the host’s immune response against *P. brasiliensis* infection (10), the accumulation of a scavenger molecule during the differentiation to the pathogenic yeast form of the fungus, as well as the up-regulation of oxidative stress-related genes, might represent an important adaptation of the pathogen to life within the vertebrate host (3, 17, 40).

Given the lack of classic genetic tools for the study of *P. brasiliensis*, such as DNA-mediated transformation and modulation of gene expression by gene knockout or RNA interference techniques, the role of 4-HPPD overexpression could be tested due only to the availability of a specific inhibitor for this enzyme. Nitisineone, or NTBC, has been used in humans to treat hereditary type I tyrosinemia, a rare pediatric disease that causes progressive liver failure and liver cancer in young children (29, 32). Our experiments have shown that the presence of increasing concentrations of NTBC in the culture media can affect, in a dose-dependent manner, both the growth of the pathogenic yeast form and its development from mycelium. Interestingly, the yeast-to-mycelium transition is not affected by concentrations of nitisineone that affect both yeast growth and mycelium-to-yeast transition. It remains to be determined if the 4-HPPD inhibitors are directly modulating the developmental transition or are toxic to cells and thus indirectly affecting development. Although we did not demonstrate a direct interaction between NTBC and *P. brasiliensis* 4-HPPD, preliminary data obtained so far show that NTBC exerts a similar inhibitory effect on the growth of other fungal pathogens, such as *Aspergillus fumigatus* and *Candida* spp. (which have been shown to contain 4-HPPD), but fails to inhibit growth and differentiation of *S. cerevisiae* (which lacks 4-HPPD) (G. Goldman et al., unpublished results). Moreover, as mentioned before, NTBC has been shown to display a broad range of activity, interacting with 4-HPPD from many different organisms, including plants, bacteria, and mammals (5, 30).

Although additional studies employing animal models are still necessary to verify whether NTBC and/or NTBC derivatives will be able to eliminate *P. brasiliensis* yeast cells in vivo, the current results raise the perspective that this drug may be an alternative to the treatment of PCM and other fungus-related diseases. If this proves to be the case, the use of NTBC and/or NTBC derivatives shall represent an entirely new approach to treatment, since currently available antifungal agents, such as azoles or equinocandins, affect other fungus-specific biochemical pathways, i.e., ergosterol and β-1,3-glucans in the cell wall biosynthesis (33). The facts that NTBC has already been approved to be used in humans and that its side effects are at least partially known to physicians may foster its eventual use as a new agent for the treatment of fungus-related infections such as PCM. Moreover, the potential of NTBC as a lead to the development of new chemotherapeutic agents has just begun to be exploited, and, judging from the results obtained with the use of compound 8, which appears to be about 40 times more potent than NTBC itself, it is tempting to speculate that derivatives of this drug may develop into a new group of fungicides capable of combating fungal infections through an entirely new pharmacological strategy, the details of which are only beginning to be unraveled.

Taken together, our results provide information regarding the process of gene expression modulation that takes place during the mycelium-to-yeast transition in *P. brasiliensis*. Many of these transcriptional alterations seem to involve genes and pathways that play key roles in the process of cellular differentiation in other fungi, and many of them have been identified as virulence related. Furthermore, since the onset of pathogenicity in dimorphic fungi is directly associated with differentiation into the yeast form, the microarray hybridization analyses also provided information regarding new potential targets to combat *P. brasiliensis* infection. The experiments employing NTBC and its derivatives as new chemotherapeutic agents against PCM confirm the potential of array-based approaches to identify new targets for the development of alternative treatments against pathogenic microorganisms.

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