The Transcriptional Response of Cryptococcus neoformans to Ingestion by Acanthamoeba castellanii and Macrophages Provides Insights into the Evolutionary Adaptation to the Mammalian Host

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Virulence of Cryptococcus neoformans for mammals, and in particular its intracellular style, was proposed to emerge from evolutionary pressures on its natural environment by protozoan predation, which promoted the selection of strategies that allow intracellular survival in macrophages. In fact, Acanthamoeba castellanii ingests yeast cells, which then can replicate intracellularly. In addition, most fungal factors needed to establish infection in the mammalian host are also important for survival within the amoeba. To better understand the origin of C. neoformans virulence, we compared the transcriptional profile of yeast cells internalized by amoebae and murine macrophages after 6 h of infection. Our results showed 656 and 293 genes whose expression changed at least 2-fold in response to the intracellular environments of amoebae and macrophages, respectively. Among the genes that were found in both groups, we focused on open reading frame (ORF) CNAG_05662, which was potentially related to sugar transport but had no determined biological function. To characterize its function, we constructed a mutant strain and evaluated its ability to grow on various carbon sources. The results showed that this gene, named PTP1 (polyol transporter protein 1), is involved in the transport of 5- and 6-carbon polyols such as mannitol and sorbitol, but its presence or absence had no effect on cryptococcal virulence for mice or moth larvae. Overall, these results are consistent with the hypothesis that the capacity for mammalian virulence originated from fungus/protozoan interactions in the environment and provide a better understanding of how C. neoformans adapts to the mammalian host.

Cryptococcus neoformans is an opportunistic pathogen often found in soils contaminated with bird excreta (1). Infection by C. neoformans, which occurs through inhalation of propagules from the environment by the host, seems to be accidental since C. neoformans is a saprophytic fungus that does not require an animal host for replication and survival (2). In the case of human exposure to C. neoformans, the establishment of infection and subsequent development of cryptococcosis depend both on the host’s immune response and the virulence of the fungus (3). One of the first lines of defense in the lung are alveolar macrophages, which are able to phagocytose C. neoformans efficiently (4). Phagocytosis is followed by phagosome acidification and fusion of lysosomes (5). However, this process does not always result in the death of yeast cells and C. neoformans can survive and replicate within macrophages in vivo, as shown by Feldmesser et al. (6).

The ability of C. neoformans to survive in the intracellular environment of phagocytes might have a critical role in disease progression and probably contributes to the propensity of the fungus to cause chronic and latent infections (3, 7, 8). However, it is known that the intracellular microenvironment of phagocytes is extremely inhospitable to internalized microorganisms due to nutritional and oxidative stress and exposure to antimicrobial peptides and hydrolytic enzymes (9, 10, 11). To survive the harsh environment of the phagosome, C. neoformans is able to reprogram its gene expression profile. Twenty-four hours after phagocytosis by murine macrophages, C. neoformans responds to carbon starvation by upregulating genes that encode sugar transporters and proteins involved in the utilization of alternative carbon sources, including enzymes of the glyoxylate cycle and fatty acid metabolism. Genes related to the oxidative stress response were also induced (4).

Since C. neoformans is a free-living fungus, its mammalian intracellular lifestyle is particularly curious, given that this organism has no obvious requirement for animal virulence in its life cycle. In this sense, the evolutionary origin and the maintenance of virulence strategies that allow for survival of C. neoformans within macrophages have been an issue of interest, and the investigation of the C. neoformans ecological niche may be informative in this regard. Steenbergen et al. (12) suggested that the competence of C. neoformans to proliferate within mammalian phagocytic cells was initially selected to confer an advantage against environmental predators. This idea was supported by the fact that interaction of C. neoformans with the amoeba Acanthamoeba castellanii results in the ingestion of the fungus followed by its intracellular replication and the accumulation of vesicles containing polysaccharide in the cytoplasm of amoeboid cells (12). This result is similar to those previously observed in the interaction of C. neoformans with macrophages. Furthermore, mutant strains defective in capsule
and phospholipase production, two important virulence factors of *C. neoformans*, are unable to survive within *A. castellanii* (12), indicating that virulence factors known to be required for the persistence of the fungus in animals can also be required for persistence in environmental predators (13, 14). Additional supportive data for this hypothesis came from the interaction of *C. neoformans* with the free-living slime mold *Dicyostelium discoideum*, which significantly increases the virulence of the fungus in a murine model of infection (15). Thus, it has been postulated that the intracellular survival strategy used by *C. neoformans* in mammalian macrophages is a consequence of evolutionary selection for fungus survival within soil protozoa.

We hypothesize that this adaptive convergence is a result of the similarity between the intracellular environments of macrophages and amoebae. Consequently, we have compared the transcriptional profiles of *C. neoformans* after 6 h of interaction with *A. castellanii* or murine macrophages. Our results suggest a conserved metabolic response of *C. neoformans* to the microenvironments of both cells.

**MATERIALS AND METHODS**

**Cell lines and media.** The H99 strain of *C. neoformans* var. *grubii* (serotype A) was used as the wild-type strain for microarray experiments. The *C. neoformans* serotype A strain KN99a was used to generate the *ptp1Δ* mutant and the *ptp1Δ:ptp1* complemented strains for the CNAG_05662 open reading frame (ORF). We also used the D1307 mutant strain deleted for the *C. neoformans* mutant library (16) purchased from ATCC. All strains were maintained at −80°C. Cultures were initiated by streaking YPD agar plates (containing 1% yeast extract, 2% Bacto peptone, and 2% dextrose [pH 6.8]) and incubating them for 2 days at 30°C, followed by seeding a single colony in YPD liquid medium and incubating it at 30°C overnight with agitation. The murine macrophage line J774.A.1 was maintained at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (11965; GibcoBRL) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 10% NCTC-109 medium (GibcoBRL; 21340) and 1% nonessential amino acids (GibcoBRL; 11140). The *Acanthamoeba castellanii* 30234 strain (ATCC) was maintained in the dark at 28°C on PYG medium (2% peptone, 0.1% yeast extract, 1.8% glucose [pH 7.2]).

**Phagocytosis assay.** Approximately 1 × 10⁵ cells of the J774.A.1 macrophage line or 2 × 10⁵ cells of *A. castellanii* were added to each well of 96-well tissue culture plates with 200 μl/well of DMEM or PYG medium, respectively. Amoebae and macrophages were cultured for 18 h at 28°C and 37°C in 5% CO₂, respectively. Subsequently, the culture supernatant was removed, the remaining cells were washed with sterile 1× phosphate-buffered saline (PBS), and 1 × 10⁶ yeast cells of *C. neoformans* were added to each well. In both cases, the effector-to-target ratio was about 5:1. For macrophage infections, the monoclonal antibody (MAb) 18B7 was included at 10 μg/ml as an opsonin. The cocultures of *C. neoformans* with amoebae or macrophages were then incubated for 15 min, 30 min, 1 h, 2 h, 4 h, or 6 h at 28 or 37°C, respectively, in 5% CO₂. At each time, the culture supernatant was discarded and wells were washed, fixed with cold methanol, and stained with Giemsa. Using optical microscopy, 100 to 200 cells of amoebae or macrophages were counted per well to determine the percentage of phagocytosis at each interval postinfection. The experiments were performed in quadruplicate. GraphPad Prism 5.0 (GraphPad Software) was used for statistical analyses. The paired two-tailed Student’s *t* test was used, and a *P* value of ≤0.05 was considered significant. In addition, multiple group comparisons were conducted by one-way analysis of variance (ANOVA) followed by the Bonferroni’s correction, as appropriate.

**RNA preparation.** For the microarray analysis, *C. neoformans* yeast cells were grown in YPD for 24 h at 30°C. The cells were harvested by centrifugation, washed twice with 1× PBS, and counted using a hemacytometer. Amoebae and macrophages were each grown in 175-cm² culture flasks (BD Molecular Biotechnology) with 75 ml of PYG medium or DMEM, respectively, until near confluence. H99 yeast cells were added to each flask at an approximate effector-to-target ratio of 5:1 (10⁶ yeast cells/flask). For macrophage infection, the MAB 18B7 was added at 10 μg/ml. Flask were incubated for 6 h at 37°C in 5% CO₂ (macrophages) or 28°C (amoebae). As controls, H99 cells were cultured *in vitro* in the same medium under the same temperature and incubation conditions as the macrophage and amoeba cocultures. At 6 h postinfection, extracellular *C. neoformans* cells and detached phagocytic cells were rinsed off with 1× PBS, and attached amoebae and macrophages were lysed by the addition of 25 mM deoxycholate. This treatment preserves fungal cells (data not shown). Yeast cells were recovered by centrifugation and used for total RNA extraction with TRIzol reagent (Invitrogen) following the manufacturer’s recommendations. To minimize contamination with genomic DNA, total RNA was further purified with the RNeasy kit (Qiagen). The concentration and purity of total RNA were assessed by measuring the 260/280-nm absorbance ratio, and its quality was determined by an Agilent 2100 bioanalyzer (Agilent Technologies) according to the manufacturer’s recommendations.

**Microarray.** The microarray assay was performed by the Genome Technology Access Center (GTAC) at Washington University in St. Louis (https://gtac.wustl.edu). First-strand cDNA was generated by oligo(dT)-primed reverse transcription (Superscript II; Invitrogen) utilizing the 3DNA Array 350 kit (Genisphere). Modified oligo-dT primers were utilized in which a fluorophore/dendrimer-specific oligonucleotide sequence was attached to the 5’ end of the dT primer. For cDNA synthesis, 1 μl of fluorophore-specific oligo(dT) primer was added to 8 μg of total RNA and the solution was incubated at 80°C for 10 min and then cooled on ice for 2 min. To each sample were added RNase inhibitor (Superase-In; Ambion) (1 μl), 5 × first-strand buffer (4 μl), deoxynucleoside triphosphate (dNTP) mix (10 mM each dATP, dCTP, dGTP, and dTTP) (1 μl), 0.1 M dithiothreitol (DTT) (2 μl), and Superscript II RNase H− reverse transcriptase (1 μl). Reverse transcription was carried out at 42°C for 2 h. The reaction was terminated by adding 0.5 M NaOH–50 mM EDTA (3.5 μl) and incubation at 65°C for 15 min and then neutralized with 1 M Tris-HCl (pH 7.5) (5 μl). For RNA expression-level comparison, samples were paired and concentrated using Microcon YM30 microconcentrators (Millipore) according to the manufacturer’s protocol. Each sample pair (~20 μl) was suspended in formamide-based hybridization buffer (vial 7; Genisphere) (26 μl), Array 50T blocker (Genisphere) (2 μl), and RNase/DNase-free water (4 μl). Two hybridizations were carried out in a sequential manner. The primary hybridization was performed by adding 48 μl of sample to the microarray under a supported glass coverslip (Erie Scientific) at 43°C for 16 to 20 h at high humidity. Prior to the secondary hybridization, slides were gently submerged into 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)−0.2% SDS (at 43°C) for 11 min, transferred to 2× SSC (at room temperature) for 11 min, transferred to 0.2× SSC (at room temperature) for 11 min, and then spun dry by centrifugation. Secondary hybridization was carried out using the complementary capture reagents provided in the 3DNA Array 350 kit (Genisphere). For each reaction, the following were added: 3DNA capture reagent with Cy3 (2.5 μl), 3DNA capture reagent with Cy5 (2.5 μl), SDS-based hybridization buffer (vial 6; Genisphere) (26 μl), and RNase/DNase-free water (21 μl). The secondary hybridization solution was incubated in the dark at 80°C for 10 min followed by 50°C for 15 min. Hybridization was performed by adding 48 μl secondary hybridization solution to the slide under a supported glass coverslip at 65°C for 3 h at high humidity in the dark. At hybridization termination, arrays were gently submerged into 2× SSC−0.2% SDS (at 65°C) for 11 min, transferred to 2× SSC (at room temperature) for 11 min, transferred to 0.2× SSC (at room temperature) for 11 min, and then spun dry by centrifugation. To prevent fluorophore degradation, the arrays were treated with Dyesaver (Genisphere). For data analysis, slides were scanned on an Axon 4000B scanner (Molecular Devices) to detect Cy3 and Cy5 fluorescence. Laser
power was kept constant for Cy3/Cy5 scans, and the photomultiplier tube (PMT) setting was varied for each experiment based on optimal signal intensity with the lowest possible background fluorescence. A low-PMT setting scan was also performed to recover signals from saturated elements. Gridding and analysis of images were performed using Genepix v6.1 (Molecular Devices). After background subtraction, median values were imported into the Partek genomics statistical analysis software. Values were log2 transformed, normalized by quantile, and readings lower than 10 were corrected to 10. For fold change and statistical validation of differences, we used two-way ANOVA, taking into account treatment and batch effects. Genes were considered differentially expressed if they presented a fold change equal to or higher than 2 and a P value of ≤0.05.

**qRT-PCR.** To validate the microarray data generated by the methodology, quantitative real-time PCR (qRT-PCR) was performed using the same RNA samples used for microarray experiments. For first-strand cDNA synthesis, equal amounts of total RNA (1 μg) were reverse transcribed (Superscript II; Invitrogen) using oligo(dT) 12–18 as primer. This cDNA was then used as the template in a qRT-PCR using SYBR green PCR reagents (Applied Biosystems) according to the manufacturer’s recommendations. The amplification assays were performed on the ABI PRISM 7900HT sequence detection system (Applied Biosystems) in 8-μl reaction mixtures containing 0.5 μl of each primer (10 μM each), 4 μl of SYBR green PCR reagents (2×) (Applied Biosystems), and 0.2 μl of cDNA. After initial denaturation at 95°C for 10 min, amplifications were performed for 40 cycles at 95°C for 15 s, 58°C for 15 s, and 72°C for 20 s. To confirm the specificity of amplification, melting curves of PCR products were analyzed. The method applied in the analysis of the data from qRT-PCR was the threshold cycle (CT) method, described by Livak and Schmittgen (17). The results were normalized to the expression of the housekeeping gene ACT1, which encodes the protein actin from *C. neoformans*. The experiment was performed in triplicate for all genes analyzed. All primers used are listed in Table 1.

**Generation of ptp1Δ mutant strain.** Targeted deletion of the *PTP1* gene was accomplished following the double-joint PCR strategy of Kim et al. (18). Briefly, we used PCR to fuse 5'- and 3'-flanking regions of the *PTP1* ORF to overlapping, incomplete segments of an expression cassette for the hygromycin phosphotransferase (HYG) gene under the control of the *C. neoformans* β-actin gene promoter. The two amplicons obtained were then simultaneously transformed in equimolar quantities into the whole wild-type locus plus flanking regions and the plasmid pJAF1 containing the G418 (NEO) resistance cassette. G418+ strains in which the HYG cassette had been expelled by the recombination event that restored the *PTP1* gene were selected by their inability to grow on hygromycin.

**Phenotype microarray.** Phenotype microarray experiments (Biologic, Hayward, CA) were done as described previously by Nielsen et al. (20). Briefly, after overnight growth in YPD liquid medium, cells were collected by centrifugation, washed with sterile distilled H2O, and resuspended at 107 cells/ml in 12% yeast nitrogen base liquid medium supplemented with histidine, leucine, and uracil. IFY-0 supplemented with 5 mg/ml ammonium sulfate, 0.85 mg/ml potassium phosphate monobasic, 0.15 mg/ml potassium phosphate dibasic, and 0.5 mg/ml magnesium sulfate was the inoculating fluid for the PM1 and PM2 phenotype microarray plates used (http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf). Cells were combined with supplemented IFY-0 at a ratio of 1:11, and 100 μl of this mixture was added to each well. All phenotype microarrays were incubated at 30°C, and the growth rate of each strain was checked by measuring the change in optical density at 492 nm (OD492) over the course of 72 h. The phenotype microarray results were confirmed by growing the *C. neoformans* *KN99a*, *ptp1Δ*, and *ptp1Δ*:ptp1 strains in minimal medium (10 mM MgSO4, 29.3 mM KH2PO4, 13 mM glycine, 3 mM thiamine [pH 5.5]) with 1% glucose or mannitol as the only carbon source. *C. neoformans* *D1307* mutant and *H99* wild-type strains were compared for the growth rate in minimal medium with 1% of the chosen carbon source, 10 mM MgSO4, 29.3 mM KH2PO4, 13 mM glycine, and 3 mM thiamine (pH 5.5). The sole carbon sources used were glucose, mannose, galactose, xylose, mannitol, sorbitol, dulcitol, ribitol, arabitol, and maltitol. Cultures were incubated for 48 h at 30°C, and growth was checked by measuring the change in optical density at 630 nm every 12 h.

**Evaluation of virulence factors expression in vitro.** We used 24-h exponential-phase cultures of the fungus growing in liquid YPD at 30°C under moderate (100-rpm) rotation. Resistance to different stressors was tested by serial, five-step 10-fold dilutions of the fungus, starting from 5 × 106 cells per agar spot. To evaluate thermostolerance, the growth rates of wild-type and mutant strains at 37°C were compared as follows. To test for resistance to cell wall-destabilizing agents, the fungus was grown on solid YPD containing Congo red (1% [mass/vol]) or calciluoc white (250 μg/ml) at 37°C or SDS (0.1% [mass/vol]) at 30°C. For resistance to salt stress, the fungus was grown on 1.5 M NaCl in solid YPD at 30°C. The ability to produce phospholipase and urease was tested by spotting 10-μl suspensions containing 104 cells onto solid YPD containing 8% (vol/vol) egg yolk (21) and Christensen’s urea agar (0.1% peptone, 0.5% NaCl, 0.2% KH2PO4, 0.1% glucose, 2% urea, 0.016% phenol red), respectively. To evaluate melanin production, *C. neoformans* strains were grown in chemically defined minimal medium (15 mM dextrose, 10 mM MgSO4, 29.4 mM KH2PO4, 13 mM glycine, 3 μM thiamine [pH 5.5]) with 1 mM l-DOPA (l-3,4-dihydroxyphenylalanine) (Sigma-Aldrich) and incubated at 30°C in the dark.

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**TABLE 1 Primers for *C. neoformans* genes used in real-time PCR experiments**

| Primer | Gene product | Sequence (5’→3’) |
|--------|--------------|-----------------|
| Act F  | Actin        | TTGTCCTCCTCCCAATCTTGCAG |
| Act R  | Actin        | CGGCGACATTTCTTCCGATA |
| Pip1 F | Polyl transporter protein 1 | TACCGTTCCCCATCTTCTCTG |
| Pip1 R | Polyl transporter protein 1 | ATACCGAAGCAGCACCACAGG |
| Cat3 F | Catalase 3   | GTAAAACCAAGCCGCAATA |
| Cat3 R | Catalase 3   | CTGATACCTTGGACCCCAGAA |
| Ths F  | Trehalose synthase | GCCCAAAAGAGTGGGATGAC |
| Ths R  | Trehalose synthase | AGAGCTCTCATGGACCCAGGA |
| Plc F  | Phospholipase C | GTCTTTGTGTCATGCGGAGCTT |
| Plc R  | Phospholipase C | TAGCAGGAGGATGAACTG |
| Gbe F  | 1,4-α-Glucan-branching enzyme | ATCCCTGTGCGAACCTTTT |
| Gbe R  | 1,4-α-Glucan-branching enzyme | CTTTGAGAATTTCGCGGAGAC |
| Aox F  | Alternative oxidase | CCCATTACACCCGGAAGGGA |
| Aox R  | Alternative oxidase | AGGAACCTTGAGGTCCTGGT |
| Erg3 F | C-5 sterol desaturase | CCCAGTCCTCTCTACCAT |
| Erg3 R | C-5 sterol desaturase | TCATACACGCGTGGATGT |
| Erg1 F | Lanosterol 14 α-demethylase | TCCATCCATCAGCATTCACC |
| Erg1 R | Lanosterol 14 α-demethylase | CCCCCTTGGAATGATGTTATG |
| Mls F  | Malate synthase | AAACATGGTTCTCGGACAG |
| Mls R  | Malate synthase | AGGTGCAGCAGAAGAACAG |

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Capsule size measurements. C. neoformans wild-type and mutant strains were grown for 24 h in YPD medium at 30°C, collected by centrifugation, washed twice with 1× PBS, and cultured for 48 h in minimal medium (10 mM MgSO₄, 29.3 mM KH₂PO₄, 13 mM glycine, 3 mM thiamine [pH 5.5]) with 1% glucose or mannitol as only carbon source. Cultures of C. neoformans were diluted in 1× PBS, and an aliquot of each sample was mixed with a drop of India ink (BD Biosciences, NJ). Inspection was performed in an Axiovert 200 M inverted microscope (Carl Zeiss Micro Imaging, NY). Capsule size was measured with ImageJ 1.39g software (National Institutes of Health, Bethesda, MD) as the distance between the capsule border and the cell wall, corresponding to the India ink exclusion zone. The size of the cell body was also measured. At least 150 yeast cells were evaluated for each experimental condition.

Measurement of mannitol secretion by C. neoformans cells. C. neoformans cells were inoculated in YPD medium and grown overnight at 30°C. On the following day, the cells were washed 3 times and inoculated at 1.8 × 10⁵ cells/ml in 10 mM potassium phosphate buffer containing glucose at 100 mM and incubated at 30°C with agitation for 3 h, as described before (22). Samples were collected at 0, 1, and 3 h, centrifuged, filtered through a 0.2-μm membrane to remove cells, and analyzed for mannitol in a Shimadzu high-performance liquid chromatography (HPLC) system (Kyoto, Japan) equipped with a refractive index detector. The analysis was performed in isocratic mode at 85°C using a Bio-Rad Aminex HPX-87P column and Milli-Q water as the mobile phase at a flow rate of 0.6 ml/min. A 4-level calibration standard curve was created for commercial glucose and mannitol. The amounts of mannitol and glucose in each sample were calculated using the LCsolution software (Shimadzu, Kyoto, Japan).

Mouse virulence studies. Groups of 6- to 8-week-old female BALB/c mice (10 mice per group) were obtained from the National Cancer Institute. Intratracheal infection was performed as previously described by Feldmesser and Casadevall (23). Briefly, strains of C. neoformans were cultured in YPD liquid medium (24 h, 30°C, 150 rpm), collected by centrifugation (1,800 × g for 10 min), washed, and resuspended in 1× PBS to a final cell concentration of 2 × 10⁸ cells/ml. Mice were intraperitoneally anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Fifty microliters of the fungal suspension was slowly injected into the previously exposed trachea. The number of live mice was monitored daily. The experiment was repeated twice. The data presented here are from a representative experiment. All animal experiments were previously approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Quantitative analysis of tissue fungal burden. Groups of 4- to 6-week-old female BALB/c mice (five mice per strain) infected intratracheally with C. neoformans strains were sacrificed by CO₂ inhalation at 15 days postinfection. The lungs and brains were removed, weighed, and homogenized in 5 ml sterile 1× PBS. Serial dilutions of the organ samples were plated on Sabouraud agar plates and incubated at 30°C for 48 h. Colony counts were performed and adjusted to reflect the total number of CFU/g tissue.

Galleria mellonella survival experiments. Galleria mellonella infection was performed as previously described by García-Rodas et al. (24). Briefly, G. mellonella larvae (Vanderhorst Wholesale, Inc., St. Marys, OH, and Mous Livebait R.J., The Netherlands) were inoculated with 10 μl of a 10⁶ cells/ml yeast suspension by the last left pro-leg using a sterile 26-gauge needle-fitted Hamilton syringe. Infected larvae were incubated at 25 or 37°C, and the number of dead caterpillars was scored daily. A group of G. mellonella larvae was inoculated with 1× PBS as a control for physical injury; another group without any manipulation was set as an untreated control. This experiment was repeated twice. The data presented here are from a representative experiment.

Statistical analyses. GraphPad Prism 5.0 (GraphPad Software) was used for statistical analyses. The paired two-tailed Student’s t test was performed for phenotype microarray, capsule size measurement, and CFU experiments. The log rank test was performed to evaluate statistical differences on survival curves. A P value of ≤0.05 was considered significant.

Microarray data accession number. The microarray data generated by this study have been deposited into the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE45027.

RESULTS
Kinetics of C. neoformans phagocytosis by amoebae and murine macrophages. We carried out an analysis of the kinetics of C. neoformans internalization by the amoeba A. castellanii and murine macrophages to ascertain the optimal time intervals for carrying out experiments. After 4 h of coculture with macrophages, more than 90% contained yeast cells (Fig. 1C). This percentage decreased after 6 h, possibly due to exocytosis of the fungus, which can occur with or without lysis of the host cell (25, 26). The percentage of phagocytosis of C. neoformans by amoebae, however, increased progressively throughout the period evaluated. After 6 h, about 30% of amoebae contained yeasts (Fig. 1C). Encapsulated C. neoformans cells are known to resist ingestion by amoeba (27), and given the low efficiency of phagocytosis by A. castellanii in vitro, 6 h was chosen as the time point for gene expression profiling of C. neoformans recovered from phagocytic cells. Figures 1A and B are representative photomicrographs of macrophages and amoebae, respectively, after 6 h of infection with C. neoformans.

Transcriptional profile of C. neoformans inside amoebae and macrophages. The microarray was used to compare the transcriptional profiles of C. neoformans cells upon interaction with A. castellanii and murine macrophages. Total RNA was extracted from fungal cells either internalized by phagocytes or grown in the corresponding culture medium (control) for 6 h. All RNA prepa-
rations satisfied the criteria of integrity, and no contamination with host cell RNA was detected (data not shown).

Analysis of the differential gene expression of *C. neoformans* during *A. castellanii* infection indicated modulation of 656 genes from a total of 7,775 genes represented in the microarray assay. (For a complete list, see the supplemental material.) Of these, 322 genes were upregulated more than 2-fold, including genes encoding proteins related to nutrient transport, general metabolism, and oxidative stress response (Fig. 2A), while 334 genes were downregulated (Fig. 2B). Among the latter were genes encoding proteins involved in transcription, translation, and ergosterol biosynthesis. Additionally, genes encoding well-established *C. neoformans* virulence factors for mammalian hosts were also modulated during amoeba infection.

A similar analysis was performed to evaluate the transcriptional profile of *C. neoformans* interacting with murine macrophages for 6 h. The results showed a total of 293 genes whose expression was modulated more than 2-fold in response to the macrophage microenvironment (for a complete list see the supplemental material). The 222 genes of *C. neoformans* with increased expression included those encoding transporters, chaperones, and proteins involved in the oxidative stress response and metabolism (Fig. 2C). Genes related to ergosterol biosynthesis and oxidative phosphorylation were suppressed, as shown in Fig. 2D.

To validate the data obtained by the microarray assay, qRT-PCR analysis was performed for some *C. neoformans* genes previously identified in our microarray studies to be modulated in response to the phagocytes’ intracellular niches. To correct for differences in RNA quantity among the samples, the gene encoding the actin protein was used as a control. The expression profiles for all genes analyzed by qRT-PCR matched the microarray results with respect to direction (up or down) of modulation (Fig. 3).
A comparative analysis of transcriptional changes in amoebae and macrophages showed that 111 genes were similarly modulated in response to both intracellular environments. This represents 38% of all genes differentially expressed by *C. neoformans* during macrophage interaction. Of these genes, 63.1% were induced, while 20.7% were repressed in both intracellular niches. Among the genes whose expression was increased in both macrophages and amoebae, those involved in nutrient transport, oxidative stress response, and general metabolism stand out. In fact, the metabolic responses of *C. neoformans* to interaction with macrophages and amoebae were very similar. Overall, our results indicate a metabolic switch from glycolysis to gluconeogenesis and fatty acid degradation for conversion to glucose via the glyoxylate cycle (Fig. 4). Conversely, genes related to ergosterol biosynthesis were suppressed after internalization of *C. neoformans* by both phagocytic cells (Table 2).

Although the majority of yeast genes (83.8%) commonly expressed during interaction with both phagocytes were similarly modulated, suggesting that the intracellular environments of amoeba and macrophages are quite comparable, 16.2% of the identified genes were differently modulated (Table 3). Most of them encode hypothetical proteins (39%), transporters (22.2%), and proteins related to metabolism (11.1%), suggesting differences in nutrient availability within phagosomes of macrophages and amoebae.

**Functional study of the *C. neoformans* ORF CNAG_05662.**

The comparison of *C. neoformans* transcriptomes upon internalization by amoeba and macrophages allowed the identification of genes potentially related to its survival inside host cells. The presumed importance of these genes in *C. neoformans* adaptation to the harsh phagosome environment translates into a potential role in virulence. Based on the expression levels of *C. neoformans* genes during interaction with host cells, some were selected for future assessment of their relevance in the establishment of cryptococcosis. Among them, the ORF CNAG_05662, which codes for a sugar transport motif (http://motif.genome.jp/) of 631 amino acids, was particularly interesting since it was strongly induced, by 17.4- and 16.8-fold in amoebae and macrophages, respectively (Table 2). Additionally, the quantitative analysis by qRT-PCR revealed induction levels on the order of 250- and 150-fold upon internalization by amoebae and macrophages, respectively (Fig. 3).

To characterize the function of the ORF CNAG_05662 and investigate its role in the virulence of the fungus, both a strain in which this gene has been mutated (ptp1Δ/H9004) and a strain in which this gene has been complemented (ptp1Δ::ptp1) were generated. Southern blotting was performed to confirm a sole insertion event in the targeted locus, and qRT-PCR was performed to confirm that the mutant strain did not express the CNAG_05662 gene, while the complemented strain did (data not shown).

Since the predicted function for the product of CNAG_05662 was related to sugar transport, a phenotype microarray was performed to compare the growth of wild-type (WT), mutant (Mut), and complemented (Rec) strains after 72 h of culture in various carbon sources. Using the PM1 and -2 assays (Biolog, Hayward, CA), we observed that the mutant strain was not able to grow when the carbon source was in the form of polyols, such as D-mannitol, D-sorbitol, dulcitol, arabitol, and adonitol (Fig. 5). This suggested that CNAG_05662 coded for a protein related to the transport of 5- and 6-carbon polyols. In contrast, the fungus was able to grow when erythritol and maltitol were used as carbon sources, suggesting that this gene is not involved in the transport of molecules of 4 and 12 carbons, respectively (Fig. 5). The microarray phenotype was validated assessing the growth curve of the wild-type (WT), mutant (Mut), and complemented (Rec) strains using glucose or mannitol as the carbon source (see Fig. S1 in the supplemental material). Alternatively, we compared the results for the mutant constructed in this work with those for a strain (D1307) obtained from a mutant library constructed by Liu et al.
### TABLE 2  
*C. neoformans* genes with similar modulation patterns after interaction of the fungus with amoebae and murine macrophages

| Category                          | Accession no.   | Predicted function                        | Fold change in: |
|-----------------------------------|-----------------|-------------------------------------------|-----------------|
|                                   |                 |                                           | Amoebae         | Macrophages    |
| Ergosterol biosynthesis           | CNAG_04687      | Stearoyl-CoA 9-desaturase                 | $-2.82949$      | $-2.5887$      |
|                                   | CNAG_01737      | C-4 methyl sterol oxidase                 | $-5.10666$      | $-2.1578$      |
| Oxidative phosphorylation         | CNAG_05626      | h-sco1                                    | $-3.39215$      | $-2.02884$     |
| Glyoxylate cycle                  | CNAG_05303      | Isocitrate lyase                          | $3.32005$       | $12.9474$      |
|                                   | CNAG_05653      | Malate synthase                           | $2.09609$       | $2.62303$      |
| Oxidative stress                  | CNAG_02147      | Cytochrome c peroxidase                   | $3.59838$       | $5.1284$       |
|                                   | CNAG_01846      | Flavoprotein                              | $3.11961$       | $2.0610$       |
|                                   | CNAG_05169      | Cytochrome $b_2$                          | $2.68905$       | $2.7162$       |
| Transcription                     | CNAG_04345      | RNA polymerase II transcription factor    | $2.59053$       | $2.40314$      |
| Transport                         | CNAG_07869      | Sugar transporter                         | $24.5501$       | $5.20518$      |
|                                   | CNAG_05662      | Sugar transporter                         | $17.4444$       | $16.8462$      |
|                                   | CNAG_01384      | bodown198                                 | $8.88579$       | $4.86666$      |
|                                   | CNAG_01936      | Sugar transporter                         | $6.84072$       | $7.29332$      |
|                                   | CNAG_03772      | Glucose transporter                       | $6.70411$       | $11.1359$      |
|                                   | CNAG_03910      | D-Xylose-proton symporter                 | $6.64142$       | $2.84406$      |
|                                   | CNAG_03060      | Multidrug resistance protein              | $6.03422$       | $2.11251$      |
|                                   | CNAG_01925      | Conserved hypothetical protein            | $4.10947$       | $2.45472$      |
|                                   | CNAG_05685      | Neutral amino acid transporter            | $3.44863$       | $2.77709$      |
|                                   | CNAG_05929      | MFS maltose permease MalP                 | $2.95817$       | $3.30233$      |
|                                   | CNAG_02288      | Succinate:fumarate antipporter            | $2.68543$       | $4.96111$      |
|                                   | CNAG_07367      | Amino acid transporter                    | $2.42433$       | $3.12233$      |
|                                   | CNAG_04795      | Adenine nucleotide transporter            | $2.26578$       | $2.83099$      |
|                                   | CNAG_07387      | Siderophore-iron transporter Str3          | $-2.04064$      | $-2.56502$     |
|                                   | CNAG_03438      | Hexose transporter                        | $-3.94741$      | $-2.56777$     |
| Pentose phosphate pathway         | CNAG_00827      | Ribose 5-phosphate isomerase              | $3.00641$       | $7.41447$      |
| Amino acid metabolism             | CNAG_03128      | Lincomycin-condensing protein lmbA        | $2.27524$       | $2.19625$      |
| Carbohydrate metabolism           | CNAG_04621      | Glycogen synthase                         | $5.22439$       | $2.17416$      |
|                                   | CNAG_03067      | Pyruvate carboxyltransferase              | $3.79568$       | $2.66549$      |
|                                   | CNAG_00393      | 1.4-α-Glucan-branching enzyme             | $3.33816$       | $2.70596$      |
|                                   | CNAG_04659      | Pyruvate decarboxylase                    | $-3.97919$      | $-2.58975$     |
| Lipid metabolism                  | CNAG_03019      | Long-chain-fatty-acid-CoA ligase          | $5.87013$       | $2.70475$      |
|                                   | CNAG_00641      | Acyl-CoA oxidase                          | $5.64239$       | $6.20667$      |
|                                   | CNAG_00490      | Acetyl-CoA C-acyltransferase              | $5.09895$       | $5.5079$       |
|                                   | CNAG_03666      | Acyl-CoA dehydrogenase                    | $5.08676$       | $5.62055$      |
|                                   | CNAG_06551      | Carnitine O-acetyltransferase             | $4.5781$        | $5.56428$      |
|                                   | CNAG_07747      | Acyl-CoA oxidase I                        | $4.54934$       | $2.41408$      |
|                                   | CNAG_02562      | Acyl-CoA dehydrogenase                    | $4.19328$       | $2.79301$      |
|                                   | CNAG_02045      | Acetoacyl-CoA synthase                    | $3.65055$       | $2.95075$      |
|                                   | CNAG_03593      | Acyl-CoA thiolesterase                    | $3.56595$       | $2.11571$      |
|                                   | CNAG_05721      | Peroxisomal hydratase-dehydrogenase-epimerase | $3.16504$   | $3.29229$      |
|                                   | CNAG_01116      | β-Ketoacyl reductase                      | $3.06954$       | $2.80628$      |
|                                   | CNAG_04392      | Sterol-binding protein                    | $2.83953$       | $3.82553$      |
|                                   | CNAG_00537      | Carnitine acetyltransferase               | $2.7752$        | $3.02984$      |
|                                   | CNAG_04238      | 2,4-Dienoyl-CoA reductase                 | $2.71898$       | $2.7285$       |
|                                   | CNAG_00371      | Enoyl-CoA hydratase                       | $2.34604$       | $3.14993$      |
|                                   | CNAG_04308      | Short-chain 3-hydroxyacyl-CoA dehydrogenase | $2.32894$   | $2.66174$      |
|                                   | CNAG_01671      | Acetylpropionyl CoA carboxylase           | $2.12004$       | $3.93367$      |
|                                   | CNAG_00499      | Carnitine/acil carnitine carrier          | $2.10977$       | $3.88855$      |
|                                   | CNAG_06628      | Aldehyde dehydrogenase                    | $2.74167$       | $6.49193$      |
|                                   | CNAG_02087      | Sphingosine N-acyltransferase             | $-7.58831$      | $-3.75872$     |

(Continued on following page)
in which the same ORF was deleted. The lack of expression of the gene in the D1307 mutant strain was confirmed by qRT-PCR (data not shown). Incubation of the D1307 strain in different carbon sources for 48 h revealed no growth when mannitol, sorbitol, dulcitol, ribitol, and arabitol were used as the sole carbon sources (see Fig. S2 in the supplemental material), confirming the function of the gene. Consequently, we have named the product of CNAG_05662 “polyol transporter protein 1” (Ptp1).

Next we assessed whether Ptp1 function was related to mannitol transport out of the cell by inducing and quantifying its secretion by the wild-type, mutant, and reconstituted strains in vitro. Our results showed no statistically significant difference in mannitol secretion levels among them (data not shown), suggesting that Ptp1 is only involved in the transport of polyols into the cell.

Role of the PTP1 gene in the virulence of C. neoformans. To assess whether expression of PTP1 is related to virulence in C. neoformans, we evaluated the ability of the mutant strain to express known virulence factors in vitro. Our results showed no differences in several phenotypes implicated in virulence, such as growth at 37°C, melanin production, and urease and phospho-

### TABLE 2 (Continued)

| Category                     | Accession no. | Predicted function                     | Fold change in: | Amoebae | Macrophages |
|------------------------------|---------------|----------------------------------------|-----------------|---------|-------------|
| Nitrogen metabolism          | CNAG_03243    | 2-Nitropropane dioxygenase              | 3.40319         | 3.0279  |             |
|                              | CNAG_05644    | 2-Nitropropane dioxygenase              | 2.16675         | 4.29356 |             |
| Secondary metabolism         | CNAG_06623    | myo-Inositol oxygenase                  | 2.52692         | 2.13304 |             |
| Redox processes              | CNAG_05299    | Oxidoreductase                          | 3.16418         | 3.5935  |             |
|                              | CNAG_00541    | Dimethylaniline monoxygenase            | −3.47134        | −2.62673|             |
|                              | CNAG_01393    | fmHP                                   | −5.14025        | −2.08856|             |
|                              | CNAG_02577    | Oxidoreductase                          | −5.3935         | −2.63942|             |
| Other                        | CNAG_01702    | Integral membrane protein               | 11.4333         | 3.68741 |             |
|                              | CNAG_05229    | Stomatin family protein                 | 5.47933         | 2.29192 |             |
|                              | CNAG_02295    | Phosphotransferase enzyme family domain-containing protein | 4.64925         | 2.60426 |             |
|                              | CNAG_07862    | Fumarate reductase                      | 3.93416         | 9.8097  |             |
|                              | CNAG_01252    | Thiosulphate sulphur transferase        | 3.08677         | 2.65488 |             |
|                              | CNAG_01794    | 2-Hydroxyacly dehydrogenase             | 2.68166         | 2.99315 |             |
|                              | CNAG_04096    | Racemase                                | 2.09515         | 2.30145 |             |
|                              | CNAG_02674    | dJ347H13.4                              | 2.05302         | 2.52509 |             |
|                              | CNAG_04103    | DUF895 domain membrane protein          | 2.02542         | 2.75068 |             |
|                              | CNAG_02264    | AFG1-family ATPase                      | −2.25762        | −2.26571|             |
|                              | CNAG_05370    | Integral membrane protein               | −4.69223        | −2.34957|             |
| Hypothetical protein         | CNAG_04837    |                                        | 7.15172         | 6.35904 |             |
|                              | CNAG_03394    |                                        | 4.22103         | 2.59755 |             |
|                              | CNAG_02405    |                                        | 3.51154         | 3.46188 |             |
|                              | CNAG_02978    |                                        | 3.17452         | 3.07828 |             |
|                              | CNAG_01847    |                                        | 3.06055         | 2.00546 |             |
|                              | CNAG_06583    |                                        | 2.76876         | 2.92177 |             |
|                              | CNAG_04394    |                                        | 2.75153         | 2.64509 |             |
|                              | CNAG_00079    |                                        | 2.74434         | 2.5312  |             |
|                              | CNAG_05784    |                                        | 2.67849         | 2.69336 |             |
|                              | CNAG_06355    |                                        | 2.65531         | 2.26161 |             |
|                              | CNAG_02044    |                                        | 2.3962          | 4.54104 |             |
|                              | CNAG_07708    |                                        | 2.33125         | 2.78776 |             |
|                              | CNAG_07912    |                                        | 2.01817         | 3.91411 |             |
|                              | CNAG_00315    |                                        | 2.00838         | 2.28288 |             |
|                              | CNAG_03587    |                                        | −2.04111        | −2.09241|             |
|                              | CNAG_00349    |                                        | −2.05716        | −2.90138|             |
|                              | CNAG_07568    |                                        | −2.19254        | −2.23732|             |
|                              | CNAG_02910    |                                        | −2.45926        | −2.39798|             |
|                              | CNAG_00814    |                                        | −4.9423         | −2.36478|             |
|                              | CNAG_07920    |                                        | −5.37886        | −3.4471 |             |
|                              | CNAG_04891    |                                        | −5.38601        | −2.13027|             |
|                              | CNAG_01803    |                                        | −5.56394        | −2.8793 |             |
|                              | CNAG_05741    |                                        | −5.82377        | −2.40385|             |
|                              | CNAG_01369    |                                        | −8.89767        | −2.03519|             |
|                              | CNAG_06590    |                                        | −11.3033        | −3.06825|             |

*Accession numbers of genes listed in the homepage of the Cryptococcus neoformans var. grubii genome assembled by the Broad Institute of MIT and Harvard (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans).*
lipase synthesis. The deletion of PTPI did not result in changes in capsule size when yeast cells were grown in glucose as the only carbon source. However, in contrast to the wild-type strain, the ptp1/H9004 mutant strain did not increase capsule size in response to mannitol (Fig. 6).

The role of Ptp1 in the virulence of C. neoformans in vivo was investigated comparing the pathogenic potentials of KN99α and ptp1Δ strains using mice and G. mellonella as infection models. The results suggest that these two strains are equivalent in their abilities to establish cryptococcosis in both standard models, since no difference in survival rates of mammalian and invertebrate hosts was observed (Fig. 7A to C). To confirm that KN99α and ptp1Δ strains cause disease in the same way, as suggested by the survival results, we checked the CFU numbers in the lungs and brains of mice at an intermediate time point in the infection (10 days). As shown in Fig. 7D and E, no differences were observed in lung and brain fungal burdens.

To assess the role of the PTPI gene C. neoformans, macrophages and amoebae were infected with both KN99α and ptp1Δ strains for 6 h, and the survival rate of internalized yeast cells was calculated by the number of fungal CFU obtained from lysed host cells. The results show that the ptp1Δ strain exhibited no significant difference in survival compared to the wild type (data not shown). Also, the abilities of macrophages and amoebae to ingest the KN99α and ptp1Δ strains were similar, as measured by the phagocytosis kinetic assay (data not shown).

### TABLE 3 C. neoformans genes with different modulation patterns after interaction of the fungus with amoeba and murine macrophages

| Category                      | Accession no. | Predicted function                             | Fold change in: |
|-------------------------------|---------------|-------------------------------------------------|-----------------|
|                               |               |                                                  | Amoebae         | Macrophages    |
| Amino acid metabolism         | CNAG_05676    | Tyrosine aminotransferase                       | −3.30706        | 3.31586        |
| Lipid metabolism              | CNAG_00984    | Glucose and ribitol dehydrogenase protein       | 3.2289          | −2.10481       |
| Redox processes               | CNAG_04085    | Oxidoreductase                                  | −4.76438        | 3.27837        |
|                               | CNAG_00876    | Ferric-chelate reductase                        | −2.39397        | 2.68318        |
| Translation                   | CNAG_03563    | Aspartate-tRNA ligase                           | 2.02203         | −2.59223       |
| Transport                     | CNAG_04758    | Ammonium transporter                            | 2.41737         | −2.39166       |
|                               | CNAG_04210    | Sugar transporter                               | −2.3879         | 2.78891        |
|                               | CNAG_03426    | GDP-mannose transporter                         | −2.94678        | 3.16533        |
|                               | CNAG_00895    | Zinc ion transporter                            | −4.53653        | 2.67154        |
| Other                         | CNAG_03759    | Conidiation-specific protein 6                  | 5.73768         | −4.86503       |
|                               | CNAG_06576    | Allergen                                        | 2.38555         | −2.92608       |
| Hypothetical protein          | CNAG_03068    |                                                  | 9.56139         | −2.24987       |
|                               | CNAG_02694    |                                                  | 4.11009         | −2.25959       |
|                               | CNAG_00848    |                                                  | 3.1508          | −2.19634       |
|                               | CNAG_02362    |                                                  | 2.74084         | −2.04115       |
|                               | CNAG_06396    |                                                  | 2.13514         | −2.57459       |
|                               | CNAG_01865    |                                                  | −2.36364        | 4.66234        |
|                               | CNAG_00813    |                                                  | −5.87379        | 4.67373        |

### FIG 5 Growth rate of KN99α (wild type [W/T]), mutant (Mut), and complemented (Rec) strains of C. neoformans in media with different polyols as sole carbon sources. Yeast cells were cultivated at 30°C for 72 h, and the growth rate was verified by monitoring for color change in the wells and by measuring the variation of optical density at 492 nm as described for the phenotype microarray assay. The t test was performed using GraphPad Prism 5. *, P < 0.05.
The capacity of the saprophytic fungus C. neoformans for mammalian pathogenesis has been suggested to arise by selection of traits that function in virulence through the interaction with soil predators (28), among which amoebae are of particular interest, since they share certain characteristics with mammalian phagocytic cells, such as macrophages. In fact, the amoeba have been shown to be predators of C. neoformans that influence its survival in soils (29, 30). Several studies have shown parallels in the interaction of C. neoformans with macrophages and amoebae (12, 27, 31). Although these reports support the environmental selection hypothesis, no study to date has focused on the molecular mechanisms central to the adaptation of C. neoformans to the intracellular environment of soil predators that may have allowed it to survive inside mammalian host cells.

If the amoeba hypothesis is correct, one might predict similar C. neoformans transcriptional profiles when it interacts with macrophages and protozoa. To test this prediction, we compared the transcriptional profiles of C. neoformans 6 h after phagocytosis by the amoeba A. castellanii and by murine macrophages. In planning our experimental design, we needed to take into account that the interactions between C. neoformans and amoeba and macrophages occur in very different environments. C. neoformans-amoeba interactions occur at ambient temperatures in soils, which are usually acidic. In contrast, C. neoformans-macrophage interactions occur at 37°C in near-neutral pHs. Thinking through our experimental options, we considered carrying out amoeba-cryptococcus interactions at higher temperatures and or macrophage-cryptococcus interactions at lower temperatures to harmonize the physical conditions but opted against such experiments because they would have represented a highly constrained comparison with little or no relevance to reality. Instead, we focused on comparing these interactions under conditions that approximated the natural interactions, fully aware that differences in temperature and media would introduce additional variables. However, by focusing only on the subset of gene responses induced or repressed by both amoeba and macrophage interactions relative to those outside, we avoided focusing on transcriptional differences resulting from different environmental conditions. We note that classic experiments such as those showing that bacteria use similar virulence genes for plants and animals also required infection of their hosts at different temperatures (32).

Our transcriptional analysis showed totals of 656 and 293 induced genes in C. neoformans ingested by amoebae and macrophages, respectively, whose expression was changed at least 2-fold relative to nonphagocytosed cells. These results allowed us to compare the adaptation of C. neoformans to the intracellular environment of the two hosts on a molecular level. It is interesting that the number of C. neoformans genes induced by the interaction with amoebae was more than two times greater than the number induced upon interaction with macrophages. Although the mechanism responsible for this difference is not understood, we note that an amoeba is a free-living organism that feeds by microbial predation and that it must encounter a very diverse microbiota in soils. Consequently, amoebae may have more versatile microbial killing and digesting mechanisms than macrophages, which would submit C. neoformans to greater stress, leading to a more extensive transcriptional response. Alternatively, the number of genes induced after the interaction with macrophages may be smaller than that observed with the amoeba because C. neoformans-macrophage experiments were carried out at 37°C and the higher temperature can itself induce stress on fungal cells. Despite these differences, the overall categorization of the protein-coding genes revealed a very similar gene expression profile of the fungus inside either phagocyte. We have analyzed the transcriptional response by focusing on gene functional categories.

Gene functional categories. (i) Genes related to the transport of ions and small molecules. Although little is known concerning the nutritional composition of the phagosome, it is assumed that this organelle is poor in carbon and amino acid sources (33, 34). This nutritional stress induces an adaptive response in intracellular pathogens to allow for survival within phagocytes (4, 9, 35, 36). For example, expression of genes encoding transporter proteins can be translated into more effective assimilation of available nutrients. In agreement with the notion that C. neoformans residence in the phagosome translates into a starvation state, our results show that about 10% of the genes modulated after internalization by amoebae and 13.7% of those genes modulated in response to the macrophage are related to nutrient transport. Fan et al. (4) also reported the transcriptional induction of various C. neoformans transporters after 2 and 24 h of cocultivation with activated macrophages. In addition, Hu et al. (37) identified a large amount of transporters potentially important for the growth of C. neoformans in the lungs of infected mice.

One of the most highly differentially expressed genes by C. neoformans in macrophages and amoebae was the ORF CNAG_05662, which was deduced to be a sugar transporter based on amino acid homology to known proteins. Functional experiments determined that this gene encoded a protein related to the transport of 5- and 6-carbon polyols, which was named PtP1 (polyol transporter protein 1). The evidence that the PtP1 gene product is involved with mannitol transport was particularly interesting since some studies have correlated the secretion of this polyl by C. neoformans with progression of pathogenesis. The production of 1,3-dmannitol by C. neoformans occurs both in vitro (38) and in vivo and contributes to the pathogenesis of meningococcal meningitis (39) and virulence in mice (40). PtP1 appeared to have a role only in the transport of mannitol into the fungal cell and was not involved in its secretion by C. neoformans. Disruption of the PtP1 gene had no effect on the expression of virulence factors by C. neoformans in vitro, such as melanin, urease, and phospholipase. The capsule size of the mutant strain was only affected during growth in mannitol as the sole carbon source.
Mannitol is a potent inducer of *C. neoformans* capsule (41), and as the mutant strain is unable to transport it into the cell, it does not respond to the induction mediated by this polyol. Furthermore, the virulence of the strain lacking PTP1 did not differ from that of the wild-type strain in vitro, in macrophages and amoeba, or in vivo, in mouse and *G. mellonella* models of infection. These results suggest that, despite its upregulation by *C. neoformans* inside phagocytic cells, disruption of this gene does not impair the establishment of fungal infection. The absence of an effect in virulence could be explained by the fact that 5- and 6-carbon polyols are unlikely to be sources of energy in vivo, since they have not been reported in animal tissues.

(ii) Genes related to *C. neoformans* metabolic adaptation to nutritional stress. Inside the phagosome, intracellular parasites adapt by modulating the expression of genes related to metabolic pathways. For example, the yeast *Candida albicans* modifies its metabolism to assimilate alternative carbon sources after phagocytosis by macrophages, which is reflected by the induction of the
gluconeogenesis, glyoxylate cycle, and β-oxidation pathways (35). Furthermore, *C. albicans* and *Paracoccidioides brasiliensis* show a strong reduction in the expression of genes involved in glycolysis in response to the glucose shortage inside the phagosome (35, 36). Similar patterns of expression were also observed for *C. neoformans* in response to the interaction with both host cells in this study. The switch from glycolysis to gluconeogenesis upon phagocytosis by amoebae and macrophages can be observed in the induction of the genes encoding the enzymes fructose-1,6-bisphosphatase (Fbp1) and phosphoenolpyruvate carboxykinase (Pck1), respectively. *C. neoformans* cells defective for the *PCK1* gene were avirulent in an animal model of infection (42).

The activation of the glyoxylate cycle is another important adaptation of many intracellular pathogens to the phagosomal microenvironment (34, 35, 43). Consistent with those observations, our results showed strong activation of the expression of its two regulatory enzymes, isocitrate lyase (Icl) and malate synthase (Ms), upon interaction of *C. neoformans* with both amoebae and macrophages. Additionally, our results suggest that acetyl coenzyme A (acetyl-CoA) generated from the oxidation of fatty acids drives the glyoxylate cycle. Transcripts related to enzymes of all steps of the *C. neoformans* β-oxidation pathway were induced upon internalization by amoebae and macrophages. Furthermore, genes related to peroxisome biogenesis, the organelle associated with cellular functions such as β-oxidation and hydrogen peroxide detoxification, were upregulated. Fatty acid oxidation is also required by other microorganisms after internalization by macrophages, such as *C. albicans* (35), and by *C. neoformans* in the lungs of infected mice (37). Taken together, our results show that *C. neoformans* cells responded similarly to nutritional stress within both amoebae and macrophages.

(iii) Genes related to oxidative/nitrosative stress and the electron transport chain. We identified seven and four *C. neoformans* genes related to the oxidative stress response that were upregulated after phagocytosis by *A. castellanii* and murine macrophages, respectively. Among them, the genes coding for catalase s and cytochrome c peroxidase are of particular significance. *C. neoformans* has four genes that encode distinct catalases, and we showed that two of them were more expressed in response to the interaction with amoebae. Although it is proposed that catalases play an important role in the adaptation of the fungus to the intracellular environment of phagocytes, none of the four isoforms is required for virulence (44), probably due to the redundancy of the antioxidant system in *C. neoformans*. This may also explain why the enzyme cytochrome c peroxidase, which also acts in the detoxification of H₂O₂, has no role in the virulence of *C. neoformans* in mice (45). However, our results showed that its expression was induced after the interaction of the fungus with both hosts, in another case of disconnection between upregulation of gene expression in the presence of the host and role in virulence.

In addition to the oxidative stress, nitrosative stress acts as an antimicrobial mechanism by phagocytic cells. In *C. neoformans*, the enzyme thioredoxin reductase (Trt1) has been associated with protection from nitrosative stress (46), and we have found its gene upregulated upon phagocytosis by both cells, again suggesting that their microenvironments are similar.

Genes related to the electron transport chain were also modulated by both phagocytes. For example, the gene encoding cytochrome c oxidase (COX1) was downregulated in response to the interaction of *C. neoformans* with *A. castellanii*. Cox1 corresponds to the complex IV of the electron transport chain, reducing oxygen into water. This process is essential for cellular energy production but requires oxygen as the final acceptor of electrons. The intracellular environment presents *C. neoformans* with low levels of oxygen, which can explain the negative regulation of *COX1*. In agreement with our data, a hypoxic environment represses *COX1* expression in *Saccharomyces cerevisiae* (47). In contrast, there was no decrease in the *COX1* expression in macrophages, which may be explained by the fact that this gene is induced at 37°C (48). The evidence that both intracellular environments are low in oxygen was supported by the upregulation of the gene *FRD*, which encodes the enzyme fumarate reductase. This enzyme uses fumarate as the final acceptor of the electron transport chain, establishing an alternative pathway for energy generation in the absence of oxygen (49). In *Mycobacterium tuberculosis*, expression of *FrdA* from the fumarate reductase complex is induced in response to interaction with macrophages (34), and in *Mycobacterium phlei*, the activity of the *Frd* complex increases 4-fold when this bacterium is grown under oxygen restriction (50).

(iv) Genes related to thermal stress. Thermotolerance is critical for the virulence of human pathogens and the trehalose synthesis pathway is crucial for the infectivity of such pathogenic fungi as *C. albicans* (51, 52) and *C. neoformans* (53). In *C. neoformans*, the gene for trehalose synthase is induced at 37°C (54) and knockout strains of *C. neoformans* for the *TPS1* and *TPS2* genes, which encode trehalose-phosphate synthase and trehalase-phosphate phosphatase, respectively, are avirulent and unable to grow at 37°C (53). Curiously, our results showed that the *TPS1* gene is induced upon interaction of *C. neoformans* with amoebae at 25°C. Moreover, the *Tps1* knockout strain of *C. neoformans* is less virulent in *Caenorhabditis elegans* at 25°C (53). These results suggest that the trehalose pathway may be involved in the response to stress conditions other than high temperature. In accordance with this, trehalose plays an important role in tolerance to desiccation (55) and hypoxia (56).

(v) Genes related to ergosterol. The biosynthesis of one molecule of ergosterol from squalene consumes 12 molecules of oxygen, which represents about 25% of the oxygen not used in the respiratory chain in *Saccharomyces cerevisiae* (57). Thus, it is expected that the concentration of oxygen in the environment affects ergosterol biosynthesis by fungi. Under hypoxic conditions, the concentration of ergosterol in the membrane of *S. cerevisiae* is only 25% of the concentration found in normoxia (58). Therefore, hypoxia in the intracellular environment of phagocytes may explain the downregulation of several genes related to ergosterol biosynthesis in *C. neoformans* after internalization by amoebae and macrophages.

(vi) Genes related to virulence. Steenbergen et al. (12) reported that strains defective in capsule synthesis and phospholipase B production were unable to survive inside amoebae, suggesting that they were required for persistence of the fungus in both mammalian and soil predator cells. In our experiments, we observed the induction of the genes *ISC1* and CAP64, which encode the enzyme C-inositol phosphosphingolipid phospholipase and a protein necessary for the synthesis of polysaccharide capsule, respectively, in response to *C. neoformans* internalization by *A. castellanii*. *ISC1* is important for *C. neoformans* survival within macrophages since it protects against nitrosative and oxidative stresses and the acidic pH found in the phagolysosome. Furthermore, *ISC1* is crucial for *C. neoformans* to spread to the central
nervous system (59). Likewise, CAP64 seems to be important for C. neoformans virulence since knockout strains are avirulent (60). However, the precise function of CAP64 in capsule formation is unknown (61). The upregulation of ISCI and CAP64 by C. neoformans in response to A. castellanii suggests a convergence of adaptive mechanisms employed by this fungus to persist in amoebae and mammalian host cells.

Another gene of C. neoformans modulated in response to interaction with A. castellanii coded for the enzyme glucosylceramide synthase (Gcs). GSC is a pathogenesis regulator that ensures the growth of C. neoformans in neutral/alkaline pH and physiological concentrations of CO₂, a condition typically found in alveolar spaces. However, GCS has no involvement in fungal growth at acidic pH, as well as in fungal survival inside macrophages (62). In our microarray data, we found a decrease in the GCS transcript level after internalization of yeasts by A. castellanii, implying that this gene is not implicated in the persistence of C. neoformans inside amoebae either. Moreover, this result suggests that the intracellular environment of A. castellanii is acidic, as described for macrophages.

Overall, our results show that the transcriptional responses of C. neoformans to the hostile microenvironments of macrophages and amoebae are similar. The transcriptional profile in response to both phagocytes suggests an adaptive pattern to the stress of ingestion and phagosomal attack. The mechanisms of adaptation to phagocytic cells included the remodeling of central carbon metabolism, the expression of specific nutrient acquisition systems, and a response to the harsh conditions of the phagosome. Analysis of a highly expressed gene led to the identification and characterization of the C. neoformans PTP1. The fact that it was highly activated but irrelevant for murine or moth virulence is consistent with the notion that the transcriptional response to phagocytosis is a general stress response, which would necessarily include many genes that are not important in virulence. Although the transcriptional modulation of a specific gene of C. neoformans within phagocytic cells itself does not necessarily indicate a role in virulence, the comparative transcriptional profile provides insights into the adaptation of the fungus to key features of the host environment. In fact, the similarities between the transcriptional responses to ingestion by amoebae and macrophages are consistent with the view that cryptococcal virulence for mammals was selected by interactions with phagocytic predators in the environment and include adaptation to the intracellular milieu of eukaryotic phagocytic cells.

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