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RESEARCH ARTICLE

Exploration of whole-genome responses of the human AIDS-associated yeast pathogen *Cryptococcus neoformans* var *grubii*: nitric oxide stress and body temperature

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Abstract *Cryptococcus neoformans* var *grubii* is an opportunistic basidiomycete yeast pathogen that is a significant cause of HIV/AIDS-related deaths worldwide. We describe a whole-genome oligonucleotide microarray for this pathogen. These arrays have been used to elucidate the transcriptional responses of the genome to heat shock as well as to two conditions relevant to human infections: body temperature and nitric oxide (NO) stress produced by the NO donor DPTA-NONOate. This analysis revealed an NO-inducible *C. neoformans*-specific four-gene family that showed a highly similar transcriptional profile to that of *FHB1*, a previously described NO dioxygenase/flavohemoglobin required for virulence. NO treatment also induced genes involved in the synthesis of the antioxidant mannitol, a polyol that accumulates in the cerebrospinal fluid of infected patients. Exposure to NO also caused increased expression of the sole *C. neoformans* var *grubii* protein with HHE/hemerythrin cation binding motifs. Notably, a similar gene in *E. coli*, *ytfE*, has been shown to be NO-inducible and protects bacterial cells from killing by NO. Genes induced by NO were highly enriched for those repressed at 37°C, indicating an unexpected interplay between temperature and NO regulation in this basidiomycete. Resources described here should facilitate future investigations of this lethal human yeast pathogen.

Keywords Fungal pathogenesis · Nitric oxide · DNA microarray · Transcriptional profiling · Annotation database · Genomic

Introduction

Cryptococcosis, the commonest systemic fungal infection in people infected with HIV, is caused by the opportunistic fungal pathogen *Cryptococcus neoformans*. In sub-Saharan Africa and Southeast Asia, where over 90% of AIDS-related deaths occur worldwide, up to 40% of AIDS patients present with *C. neoformans* infections (Corbett et al. 2002; Amornkul et al. 2003). The most lethal manifestation of cryptococcosis is meningoencephalitis. The life expectancy of individuals with untreated cryptococcal meningitis is less than one month (Bicanic and Harrison 2004).

As with other fungal pathogens, antifungal treatment options are limited to a handful of drugs. The current recommended therapeutic regimen for cryptococcal meningitis is initial induction therapy with a combination of amphotericin B and flucytosine followed by a maintenance phase with fluconazole (Bicanic and Harrison 2004). Both amphotericin B and flucytosine have significant toxicities and treatment failure is not uncommon. Even when recommended treatment is administered, three-month mortality rates for cryptococcosis range between 10 and 20% (Lortholary et al. 2006; Dromer et al. 2007). Moreover, the high costs of these drugs (amphotericin B must be administered intravenously) and limited availability in developing countries

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where HIV infection is common often make treatment impossible. Mortality rates in sub-Saharan Africa often reach close to 100% (Mwaba et al. 2001; French et al. 2002). Therefore, the identification of additional drug targets as well as vaccine candidates is desirable.

C. neoformans is a basidiomycetous yeast, which places it evolutionarily distal to the model fungal organisms *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, both of which are ascomycetous yeasts. As an experimental system, it has haploid genetics, facile gene knockout technology, and the existence of faithful animal models of infection (Hull and Heitman 2002; Idnurm et al. 2005). It therefore has the potential to become a powerful model for studies of basidiomycete biology and fungal pathogenesis mechanisms. Consequently, implementation of genome-based tools developed in the model yeast *S. cerevisiae* could add significant leverage to *C. neoformans* as an experimental system.

C. neoformans exists as three varieties which have diverged from each other by as much as 50 million years of evolution. *C. neoformans* var *grubii* (primarily serotype A) is responsible for almost all infections of HIV positive patients (Canteros et al. 2002; French et al. 2002; Banerjee et al. 2004). *C. neoformans* var *neoformans* (primarily serotype D) is more commonly found in Europe and is less likely to cause severe disease (Dromer et al. 2007). The third variety, *Cryptococcus gattii* (primarily serotypes B and C), is now considered a separate species and is predominantly a primary pathogen, infecting the immunocompetent. The genome sequencing of five strains has been completed to at least 5× coverage. Two *C. neoformans* var *neoformans* serotype D inbred laboratory strains, JEC21 and its parent strain B-3501, were sequenced by the TIGR and Stanford Centers, respectively (Loftus et al. 2005). In addition, using a combination of cDNA sequencing and bioinformatics, gene models for B-3501 and JEC21 were developed (Loftus et al. 2005). Two *C. gattii* strains, WM276 and R265, have been sequenced by the University of British Columbia and the Broad Institute, respectively. The sequence of the *C. neoformans* var *grubii* serotype A human clinical isolate called H99 was deposited into GenBank by the Broad Institute in June of 2003. Since most infections of humans and almost all infections of HIV positive patients are caused by serotype A strains, H99 is often the strain of choice for studies of pathogenesis.

To facilitate whole-genome analysis for a pathogenic serotype A strain, we designed and manufactured whole-genome DNA microarrays for *C. neoformans* var *grubii*. We report the transcriptional responses of this pathogen to two conditions relevant to virulence: body temperature and nitric oxide stress. These studies provide a glimpse into the whole-genome transcriptional responses of this important AIDS pathogen.

Materials and methods

DNA sequences

H99 sequence scaffolds were obtained from the *C. neoformans* Sequencing Project website of the Broad Institute of MIT and Harvard (http://www.broad.mit.edu).

Development of gene models for *C. neoformans* var *grubii* strain H99

The starting point for annotation was the sequence scaffolds released in June of 2003 by the Broad Institute for the H99 strain. Two routes were chosen to develop gene models. The first approach was based on BLASTX (Altschul et al. 1997) and defined genes based on homology to entries in the nonredundant protein database. The second approach took advantage of gene models based on the genome sequences of the serotype D laboratory strains JEC21 and B-3501 that were developed by TIGR and Stanford centers through large-scale cDNA sequence analysis and the use of gene prediction software including TWINSCAN (Tenney et al. 2004). Thus, in our second approach, rather than performing *de novo* predictions for the H99 sequence, we attempted to utilize the JEC21/B-3501 gene models (Loftus et al. 2005).

To identify genes using BLASTX, we concatenated contig sequences into scaffolds and computationally broke up each scaffold into 1 kb segments which overlapped each other by 0.5 kb. These were locally compared to the nonredundant protein database using BLASTX. Hits with an E value of less than 10^{-3} were considered significant. Then in an iterative process, we grouped together adjacent windows that shared one or more BLAST hits. This process identified 6,044 homology blocks or candidate genes.

We next used the mRNA alignment program SPIDEY (Wheelan et al. 2001) to attempt to align the H99 genomic scaffolds with predicted cDNAs from the serotype D strains. Using SPIDEY, we were able to align 6,478 of 6,574 predicted cDNAs (including predicted splice variants) from JEC21/B-3501 with the H99 genomic sequence. However, we noticed that many of the predicted mRNAs from H99 did not have ORFs as large as the predicted JEC21 ORFs. To remedy this, we generated custom scripts to adjust ad hoc the alignments determined by SPIDEY. These scripts produced modified predictions that corrected for consensus splice sites and in-frame mRNAs. This resulted in improved alignments by several criteria. First, we determined that the homologies of predicted proteins between the serotype D and A sequences were increased. Second, in order to verify some of these adjusted predictions, we amplified cDNAs from H99 for 12 genes with such altered predictions and directly sequenced the PCR

products. For all 12 genes, our modified predictions exactly matched the sequences obtained (data not shown). Third, we compared our 36,024 splice site predictions to the available EST data available for H99 (http://www.genome.ou.edu/cneo.html). Of the predicted regions identified in the EST data (2,204), perfect matches were seen in 91% (2,037) of the cases. This is likely to be an underestimate of accuracy since splice variants not present in the EST database would be scored as discrepancies. In cases where we adjusted the predictions made by SPIDEY ad hoc, the EST matched the adjusted prediction in 84% of cases and the original in only 6.4% of cases (785 splice junctions identified). Taken together, these data provide objective experimental support for the computational mRNA predictions as well as an estimate of their accuracy. In total, we identified 6,528 genes and, taking into account potential splicing variants, 6,980 gene models.

Web-based database implementation of gene models

The gene models described above were stored in a relational database. Generic and gene-specific names were generated for each gene model as well as additional information computed from the predicted protein sequences. The database is accessible though a set of linked public web pages (http://cryptogenome.ucsf.edu).

Gene naming conventions

In this paper, we refer to the generic names described by the Stanford B-3501 serotype D genome annotation (GenBank accession: AAEY00000000). These are in the form CNXY, where X indicates the chromosome (A for chromosome I, B for chromosome II, etc.) and Y indicate the gene position. In addition, if a gene has a homolog in *S. cerevisiae* with a BLASTP expect value of less than 10^{-4}, we refer to it by the name of the best *S. cerevisiae* homolog. In some cases, the same gene in *S. cerevisiae* was the closest homolog to multiple genes in *C. neoformans*, possibly due to expansion of gene families in *C. neoformans*. To generate unique specific names for these sets of genes, we added two digits to the *S. cerevisiae* gene name and numbered the *C. neoformans* homologs sequentially. For example, the gene *STL1* is the closest *S. cerevisiae* homolog to 12 *C. neoformans* genes. We have therefore assigned these 12 genes the specific names *STL101–STL112*.

Genes without specific intron–exon models

For two classes of genes, we were unable to compute gene models. One class were those for which serotype D gene models exist, but no alignment was obtained using SPIDEY. These are likely to include genes that have diverged more rapidly since the divergence of the serotype A and D lineages from the common ancestor. A second class were those where no serotype D gene model exists but whose existence was supported by the sliding-window BLASTX analysis described above. Of the 6,528 genes, we were unable to compute models for 497 genes. Of these, 152 lacked a serotype D BLASTX hit with an expect value of 10^{-4} or lower. These 152 are currently listed in the database without specific names pending nomenclature assignment. cDNA sequencing efforts should yield gene models for many of the 497 genes that currently lack gene models (Tenney et al. 2004).

During the course of this work, an annotation for the H99 sequence was posted by the Broad Institute on the web on 17 February 2006 (http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans). The annotation used GENEWISE, TWINSCAN and GLEAN software and reports an accuracy of 90.9% in intron prediction using the available EST data. While our annotations were generated using different methods, the almost identical level of success in intron prediction indicates that these two annotations offer gene models of apparently similar accuracies.

Culture growth for heat shock experiments

The H99 strain was grown overnight to saturation in YPAD (1%yeast extract, 2% Bacto peptone, 2% glucose, 0.015% L-tryptophan, 0.004% adenine), diluted to an optical density at 600 nm (OD_{600}) of 0.1, grown to OD_{600} 1.0, and shifted to YPAD media that was pre-warmed to 42°C. Cultures were harvested at 0, 10, 20, and 30 min. after the shift to 42°C.

Culture growth for temperature experiments

The H99 strain was grown to mid-log phase in YPAD. Cells were then collected by centrifugation and resuspended in YPAD pre-warmed to either 30 or 37°C. Cultures were grown to an OD_{600} of 1.0 and harvested by centrifugation. Four replicate cultures were grown at each temperature.

Culture growth for NO experiments

The H99 strain was grown overnight to saturation in YPAD at 37°C to mimic body temperature. Cultures were diluted to an OD_{600} of 0.1 in YPAD (pre-warmed to 37°C) that was buffered to a pH of 7.5 with 50 mM MOPS. Cells were grown at 37°C to an OD_{600} of 1.0 and then treated with 0, 0.2, 0.5, 1, 2, and 5 mM of the NO donor DPTA-NONOate for an additional 2 h prior to harvesting. A single sample was collected per dosage. The concentrations of DPTA-NONOate, initial pH, and timepoint for sample

collection were identified empirically as maximizing the response to the NO donor used. In pilot studies, a concentration of 0.5 mM DPTA-NONOate was shown to have no effect on the growth of wildtype H99, but inhibited the growth of the *fhb1Δ* mutant. Higher concentrations were found to inhibit the growth of both H99 and the *fhb1Δ* mutant.

RNA isolation

Cultures were harvested by centrifugation and snap freezing. Total RNA was isolated using the TRIzol reagent (Invitrogen) and the manufacturer's instructions. For the heat-shock experiments, polyA+ RNA was selected using an Oligotex kit (Qiagen).

Microarray hybridization and data analysis

Oligonucleotide microarrays were printed on polylysine-coated glass microscope slides using a custom robot and silicon tips. Our microarray design is available for download as Table S1. Note that genes for which no name could be assigned are described in the Figures by their "CNO" unique ID numbers which can be found in Table S1. Probes were synthesized using dT_{20} primers and 10 μg of total RNA, except in the case of the heat shock experiments, which used 2 μg of polyA+ RNA. Standard procedures were used for probe labeling, hybridzation and washes (DeRisi et al. 1997). Detailed protocols can be found at http://www.microarrays.org. Hybridization signals were visualized using an Axon 4000B scanner and GenePix software (Axon Instruments). Array signals were bulk-normalized, filtered for flagged and low-intensity spots using NOMAD software (available at http://www.ucsf-nomad.sourceforge.net/). Data was analyzed using either SAM (available at http://www-stat.stanford.edu/~tibs/SAM/) or Cluster (available at http://www.rana.lbl.gov/EisenSoftware.htm) and Java TreeView (available at http://www.jtreeview.sourceforge.net/). Data were filtered for minimum fold-changes prior to hierarchical clustering as described in the figure legends. Significance analysis of microarrays (SAM) was performed as described (Tusher et al. 2001), using the One-class Response, K-nearest neighbors settings (10 neighbors) and the default Random Number Seed (1,234,567). Delta values were selected to calculate false discovery rates (FDRs) and those yielded a FDR of <1% were chosen (Tusher et al. 2001). For time-course or dose-response experiments, samples were labeled with Cy3 dye and hybridized to a pooled reference sample consisting of equal amounts of cDNA from each sample labeled with Cy5 dye. The data were then normalized by dividing by the zero timepoint or dose. For the steady-state temperature experiment, four replicates (for two of the samples, dye-flips were performed to avoid dye-specific artifacts) were analyzed using SAM as described above. Data described in this paper are available for download as Table S2. In addition, additional annotation for each gene can be found by entering the corresponding gene name into the search window at http://cryptogenome.ucsf.edu.

Quantitative RT-PCR

Microarray results were confirmed utilizing quantitative RT-PCR. The same total RNA used for microarray analysis was treated with DNase I (Roche) according to the manufacturer's instructions. cDNA was then synthesized from the DNase I-treated total RNA using Superscript III Reverse Transcriptase (Invitrogen) and oligo dT primers. Approximately 0.2 ng of cDNA was used as template in a QPCR reaction containing SYBR Green dye (Molecular Probes). Fluorescent signal was measured on an Opticon DNA Engine PCR machine (MJ Research). For each primer set, standard curves were generated using fivefold sequential dilutions of cDNA to account for differences in priming efficiencies. For each sample, values obtained were normalized to the levels of actin (*ACT1*) in that sample. The primers used in these experiments can be found in Table S3.

Annotation translation table

Table S4 cross-references our oligonucleotide names (CNO#) and gene names with the Broad Institute annotations of the H99 serotype A genome sequence (CNAG#) as well as with the gene names assigned by Stanford to the B3501 serotype D genome sequence.

Results

C. neoformans var *grubii* whole-genome oligonucleotide microarray design

To design whole genome DNA microarrays for the H99 strain, we generated gene models (see "Materials and methods") and then used these as inputs to ArrayOligoSelector software (Bozdech et al. 2003). A single oligonucleotide (70-mer) was chosen for each gene model. Oligonucleotides were designed to minimize potential cross hybridization. For genes identified by BLASTX for which we have no gene model, an oligonucleotide was chosen from a region of genomic DNA with the best BLAST hit with the NR database. Oligonucleotides were custom-synthesized and then printed on polylysine-coated glass sides as described (DeRisi et al. 1997).

Elucidation of the heat-shock response of *C. neoformans* var *grubii*

We first examined a highly conserved and well-characterized stress response, the heat shock response. We shifted exponential cultures of H99 from 30 to 42°C for 0, 10, 20, or 30 min, and prepared total RNA from these cultures. Transcript profiling of labeled probe derived from these samples was performed by hybridization to the whole-genome microarrays described above (see "Materials and methods"). Hierarchical clustering and annotation of the data is shown in Fig. 1.

Inspection of the identities of the induced genes revealed that *C. neoformans* homologs of numerous heat shock genes (e.g., *KAR2*, *HSC8202*, *HSP104*, *HSP78*, *SSA4*, *STI101*, *SSE2, LHS1*) were induced as well as genes encoding components of the thioredoxin system (*TRR1*, *TRX3*). A gene encoding a subunit of RNA polymerase II, *RPB7*, which is heat shock inducible in *S. cerevisiae* (Gasch et al. 2000), was also induced. Notable among the repressed genes were

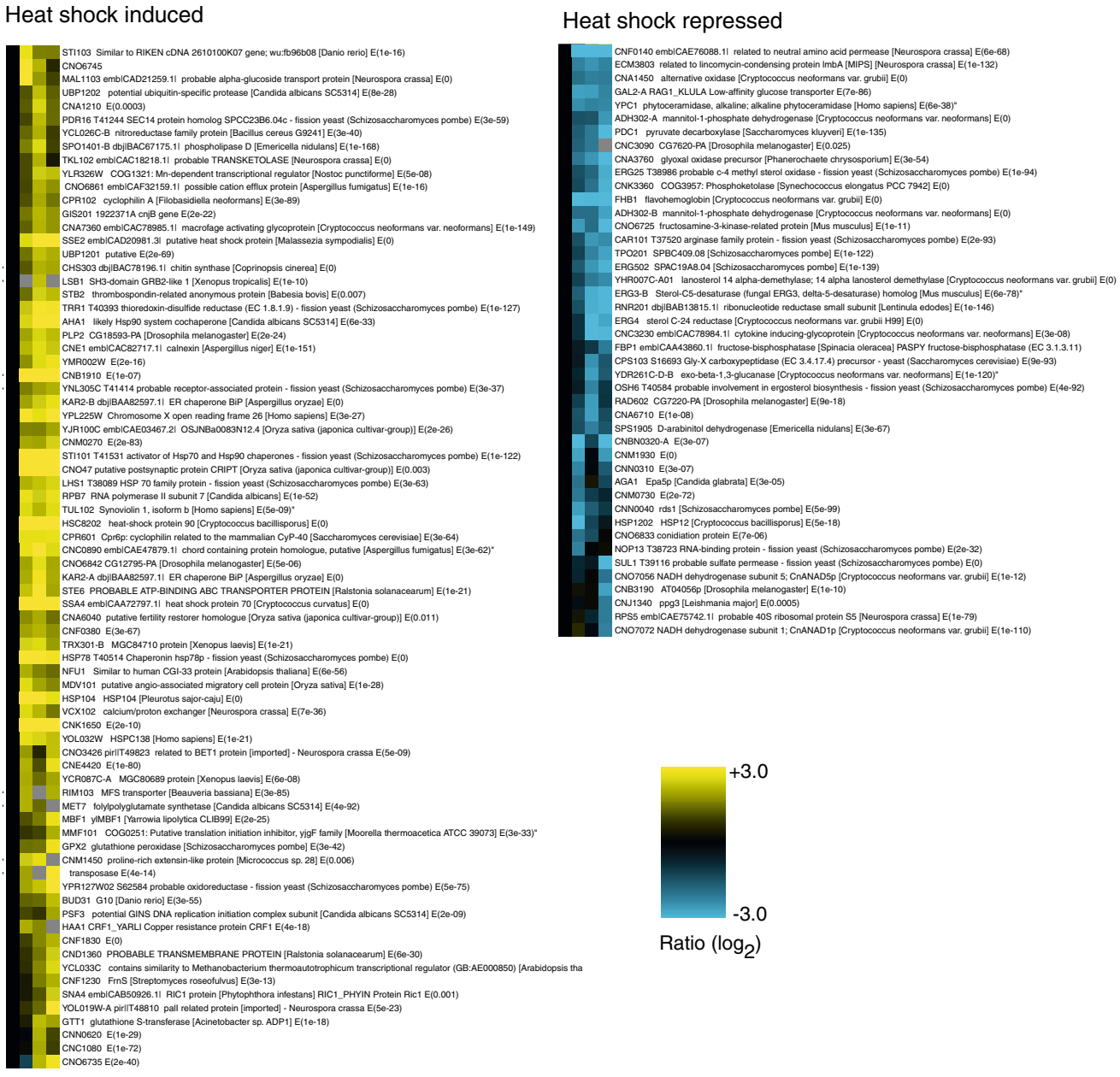


Fig. 1 *C. neoformans* var *grubii* heat shock response. Shown is a time course of gene expression changes of cultures shifted from 30 to 42°C for 0, 10, 20 or 30 min. A minimum twofold change in mRNA levels for at least one timepoint was chosen to filter the data prior to hierarchical clustering

those involved in ergosterol biosynthesis (*ERG3*, *ERG4*, *ERG502*, *ERG25*, *OSH6*), a finding that would be consistent with the increased membrane fluidity at 42°C. Notably, *ERG11* encodes lanosterol demethylase, the target of fluconazole, the major drug used to prevent relapses of cryptococcal meningitis (Marichal et al. 1999). As described previously, a *C. neoformans* gene encoding a flavohemoglobin nitric oxide dioxygenase, *FHB1*, is repressed upon a shift of cultures to higher temperatures (Kraus et al. 2004). Overall, the *C. neoformans* heat shock response appears to generally resemble those of other organisms, consistent with the highly conserved nature of the heat shock response and the need to induce conserved protein chaperones.

Genomic response to temperature

The ability to grow at body temperature would seem to be an essential characteristic of most human pathogens, and it has been widely proposed that this trait constitutes a necessary virulence factor for fungal pathogens since many non-pathogenic fungi do not proliferate at body temperature. Therefore, we examined the mRNA profiles of *C. neoformans* grown continuously at 30 versus 37°C to identify genes that are differentially regulated between the two temperatures. Samples were grown in YPD and harvested at optical densities of 1.0. Four replicate cultures were grown at each temperature (see "Materials and methods" for additional details). We used the SAM statistical tool (Tusher et al. 2001) to identify genes that were differentially expressed at the two temperatures (see "Materials and methods"). This analysis, which involves data permutation and a series of t-tests, identified 60 genes (Fig. 2). Of these, only 7 showed increased expression at 37°C. A much larger number, 53, displayed decreased expression at 37°C. The relationship of these genes to those induced by heat shock and nitrosative stress will be described below.

Genomic response to nitric oxide stress

A key cell type in the innate immune system is the macrophage. Macrophages ingest and kill microbial pathogens and serve to present their antigens to lymphocytes. An important mechanism by which macrophages kill microbes is the synthesis of nitric oxide (NO) by the iNOS protein. Pathogens have mechanisms to respond to attack by NO. For instance, many microbes express flavohemoglobins that inactivate NO via their potent nitrogen oxide dioxygenase activity. A role for NO resistance for pathogenesis by *C. neoformans* is supported by the observation that strains lacking the *FHB1* flavohemoglobin gene display a reduced virulence (de Jesus-Berrios et al. 2003).

To determine what genes are induced by NO in *C. neoformans*, we chose to use a specific donor of NO, Dipropylenetriamine-NO (also known at DPTA-NONOate). Although acidified nitrite has been used to assess the resistance of *C. neoformans* to NO in previous studies (Missall et al. 2005), recent work has shown that nitrite has NO-independent effects on human cells (Bryan et al. 2005). Moreover, the pathway by which nitrite produces NO at low pH involves the production of nitrous acid, a toxic compound which could also have NO-independent effects (Nathan and Shiloh 2000). We therefore performed dose-response experiments using DPTA-NONOate (Yamamoto and Bing 2000). The transcript profile of cultures after 2 h of treatment in buffered media was obtained by microarray hybridization (see "Materials and methods"). Hierarchical clustering analysis revealed a cluster of 24 genes induced in a dose-dependent manner by DPTA-NONOate (Fig. 3). As expected from previous studies, we observed a strong induction of the *FHB1* mRNA. Other notable induced genes include *CAT3*, encoding a catalase homolog and *AOX1/CNA1450* which encodes an alternative oxidase. Several potential oxidoreductases are also induced including *YMR226C02* and *CNC3900*. *CNA2870*, another gene strongly induced by DPTA-NONOate treatment, is a homolog of *ytfE*, a gene in *E. coli* that is transcriptionally induced by NO and required for resistance to NO killing in vitro (Justino et al. 2005). Finally, *MPD1/ADH302*, which encodes an ortholog of the *C. neoformans* var neoformans mannitol-1-phosphate dehydrogenase that synthesizes the polyol mannitol (Suvarna et al. 2000), was also induced by treatment with DPTA-NONOate.

A novel four-gene family is strongly induced by NO treatment. These proteins are of low molecular weight (10–13 kDa) and highly related to each other (protein sequences are aligned in Fig. 4). These are *CNM1930*, *CNN0310*, *CNN0320* (two splice isoforms are shown in Fig. 4, *CNN0320-A* and *CNN0320-B*), and *CNN1140*. Members of this family show a highly similar transcriptional profile to that of *FHB1* across all conditions tested except for *CNN1140* and the *CNN0320-B* predicted splice isoform. Unlike the other genes, these two are not strongly repressed upon heat shock treatment, but are repressed at 37°C relative to 30°C. Quantitative RT-PCR was used to confirm the changes in RNA transcript level during NO treatment for several of the genes detected by these microarrays (Fig. 5).

Heirarchical clustering reveals a relationship between the temperature and NO regulation

To determine relationships between the genes controlled by heat shock, steady-state temperature, and DPTA-NONOate, we examined the entire dataset using hierarchical clustering. As shown in Fig. 6, there are substantial overlaps between the three datasets. Most apparent is that genes that display a dose-dependent induction by NO treatment

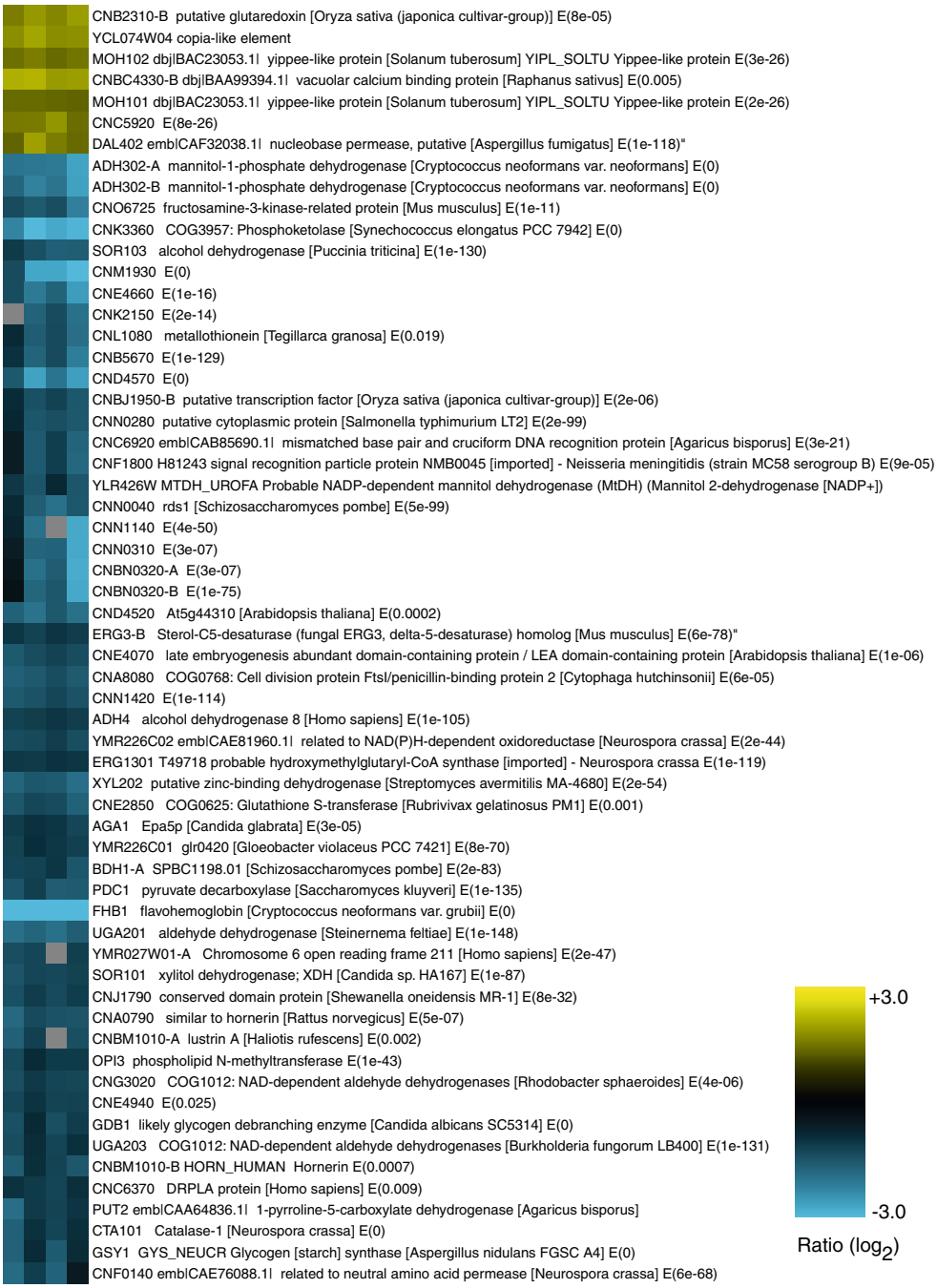


Fig. 2 Temperature-regulated gene expression. Shown are gene expression comparisons of four pairs of replicate cultures in which one culture was grown continuously at 30°C while the other was grown continuously at 37°C. Shown are genes that were significantly different as determined by SAM analysis (see "Materials and methods")

(indicated by blue annotations in Fig. 6) are generally repressed by high temperature and heat-shock.

Discussion

Studies of *Cryptococcus neoformans* are motivated by its importance as an opportunistic human fungal pathogen and its experimental tractability. The development of facile gene disruption methods and faithful animal models of infection make it possible to test the role of individual genes in virulence. The release of genome sequences for *C. neoformans* serotypes advances it as an experimental system; however, the utility of these sequences depends on their translation into genomic resources. To this end, we generated whole-genome DNA microarrays for *C. neofor-*

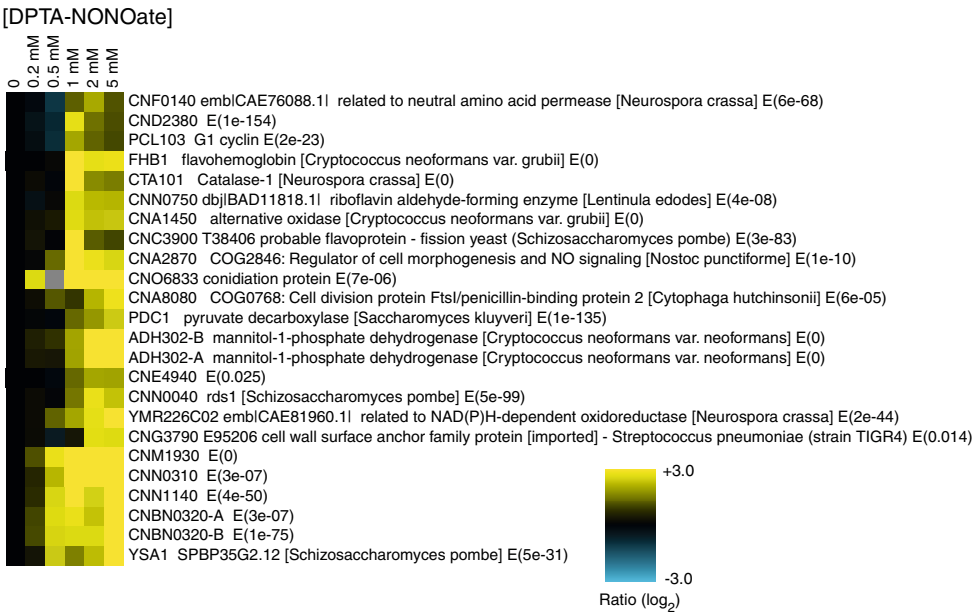


Fig. 3 NO-induced genes. Shown is a cluster of genes that display dose-dependent increases in expression in response to treatment of cultures with the NO donor DPTA-NONOate. A minimum twofold increase for at least one dose was chosen to filter the data prior to hierarchical clustering

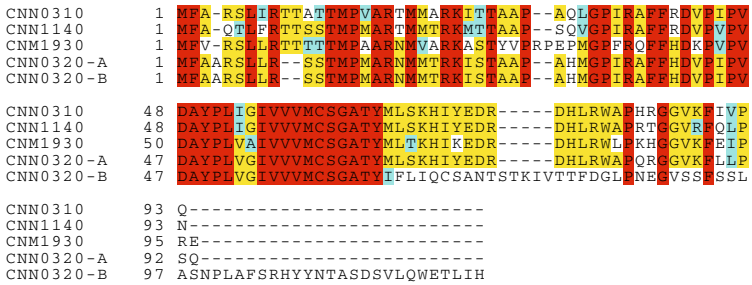


Fig. 4 *C. neoformans*-specific four-gene family induced by NO. Shown is a CLUSTALW alignment of the four-gene family described in the text

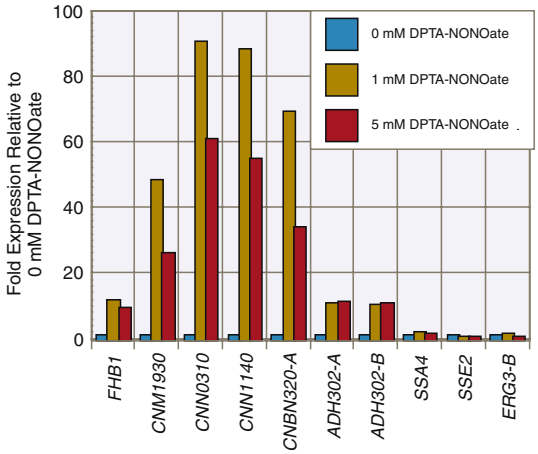


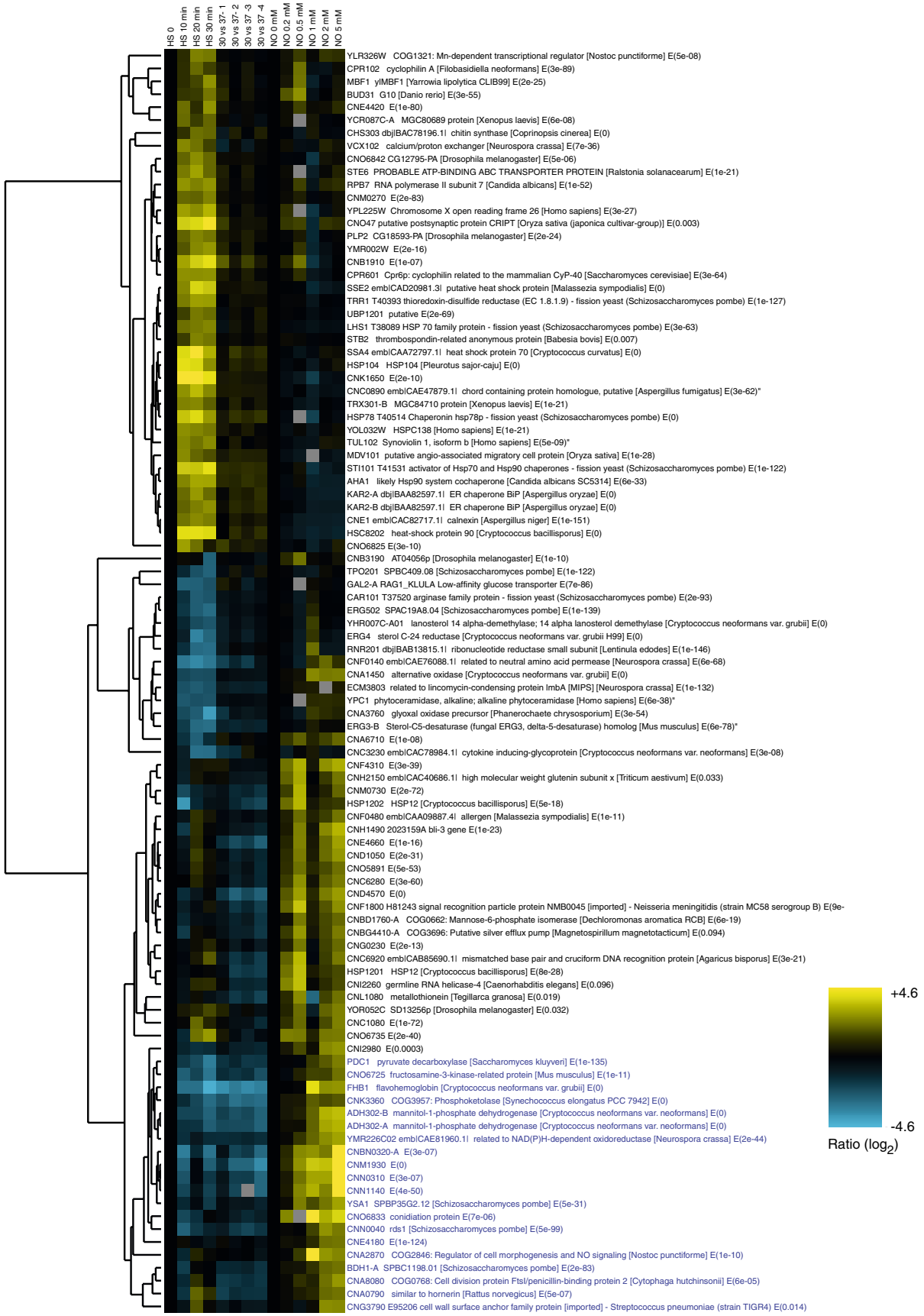
Fig. 5 Quantitiave RT-PCR confirms expression changes induced by NO in genes detected by microarray. Quantitiave RT-PCR was performed on total RNA collected from cells treated with the indicated doses of DPTA-NONOate. Expression values were normalized to the value of actin (*ACT1*) in the sample and the fold expression relative to no DPTA-NONOate treatment was calculated. *FHB1*, *CNM1930*, *CNN0310*, *CNN1140*, *CNBN320-A*, *ADH302-A*, and *ADH302-B* were identified in our microarray experiments as being induced by NO treatment. Expression of *SSA4*, *SSE2*, and *ERG3-B* was unchanged by NO treatment in our microarrays

mans var *grubii*. We examined the transcriptional responses of the genome to two conditions relevant to infection: body temperature and nitric oxide stress.

A glimpse into the whole-genome transcriptional responses of the *C. neoformans* var *grubii* genome

Heat shock and temperature. We first characterized the *C. neoformans* heat shock response, which revealed that, as in other organisms, this response involves the induction of proteins such as chaperones that deal with unfolded and otherwise damaged proteins produced by a temperature shift. In addition, we observed reduced mRNA accumulation for genes involved in ergosterol biosynthesis, likely due to a reduced need for sterols in membrane fluidity at high temperatures. Notably, in a study of temperature stress in *A. fumigatus*, genes involved in ergosterol biosynthesis were also repressed relative to 30°C (Nierman et al. 2005). Ergosterol biosynthesis is the target for most

Fig. 6 Heirarchical cluster diagram of gene expression studies described in this paper. Cluster of genes induced by NO in a dose-dependent manner are indicated with *blue* annotations ▶


HS 0
HS 10 min
HS 20 min
HS 30 min
30 vs 37- 1
30 vs 37- 2
30 vs 37 -3
30 vs 37 -4
NO 0 mM
NO 0.2 mM
NO 0.5 mM
NO 1 mM
NO 2 mM
NO 5 mM
YLR326W COG1321: Mn-dependent transcriptional regulator [Nostoc punctiforme] E(5e-08)
CPR102 cyclophilin A [Filobasidiella neoformans] E(3e-89)
MBF1 yIMBF1 [Yarrowia lipolytica CLIB99] E(2e-25)
BUD31 G10 [Danio rerio] E(3e-55)
CNE4420 E(1e-80)
YCR087C-A MGC80689 protein [Xenopus laevis] E(6e-08)
CHS303 dbj|BAC78196.1| chitin synthase [Coprinopsis cinerea] E(0)
VCX102 calcium/proton exchanger [Neurospora crassa] E(7e-36)
CNO6842 CG12795-PA [Drosophila melanogaster] E(5e-06)
STE6 PROBABLE ATP-BINDING ABC TRANSPORTER PROTEIN [Ralstonia solanacearum] E(1e-21)
RPB7 RNA polymerase II subunit 7 [Candida albicans] E(1e-52)
CNM0270 E(2e-83)
YPL225W Chromosome X open reading frame 26 [Homo sapiens] E(3e-27)
CNO47 putative postsynaptic protein CRIPT [Oryza sativa (japonica cultivar-group)] E(0.003)
PLP2 CG18593-PA [Drosophila melanogaster] E(2e-24)
YMR002W E(2e-16)
CNB1910 E(1e-07)
CPR601 Cpr6p: cyclophilin related to the mammalian CyP-40 [Saccharomyces cerevisiae] E(3e-64)
SSE2 emb|CAD20981.3| putative heat shock protein [Malassezia sympodialis] E(0)
TRR1 T40393 thioredoxin-disulfide reductase (EC 1.8.1.9) - fission yeast (Schizosaccharomyces pombe) E(1e-127)
UBP1201 putative E(2e-69)
LHS1 T38089 HSP 70 family protein - fission yeast (Schizosaccharomyces pombe) E(3e-63)
STB2 thrombospondin-related anonymous protein [Babesia bovis] E(0.007)
SSA4 emb|CAA72797.1| heat shock protein 70 [Cryptococcus curvatus] E(0)
HSP104 HSP104 [Pleurotus sajor-caju] E(0)
CNK1650 E(2e-10)
CNC0890 emb|CAE47879.1| chord containing protein homologue, putative [Aspergillus fumigatus] E(3e-62)"
TRX301-B MGC84710 protein [Xenopus laevis] E(1e-21)
HSP78 T40514 Chaperonin hsp78p - fission yeast (Schizosaccharomyces pombe) E(0)
YOL032W HSPC138 [Homo sapiens] E(1e-21)
TUL102 Synoviolin 1, isoform b [Homo sapiens] E(5e-09)"
MDV101 putative angio-associated migratory cell protein [Oryza sativa] E(1e-28)
STI101 T41531 activator of Hsp70 and Hsp90 chaperones - fission yeast (Schizosaccharomyces pombe) E(1e-122)
AHA1 likely Hsp90 system cochaperone [Candida albicans SC5314] E(6e-33)
KAR2-A dbj|BAA82597.1| ER chaperone BiP [Aspergillus oryzae] E(0)
KAR2-B dbj|BAA82597.1| ER chaperone BiP [Aspergillus oryzae] E(0)
CNE1 emb|CAC82717.1| calnexin [Aspergillus niger] E(1e-151)
HSC8202 heat-shock protein 90 [Cryptococcus bacillisporus] E(0)
CNO6825 E(3e-10)
CNB3190 AT04056p [Drosophila melanogaster] E(1e-10)
TPO201 SPBC409.08 [Schizosaccharomyces pombe] E(1e-122)
GAL2-A RAG1_KLULA Low-affinity glucose transporter E(7e-86)
CAR101 T37520 arginase family protein - fission yeast (Schizosaccharomyces pombe) E(2e-93)
ERG502 SPAC19A8.04 [Schizosaccharomyces pombe] E(1e-139)
YHR007C-A01 lanosterol 14 alpha-demethylase; 14 alpha lanosterol demethylase [Cryptococcus neoformans var. grubii] E(0)
ERG4 sterol C-24 reductase [Cryptococcus neoformans var. grubii H99] E(0)
RNR201 dbj|BAB13815.1| ribonucleotide reductase small subunit [Lentinula edodes] E(1e-146)
CNF0140 emb|CAE76088.1| related to neutral amino acid permease [Neurospora crassa] E(6e-68)
CNA1450 alternative oxidase [Cryptococcus neoformans var. grubii] E(0)
ECM3803 related to lincomycin-condensing protein lmbA [MIPS] [Neurospora crassa] E(1e-132)
YPC1 phytoceramidase, alkaline; alkaline phytoceramidase [Homo sapiens] E(6e-38)"
CNA3760 glyoxal oxidase precursor [Phanerochaete chrysosporium] E(3e-54)
ERG3-B Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog [Mus musculus] E(6e-78)"
CNA6710 E(1e-08)
CNC3230 emb|CAC78984.1| cytokine inducing-glycoprotein [Cryptococcus neoformans var. neoformans] E(3e-08)
CNF4310 E(3e-39)
CNH2150 emb|CAC40686.1| high molecular weight glutenin subunit x [Triticum aestivum] E(0.033)
CNM0730 E(2e-72)
HSP1202 HSP12 [Cryptococcus bacillisporus] E(5e-18)
CNF0480 emb|CAA09887.4| allergen [Malassezia sympodialis] E(1e-11)
CNH1490 2023159A bli-3 gene E(1e-23)
CNE4660 E(1e-16)
CND1050 E(2e-31)
CNO5891 E(5e-53)
CNC6280 E(3e-60)
CND4570 E(0)
CNF1800 H81243 signal recognition particle protein NMB0045 [imported] - Neisseria meningitidis (strain MC58 serogroup B) E(9e-
CNBD1760-A COG0662: Mannose-6-phosphate isomerase [Dechloromonas aromatica RCB] E(6e-19)
CNBG4410-A COG3696: Putative silver efflux pump [Magnetospirillum magnetotacticum] E(0.094)
CNG0230 E(2e-13)
CNC6920 emb|CAB85690.1| mismatched base pair and cruciform DNA recognition protein [Agaricus bisporus] E(3e-21)
HSP1201 HSP12 [Cryptococcus bacillisporus] E(8e-28)
CNI2260 germline RNA helicase-4 [Caenorhabditis elegans] E(0.096)
CNL1080 metallothionein [Tegillarca granosa] E(0.019)
YOR052C SD13256p [Drosophila melanogaster] E(0.032)
CNC1080 E(1e-72)
CNO6735 E(2e-40)
CNI2980 E(0.0003)
PDC1 pyruvate decarboxylase [Saccharomyces kluyveri] E(1e-135)
CNO6725 fructosamine-3-kinase-related protein [Mus musculus] E(1e-11)
FHB1 flavohemoglobin [Cryptococcus neoformans var. grubii] E(0)
CNK3360 COG3957: Phosphoketolase [Synechococcus elongatus PCC 7942] E(0)
ADH302-B mannitol-1-phosphate dehydrogenase [Cryptococcus neoformans var. neoformans] E(0)
ADH302-A mannitol-1-phosphate dehydrogenase [Cryptococcus neoformans var. neoformans] E(0)
YMR226C02 emb|CAE81960.1| related to NAD(P)H-dependent oxidoreductase [Neurospora crassa] E(2e-44)
CNBN0320-A E(3e-07)
CNM1930 E(0)
CNN0310 E(3e-07)
CNN1140 E(4e-50)
YSA1 SPBP35G2.12 [Schizosaccharomyces pombe] E(5e-31)
CNO6833 conidiation protein E(7e-06)
CNN0040 rds1 [Schizosaccharomyces pombe] E(5e-99)
CNE4180 E(1e-124)
CNA2870 COG2846: Regulator of cell morphogenesis and NO signaling [Nostoc punctiforme] E(1e-10)
BDH1-A SPBC1198.01 [Schizosaccharomyces pombe] E(2e-83)
CNA8080 COG0768: Cell division protein FtsI/penicillin-binding protein 2 [Cytophaga hutchinsonii] E(6e-05)
CNA0790 similar to hornerin [Rattus norvegicus] E(5e-07)
CNG3790 E95206 cell wall surface anchor family protein [imported] - Streptococcus pneumoniae (strain TIGR4) E(0.014)
+4.6
-4.6
Ratio (log2)

antifungal drugs (including amphotericin B and azoles). The apparently reduced activity of this pathway after a temperature shift may limit the effectiveness of such drugs in febrile patients (although it should be noted that temperatures exceeding 40°C are unlikely to occur in human patients).

We next examined steady-state transcripts in exponential cells cultured at 30 versus 37°C, since it has been widely proposed that growth at high temperature may constitute a specific specialization of human fungal pathogens. Statistical analysis revealed differences that were reproducible albeit quantitatively more subtle than those seen upon heat shock. No obvious broad patterns of gene functions were apparent in the high temperature-regulated gene sets. Whether any of these genes are related to the ability of *C. neoformans* to survive high-temperature remains to be determined. Most of these genes have homologs in non-pathogenic yeast such as *S. cerevisiae*. A handful of *C. neoformans*-specific genes are temperature-regulated; these are therefore candidates for specialized genes involved in high-temperature growth.

A previous study examined temperature-dependent gene expression in the H99 strain using a partial-genome shotgun DNA microarray containing random genomic fragments (Kraus et al. 2004). We found little overlap between the genes identified in this study as being induced by heat shock or being differentially expressed at steady state at 37 versus 30°C and those identified by Kraus et al. as being regulated by temperature. This could be due to differences in the type of microarray used and the experimental design. Kraus et al. utilized shotgun microarrays which, in some cases, can make it difficult to determine whether a spot corresponds uniquely with a single gene. Furthermore, many of the genes described as temperature regulated by Kraus et al. were identified as such if their expression changed at any point during a 12 h. time course after a shift from 25 to 37°C. These include genes, such as *RDS1* (possible stress response gene), *ILV2* and *ILV5* (amino acid biosynthesis), and *URA2* (pyrimidine biosynthesis), which display changes in expression very late in the time course (9–12 h after the shift to 37°C). However, data for control cultures grown to the same densities at 25°C were not shown. It seems possible that some of the observed profiles, such as the repression of genes involved in amino acid and pyrimidine biosynthesis, may therefore be due to changes unrelated to temperature that occurred during the time course, such as nutrient depletion. Our temperature shift experiments were done over a short 30 min period that should have mitigated such effects and the steady-state temperature data presented here compare exponential cultures grown to the same optical density.

Nitrosative stress. We have described the genomic response to nitrosative stress, a major mechanism by which pathogens are killed by macrophages during infection. Recent microarray studies of NO responses in the non-pathogenic fungus *S. cerevisiae* and in the ascomycete human pathogenic fungi *H. capsulatum* and *C. albicans* revealed their genomic responses to nitrosative stress (Hromatka et al. 2005; Nittler et al. 2005; Sarver and DeRisi 2005). All three fungi displayed induction of their respective flavohemoglobin/nitric oxide dioxygenase gene. Correspondingly, we observed induction of the corresponding *C. neoformans gene*, *FHB1*. In *H. capsulatum* and *C. albicans*, an alternative oxidase gene, *AOX1* is induced by NO, which we also observed in *C. neoformans*. Consistent with the idea that the genes induced by NO play a role in infection, previous studies of knockouts of *FHB1* and *AOX1* demonstrates that both genes are necessary for full virulence (Akhter et al. 2003; de Jesus-Berrios et al. 2003). Aside from the induction of *FHB1* and *AOX1* in response to NO treatment of cultures, the response in *C. neoformans* appears divergent. Three notable features that distinguish the NO response of *C. neoformans* from those reported in other fungi are 1) the induction of a previously undescribed gene, *CNA2870*, related to the *E. coli* NO resistance gene *ytfE*, 2) the induction of a mannitol dehydrogenase, *MPD1/ADH302* thought to be involved in the biosynthesis of the polyol mannitol (Suvarna et al. 2000), a potent antioxidant, and 3) the induction of a *C. neoformans*-specific family of four genes that were previously undescribed.

Both *ytfE* and CNA2870 contains two HHE or hemerythrin cation binding motifs (Yeats et al. 2003). By analogy to the heme-binding flavohemoglobins, these may be Fe(II)-binding proteins involved in the detoxification of NO. The induction of both *ytfE* and *CNA2870* by NO and the role of *ytfE* in NO resistance in *E. coli* is consistent with this possibility (Justino et al. 2005). Our preliminary studies of a knockout mutant of *CNA2870* have not uncovered a role for this protein in NO resistance (C. Chen and H. D. M., unpublished observations). Moreover, this mutant did not display a defect in virulence in an ongoing signature-tagged mutagenesis screen (O. Liu and H. D. M., unpublished observations). Thus either *CNA2870* does not play a role in NO detoxification or virulence, or, more likely, it is redundant with other factors induced by NO such as *FHB1*.

Mannitol synthesis has long been known to be a characteristic of *C. neoformans*, and an enzyme capable of synthesizing mannitol, Mpd1/Adh302, has been purified and cloned (Suvarna et al. 2000). Mannitol is produced during infection, and cerebrospinal fluid of infected patients contain concentrations in the range of milligrams per ml (Megson et al. 1996). Although mannitol synthesis has been proposed to be important for pathogenesis, no genetic studies have been reported that support this hypothesis. Our annotation shows that two genes, *CNJ0590* and *ADH301*, display strong homology to *MPD1/ADH302*, raising the

possibility that these genes function redundantly to produce mannitol. Thus, the generation of double or triple knockout strains may be necessary to test the role of mannitol in NO resistance and *C. neoformans* virulence.

As described in the Results, we identified a four gene family whose mRNAs are induced in response to NO treatment of cultures. Intriguingly, these genes are specific to *C. neoformans*, and are even missing from the sequenced genomes of two basidiomycete relatives of *C. neoformans*: *Ustilago maydis* and *Phanerochaete chrysosporium.* As such, they could represent a pathogen-specific specialization involved in virulence. Genetic and biochemical investigation of this family be will be required to test their potential role in virulence and to determine the precise molecular function of these proteins.

After this work was completed, the whole-genome transcriptional response of the serotype A strain H99 to a single dose of acidified nitrite was recently reported (Missall et al. 2006). Prominent changes were seen in the expression of amino acid biosynthetic genes as well as genes involved in respiration, cell wall synthesis, transport, and stress responses. Overall, 205 genes were reported to be reduced in expression while 216 increased in expression. In contrast, our study identified only 25 genes that displayed a dose-dependent induction by DPTA-NONOate, and we found no genes that displayed a dose-dependent reduction in expression (data not shown). The simpler transcriptional profile for the *C. neoformans* var *grubii* response to NO obtained in this study could be due to two factors. First, our experiments were performed in rich media while the previous study utilized minimal media. This may explain the lack of induction in our studies of many metabolic genes such as the amino acid biosynthetic genes. Second, we utilized multiple doses of a more specific NO donor (DPTA-NONOate) in this study versus the choice of a single concentration of acidified nitrite in the other study. (Missall et al. 2006).

We found that NO-induced genes display reduced expression at higher temperatures. Although the reason for this correlation is not obvious, it is consistent with the possibility that the genes identified here respond in distinct ways to a wide range of environmental inputs and thus may play roles in multiple biological responses. Future genetic analysis of their regulation and functions should shed light on this issue.

Finally, it is important to note that the relevance of the work presented here to the responses of *C. neoformans* in vivo will ultimately require accurate profiling of pathogen responese in a mammalian host. The microarray and annotation resources described represent a first step towards understanding the genomic responses of the host-pathogen relationship.

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