

## Identification of *Cryptococcus neoformans* Temperature-Regulated Genes with a Genomic-DNA Microarray†

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**The ability to survive and proliferate at 37°C is an essential virulence attribute of pathogenic microorganisms. A partial-genome microarray was used to profile gene expression in the human-pathogenic fungus *Cryptococcus neoformans* during growth at 37°C. Genes with orthologs involved in stress responses were induced during growth at 37°C, suggesting that a conserved transcriptional program is used by *C. neoformans* to alter gene expression during stressful conditions. A gene encoding the transcription factor homolog Mga2 was induced at 37°C and found to be important for high-temperature growth. Genes encoding fatty acid biosynthetic enzymes were identified as potential targets of Mga2, suggesting that membrane remodeling is an important component of adaptation to high growth temperatures. *mga2*Δ mutants were extremely sensitive to the ergosterol synthesis inhibitor fluconazole, indicating a coordination of the synthesis of membrane component precursors. Unexpectedly, genes involved in amino acid and pyrimidine biosynthesis were repressed at 37°C, but components of these pathways were found to be required for high-temperature growth. Our findings demonstrate the utility of even partial-genome microarrays for delineating regulatory cascades that contribute to microbial pathogenesis.**

Pathogenic microorganisms must be able to survive at the physiological temperature of the host in order to proliferate and cause disease. A hallmark of human-pathogenic fungi is their ability to grow at the human body temperature of 37°C. The ability to grow at temperatures as high as 42°C is an established virulence factor for pathogenic *Saccharomyces cerevisiae* strains (42), and growth at 37°C is associated with a mycelium- to yeast-form switch that is essential for the virulence of the human pathogens *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii* (8, 41). Thermally regulated dimorphism occurs in *Cryptococcus neoformans* during mating and haploid fruiting and in self-fertile diploid strains but is not a requirement for pathogenesis (4, 47). Although a temperature-dependent morphogenetic switch is not essential for the virulence of *C. neoformans*, the ability to grow at 37°C is considered an established virulence attribute (9). At least 38 *Cryptococcus* species other than *C. neoformans* have been described, and *C. neoformans* is the only species that can consistently grow at mammalian body temperatures and is the only common human pathogen (9).

*C. neoformans* is a basidiomycete that is the most common cause of fungal meningitis. *C. neoformans* infects both immunocompetent and immunocompromised hosts, but disease is generally expressed when the host has impaired immunity. Virulence factors for *C. neoformans* that have been genetically defined include (i) the ability to synthesize the antioxidant

pigment melanin, (ii) the production of an antiphagocytic polysaccharide capsule, (iii) urease and phospholipase production, and (iv) the ability to survive and proliferate at 37°C (13, 14, 37, 45). Proteins that have been identified in *C. neoformans* as being essential or important for growth at high temperatures include Ras1, the p21-activated kinase (PAK) kinase Ste20, the phospholipid-binding protein Cts1, the vacuolar ATPase Vph1, the thiol peroxidase Tsa1, the cell integrity mitogen-activated protein (MAP) kinase Mpk1, and the Ca<sup>2+</sup>/calmodulin-dependent phosphatase calcineurin (3, 21, 22, 34, 43, 45, 57). Deletion or disruption of the genes encoding each of these proteins results in either attenuation or complete loss of virulence in mammalian models of cryptococcosis.

The increasing availability of entire genome sequences has resulted in the proliferation of large-scale techniques for studying gene function. For fungal systems where complete genome sequences are not yet available, these techniques have been adapted to provide valuable information about gene expression and function. A major strength of these approaches is that they can provide information that may be difficult to obtain by traditional techniques of genetic analysis; they can also establish a foundation for further study when complete genome sequences become available. cDNA library subtraction and serial analysis of gene expression (SAGE) have been used to identify *C. neoformans* genes that are preferentially transcribed under conditions of high-temperature growth and in animal models of infection (2, 46, 49, 50, 52). Furthermore, DNA microarrays were adapted for studying phase-regulated gene expression in *H. capsulatum* prior to completion of the genome sequence (30).

In this study, we used a similar approach and constructed a shotgun genomic-DNA microarray to assess *C. neoformans* transcription during growth at the human physiological tem-

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perature of 37°C. Implementation of this 6,274-element array resulted in the identification of 239 genes that displayed differential expression during growth at 37 and 25°C. One differentially regulated gene, *MGA2*, encodes a transcription factor homolog that is required for normal growth in vitro at a range of growth temperatures. Microarray experiments were performed to identify candidate target genes of the *MGA2* transcription factor which encode orthologs of components of the fatty acid biosynthesis machinery. *mga2Δ* mutants are hypersensitive to the ergosterol synthesis inhibitor fluconazole, suggesting a coordination between the metabolism of fatty acid membrane components and the metabolism of sterol membrane components. In addition, we report the unexpected finding that the transcription of genes in amino acid and pyrimidine biosynthetic pathways decreases at 37°C, yet mutations in components of these pathways result in a growth defect at high temperatures. Our studies highlight the potential of genomic approaches for revealing molecular principles of microbial pathogenesis.

## MATERIALS AND METHODS

**Strains and media.** The strains used were as follows: H99 (*MATα*), JF99 (*MATα ura5*), PK23 (*MATα ura5 [URA5]*), PK28 (*MATα mga2Δ::nat*), and AI34 (*MATα clc1Δ::nat*). The growth medium used was 2% yeast extract–1% peptone–2% dextrose (YPD). For solid media, 2% agar was added. Nourseothricin was added to solid media at 100 μg/ml, and fluconazole was added at 10 mg/ml.

An allele to delete the *MGA2* gene and replace it with the nourseothricin resistance cassette by homologous recombination was generated as previously described (16, 23). The construct was transformed into strain H99 by biolistic transformation, and nourseothricin-resistant transformants were screened for deletion of the *MGA2* open reading frame (ORF) by PCR and Southern analysis of genomic DNA. The *MGA2* gene was reintroduced by transforming a plasmid containing the entire *MGA2* gene plus 1 kb each of 5' and 3' flanking DNAs linked to a cassette that confers resistance to G418 (23). Transformants were selected on G418 and tested for the ability to grow in the presence of 10 μg of fluconazole/ml.

**Array construction, sequencing, and annotation.** The array consists of two components: cDNA clones from strains JEC21 and H99 amplified by PCR with gene-specific primers (130 elements) and shotgun H99 genomic clones (6,144 elements). The H99 genomic library was constructed as follows: 3 to 5 μg of genomic DNA was sheared with a Hydroshear device (Gene Machines) to generate 1.5- to 3-kb DNA fragments, which were cloned as described previously (39), picked and placed in Luria-Bertani-Hogness medium, and stored at –80°C. For sequencing, clones were grown in 96-well plates containing Terrific broth medium in a Higo orbital shaker (Gene Machines), and DNA was isolated by using a RevPrep robot (Gene Machines). Sequencing reactions were performed by using an MJ Research thermal cycler with standard BigDye chemistry (Applied Biosystems). Samples were analyzed on a PE3700 96-capillary sequencer, and data were analyzed and assembled by using the Pare/Phrased sequence package.

PCRs to amplify genomic library inserts were performed by using 64 96-well plates containing bacterial cultures, each of which harbored an independent library transformant. A total of 2 μl of bacterial stock was used for PCR amplification of inserts with vector-specific primers. PCR products were analyzed on 1% agarose gels, precipitated, washed, and printed on polylysine-coated glass slides at the Duke Center for Genome Technology.

Array annotation was performed by comparing array clone sequences with The Institute for Genomic Research (TIGR) JEC21 genome database by using BLASTx (21 January 2004 version, *C. neoformans* Genome Project). Array clones were assigned JEC21 locus names only when a single BLAST match with an expect value (E value) of between  $10^{-50}$  and 0 was found. The median E value for all array element sequences compared to JEC21 sequences was  $10^{-104}$ ; however, a large majority of the highly scoring segment pairs produced an E value of 0. Complete annotations, including E values and accession numbers of orthologs, were completed only for elements of interest based on hybridization data. Gene names were assigned to some array clones based on the names of orthologs from other fungal species.

**Culture conditions and RNA preparation.** H99 cultures for the 37°C time course were inoculated into YPD medium and grown at 25°C for 16 h with shaking. The cultures were diluted to an optical density at 600 nm ( $OD_{600}$ ) of 0.2 and returned to 25°C until the culture density reached an  $OD_{600}$  of 0.8 (approximately 4 h). A portion of the cultures was harvested by centrifugation at this time, which was considered the 0-h time point. Cells were diluted in prewarmed YPD medium to an  $OD_{600}$  of 0.1 and incubated for 12 h. Portions of the cultures were harvested every 3 h during the time course. For steady-state experiments, the cultures were inoculated into YPD medium and grown for 16 h at 25 or 37°C, diluted to an  $OD_{600}$  of 0.2 with fresh YPD medium, and incubated at 25 or 37°C until an  $OD_{600}$  of 0.8 was reached. For the comparison of wild-type and *mga2Δ* mutant cells, cultures from isogenic strains H99 (wild type) and PK28 (*mga2Δ::nat*) were inoculated into YPD medium, grown for 16 h at 30°C, diluted to an  $OD_{600}$  of 0.2 with fresh YPD medium, and incubated at 30°C until an  $OD_{600}$  of 0.8 was reached. Cells from all experiments were harvested by centrifugation, frozen in ethanol-dry ice, and lyophilized for RNA preparation. RNA was prepared by using TRIzol reagent (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions.

**Microarray hybridization.** Fluorescence-labeled cDNA was generated by incorporating amino-allyl-dUTP during reverse transcription of 10 μg of total RNA. Cy3 or Cy5 dye (Amersham, Piscataway, N.J.) was coupled to the amino-allyl group as described previously (18). For the 37°C time course, equal amounts of samples from all time points were pooled to generate a reference sample, which was labeled with Cy3. A sample from each time point was individually labeled with Cy5 and competitively hybridized against the reference sample. For steady-state experiments, samples from cells grown at 25°C were labeled with Cy3 and samples from cells grown at 37°C were labeled with Cy5. Dyes were switched for the reverse fluor control. For the comparison of wild-type and *mga2Δ* mutant cells, three hybridizations were performed by using wild-type cDNA labeled with Cy5 and *mga2Δ* cDNA labeled with Cy3. In parallel, reverse fluor control hybridization was performed for each of these hybridizations.

**Data analysis.** Arrays were scanned on a GenePix 4000B scanner (Axon Instruments, Foster City, Calif.) and analyzed by using GenePix Pro (version 4.0), Cluster, TreeView, and CryptoArray, a Microsoft Excel macro for normalizing and formatting data (19). Data were normalized for each array element by dividing the background-corrected median pixel intensity of each spot by the sum of the median intensities of all spots on the array (6). This normalization was performed individually for data generated at each wavelength. Gene expression ratios were determined by dividing the normalized intensity in the red channel (635 nm) by the intensity in the green channel (532 nm) for each element. CryptoArray also parses the data to exclude ribosomal DNA and mitochondrial DNA elements and provides the data in the appropriate format for Cluster. For the time course experiments, ratio measurements for each time point were divided by the corresponding ratio measurements for the 0-h time point to cancel the pooled reference sample.

**Northern analysis.** Samples of 10 μg of total RNA were separated on 1% agarose gels containing 1.85% formaldehyde and transferred to Nytran SuPer-Charge membranes (Schleicher & Schuell, Keene, N.H.). The probes used were generated by PCR amplification of cDNA or genomic DNA with gene-specific primers. For genes from the genomic-DNA portion of the array (*MGA2*, *SLG1*, *CLC1*, and *RDS1*), ORF-specific probes were designed and used for Northern analysis. For known *C. neoformans* genes included on the microarray (*LLV5*, *SMG1*, *FHB1*, and *AOX1*), the same PCR products that were printed on the array were used as probes for Northern analysis. Probe labeling and hybridization were performed as described previously (29).

## RESULTS

**Array construction.** To assess *C. neoformans* gene expression on a large scale, we constructed a 6,274-element shotgun genomic-DNA microarray by using PCR to amplify clones from a library of genomic-DNA fragments. Genomic libraries that were constructed for the *C. neoformans* serotype A strain H99 sequencing project were used as a platform for PCR amplification of genomic DNA. These libraries contain genomic-DNA inserts ranging from 1.6 to 3.2 kb in length. Inserts of this size are likely to contain coding regions and therefore are suitable for use as microarray probe elements. A total of 6,144 genomic clones were amplified and comprise approximately 12 Mb of *C. neoformans* genomic DNA. Two different methods

were used to estimate that this portion of the array represents approximately  $0.5\times$  coverage of the 19-Mb *C. neoformans* genome (38, 59). Both ribosomal DNA and mitochondrial DNA clones are present in the genomic library and therefore are also present on the microarray (139 and 325 elements, respectively). For designation of genes within each clone, the annotation information provided by the sequencing project for closely related *C. neoformans* strain JEC21 was an essential resource. We performed BLASTx searches of each array element against the JEC21 genome database, and a majority of array elements yielded only one significant BLAST match. Based on the sizes of the inserts in the genomic library, it is possible that multiple ORFs are present on a given array element. Elements predicted to contain more than one ORF by the BLASTx comparison to the JEC21 genome database were excluded from further analysis. In addition to the genomic-DNA probes, 130 PCR products, each containing a single known *C. neoformans* gene, were also included on the array (see Table S1 in the supplemental material).

**Assessing temperature-regulated transcription.** We used two approaches to identify genes that display differential expression during growth at 25 and 37°C. First, we shifted *C. neoformans* cultures from 25 to 37°C and assessed gene expression at multiple time points. Time course experiments were performed twice, and representative results are shown in the cluster analysis in Fig. 1. Second, we compared steady-state gene expression in cultures of cells actively growing in logarithmic phase at exclusively 25 or 37°C. These experiments were performed in triplicate, and a reverse fluor control was used in the second trial. By using both temperature shift and steady-state experiments, we reasoned that genes required for both the initiation and the maintenance of growth at 37°C would be revealed in the microarray data analysis. For each experiment, samples were collected and total RNA was prepared. cDNAs were generated from the total RNA samples and differentially labeled with Cy3 (pooled reference sample for the time course experiment and 25°C samples for the steady-state experiment) or Cy5 (individual time point samples for the time course experiment and 37°C samples for the steady-state experiment). These cDNAs were subjected to competitive hybridization on the genomic-DNA microarray. The data for the time course and steady-state experiments are provided in Table S2 in the supplemental material.

Initial annotation of the elements on the microarray was performed by using BLASTx searches of the *C. neoformans* JEC21 genome annotation database. For the 239 elements that showed differential expression in at least one experiment, a more complete analysis of the clone identity was performed by using BLASTx searches of the nonredundant database at the National Center for Biotechnology Information. For most cases, our annotation of these clones was similar to that of the corresponding locus in the *C. neoformans* JEC21 genome annotation database. Genes with no published name were assigned one if other fungal orthologs had the same designation or were assigned a JEC21 locus designation if no consensus gene name exists. Several clones identified in our experiments did not have significant BLAST matches to the JEC21 genome database or any other database, and these clones were not included in the list of differentially expressed genes.

**Genes induced at 37°C.** Table 1 summarizes the genes that were induced during growth at 37°C compared to 25°C. The induction values reported represent the values at which the greatest difference between the 37°C culture and the 25°C culture was observed in either the time course or the steady-state experiment. Genes with orthologs that have known roles in responding to stress were induced at 37°C, including an ortholog of a WSC domain protein gene (*SLG1*), a chitin synthase gene, a trehalose synthase gene, a trehalose-associated protein kinase gene (*RIM15*), and genes involved in promoting resistance to reactive oxygen species (catalases and oxidases). In *S. cerevisiae*, these and other related genes are induced by heat shock and other types of cellular stress as part of a general stress response (25).

We performed a cluster analysis to group genes according to similarities in expression profiles (Fig. 1) (19). Genes whose expression changed at least threefold in at least one experiment were analyzed by hierarchical clustering. Cluster 1 contains genes that were induced in both the time course and the steady-state experiments and includes the WSC domain ortholog *SLG1* and the *MGA2* gene, encoding a transcription activator homolog. WSC domain proteins are a family of cell surface proteins that are important for adaptation to heat stress and may act to sense stress-induced perturbations of the cell surface and to signal via the protein kinase C-activated cell integrity MAP kinase pathway (27, 55). Cluster 2 contains genes that were induced at 37°C in the time course experiment, with peak induction occurring at late time points after the shift to 37°C (9 and 12 h). Genes in this cluster include an ortholog of the *S. pombe* stress response gene *rds1*<sup>+</sup>. The expression of *rds1*<sup>+</sup> is induced in response to heat stress in *S. pombe*, but no function has as yet been reported for this gene (40). Putative orthologs of *rds1*<sup>+</sup> are present in the *Neurospora crassa*, *Magnaporthe grisea*, *Aspergillus nidulans*, *Fusarium graminearum*, and *Ustilago maydis* genomes (24; Center for Genome Research, www.broad.mit.edu). Cluster 3 includes multiple clones containing the *CLC1* gene, which displays strong induction at 37°C at early time points but less pronounced differences in gene expression at later time points and in the steady-state experiment. *CLC1* encodes a voltage-gated chloride channel important for melanin synthesis, and no role for *CLC1* in high-temperature growth has been reported (31, 61). Northern analysis confirmed that the *MGA2*, *SLG1*, *CLC1*, *RDS1*, and *SMG1* genes were more highly expressed at 37°C than at 25°C in either the time course experiment or the steady-state experiment (Fig. 2).

**Genes repressed at 37°C.** Table 1 summarizes the genes that were repressed during growth at 37°C compared to 25°C. Strikingly, genes that participate in amino acid biosynthesis (*SPE3-LYS9*, *ILV2*, and *ILV5*) and pyrimidine biosynthesis (CTP synthase; *URA2*) displayed significantly reduced expression during growth at 37°C compared to 25°C. We also observed a strong reduction in the expression of *FHB1*, encoding a flavohemoglobin important for resistance to nitrosative stress (17). Furthermore, three different ribosomal protein genes were repressed at 37°C compared to 25°C. The *AOX1* gene, encoding an alternative oxidase, was previously identified by a cDNA library subtraction technique as a gene that is preferentially transcribed at 37°C (2). However, we found that the expression of the *AOX1* gene was reduced both at early time points after

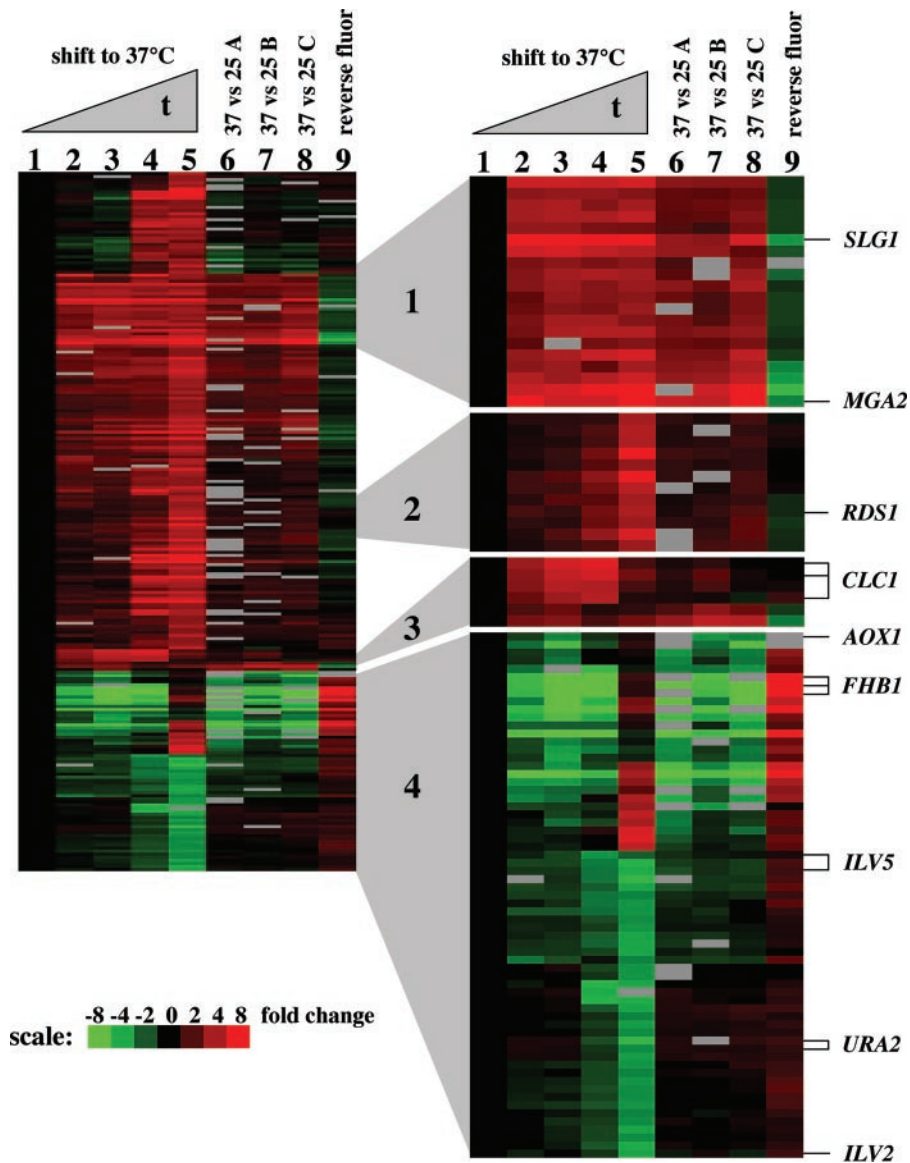


FIG. 1. Gene expression profiles in temperature shift and steady-state microarray experiments. Elements whose expression changed at least threefold in at least one time point after the shift to 37°C or in the steady-state experiment were analyzed by hierarchical clustering. Each lane represents a microarray experiment, and each row represents the expression of an element on the array. For the temperature shift experiment, cDNAs from the 0-, 3-, 6-, 9-, and 12-h time points were labeled with Cy5 (red), and a reference sample consisting of equal amounts of samples from all time points was labeled with Cy3 (green). A sample from each time point was competitively hybridized against the reference sample on the microarray. The time course experiment was normalized against the 0-h time point as described in Materials and Methods. In the 37 versus 25°C experiments, the 37°C samples were labeled with Cy5 and the 25°C samples were labeled with Cy3. The reverse fluor experiment was carried out with samples labeled "B," and the dye labeling was reversed. For all experiments, red indicates increased gene expression and green indicates decreased gene expression. Missing data are represented in gray. Genes with more than two data points missing were removed from the cluster analysis. Clusters of interest have been enlarged and are discussed in the text.

the shift from 25 to 37°C and at 37°C compared to 25°C in the steady-state experiment (Fig. 1, cluster 4). The expression of *AOX1* was greater after 12 h of growth at 37°C than at the 0-h time point, indicating that growth phase or culture density influences the expression of *AOX1*. Northern analysis confirmed that *FHB1*, *AOX1*, and *ILV5* were expressed at lower levels during growth at 37°C than during growth at 25°C in the time course experiment or the steady-state experiment (Fig. 2).

**Correlation of gene expression profile and mutant phenotype.** One goal of this study was to identify genes important for

survival at 37°C based on expression profiles after a shift to 37°C or in a steady-state experiment. We identified a gene, *MGA2*, encoding a transcription factor homolog, whose expression was markedly induced after the shift from 25 to 37°C. In *S. cerevisiae*, Mga2 and its paralog, Spt23, are involved in regulating transcription in response to cold shock and hypoxia (32, 44). Both transcription factors activate the expression of *OLE1*, encoding a fatty acid desaturase, suggesting that cell and/or organelle membranes may require remodeling for responses to growth temperature changes (60).

TABLE 1. Temperature-regulated genes

Effect on gene at 37°C	Gene name <sup>a</sup>	Annotation	E value <sup>b</sup>	Genbank accession no. of ortholog	Fold change <sup>c</sup>
Induction	CNC05450	Putative short-chain dehydrogenase	6.6E-38	CAB86467	16
	CNA06780	WSC domain protein ( <i>SLG1</i> )	1.2E-63		12
	CNL00800	Amino acid permease	3.1E-102	CAA65074	11
	<i>MGA2</i>	Transcriptional activator	2.9E-20	CAB92703	11
	CNC06420	Hypothetical protein	1.0E-73	CAC36937	8.2
	CND03740	Catalase, putative	8.0E-136	AAG45152	7.9
	CNE01110	Predicted protein	2.7E-80	CAD37156	7.5
	<i>PPS1</i>	Putative dual-specificity phosphatase	2.4E-42	CAD21139	7
	<i>THR4</i>	Threonine synthase		AAK83373	7
	<i>CLC1</i>	Voltage-gated chloride channel		AAO73005	6.3
	CNA02540	S1/P1 nuclease	2.2E-29	BAB96801	6
	<i>GLT1</i>	Glutamate synthase	0E+00	CAA61505	5.8
	CNH04480	Trehalose synthesis	1.3E-90	BAA31349	5.3
	<i>DUR3</i>	Urea transporter	1.1E-85	AAB65069	5.1
	CNI03330	O-Methyl transferase	1.2E-36	AAK44417	4.9
	<i>LYS2</i>	Amino adipate semialdehyde dehydrogenase	6.4E-12	CAA85072	4.9
	CND06130	Unknown			4.5
	<i>SMG1</i>	Putative aryl alcohol oxidase		AAM83033	4.5
	CNI01590	Superoxide dismutase	1.2E-69	AAK82369	4.5
	CNF03810	Unknown			4.4
	CNA04360	Unknown			4.3
	CNA07560	Unknown			4.2
	CNA05600	Catalase A related	1.7E-170	AAK15808	4.2
	<i>RDS1</i>	Stress response protein	6.9E-98	CAB52275	4.2
	CNE00600	Carboxypeptidase F	5.9E-172	CAA56075	4.1
	CHD02910	Malate synthase	7.0E-185	AABD01988	4.1
	CNB05770	Putative ABC transporter	0E+00	CAD10327	4
	CNM01770	Similar to benzodiazepine receptor	8.1E-17	CAA22182	4
	CNL01900	Unknown			3.9
	CNK00270	UDP-N-acetyl-glucosamine-1-P transferase	6.9E-98	CAA67366	3.9
	CNB00750	Unknown			3.7
	CNB02490	Putative short-chain dehydrogenase	5.8E-62	AAK89074	3.7
	CNB04620	Similar to HSCARG protein	6.5E-31	AAH30039	3.7
	CNE04710	Unknown			3.6
	CNC00290	Putative multiple-drug resistance protein	1.3E-128	CAD29613	3.6
	CNB00710	Unknown			3.5
	CNF04870	Proline oxidase	2.6E-21	AAB88789	3.4
	CNH02020	Conserved hypothetical protein	8.5E-19	CAD37054	3.4
	CND01100	12-Oxophytodienoate reductase	2.6E-68	CAB43506	3.4
	<i>RIM15</i>	Trehalose-associated protein kinase	9.6E-131	CAD27468	3.3
	<i>PMAI</i>	Plasma membrane ATPase	1.2E-285	AAK94188	3.2
	CNE00040	Putative transketolase	5E-38	AAD36826	3.1
	CNF05280	Unknown			3.1
	CNF00410	Unknown			3.1
	CNA05300	Chitin synthase 6	2.1E-302	AAB84285	3.1
	CNI00390	C <sub>2</sub> H <sub>2</sub> zinc finger protein	2.4E-14	CAB63541	3
	CNFp2250	Unknown			3
	CNH03900	Unknown			3
	<i>CHL1</i>	DNA helicase	1.1E-122	AAB18749	3
	Repression	<i>FHB1</i>	Flavohemoglobin		AAP41066
CNG00908		Unknown			-11
CNB05100		Unknown			-8.3
<i>AOX1</i>		Alternative oxidase		AAM22475	-7.1
CNA01720		Unknown			-5.3
<i>ILV5</i>		Acetohydroxyacid reductoisomerase			-4.5
CNC06370		Hypothetical WD-repeat protein	3.6E-101	CAB11489	-4.5
CNH00300		Ribosomal protein P.0	5.0E-88	AAK11262	-3.9
<i>ILV2</i>		Acetolactate synthase		AAR29084	-3.8
CNC00200		CTP synthase	1.4E-193	CAA15716	-3.7
CND01770		Ribosomal protein S26E	2.9E-35	AAC95384	-3.6
<i>RPL22</i>		Ribosomal protein L22		AAN75181	-3.5
<i>PKR1</i>		Protein kinase A regulatory subunit		AAG30146	-3.4
CND04910		Putative dityrosine transporter	4.6E-41	CAD70885	-3.4
CNM01290		Unknown			-3.4
CNE02070		HpcH/HpaI aldolase family	1.2E-38	PF03328	-3.4
<i>PAN6</i>		Pantothenate biosynthesis		AAN75165	-3.3
CNK02850		Putative allantoin transporter	4.8E-58	CAA22656	-3.3
<i>URA2</i>		Pyrimidine biosynthesis	0E+00	CAA91130	-3.2
CNC04280		Pumilio family RNA binding protein	1.6E-69	CAB03616	-3.2
CND04330		Cdc20/fizzy	9.0E-101	CAB57442	-3.2
<i>SPE3-LYS9</i>		Chimeric spermidine synthase-saccharopine dehydrogenase		AAK83327	-3.2

<sup>a</sup> Named genes include known genes with specifically amplified PCR products included on the array or genomic clones of previously identified *C. neoformans* genes (no E value is provided) and clones with significant homology to other fungal orthologs that have a gene name. Genes that do not fit these criteria were given the *C. neoformans* JEC21 annotation database locus name.

<sup>b</sup> E value comparing *C. neoformans* gene and its ortholog, using information from BLASTx searches of the nonredundant database at the National Center for Biotechnology Information and from the *C. neoformans* JEC21 annotation database (<http://www.tigr.org/tdb/e2k1/cna1/cna1.shtml>).

<sup>c</sup> Peak fold induction ratio from the time course and steady-state experiments. Genes displaying both induction and repression over the course of the experiments were categorized as induced or reduced based on cluster analysis (Fig. 1).

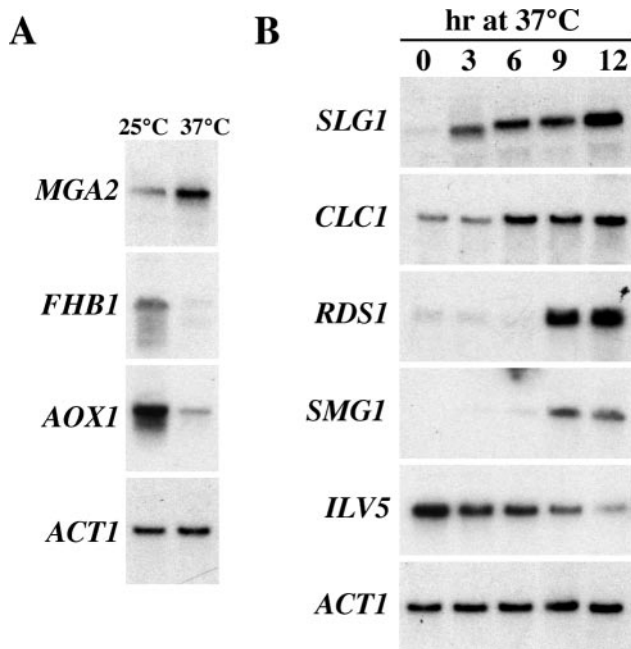


FIG. 2. Northern analysis of temperature-regulated expression. (A) Total RNA from 25°C samples and 37°C samples was probed with gene-specific probes. (B) Total RNA from 0-, 3-, 6-, 9-, and 12-h time points was probed with gene-specific probes. *ACT1* was included as a loading control in both experiments.

The *C. neoformans* ortholog of *MGA2* was deleted by homologous recombination, and its role in promoting high-temperature growth was assessed. Compared to the isogenic wild-type strain H99, the *mga2Δ* mutant strain displayed slow growth at 25 and 30°C (Fig. 3A). The *mga2Δ* growth defect was more pronounced at 37 and 39°C (Fig. 3), suggesting that this transcription factor homolog plays a significant role in promoting high-temperature growth. Because fatty acid metabolism and sterol biosynthesis are coordinated in *S. cerevisiae* (11, 36), we tested whether the *mga2Δ* mutation had an effect on *C. neoformans* growth in the presence of the ergosterol biosynthesis inhibitor fluconazole. The *mga2Δ* mutant strain was hypersensitive to 10 μg of fluconazole/ml, while the growth of wild-type strain H99 was unaffected (Fig. 4A). In a genetic cross with a wild-type *MATa* strain, the phenotypes conferred by the *mga2Δ* mutation segregated in a 1:1 ratio of wild type to mutant, and all mutant phenotypes cosegregated 100% with the nourseothricin resistance marker (data not shown). Reintroduction of the *MGA2* gene into the *mga2Δ* mutant restored its ability to grow in the presence of 10 μg of fluconazole/ml and complemented the growth defects (Fig. 4B and data not shown). The hypersensitivity of the *mga2Δ* mutant to fluconazole likely was due to defects in sterol metabolism, because the *mga2Δ* mutant was also hypersensitive to fenpropimorph, another sterol synthesis inhibitor, but not to the protein synthesis inhibitor cycloheximide (data not shown). These findings suggest that the mechanisms coordinating fatty acid metabolism and sterol biosynthesis are conserved in *C. neoformans*.

The microarray results indicated that multiple genes in two biosynthetic pathways, pyrimidine biosynthesis and isoleucine

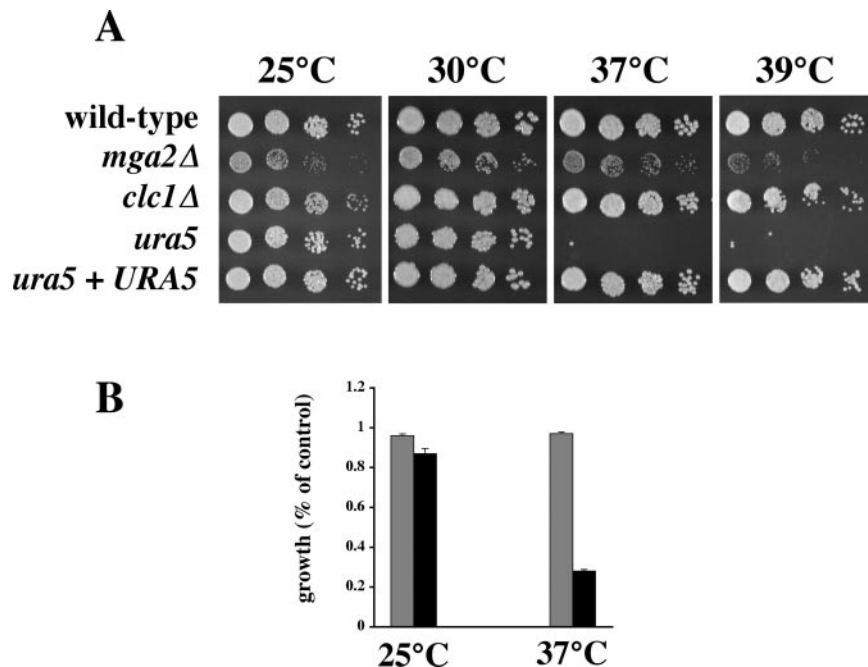


FIG. 3. Phenotypic analysis of mutant strains. (A) Fivefold serial dilutions of cultures of H99 (wild type), PK28 (*mga2Δ*), AI34 (*clc1Δ*), JF99 (*ura5*), and PK23 (*ura5* mutant JF99 with *URA5* reintroduced) were inoculated into YPD medium and grown for 3 days at the indicated temperatures. (B) Cultures of H99 (wild type) (gray bars) and PK28 (*mga2Δ*) (black bars) were inoculated into YPD medium to an  $OD_{600}$  of 0.05 and incubated at 25 or 37°C for 24 h. Growth was determined by comparing the  $OD_{600}$  of each experimental culture to that of a control culture of the same strain grown at 30°C for 24 h. Error bars represent the standard deviation of the mean from three independent experiments.

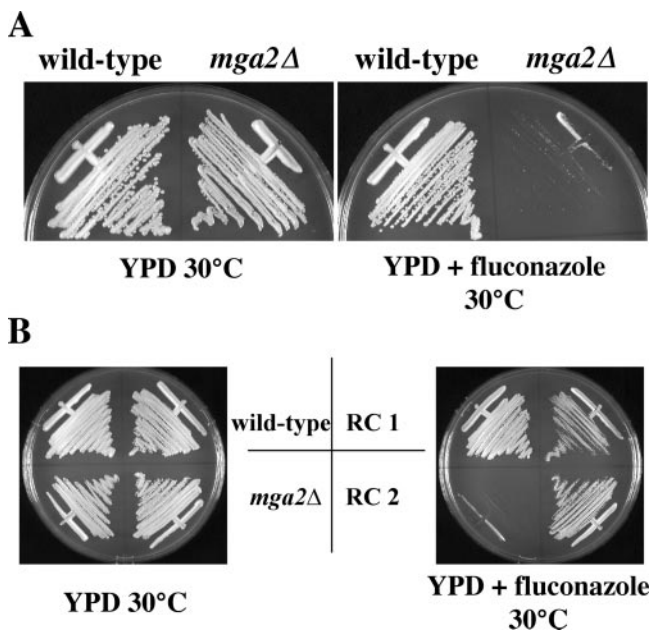


FIG. 4. Deletion of *MGA2* results in hypersensitivity to the ergosterol biosynthesis inhibitor fluconazole. (A) Cells from wild-type (H99) and *mga2Δ* mutant (PK28) strains were inoculated into YPD medium and YPD medium containing 10  $\mu$ g of fluconazole/ml and grown at 30°C for 3 days. (B) Reintroduction of *MGA2* into the *mga2Δ* mutant restores growth in the presence of fluconazole. Strain PK28 (*mga2Δ::nat*) was transformed with a plasmid containing the *MGA2* gene and a cassette that confers resistance to G418. Transformants were selected on G418 at 30°C. Two independent transformants (RC1 and RC2) were tested for growth in the presence of 10  $\mu$ g of fluconazole/ml.

or valine biosynthesis, were repressed after a shift in growth temperature from 25 to 37°C (Table 1). Interestingly, disruption of *ILV2* (encoding acetolactate synthase) confers a 37°C growth defect (33). Therefore, we examined whether a mutation in the pyrimidine biosynthetic pathway also resulted in an inability to grow at 37°C. Strain JF99 was isolated as a spontaneous 5-fluoroorotic acid (5-FOA)-resistant mutant of wild-type strain KN99a, which is isogenic with strain H99. 5-FOA is converted into the toxic compound 5-fluorouracil when the pyrimidine synthetic pathway is intact, and growth on 5-FOA occurs only when pyrimidine synthesis is defective. Strain JF99 displayed a marked growth defect compared to strain H99 when incubated at 37°C, and this growth defect at 37°C was complemented by reintroduction of the *URA5* gene (Fig. 3A). These findings demonstrate that the growth defect is due to a mutation in *URA5* and not to secondary mutations. Similar results were obtained with six other independent 5-FOA-resistant isolates (data not shown).

The *CLC1* gene encodes a voltage-gated chloride channel implicated in melanin biosynthesis and ion homeostasis (31, 61). Based on the microarray results, *CLC1* was strongly induced after a shift from 25 to 37°C (Fig. 1). This induction was confirmed by Northern analysis (Fig. 2B). Disruption of the *CLC1* gene had no effect on the ability of *C. neoformans* to grow at 37°C (Fig. 3A).

**Identifying putative targets of Mga2.** An important application of microarray technology is the identification of novel

transcription factors and the definition of their targets. Here we sought to identify targets of the Mga2 transcription factor homolog. Microarray analysis of wild-type gene expression compared to *mga2Δ* mutant gene expression was performed (Table 2; see Table S3 in the supplemental material). Notably, the orthologs of the *S. cerevisiae* *FAS1* and *ACC1* genes, encoding the beta subunit of fatty acid synthase and acetyl coenzyme A decarboxylase, respectively, displayed significantly reduced expression in the *mga2Δ* mutant. Cluster analysis of three independent wild-type and *mga2Δ* mutant microarray experiments and the corresponding reverse fluor control experiments revealed similar expression patterns for the *C. neoformans* *FAS1* and *ACC1* genes (Fig. 5A). The reduction in *FAS1* and *ACC1* expression was confirmed by Northern analysis (Fig. 5B). These results further support the hypothesis that Mga2 regulates fatty acid biosynthesis in *C. neoformans*.

Among the genes that were induced in the *mga2Δ* mutant strain compared to the wild-type strain were several whose products are implicated in regulating polarized cell growth. The expression of two homologs of small GTPase effectors was significantly higher in the *mga2Δ* mutant strain than in the wild-type strain (Table 2). GTPase modules play important roles in directing polarized growth in *S. cerevisiae* and other fungi (10, 26, 58). In addition, the expression of *PAK1*, encoding a PAK kinase important for filamentous growth during *C. neoformans* mating, was higher in the *mga2Δ* mutant strain than in the wild-type strain (57). Taken together, these results indicate that cells lacking Mga2 are defective in the regulation of polarized cell growth. Cultures of wild-type and *mga2Δ* mutant cells were grown in YPD medium at 37°C for 24 h and examined by microscopy (Fig. 5C). Approximately 50% of *mga2Δ* mutant cells displayed defective morphology, including multiple buds and misshapen buds ( $n = 200$ ). Less than 1% of wild-type cells grown under the same conditions displayed defects in bud morphology. These findings support the hypothesis that Mga2 plays a role in the morphogenesis of *C. neoformans*.

## DISCUSSION

We developed and implemented a genomic-DNA microarray to identify genes that display differential expression during growth at 25 or 37°C in the human-pathogenic fungus *C. neoformans* by using an approach similar to that taken by Sil and colleagues to study phase-regulated gene expression in *H. capsulatum* (30). When this work began, sequencing projects were ongoing for three related varieties of *C. neoformans*, but no finished genome sequence was available. In conjunction with sequence and annotation information provided by these sequencing projects, our microarray data allow the identification of genes whose expression is regulated by growth temperature. In addition, microarray experiments were used to identify possible targets of a previously uncharacterized transcription factor homolog in *C. neoformans*. Our studies illustrate how even partial-genome microarrays can be used to identify genes with altered expression patterns and delineate regulatory networks.

A goal of this study was to identify genes important for growth at 37°C, a crucial virulence attribute of *C. neoformans*. Analysis of gene expression changes that occur after a shift from 25 to 37°C and the steady-state comparison of 25°C growth versus 37°C growth revealed important features about

TABLE 2. Gene expression in wild-type versus *mga2Δ* mutant strains

Gene expression in <i>mga2Δ</i> mutant	Gene name	Annotation	E value	Genbank accession no. of ortholog	Fold change <sup>a</sup>	
Repression	<i>FAS1</i>	Fatty acid synthase, beta subunit	0E+00	AAB41494	-4.5	
	CNL00860	Nuclear pore complex protein	1.5E-70	AAC27297	-4.3	
	CNK02060	Unknown			-3.5	
	CNJ03370	Ribosomal protein L11	3.3E-68	BAA31552	-3.1	
	CNH00590	C1 tetrahydrofolate synthase	1.3E-288	AAG11417	-3.1	
	CNI01120	Ribosomal protein S7	9.5E-47	AAB94301	-3	
	<i>LEU1</i>	2-Isopropylmalate synthase	4.4E-148	CAA20723	-3	
	CNC03960	Phosphate transporter	3.1E-187	BAB43910	-2.9	
	CND02650	Chorismate synthase	6.1E-122	CAA68214	-2.9	
	<i>ACC1</i>	Acetyl CoA carboxylase	0E+00	CAA86983	-2.9	
	CND05600	Pyruvate dehydrogenase	2.0E-116	CAB97287	-2.8	
	CNB00560	Histone H2B	6.4E-40	CAD60694	-2.6	
	CNE04690	Putative major facilitator superfamily transporter	7.7E-83	CAD70735	-2.4	
	CNE02420	Elongation factor 3		AAK26245	-2.2	
	Induction	<i>LYS2</i>	Aminoacidipate semialdehyde dehydrogenase	6.4E-12	CAA85072	6.7
		CNB02680	Monosaccharide transporter	7.5E-188	AAL89822	5.6
		CND03740	Catalase, putative	8.0E-136	AAG45152	4.6
CNF00610		Calnexin	1.5E-143	CAC82717	4.2	
CNJ00670		Unknown			4.1	
CNG03520		Unknown			3.7	
CNG04460		Unknown			3.7	
CNG01070		Putative coiled-coil protein	2.3E-28	CAB88270	3.4	
CNG03440		Vacuolar sorting protein	1.0E-33	AAB49810	3.4	
CNG04330		U5 snRNP 200-kDa helicase	0E+00	BAA34508	3.3	
<i>PAK1</i>		PAK kinase		AAL58841	3.3	
CNG01610		Chitin synthase related	0E+00	AAF04279	3	
CNM01490		Regulator of purine utilization	4.8E-28	CAA58838	3	
CNG02710		LIM and Rho GAP domain protein	7.6E-136	CAA17694	2.9	
<i>MDR1</i>		Multidrug resistance protein 1		AAC49889	2.9	
CNG01350		GTPase-activating protein	1.3E-65	AAB84002	2.9	
CNG04670		Nucleoporin repeat protein	2.1E-28	CAB53357	2.9	
CNA07770		Exo-1,3-beta-glucanase	1.4E-11	AAF65310	2.8	
CNF03200		Unknown			2.8	
CNA08250		Unknown			2.8	
CNF03070		ATM/ATR PI-3 kinase	4.4E-166	CAB40165	2.8	
CNB05770		Putative ABC transporter	0E+00	CAA54692	2.8	
CNG06090		Hypothetical protein	2.1E-65	BAA07526	2.7	
CNC06090		Unknown			2.7	
<i>HSP104</i>		Heat shock protein 104	1.8E-202	AAK60625	2.7	
CNK02930		Unknown			2.5	
CNF04580		Conserved hypothetical protein	1.4E-289	CABB91226	2.2	

<sup>a</sup> Average fold change in expression in three independent trials of the *mga2Δ* mutant compared to wild-type strain H99.

how this fungus adapts to alterations in growth environment. Genes that are important for stress responses are induced upon a shift from 25 to 37°C. Among the induced genes are orthologs of trehalose synthase, catalase, superoxide dismutase, and a presumptive sensor of cell wall stress. It is important to note that for those genes that were induced only at later time points after the shift from 25 to 37°C, induction may be the result of long-term adaptation to growth at 37°C or an indirect consequence of increased cell density. An example of this phenomenon is the pattern of expression of the alternative oxidase *AOX1*, which is initially repressed after a shift to 37°C but then is strongly induced by 12 h after the shift. *FHB1*, encoding flavohemoglobin, displays a similar pattern of expression as *AOX1*. Both *FHB1* and *AOX1* play roles in responding to nitrosative and oxidative stresses, respectively (2, 17). It is unclear why these genes would be initially repressed after

exposure to heat stress; however, the regulation of these genes or their products at posttranscriptional levels in response to environmental changes is possible.

Heat shock induced gene expression is a phenomenon observed in both *S. cerevisiae* and *Candida albicans* (20, 25). These two fungi differ in that *S. cerevisiae* undergoes a general stress response, involving an extensive gene expression program that is initiated regardless of the specific stress encountered, whereas *C. albicans* does not. Our studies suggest that it is possible to test whether a general stress response exists in *C. neoformans* by using the genomic microarray. One important component of the response to high-temperature growth in both *S. cerevisiae* and *C. albicans* is the induction of heat shock proteins that act as molecular chaperones. Our array contains orthologs of the heat shock proteins *HSP78* and *HSP104*, and these genes display an approximately twofold increase in ex-

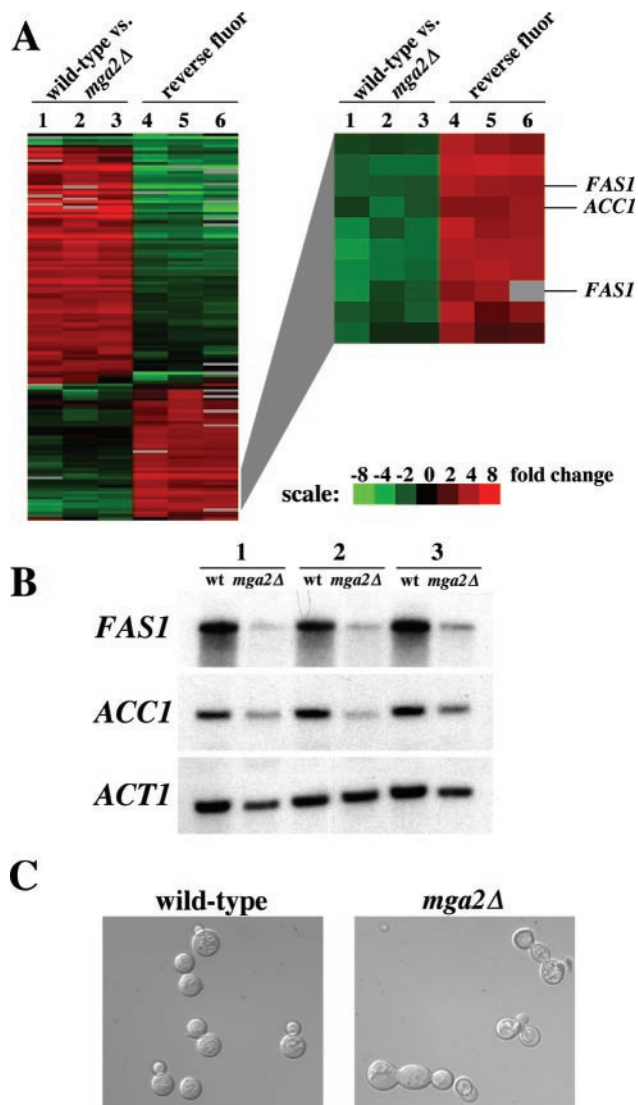


FIG. 5. Gene expression in wild-type and *mga2Δ* mutant strains. (A) Three independent microarray experiments and the corresponding reverse fluor control experiments were performed with cDNAs from wild-type (Cy3-labeled) and *mga2Δ* (Cy5-labeled) strains. Elements whose expression changed at least threefold in at least one experiment were analyzed by hierarchical clustering. Each lane represents one experiment, and each row represents gene expression. A cluster of interest containing the *FAS1* and *ACC1* genes is enlarged. Green color in lanes 1 to 3 represents decreased gene expression. (B) Northern analysis of wild-type (wt) and *mga2Δ* mutant gene expression. Three independent samples of total RNA from wild-type (H99) and *mga2Δ* mutant (PK28) strains were probed with *FAS1*- and *ACC1*-specific probes. *ACT1* was included as a loading control. (C) Morphogenesis of wild-type and *mga2Δ* mutant strains. Cultures of wild-type (H99) and *mga2Δ* mutant (PK28) strains were inoculated into YPD medium and incubated at 37°C for 24 h. Differential interference contrast images were captured at a magnification of  $\times 1,000$  with a Zeiss Axioskop 2 Plus microscope equipped with an AxioCam MRM digital camera.

pression in the 3-h time point after the shift from 25°C growth to 37°C growth (data not shown). An ortholog of the SSA family of heat shock chaperones showed no significant change in expression. The failure to detect induction of these heat shock protein genes to similar levels as in *S. cerevisiae* is likely

due to differences in experimental design, in that the first time point taken in our study was 3 h after the temperature shift, rather than 10 to 30 min, as in the *S. cerevisiae* and *C. albicans* studies.

Recently, Steen et al. analyzed temperature-regulated transcription in *C. neoformans* using SAGE (49). In that report, gene expression at 25 and 37°C was studied for both the serotype D strain B3501A and the serotype A strain H99. Although SAGE and microarray hybridization use different technologies to probe gene expression, it is reasonable to expect overlap in the microarray data and the SAGE data. We find similarities in the genes whose expression is higher at 37°C than at 25°C in our transcriptional profiling of H99 and the SAGE report analyzing the serotype D strain B3501A. Most notably, the genes encoding homologs of an aryl alcohol oxidase (*SMG1*) and a peripheral benzodiazepine receptor were more highly expressed at 37°C in both studies. Among genes that are repressed at 37°C, the ribosomal proteins P0 and S26 were found in our study and the SAGE analysis of both temperature-regulated transcription and gene expression during experimental cryptococcal meningitis, suggesting that alterations in protein synthesis are important for tolerating high-temperature growth and in vivo growth (49, 50).

That differences exist in our study and the SAGE studies is also not surprising given differences in experimental design. An advantage of microarray studies is the ability to analyze changes in gene expression over a time course, while the SAGE experiments described by Steen et al. (49) were performed at a single time point. Additionally, Steen et al. found significant differences in the patterns of expression for strains B3501A and H99 in their study. Transcriptional analysis of strain B3501A reveals that genes involved in lipid metabolism, such as genes encoding  $\Delta 9$  fatty acid desaturase and fatty acid synthase, were more highly expressed at 25°C. We found that a transcription factor homolog that may target lipid metabolism genes is strongly induced at 37°C (see below). These differences could reflect unique properties of *C. neoformans* serotype D strains such as B3501A relative to the more thermotolerant serotype A strain H99. However, changes in membrane composition and fluidity are likely to occur during growth at either low- or high-temperature extremes (1, 48, 51).

Microarray analysis identified a gene, *MGA2*, that shows significantly higher expression during growth at 37°C and that is also important for normal growth at high temperatures. However, this approach does not appear to be generally applicable. In *S. cerevisiae*, genes that protect against DNA damage are not significantly induced in the presence of DNA-damaging agents (7). Of the previously identified genes important for *C. neoformans* high-temperature growth (*CNA1*, *CNB1*, *CTS1*, *STE20*, *MPK1*, and *RAS1*), none displayed significantly higher gene expression during growth at 37°C than during growth at 25°C. *MPK1* encodes a MAP kinase in the protein kinase C-mediated cell integrity pathway and therefore might be expected to display an increase in expression upon a temperature shift; however, no such increase in expression was observed. Furthermore, *CLC1* was induced strongly in the earliest time points after the shift to 37°C (Fig. 1); however, a *clc1Δ* mutant was not affected in its ability to tolerate high-temperature growth (Fig. 3A). Strikingly, we observed a significant reduction in expression of genes involved in isoleucine

or valine biosynthesis and pyrimidine biosynthesis after a shift to 37°C, yet mutations inactivating each of these pathways result in a high-temperature growth defect. This is an unexpected result, and an explanation may be that the *ilv2Δ* and *ura5Δ* mutants accumulate a toxic intermediate at 37°C but not at 25°C or that the uptake of essential nutrients is significantly altered by the growth temperature, the presence of the mutations, or both. This expression pattern may not occur for all amino acid biosynthetic pathways, as genes encoding homologs of lysine and threonine biosynthetic enzymes are induced rather than repressed at 37°C (Table 1). These results suggest caution may be warranted in the use of *URA5* as a selectable marker and in the interpretation of experiments using *ura5* mutant strains. Interestingly, we found no evidence of temperature-sensitive growth defects in serotype D *ura5* mutants, whereas serotype A *ura5* mutants are clearly temperature sensitive (A. Idnurm, P. Kraus, and J. Heitman, unpublished data). Although identification of genes that display differential expression may not in all cases reveal physiologically relevant patterns of gene expression, microarray analysis can be used effectively as a screen to prioritize candidates for further study. Hence, an essential component of studies with the *C. neoformans* genomic microarray is incorporation of information regarding phenotypic consequences of alterations in gene expression.

Long-chain fatty acids are used by eukaryotic cells as an energy source as well as for building blocks of cell and organelle membrane lipids. *S. cerevisiae* has served as an excellent model system for studying fatty acid metabolism, and considerable information is known about how fatty acids are synthesized, transported, and degraded (54). An important component of the regulation of fatty acid metabolism in yeast cells is the activation of gene expression, with signals such as glucose and unsaturated fatty acids acting to induce the expression of a panoply of target genes. The expression of genes involved in saturated fatty acid biosynthesis, including *ACC1* and *FAS1*, is coregulated (12). Our results suggest that these genes are also coregulated in *C. neoformans*, and a transcription factor homolog that may target these genes was identified. *C. neoformans* Mga2 is homologous to two *S. cerevisiae* transcription factors, Mga2 and Spt23. These factors activate expression of *OLE1*, encoding Δ9 desaturase, in response to low oxygen levels and low growth temperatures (32, 60). Other targets of these factors in yeast cells, if any, are unknown. Membrane composition is altered when *S. cerevisiae* cells are exposed to temperature extremes, and activation of desaturases is thought to provide increased membrane fluidity (48, 51). In *C. neoformans*, increased production of long-chain fatty acids may be necessary for tolerating high-temperature growth and could explain the increased expression of a factor regulating their production. It is important to note that expression of the potential targets of Mga2, *FAS1* and *ACC1*, was not significantly higher at 37°C than at 25°C (Table 1; see Table S2 in the supplemental material). Mga2 and Spt23 are synthesized as precursors that are anchored to the endoplasmic reticulum membrane and then activated by ubiquitin- or proteasome-dependent processing (28). Hence, an increase in the expression of the *MGA2* and *SPT23* genes may not result in a concomitant increase in activity of the transcription factors. *S. cerevisiae* *MGA2* expression is induced by the unfolded protein

response, suggesting that additional pathways may be targeted by this transcription factor (53). Increased expression of *MGA2* in *C. neoformans* may be a consequence of the unfolded protein response or endoplasmic reticulum-mediated protein degradation, both of which are activated by heat stress in *S. cerevisiae* (25, 53). Nevertheless, *C. neoformans* *MGA2* plays an important role in cell growth, particularly at high growth temperatures.

Fatty acid metabolism is coordinated with sterol synthesis in yeast cells, and antifungal drugs that inhibit ergosterol biosynthesis may also have secondary effects on the enzymes that catalyze fatty acid biosynthesis (11, 35, 36). The *C. neoformans* *mga2Δ* mutant is hypersensitive to the ergosterol synthesis inhibitor fluconazole, indicating a conserved link between fatty acid and sterol metabolism. Membrane remodeling via fatty acid biosynthesis, saturation-desaturation, and sterol metabolism is likely to be an important component of the regulatory mechanisms enabling responses to stress in *C. neoformans*. Interestingly, the sterol composition of *C. neoformans* can be altered after passage through a mammalian host (15). Additionally, lipids and sterols are important components of polarized cell growth in *S. cerevisiae* and *S. pombe*, and our findings suggest that this relationship is conserved in *C. neoformans* (5, 56). The effects of improper regulation of fatty acid and sterol metabolism in *C. neoformans* influence the ability to undergo normal morphogenesis, which is essential for growth, mating, and haploid fruiting (4). The wiring of regulatory pathways that respond to stress, coordinate fatty acid and sterol synthesis, and control cellular morphogenesis likely differs between *S. cerevisiae* and *C. neoformans* and may differ among the closely related varieties of *C. neoformans*. Detailed examination of these pathways will provide additional insight into the biology underlying stress responses and potentially reveal new areas to pursue for antifungal therapy.

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