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Genome-Wide Transcriptional Profiling of the Cyclic AMP-Dependent Signaling Pathway during Morphogenic Transitions of *Candida albicans*▽†

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***Candida albicans* is an opportunistic human fungal pathogen that causes systemic candidiasis as well as superficial mucosal candidiasis. In response to the host environment, *C. albicans* transitions between yeast and hyphal forms. In particular, hyphal growth is important in facilitating adhesion and invasion of host tissues, concomitant with the expression of various hypha-specific virulence factors. In previous work, we showed that the cyclic AMP (cAMP) signaling pathway plays a crucial role in morphogenic transitions and virulence of *C. albicans* by studying genes encoding adenylate cyclase-associated protein (*CAP1*) and high-affinity phosphodiesterase (*PDE2*) (Y. S. Bahn, J. Staab, and P. Sundstrom, Mol. Microbiol. 50:391–409, 2003; and Y. S. Bahn and P. Sundstrom, J. Bacteriol. 183:3211–3223, 2001). However, little is known about the downstream targets of the cAMP signaling pathway that are responsible for morphological transitions and the expression of virulence factors. Here, microarrays were probed with RNA from strains with hypoactive (*cap1*/*cap1* null mutant), hyperactive (*pde2*/*pde2* null mutant), and wild-type cAMP signaling pathways to provide insight into the molecular mechanisms of virulence that are regulated by cAMP and that are related to the morphogenesis of *C. albicans*. Genes controlling metabolic specialization, cell wall structure, ergosterol/lipid biosynthesis, and stress responses were modulated by cAMP during hypha formation. Phenotypic traits predicted to be regulated by cAMP from the profiling results correlated with the relative strengths of the mutants when tested for resistance to azoles and subjected to heat shock stress and oxidative/nitrosative stress. The results from this study provide important insights into the role of the cAMP signaling pathway not only in morphogenic transitions of *C. albicans* but also for adaptation to stress and for survival during host infections.**

In most kingdoms of life, cyclic AMP (cAMP) functions as a second messenger that plays a pivotal role in controlling a variety of cellular responses, depending on the organism. cAMP is universally produced through cyclization of ATP catalyzed by adenylyl cyclases (ACs) with the release of inorganic pyrophosphate. In mammals, two classes of ACs exist, transmembrane ACs and soluble ACs (sACs). The transmembrane Acs mainly are activated by heterotrimeric G proteins and G-protein-coupled receptors in response to a variety of hormones and neurotransmitters (39). In contrast, the sACs are insensitive to G protein signaling and instead are uniquely stimulated by bicarbonate and calcium, governing sperm maturation and pH regulation in epididymis and tumor necrosis factor activation of granulocytes (39). Pulses of cAMP trigger related signaling events via cAMP-dependent protein kinase A (PKA) that comprises two catalytic subunits and two inhibitory regulatory subunits. cAMP binds to and causes conformational changes in PKA regulatory subunits, releasing activated PKA catalytic subunits (40, 75, 82). Activated PKA subsequently phosphorylates other protein kinases, transcription factors, and other substrates to control various physiological processes. This process is generally under negative feedback regulation by phosphodiesterases (PDEs) that degrade cAMP to AMP (50).

In eukaryotic microorganisms, the cAMP signaling pathway has been most extensively studied in the model yeast *Saccharomyces cerevisiae*. These studies have revealed the presence of subtle differences between fungi and higher eukaryotes in cAMP signaling pathways. The interaction of the sAC with G proteins is one example; the sAC (Cdc35/Cyr1) is regulated by the G protein Ras1 or Ras2, Gpa2 coupled with Gpb1/2 (Gpa2-binding proteins), and a G-protein-coupled receptor, Gpr1, in *S. cerevisiae* (47, 64). cAMP produced by ACs activates PKA composed of catalytic subunits (Tpk1/2/3) and a regulatory subunit (Bcy1) for controlling vegetative growth and pseudohyphal development (47, 64).

In the pathogenic fungi, the cAMP signaling pathway has evolved to control major virulence attributes. In *Cryptococcus neoformans*, which causes fungal meningoencephalitis, disruption of an AC gene (*CAC1*) disables the production of two major virulence factors without affecting viability: the antiphagocytic polysaccharide capsule, which also interferes with

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normal functions of host cells, and melanin, which acts as an antioxidant (2). The virulence of *C. neoformans* also is significantly attenuated by disrupting or mutating other genes that function in the cAMP signaling pathway, such as *GPA1* (Gα protein), *PKA1* (PKA catalytic subunit), and *ACA1* (AC-associated protein) (1, 4, 20, 29). In the rice blast fungus *Magnaporthe grisea*, deletion of the AC gene (*MAC1*) abolishes the ability of cells to form appressoria for penetration of plant hosts and reduces growth (17).

In the ascomycetous, pathogenic fungus *Candida albicans*, genes that are regulated by the cAMP-dependent signaling pathway are important determinants of morphology and virulence. *C. albicans*, a part of the normal human microbial flora colonizing mucocutaneous surfaces of the oral cavity, the gastrointestinal tract, and the vaginal cavity, often causes superficial and systemic candidiasis, particularly in the presence of weakened immune systems. The presence of yeast, hyphal, and pseudohyphal morphologies typically is observed in candidiasis caused by *C. albicans* (74). Hyphal growth forms are proadherent and proinvasive, properties that are partly conferred by surface proteins such as Hwp1 and Als3 (56, 58, 72), whereas the virulence attributes of yeast and pseudohyphal growth forms are not as well characterized. The proinvasive and proadhesive surface protein, Hwp1, is hypha specific and mediates covalent attachment of hyphae to host epithelial cells by functioning as a substrate for mammalian transglutaminases (71–73). The possibility that yeast growth forms play a role in dissemination is suggested by the presence of Ywp1, which appears to decrease adherence (26). Disruption of the single AC gene in *C. albicans*, *CaCDC35*, reduces normal growth and restricts morphology to the yeast form (62). Consequently, the homozygous *cacdc35*/*cacdc35* mutant is avirulent in animal models of both vaginal and systemic infections. Downstream of CaCdc35, Tpk1/2 and Sra1 have been identified as the catalytic and regulatory subunits, respectively, of PKA, mediating cAMP signaling (10, 68, 70). Both Tpk1 and Tpk2 promote hypha formation, but each responds to different germ-tube-inducing signals (10, 68). Overexpression of the *SRA1* gene represses filamentous growth, possibly by inhibiting the activation of Tpk1/2 (70). The transcription factors Efg1 and Flo8 are determinants of morphology and also are downstream targets of PKA in *C. albicans* (9, 14, 48, 68). Efg1 contains a putative PKA phosphorylation site (T206) that is essential for hypha formation (9). Flo8 physically interacts with Efg1, governing hyphal development (14). Homozygous *efg1*/*efg1* and *flo8*/*flo8* mutants are avirulent in an animal model of systemic candidiasis (14, 48).

Previously, we characterized two cAMP signaling pathway genes, *CAP1* and *PDE2*, encoding the AC-associated protein Cap1 and the high-affinity Pde2p, respectively (5, 6). Cap1 stimulates synthesis of cAMP, whereas Pde2p degrades cAMP. The *cap1*/*cap1* mutant is only partially inactivated in the cAMP pathway, in that basal levels of cAMP are equivalent to those of the wild type (WT). Budding growth of both *cap1*/*cap1* and *pde2*/*pde2* mutants is normal, except that buds are elongated in the latter case. In standard germ-tube-inducing conditions, the hypofilamentous *cap1*/*cap1* mutant exhibits budding growth, whereas the hyperfilamentous *pde2*/*pde2* mutant forms germ tubes more rapidly, and these germ tubes are longer at each time point than germ tubes produced by the WT strain. Consistent with these results, a strain that overexpresses *PDE2* is nonfilamentous in most germ-tube-inducing conditions, including serum (5). Although *pde2*/*pde2* mutants are hyperfilamentous, they are not hypervirulent; both hypofilamentous (*cap1*/*cap1*) and hyperfilamentous (*pde2*/*pde2*) mutants are equally attenuated in a mouse model of systemic candidiasis (5), suggesting that a balanced mixture of morphologies reflects an optimal combination of expressed genes at appropriate levels for virulence. Knowledge about the molecular mechanisms of candidiasis would be advanced by the identification of downstream targets that are modulated by the cAMP signaling pathway because of their probable effect on the virulence of *C. albicans*.

Genes that are coregulated with *HWP1* are of interest because of the possible cooperation of their products with Hwp1 in adherence and invasion. Genes that are down-regulated could represent genes that counteract adherent and invasive properties of hyphae. Using three isogenic *C. albicans* strains, UnoPP-1 (the WT control strain), CAC1-1A1E1 (the hypoactive cAMP signaling *cap1*/*cap1* mutant), and BPS15 (the hyperactive cAMP signaling *pde2*/*pde2* mutant) (5), we performed a genome-wide screening using DNA microarrays to detect genes modulated by the cAMP signaling pathway. Here, we demonstrate that the cAMP signaling pathway confers metabolic specialization to growth forms (yeast or hypha), reorganizes the architecture of the cell wall surface as well as ergosterol/lipid biosynthesis, and modulates stress responses during germ tube induction in *C. albicans*. Furthermore, several previously uncharacterized downstream targets that were up-regulated or down-regulated by the cAMP signaling pathway also were identified.

MATERIALS AND METHODS

***C. albicans* strains and media.** The *C. albicans* strains used in this study are described in Table 1. For yeast and germ tube cultures, YNB medium (yeast nitrogen base minimal medium containing 50 mM glucose and 0.0002% biotin) and M199 (medium 199 containing 150 mM HEPES [pH 7.0]; Gibco-BRL) were used, respectively.

Growth conditions, total RNA isolation, and preparation of labeled cDNA. Yeasts for germ tube induction were grown to mid-logarithmic phase in YNB at 25°C to an optical density at 600 nm of 0.8 to 1.0. For germ tube induction, yeasts were briefly sonicated, washed, resuspended in phosphate-buffered saline (PBS), and inoculated at a concentration of 5×10^6 cells/ml into prewarmed M199 and incubated at 37°C for 1.5 h. This time point was chosen to assess gene expression profiles that followed the transient peak of cAMP at 1 h as determined previously (6). At 1.5 h of incubation, the morphologies of the two mutants and the WT were markedly different, in that the *pde2*/*pde2* mutant formed germ tubes that were longer than those of the WT strain, whereas the *cap1*/*cap1* mutant exhibited budding yeast growth (Fig. 1). Indirect immunofluorescence using anti-Hwp1 antibodies showed abundant expression of Hwp1 on true germ tubes formed by the WT strain and the *pde2*/*pde2* mutant (5). Parallel cultures of the *pde2*/*pde2* mutant and WT strain as well as the *cap1*/*cap1* mutant incubated at 25°C grew as yeasts.

To identify cAMP-dependent genes induced during morphogenesis, experimental RNA was prepared from each strain after incubation in germ-tube-inducing conditions for 1.5 h at 37°C. A single reference RNA preparation consisted of equal amounts of pooled RNA from the three cultures incubated in M199 for 1.5 h at 25°C. RNA was isolated using the hot-acid–phenol method (3), treated with DNase I (Promega) for 1 h at 37°C, and stored at −80°C.

For budding yeast growth conditions, cAMP-dependent gene expression profiles were generated using experimental RNA prepared from mid-logarithmic-phase yeast cultures of each strain. The single reference RNA preparation consisted of equal amounts of pooled RNA from the same three cultures.

For both the identification of cAMP-regulated genes during morphogenesis

TABLE 1. *C. albicans* strains used in this study

Strain	Genotype	Parent strain	Reference
SC5314	WT		25
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	SC5314	22
UnoPP-1	Same as CAI4, but Δ*eno1*::*URA3*/*ENO1*	CAI4	60
CAC1-1A	Same as CAI4, but *cap1*::*hisG*/*cap1*::*hisG-URA3-hisG*	CAC1-1	6
CAC1-1A1	Same as CAI4, but *cap1*::*hisG*/*cap1*::*hisG*	CAC1-1A	6
CAC1-1A1E1	Same as CAC1-1A1, but Δ*eno1*::*URA3*/*ENO1*	CAC1-1A1	5
CACRE1	Same as CAI4, but *CAP1*/*cap1*::*hisG* Δ*eno1*::*URA3*/*ENO1*	CAC1-1A1	6
BPS1	Same as CAI4, but *PDE2*/*pde2*::*hisG-URA3-hisG*	CAI4	5
BPS2	Same as CAI4, but *PDE2*/*pde2*::*hisG*	BPS1	5
BPS4	Same as CAI4, but *pde2*::*hisG URA3 hisG*/*pde2*::*hisG*	BPS2	5
BPS7	Same as CAI4, but *pde2*::*hisG*/*pde2*::*hisG*	BPS4	5
BPS9	Same as CAI4, but *PDE2*/*pde2*::*hisG* Δ*eno1*::*URA3*/*ENO1*	BPS7	5
BPS15	Same as BPS7, but Δ*eno1*::*URA3*/*ENO1*	BPS7	5
BPS16	Same as CAC1-1A1, but *PDE2*/*pde2*::*hisG-URA3-hisG*	CAC1-1A1	5
BPS17	Same as CAC1-1A1, but *PDE2*/*pde2*::*hisG*	BPS16	5
BPS18	Same as CAC1-1A1, but *pde2*::*hisG URA3 hisG*/*pde2*::*hisG*	BPS17	5
BPS20	Same as CAC1-A1, but *pde2*::*hisG*/*pde2*::*hisG*	BPS18	5
BPS27	Same as BPS20, but Δ*eno1*::*URA3*/*ENO1*	BPS20	5

and unperturbed budding growth, cDNA from each of the three experimental RNAs (labeled with Cy5) and the single reference RNA (labeled with Cy3) was prepared. Experiment 1 consisted of three arrays, each incubated with a mixture of one of the experimental cDNAs and the reference cDNA. Experiment 2 was a technical replicate of experiment 1. A biological replicate was generated by growing new cultures from each strain to prepare cDNA for labeling and hybridizations in experiments 3 and 4.

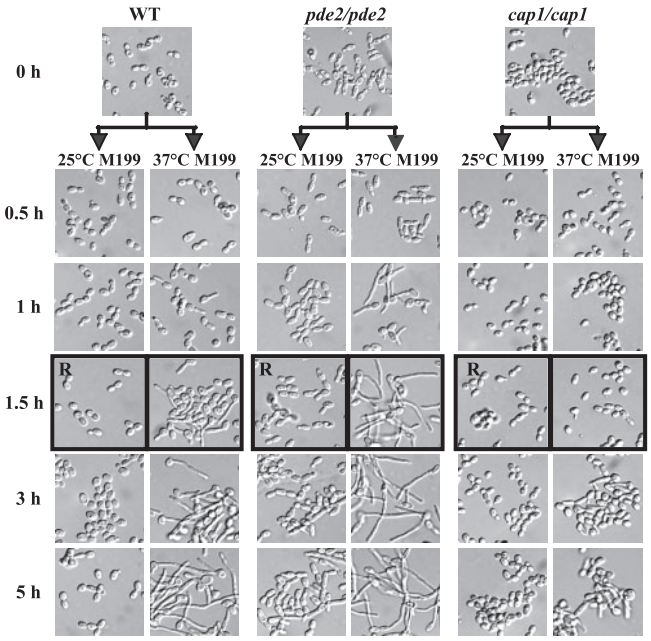


FIG. 1. Morphologies of the WT, hyperfilamentous *pde2*/*pde2* mutant, and budding *cap1*/*cap1* mutant used to prepare RNA in profiling experiments. Cells were grown at 25°C in YNB to mid-logarithmic phase, reinoculated at a concentration of 5×10^6 cells/ml into M199, and further incubated for 5 h at 25°C for yeast growth or at 37°C for germ tube induction. At each time point, cells were sampled and their morphologies were observed by microscopy. Total RNAs were isolated from a 1.5-h culture of each strain in M199 at 25 or 37°C (black-outlined boxes). True hyphal formation of the WT and *pde2*/*pde2* mutant was confirmed by an indirect immunofluorescence assay with hypha wall protein Hwp1-specific antibodies as described before (5). The reference RNA was pooled from RNA prepared from each strain at 25°C (denoted as R). The proportion of cells with germ tubes after 1 h of incubation in M199 at 37°C was nearly 100% for the *pde2*/*pde2* (Δ*pde2*; strain BPS15) mutant, 20 to 30% for the WT strain (UnoPP-1), and 0% for the *cap1*/*cap1* (Δ*cap1*; CAC1-1A1E1) mutant.

***C. albicans* DNA microarray.** *C. albicans* microarrays were spotted in the laboratory of Judith Berman (University of Minnesota; http://www.cbs.umn.edu//labs/berman/) from PCR products generated in the Berman laboratory and the laboratory of Lois Hoyer (University of Illinois at Urbana—Champagne). These *C. albicans* microarrays were constructed using PCR products consisting of 6,175 unique open reading frames (ORFs) (>100 amino acids). Each PCR product was spotted at least twice. Pre- and postprocessing of microarray slides, preparation of Cy3- or Cy5-labeled cDNA probes, and hybridization were performed as described before (18, 21). Array slides were scanned using an Affymetrix 428 scanner (Santa Clara, CA).

Data filtering and analysis. Image analysis of the scanned TIF file was performed using Scanalyze (version 2.51; Eisen Lab and Stanford University) and Gene Traffic (version 3.2-12; Iobion). Flagging of invalid spots occurred manually in Scanalyze and automatically in Gene Traffic. Names for the hybridization groups used in the analysis were chosen to be cap1 for the *cap1*/*cap1* mutant, WT for the UnoPP-1 strain, and pde2 for the *pde2*/*pde2* mutant. Differences in transcript levels between strains were termed fold changes. Data were normalized using the global intensity method and were filtered to exclude genes that did not have at least two valid spots in each hybridization group (cap1, WT, and pde2). The data from Gene Traffic were imported into Excel 2000 (Microsoft).

The spot average $\log_2$ Cy5/Cy3 ratio was calculated for each gene. For the experiments in which germ tube growth conditions were used, the spot average $\log_2$ Cy5/Cy3 ratio approximated the increase of a transcript in germ-tube-inducing conditions relative to that of yeast-inducing conditions and was termed fold induction. Genes that appear in Tables 2 and 3, as well as Tables S3 and S4 in the supplemental material, all were tested for significance using significance analysis of microarrays (SAM; version 2.23b; Stanford University) and were required to have SAM-generated q values of less than 0.5. A result of $q < 0.5$ indicated a false discovery rate of <0.5% for each gene. When significant duplicates of the same gene were present in a table, their $\log_2$ ratios were averaged. The complete microarray data sets are available in Table S1 in the supplemental material. Genes appearing in each table also were required to meet the specific criteria outlined below.

Comparison of WT, *pde2*/*pde2*, and *cap1*/*cap1* expression profiles. For the identification of genes up-regulated by the cAMP signaling pathway in germ-tube-inducing conditions (Table 2), criteria were applied as described below to identify positively regulated genes that had average $\log_2$ Cy5/Cy3 ratios in the order pde2 ≥ WT > cap1. The difference in $\log_2$ Cy5/Cy3 ratios between strains was termed fold change. The criteria (criteria set A) for positively regulated, cAMP-dependent genes following 1.5 h in germ-tube-inducing conditions were selected so that genes that are known to be cAMP regulated, including *HWP1*, *ECE1*, and *RBT1* (8, 12, 73), were identified (Fig. 2). The difference between the average $\log_2$ Cy5/Cy3 ratios of the WT and cap1 or pde2 and cap1 was required to be ≥0.6, which was equivalent to a minimum 1.5-fold change. The $\log_2$ Cy5/Cy3 ratio for the WT was required to be greater than or equal to 0.6 (≥1.5-fold induction). These criteria guarantee that genes on this list are induced

TABLE 2. Genes up-regulated by the cAMP signaling pathway in germ-tube-inducing conditions[a]

Gene	ORF identity		Fold change between:			Function of gene product
	ORF6	ORF19	WT / cap1	pde2 / cap1	pde2 / WT	
Hypha-specific or cell surface proteins						
ALS3	1377	1816	3.01	5.97	1.98	Cell surface adhesin, hypha induced
ECE1	2886	3374	3.03	8.65	2.85	Unknown, hypha induced
RBT1	4889	1327	3.63	5.09	1.40	Unknown, repressed by Tup1
ECE99	2929	3384	2.54	3.93	1.55	Unknown, homologous to Rbt1
PGA45	1247	2451	2.00	3.89	1.94	Putative GPI-anchored protein of unknown function
HWP1	4883	1321	2.39	3.62	1.51	Cell surface adhesin, human transglutaminase substrate
PDC11	4386	2877	3.58	3.36	0.94	Similar to pyruvate decarboxylase; antigenic; located at the cell surface of hyphae, but not yeast
Cytoskeleton/microtubules						
STU11	6222	6610	2.15	2.96	1.38	Structural constituent of cytoskeleton, mitotic spindles
Ergosterol biosynthesis and lipid metabolism						
ERG1	2816	406	2.31	3.94	1.70	Squalene monooxygenase
ERG251	2054	4631	2.25	3.89	1.73	Homologous to C-4 methyl sterol oxidase encoded by *ERG25*
SUR2	4041	5818	3.08	3.02	0.98	Predicted enzyme of sphingolipid biosynthesis
ERG11	2866	922	3.29	2.99	0.91	Lanosterol 14 α-demethylase
Fermentation						
PDC12	7907	4608	2.66	3.00	1.13	Putative pyruvate decarboxylase, fungus specific
ADH2	6337	5113	2.28	2.18	0.95	Putative alcohol dehydrogenase, fungus specific
Glycolysis or gluconeogenesis						
HGT8	2377	2021	2.60	3.39	1.30	Hexose transport
CDC19	5754	3575	2.28	2.95	1.29	Pyruvate kinase
HGT7	2376	2023	2.98	2.91	0.98	Hexose transport
PFK1	3504	3967	2.95	2.52	0.85	Alpha subunit of phosphofructokinase
HGT6	2379	2020	2.74	2.49	0.91	Putative glucose transporter of the major facilitator superfamily
GPM2	4922	1067	2.47	2.25	0.91	Phosphoglycerate mutase
GPM1	7274	903	2.22	2.22	1.00	Phosphoglycerate mutase
TPI1	8886	1306	1.85	2.16	1.17	Triose-phosphate isomerase
HGT7	1210	2023	1.90	2.16	1.14	Hexose transport
TDH3	8817	6814	1.69	2.09	1.23	Glyceraldehyde 3-phosphate dehydrogenase
PFK2	5274	3967	2.26	1.93	0.86	Beta subunit of phosphofructokinase
Heme binding/biosynthetic pathway						
ORF6.2323	2323	1034	1.89	2.76	1.46	Homologous to *S. cerevisiae* Dap1p, caspofungin repressed
Oxidoreductase						
IFE2	3827	5288	1.97	2.92	1.48	Alcohol dehydrogenase, Efg1 regulated
Stress-associated protein						
PHO15	7257	4444	2.22	1.96	0.88	4-Nitrophenyl phosphatase, induced by heavy metal
Unknown						
UCF1	1464	1354	10.35	10.29	0.99	Down-regulation correlates with fluconazole resistance
ORF6.6008	6008	2372	1.65	3.06	1.85	Unknown, similar to the Tca2 (pCal) retrotransposon
SEC65	3806	2557	1.96	2.76	1.41	Unknown, potential signal recognition particle
ORF6.294	294	2452	1.41	2.75	1.96	Unknown, transcriptionally regulated by iron
ORF6.4769	4769	2371	2.13	2.67	1.26	Unknown, similar to the Tca2 (pCal) retrotransposon
YCL019W	4770	2372	2.00	2.56	1.28	Unknown, similar to the Tca2 (pCal) retrotransposon
ORF6.7250	7250	3262	2.26	2.55	1.13	Unknown
ORF6.162	162	8979	2.09	2.44	1.17	Unknown, expression greater at high iron concentrations
ORF6.7932	7932	7006	1.85	2.05	1.11	Unknown
YFR044C	5132	3915	2.16	1.91	0.88	Unknown, Hog1 induced

[a] Significant duplicates were averaged.

TABLE 3. Genes down-regulated by the cAMP signaling pathway in germ-tube-inducing conditions[a]

Gene	ORF identity		Fold change between:			Function of gene product
	ORF6	ORF19	cap1/WT	cap1/pde2	WT/pde2	
Aldehyde metabolism						
ALD4	5499	6306	1.49	2.56	1.72	Aldehyde dehydrogenase (NAD) activity
IFD7	425	629	1.64	2.21	1.35	Similar to aryl-alcohol dehydrogenases
ALD5	6640	5806	1.37	2.21	1.61	Aldehyde dehydrogenase
Amino acid/protein biosynthesis						
LYS22	4198	4506	2.56	2.60	1.01	Putative homocitrate synthase, fungus specific
RPP0	7941	7015	1.21	2.54	2.11	Putative ribosomal protein
CSP37	2388	2531	2.46	2.48	1.01	Protein complex assembly
RPL12	6437	1635	1.84	2.41	1.31	Predicted ribosomal protein
RPL10A	3768	3465	2.16	2.36	1.09	Predicted ribosomal protein
ILV5	3699	88	1.26	2.33	1.85	Keto-acid reductoisomerase
ACO1	7870	6385	2.06	2.28	1.10	Protein described as aconitase, glutamate biosynthesis
RPL23A	4364	3504	1.92	1.99	1.03	Putative ribosomal protein
HOM6	6665	2951	1.28	1.95	1.52	5-Amino-6-(5-phosphoribosylamino)uracil reductase
ASN1	5930	198	1.95	1.95	1.00	Asparagine synthetase, soluble protein in hyphae
RPP1A	4873	2992	1.71	1.95	1.14	Conserved acidic ribosomal protein
IDH1	7385	4826	1.45	1.78	1.22	Isocitrate dehydrogenase (NAD^+) activity
HIP1	1878	3195	1.30	1.76	1.35	Amino acid permease activity, amino acid transport
TUF1	7591	6047	1.65	1.69	1.02	Putative translation elongation factor
Arginine catabolism						
CAR2	4510	5641	1.66	1.89	1.14	Unknown, alkaline up-regulated
Axial bud site selection						
BUD3	8474	7079	1.11	1.58	1.42	Axial bud site selection
Cell surface protein						
ALS4	3075	4556	2.71	3.03	1.12	Member of the ALS[b] GPI-anchored protein family
ALS2	2999	1097	2.44	4.17	1.71	Member of the ALS GPI-anchored protein family
Endoplasmic reticulum protein						
YET3	6421	1564	1.54	1.84	1.20	Unknown, Yet3p analog
Fatty acid catabolism						
ARD1	5515	6322	1.64	2.01	1.23	D-Arabitol dehydrogenase, NAD dependent
Glycine metabolism						
SHM2	2091	5750	1.40	2.43	1.73	Cytoplasmic serine hydroxymethyltransferase
Metabolism						
MAM33	8087	7187	1.42	2.35	1.65	Possible role in aerobic respiration
Mitochondria						
MRH4	4978	3481	1.94	2.55	1.32	Mitochondrial RNA helicase
RSM23	4979	3480	2.27	1.99	0.87	Ribosomal small subunit of mitochondria
Nucleotide biosynthesis						
TFS1	1861	1974	2.08	2.47	1.19	Transcription is regulated upon yeast-hypha switch
URA4	1407	1977	1.68	1.77	1.06	Nucleotide biosynthesis
Purine base metabolism						
ADE4	5084	1233	1.81	1.87	1.04	Unknown, flucytosine induced
Stress-associated protein						
HSP12	2761	4216	3.58	4.66	1.30	Heat shock protein, cell adhesion (oxidative stress response)
CCP1	957	238	2.35	2.95	1.25	Cytochrome *c* peroxidase (oxidative stress response)

Continued on following page

TABLE 3—*Continued*

Gene	ORF identity		Fold change between:			Function of gene product
	ORF6	ORF19	cap1/WT	cap1/pde2	WT/pde2	
MCR1	4367	3507	1.87	2.91	1.55	Cytochrome b_5 reductase
SOD2	4731	3340	1.47	2.41	1.64	Mn-superoxide dismutase (oxidative stress response)
Transporter						
MUP1	7662	5280	1.64	2.14	1.30	Alkaline up-regulated by Rim101p, sulfur amino acid transport
YOR1	2531	1783	1.51	2.07	1.37	ATPase, ATP binding
CHS7	3622	2444	1.28	1.99	1.56	Endoplasmic-reticulum-to-Golgi-vesicle-mediated transport
MIR1	3051	4885	1.16	1.90	1.64	Putative mitochondrial phosphate transporter
Unknown						
MAP2	7653	6507	1.01	1.86	1.83	Unknown
TOM40	7636	6524	1.41	1.81	1.29	Unknown, protein transporter activity

[a] Significant duplicates were averaged.
[b] ALS, agglutinin-like sequence.

by WT levels of cAMP and are not induced by below-normal levels of cAMP. Genes fitting the pattern pde2 > WT > cap1 were kept as positively regulated genes and were subjected to SAM analysis. Additional criteria (criteria set B) were established to account for those genes that are maximally regulated with normal levels of cAMP but do not increase in expression above a threshold level of cAMP. This was accomplished by selecting genes that had similar Cy5/Cy3 ratios for the WT and the *pde2*/*pde2* mutant, which were those genes that were found to have a covariance of ≤0.75 after taking spot averages for $\log_2$ Cy5/Cy3 ratios from combined WT and pde2 hybrid groups. Genes for which the pde2 and WT Cy5/Cy3 ratios are equivalent (pde2 ≈ WT) and with averages (pde2 plus WT) of at least twofold (equal to 1 in terms of the $\log_2$ ratio) greater than that of cap1 were kept as positively regulated genes in germ-tube-inducing conditions. Genes found to meet criteria set A or B (or both) were listed in Table 2.

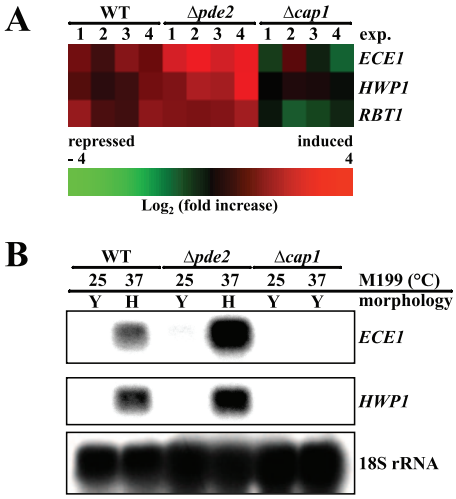


FIG. 2. Expression patterns of known hypha-specific genes. (A) Microarray data showing the fold induction of *ECE1*, *HWP1*, and *RBT1* in the WT (UnoPP-1), the *pde2*/*pde2* (Δ*pde2*; BPS15), and the *cap1*/*cap1* (Δ*cap1*; CAC1-1A1E1) strains under hypha-inducing (M199 at 37°C for 1.5 h) conditions relative to induction levels under yeast growth conditions (M199 at 25°C for 1.5 h), as described in Materials and Methods. (B) Northern blot showing *ECE1* and *HWP1* expression. Total RNA samples that also were used for the microarray experiments were separated by electrophoresis in a formaldehyde agarose gel, transferred to a nitrocellulose membrane, and probed with ^{32}P-labeled DNA probes for *ECE1*, *HWP1*, and 18S rRNA as a loading control. The membrane was exposed to X-ray film for 1 and 8 h for the detection of *HWP1* and *ECE1* mRNA, respectively, and for 4 h for the detection of 18S rRNA. exp., experiment. Y, yeast growth conditions; H, hyphal growth conditions.

For the identification of genes down-regulated by the cAMP signaling pathway in germ-tube-inducing conditions (Table 3), criteria were applied as described below to identify negatively regulated genes that had average $\log_2$ Cy5/Cy3 ratios in the order cap1 > WT ≥ pde2. The criteria (criteria set C) for negatively regulated, cAMP-dependent genes following 1.5 h in germ-tube-inducing conditions were established by inversing the logic used to find positively regulated genes. The difference between the average $\log_2$ Cy5/Cy3 ratios between WT and cap1 or between pde2 and cap1 was required to be less than 0.6. The $\log_2$ Cy5/Cy3 ratio for the WT was required to be less than or equal to 0.6 (≤1.5-fold). These criteria guarantee that genes in Table 3 are not induced by WT or by above-normal levels of cAMP (which were found in the *pde2*/*pde2* strain) but are possibly induced by below-normal levels of cAMP (which was found in the *cap1*/*cap1* strain) when the list contains only genes that fit the order of cap1 > WT > pde2. Genes fitting the pattern cap1 > WT > pde2 were kept as negatively regulated genes and were subjected to SAM analysis. Additional criteria (criteria set D) were established to account for those genes that have minimal expression with normal or above-normal levels of cAMP. To select genes that had similar ratios for WT and pde2, those genes found to have a covariance of ≤0.75 after taking spot averages for $\log_2$ Cy5/Cy3 ratios from combined WT and pde2 hybrid groups were identified. Genes with equivalent pde2 and WT $\log_2$ Cy5/Cy3 ratios (pde2 ≈ WT) and with averages (pde2 plus WT) of at least twofold (equal to 1 in the $\log_2$ ratio) less than that of cap1 were kept as negatively regulated genes in germ-tube-inducing conditions. Genes found to meet criteria set C or D (or both) were listed in Table 3.

Criteria and analysis methods for the identification of transcripts that were up-regulated or down-regulated by the cAMP signaling pathway during normal budding growth (mid-logarithmic phase) are described in the supplemental material.

Northern blot analysis. Northern blot analyses were performed as described before (6), using the same total RNA from each strain that was used to prepare labeled cDNA for the microarray experiments. Gene-specific probes were prepared by PCR using genomic DNA from strain UnoPP-1 as the template and the primer pairs shown in Table S2 in the supplemental material. The probe for *HWP1* was the gel-purified 609-bp *HWP1* insert of a phagemid clone (pBluescript+13) (73). To ensure that the lanes were loaded with equivalent amounts of RNA, a 687-bp PCR product representing the 18S rRNA gene of *C. albicans* SC5314 was generated and used as a probe (6).

Sensitivity to heat shock, high salt concentrations, and osmotic stress. To assess heat shock sensitivity, yeast extract-peptone-dextrose (YPD) plates spotted with cell suspensions (1 μl) of each strain, ranging in concentration from 10^8 to 10^4 cells/ml, were incubated at 55°C for 0, 10, 12, 15, and 20 min, followed by

immediate transfer to an incubator set at 30°C. Plates were observed after 24 and 48 h of incubation at 30°C and photographed. To examine the sensitivity to high salt concentrations and osmotic stress, the serially diluted cell suspensions ($10^4 \sim 10^7$ cells/ml) were spotted on YPD plates containing 1.5 M NaCl, 1.5 M KCl, and 2 M sorbitol, incubated at 30°C, and photographed.

Sensitivity to oxidative and nitrosative stress. To test responses to oxidative and nitrosative stress, H_2O_2 (Sigma), menadione (Sigma), sodium nitroprusside (SNP; Sigma), 3-morpholinosydnonimine (SIN-1; Calbiochem), and a peroxynitrite (Calbiochem) were used. YNB plates containing the indicated concentrations of each agent were spotted with 2 μl of 10^6 cells/ml of each strain and were incubated at 30°C for 2 days prior to visual assessment of growth and photography. For quantitative measurements (81), cells were diluted to 10^4 cells/ml with 1× PBS and were incubated at 37°C for 3 h with the indicated concentrations of the aforementioned agents. Fifty microliters of each sample then was cultured on YPD plates for 48 h at 30°C to measure the number of CFU. The percentage of survival of *C. albicans* strains in the presence of each agent at the indicated concentrations was calculated by the following formula: [(CFU of a strain incubated in PBS having the indicated concentration of an agent)/(CFU of the strain incubated in PBS only)] × 100.

Antifungal susceptibility test. The Etest (AB Biodisk) was performed to measure the fluconazole sensitivity of *C. albicans cap1*/*cap1* and *pde2*/*pde2* mutant strains by following the manufacturer's directions. Briefly, each yeast strain was grown at 30°C for 48 h in Sabouraud dextrose agar (Fisher Scientific) and resuspended in 0.85% NaCl to achieve a turbidity of 0.5 McFarland. Resuspended cells were evenly distributed onto solid RPMI 1640 medium (containing 2% glucose, morpholinepropanesulfonic acid [Sigma], 1.5% Bacto agar [Fisher Scientific]) by using a sterile swab and dried for at least 15 min. Fluconazole Etest strips (AB Biodisk) then were applied to the lawn of cells on the agar surface, and the plates were incubated at 35°C in a moist incubator until growth was clearly evident after 24 to 48 h. The MIC for each strain was determined using guidelines provided by the manufacturer.

RESULTS

Identification of genes modulated by the cAMP signaling pathway in germ-tube-inducing conditions. Modulation of cAMP levels by interfering with synthesis or breakdown as in the *cap1*/*cap1* and *pde2*/*pde2* mutants, respectively, affects many cellular characteristics, notably morphology, invasiveness, and resistance to nutritional starvation (5, 6). To identify genes with expression levels that correlated with cAMP levels by using the *cap1*/*cap1* cAMP hypomorph and the cAMP *pde2*/*pde2* hypermorph, we performed genome-wide transcriptional profiling experiments. Comparing the *pde2*/*pde2* and *cap1*/*cap1* mutant transcript profiles to that of WT is useful for identifying cAMP-regulated genes that influence cell metabolism and structure during the formation of nascent germ tubes. The transcriptional profiles of these two strains were compared to each other and to that of the WT.

To evaluate the reproducibility of cell cultures, RNA isolation, and sample processing, two arrays were hybridized with two separate batches of Cy3-labeled pooled RNA prepared from cultures growing as yeasts. Approximately 90% of the ORFs were within a twofold variation in normalized median intensities, as shown in the scatter plot in Fig. S1 in the supplemental material. To obtain another indication of reproducibility, the fold inductions of each gene in the two independent cultures were compared. Results of duplicate experiments in each independent culture were averaged. Approximately 90% of values differed by less than twofold. Overall, the control experiments indicated that the experimental conditions and sample preparations were reproducible.

The ability of the arrays to accurately reflect differences in the levels of cAMP-dependent transcripts under germ-tube-inducing conditions was provided by the signal intensity changes of known hypha-specific genes, such as *HWP1*, *ECE1*, and *RBT1* (8, 12, 73). In particular, our previous study showed that the *HWP1* transcript is markedly down-regulated in the *cap1*/*cap1* mutant (5). *HWP1*, *ECE1*, and *RBT1* were up-regulated by the activation of cAMP signaling in germ-tube-inducing conditions in the order pde2 > WT > cap1 in both microarray and Northern blot analyses (Fig. 2). These data indicate that our experimental scheme for microarray analysis was appropriate to perform a genome-wide search of cAMP-dependent genes modulated under germ-tube-inducing conditions.

Genes positively regulated by the cAMP signaling pathway in germ-tube-inducing conditions. Seventy-seven genes were differentially modulated by the cAMP signaling pathway in germ-tube-inducing conditions. The modulation of 37 genes was increased in both the WT and *pde2*/*pde2* mutants relative to that of the *cap1*/*cap1* mutant (Table 2). Conversely, 40 genes were found to be down-regulated (Table 3). The major portion of cAMP-dependent genes up-regulated during filamentous growth included cell surface proteins (18.9%) and those involved in glycolysis/gluconeogenesis (27.0%), ergosterol biosynthesis (10.8%), and fermentation (5.4%) (Table 2).

Six genes (*HWP1*, *ALS3*, *RBT1*, *ECE99*, *PGA45*, and *PDC11*) encoding known (12, 30, 73) or putative cell surface proteins were found to be up-regulated in hypha-inducing conditions in a cAMP-dependent manner (Table 2). With one exception, these genes exhibited reciprocal responses to hyper- or hypoactivation of the cAMP pathway. *PDC11*, which encodes pyruvate decarboxylase, was not elevated in the *pde2*/*pde2* mutant above the level of the WT and appeared more similar to genes involved in glycolysis (see below). In contrast, the hypha-specific surface protein gene, *HYR1* (7), was found to be hypha specific but cAMP independent, in that the fold induction in the WT (1.6) was approximately equivalent to that in the *cap1*/*cap1* (1.7) and *pde2*/*pde2* (1.6) mutants. In addition, several genes involved in ergosterol biosynthesis (*ERG1*, *ERG11*, and *ERG251*) and lipid metabolism (*SUR2*) were positively regulated by cAMP signaling during germ tube induction. Overall, these results indicated that during germ tube induction, pulses of cAMP change ergosterol and lipid contents in the cell membrane, in addition to remodeling the cell wall architecture with hyphal surface proteins.

Ten genes involved in gluconeogenesis or glycolysis (*TDH3*, *TPI1*, *CDC19*, *HGT6*, *HGT7*, *HGT8*, *PFK1*, *PFK2*, *GPM1*, and *GPM2*) (51, 57, 60, 69, 83) and two genes involved in fermentation (*PDC12* and *ADH2*) were found to be up-regulated by the cAMP signaling pathway. Whereas these genes were decreased in the *cap1*/*cap1* mutant, their expression levels in the *pde2*/*pde2* mutant appeared similar to those of the WT, indicative of maximal expression in the presence of WT levels of cAMP. The role of the Ras/cAMP signaling pathway in glucose transport and the activation of glycolysis has been reported for *S. cerevisiae* (11, 67, 76). The upregulation of genes involved in glycolysis and fermentation is consistent with physiological studies performed three decades ago that demonstrated that *C. albicans* transitions from aerobic respiration to anaerobic glycolysis and fermentation when placed in germ-tube-inducing conditions (44).

Among the uncharacterized ORFs without significant homologies to any other genes, one ORF (YER067W; ORF6.1464) was

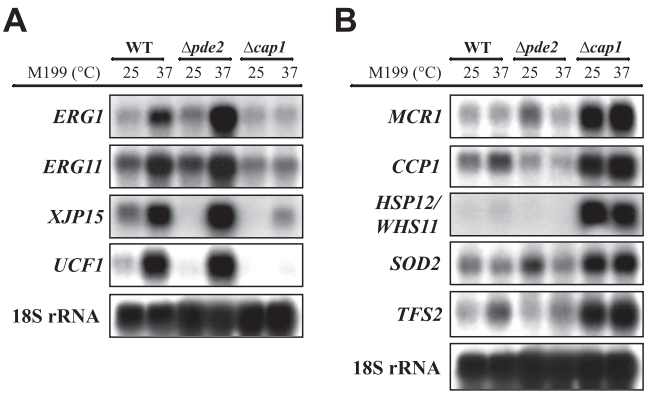


FIG. 3. Verification of expression patterns of genes modulated by the cAMP signaling pathway during bud-hypha transitions by Northern blot analysis. Northern blot analysis was performed with gene-specific PCR probes (genes positively regulated [A] and negatively regulated [B]) as described in the legend to Fig. 2B and Materials and Methods. Several replicated membranes were prepared using the same sample of total RNA and were used repeatedly for multiple probes after stripping the membrane as previously described (63). In general, the membrane was exposed to X-ray film for 1 to 2 days for detection of the mRNA of each probe, except for *ERG1*, *ERG11*, *UCF1*, and *MCR1* probes, which required only 6 to 7 h of exposure, and for the *XJP15* probe, which required only 1 h of exposure. The membrane probed with the control 18S rRNA was exposed for 4 h.

named *UCF1* (*u*p-regulated by *c*AMP in *f*ilamentous growth). The expression pattern determined from the array was further proved by Northern blot analysis (Fig. 3). The *UCF1* gene encodes a hypothetical protein of 201 amino acids without any conserved domains. Three ORFs (ORF6.6008, ORF6.4769, and ORF6.4770) homologous to the Tca2 retrotransposon were up-regulated. Although their roles in filamentous growth of *C. albicans* are unknown, it has been reported that transcription of retrotransposon-like elements is greatly up-regulated during a temperature shift from 25 to 37°C (16).

Genes negatively regulated by the cAMP signaling pathway in germ-tube-inducing conditions. Among the 40 genes that were down-regulated by the cAMP signaling pathway in germ-tube-inducing conditions (Table 3), two groups of genes were notable: those involved in amino acid/protein biosynthesis (35.0%) and those involved in the stress response (10.0%). Several genes encoding constituents of the ribosome (*RPL10A*, *RPP1A*, *RPP0*, *RPL12*, and *RPL23A*) and genes involved in protein or amino acid biosynthesis (*ILV5*, *ACO1*, *ASN1*, *IDH1*, *HIP1*, *LYS22*, and *HOM6*) were negatively regulated by the cAMP signaling pathway. These genes showed highest expression in the *cap1*/*cap1* mutant, whereas the *pde2*/*pde2* mutant appeared equivalent to the WT in terms of expression, with three exceptions (*RPP0*, *ILV5*, and *HOM6*). The negative regulation of the genes in this group is consistent with transitioning from respiration to aerobic glycolysis, which has been previously described to occur during the bud-hypha transition (44), and with the induction of filamentous growth during amino acid starvation through the interaction of CaGcn4 with the Ras/cAMP signaling pathway in *C. albicans* (78).

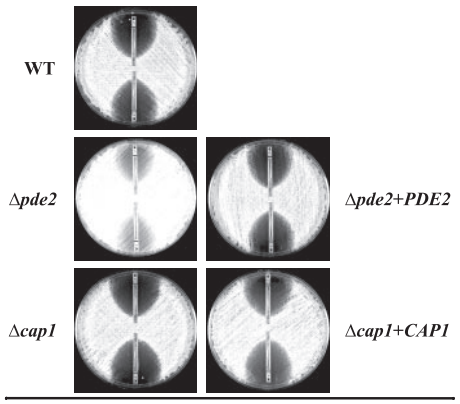
Of the four stress defense genes negatively regulated by cAMP (*MCR1*, *SOD2*, *HSP12* [*WHS11*], and *CCP1*), all are known to be involved in the response to oxidative stress in *S. cerevisiae* (15, 23, 46, 59, 79). *S. cerevisiae* strains with mutations in *MCR1*, *SOD2*, and *CCP1* genes show hypersensitivity to H_2O_2 and menadione, an O_2^- generator (15, 23, 46). *C. albicans* Sod2 regulates sensitivity to various stresses, including redox-cycling agents, high temperature, ethanol, and high concentrations of sodium and potassium (32). The negative regulation of *C. albicans CCP1* by the cAMP pathway is consistent with that in *S. cerevisiae* in which *CCP1* is regulated by *POS9*/*SKN7* (15); *POS9*/*SKN7* is negatively regulated by the cAMP pathway. In *S. cerevisiae*, *HSP12* is involved in responses to heat shock, osmotic shock, and oxidative damage (59, 79).

TFS1 encodes a putative phosphatidylethanolamine-binding protein homologous to a human phosphatidylethanolamine-binding protein (Raf kinase inhibitor protein) that inhibits the Ras/cAMP signaling pathway by G-protein-mediated inhibition of AC (42). In *S. cerevisiae*, Tfs1 was discovered as a multicopy suppressor of the *cdc25-1* mutation with a connection to the Ras/cAMP signaling pathway (61) and is a high-affinity inhibitor of carboxypeptidase Y (13). The role of Tfs1 may be conserved in fungal pathogens; recently, the *C. neoformans TFS1* homologue, *OVA1*, was shown to be upregulated in the *pka1* mutant by SAGE (serial analysis of gene expression) analysis and to negatively regulate the cAMP pathway (31).

cAMP-dependent genes modulated during unperturbed yeast growth. During logarithmic growth in yeast form, cAMP levels of the *cap1*/*cap1* and *pde2*/*pde2* mutants were lower and higher, respectively, than those of the WT (5). Having discovered that the cAMP signaling pathway controlled expression of hypha-specific genes such as *HWP1*, we wondered whether the other genes identified during hyphal growth were similar to *HWP1* in having low expression levels during yeast growth or whether the cAMP pathway regulated additional genes during budding yeast growth. Among the 246 genes differentially modulated by the cAMP signaling pathway in unperturbed budding growth of *C. albicans*, 104 and 142 were positively or negatively regulated, respectively, by the cAMP signaling pathway (Tables S3 and S4 in the supplemental material).

Of the 104 genes positively regulated by cAMP signaling in budding growth, only 10 genes (*HGT7*, *PFK1*, *GPM1*, *TPI1*, *PDC11*, *ERG251*, *PDC12*, *ADH2*, *PHO15*, and *UCF1*) also were found to be positively regulated by cAMP signaling in germ-tube-inducing conditions as well (Table 2; also see Table S3 in the supplemental material). Of the 142 genes negatively regulated by the cAMP pathway in yeast growth, only seven (ORF6.2999, *CCP1*, *ALS4*, *YET3*, *MAM33*, *HSP12*, and *SOD2*) were negatively regulated by cAMP signaling in germ-tube-inducing conditions (Table 3; also see Table S4 in the supplemental material). Overall, glycolysis and fermentation were up-regulated by cAMP, whereas the stress response was down-regulated by cAMP, regardless of morphology. Respiration was down-regulated in hyphae but not in yeast by cAMP. Therefore, respiration and fermentation are more balanced in yeast growth than in hyphal growth, in which anaerobic fermentation prevails.

Northern blot analysis of cAMP-dependent genes regulated during bud-hypha transitions of *C. albicans*. Because of the importance of genes involved in ergosterol biosynthesis and the stress response in candidiasis, we wanted to determine if the profiling results were predictive of strain differences in response to azole drugs and stress-response-inducing agents.



Strains	MIC (mg/ml)
WT (UnoPP-1)	0.125
Δ*pde2* (BPS15)	1.0
Δ*pde2*+*PDE2* (BPS9)	0.125
Δ*cap1* (CAC1-1A1E1)	0.094
Δ*cap1*+*CAP1* (CACRE1)	0.125

FIG. 4. Increased azole resistance by hyperactivation of the cAMP pathway of *C. albicans*. Susceptibilities to fluconazole were determined by the Etest strip method for the following isogenic strains: WT (UnoPP-1), *pde2*/*pde2* (Δ*pde2*; BPS15), *pde2*/*PDE2* reintegrants (Δ*pde2*+*PDE2*; BPS9), *cap1*/*cap1* (Δ*cap1*; CAC1-1A1E1), and *cap1*/*CAP1* reintegrants (Δ*cap1*+*CAP1*; CACRE1). The plates were photographed, and the fluconazole MICs for each strain were determined after 48 h of incubation at 35°C.

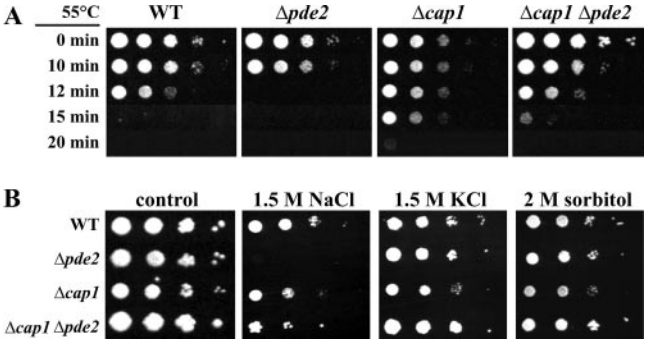


FIG. 5. Effect of heat shock, high salt concentrations, and osmotic shock on the viability of the WT and the cAMP signaling mutants. The WT (Unopp-1) strain and the mutants *cap1*/*cap1* (Δ*cap1*; CAC1-1A1E1), *pde2*/*pde2* (Δ*pde2*; BPS15), and *cap1*/*cap1 pde2*/*pde2* (Δ*cap1* Δ*pde2*; BPS27) were tested for (A) heat shock sensitivity (55°C) or (B) high salt concentrations (1.5 M NaCl or KCl) and high osmolarity (2 M sorbitol).

First, we verified the differential expression of these genes by Northern blot analysis (Fig. 3). In agreement with the microarray analysis, *ERG1* and *ERG11* mRNA levels of the *pde2*/*pde2* mutant and the WT strain were increased in hyphal growth conditions compared to those of yeast growth conditions. Expression levels of *ERG1* and *ERG11* were very low in the *cap1*/*cap1* mutant and were not increased in hyphal growth conditions (Fig. 3A). Expression of *ERG251* also was induced in the *cap1*/*cap1* mutant, but to a much lower level than that in the WT and *pde2*/*pde2* strains.

The stress response transcripts identified in the microarray analysis (*MCR1*, *SOD2*, *CCP1*, and *HSP12*) also were confirmed to be highly up-regulated in the hypoactive cAMP signaling *cap1*/*cap1* mutant compared to regulation of the WT and *pde2*/*pde2* mutant strains (Fig. 3B). *HSP12* was expressed only in the *cap1*/*cap1* mutant.

Hyperactivation of the cAMP pathway confers decreased sensitivity to azoles. Because the expression of *ERG11* has been reported to be increased in azole-resistant clinical isolates of *C. albicans* (49), we predicted that increased expression of *ERG* genes with potential membrane remodeling in the *pde2*/*pde2* mutant would be accompanied by decreased sensitivity to azole drugs. To gain support for this hypothesis, antifungal susceptibility was determined for the WT, *pde2*/*pde2*, and *cap1*/*cap1* strains. As predicted, the *pde2*/*pde2* mutant with increased expression of *ERG1* and *ERG11* was eightfold more resistant (MIC = 1.0 μg/ml) to fluconazole than the WT (MIC = 0.125 μg/ml) (Fig. 4). In contrast, the *cap1*/*cap1* mutant was slightly more sensitive to fluconazole (MIC = 0.094 μg/ml) than the WT. Reintegration of the WT *CAP1* and *PDE2* genes restored normal fluconazole susceptibility to both *cap1*/*cap1* and *pde2*/*pde2* mutants (MIC = 0.125 μg/ml). These important fluconazole susceptibility test results demonstrated for the first time for *C. albicans* that the expression of *ERG* genes, in addition to the expression of other genes that affect the composition of cell membranes, can be influenced by cAMP levels. We speculate that the presence of increased ergosterol levels leads to decreased sensitivity to the azole fluconazole.

Down-regulation of the cAMP signaling pathway increases resistance to heat shock, high salt concentrations, and oxidative and nitrosative stress. Based on results from the microarray and Northern blot analyses, we hypothesized that the activation of the cAMP signaling is inversely related to stress resistance. Therefore, we compared the viabilities of the WT strain to those of the *cap1*/*cap1*, *pde2*/*pde2*, and *cap1*/*cap1 pde2*/*pde2* mutants in the presence of heat shock, high salt concentrations, osmotic shock, and oxidative/nitrosative damage. The *cap1*/*cap1* mutant was more resistant to heat shock at 55°C than the WT and *cap1*/*cap1 pde2*/*pde2* strains (Fig. 5A). In contrast, the *pde2*/*pde2* mutant was more sensitive to heat shock than the WT and the *cap1*/*cap1 pde2*/*pde2* strains (Fig. 5A), indicative of an inverse relationship between heat shock and cAMP levels. The *pde2*/*pde2* mutant was more sensitive to sodium salt (1.5 M NaCl) than to potassium salt (1.5 M KCl) and sorbitol (2 M). Disruption of *CAP1* restored normal resistance to high-sodium salt (Fig. 5B). The *cap1*/*cap1*, WT, and *cap1*/*cap1 pde2*/*pde2* strains did not differ in response to the presence of NaCl or KCl or to high osmolarity.

To further investigate the role of the cAMP signaling pathway in response to oxidative/nitrosative stress that was predicted by the microarray analysis, cells were treated with H_2O_2 and menadione, an O_2^- generator. The *pde2*/*pde2* and the *cap1*/*cap1* mutants were more sensitive and resistant, respectively, to both H_2O_2 and menadione than the WT and *cap1*/*cap1 pde2*/*pde2* strains (Fig. 6A). The heightened sensitivity of a *C. albicans pde2*/*pde2* mutant to oxidative stress as well as to heat shock and high salt concentrations also was described in a recent study (84). The results showed that down-regulation of the cAMP signaling pathway promotes the resistance of *C. albicans* to reactive oxygen species (ROS)-mediated oxidative stress, whereas hyperactivation of the pathway has the opposite effect.

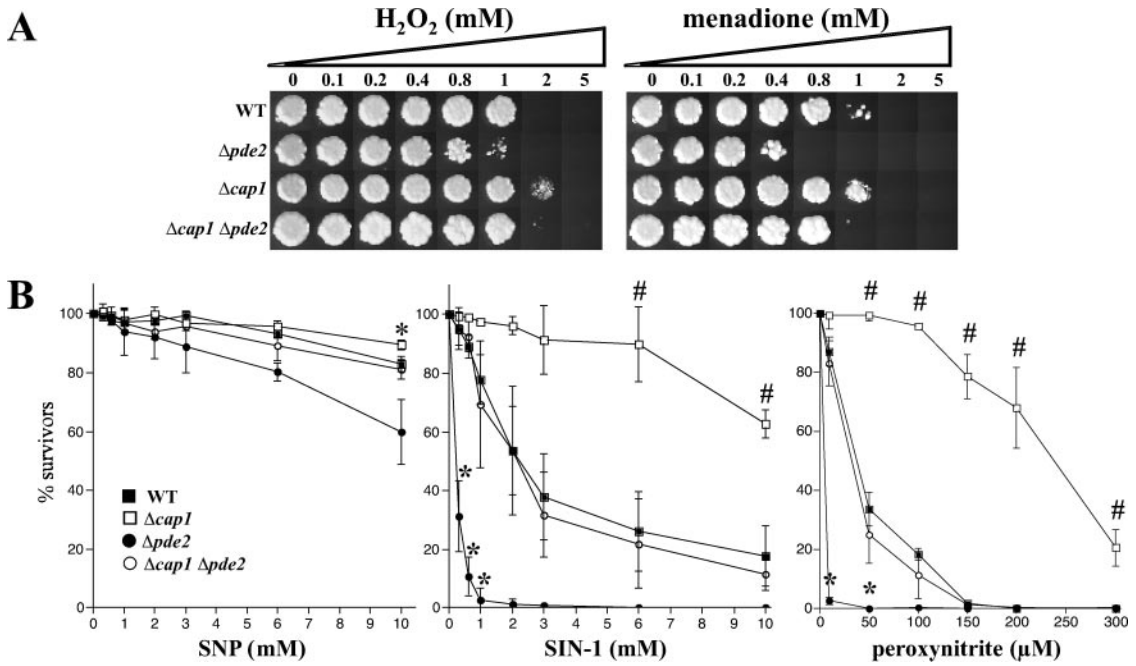


FIG. 6. Effect of hydrogen peroxide, superoxide, nitric oxide, and peroxynitrite on the viability of the WT and cAMP signaling mutants. (A) Volumes of 2 μl each of 10^6 cells/ml of the *C. albicans* strains described in the legend to Fig. 5 were spotted on YNB plates containing the indicated concentrations of H_2O_2 or menadione, a O_2^- generator, were incubated for 2 days, and were photographed. (B) Each strain was incubated at 37°C for 3 h with the indicated concentrations of SNP, SIN-1 (an $ONOO^-$ generator), or $ONOO^-$ itself. Error bars indicate the standard deviations from two independent experiments. The significant difference in percent survival between the *pde2*/*pde2* mutant and the other strains was denoted with an asterisk. The significant difference in percent survival between the *cap1*/*cap1* mutant and the other strains was denoted with a pound sign ($P < 0.05$ using Bonferroni's multiple comparison test [Prism 2.0b; GraphPad Software]).

SNP, an NO generator, was used to test whether *C. albicans* employs the cAMP signaling pathway to respond to nitrosative stress. In agreement with the previous results of Vazquez-Torres et al., we found that NO itself does not have strong candidacidal activity (81). However, at a concentration of 10 mM, the *pde2*/*pde2* mutant was slightly more susceptible to SNP than the *cap1*/*cap1* mutant (Fig. 6B). The data indicate that *C. albicans* is resistant to NO, but elevated cAMP levels increase susceptibility to NO slightly (Fig. 6B).

Peroxynitrite ($ONOO^-$), which results from the reaction of NO and O_2^-, has more potent candidacidal activity than either NO or O_2^- alone (81). The strains described above were tested for sensitivity to SIN-1 (an $ONOO^-$ generator) and to $ONOO^-$. SIN-1 more effectively killed WT *C. albicans* than did SNP (Fig. 6B). Here, we found that the *pde2*/*pde2* mutant was much more sensitive to SIN-1 than the WT and *cap1*/*cap1 pde2*/*pde2* strains, whereas the *cap1*/*cap1* mutant was more resistant. The concentration leading to approximately 50% killing of the WT strain after 3 h of incubation was 2 mM SIN-1, whereas a concentration of only 0.3 mM SIN-1 was required for approximately 70% killing of the *pde2*/*pde2* mutant (Fig. 6B). In contrast, the *cap1*/*cap1* mutant was highly resistant to SIN-1, in that survival was approximately 70% even at a concentration of 10 mM SIN-1 for the 3-h incubation period. $ONOO^-$ killed these strains more effectively than SIN-1 (Fig. 6B). Approximately 80% of the WT strain was killed by $ONOO^-$ at a concentration of 100 μM after 3 h of incubation, whereas approximately 6 mM of SIN-1 was required for killing a similar number of cells. As for the results using SIN-1, the *pde2*/*pde2* and *cap1*/*cap1* mutants showed much higher sensitivity and resistance, respectively, to $ONOO^-$ than the WT and *cap1*/*cap1 pde2*/*pde2* strains.

The steady-state mRNA levels of the stress response genes

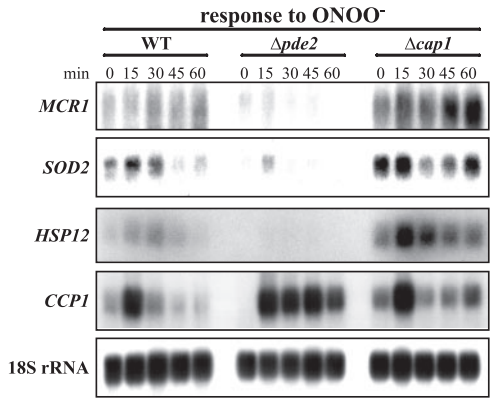


FIG. 7. Northern blot analysis of oxidative/nitrosative stress defense genes in response to peroxynitrite. Total RNAs isolated from *C. albicans* strains (WT [UnoPP-1], *cap1*/*cap1* mutant [Δ*cap1*; CAC1-1A1E1], and *pde2*/*pde2* mutant [Δ*pde2*; BPS15]) exposed to 50 μM of peroxynitrite for each indicated time of incubation were used for Northern blot analysis. A single membrane was stripped and repeatedly probed with stress-related genes (*MCR1*, *CCP1*, *SOD2*, and *HSP12*) as previously described. The membrane was exposed to X-ray film for 1 day (*MCR1*) or 2 days (*SOD2*, *HSP12*, and *CCP1*). The membrane probed with the control 18S rRNA was exposed for 4 h.

in response to peroxynitrite were determined by Northern blotting (Fig. 7). Expression of the *MCR1*, *SOD2*, and *HSP12* genes generally was higher in the *cap1*/*cap1* mutant than in the WT, but was lower in the *pde2*/*pde2* mutant, after 1 h of incubation (Fig. 7). However, the expression kinetics of the *MCR1*, *SOD2*, and *HSP12* genes were different from one another (Fig. 7). The kinetics of *CCP1* expression exhibited a pattern of expression different from that of *MCR1*, *SOD2*, and *HSP12*. The basal expression level of *CCP1* at time zero was lower in the *pde2*/*pde2* strain, but higher in the *cap1*/*cap1* strain, than that in the WT. In all strains, however, *CCP1* expression peaked at 15 min after exposure to $ONOO^-$ (Fig. 7). The expression level of *CCP1* remained elevated until 60 min in the *pde2*/*pde2* mutant, while it rapidly decreased after 15 min in the WT and *cap1*/*cap1* mutant (Fig. 7). In general, the data indicate that the genes involved in oxidative stress defense identified through the microarray analysis also were differentially regulated in response to $ONOO^-$. Taking these results together, the cAMP signaling pathway negatively modulates oxidative/nitrosative stress responses of *C. albicans*.

DISCUSSION

Several major insights into the function of the cAMP signaling pathway during germ-tube-inducing conditions were revealed in this study. Although other studies have addressed the role of the cAMP signaling pathway in *C. albicans* using *ras1*/*ras1*, *cdc35*/*cdc35*, or *efg1*/*efg1* mutants to study hypoactivation and a *pde2*/*pde2* mutant to study hyperactivation (19, 27, 45, 54, 84), this study is unique in its simultaneous use of mutants that directly affect the synthesis and the degradation of cAMP. Comparison of the *cap1*/*cap1* and *pde2*/*pde2* mutants to the WT permitted the assessment of each transcript in the presence of high and low concentrations of cAMP to confirm that expression levels were correlated with cAMP levels. Additional advantages of using the *cap1*/*cap1* and *pde2*/*pde2* mutants, as opposed to mutants in other genes that affect cAMP levels, are the reduced potential for interference of cross-talk from other pathways and the ability to assess the effects of cAMP in the absence of gross differences in cell growth rates. Our study also differed from others in focusing on the initiation phase of germ tube formation by examining changes in gene expression following the peak in cAMP at 1 h (6) during the formation of nascent germ tubes, whereas previous studies (27, 84) have focused on the maintenance phase of hyphal growth. Despite the differences in experimental designs, general agreement was found among the studies that are included in the following discussion. However, the experimental design of this study led to several important findings that were not reported in other studies.

The largest single group of transcripts found to be increased by cAMP in hyphal growth conditions in this study included those involved in glycolysis and fermentation. These findings suggest a molecular explanation for the results of historical studies showing that metabolic specialization from aerobic respiration to fermentative metabolism occurs in *C. albicans* during filamentation, with increased production of ethanol and less consumption of oxygen (44). Land et al. demonstrated that the addition of glycolytic inhibitors blocks the filamentation of *C. albicans* in germ-tube-inducing proline medium (44), indicating that the glycolytic pathway is required for filamentous growth. Our finding that genes involved in mitochondrial function and amino acid biosynthesis were significantly down-regulated by cAMP during germ tube induction is consistent with decreased mitochondrial activity and tricarboxylic acid (or Krebs) cycle activity during filamentous growth (44), because amino acids are precursors for key metabolites in the cycle. Findings from profiling studies using *efg1*/*efg1* mutants suggest that the up-regulation of genes in the glycolytic pathway occurs through Efg1p (19, 27). Changes in metabolic specialization, in concert with morphological transitions, accompanied by modulation of cAMP levels may be a common technique employed by *C. albicans* in response to various host environments. In a separate genomic profiling study, metabolic specialization also was predicted to occur during white-opaque phenotypic switching of *C. albicans* (43).

The correlation between the reduction of cAMP signaling and increased expression of stress defense genes, as well as the sensitivity to stress in the hyperactivation of cAMP signaling, which also was noted in previous studies (27, 84), led us to compare the three strains in terms of their resistance to stress. A major finding from our studies is that cAMP signaling governs susceptibility to peroxynitrite and superoxide radicals, which are important candidacidal molecules produced by the host. In humans, peroxynitrite is produced by neutrophils (polymorphonuclear leukocytes) (24) and alveolar macrophages and monocytes (77), whereas superoxide radicals are produced by most professional phagocytes, including neutrophils, eosinophils, monocytes, and macrophages (52). These are considered important candidacidal effector cells that play a major role in the host defense against candidiasis (80, 81). Murine macrophages are one of the major sources of ROS and reactive nitrogen species (RNS) in response to *Candida* infection (81). The β-glucan of *C. albicans* cell walls induces macrophages to produce O_2^-, which reacts with NO to produce $ONOO^-$ (38, 81). Our data highlight the importance of the ability of *C. albicans* to down-regulate the cAMP signaling pathway as a mechanism for counteracting a major host defense through sensing and detoxifying the caustic stress from $ONOO^-$ by the expression of various oxidative/nitrosative stress defense genes.

The cAMP regulation of stress defense genes in *C. albicans* and *S. cerevisiae* is similar in some respects and different in others. The *C. albicans pde2*/*pde2* mutant exhibited phenotypes comparable to those of the *S. cerevisiae pde2* mutant in sensitivity to heat shock and H_2O_2 (15, 66). In addition, *S. cerevisiae pde2* mutants are sensitive to nitrogen starvation in the presence of cAMP (85). In this work and in our previous studies, we showed that the *C. albicans pde2*/*pde2* mutant is more sensitive to heat shock, nutritional starvation, and H_2O_2 than the WT (5).

In contrast to the similar behaviors of the *PDE2* genes of *C. albicans* and *S. cerevisiae* regarding sensitivity to environmental stress, differences exist in the functions of their *CAP1* genes. Removal of *CAP1* from *S. cerevisiae* but not *C. albicans* results in an inability to grow in minimal media at high temperature and in sensitivity to nutritional starvation (22, 25). The role of Cap1 in oxidative and nitrosative stress responses of *S. cerevisiae* has not been reported. The reason(s) for the different phenotypes between the *C. albicans cap1*/*cap1* and *S. cerevisiae*

cap1 null mutants is unknown, but structurally conserved, functionally unique C-terminal regions of Cap proteins of the two organisms may be important (6). Other studies also found differences between *S. cerevisiae* and *C. albicans* in response to oxidative damage (34, 35). *C. albicans* was found to be more resistant to oxidative stress than the nonpathogenic *S. cerevisiae* (35). Although many signaling components in the pathway are conserved between the two organisms, it is possible that the function or expression levels of upstream receptors or downstream targets in response to common environmental stresses have diverged. For example, *C. albicans* strains with disruptions in Ca*MSN2* and Ca*MSN4* genes are as resistant to H_2O_2 as the WT (55), whereas *S. cerevisiae* strains with mutations in *MSN2* and *MSN4* are hypersensitive to H_2O_2 (28).

Our findings correlating *ERG* family gene expression with filamentous growth are consistent with those of other reports in the literature. Jung et al. reported that the ergosterol content of the *pde2*/*pde2* mutant (WH2-3U) exceeds that of the reference WT strain (CAF2-1) by 54% (36). Furthermore, Murad et al. have shown that *ERG3*, *ERG7*, *ERG8*, and *ERG25* are up-regulated in the hyperfilamentous *nrg1*/*nrg1* and *tup1*/*tup1* mutants compared to the expression of the WT strain in partial DNA microarray experiments (53). The importance of the increased expression of genes involved in ergosterol biosynthesis in the *pde2*/*pde2* mutant was shown by the increased resistance to fluconazole relative to that of the WT. Previously reported results (36, 53) and our data are consistent with other studies regarding the involvement of the cAMP signaling pathway in responses to azole drugs in *C. albicans* (33, 65) and in *S. cerevisiae* (41). Since ergosterol biosynthesis constitutes a target for a major class of antifungal compounds, the azoles, it would be interesting to further investigate how genes involved in ergosterol biosynthesis are transcriptionally regulated by the cAMP signaling pathway during filamentous growth of *C. albicans*.

The major impetus for this study was our earlier work showing that *HWP1* was regulated by the cAMP signaling pathway (5). *HWP1* also was found to be down-regulated in a *C. albicans cdc35*/*cdc35* mutant (27). The identification of cell wall protein genes that are coregulated with *HWP1* and that would contribute to the structure and function of the cell walls of hyphal growth forms was an important goal. Als3p functions as a fungal invasin, which is required for *C. albicans* to bind to multiple host cell surface proteins, including N-cadherins on endothelial cells and E-cadherin on oral epithelial cells (58). Rbt1 also encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein. Hwp1, Als3, and Rbt1 are known to be important virulence factors (12, 72, 86), indicating that cAMP signaling affects the virulence of *C. albicans* by controlling the expression of virulence genes as well as morphogenesis. *PGA45* and *PDC11* are predicted to be cell surface proteins; however, their cellular functions remain to be elucidated.

Other studies that have employed *efg1*/*efg1* mutants to identify genes regulated by the cAMP signaling pathway (27, 45) are in general agreement with ours regarding cell wall protein genes, except for *HYR1* (see below). Using a partial array, Lane et al. reported that *HWP1*, *ECE1*, *HYR1*, and *RBT1* are up-regulated in a hypha-specific fashion and are *EFG1* dependent (45). In the case of *HYR1*, the regulation by *EFG1*, and not by *CAP1* or *PDE2*, suggests that *EFG1* is involved in multiple signaling pathways, as previously suggested (27). In contrast, the study by Wilson et al. found decreased expression levels of *HWP1* and *ERG11* in their *pde2*/*pde2* mutants compared to those of the WT (84). The different methodologies, which also included different growth media (alpha minimum essential medium versus M199 in this study) and time points for RNA collection based on our focus on the initiation stage of germ tube formation and their focus on the maintenance phase, as well as differences in data analysis undoubtedly are responsible for the contrasting results for genes such as *HWP1* and *ERG11*.

Besides the aforementioned hypha-specific genes, Nantel et al. uncovered several genes with unknown function that are induced (*IHD1* and *IHD2*) or repressed (*RHD1*, *RHD2*, and *RHD3*) during hyphal development, but the involvement of the cAMP pathway was not addressed (54). Although *IHD2* was not differentially regulated between the WT, *pde2*/*pde2*, and *cap1*/*cap1* mutants in our studies, the fold induction of *IHD1* was found to be much higher in *pde2*/*pde2* mutants (3.4-fold) than the WT (1.3-fold) or the *cap1*/*cap1* mutant (1.2-fold), suggesting that *IHD1* is hypha specific and cAMP dependent. Regarding the *RHD* genes, the fold induction of *RHD1* and *RHD2* genes was less than 1.0 in all three strains, implying that the expression of these genes is repressed during bud-hypha transitions but is not cAMP dependent.

A comparison of our results to those of Nantel et al. (54) revealed additional genes that are regulated in hyphal growth but that are independent of cAMP signaling. *DDR48*, *PFY1*, *PTP3*, and *SNZ1* were induced during filamentous growth of the WT strain but were not cAMP regulated (data not shown). Other signaling pathways, such as those regulating Rfg1/Nrg1/Tup1 repressors, also may be involved in the expression of cAMP-independent genes during hyphal growth, although connections between cAMP and Rfg1/Nrg1/Tup1 pathways in *C. albicans* cannot be ruled out at this point. Kadosh and Johnson reported that about half of the genes induced during morphogenic transitions in serum and high temperature are controlled by Rfg1, Nrg1, and Tup1 repressors (37). Previous studies demonstrated that the *HGC1* gene encoding a hypha-specific G1 cyclin-related protein is transcriptionally regulated by Cdc35, Efg1, and Flo8, indicating that Hgc1p is a downstream target of the cAMP/PKA pathway (14, 87). Although the regulation of *HGC1* (ORF6.3156) in germ-tube-inducing conditions had the correct trend in that the descending order of fold inductions was *pde2*/*pde2* (1.43-fold), WT (0.99-fold), and *cap1*/*cap1* (0.54-fold), *HGC1* did not meet the criteria for this study. This discrepancy could result from the different germ-tube-inducing conditions used in the previous study (YPD and serum at 37°C) and this one (M199 at 37°C).

In yeast-inducing conditions, our data showed that glycolysis, fermentation, and ergosterol biosynthesis pathways also were controlled by cAMP. However, none of the hypha-specific cell wall protein genes were found to be up-regulated in yeasts; instead, a different set of cell surface protein genes was up-regulated by cAMP. Remodeling of the cell wall appears to be differentially modulated by cAMP depending upon morphology. Also, ergosterol biosynthesis was found to be up-regulated by cAMP in both morphologies, which is consistent with our results showing that cAMP mutants grown in yeast conditions exhibited differential azole sensitivity.

Several limitations should be noted regarding our study for identifying cAMP-dependent genes modulated during the filamentous growth of *C. albicans*. Since we used a single germ-tube-inducing condition (M199), genes that are regulated by cAMP in other germ-tube-inducing media (i.e., Lee's medium [pH 6.8, 37°C] or serum-containing medium) may not have been identified. In addition, cAMP-dependent genes with expression changes at time points other than 1.5 h would not be detected by the methods of this study. Third, the cutoff value for fold induction (1.5-fold) may exclude genes that are indeed modulated by cAMP signaling during filamentous growth but were below the fold induction threshold, as in the case of *RBT4* and *HGC1* described above. Finally, the microarray slides we used did not include all of the ORFs of *C. albicans*. Some cAMP-dependent genes could not be identified by this study because of their absence on the array.

In summary, in response to certain environmental signals, *C. albicans* triggers the activation of the cAMP signaling pathway to change its morphology from the yeast form to the hyphal form. During this process, activated cAMP signaling not only stimulates specific metabolic processes, including glycolysis/fermentation and ergosterol/lipid metabolism, but also reorganizes the cell surface architecture with the expression of hypha-specific cell wall genes, which are capable of enhancing the ability of the organism to colonize and invade host tissues. However, the activation of cAMP signaling also results in the repression of various stress defense genes potentially implicated in defending the organism from the host immune response and resisting harsh environments found in the host. Our study shows that both hyperactivation and hypoactivation of the cAMP signaling pathway have a major effect on transcriptional levels of various cellular targets, although distinct sets of genes are influenced by the morphology and growth conditions of *C. albicans*. Considering the major role of the cAMP signaling pathway in the virulence of *C. albicans* (5, 62), future functional characterization of potential cAMP-dependent target genes identified in this study will contribute to the development of new antifungal strategies for the prevention and treatment of candidiasis.

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