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. Here, microarrays were probed with RNA from strains 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 CAP1 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 CAP1 signaling pathways to provide insights into the molecular mechanisms of virulence that are regulated by CAP1 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 CAP1 during hyphal formation. Phenotypic traits predicted to be regulated by CAP1 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 valuable insights into the role of the CAP1 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, membrane ACs and soluble ACs (sACs). The transmembrane ACs 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 calcium and, more recently, by cAMP produced in mammalian cells by adenylate cyclases (ACs) (40-75, 82). Activated PKA subsequently phosphorylates other protein kinases, transcription factors, and other substrates to control various physiological processes.

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 cyclic AMP signaling pathways. The interaction of the sAC with G proteins is one example; the sAC (Cdc35/Cyr1) is regulated by 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 (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).
normal functions of host cells, and melanin, which acts as an antioxidant (2). The virulence of \textit{C. neoformans} also is significant attenuated by disrupting or mutating other genes that function in the cAMP signaling pathway, such as \textit{GPA1} (G protein), \textit{PKA1} (PKA catalytic subunit), and \textit{ACA1} (AC-associated protein) (1, 4, 20, 29). In the rice blast fungus \textit{Magnaporthe grisea}, deletion of the AC gene (\textit{MAC1}) abolishes the ability of cells to form appressoria for penetration of plant hosts and reduces growth (17).

In the ascomycete, pathogenic fungus \textit{Candida albicans}, genes that are regulated by the cAMP-dependent signaling pathway are important determinants of morphology and virulence. \textit{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 \textit{C. albicans} (74). Hyphal growth forms are broad-herent 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 \textit{C. albicans}, \textit{CaCDC35}, reduces normal growth and restricts morphology to the yeast form (62). Consequently, the homozygous \textit{cadc35/cadc35} mutant is avirulent in animal models of both vaginal and systemic infections. Downstream of \textit{CaCdc35}, \textit{Tpk1/2} and \textit{Sra1} have been identified as the catalytic and regulatory subunits, respectively, of PKA, mediating cAMP signaling (10, 68, 70). Both \textit{Tpk1} and \textit{Tpk2} promote hyphal formation, but each responds to different germ-tube-inducing signals (10, 68). Overexpression of the \textit{SRA1} gene represses filamentous growth, possibly by inhibiting the activation of \textit{Tpk1/2} (70). The transcription factors \textit{Efg1} and \textit{Flo8} are determinants of morphology and also are downstream targets of PKA in \textit{C. albicans} (9, 14, 48, 68). \textit{Efg1} contains a putative PKA phosphorylation site (T206) that is essential for hypha formation (9). \textit{Flo8} physically interacts with \textit{Efg1}, governing hyphal development (14). Homozygous \textit{efg1/efg1} and \textit{flo8/flo8} mutants are avirulent in an animal model of systemic candidiasis (14, 48).

Previously, we characterized two cAMP signaling pathway genes, \textit{CAP1} and \textit{PDE2}, encoding the AC-associated protein \textit{Cap1} and the high-affinity Pde2p, respectively (5, 6). \textit{Cap1} stimulates synthesis of cAMP, whereas \textit{Pde2} degrades cAMP. The \textit{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 \textit{cap1/cap1} and \textit{pde2/pde2} mutants is normal, except that buds are elongated in the latter case. In standard germ-tube-inducing conditions, the hypofilamentous \textit{cap1/cap1} mutant exhibits budding growth, whereas the hyperfilamentous \textit{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 \textit{PDE2} is nonfilamentous in most germ-tube-inducing conditions, including serum (5). Although \textit{pde2/pde2} mutants are hyperfilamentous, they are not hypervirulent; both hypofilamentous (\textit{cap1/cap1}) and hyperfilamentous (\textit{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 \textit{C. albicans}.

Genes that are coregulated with \textit{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 \textit{C. albicans} strains, \textit{UnoPP-1} (the WT control strain), \textit{CAC1-1A1E1} (the hypofilamentous \textit{cAMP} signaling \textit{cap1/cap1} mutant), and \textit{BPS15} (the hyperfilamentous \textit{cAMP} signaling \textit{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), reorients the architecture of the cell wall surface as well as ergosterol/lipoxygen synthesis, and modulates stress responses during germ tube induction in \textit{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**

\textit{C. albicans} strains and media. The \textit{C. albicans} strains used in this study are described in Table 1. For yeast and germ tube cultures, \textit{YNB} medium (yeast nitrogen base minimal medium containing 50 mM glucose and 0.0002% \\
biotin) and \textit{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 \textit{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 \textit{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 \textit{pde2/pde2} mutant formed germ tubes that were longer than those of the WT strain, whereas the \textit{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 \textit{WT} and the \textit{pde2/pde2} mutant, whereas budding yeast tubes formed by \textit{cap1/cap1} exhibited budding yeast growth (Fig. 1). Indirect immunofluorescence using anti-Hwp1 antibodies showed abundant expression of Hwp1 on true germ tubes formed by \textit{WT} and the \textit{pde2/pde2} mutant, whereas budding yeast tubes formed by \textit{cap1/cap1} exhibited budding yeast growth (Fig. 1). Indirect immunofluorescence using anti-Hwp1 antibodies showed abundant expression of Hwp1 on true germ tubes formed by \textit{WT} and the \textit{pde2/pde2} mutant, whereas budding yeast tubes formed by \textit{cap1/cap1} exhibited budding yeast growth (Fig. 1). Indirect immunofluorescence using anti-Hwp1 antibodies showed abundant expression of Hwp1 on true germ tubes formed by \textit{WT} and the \textit{pde2/pde2} mutant, whereas budding yeast tubes formed by \textit{cap1/cap1} exhibited budding yeast growth (Fig. 1).
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.

**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-Champaign). 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 post-processing 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). Flanking 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 log2 Cy5/Cy3 ratio was calculated for each gene. For the experiments in which germ tube growth conditions were used, the spot average log2 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 log2 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 log2 Cy5/Cy3 ratios in the order pde2 ≳ WT > cap1. The difference in log2 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 log2 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 log2 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

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### Table 1. C. albicans strains used in this study

| Strain     | Genotype | Parent strain | Reference |
|------------|----------|---------------|-----------|
| SC5314     | WT       | SC5314        | 25        |
| CAI4       | Δura3::imm434/Δura3::imm434 | CAI4        | 22        |
| UnoPP-1    | Same as CAI4, but Δeno1::URA3/ENO1 | CAI4        | 60        |
| CAC1-1A    | Same as CAI4, but cap1::hisG/cap1::hisG | CAC1-1A    | 6         |
| CAC1-1A1   | Same as CAC1-1A, but Δeno1::URA3/ENO1 | CAC1-1A1   | 5         |
| CAC1-1A1E1 | Same as CAC1-1A1, but Δeno1::URA3/ENO1 | CAC1-1A1E1 | 5         |
| CACRE1     | Same as CAI4, but ΔCAP1/cap1::hisG | CAC1-1A    | 6         |
| BPS1       | Same as CAI4, but PDE2/pde2::hisG/URAS3-hisG | CAI4        | 5         |
| BPS2       | Same as CAI4, but PDE2/pde2::hisG | BPS1        | 5         |
| BPS4       | Same as CAI4, but pde2::hisG | BPS2        | 5         |
| BPS7       | Same as CAI4, but pde2::hisG | BPS4        | 5         |
| BPS9       | Same as CAI4, but PDE2/pde2::hisG | BPS7        | 5         |
| BPS15      | Same as BPS7, but Δeno1::URA3/ENO1 | BPS7        | 5         |
| BPS16      | Same as CAC1-1A1, but PDE2/pde2::hisG | BPS16       | 5         |
| BPS17      | Same as CAC1-1A1, but PDE2/pde2::hisG | BPS17       | 5         |
| BPS18      | Same as CAC1-1A1, but pde2::hisG | BPS18       | 5         |
| BPS20      | Same as CAC1-1A, but pde2::hisG | BPS20       | 5         |
| BPS27      | Same as BPS20, but Δeno1::URA3/ENO1 | BPS20       | 5         |

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, re inoculated at a concentration of 5 × 106 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 hyphal 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 (Δpde2; strain BPS15) mutant, 20 to 30% for the WT strain (UnoPP-1), and 0% for the cap1/cap1 (Δcap1; CAC1-1A1E1) mutant.
| Gene          | ORF identity | Fold change between: | Function of gene product                                                                 |
|--------------|--------------|----------------------|------------------------------------------------------------------------------------------|
|              | ORF6         | ORF19                | WT  | pde2 | pde2 | cap1 | cap1 | WT  |                  |
| 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 |
| HP1          | 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 |          |                      |     |      |     |      |      |     | Structural constituent of cytoskeleton, mitotic spindles |
| STU11        | 6222         | 6610                 | 2.15 | 2.96 | 1.38 |      |      |     |                           |
| Ergosterol biosynthesis and lipid metabolism |          |                      |     |      |     |      |      |     |                           |
| ERG1         | 2816         | 406                  | 2.31 | 3.94 | 1.70 |      |      |     | Squalene monoxygenase          |
| ERG251       | 2054         | 4631                 | 2.25 | 3.89 | 1.73 |      |      |     | Homologous to C4 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 alpha-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               |
| HG77         | 2376         | 2023                 | 2.98 | 2.91 | 0.98 |      |      |     | Hexose transport               |
| PKI          | 3504         | 3967                 | 2.95 | 2.52 | 0.85 |      |      |     | Alpha subunit of phosphofructokinase |
| HG76         | 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 |      |      |     | Putative phosphoglycerate mutase |
| GPM1         | 7274         | 903                  | 2.22 | 2.22 | 1.00 |      |      |     | Phosphoglycerate mutase         |
| TPI          | 8886         | 1306                 | 1.85 | 2.16 | 1.17 |      |      |     | Triose-phosphate isomerase      |
| HG77         | 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, trancriptionally regulated by iron |
| ORF6.4769    | 4769         | 2372                 | 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         |

* Significant duplicates were averaged.
## Table 3. Genes down-regulated by the cAMP signaling pathway in germ-tube-inducing conditions

| Gene | ORF identity | Fold change between: | Function of gene product |
|------|--------------|----------------------|-------------------------|
|      | ORF6         | ORF19    | cap1 | cap1 | WT | cap1 | WT |    |
|      |              |          | WT   | pde2 |     | pde2 |     |    |
| Aldehyde metabolism |              |          |      |      |    |
| ALD4  | 5499         | 6306     | 1.49 | 2.56 | 1.72 | 2.56 | 1.72 | Aldehyde dehydrogenase (NAD) activity |
| IFD7  | 425          | 629      | 1.64 | 2.21 | 1.35 | 2.21 | 1.35 | Similar to aryl-alcohol dehydrogenases |
| ALD5  | 6640         | 5806     | 1.37 | 2.21 | 1.61 | 2.21 | 1.61 | Aldehyde dehydrogenase |
| Amino acid/protein biosynthesis |              |          |      |      |    |
| LYS22 | 4198         | 4506     | 2.56 | 2.60 | 1.01 | 2.60 | 1.01 | Putative homocitrate synthase, fungus specific |
| RPP0  | 7941         | 7015     | 1.21 | 2.54 | 2.11 | 2.54 | 2.11 | Putative ribosomal protein |
| CSP37 | 2388         | 2531     | 2.46 | 2.48 | 1.01 | 2.48 | 1.01 | Protein complex assembly |
| RPL12 | 6437         | 1635     | 1.84 | 2.41 | 1.31 | 2.41 | 1.31 | Predicted ribosomal protein |
| RPL10A| 3768         | 3465     | 2.16 | 2.36 | 1.09 | 2.36 | 1.09 | Predicted ribosomal protein |
| ILV5  | 3699         | 88       | 1.26 | 2.33 | 1.85 | 2.33 | 1.85 | Keto-acid reductoisomerase |
| ACO1  | 7870         | 6385     | 2.06 | 2.28 | 1.10 | 2.28 | 1.10 | Protein described as aconitase, glutamate biosynthesis |
| RPL23A| 4364         | 3504     | 1.92 | 1.99 | 1.03 | 1.99 | 1.03 | Putative ribosomal protein |
| HOM6  | 6665         | 2951     | 1.28 | 1.95 | 1.52 | 1.95 | 1.52 | 5-Amino-6-(5-phosphoribosylamino)uracil reductase |
| ASN1  | 5930         | 198      | 1.95 | 1.95 | 1.00 | 1.95 | 1.00 | Asparagine synthetase, soluble protein in hyphae |
| RPL1A | 4873         | 2992     | 1.71 | 1.95 | 1.14 | 1.95 | 1.14 | Conserved acidic ribosomal protein |
| IDH1  | 7385         | 4826     | 1.45 | 1.78 | 1.22 | 1.78 | 1.22 | Isocitrate dehydrogenase (NAD⁻) activity |
| HPI1  | 1878         | 3195     | 1.30 | 1.76 | 1.35 | 1.76 | 1.35 | Amino acid permease activity, amino acid transport |
| TUF1  | 7591         | 6047     | 1.65 | 1.69 | 1.02 | 1.69 | 1.02 | Putative translation elongation factor |
| Arginine catabolism |              |          |      |      |    |
| CAR2  | 4510         | 5641     | 1.66 | 1.89 | 1.14 | 1.89 | 1.14 | Unknown, alkaline up-regulated |
| Axial bud site selection |              |          |      |      |    |
| BUD3  | 8474         | 7079     | 1.11 | 1.58 | 1.42 | 1.58 | 1.42 | Axial bud site selection |
| Cell surface protein |              |          |      |      |    |
| ALS4  | 3075         | 4556     | 2.71 | 3.03 | 1.12 | 3.03 | 1.12 | Member of the ALS GPI-anchored protein family |
| ALS2  | 2999         | 1097     | 2.44 | 4.17 | 1.71 | 4.17 | 1.71 | Member of the ALS GPI-anchored protein family |
| Endoplasmic reticulum protein |              |          |      |      |    |
| YET3  | 6421         | 1564     | 1.54 | 1.84 | 1.20 | 1.84 | 1.20 | Unknown, Yet3p analog |
| Fatty acid catabolism |              |          |      |      |    |
| ARD1  | 5515         | 6322     | 1.64 | 2.01 | 1.23 | 2.01 | 1.23 | α-Arabitol dehydrogenase, NAD dependent |
| Glycine metabolism |              |          |      |      |    |
| SHM2  | 2091         | 5750     | 1.40 | 2.43 | 1.73 | 2.43 | 1.73 | Cytoplasmic serine hydroxymethyltransferase |
| Metabolism |              |          |      |      |    |
| MAM33 | 8087         | 7187     | 1.42 | 2.35 | 1.65 | 2.35 | 1.65 | Possible role in aerobic respiration |
| Mitochondria |              |          |      |      |    |
| MRH4  | 4978         | 3481     | 1.94 | 2.55 | 1.32 | 2.55 | 1.32 | Mitochondrial RNA helicase |
| RSM23 | 4979         | 3480     | 2.27 | 1.99 | 0.87 | 1.99 | 0.87 | Ribosomal small subunit of mitochondria |
| Nucleotide biosynthesis |              |          |      |      |    |
| TFS1  | 1861         | 1974     | 2.08 | 2.47 | 1.19 | 2.47 | 1.19 | Transcription is regulated upon yeast-hypha switch |
| URA4  | 1407         | 1977     | 1.68 | 1.77 | 1.06 | 1.77 | 1.06 | Nucleotide biosynthesis |
| Purine base metabolism |              |          |      |      |    |
| ADE4  | 5084         | 1233     | 1.81 | 1.87 | 1.04 | 1.87 | 1.04 | Unknown, flucytosine induced |
| Stress-associated protein |              |          |      |      |    |
| HSP12 | 2761         | 4216     | 3.58 | 4.66 | 1.30 | 4.66 | 1.30 | Heat shock protein, cell adhesion (oxidative stress response) |
| CCP1  | 957          | 238      | 2.35 | 2.95 | 1.25 | 2.95 | 1.25 | Cytochrome c peroxidase (oxidative stress response) |

Continued on following page
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 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 log2 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 log2 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.

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 log2 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 inverting the logic used to find positively regulated genes. The difference between the average log2 Cy5/Cy3 ratios between WT and cap1 or between pde2 and cap1 was required to be less than 0.6. The log2 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 log2 Cy5/Cy3 ratios from combined WT and pde2 hybrid groups were identified. Genes with equivalent pde2 and WT log2 Cy5/Cy3 ratios (pde2 = WT) and with averages (pde2 plus WT) of at least twofold (equal to 1 in the log2 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 materials.

**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 UN0P-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 (pBlue-script +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-pektoine-dextrose (YPD) plates spotted with cell suspensions (1 μl) of each strain, ranging in concentration from 10^6 to 10^7 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 (10³ – 10⁸ 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, H₂O₂ (Sigma), menadione (Sigma), sodium nitroprusside (SNP; Sigma), 3-morpholinophosphonodiacetic acid (Sigma; Calbiochem), and peroxynitrite (Calbiochem) were used. YNB plates containing the indicated concentrations of each agent were spotted with 1 μl of 10⁶ 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 10⁶ cells/ml with 1X 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 mutants 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, morpholinopropansulfonic 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 C. albicans 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, 50, 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, *TP1*, 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
were up-regulated. Although their roles in filamentous growth conserved domains. Three ORFs (ORF6.6008, ORF6.4769, codes a hypothetical protein of 201 amino acids without any The expression pattern determined from the array was further

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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 (MCRL, 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

| Strains | MIC (μg/ml) |
|--------|------------|
| WT     | 0.125      |
| Δpde2  | 1.0        |
| Δpde2−PDE2 (BPS9) | 0.125 |
| Δcap1 (CAC1-1A1E1) | 0.094 |
| Δcap1−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 (Δpde2; BPS15), pde2/PDE2 reintegrants (Δpde2+PDE2; BPS9), cap1/cap1 (Δcap1; CAC1-1A1E1), and cap1/CAP1 reintegrants (Δcap1−CAP1; CACRE1). The plates were photographed, and the fluconazole MICs for each strain were determined after 48 h of incubation at 35°C.

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 H₂O₂ and menadione, an O₂⁻ generator. The pde2/pde2 and the cap1/cap1 mutants were more sensitive and resistant, respectively, to both H₂O₂ 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.
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 \( \text{pde2/pde2} \) mutant was slightly more susceptible to SNP than the \( \text{cap1/cap1} \) 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 \( \text{pde2/pde2} \) mutant was much more sensitive to SIN-1 than the WT and \( \text{cap1/cap1} \) 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 \( \text{pde2/pde2} \) mutant (Fig. 6B). In contrast, the \( \text{cap1/cap1} \) 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 \( \mu M \) 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 \( \text{pde2/pde2} \) and \( \text{cap1/cap1} \) mutants showed much higher sensitivity and resistance, respectively, to ONOO\(^{-}\) than the WT and \( \text{cap1/cap1} \) \( \text{pde2/pde2} \) strains.

The steady-state mRNA levels of the stress response genes

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 \( \mu l \) 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\(_2\)O\(_2\) 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 \( \text{pde2/pde2} \) mutant and the other strains was denoted with an asterisk. The significant difference in percent survival between the \( \text{cap1/cap1} \) 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]).

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], \( \text{cap1/cap1} \) mutant [\( \Delta \text{cap1} \); CAC1-1A1E1], and \( \text{pde2/pde2} \) mutant [\( \Delta \text{pde2} \); BPS15]) exposed to 50 \( \mu M \) 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 (\( \text{MCR1} \), \( \text{SOD2} \), \( \text{HSP12} \), and \( \text{CCP1} \)) as previously described. 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 O₂⁻, 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 H₂O₂ (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 H₂O₂ 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 \textit{S. cerevisiae} and \textit{C. albicans} in response to oxidative damage (34, 35). \textit{C. albicans} was found to be more resistant to oxidative stress than the nonpathogenic \textit{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, \textit{C. albicans} strains with disruptions in Ca\textit{MSN2} and Ca\textit{MSN4} genes are as resistant to H$_2$O$_2$ as the WT (55), whereas \textit{S. cerevisiae} strains with mutations in \textit{MSN2} and \textit{MSN4} are hypersensitive to H$_2$O$_2$ (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 \textit{pde2/pde2} mutant (WH2-3U) exceeds that of the reference WT strain (CAF2-1) by 54% (36). Furthermore, Murad et al. have shown that \textit{ERG3}, \textit{ERG7}, \textit{ERG8}, and \textit{ERG25} are up-regulated in the hyperfilamentous \textit{nrg1/nrg1} and \textit{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 \textit{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 \textit{C. albicans} (33, 65) and in \textit{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 \textit{C. albicans}.

The major impetus for this study was our earlier work showing that \textit{HWPI} was regulated by the cAMP signaling pathway (5). \textit{HWPI} also was found to be down-regulated in a \textit{C. albicans} cdc35/cdc35 mutant (27). The identification of cell wall protein genes that are coregulated with \textit{HWPI} 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 \textit{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 contribute to the structure and function of the cell walls of \textit{C. albicans} (45). In the case of \textit{HYRI}, the regulation by \textit{EFG1}, and not by \textit{CAP1} or \textit{PDE2}, suggests that \textit{EFG1} is involved in multiple signaling pathways, as previously suggested (27). In contrast, the study by Wilson et al. found decreased expression levels of \textit{HWPI} and \textit{ERG11} in their \textit{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 \textit{HWPI} and \textit{ERG11}.

Besides the aforementioned hypha-specific genes, Nantel et al. uncovered several genes with unknown function that are induced (\textit{IHD1} and \textit{IHD2}) or repressed (\textit{RHD1}, \textit{RHD2}, and \textit{RHD3}) during hyphal development, but the involvement of the cAMP pathway was not addressed (54). Although \textit{IHD2} was not differentially regulated between the WT, \textit{pde2/pde2}, and cap1/cap1 mutants in our studies, the fold induction of \textit{IHD1} was found to be much higher in \textit{pde2/pde2} mutants (3.4-fold) than the WT (1.3-fold) or the \textit{cap1/cap1} mutant (1.2-fold), suggesting that \textit{IHD1} is hypha specific and cAMP dependent. Regarding the \textit{RHD} genes, the fold induction of \textit{RHD1} and \textit{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 \textit{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 \textit{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 \textit{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 \textit{HGC1} (ORF6.3156) in germ-tube-inducing conditions had the correct trend in that the descending order of fold inductions was \textit{pde2/pde2} (1.43-fold), WT (0.99-fold), and cap1/cap1 (0.54-fold), \textit{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 HGCI 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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