JOURNAL OF BACTERIOLOGY, Apr. 2002, p. 2058–2061 Vol. 184, No. 7
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.7.2058–2061.2002

Conserved Serine/Threonine Kinase Encoded by *CBK1* Regulates Expression of Several Hypha-Associated Transcripts and Genes Encoding Cell Wall Proteins in *Candida albicans*

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Received 20 August 2001/Accepted 12 January 2002

The opportunistic fungal pathogen, *Candida albicans*, is reported to have several potential virulence factors. A potentially significant factor is the ability to undergo morphological transition from yeast to hypha. This alteration of form is accompanied by many changes within the cell, including alterations in gene expression and cell wall composition. We have isolated a gene that encodes a highly conserved serine/threonine kinase that appears to be involved in the regulation of proteins associated with the cell wall. We have assigned the designation *CBK1* (cell wall biosynthesis kinase 1) to this gene. Mutants lacking *CBK1* form large aggregates of round cells under all growth conditions and lack the ability to undergo morphological differentiation. Additionally, these mutants show an altered pattern of expression of several transcripts encoding proteins associated with the cell wall. The results suggest that the kinase encoded by *CBK1* plays a general role in the maintenance and alteration of the cell wall of *C. albicans* in all morphologies.

Candida albicans is a member of the gastrointestinal normal microbiota of humans. This fungus is also a significant opportunistic pathogen capable of causing a wide range of infections in the compromised host (20). Advances in the understanding of the processes responsible for virulence in *C. albicans* have often come from the isolation and characterization of homologs responsible for similar processes in other fungi.

The corn pathogen *Ustilago maydis* alternates between the yeast and hyphal phases of growth during the infectious process. It was reported that a conserved serine/threonine kinase encoded by the *UKC1* gene affected morphogenesis and virulence (7). Disruption of *UKC1* resulted in strains that grew more slowly than wild-type strains, exhibited aberrant cellular morphology, produced more pigment, and had lost virulence. The homolog from *Neurospora crassa*, encoded by the *cot-1* gene, is necessary for maintenance of normal cellular and colonial growth. Strains containing a temperature-sensitive *cot-1* mutation showed compact growth, excessive branching, and lack of conidiation at the nonpermissive temperature (28). In the fission yeast, *Schizosaccharomyces pombe*, *orb6* encodes this conserved kinase. *orb6* is an essential gene in *S. pombe* that is involved in the maintenance of asymmetrical growth and reorganization of actin after mitosis (27).

We report here the isolation of the *C. albicans* homolog, which we have designated *CBK1* (cell wall biosynthesis kinase) after the *Saccharomyces cerevisiae* homolog (22). *CaCBK1* shows extensive homology to the other members of this family of kinases, a subgroup of the "AGC" kinases (11), within the defined kinase domain. *cbk1* null mutants demonstrate aberrant morphology and show an altered pattern of expression of several genes encoding cell wall proteins and altered expression of two hypha-related transcripts. The results suggest that, like the other homologs, Cbk1p is involved in the maintenance and regulation of cellular morphology.

Isolation and identification of the *UKC1* homolog. Sequence data for *C. albicans* were obtained from the Stanford DNA Sequencing and Technology Center website (http://www-sequence.stanford.edu/group/candida/ [revision date, Jan. 2001; last date accessed, July 2001]). A TBLASTN search of the *C. albicans* genome sequence database with the *U. maydis* Ukc1p sequence identified a single homologous sequence. Homology searches were conducted using the BLAST algorithm (1). An 826-bp fragment beginning 213 bp 5′ to the start site of *CBK1* was amplified by PCR, and this product was used as a probe for hybridization screening of a λGEM-12 genomic library (4). A 5.5-kb *Eco*RI insert containing the entire *CBK1* open reading frame (ORF) was subcloned into pUC18, generating the plasmid pMM1. A partial sequence of the *CBK1* ORF was determined by cycle sequencing with AmpliTaq DNA polymerase, the ABI Prism Ready Reaction Kit (Perkin-Elmer), and custom oligonucleotide primers. The sequence obtained from pMM1 was identical to the ORF designated YLN161 of Contig6-2278 that is available on the Stanford DNA Sequencing and Technology Center website (http://www-sequence.stanford.edu/group/candida/).

A BLASTP comparison with the GenBank database revealed that the predicted protein showed extensive homology with Cbk1p from *S. cerevisiae* (22), Ukc1p from *U. maydis* (7), orb6p from *S. pombe* (27), cot-1p from *N. crassa* (28), and multiple other members of the AGC family of eukaryotic kinases (5, 6, 11, 12).

To determine the role *CBK1* plays in *C. albicans*, a *cbk1* null mutant was constructed by replacing a 2,167-bp segment of the *CBK1* coding region with a *his*G-*URA3*-*his*G cassette (Fig. 1A). A 3.5-kb *Eco*RI-*Hin*dIII fragment from pMM1 was subcloned into like sites in pUC18. The resulting plasmid, pMM2, was digested with *Xba*I and filled in using Klenow DNA polymer-

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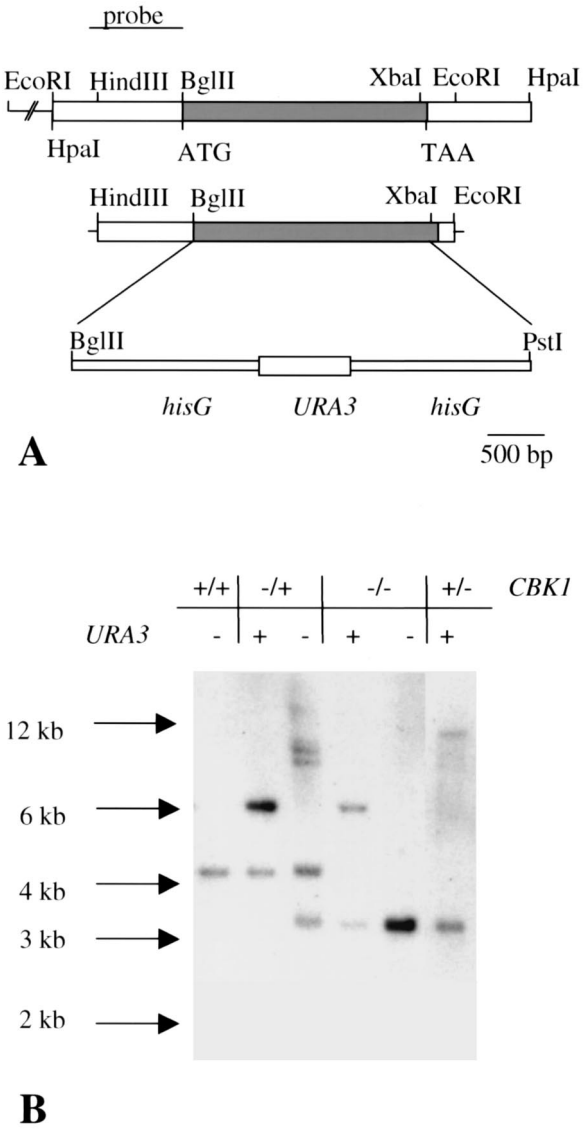

FIG. 1. Disruption of *C. albicans CBK1*. (A) Partial restriction map of the *CBK1* locus. The *Hin*dIII/*Eco*RI fragment was subcloned into pUC18, generating pMM1. The *Bgl*II/*Xba*I fragment of the *CBK1* locus was replaced with the *Bgl*II/*Pst*I *hisG-URA3-hisG* fragment to construct the disruption cassette. (B) Southern blot of genomic DNA digested with *Hpa*I and hybridized with a *CBK1*-specific probe. Genotypes of the strains are indicated above each lane.

ase to create a blunt end. The blunt-ended linearized plasmid was then digested with *Bgl*II. This resulted in the deletion of the *CBK1* ORF from position −1 to 2161. This region was replaced with a *Bgl*II-*Pst*I fragment from pMB7 (9), containing the *his*G-*URA3*-*his*G cassette. The *Pst*I end was made flush with Klenow DNA polymerase prior to ligation. This plasmid, pMM3, was cut with *Xmn*I and *Bbs*I to liberate the disruption cassette with 151 bp 5′ and 166 bp 3′ of *CBK1*. Eight micrograms of digested plasmid DNA was used to transform *C. albicans* strain CAI4 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434) by using a lithium acetate-mediated procedure (10). Reconstitution of the *cbk1* null strain was achieved by integration of plasmid pMM4. This plasmid was constructed by cloning an *Aat*I-*Nar*I fragment from pSMS44 (24), containing the entire *URA3* ORF and surrounding sequences, into pMM2 digested with the same enzymes. This plasmid was linearized at a *Bsr*GI site located 566 bp upstream of the *CBK1* ORF and it was used to transform strain CAMM 292-27.

A *Bbs*I-*Xmn*I fragment containing the cassette flanked by portions of *CBK1* was used to transform strain CAI4 (9) to uridine prototrophy. One representative transformant, termed CAMM-29 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G-*URA3-his*G/*CBK1*), was plated on 5-fluoroorotic acid (5-FOA) to select for intrachromosomal recombination between the repetitive *his*G sequences, resulting in the loss of the *URA3* selectable marker (9). The resulting strain, CAMM-292 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G/*CBK1*), was transformed with the disruption cassette to delete the second allele. The *cbk1* null mutant strain was named CAMM-292-4 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G/*cbk1*-Δ1::*his*G-*URA3-his*G). This strain was plated on 5-FOA to isolate the Uri$^-$ null mutant strain, CAMM-8 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G/*cbk1*-Δ1::*his*G). Proper integration events were verified in each strain by Southern blot analysis. Southern blot analysis was performed by standard methods (2). Blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) (5 and 10 min) and twice with 0.1× SSC, 0.1% SDS (15 min). All washes and hybridizations were performed at 68°C.

Genomic DNA was isolated from all representative isolates, digested with *Hpa*I, and hybridized with a *Hin*dIII-*Bgl*II fragment upstream of *CBK1* (Fig. 1B). The parental strain displayed a single hybridizing band of approximately 4.3 kb. The heterozygous Uri$^+$ *cbk1*/*CBK1* strain exhibited one band of 4.3 kb and a second band of approximately 6.3 kb, the predicted size of the replaced allele. One hybridizing band of approximately 3.1 kb, corresponding to the *cbk1*::*his*G allele, and a second band of 6.3 kb were exhibited in the null mutant strain. The *cbk1*/*cbk1* Uri$^-$ strain, CAMM-8, was transformed with *Bsr*GI-linearized plasmid pMM4 to reintroduce a wild-type allele of *CBK1*. The revertant strain was named CAMM-81 and exhibited the predicted 11.1-kb hybridizing band indicative of a proper integration event. Lack of expression of *CBK1* message in the null mutant and detection of a hybridizing band in the revertant strain, determined by Northern blot analysis, confirmed the validity of the Southern blot analyses (data not shown).

CBK1 is required for maintenance of normal cellular morphology. The effects of deletion of *CBK1* were analyzed using various liquid and agar-solidified media. Cells were grown to stationary phase in yeast-peptone-dextrose (YPD) at 30°C, and 10^6 cells were spotted on agar plates. The null mutant lacked the ability to form lateral hyphae at 37°C on medium 199 buffered at pH 7.0 and on Lee's medium (14) plates (Fig. 2). The heterozygous strain showed a decrease in hyphal radiation under these conditions (Fig. 2). Filamentation by the null mutant was also absent on spider medium (15) and 10% serum plates incubated at 37°C (data not shown). In addition to lacking hyphae, colonies of the *cbk1* strain had irregular borders and a rough appearance. This phenotype was noted on all media mentioned above at both 30 and 37°C (data not shown). In liquid Lee's medium at both 30 and 37°C, the null mutant strain formed large aggregations of cells (Fig. 2). This aggre-

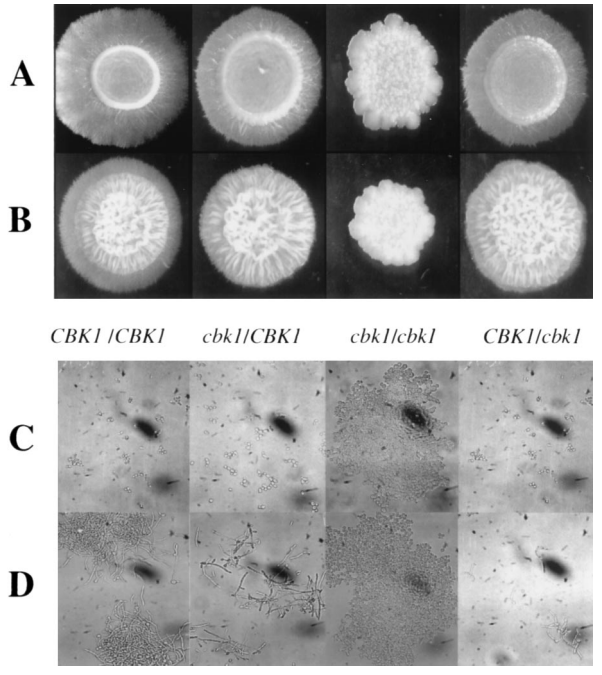

FIG. 2. Effects of *CBK1* deletion on colonial and cellular morphology. (A and B) Strains were spotted on medium 199 (A) or Lee's medium (B) plates and incubated at 37°C for 5 days. (C and D) Strains were grown overnight in YPD at 25°C and then inoculated into the Lee's medium at 25°C (C) or 37°C (D) for 4 h.

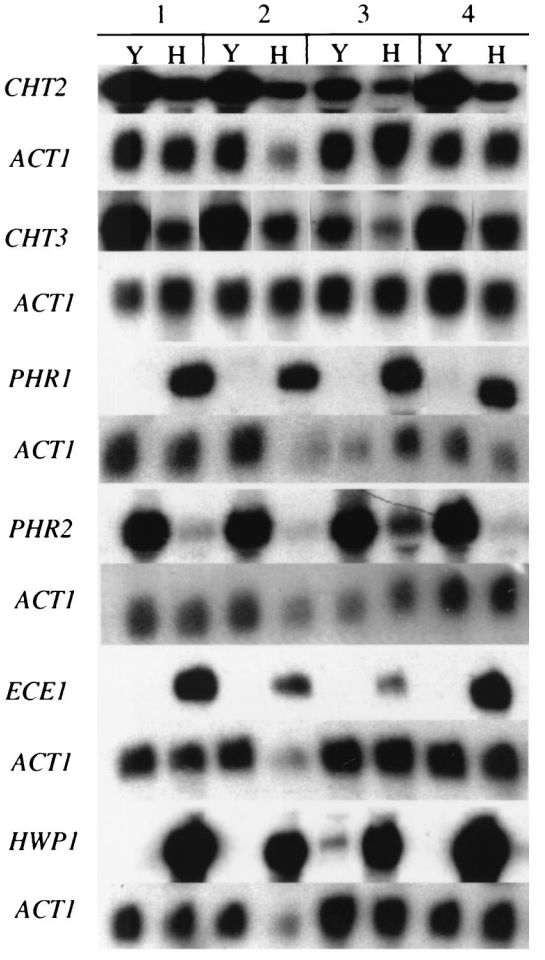

FIG. 3. Effect of *CBK1* mutations on gene expression. Total RNAs were isolated from CAI-4, CAMM-29, CAMM-292-4, and CAMM-81 (lane groupings 1 to 4, respectively) as mentioned in Materials and Methods. Cultures were incubated under conditions which favor yeast (medium 199 at 25°C and pH 4.0) (Y) or hyphae (medium 199 at 37°C and pH 7.0) (H). Hybridization probes are listed on the left.

gation phenotype was also noted at 37°C in liquid medium 199 at pH 7.0, 10% serum, and YPD and at 25°C in YPD, 10% serum, and medium 199 at pH 4.0 (data not shown). These aggregates could not be disrupted by the addition of SDS or EDTA, suggesting a physical attachment between cells. When grown under conditions that normally lead to yeast growth, the heterozygous strain grew essentially normally; however, more pseudohyphae were present at 37°C than in the control strain (Fig. 2). Reintroduction of the wild-type gene into the null mutant reverted all associated phenotypes. These results were reproduced with a set of two independently constructed mutants.

Altered expression pattern of cell wall- and hypha-related genes. The phenotype associated with disruption of *CBK1* in *C. albicans* is similar to that caused by disruption of the *CBK1* gene in *S. cerevisiae*. It has been reported that in *S. cerevisiae cbk1* mutants, expression of the gene encoding chitinase, *CTS1*, is altered (22). Furthermore, disruption of *CTS1* results in strains that form aggregations of cells under all culture conditions (13). *C. albicans* has three genes potentially encoding chitinases, but only two, *CHT2* and *CHT3*, have been shown to be expressed (16, 17). The possibility that altered expression of these two genes is at least partially responsible for the *cbk1* null mutant phenotype was examined by Northern blot analysis.

RNA was prepared by growing cultures to stationary phase at 30°C in YPD. Cells from these cultures were used to inoculate 500 ml of medium 199 buffered at pH 7.0 or pH 4.0, prewarmed to either 25 or 37°C, at an initial density of 4×10^6 cells per ml. Cultures were incubated at either 25 or 37°C for 3 h on a rotary shaker at 180 rpm. RNA was extracted from the cultures as previously described (2). Blotting and hybridization were conducted as described above for Southern blot analyses. Blots were hybridized with one of the following probes: a 1,063-bp *Aat*II-*Nde*I fragment from within the ORF of *PHR1*; a 1,257-bp *Bam*HI-*Nhe*I fragment of *PHR2*; a 1,078-bp PCR product from within the ORF of *CHT2*; an 804-bp PCR product from the ORF of *CHT3*; a 984-bp *Sty*I-*Xba*I fragment of the *CBK1* ORF; a 1.6-kb *Bam*HI-*Eco*RV fragment of the *ECE1* ORF; or a 4.3-kb *Eco*RI fragment containing the entirety of the *HWP1* ORF. All probes were labeled by random priming using [α-^{32}P]dCTP and Ready-to-go DNA labeling beads (Amersham Pharmacia Biotech).

The *CBK1* wild-type control strain showed the expected pattern of *CHT2* and *CHT3* expression (Fig. 3). The *cbk1* null mutant strain, CAMM-292-4, showed decreased expression of both genes under these conditions compared to the control strain. The *cbk1*/*CBK1* strain showed a level of expression of both genes similar to that of the control strain. While there was a reduction in the level of expression of the two chitinase-

encoding genes in the *cbk1* null mutant strain, abundant mRNA for both was apparent.

It has been noted in *S. cerevisiae* strains lacking a functional *CBK1* gene that the expression of more than 50 genes is altered by more than 2.2-fold (3). Because the decrease in the level of expression of *CHT2* and *CHT3* was not considered likely to be the sole cause of the complex morphological phenotype seen in the *cbk1* strain, the expression of several genes encoding known cell wall proteins was analyzed. *PHR1* and *PHR2* are regulated by external pH and are expressed in a reciprocal pattern to each other (19, 24). It has recently been suggested that the homologous proteins encoded by *PHR1* and *PHR2* are putative glycosidases necessary for proper construction of the cell wall (8, 18). The *cbk1* null mutant strain showed a pattern of expression of *PHR1* identical to that of the control strain (Fig. 3). However, the *cbk1*/*cbk1* strain showed an increased level of expression of *PHR2* at pH 7.0 when compared to the control strain (Fig. 3). Deletion of *CBK1* had no effect on the expression of *PRR1* and *RIM101*/*PRR2*, two known regulators of *PHR1* and *PHR2* expression (data not shown) (21, 23). Additionally, *CBK1* expression is unaffected in *prr1* and *rim101*/*prr2* null mutant strains (data not shown).

Expression of the hypha-associated transcripts, *ECE1* (4) and *HWP1* (25, 26), was also analyzed in the *cbk1* null mutant. Both *ECE1* and *HWP1* are expressed under conditions that lead to hyphal growth. The cellular location of Ece1p has not been identified, but Hwp1p has been shown to be a cell wall protein (26). Under conditions that led to hyphal growth of the control strain, both *ECE1* and *HWP1* showed a high level of induction (Fig. 3). The *cbk1* strain showed a lack of complete induction of *ECE1* at pH 7.0 and 37°C but, like the control strain, it lacked expression at pH 4.0 and 25°C. *HWP1* expression in the *cbk1* null mutant strain grown at pH 7.0 and 37°C was essentially identical to that of the control strain, but it showed a lack of complete repression under normally noninducing conditions. Reintroduction of a functional copy of *CBK1* in the revertant strain, CAMM-81, restored a normal pattern of expression of *CHT2*, *CHT3*, *ECE1*, *HWP1*, and *PHR2*. Whether Cbk1p affects expression of these genes directly or indirectly is not known, and further analysis will be necessary to elucidate its function in the maintenance of normal morphological development.

This work was supported by Public Health Service grant GM47727 from the National Institutes of Health.

We recognize the valuable assistance of Christopher Herbert.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Bidlingmaier, S., E. L. Weiss, C. Seidel, D. G. Drubin, and M. Snyder.** 2001. The Cbk1p pathway is important for polarized cell growth and cell separation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **21:**2449–2462.
4. **Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd.** 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. Infect. Immun. **61:**3648–3655.
5. **Brook, J. D., M. E. McCurrach, H. G. Harley, A. J. Buckler, D. Church, H. Aburatani, K. Hunter, V. P. Stanton, J. P. Thirion, T. Hudson, et al.** 1992. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell **68:**799–808.
6. **Buhr, T. L., S. Oved, G. M. Truesdell, C. Huang, O. Yarden, and M. B. Dickman.** 1996. A kinase-encoding gene from *Colletotrichum trifolii* complements a colonial growth mutant of *Neurospora crassa*. Mol. Gen. Genet. **251:**565–572.
7. **Dürrenberger, F., and J. Kronstad.** 1999. The *UKC1* gene encodes a protein kinase involved in morphogenesis, pathogenicity and pigment formation in *Ustilago maydis*. Mol. Gen. Genet. **261:**281–289.
8. **Fonzi, W. A.** 1999. *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of beta-1,3- and beta-1,6-glucans. J. Bacteriol. **181:**7070–7079.
9. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain constructions and gene mapping in *Candida albicans*. Genetics **134:**717–728.
10. **Geitz, D., A. St. Jean, R. A. Woods, and R. H. Scheistl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
11. **Hunter, T., and G. D. Plowman.** 1997. The protein kinases of budding yeast: six score and more. Trends Biochem. Sci. **22:**18–22.
12. **Justice, R. W., O. Zilian, D. F. Woods, M. Noll, and P. J. Bryant.** 1995. The *Drosophila* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. **9:**534–546.
13. **Kuranda, M. J., and P. W. Robbins.** 1987. Cloning and heterologous expression of glycosidase genes from *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **84:**2585–2589.
14. **Lee, K. L., H. R. Buckley, and C. C. Campbell.** 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. Sabouraudia **13:**148–153.
15. **Liu, H., J. Kohler, and G. R. Fink.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726.
16. **McCreath, K. J., C. A. Specht, and P. W. Robbins.** 1995. Molecular cloning and characterization of chitinase genes from *Candida albicans*. Proc. Natl. Acad. Sci. USA **92:**2544–2548.
17. **McCreath, K. J., C. A. Specht, Y. Liu, and P. W. Robbins.** 1996. Molecular cloning of a third chitinase gene (*CHT1*) from *Candida albicans*. Yeast **12:**501–504.
18. **Mouyna, I., T. Fontaine, M. Vai, M. Monod, W. A. Fonzi, M. Diaquin, L. Popolo, R. P. Hartland, and J. P. Latge.** 2000. Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. J. Biol. Chem. **275:**14882–14889.
19. **Mühlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. Mol. Cell. Biol. **17:**5960–5967.
20. **Odds, F. C.** 1988. Candida and candidosis. A review and bibliography, 2nd ed. Bailliere Tindal, London, United Kingdom.
21. **Porta, A., A. M. Ramon, and W. A. Fonzi.** 1999. *PRR1*, a homolog of *Aspergillus nidulans* palF, controls pH-dependent gene expression and filamentation in *Candida albicans*. J. Bacteriol. **181:**7516–7523.
22. **Racki, W. J., A. M. Becam, F. Nasr, and C. J. Herbert.** 2000. Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in *Saccharomyces cerevisiae* EMBO J. **19:**4524–4532.
23. **Ramon, A. M., A. Porta, and W. A. Fonzi.** 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR2*. J. Bacteriol. **181:**7524–7530.
24. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. *PHR1*, a pH-regulated gene of *Candida albicans* is required for morphogenesis. Mol. Cell. Biol. **15:**601–613.
25. **Sharkey, L. L., M. D. McNemar, S. M. Saporito-Irwin, P. S. Sypherd, and W. A. Fonzi.** 1999. *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1*, and *RBF1*. J. Bacteriol. **181:**5273–5279.
26. **Staab, J. F., and P. Sundstrom.** 1998. Genetic organization and sequence analysis of the hypha-specific cell wall protein gene *HWP1* of *Candida albicans*. Yeast **14:**681–686.
27. **Verde, F., D. J. Wiley, and P. Nurse.** 1988. Fission yeast *orb6*, a ser/thr protein kinase related to mammalian rho kinase and myotonic dystrophy kinase, is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proc. Natl. Acad. Sci. USA **95:**7526–7531.
28. **Yarden, O., M. Plamann, D. J. Ebbole, and C. Yanofsky.** 1992. *cot*-1, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J. **11:**2159–2166.