

Comparison of the Roles of Calcineurin in Physiology and Virulence in Serotype D and Serotype A Strains of *Cryptococcus neoformans*

M. CRISTINA CRUZ,¹ REY A. L. SIA,¹ MICHAEL OLSON,² GARY M. COX,³
AND JOSEPH HEITMAN^{1,2,3,4,5*}

Departments of Genetics,¹ Pharmacology and Cancer Biology,² Microbiology,⁴ and Medicine,³ and the Howard Hughes Medical Institute,⁵ Duke University Medical Center, Durham, North Carolina 27710

Received 1 July 1999/Returned for modification 3 September 1999/Accepted 7 November 1999

The calcineurin gene was cloned and disrupted in serotype D strains of *Cryptococcus neoformans*. Serotype A and serotype D calcineurin mutants were inviable at 37°C and avirulent in mice, whereas only serotype A mutants were cation stress sensitive. Thus, calcineurin plays conserved and divergent roles in serotype A and serotype D strains.

Cryptococcus neoformans is an encapsulated basidiomycete that is the most common cause of systemic mycosis in AIDS patients. *C. neoformans* strains are classified into five serotypes (A, B, C, D, and AD) and two varieties: *C. neoformans* var. *neoformans* (serotypes A, D, and AD) and *C. neoformans* var. *gattii* (serotypes B and C). Serotype A and serotype D strains exhibit significant variation and may represent distinct varieties that have diverged in ~18 million years of evolution (8, 9, 16, 19, 25). *C. neoformans* virulence factors include the capsule (3–5), melanin (22), prototrophy (17), and growth at 37°C (14). The protein phosphatase calcineurin is required for *C. neoformans* growth at 37°C and virulence (14).

Calcineurin is a Ca²⁺-calmodulin-activated phosphatase with catalytic and regulatory subunits (10). Calcineurin is the target of the T-cell-specific immunosuppressants cyclosporine (CsA) and tacrolimus (FK506). CsA and FK506 suppress the immune system by inhibiting calcineurin and preventing gene expression during T-cell activation. The antifungal activities of CsA and FK506 are mediated by a similar mechanism involving fungal homologs of calcineurin and the drug-binding proteins cyclophilin A and FKBP12 (1, 2, 6, 7, 14, 15).

Calcineurin has been identified from several fungi and regulates cell cycle progression in *Aspergillus nidulans* (21), hyphal elongation and growth in *Neurospora crassa* (11, 20), and mating and cytokinesis in *Schizosaccharomyces pombe* (18, 28). In *Saccharomyces cerevisiae*, calcineurin is required for recovery from pheromone arrest (12, 27) and regulates cation homeostasis and cell wall biosynthesis via the transcription factor Crz1 (1, 13, 23, 24).

The calcineurin gene has been identified, sequenced, and disrupted by homologous recombination in *C. neoformans* serotype A strain H99 (14). Calcineurin is essential for growth at 37°C, virulence in a rabbit model of cryptococcal meningitis, and cation homeostasis (14). Here, we isolated and disrupted the gene encoding the calcineurin A catalytic subunit (*CNAI*) from the congeneric serotype D strains of *C. neoformans* and compared the functions of calcineurin in serotype A and serotype D strains.

Identification, sequence, and disruption of the serotype D calcineurin A *CNAI* gene. The *CNAI* gene encoding calcineurin A was cloned from the serotype D strain JEC21 by PCR amplification with primers to conserved sequences in the serotype A *CNAI* gene. A 1.8-kb *CNAI* gene fragment was sequenced, revealing identity to calcineurin genes, and used in Southern blot analysis to show that the *CNAI* gene is contained on an 8-kb *EagI* fragment. The gene was recovered from a size-selected *EagI* genomic library and sequenced. There were seven amino acid differences between the calcineurin A protein in serotype A and serotype D.

To disrupt the serotype D *CNAI* gene, the *ADE2* gene was inserted at an *HpaI* site in the *CNAI* gene. The *cnal::ADE2* allele was transformed with a biolistic apparatus into serotype D *MAT α ade2* strain JEC50 and *MAT α ade2 ura5* strain JEC156. A total of 3 of 200 Ade⁺ transformants (1.5%) were viable at 24°C, grew poorly at 30°C, and were inviable at 37°C. The *cnal* mutation confers a more severe growth defect at 30°C in serotype D than in serotype A strains, consistent with findings that FK506 and CsA are more toxic at 30°C to serotype D than to serotype A strains (14). Southern blotting confirmed that the *CNAI* gene was replaced by the *cnal::ADE2* allele without ectopic integration in all three isolates (data not shown). In one isolate, the wild-type *CNAI* gene was precisely replaced by the *cnal::ADE2* allele by a double crossover. In two other isolates, tandem integrations had occurred at the *CNAI* locus. By an overlay blot with ¹²⁵I-calmodulin, calcineurin was expressed in *CNAI* wild-type strains but not in the *cnal::ADE2* mutant strains (data not shown). Genetic crosses and analysis of basidiospores by micromanipulation revealed that the *ADE2* gene was integrated into a single genomic locus. All Ade⁺ meiotic segregants exhibited the *cnal* temperature-sensitive growth defect, and all Ade⁻ segregants grew at 37°C, indicating that the *cnal* mutation is linked to the temperature-sensitive defect and the *ADE2* gene.

Comparison of calcineurin *cnal* mutant phenotypes in serotype A and serotype D strains. The growth of serotype A and serotype D wild-type and *cnal* mutant strains was compared using a quantitative dilution assay for viability at 37°C and sensitivity to Na⁺ and Li⁺ (Fig. 1). At 25°C, growth of serotype A and serotype D *cnal* mutants was comparable to that of wild-type strains. At 30°C, viability of serotype D *cnal* mutants was severely reduced compared to the wild-type strain; growth of wild-type and *cnal* mutant serotype A strains did not differ

* Corresponding author. Mailing address: Box 3546, 322 CARL Bldg., Research Dr., Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-2824. Fax: (919) 684-5458. E-mail: heitman001@mc.duke.edu.

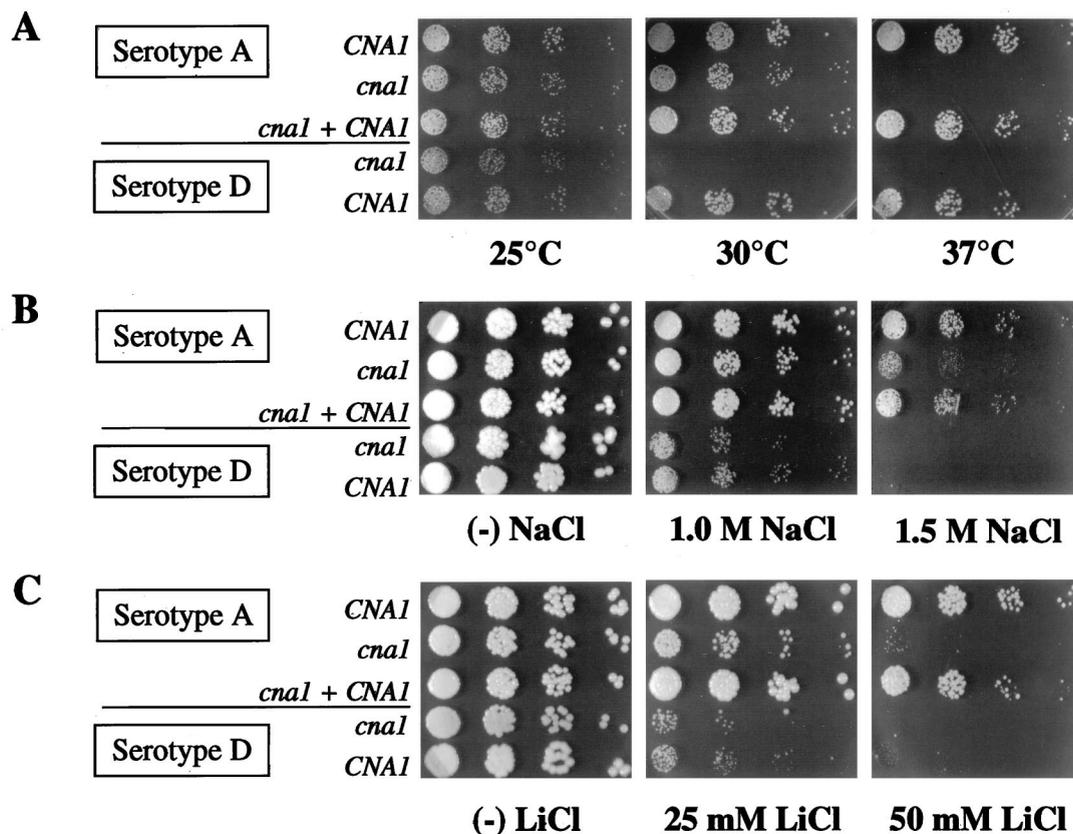


FIG. 1. Growth properties of serotype A and serotype D calcineurin mutants of *C. neoformans*. Isogenic serotype A *CNAI* wild-type (H99), calcineurin A *cnaI* mutant (AO4), and *cnaI* plus *CNAI* reconstituted (AO10) strains and isogenic serotype D *CNAI* wild-type (JEC21) and *cnaI* mutant (MCC3) strains were grown in YPD liquid medium and spotted onto YPD medium to compare growth at different temperatures (25, 30, and 37°C) (A), on medium containing 0 (-), 1, or 1.5 M NaCl (B) and 0 (-), 25, or 50 mM LiCl (C). Each dilution contained (from left to right) 1,250, 250, 50, and 10 cells of each strain. Cells shown in panels B and C were incubated for 72 h at 25°C.

at 30°C. At 37°C, both serotype A and serotype D *cnaI* mutant strains were inviable. Reintroduction of the *CNAI* gene restored growth at 37°C; thus, calcineurin is required for growth at 37°C for both serotype A and serotype D strains.

Growth of serotype A *cnaI* mutants was impaired compared to that of the *CNAI* wild-type or *cnaI* plus *CNAI* strains on 1.5 M NaCl (Fig. 1B). Serotype D *cnaI* mutants showed only a slight reduction in the number of CFU on 1 M NaCl. The growth of wild-type serotype D strains was completely inhibited on 1.5 M NaCl, whereas serotype A wild-type strain H99 was viable (Fig. 1B). Thus, the congenic serotype D strains are more sensitive to cation stress than serotype A strain H99, and the *cnaI* mutation has little effect on cation resistance in serotype D.

Growth of serotype A *cnaI* mutants was compromised on yeast extract-peptone-dextrose (YPD) medium with 50 mM LiCl compared to the wild-type or *cnaI* plus *CNAI* reconstituted strains (Fig. 1C). Growth of serotype D *cnaI* mutants was not reduced on 25 mM LiCl compared to wild-type strains. Both the wild-type and the *cnaI* mutant serotype D strains were inviable on 50 mM LiCl, in contrast to serotype A strain H99 (Fig. 1C).

Calcineurin is required for virulence of serotype A and serotype D strains in mice. We tested if calcineurin is required for virulence of serotype D strains of *C. neoformans* in the murine model of cryptococcosis. Each animal was infected with 10^7 *C. neoformans* cells by lateral tail vein injection. Ten ani-

mals were analyzed for each strain, and survival was monitored as the endpoint.

Infection of BALB/c mice with the serotype D *CNAI* wild-type strain JEC21 resulted in the death of 50% of infected animals by day 153 and 80% by day 195. In comparison, all 10 mice infected with the isogenic *cnaI* mutant strain MCC2 survived to day 238 (Fig. 2A). No viable fungal cells could be cultured from the brains or lungs of mice infected with the serotype D *cnaI* mutant strain, indicating that the mutant had been eradicated (results not shown).

Comparable studies were conducted with the serotype A *CNAI* wild-type, *cnaI* mutant, and *cnaI* plus *CNAI* reconstituted strains (Fig. 1B). Infection with *CNAI* wild-type strain H99 resulted in 50% mortality by day 8 and 100% mortality by day 20. The *cnaI* mutation severely attenuated virulence, and all 10 mice infected with the *cnaI* mutant survived to day 100. Reintroduction of the wild-type *CNAI* gene restored the virulence of the *cnaI* mutant, albeit not to the wild-type level, resulting in 100% mortality by day 38. Thus, calcineurin is required for virulence of serotype A strain H99 in two different animal models.

Studies conducted with C5-deficient mice resulted in 100 and 70% mortality at day 120 following infection with wild-type strains JEC20 or JEC21, whereas 100% of mice infected with serotype D *MATa* or *MATa cnaI* mutant strains survived to day 120 (Fig. 2C and D). Thus, calcineurin is required for the

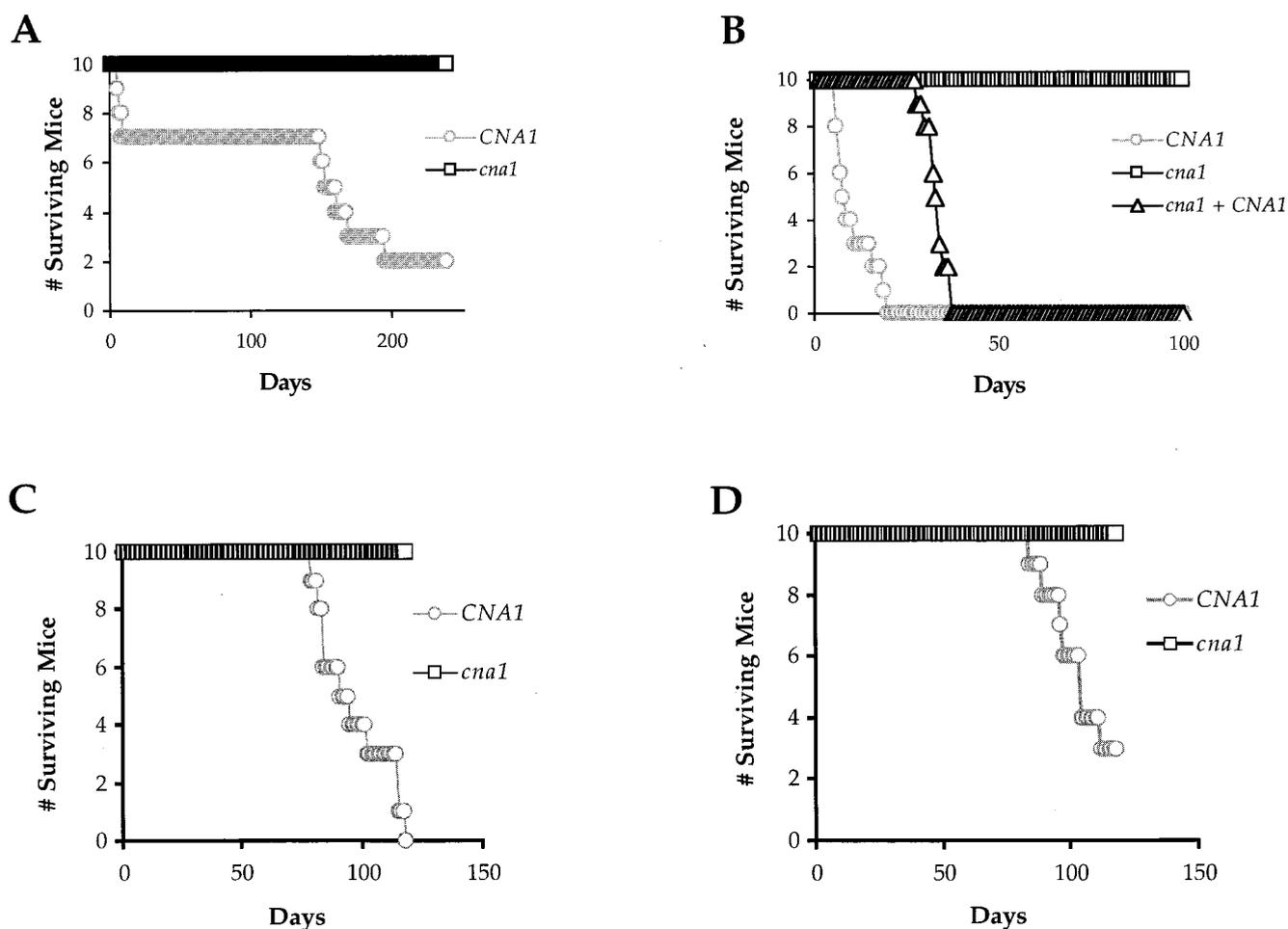


FIG. 2. Calcineurin is required for virulence of *C. neoformans* serotype D and serotype A strains in mice. BALB/c mice (10 for each strain) received injections in the lateral tail vein of 10^7 cells of the prototrophic *CNA1* wild-type *MAT α* serotype D strain (JEC21) or the congenic *MAT α* *cna1* mutant strain (MCC2) lacking calcineurin A (A) or the isogenic serotype A *CNA1* wild-type (H99), *cna1* mutant (AO4), and *cna1* plus *CNA1* reconstituted (AO10) strains (B). C5-deficient mice (10 each) received injections in the lateral tail vein of 10^7 cells of prototrophic *CNA1* wild-type *MAT α* serotype D strain (JEC20) or the isogenic *MAT α* *cna1* mutant strain (MCC10) lacking calcineurin (C) or the prototrophic *CNA1* wild-type *MAT α* serotype D strain (JEC21) or the congenic *MAT α* *cna1* mutant (MCC5) lacking calcineurin (D). Mice were observed twice daily and the number of mice surviving versus time were plotted.

virulence of both *MAT α* and *MAT α* serotype D strains in both immunocompetent and immunodeficient mice.

Summary and conclusions. Serotype A calcineurin A mutants are hypersensitive to Na^+ and Li^+ compared to the isogenic wild-type strain (14). These findings suggest a role for calcineurin analogous to that in *S. cerevisiae*, where calcineurin controls expression of the Pmr2 ion pump that effluxes Na^+ and Li^+ (26). In contrast, in the *C. neoformans* serotype D strain, calcineurin does not appear to regulate cation homeostasis because calcineurin mutants were as sensitive to cations as were isogenic wild-type strains. In fact, the wild-type congenic serotype D strains were inherently more sensitive to cation stress than serotype A strain H99, indicating significant physiological differences between serotype A and serotype D strains under stress growth conditions. Our findings provide further support for the classification of serotype A and serotype D strains into distinct varieties of *C. neoformans*: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (9).

Calcineurin is required for virulence of serotype D and serotype A strains in both rabbits and mice. Calcineurin is the target for the immunosuppressive antifungal agents CsA and

FK506 and thus is an attractive target for antifungal agents (6, 7, 14, 15). The role of calcineurin in growth at 37°C and virulence in *C. neoformans* could not have been predicted from model organisms, because calcineurin is not required for growth at 37°C in *S. cerevisiae* and is only required for normal growth at 22°C but not at 37°C in *S. pombe* (28). Thus, studies of pathogens are necessary to establish the functions of specific genes in virulence.

Nucleotide sequence accession number. The nucleotide sequence of the serotype D calcineurin A *CNA1* gene has been deposited in the GenBank database under accession no. AF1559511.

We thank Tony Means and Elizabeth McDougal for generously providing ^{125}I -calmodulin.

This work was supported by grants RO1 AI39115 and AI42159 from NIAID (to J.H.), supplement AI41937-S1 from the NIAID (to M.C.C. and J.H.), and PO1 grant AI44975 to the Duke University Mycology Research Unit. Joseph Heitman is an associate investigator of the Howard Hughes Medical Institute and a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology.

M.C.C. and R.A.L.S. contributed equally to this work.

REFERENCES

1. Breuder, T., C. S. Hemenway, N. R. Movva, M. E. Cardenas, and J. Heitman. 1994. Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. *Proc. Natl. Acad. Sci. USA* **91**:5372–5376.
2. Cardenas, M. E., R. S. Muir, T. Breuder, and J. Heitman. 1995. Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. *EMBO J.* **14**:2772–2783.
3. Chang, Y. C., and K. J. Kwon-Chung. 1994. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol. Cell. Biol.* **14**:4912–4919.
4. Chang, Y. C., and K. J. Kwon-Chung. 1998. Isolation of the third capsule-associated gene, *CAP60*, required for virulence in *Cryptococcus neoformans*. *Infect. Immun.* **66**:2230–2236.
5. Chang, Y. C., L. A. Penoyer, and K. J. Kwon-Chung. 1996. The second capsule gene of *Cryptococcus neoformans*, *CAP64*, is essential for virulence. *Infect. Immun.* **64**:1977–1983.
6. Cruz, M. C., L. M. Cavallo, J. M. Görlach, G. Cox, J. R. Perfect, M. E. Cardenas, and J. Heitman. 1999. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in *Cryptococcus neoformans*. *Mol. Cell. Biol.* **19**:4101–4112.
7. Cruz, M. C., M. D. Poeta, P. Wang, R. Wenger, G. Zenke, V. F. J. Quesniaux, N. R. Movva, J. R. Perfect, M. E. Cardenas, and J. Heitman. 2000. Immunosuppressive and nonimmunosuppressive cyclosporin analogs are toxic to the opportunistic fungal pathogen *Cryptococcus neoformans* via cyclophilin-dependent inhibition of calcineurin. *Antimicrob. Agents Chemother.* **44**:143–149.
8. Franzot, S. P., B. C. Fries, W. Cleare, and A. Casadevall. 1998. Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *J. Clin. Microbiol.* **36**:2200–2204.
9. Franzot, S. P., I. F. Salkin, and A. Casadevall. 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J. Clin. Microbiol.* **37**:838–840.
10. Hemenway, C. S., and J. Heitman. 1999. Calcineurin: structure, function, and inhibition. *Cell Biochem. Biophys.* **30**:115–151.
11. Kothe, G. O., and S. J. Free. 1998. Calcineurin subunit B is required for normal vegetative growth in *Neurospora crassa*. *Fungal Genet. Biol.* **23**:248–258.
12. Moser, M. J., J. R. Geiser, and T. N. Davis. 1996. Ca^{2+} -calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca^{2+} -calmodulin-dependent protein kinase. *Mol. Cell. Biol.* **16**:4824–4831.
13. Nakamura, T., Y. Liu, D. Hirata, H. Namba, S.-I. Harada, T. Hirokawa, and T. Miyakawa. 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. *EMBO J.* **12**:4063–4071.
14. Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman. 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J.* **16**:2576–2589.
15. Odom, A., M. D. Poeta, J. Perfect, and J. Heitman. 1997. The immunosuppressant FK506 and its nonimmunosuppressive analog L-685,818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. *Antimicrob. Agents Chemother.* **41**:156–161.
16. Perfect, J. R., B. B. Magee, and P. T. Magee. 1989. Separation of chromosomes of *Cryptococcus neoformans* by pulsed field gel electrophoresis. *Infect. Immun.* **57**:2624–2627.
17. Perfect, J. R., D. L. Toffaletti, and T. H. Rude. 1993. The gene encoding phosphoribosylaminoimidazole carboxylase (*ADE2*) is essential for growth of *Cryptococcus neoformans* in cerebrospinal fluid. *Infect. Immun.* **61**:4446–4451.
18. Plochocka-Zulinska, D., G. Rasmussen, and C. Rasmussen. 1995. Regulation of calcineurin gene expression in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **270**:24794–24799.
19. Polacheck, I., and G. A. Lebens. 1989. Electrophoretic karyotype of the pathogenic yeast *Cryptococcus neoformans*. *J. Gen. Microbiol.* **135**:65–71.
20. Prokisch, H., O. Yarden, M. Dieminger, M. Tropschug, and I. B. Barthelmeß. 1997. Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical Ca^{2+} gradient. *Mol. Gen. Genet.* **256**:104–114.
21. Rasmussen, C., C. Garen, S. Brining, R. L. Kincaid, R. L. Means, and A. R. Means. 1994. The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. *EMBO J.* **13**:2545–2552.
22. Salas, S. D., J. E. Bennett, K. J. Kwon-Chung, J. R. Perfect, and P. R. Williamson. 1996. Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans*. *J. Exp. Med.* **184**:377–386.
23. Stathopoulos, A. M., and M. S. Cyert. 1997. Calcineurin acts through the *CRZ1/TCN1*-encoded transcription factor to regulate gene expression in yeast. *Genes Dev.* **11**:3432–3444.
24. Stathopoulos-Gerontides, A., J. J. Guo, and M. S. Cyert. 1999. Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. *Genes Dev.* **13**:798–803.
25. Sudarshan, S., R. C. Davidson, J. Heitman, and J. A. Alspaugh. 1999. Molecular analysis of the *Cryptococcus neoformans ADE2* gene, a selectable marker for transformation and gene disruption. *Fungal Genet. Biol.* **27**:36–48.
26. Wieland, J., A. M. Nitsche, J. Strayle, H. Steiner, and H. K. Rudolph. 1995. The *PMR2* gene cluster encodes functionally distinct isoforms of a putative Na^+ pump in the yeast plasma membrane. *EMBO J.* **14**:3870–3882.
27. Withee, J. L., J. Mulholland, R. Jeng, and M. S. Cyert. 1997. An essential role of the yeast pheromone-induced Ca^{2+} signal is to activate calcineurin. *Mol. Biol. Cell.* **8**:263–277.
28. Yoshida, T., T. Toda, and M. Yanagida. 1994. A calcineurin-like gene *ppb1⁺* in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. *J. Cell Sci.* **107**:1725–1735.

Editor: T. R. Kozel