Vol. 10, No. 9

NOTE

Cell Wall Chitosan Is Necessary for Virulence in the Opportunistic Pathogen *Cryptococcus neoformans*[▽]

Lorina G. Baker,[1] Charles A. Specht,[2] and Jennifer K. Lodge[1]*

Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110,[1] and Department of Medicine, University of Massachusetts, 364 Plantation Street, Worcester, Massachusetts 01605[2]

***Cryptococcus neoformans* is an opportunistic fungal pathogen that causes meningoencephalitis. Its cell wall is composed of glucans, proteins, chitin, and chitosan. Multiple genetic approaches have defined a chitosan-deficient syndrome that includes slow growth and decreased cell integrity. Here we demonstrate chitosan is necessary for virulence and persistence in the mammalian host.**

Cryptococcus neoformans is an opportunistic fungal pathogen that causes cryptococcosis in immunocompromised individuals. The incidence of cryptococcosis is rising in direct proportion to the spread of human immunodeficiency virus (for review see, Heitman et al. [10]). It is estimated that worldwide, over 1 million people develop life-threatening cryptococcal meningitis and that an estimated 700,000 die every year from cryptococcosis (18). Current antifungal therapies for treatment of cryptococcosis are inadequate: amphotericin B is effective, but toxic; fluconazole is fungistatic, having high relapse rates; flucytosine (5-FC) incurs frequent resistance (19–21, 25). Components of the fungal cell wall are attractive antifungal drug targets because they are absent in the host. The echinocandins, for example, target β-(1,3)-glucan synthase, an enzyme essential for the synthesis of a major component in the fungal cell wall (13, 24). Echinocandins are well-tolerated by patients but are not effective against *C. neoformans* (14).

The cell wall of *C. neoformans* is an essential organelle that provides cellular structure and integrity. An indispensable component of this cell wall that contributes to its strength and integrity is chitin (1). Chitin is a linear polymer of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) synthesized by the chitin synthase/regulator (Chs/Csr) system. Chitin can then be enzymatically deacetylated to chitosan by chitin deacetylases (EC 3.5.1.41). The cell wall of *C. neoformans* contains polymers of both chitin and chitosan, and the quantity and ratio can be dynamic depending on the growth phase (4, 5). In *C. neoformans*, only the isoforms Chs3 and Csr2 produce the majority of vegetative cell wall chitin, which is converted to chitosan (despite there being eight chitin synthase genes [*CHS*] and three synthase regulator genes [*CSR*]) (5). *C. neoformans* also expresses three chitin deacetylases, Cda1, Cda2, and Cda3, that can each produce chitosan. Strains of *C. neoformans* deleted for *CHS3*, *CSR2*, or all three chitin deacetylases are

deficient in chitosan production and have a common set of phenotypes, including sensitivity to a variety of cell wall inhibitors. This indicates that chitosan is essential for the proper maintenance of cell wall integrity in *C. neoformans*, and Chs3, Csr2, and the chitin deacetylases are the main proteins that contribute to its formation (4, 5). Chitosan deficiency also affects two virulence factors of *C. neoformans*, melanin and capsule. The *chs3Δ*, *csr2Δ*, and *cda1Δ cda2Δ cda3Δ* strains "leak" melanin, and under certain conditions their capsule is enlarged and less uniform (4, 5). These three strains, *chs3Δ*, *csr2Δ*, and *cda1Δ cda2Δ cda3Δ*, represent three distinct deletions of proteins with different activities, which independently lead to chitosan deficiency.

We hypothesized that chitosan would be an important structural component for *C. neoformans* growth in the mammalian host, based on the impaired cell wall observed in chitosan-deficient mutants *in vitro*. In this report we show that chitosan is produced by *Cryptococcus* during murine infection and that strains deficient in chitosan due to mutations in three distinct genetic processes are avirulent and are rapidly cleared by the mammalian host. For each of the chitosan-deficient lines, at least two independently derived isolates were obtained and tested. The data were similar for the two independently derived isolates in all three lines; therefore, data shown here for each chitosan-deficient strain were derived from one of the isolates.

***C. neoformans* produces chitosan during *in vivo* growth.** *In vitro* analyses indicated that the cell wall integrity of strains that produce little to no chitosan is compromised, but the strains are still viable (4, 5). This suggests that chitosan is not essential for *in vitro* growth; however, we postulated that the loss of cell wall chitosan could impact the ability of *C. neoformans* to survive within the host. To begin addressing this question, we considered that if chitosan is important for growth *in vivo*, then the cellular content of it would be similar to amounts measured for cultures grown *in vitro*. Therefore, we first established whether chitosan was produced by *C. neoformans* during growth in the mammalian host. Mice were inoculated intranasally with 10^6 wild-type (KN99) cells, and lungs from each mouse were harvested 16 days postinfection (p.i.), homoge-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8230, St. Louis, MO 63104. Phone: (314) 286-2125. Fax: (314) 362-7325. E-mail: lodgejk@wustl.edu.
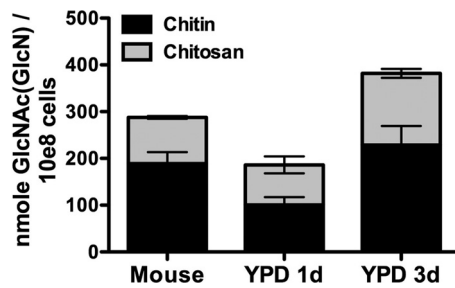
FIG. 1. Chitosan and chitin levels from KN99 recovered from mouse lungs 16 days p.i. Chitosan and chitin levels from mouse lungs were compared to levels measured for KN99 grown in YPD at 30°C for 1 day and 3 days from a starting optical density at 600 nm of 0.10. Error bars indicate standard deviations for averaged measurements from four mice, and five 1-day YPD and four 3-day YPD cultures.

FIG. 2. Growth curves of strains in liquid YNB at 30°C (A) or 37°C (B). The inset of panel B shows csr2Δ, chs3Δ, cda1Δ cda2Δ cda3Δ, and trx1Δ strains on an expanded y axis. Strains are listed on the right side of panel A. The x axis is in hours. Data consist of three bioreplicates, each with two technical replicates, with standard errors shown.

nized in phosphate-buffered saline, and then extracted in 1 M KOH at 80°C for 1.5 h. Deacetylation of chitin under the conditions used for alkaline extraction was not detectable in control experiments (data not shown). Pellets containing the cryptococci were collected by centrifugation from each lung extract along with small amounts of lung material. The pellets were suspended in 1 ml 0.1 M KOH. A 10-μl aliquot was removed, stained with Solophenyl flavine (11) to facilitate counting using a hemacytometer when viewed by fluorescence microscopy (fluoroscein isothiocyanate filters). Each sample, containing approximately 2×10^8 cryptococci, was divided in two, and the chitosan in one half was converted to chitin by acetylation with acetic anhydride and the other half remained untreated. The chitin in both halves was measured, and the chitosan amount was calculated as the difference between the two measurements as previously described (4). The chitosan and chitin data, normalized to the number of cryptococcus cells from each sample, were found to be comparable to KN99 cultured in yeast extract-peptone-dextrose (YPD) at 30°C for 1 day and 3 days (Fig. 1). Chitin and chitosan were not detected from uninfected lungs (data not shown). After 16 days in the host lung, C. neoformans cell number increased by 200-fold (about 8 doublings). Chitin and chitosan levels are dynamic during growth in YPD (Fig. 1), so direct comparisons to any specific in vitro condition are difficult (5). These data demonstrated that chitosan was produced by C. neoformans during in vivo growth and implied that it may be important during infection of the mammalian host.

Strains with reduced chitosan grow slowly at 37°C. The ability to grow at host temperature is a feature indicative of a successful mammalian pathogen. Previous reports on the three chitosan-deficient strains, chs3Δ, csr2Δ, and cda1Δ cda2Δ cda3Δ, indicated that chs3Δ and csr2Δ are temperature sensitive when grown in vitro and that cda1Δ cda2Δ cda3Δ is not (4, 5). However, these experiments were performed on solid media. The inability of the chs3Δ and csr2Δ strains to grow on a plate may have indicated that the cells were dead or that they had entered a state of stasis, in which they were still viable but unable to divide. To distinguish between these two possibilities, we investigated the growth rate of the chitosan-deficient strains in liquid yeast nitrogen base with 2% glucose (YNB) medium, pH 4, at either 30°C or 37°C. Strains deficient in cellular chitosan, chs3Δ, csr2Δ, and cda1Δ cda2Δ cda3Δ, grew

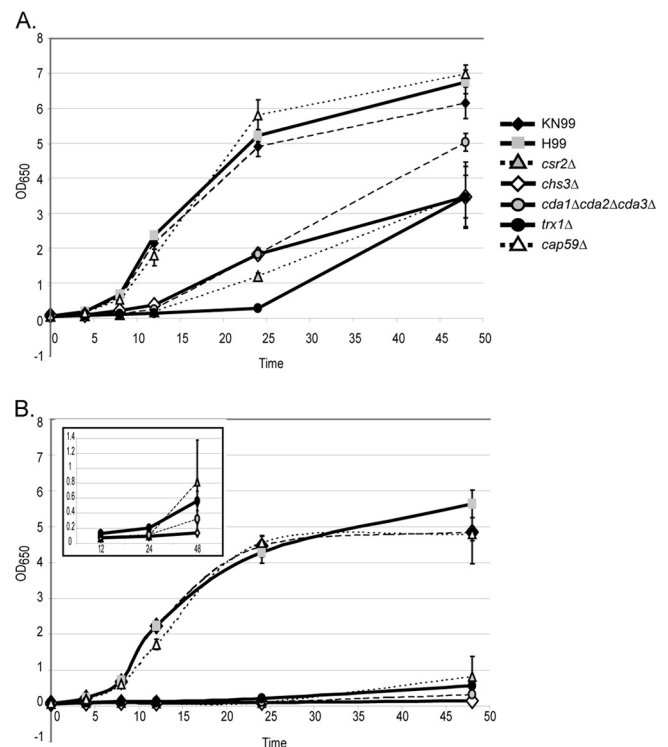more slowly than the wild type at both temperatures and had a greater growth defect at 37°C. They experienced a long lag phase compared to the wild type, anywhere between 12 h and 24 h at both temperatures, before undergoing a doubling event over the next 24-hour period (Fig. 2). These data demonstrate that although the chitosan-deficient strains grow more slowly, they are viable at 37°C and will grow at the mammalian host temperature.

Lack of cellular chitosan attenuates virulence. A popular hypothesis is that strains that grow slowly at host temperature will be avirulent. However, Missall et al. observed that a strain deleted for a thioredoxin, TRX1, that grows slowly at 37°C was still virulent (15). We compared the growth rate of the trx1Δ mutant at both 30°C and 37°C in YNB and found that it was very similar to what we observed with the three chitosan-deficient strains (Fig. 2). These data suggest that slow growth at 37°C does not necessarily predict avirulence and that testing the chitosan-deficient strains for virulence might provide valuable insights.

Our initial hypothesis was that chitosan would be important for virulence, based on the in vitro cell wall sensitivities of the deficient strains and on the observation that wild-type C. neoformans produces chitosan during infection. To determine if loss of cellular chitosan affects virulence, we used an inhalation mouse model as previously described (8, 15) with the following exceptions; mice (CBA/J; Jackson Laboratories) were inoculated with 1×10^5 cells per strain tested and sacrificed when they reached 75% of their original body weight. All mice inoculated with the wild-type strain
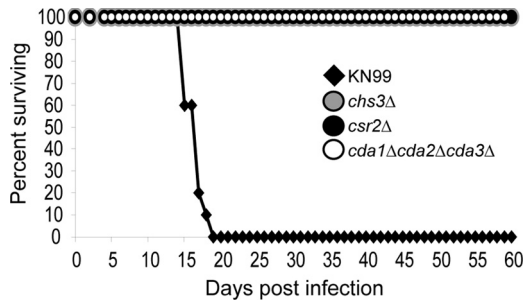
FIG. 3. Virulence of chitosan-deficient strains. Strains are listed. Data represent results for two independent isolates per deletion strain.

became ill and were sacrificed by 19 days p.i. None of the mice infected with the chitosan-deficient strains showed loss of weight or displayed symptoms of illness throughout the 60-day experiment, supporting the hypothesis that chitosan was necessary for virulence (Fig. 3). A second, independently generated mutant line for each of the deletion strains was also tested in mice, with

identical results (data not shown). Taken together, these data indicated that chitosan is necessary for the virulence of *C. neoformans.*

Chitosan-deficient strains are quickly eradicated from the lungs. We sought to determine if the *Cryptococcus* strains were avirulent, because they were more susceptible to killing and clearance by the host. To test this, four mice were infected with two independently generated strains of each mutation as described above, and lung tissue harvested at 2 h, 8 h, 24 h, 48 h, 7 days, and 15 days p.i. For the determination of CFU, one lung from each mouse was placed in 2.5 ml of 1× phosphate-buffered solution (pH 7), homogenized, serially diluted, and plated onto YPD supplemented with 100 μg/ml streptomycin and ampicillin. The second lung of each mouse was preserved for histology preparation in 5 ml of 10% buffered formalin (see below). We found that the fungal burden of the *trx1*Δ strain, which grows slowly at 37°C, was similar to its wild-type (H99) progenitor. Only at very early time points (2 to 24 h) were live cells recovered from mice infected with chitosan-deficient strains, and no CFU were recovered from tissue infected with these strains past 24 h p.i. (Fig. 4A). These data demonstrated
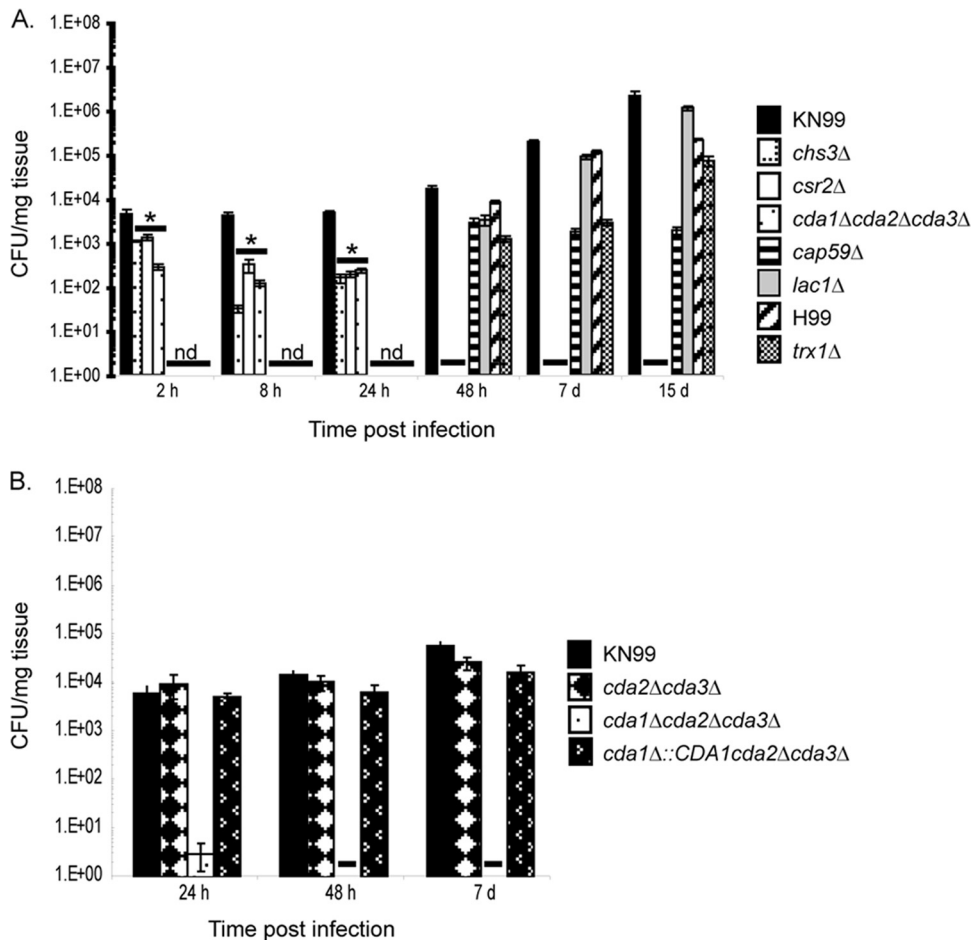

FIG. 4. Fungal burden over time for selected strains. Data are from four mice per strain. Strains are listed to the right of each graph. nd, not determined; a dash indicates no live cells were recovered. (A) ∗, significant difference between deletion strains and wild type at 2 h (*chs3*Δ, $P = 0.009$; *csr2*Δ, $P = 0.010$; *cda1*Δ *cda2*Δ *cda3*Δ, $P = 0.003$); $P < 0.001$ for the 8-h and 24-h time points based on one-way analysis of variance with Dunnett's post hoc test. Data represent one of two independent isolates for each chitosan-deficient strain after the 24-h time point. Standard errors are shown. (B) Fungal burden of the complemented strain. Wild-type and *cda2*Δ *cda3*Δ controls were included.

that chitosan is necessary for the viability of *Cryptococcus* within the host and suggest that it is important for the establishment of a successful infection.

Chitosan-deficient strains are cleared faster than other avirulent strains. Two well-established virulence factors of *C. neoformans* are its abilities to produce a polysaccharide capsule and the pigment melanin (7, 22). Both of these virulence factors are associated with the cell wall. The deletions *cap59Δ* (an acapsular strain) and *lac1Δ* (a strain lacking melanin production) each lead to the complete loss of virulence of *C. neoformans* (7, 22). Given that the chitosan-deficient strains are also avirulent, we sought to determine if their clearance patterns were similar to the acapsular and melanin-deficient strains. The fungal burdens of mice infected with the chitosan-deficient strains were much lower than those of acapsular or melanin-deficient strains, as only *cap59Δ* and *lac1Δ* cells were recovered from infected tissue 48 h to 15 days p.i. (Fig. 4A). These data indicate that the loss of chitosan renders *Cryptococcus* more susceptible to killing by the host than does the loss of capsule or melanin.

Complementation of a chitosan-deficient strain restores fungal burden to wild-type levels. To date, we have been unable to complement the *chs3Δ* and *csr2Δ* strains. Several attempts were made to biolistically reintroduce a wild-type copy of *CHS3* or *CSR2* into their respective deletion strains. In addition to our normal transformation procedures, we tried two modifications, including reduction in the pressure at which the DNA-covered gold particles were delivered and inclusion of sorbitol in the transformation recovery plates. Furthermore, we tried to introduce the complementation DNA by using an electroporation method both with and without sorbitol in the recovery plates (9). No colonies were ever recovered from any of these transformation attempts, while control strains generally produced hundreds of colonies. This indicated that the cell walls of the *chs3Δ* and *csr2Δ* strains were compromised to a point that they could not survive any of the transformation procedures.

Interestingly, the *cda1Δ cda2Δ cda3Δ* strain was transformable, and we were able to complement it by integrating a wild-type copy of *CDA1* into the *cda1* locus. The *cda1Δ cda2Δ cda3Δ* makes more chitin than either the *chs3Δ* or *csr2Δ* strain (4), and this additional chitin may provide enough structural integrity to the cell wall to permit transformation (4).

A *cda2Δ cda3Δ* strain, which still contains a wild-type copy of *CDA1* and makes near-wild-type levels of chitin and chitosan (4), had a clearance pattern similar to the wild type and was used as a positive control for the *cda1Δ::CDA1 cda2Δ cda3Δ* strain. The fungal burden of *cda1Δ::CDA1 cda2Δ cda3Δ* was similar to both *cda2Δ cda3Δ* and wild-type strains (Fig. 4B). These data suggest that the complete lack of chitosan is necessary for the rapid killing of *C. neoformans* in the mammalian host.

The host readily cleared nonviable cells of chitosan-deficient strains from lung tissue. Because the fungal burden assay provides only live cell counts, we determined if the nonviable cells were also being readily cleared from the host. Lung tissue was harvested for histology as described above. Histology preparations of stepwise, longitudinal lung sections stained with Grocott's methenamine silver (GMS) were made by the Department of Pathology core facility at Washington University
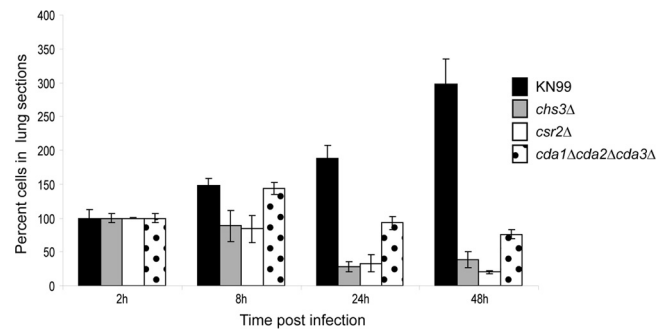

FIG. 5. Average cryptococcal cell numbers in longitudinal lung sections. Cryptococcal cells in three stepwise lung sections from each mouse were stained with Grocott's methenamine silver (GMS). All cells in lung sections were counted by viewing with a light microscope. The number of cells for time points 8 to 48 h of each strain were calculated as a percentage of cells counted for each respective 2-h time point. Error bars indicate standard deviations between the four mice (12 lung sections) in each group.

School of Medicine (St. Louis, MO). We observed cells in all samples up to 48 h p.i. (Fig. 5). The chitosan-deficient strains showed decreased cell numbers after 8 h, suggesting that clearance of the nonviable cells was occurring.

Chitosan is likely critical for virulence of *C. neoformans*. Our results are consistent with and suggestive of the notion that chitosan-deficient cell walls make fungal cells more vulnerable to host defense mechanisms, and they indirectly suggest a role for this cell wall component in virulence. The result that chitosan-deficient mutants with genetic defects in three distinct proteins were all avirulent and rapidly killed by the host lends strength to the argument that chitosan deficiency was directly responsible. It is possible that destabilization of the cell wall due to lack of chitosan renders *C. neoformans* highly susceptible to host killing and clearance (23). Alternatively, a lack of chitosan could expose other pathogen-associated molecular patterns (PAMPs) and increase the host response to the mutant *Cryptococcus*. Finally, host chitinases are induced by *C. neoformans* and other fungal pathogens, and converting chitin to chitosan may be a mechanism to protect the fungus from detrimental effects of chitinases.

Proteins that produce chitosan may be good antifungal drug targets. This is the first report to demonstrate the importance of cell wall chitosan for virulence of a human fungal pathogen. Other opportunistic fungal pathogens have been reported to produce chitosan. *Aspergillus fumigatus* produces chitosan in its mycelia and conidia (16), while *Mucor rouxii* generates significant amounts of chitosan in the cell wall of structures found throughout its life cycle (3, 6, 12, 17, 26). Additionally, a chitin deacetylase gene was shown to be highly transcribed in the yeast phase of *Paracoccidioides brasiliensis* (2). It has yet to be determined if loss of chitosan will have the same effect on other pathogenic fungi as it does on *C. neoformans*. Many fungal pathogens produce chitosan in some phase of their life cycles, but here chitosan has been shown to be essential for cryptococcal virulence. We postulate that the proteins that generate chitosan could prove to be excellent antifungal drug targets.

REFERENCES

1. **Adams, D. J.** 2004. Fungal cell wall chitinases and glucanases. Microbiol. **150:**2029–2035.
2. **Andrade, R., et al.** 2006. Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells. BMC Genomics **7:**208.
3. **Arcidiacono, S., and D. L. Kaplan.** 1992. Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. Biotech. Bioeng. **39:**281–286.
4. **Baker, L. G., C. A. Specht, M. J. Donlin, and J. K. Lodge.** 2007. Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in *Cryptococcus neoformans*. Eukaryot. Cell **6:**855–867.
5. **Banks, I. R., et al.** 2005. A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen *Cryptococcus neoformans*. Eukaryot. Cell **4:**1902–1912.
6. **Bartnicki-Garcia, S.** 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. **22:**87–108.
7. **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.
8. **Cox, G. M., J. Mukherjee, G. T. Cole, A. Casadevall, and J. R. Perfect.** 2000. Urease as a virulence factor in experimental cryptococcosis. Infect. Immun. **68:**443–448.
9. **Gerik, K. J., S. R. Bhimireddy, J. S. Ryerse, C. A. Specht, and J. K. Lodge.** 2008. Protein kinase C1 (PKC1) is essential for protection against both oxidative and nitrosative stress, cell integrity, and normal manifestation of virulence factors in the pathogenic fungus *Cryptococcus neoformans*. Eukaryot. Cell **7:**1685–1698.
10. **Heitman, J., T. Kozel, J. Kwon-Chung, J. Perfect, and A. Casadevall.** 2011. Cryptococcus: from human pathogen to model yeast, p. 620. ASM Press, Washington D. C.
11. **Hoch, H. C., C. D. Galvani, D. H. Szarowski, and J. N. Turner.** 2005. Two new fluorescent dyes applicable for visualization of fungal cell walls. Mycologia **97:**580–588.
12. **Kafetzopoulos, D., A. Martinou, and V. Bouriotis.** 1993. Bioconversion of chitin to chitosan: purification and characterization of chitin deacetylase from *Mucor rouxii*. Proc. Natl. Acad. Sci. U. S. A. **90:**2564–2568.
13. **Kauffman, C. A.** 2006. Clinical efficacy of new antifungal agents. Curr. Opin. Microbiol. **9:**483–488.
14. **Krishnarao, T., and J. Galgiani.** 1997. Comparison of the *in vitro* activities of the echinocandin LY303366, the pneumocandin MK-0991, and fluconazole against *Candida* species and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **41:**1957–1960.
15. **Missall, T. A., and J. K. Lodge.** 2005. Function of the thioredoxin proteins in *Cryptococcus neoformans* during stress or virulence and regulation by putative transcriptional modulators. Mol. Microbiol. **57:**847–858.
16. **Mouyna, I., and T. Fontaine.** 2009. Cell wall of *Aspergillus fumigatus*: a dynamic structure, p. 169–183. *In* J. P. Latge and W. J. Steinbach (ed.), *Aspergillus fumigatus* and aspergillosis. ASM Press, Washington, DC.
17. **Orlowski, M.** 1991. *Mucor* dimorphism. Microbiol. Rev. **55:**234–258.
18. **Park, B. J., et al.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525–530.
19. **Paugam, A., et al.** 1994. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. Clin. Infect. Dis. **19:**975–976.
20. **Powderly, W. G., et al.** 1995. A randomized trial comparing fluconazole with clotrimazole troches for the prevention of fungal infections in patients with advanced human immunodeficiency virus infection. N. Engl. J. Med. **332:**700–705.
21. **Saag, M. S., et al.** 2000. Practice guidelines for the management of cryptococcal disease. Infectious Diseases Society of America. Clin. Infect. Dis. **30:**710–718.
22. **Salas, S., J. Bennett, K. Kwon-Chung, J. Perfect, and P. Williamson.** 1996. Effect of the laccase gene *CNLAC1* on virulence of *Cryptococcus neoformans*. J. Exp. Med. **184:**377–386.
23. **Vicencio, A. G., et al.** 2008. Pulmonary cryptococcosis induces chitinase in the rat. Respir. Res. **9:**40.
24. **Walsh, T. J.** 2002. Echinocandins: an advance in the primary treatment of invasive candidiasis. N. Engl. J. Med. **347:**2070–2072.
25. **White, M. H., and D. Armstrong.** 1994. Cryptococcosis. Infect. Dis. Clin. North Am. **8:**383–398.
26. **White, S. A., P. R. Farina, and I. Fulton.** 1979. Production and isolation of chitosan from *Mucor rouxii*. Appl. Environ. Microbiol. **38:**323–328.