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OBSERVATION
Host-Microbe Biology

Host Carbon Dioxide Concentration Is an Independent Stress for *Cryptococcus neoformans* That Affects Virulence and Antifungal Susceptibility

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ABSTRACT The ability of *Cryptococcus neoformans* to cause disease in humans varies significantly among strains with highly related genotypes. In general, environmental isolates of pathogenic species such as *Cryptococcus neoformans* var. *grubii* have reduced virulence relative to clinical isolates, despite having no differences in the expression of the canonical virulence traits (high-temperature growth, melanization, and capsule formation). In this observation, we report that environmental isolates of *C. neoformans* tolerate host CO_2 concentrations poorly compared to clinical isolates and that CO_2 tolerance correlates well with the ability of the isolates to cause disease in mammals. Initial experiments also suggest that CO_2 tolerance is particularly important for dissemination of *C. neoformans* from the lung to the brain. Furthermore, CO_2 concentrations affect the susceptibility of both clinical and environmental *C. neoformans* isolates to the azole class of antifungal drugs, suggesting that antifungal testing in the presence of CO_2 may improve the correlation between *in vitro* azole activity and patient outcome.

IMPORTANCE A number of studies comparing either patient outcomes or model system virulence across large collections of *Cryptococcus* isolates have found significant heterogeneity in virulence even among strains with highly related genotypes. Because this heterogeneity cannot be explained by variations in the three well-characterized virulence traits (growth at host body temperature, melanization, and polysaccharide capsule formation), it has been widely proposed that additional *C. neoformans* virulence traits must exist. The natural niche of *C. neoformans* is in the environment, where the carbon dioxide concentration is very low (~0.04%); in contrast, mammalian host tissue carbon dioxide concentrations are 125-fold higher (5%). We have found that the ability to grow in the presence of 5% carbon dioxide distinguishes low-virulence strains from high-virulence strains, even those with a similar genotype. Our findings suggest that carbon dioxide tolerance is a previously unrecognized virulence trait for *C. neoformans*.

KEYWORDS *Cryptococcus neoformans*, fluconazole, mycology, pathogenesis

Cryptococcus neoformans is one of the most important human fungal pathogens and causes meningoencephalitis (CME). Recent estimates indicate that 223,000 new cases of CME occur each year with an annual mortality of 181,000 (1); the majority of CME disease affects people infected with HIV (2). *Cryptococcus* species are environmental yeasts that occupy a variety of niches, and therefore, *Cryptococcus* must transition from an environmental niche to the mammalian host to cause disease (3). Most strains

Citation Krysan DJ, Zhai B, Beattie SR, Misel KM, Wellington M, Lin X. 2019. Host carbon dioxide concentration is an independent stress for *Cryptococcus neoformans* that affects virulence and antifungal susceptibility. mBio 10:e01410-19. https://doi.org/10.1128/mBio.01410-19.

Editor Tamara L. Doering, Washington University School of Medicine

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This article is a direct contribution from a Fellow of the American Academy of Microbiology. Solicited external reviewers: J. Andrew Alspaugh, Duke University Medical Center; Robert Cramer, Geisel School of Medicine at Dartmouth.

Received 3 June 2019
Accepted 11 June 2019
Published 2 July 2019

isolated from the environment are much less virulent in animal models than strains isolated from human patients. For example, Litvintseva and Mitchell (4) found that only one out of 10 environmental strains caused mortality in a murine model of cryptococcosis by 60 days while 5/7 clinical strains caused lethal infection by 40 days. *In vitro*, all of the strains grew at 37°C and generated comparable levels of capsule and melanin (4).

Recently, Mukaremera et al. systematically characterized a set of genetically similar strains associated with highly variable patient outcomes (5). The virulence trends observed in patients were recapitulated in the inhalational murine model, providing important validation of the model as predicative of clinical virulence. Like the work of Litvintseva and Mitchell (4), extensive phenotyping of the strains did not reveal an *in vitro* phenotype that correlated with virulence (5). These data strongly indicate that uncharacterized virulence properties beyond the "big three" of host body temperature tolerance, melanization, and capsule formation play an important role in determining the virulence potential of a given cryptococcal strain (6).

We hypothesized that the host environment may contain additional stresses under which clinical/pathogenic isolates are fitter than environmental/nonpathogenic isolates. One dramatic difference between terrestrial and host environments is the concentration of carbon dioxide (CO_2): ambient air is ~0.04% CO_2 while the CO_2 concentration in mammalian tissues is 125-fold higher (5%). In ambient air, *Cryptococcus*, like many yeasts, expresses carbonic anhydrase (Can2) which catalyzes the generation of essential HCO_3^- under low CO_2 concentrations. At 5% CO_2 in the host, *CAN2* expression is repressed presumably due to sufficient levels of HCO_3^- produced by dissolved CO_2. Consequently, *CAN2* is dispensable for growth at 5% CO_2 and virulence in a murine host (7). Host CO_2 concentrations, however, have a profound effect on *C. neoformans* biology because they induce capsule formation (8). Indeed, our hypothesis that host concentrations of CO_2 are a significant stress to *C. neoformans* is supported by the fact that Granger et al. noted that the growth of cultures slowed considerably after being shifted to host CO_2 concentrations to induce capsule formation (9). In addition, *C. neoformans* strains lacking calcineurin, a key stress response regulator, are hypersensitive to CO_2 (10). Finally, Bahn et al. found that elevated concentrations of CO_2 inhibit *C. neoformans* mating (7). Taken together, tolerance of host CO_2 concentrations seemed to us a potentially important independent trait of *C. neoformans* strains that cause disease in mammals.

To test the hypothesis that CO_2 tolerance may play a role in distinguishing between clinical and environmental strains of *C. neoformans*, we examined the CO_2 fitness of a set of 12 strains that had been previously characterized in the mouse pulmonary infection model by Litvintseva and Mitchell (4). The clinical and reference strain H99 as well as three other clinical strains grew similarly in the presence and absence of CO_2 on RPMI medium buffered to pH 7 with morpholinepropanesulfonic acid (MOPS) while all but one of the environmental strains displayed a growth defect in 5% CO_2 (Fig. 1A). The serotype D reference strain JEC21 is also CO_2 sensitive relative to H99 and the clinical isolates. Of 10 additional clinical strains isolated from patients at Duke University (gift of John Perfect), 8 had similar growth at ambient and host CO_2 concentrations (see Fig. S1 in the supplemental material). Of the 12 strains that were examined by Litvintseva and Mitchell in animal models (4), only those that were CO_2 tolerant caused disease by 60 days. The only environmental strain to be CO_2 tolerant was also the only one to cause disease.

To obtain a more quantitative measure of the *in vitro* fitness advantage of a CO_2-tolerant strain over a CO_2-sensitive strain, we carried out a competition experiment in which the mCherry-labeled H99 strain and unlabeled, environmental strain A4-34-6 were cocultured as a 1:1 mixture in ambient air or 5% CO_2; the ratio of H99 to A4-34-6 was determined by microscopy. A4-34-6 has a 5-fold fitness defect relative to H99 in ambient air, and that defect is increased to 50-fold in 5% CO_2 (Fig. 1B). To determine if CO_2 tolerance is recessive or dominant, the previously generated diploid AD hybrid was compared to its parental strains serotype A H99 and serotype D JEC21 (11). The diploid AD hybrid is CO_2 tolerant, indicating that the trait is dominant (Fig. 1C).

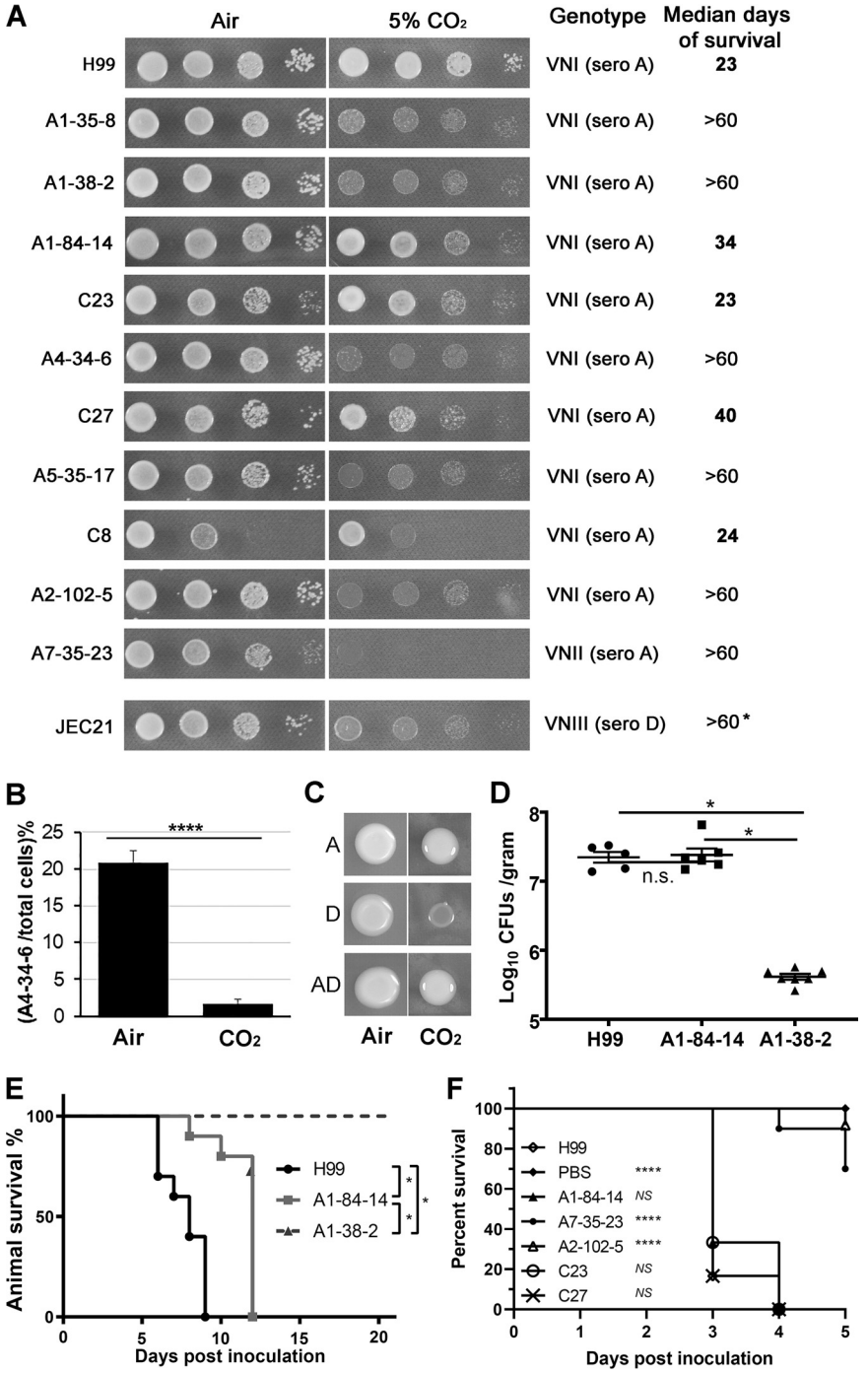

FIG 1 *In vitro* CO_2 tolerance correlates with cryptococcal virulence in insect and mammalian hosts. (A) Clinical and environmental *C. neoformans* isolates were cultured on RPMI medium buffered to pH 7 with MOPS at 37°C in ambient air (~0.04% CO_2) or in 5% CO_2. The information about the genotype of the strains and the median survival days of mice infected by these strains intranasally was obtained from a previous study (4). (B) Competition assay of isolate A4-34-6 and mCherry-labeled H99 cultured on RPMI medium buffered to pH 7 with 165 mM MOPS in ambient air or in 5% CO_2. $P < 0.00001$, Student's *t* test. (C) Cell suspensions of H99 (A), JEC21 (D), and XL1462 (AD hybrid) at equal concentrations were spotted onto RPMI medium and incubated at 37°C in ambient air or in 5% CO_2. (D and E) CD-1 mice were inoculated with H99, CO_2-tolerant environmental strain A1-84-14, and CO_2-sensitive strain A1-38-2 (5 to 7 animals per group) by tail vein injection. Five animals were sacrificed at 5 days for brain fungal burden, and 10 animals per group were monitored for morbidity until day 21 (D); asterisks indicate statistically significant difference between indicated groups by Student's *t* test of log-transformed data. Survival curves were analyzed by Kaplan-Meier/log rank test (E). (F) The great wax moth *Galleria mellonella* larvae in the final-instar larval stage (10 to 15 per group) were injected with the indicated clinical or environmental isolates via the last left proleg. The survival rate of the infected larvae over days postinoculation is shown. NS, nonsignificant; ****, $P < 0.0001$ compared to H99 by log rank test.

Consistent with other yeasts (12), CO_2 is fungistatic and dose dependent (data not shown). Because CO_2 and HCO_3^- are substrates in reactions of central carbon metabolism (12), we wondered if increasing the glucose concentration of RPMI from 0.2% to 2% would affect CO_2 sensitivity; however, it had no effect on the growth of the sensitive strains (Fig. S1).

As reported by Litvintseva and Mitchell (4), all clinical strains caused lethal infections in mice within 40 days while the only environmental strain to cause a lethal infection within 60 days was the CO_2-tolerant strain A1-84-14 (Fig. 1A). To determine if the virulence differences between the CO_2-tolerant and CO_2-sensitive strains were dependent on the infection model, the environmental CO_2-tolerant and CO_2-sensitive strains were compared in both the intravenous model of disseminated murine cryptococcosis and the *Galleria mellonella* model. Five days postinfection (Fig. 1D), the fungal brain burden was 2 $\log_{10}$ CFU/g lower in the CO_2-sensitive environmental strain than the CO_2-tolerant environmental strain, while the CO_2-tolerant environmental strain was similar to the reference strain H99. Consistent with the pulmonary infection model data, the median survival of mice infected with the CO_2-tolerant environmental strain is modestly longer than that of mice infected with the highly virulent H99 reference strain (Fig. 1E). Mice infected with the CO_2-sensitive strain, in contrast, were asymptomatic for an additional week. The fungal burden for the CO_2-sensitive-strain-infected mice had increased 1.7 $\log_{10}$ over the 16 days (Fig. S3), indicating that the environmental strain replicated very slowly in the brain. Finally, *G. mellonella* larvae infected with CO_2-sensitive strains showed prolonged survival relative to larvae infected with CO_2-tolerant strains (Fig. 1F). Taken together, these data strongly support a correlation between CO_2 tolerance and virulence in multiple models of cryptococcal infection.

CO_2 levels affect the composition and function of cellular membranes in a variety of biological systems, which is proposed to be one possible mechanism of CO_2-mediated growth inhibition (12, 13). The two most important anticryptococcal drugs, amphotericin B and fluconazole, affect membrane-related processes (14), and thus, we hypothesized that CO_2 may modulate antifungal susceptibility. The MIC was determined using Etest strips on solid agar RPMI medium buffered to pH 7 with 165 mM MOPS. The MIC for amphotericin B was not affected by CO_2 in any of the strains tested ($\leq$2-fold change between ambient air and 5% CO_2 [Fig. 2A and Fig. S4B]). In contrast, the fluconazole MIC decreased approximately 8- to 10-fold (4 μg/ml to 0.5 μg/ml) for H99, CO_2-tolerant clinical isolates (C23 and C27), and environmental (A1-84-14) isolates in the presence of 5% CO_2 (Fig. 2B and Fig. S4B). The MICs of both itraconazole (Fig. 2B) and voriconazole (Fig. S4B) are decreased at 5% CO_2, indicating that the effect is not limited to fluconazole. Five percent CO_2 has no effect on the MIC of fluconazole against the *C. albicans* reference strain SC5314 (2 μg/ml). The effect of CO_2 is also not specific to ergosterol biosynthesis inhibitors in that the sphingolipid biosynthesis inhibitor myriocin is also more active at host CO_2 levels than in ambient air (Fig. 2B).

The physiological mechanism underlying the differential sensitivity of *Cryptococcus* strains to host levels of CO_2 awaits further study. A variety of potential mechanisms for the fungistatic and bacteriostatic effect of CO_2 have been proposed, including altered membrane fluidity and inhibition of biosynthetic reactions involving CO_2 (12). Despite these outstanding mechanistic questions, the effects of host concentrations of CO_2 on *C. neoformans* virulence and antifungal susceptibility have two important implications. First, CO_2 tolerance appears to be an independent virulence feature of pathogenic strains of *C. neoformans* that correlates with variations in mammalian virulence. It will be interesting to expand this analysis to determine if CO_2 tolerance contributes to the variation in patient outcome (5). Based on fungal burden data reported by Litvintseva and Mitchell (4), the CO_2-sensitive strains cause a significantly lower lung burden than CO_2-tolerant strains while the brain burden for CO_2-sensitive strains is almost undetectable at postinfection day 60. Although the CO_2-sensitive strain has reduced virulence in the intravenous model, which rapidly establishes central nervous system (CNS) infection, it is able to replicate within the brain. Taken together, these preliminary and

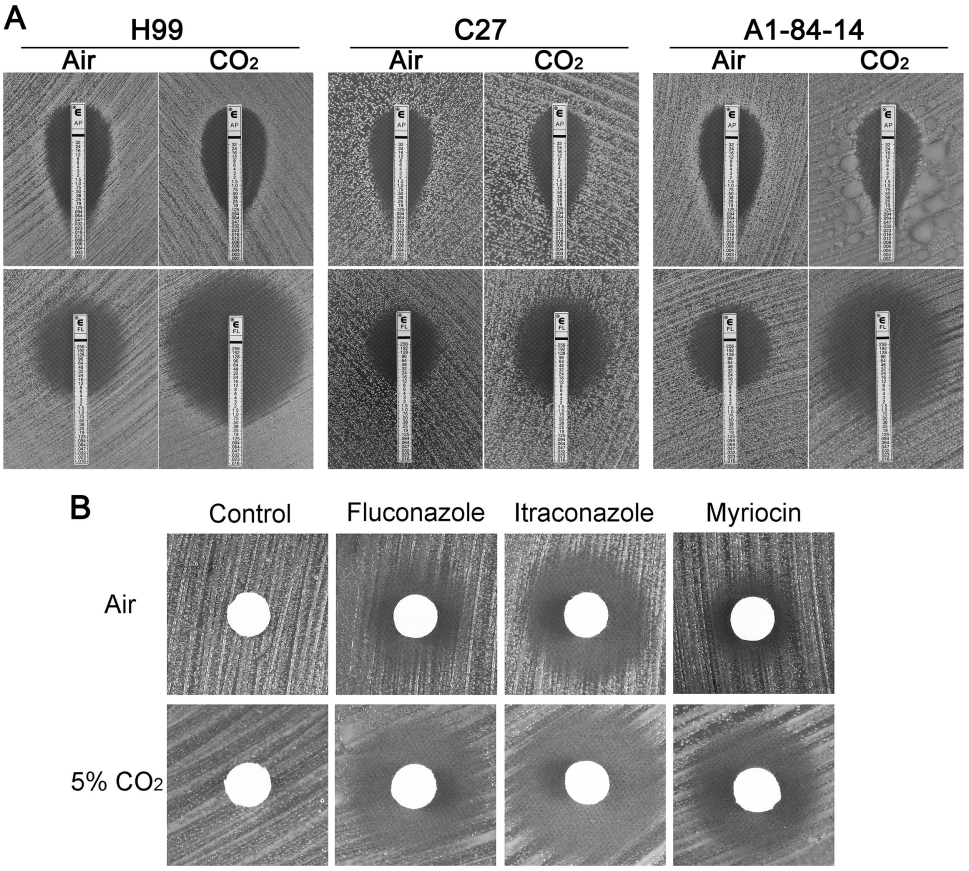

FIG 2 CO_2 affects cryptococcal susceptibility to antifungals. (A) The CO_2-tolerant cryptococcal isolates, including the reference and clinical isolate H99, the clinical isolate C27, and the environmental isolate A1-84-14, are more susceptible to fluconazole in 5% CO_2 than ambient air, but amphotericin B susceptibility is unaffected. Suspensions of *C. neoformans* isolates were spread onto RPMI agar medium. Etest strips with fluconazole (FL) or amphotericin B (AP) were placed on top of the air-dried yeast lawn. The cells were then incubated at 37°C in ambient air or in 5% CO_2. (B) H99 is more susceptible to fluconazole, itraconazole, and myriocin in 5% CO_2 by disk diffusion assay. H99 cells were spread onto RPMI agar medium. Disks containing fluconazole (4 μg), itraconazole (4 μg), myriocin (0.8 μg), or DMSO (control) were air dried and placed on top of the yeast lawn. Cells were incubated at 37°C for 2 days in ambient air or in 5% CO_2. The size of the halo surrounding the disks correlates with cryptococcal susceptibility to the drugs.

previously published data (4) indicate that CO_2 tolerance may play a more important role in dissemination from the lung than in replication within the brain.

Second, the profound effect of host CO_2 concentrations on *in vitro* azole activity identifies a potential limitation of current antifungal drug susceptibility testing conditions in predicting the outcomes of patients treated with azoles. Indeed, the lack of correlation between MICs generated by standardized antifungal susceptibility testing assays and clinical outcomes for cryptococcal infections has been well described, and a number of potential explanations for this discrepancy have been proposed (15). Our data suggest that CO_2 is likely to be an important factor in drug susceptibility. Although the mechanism of this effect will require additional investigation, it is unlikely that high CO_2 concentrations directly reduce ergosterol levels because strains with reduced ergosterol content typically have reduced susceptibility to amphotericin B, a drug that binds directly to ergosterol. Regardless of the mechanism, the use of host CO_2 concentrations may represent a simple adjustment that could improve the correlation between MIC and clinical outcome.

Strains and growth conditions. Media were prepared using standard recipes (16). Reference strains H99 and JEC21 were from stocks in the Krysan and Lin labs. Environmental and clinical strains were generous gifts from Anastasia Litvintseva, Tom Mitchell,

and John Perfect. Strains were stored at −80°C in 15% glycerol. Freshly streaked-out yeast cells were grown on yeast extract-peptone-2% dextrose (YPD) medium at 30°C. For spotting assays, the cells were washed, adjusted to the same cell density, and serially diluted. Serial dilutions (3 μl) were then spotted onto agar plates of RPMI without glutamine (buffered to pH 7 with 165 mM MOPS) and incubated at 37°C in ambient air or at 5% CO_2. For the glucose supplementation assay, 2% glucose was added to RPMI medium buffered to pH 7 with 165 mM MOPS. H99 was labeled with mCherry by integrating plasmid pH3mCHSH2 (Addgene) into the so-called Safe-Haven 2 (SH2) locus described by Upadhya et al. (17). Transiently expressed Cas9 (18) was used to generate a double-strand break in the SH2 region, and pH3mCHSH2, linearized with ApaI, was used as the repair construct. After selection on neomycin-containing plates, colonies that remained Neo^+ and fluorescent were isolated.

Competition assay. mCherry-labeled H99 and A4-34-6 were grown overnight in liquid YPD at 30°C. The stationary-phase cells were adjusted to identical cell density, and a combined inoculum with equal numbers of cells was spotted on RPMI plates as described above. After 2 days in either ambient air or 5% CO_2, samples of cells from different regions of the colony were collected and imaged in bright field (total cell number) and red channel using a Nikon epifluorescence microscope with a Cool Snap HQ2 camera and Nikon Elements image acquisition and analysis software. The ratio of nonfluorescent to total cells was used to generate a competitive index (>100 cells counted for each data point). The reported data are from three biological replicates with three technical replicates.

Murine model of cryptococcosis. *C. neoformans* H99, A1-84-18, and A1-38-2 were cultured in YPD for 48 h at 30°C. Harvested cells were washed three times with sterile phosphate-buffered saline (PBS), enumerated with a hemocytometer, and diluted to 1.9×10^6 CFU/ml in sterile PBS. CD-1 females (Envigo), 25 to 30 g, were inoculated with 3.8×10^5 CFU (200 μl) by tail vein injection. For fungal burden, brains were harvested 5 days postinoculation and homogenized in sterile PBS (1 ml), and 10-fold dilutions were plated on YPD. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. For virulence studies, mice were monitored for 21 days following *C. neoformans* inoculation. Percent survival was plotted on a Kaplan-Meier curve, and a log rank test was used to assess statistical significance of the curves.

Ethics statement. The *Guide for the Care and Use of Laboratory Animals* of the National Research Council (19) was strictly followed for all animal experiments. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee at the University of Iowa (protocol no. 7102064).

***Galleria mellonella* larva model of cryptococcosis.** The larvae of the great wax moth *G. mellonella* in the final-instar larval stage were obtained from Vanderhorst, Inc. (St. Marys, OH, USA). The *G. mellonella* larvae (0.3 to 0.4 g) were used for inoculation as previously described (20). Briefly, 1×10^5 *C. neoformans* cells in PBS (5 μl) were injected into the hemocoel of each wax moth via the last left proleg. After injection, the wax moth larvae were incubated at 37°C in the dark. For each experiment, 10 to 15 wax moth larvae per group were infected and monitored for survival. Kaplan-Meier curves were analyzed using log rank test to determine statistical significance for differences between groups.

Etest and disk diffusion assay. Briefly, yeast cells at a cell density of approximately 5×10^6 were spread onto RPMI 1640 agar medium with L-glutamine and without sodium bicarbonate. The plates were allowed to dry. In disk diffusion assays, Whatman paper disks (7 mm) containing dimethyl sulfoxide (DMSO), fluconazole, itraconazole, and myriocin at indicated concentrations were dried and placed on the agar surface. Etest strips (bioMérieux) with amphotericin B, fluconazole, or voriconazole were placed on the agar surface. The plates were incubated at 37°C in ambient air or at 5% CO_2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01410-19.

FIG S1, TIF file, 0.3 MB.

FIG S2, TIF file, 0.3 MB.

FIG S3, TIF file, 1.6 MB.

FIG S4, TIF file, 1.8 MB.

ACKNOWLEDGMENTS

We thank Zhiyao Yang for performing *Candida albicans* susceptibility testing.

We gratefully acknowledge the financial support from the Biology Department of Texas A&M University, the Microbiology Department of the University of Georgia (startup funds to X.L.), and the Department of Pediatrics (startup funds for D.J.K.). Xiaorong Lin holds an Investigator Award in the Pathogenesis of Infectious Disease from the Burroughs Wellcome Fund (1012445 to X.L.).

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