Chitosan Biosynthesis and Virulence in the Human Fungal Pathogen *Cryptococcus gattii*

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ABSTRACT *Cryptococcus gattii* R265 is a hypervirulent fungal strain responsible for the recent outbreak of cryptococcosis in Vancouver Island of British Columbia in Canada. It differs significantly from *Cryptococcus neoformans* in its natural environment, its preferred site in the mammalian host, and its pathogenesis. Our previous studies of *C. neoformans* have shown that the presence of chitosan, the deacetylated form of chitin, in the cell wall attenuates inflammatory responses in the host, while its absence induces robust immune responses, which in turn facilitate clearance of the fungus and induces a protective response. The results of the present investigation reveal that the cell wall of *C. gattii* R265 contains a two- to threefold larger amount of chitosan than that of *C. neoformans*. The genes responsible for the biosynthesis of chitosan are highly conserved in the R265 genome; the roles of the three chitin deacetylases (CDAs) have, however, been modified. To deduce their roles, single and double *CDA* deletion strains and a triple *CDA* deletion strain were constructed in a R265 background and were subjected to mammalian infection studies. Unlike *C. neoformans* where Cda1 has a discernible role in fungal pathogenesis, in strain R265, Cda3 is critical for virulence. Deletion of either *CDA3* alone or in combination with another CDA (*cda1Δ3Δ* or *cda2Δ3Δ*) or both (*cda1Δ2Δ3Δ*) rendered the fungus avirulent and cleared from the infected host. Moreover, the *cda1Δ2Δ3Δ* strain of R265 induced a protective response to a subsequent infection with R265. These studies begin to illuminate the regulation of chitosan biosynthesis of *C. gattii* and its subsequent effect on fungal virulence.

IMPORTANCE The fungal cell wall is an essential organelle whose components provide the first line of defense against host-induced antifungal activity. Chitosan is one of the carbohydrate polymers in the cell wall that significantly affects the outcome of host-pathogen interaction. Chitosan-deficient strains are avirulent, implicating chitosan as a critical virulence factor. *C. gattii* R265 is an important fungal pathogen of concern due to its ability to cause infections in individuals with no apparent immune dysfunction and an increasing geographical distribution. Characterization of the fungal cell wall and understanding the contribution of individual molecules of the cell wall matrix to fungal pathogenesis offer new therapeutic avenues for intervention. In this report, we show that the *C. gattii* R265 strain has evolved alternate regulation of chitosan biosynthesis under both laboratory growth conditions and during mammalian infection compared to that of *C. neoformans*.

KEYWORDS *Cryptococcus gattii*, R265, chitin, chitosan, chitosan regulation, protection, vaccine, virulence

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Chitosan is a virulence factor in Cryptococcus gattii, and one chitin deacetylase (CDA3) is critical for chitosan production under host conditions.

Cryptococcosis is an invasive fungal infection caused mainly by the *Cryptococcus neoformans* and *Cryptococcus gattii* species. *Cryptococcus* is ubiquitous with worldwide distribution and causes infections in a wide variety of host species, such as plants,

birds, and mammals (1, 2). Infections caused by *Cryptococcus* may lead to cryptococcal meningitis and is estimated to cause more than 200,000 deaths annually (3). *C. neoformans* (including serotypes A and D) is an opportunistic pathogen and mainly causes disease in immunocompromised patients (4, 5). However, cryptococcal infections have also been reported even in healthy individuals (non-HIV) with or without underlying risk factors (6, 7). Unlike *C. neoformans*, its sibling species *C. gattii* (serotypes B and C) is recognized as a primary pathogen, as it predominately causes infections in immunocompetent individuals (5, 8–10). *C. gattii* was initially considered to be endemic to tropical and subtropical regions, especially Australia until it attracted attention with a major outbreak on Vancouver Island, British Columbia, Canada, in 1999 (11). Based on the global molecular epidemiologic survey employing a wide variety of molecular techniques, five distinct genetic groups (VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, VGIV/AFLP7, and VGIV/AFLP10) within the *C. gattii* species complex were described (12). The *C. gattii* R265 strain belongs to the VGII subtype and was the strain associated with the outbreak in British Columbia in 1999 (13). The fungus subsequently spread to the Pacific Northwest of the United States (14–16). Infections due to *C. gattii* R265 are associated with an 8 to 20% mortality rate in spite of antifungal therapies. With advances in genotyping, *C. gattii* distribution has been revised. It is predominantly isolated from environmental sources and is currently found in a variety of climates, including humid and arid conditions and associated with 53 different tree species across six continents (10). It has also been isolated from diverse groups of organisms, including cats, dogs, marine mammals, koalas, deer, ferrets, llamas, horses, birds, and insects (10). This distribution to diverse conditions of environment and host species demonstrates its adaptability.

Significant differences in the ecological, morphological, biochemical, molecular, pathological, and clinical features exist between *C. neoformans* and *C. gattii* species complex (12, 17). For example, the difference in the assimilation of nitrogen and carbon sources between *C. neoformans* and *C. gattii* species has been exploited to formulate a one-step diagnostic media for their identification (18). Several differences in the nature of the host immune response elicited by *C. gattii* R265 compared to *C. neoformans* have been attributed to its capacity to cause disease in persons with apparently normal immune systems. *C. gattii* R265 has been shown to proliferate better in macrophages compared to *C. neoformans* due to their increased resistance to reactive oxygen species (ROS) inside macrophages and to their tubular mitochondrial morphology (19). At the genomic level, strain R265 has shown to evolve by expanding stress-related heat shock proteins, which may offer better fitness inside the host (20). *C. gattii* R265 readily proliferates in the lung and disseminates poorly to the brain, suggesting that unlike *C. neoformans*, the major target organ of *C. gattii* R265 is the host lung (19, 21). *C. gattii* R265 has been shown to trigger a dampened immune response in the lung with reduced infiltration of macrophages, neutrophils, and Th1/Th17 lymphocytes, and it inhibits host dendritic cell maturity (22–25). Both *C. neoformans* and *C. gattii* R265 share a similar suite of virulence factors, yet they differ in the nature of pathogenesis in mammalian hosts.

Chitosan is one of the critical components of the cell wall of *C. neoformans* and has been shown to be essential for its virulence (26). The genes coding for enzymes that are responsible for the production of chitosan in *C. neoformans* have been identified and characterized (27). Out of the eight potential chitin synthase genes in the genome, chitin synthase 3 (Chs3) coded by *CHS3* and chitin synthase regulator 2 (Csr2) coded by the *CSR2* gene are critical for the production of chitosan (27). There are four potential chitin deacetylase (*CDA*) genes in the genome of *C. neoformans*; three have been shown to possess chitin deacetylase activity in vegetatively growing cells (28). Chitosan-deficient strains of *C. neoformans* induce significant host immune responses during mammalian infection and clearance of the fungus (26, 29). These results suggest that the presence of chitosan may influence the cell wall architecture, thereby shielding pathogen-associated molecular patterns (PAMPs) from being recognized by host immune cells. When grown in yeast extract-peptone-dextrose (YPD) culture medium, all

TABLE 1 Identification of chitin deacetylases in *C. gattii* R265 genome

Locus tag	*C. gattii* protein	E value for the following query:		
		C. neoformans Cda1p	*C. neoformans* Cda2p	*C. neoformans* Cda3p
CNBG_1745	Cda1	0.0	4.54E−37	4.58E−36
CNBG_0964	Cda2	2.07E−35	0.0	3.63E−29
CNBG_0806	Cda3	1.01E−37	2.34E−34	0.0

three Cda proteins appear to be functionally redundant. However, they are differentially regulated in the lungs of the infected host with Cda1 being preferentially expressed (30). Accordingly, either *cda1Δ* or the *cda* mutant with abolished chitin deacetylase activity were found to be avirulent, suggesting that Cda1 alone with its chitin deacetylase activity is sufficient to render the yeast cells fully virulent during a murine infection of CBA/J mice. Deletion of either Cda2 or Cda3 did not affect the virulence of the yeast strains (30). These results indicate that in *C. neoformans*, chitosan and the mechanisms of its production are the critical mediators of fungal pathogenesis and virulence.

The mechanisms responsible for differences in the pathogenicity and virulence of *C. neoformans* and *C. gattii* are not understood. We sought to determine whether there are any differences in either the level of cell wall chitosan or in the regulation of its biosynthesis between these two species. Here, we report the identification of genes present in *C. gattii* R265 genome that are either responsible for the production of chitosan during growth under vegetative conditions or that contribute to chitosan biosynthesis during mammalian infection. We found significant differences in the amount and regulation of chitosan biosynthesis between *C. gattii* R265 and *C. neoformans*. First, the cell walls of *C. gattii* R265 had more than double the amount of chitosan compared to *C. neoformans* when either grown in culture or in infected mice. We targeted homologs of *C. neoformans* chitosan biosynthetic genes in the *C. gattii* R265 genome for gene deletion and subjected the respective deletion strains to various *in vitro* phenotypic assays and to mouse virulence studies. For *C. neoformans*, Cda1 played an important role in the synthesis of chitosan, while Cda3 was found to be dispensable during murine infection. Interestingly for *C. gattii* R265, we found that Cda3 plays a critical role in fungal virulence, while the deletion of Cda1 did not affect fungal virulence across different mouse strains. The results of these studies will provide a framework to further design strategies to dissect the molecular mechanisms of chitosan in fungus-induced host immune response and virulence.

RESULTS

Identification of *C. gattii* R265 genes potentially involved in the synthesis of chitosan. In *C. neoformans*, the conversion of chitin to chitosan is catalyzed by Cda1, Cda2, and Cda3 (28, 30). We utilized BLASTp homology with *C. neoformans* Cda1, Cda2, and Cda3 to identify the *C. gattii* chitin deacetylases in the *C. gattii* R265 genome. This search yielded CNBG_1745 (Cda1), CNBG_9064 (Cda2), and CNBG_0806 (Cda3) as the *C. gattii* homologs of *C. neoformans* (Table 1). At the protein level, Cda1, Cda2, and Cda3 are 85%, 83%, and 85% identical and 92%, 90%, and 90% similar, respectively, between the two species. All three Cda proteins of *C. gattii* have similar predicted sequence features to those of *C. neoformans* Cda proteins in having N-terminal signal sequences, S/T-rich regions, and glycosylphosphatidylinositol (GPI) anchor sites, as well as conserved amino acids for catalysis. Pairwise protein sequence alignments are shown in Fig. S1 in the supplemental material.

***C. gattii* R265 cells produce significantly larger amount of chitosan in the cell wall compared to *C. neoformans* under YPD growth conditions.** We have shown for *C. neoformans* that the amount of cell wall chitosan significantly influences host immune response during infection (26, 29). Various studies have demonstrated that strain R265 elicits different types of immune response either in the host or when incubated with immune cells under *in vitro* conditions compared to the response induced by *C. neoformans* cells (22–25). Therefore, we were curious to see whether
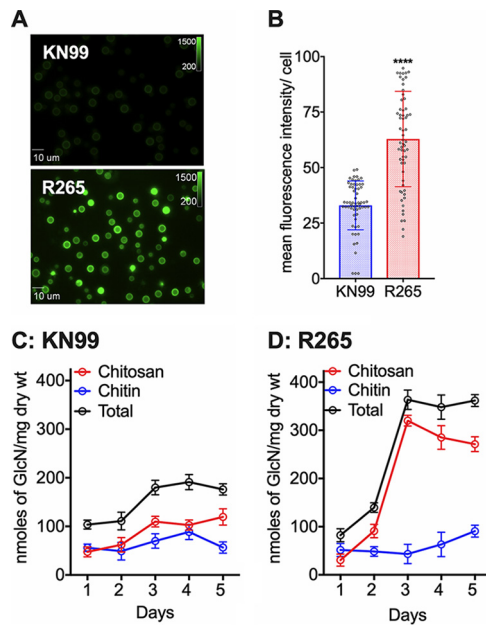
FIG 1 *C. gattii* R265 cells produce significantly larger amounts of chitosan in the cell wall compared to *C. neoformans* under YPD growth conditions. (A) Wild-type R265 and KN99 strains were grown in YPD for 5 days at 30°C and stained with Eosin Y to detect cell wall chitosan. Staining intensity was assessed using epifluorescence microscopy with identical exposures for all images. (B) Fluorescent levels for 60 individual cells (represented in panel A) were quantified using ImageJ (Fiji). The two-tailed unpaired *t* test with Welch's correction was used to compare mean values of the wild type. Means represent the fluorescence intensity levels from three independent experiments ($n = 3$). ****, $P < 0.0001$. Error bars represent standard errors of the means. (C) Quantitative determination of cell wall chitosan and chitin of strain KN99 by the MBTH assay. Cells were grown in YPD for 1 to 5 days, collected, washed, and used for the assay. Data represents the averages of three biological experiments and are expressed as nanomoles of glucosamine per milligram (dry weight) of yeast cells. (D) Quantitative determination of cell wall chitosan and chitin of R265 were determined as in panel C.

there is a difference in the amount of chitosan between KN99, a hypervirulent strain of *C. neoformans* (31), and R265. To determine the amount of chitosan, we grew both KN99 and R265 cells in YPD at 30°C. We have previously reported the specific affinity of an anionic dye Eosin Y to chitosan in *C. neoformans* (28). Therefore, we stained wild-type *C. neoformans* KN99 and *C. gattii* R265 cells after 5 days of culture. As shown in Fig. 1A, we observed a dramatic increase in the binding of Eosin Y to R265 cells compared to KN99 cells. This difference was further quantified by measuring the mean fluorescence intensity (MFI) per cell using ImageJ (Fig. 1B). The MFI/cell of strain KN99 was 32.9 compared to 62.9 for strain R265. We then measured the total amount of chitin and chitosan biochemically employing the 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay as described in Materials and Methods. Wild-type KN99 and R265 cells were grown for 1 to 5 days. At different days of growth, the cellular chitosan was quantified by the MBTH assay. As shown in Fig. 1C and D, at day 2, R265 cells started to show increased amount of chitosan compared to KN99, and this increase peaked at day 3 of growth before it started to decrease. At its peak, the amount of chitosan as expressed as nanomoles of glucosamine per milligram (dry weight) of the cell wall material in the R265 cells had increased by threefold compared to the amount in the KN99 cells. The levels of chitin in the R265 cells remained almost constant throughout the growth period, and their levels were comparable to those in the KN99 cells. The sharp increase in the amount of chitosan observed in R265 on day 3 was not observed in KN99 cells which showed a slight increase in the chitosan amount (Fig. 1C and D). These data indicate that outbreak R265 cells produce significantly larger amounts of chitosan compared to those produced by KN99 cells under YPD growth conditions.

C. gattii R265 cells produce significantly larger amount of chitosan compared to *C. neoformans* under host conditions. To determine whether the increased
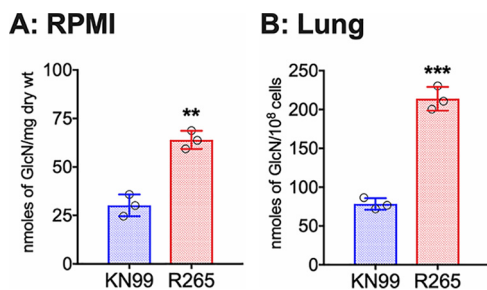
A: RPMI

B: Lung

FIG 2 *C. gattii* R265 cells produce significantly larger amount of chitosan in the cell wall compared to *C. neoformans* under host conditions. (A) Chitosan levels of strains grown in RPMI 1640 containing 10% FBS and 5% CO_2 at 37°C for 5 days. Strains were grown in YPD for 2 days. Yeast cells were harvested, washed with PBS, inoculated at 500 cells/μl in RPMI 1640 containing 10% FBS, and incubated for 5 days at 37°C in the presence of 5% CO_2. At the end of incubation, chitosan was measured by the MBTH assay and expressed as nanomoles of glucosamine per milligram (dry weight) of cells. Data represent the averages from three biological experiments. (B) Chitosan levels of strains growing in the murine lung. Mice (CBA/J) (three mice per group) were intranasally inoculated with 10^7 CFU of each strain. On day 7 postinfection (PI), the lungs were excised and homogenized, and the lung tissue was removed by alkaline extraction, leaving the fungal cells to be harvested, counted, and subjected to the MBTH assay. Data are expressed as nanomoles of glucosamine per 10^8 cells. Significant differences between the groups were compared by two-tailed unpaired *t* test with Welch's correction. Error bars represent standard errors of the means. ***, $P < 0.0062$; **, $P < 0.001$.

amount of chitosan was also seen under host conditions, we first measured chitosan of KN99 and R265 cells cultured in tissue culture conditions and then for *Cryptococcus* isolated from infected mouse lungs. Wild-type KN99 and R265 cells were first grown in YPD medium at 30°C and then transferred to RPMI 1640 medium containing 10% fetal bovine serum (FBS), 5% CO_2 at 37°C for 5 days. The cells were then harvested, and the MBTH assay was used to quantify the chitosan content. As shown in Fig. 2A, R265 cells have a mean value of 63.9 versus 30.2 of KN99 cells as expressed as nanomoles of glucosamine sugar per milligram (dry weight) of the cell wall material. Next, chitosan was determined after isolating the yeast cells from infected mouse lungs. Similar to YPD and RPMI 1640 culture conditions, R265 cells showed significantly higher cell wall chitosan compared to KN99 cells with mean values of 213.9 and 78.3, respectively, as expressed as nanomoles of glucosamine sugar per 10^8 cells (Fig. 2B). These results suggest that the increase in cell wall chitosan of *C. gattii* R265 over *C. neoformans* KN99 is a phenotype that is preserved when these strains are grown in culture and during mouse infection.

The deletion of *CDA1* in *C. gattii* R265 causes a decrease in cell wall chitosan under YPD growth conditions. We generated single CDA deletion mutants of *C. gattii* R265 by biolistic transformation. The strains generated and used in this study are listed in Table 2. The deletion cassettes for each gene deletion were generated by overlap

TABLE 2 Strains used and generated in this study

| Strain	Resistance marker(s)	Background	Designation
R265			JLCG924
cda1Δ	G418	JLCG924	WLCG1156
cda1Δ–2	G418	JLCG924	WLCG1157
cda2Δ	NAT	JLCG924	WLCG1159
cda3Δ	HYG	JLCG924	WLCG1162
cda3Δ::CDA3	G418	WLCG1162	JLCG951
cda1Δ2Δ	G418/NAT	WLCG1156	WLCG1169
cda1Δ2Δ–2	G418/HYG	WLCG1156	WLCG1171
cda1Δ3Δ	G418/HYG	WLCG1162	WLCG1197
cda1Δ3Δ–2	G418/HYG	WLCG1163	WLCG1198
cda2Δ3Δ	NAT/HYG	WLCG1162	WLCG1166
cda2Δ3Δ–2	NAT/HYG	WLCG1162	WLCG1167
cda1Δ2Δ3Δ	G418/NAT/HYG	WLCG1166	WLCG1190
cda1Δ2Δ3Δ–2	G418/NAT/HYG	WLCG1166	WLCG1194
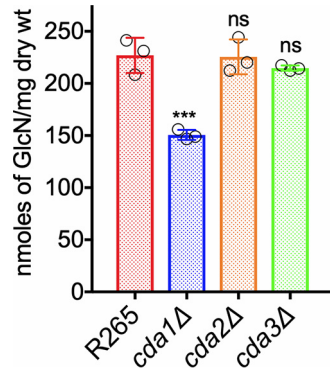
FIG 3 The deletion of *CDA1* in strain R265 displays a decrease in cell wall chitosan in cells grown in YPD. Chitosan levels of strains grown in YPD for 5 days were determined. The amount of chitosan in the cell wall of the strains was quantified by the MBTH assay. Data represent the averages from three biological experiments with two technical replicates and are expressed as nanomoles of glucosamine per milligram (dry weight) of yeast cells. Significant differences between the groups were compared by one-way ANOVA followed by Dunnett's multiple-comparison test. ***, $P < 0.0002$ comparing wild-type R265 with any other strain. ns, not significant.

PCR and biolistically transformed into R265 cells. The primers used to generate these deletion cassettes are listed in Table S1 in the supplemental material. All the isolates were characterized by diagnostic PCR screening and Southern blot hybridization. After growing the strains in YPD for 5 days, we measured chitosan by MBTH assay. We found that deletion of the *CDA1* gene decreased the chitosan amount by 33% compared to that in wild-type R265. However, deletion of either *CDA2* or *CDA3* did not affect the total amount of chitosan, as shown in Fig. 3. This is different from what we have previously described for *C. neoformans*, where there was no significant difference in chitosan in any of the single CDA deletion strains (28, 30). This suggests that there are differences in the regulation of chitin deacetylation between the two species, with R265 Cda1 having a more significant role in chitosan production when the cells were cultured in YPD.

R265 Cda3 is critical for fungal virulence. Next, we assessed the CDA single-deletion mutant strains for virulence by employing the murine intranasal infection model. We infected CBA/J mice with 10^5 wild-type cells or with cells of the corresponding single CDA deletion strains. The virulence was assayed as described in Materials and Methods. We found that deletion of either *CDA1* or *CDA2* did not affect virulence (Fig. 4A). The virulence of the *cda1Δ* mutant was reproduced using a second isolate and
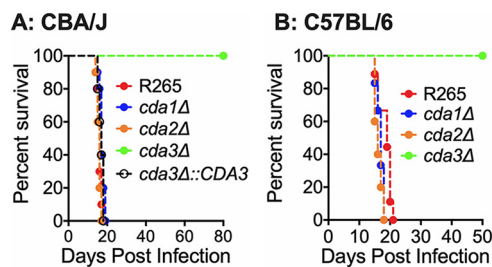

FIG 4 *C. gattii cda3Δ* displays severely attenuated virulence in CBA/J and C57BL/6 mouse models of infection. (A) CBA/J mice (6 to 8 weeks old, female) were infected intranasally with 10^5 CFU of each strain. Survival of the animals was recorded as mortality of mice for 80 days PI. Mice that lost 20% of the body weight at the time of inoculation were considered ill and sacrificed. Data are representative of two independent experiments with five animals for each strain. (B) C57BL/6 mice (4 to 6 weeks old, female) were infected with 10^4 CFU of each strain by intratracheal inoculation. Survival of the animals was recorded as mortality of mice for 50 days PI. Mice that lost 20% of the body weight at the time of inoculation were considered ill and sacrificed. Data are representative of one experiment with 10 animals for each strain. Virulence was determined using Mantel-Cox curve comparison with statistical significance determined by log rank test. $P < 0.0001$ comparing strain KN99 with *cda3Δ* strain.
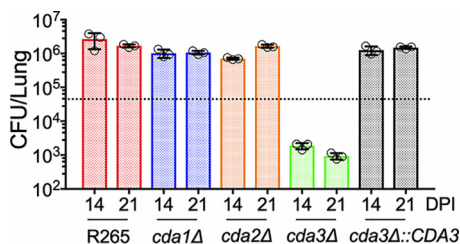
FIG 5 There is a slow but gradual clearance of *C. gattii cda3Δ* in CBA/J mice. Fungal burden in the lungs of CBA/J mice infected with indicated strains at different days postinfection (DPI). Data are from three mice per group at each time point. The dotted line indicates the CFU of the initial inoculum for each strain.

is shown in Fig. S2 to further confirm the absence of a role for Cda1 in the virulence of *C. gattii* R265. Interestingly, we found that R265 Cda3 is essential for fungal virulence. The virulence defect of the *cda3Δ* mutant was completely restored in a *CDA3* complemented strain (Fig. 4A). Since different mouse models show various degrees of sensitivity to *Cryptococcus* infection, we wanted to verify whether the associations between the absence of CDA genes, amount of cell wall chitosan, and virulence of the specific CDA deletion strain can be recapitulated in C57BL/6 mice, which are routinely employed for diverse immunological studies using readily available mutants. Similar to the results obtained with CBA/J mice, we found that only the R265 *cda3Δ* mutant is specifically avirulent when tested following orotracheal inoculation of 10e4 CFU of yeast (Fig. 4B). The avirulent phenotype in both mouse strains was accompanied by the complete clearance of the mutant strain from the infected host at the endpoint of the survival study (Fig. S3). This was rather surprising because we have previously seen that for strain KN99, Cda1 plays a major role in fungal virulence without the contribution of Cda3 to pathogenesis and that *C. neoformans cda1Δ* mutant cells persist in the mouse lungs at low levels, even though the strain does not cause disease (30).

The virulence phenotype of the single CDA deletion strains followed their ability to grow in the infected lung. As shown in Fig. 5, we found that CBA/J mice infected with either *cda1Δ* or *cda2Δ* strains had lung fungal burdens similar to those of R265 on day 14 and 21 postinfection (PI). On the other hand, mice infected with the *cda3Δ* strain showed slow and gradual clearance of the mutant strain. The fungal growth in the lungs of the mice infected with the *cda3Δ* strain was restored to wild-type R265 levels in a *CDA3* complemented strain (Fig. 5, *cda3Δ::CDA3*).

Fungal virulence of different CDA deletion strains is directly correlated with their ability to produce chitosan under host-mimicking growth conditions. Recently, we have shown that in *C. neoformans*, Cda1 is essential for fungal virulence, and this avirulent phenotype is associated with the inability of the mutant to produce wild-type levels of chitosan when grown under host-mimicking conditions, such as RPMI 1640 medium containing 10% FBS, 5% CO_2, and 37°C (30). Therefore, we wanted to test whether such defects are also responsible for the avirulent phenotype of R265 mutants that do not harbor the *CDA3* gene in the genome. As shown in Fig. 6, the loss of Cda3 resulted in a 77% decrease in the amount of chitosan produced compared to wild-type R265. Even though the amount of chitosan in the *cda1Δ* strain showed 33% reduction compared to wild-type R265 (Fig. 6), this decrease was similar to what we observed when the cells were grown in YPD culture conditions as well (Fig. 3). These data indicate that in strain R265, Cda1-mediated deacetylation of chitin is not influenced by growth conditions and is not critical for virulence. However, Cda3 is responsible for the majority of the deacetylation either in RPMI 1640 culture conditions or in infected mice and thus contributes significantly to fungal proliferation in the host. Taken together, these results confirmed the importance of cell wall chitosan to fungal virulence and suggests that a certain threshold amount of chitosan needs to be maintained in the cell wall to sustain infection.
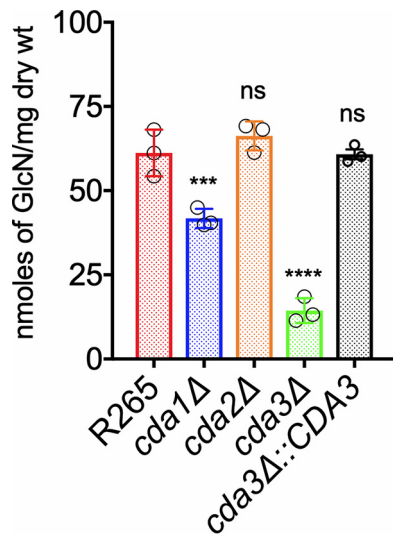
FIG 6 *C. gattii* Cda3 plays a major role in the synthesis of chitosan under host infection conditions. Chitosan levels of strains grown in RPMI 1640 containing 10% FBS and 5% CO_2 at 37°C for 5 days. The indicated strains were grown in YPD for 48 h. Yeast cells were harvested, washed with PBS, and inoculated at 500 cells/μl in RPMI 1640 containing 10% FBS and incubated for 5 days at 37°C in the presence of 5% CO_2. At the end of incubation, chitosan was measured by the MBTH assay and expressed as nanomoles of glucosamine per milligram (dry weight) of cells. Data represent the averages from three biological experiments. Significant differences between the groups were compared by one-way ANOVA, followed by Dunnett's multiple-comparison test (***, $P < 0.0002$, *, $P < 0.0280$ comparing KN99 with any other strain; ns, not significant).

Double and triple CDA mutants of *C. gattii* R265 displayed varied amounts of chitosan under YPD and host-mimicking conditions. We generated three double CDA deletion strains and a triple CDA gene deletion strain in *C. gattii* R265 by biolistic transformations as indicated in Table 2. We subjected these strains to chitosan quantification after growing them either in YPD or in host-mimicking conditions of RPMI 1640 medium containing 10% FBS in the presence of 5% CO_2 at 37°C. When we compared the chitosan amounts among the CDA double deletion strains, we found that the cell wall chitosan amount was significantly decreased in all the double deletion strains grown under YPD culture conditions (cda1Δ2Δ, cda1Δ3Δ, and cda2Δ3Δ strains in Fig. 7A). The decrease in the chitosan amount was more pronounced in the absence of Cda1 in combination with either Cda2 or Cda3: 63% reduction for the cda1Δ2Δ strain and 54% reduction for the cda1Δ3Δ strain compared to wild-type R265. The amount of chitosan in the cda2Δ3Δ strain showed a reduction of only 26% compared to wild-type R265. These results are consistent with the major role of Cda1 in chitosan production in strain R265 when the cells were grown under YPD conditions (Fig. 3). When we deleted all three CDAs to generate a cda1Δ2Δ3Δ strain, the chitosan amount decreased to almost negligible amounts, suggesting that in spite of differences in the roles of individual CDAs in strains KN99 and R265, the deletion of all three CDAs was sufficient to render the mutant chitosan deficient (Fig. 7A). When the strains were grown under host-mimicking conditions (RPMI 1640 plus 10% FBS grown with CO_2 at 37°C), any strain in which CDA3 was deleted in combination with either CDA1 or CDA2 (cda1Δ3Δ or cda2Δ3Δ strain in Fig. 7B) produced negligible amounts of chitosan similar to the cda1Δ2Δ3Δ strain, further indicating the importance of R265 Cda3 in the production of chitosan in the host and its subsequent effect on fungal virulence.

Chitosan-deficient R265 cells were sensitive to cell wall stressors and had normal capsule- and melanin-producing abilities. We subjected the single CDA deletion strains and the triple CDA deletion strain of *C. gattii* R265 to a panel of cell wall stressors (0.005% SDS, 1 mg/ml Calcofluor white, 0.5 mg/ml caffeine, 0.4% Congo red) added to YPD agar medium; these stressors are routinely employed to determine cell wall integrity. As shown in Fig. S4, only the chitosan-deficient cda1Δ2Δ3Δ strain was
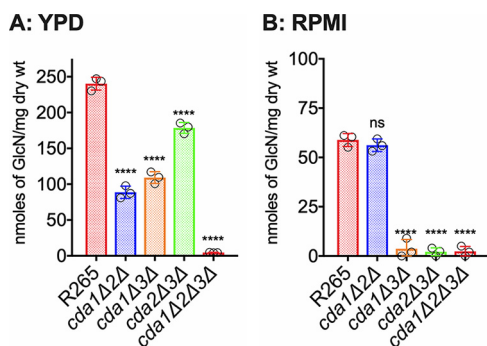
A: YPD

B: RPMI

FIG 7 The deletion of *C. gattii* *CDA1*, *CDA2*, and *CDA3* results in a strain that is completely chitosan deficient. *C. gattii* Cda1 in combination with Cda2 or Cda3 plays a major role in chitosan synthesis in vegetative growing conditions. While *C. gattii* Cda3 in combination with Cda1 or Cda2 results in a stain that is completely chitosan-deficient. (A) Chitosan levels of strains grown in YPD. The indicated strains were grown in YPD for 5 days. The amount of chitosan in the cell wall of the strains was quantified by the MBTH assay. Data are the averages for three biological experiments and are expressed as nanomoles of glucosamine per milligram (dry weight) of yeast cells. (B) Chitosan levels of strains grown in RPMI 1640 containing 10% FBS and 5% CO_2 at 37°C for 5 days. The indicated strains were grown in YPD for 48 h. Yeast cells were harvested, washed with PBS, and inoculated at 500 cells/μl in RPMI 1640 containing 10% FBS and incubated for 5 days at 37°C in the presence of 5% CO_2. At the end of incubation, chitosan was measured by the MBTH assay and expressed as nanomoles of glucosamine per milligram (dry weight) of cells. Data represent the averages for three biological experiments. Significant differences between the groups were compared by one-way ANOVA, followed by Dunnett's multiple-comparison test (****, $P <$ 0.0001, comparing KN99 with any other strain; ns, not significant).

sensitive to the various cell wall stressors. None of the CDA deletion mutants were sensitive to temperature when their growth at 30°C was compared to growth at 37°C on YPD agar medium. The deletion of CDA genes in strain R265 either individually or in combination did not affect their ability to produce either capsule (Fig. S5) or melanin as shown in Fig. S6. In contrast to *C. neoformans* mutants (26), none of the CDA mutants of R265 displayed "leaky melanin" phenotype.

R265 *CDA1* and *CDA2* are dispensable for virulence even when both genes were deleted. We next subjected the double and triple CDA gene deletion strains to tests of fungal virulence either by intranasal infection of CBA/J mice or by orotracheal inoculation of C57BL/6 mice. We found that deletion of *CDA1* and *CDA2* did not affect the virulence in either CBA/J or C57BL/6 mice (*cda1Δ2Δ* strain in Fig. 8A and B). Consistent with the role of *C. gattii* R265 Cda3 in virulence, double CDA deletion strains in which *CDA3* is deleted in combination with either *CDA1* or *CDA2* showed a major defect in virulence (*cda1Δ3Δ* and *cda2Δ3Δ* strains in Fig. 8A and B). These results further point to the importance of just Cda3 in fungal virulence for strain R265. The mutant
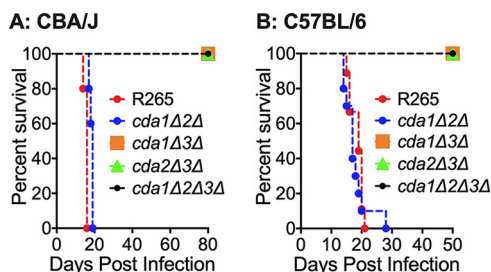
A: CBA/J

B: C57BL/6

FIG 8 Deletion of *C. gattii* *CDA3* in combination with any of the other two CDAs results in severe attenuation of virulence in CBA/J and C57BL/6 mouse models of infection. (A) CBA/J mice (6 to 8 weeks old, female) were infected intranasally with 10^5 CFU of each strain. Survival of the animals was recorded as mortality of mice for 80 days postinfection (PI). Mice that lost 20% of the body weight at the time of inoculation were considered ill and sacrificed. Data are representative of two independent experiments with five animals for each strain. (B) C57BL/6 mice (4 to 6 weeks old, female) were infected intratracheally with 10^4 CFU of each strain. Survival of the animals was recorded as mortality of mice for 50 days PI. Mice that lost 20% of the body weight at the time of inoculation were considered ill and sacrificed.
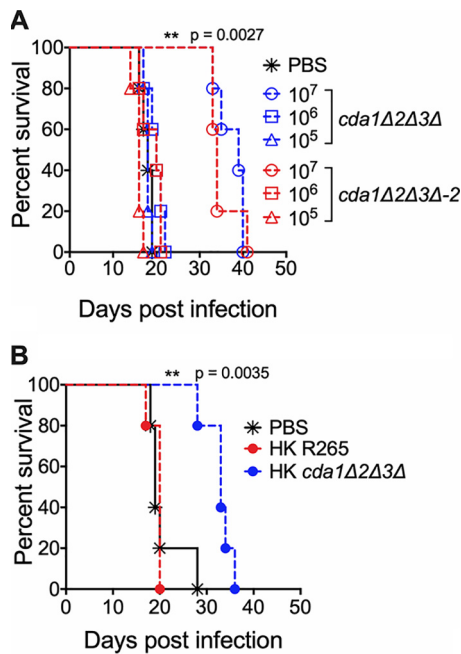
FIG 9 Vaccination of CBA/J mice with 10^7 CFU of live or heat-killed (HK) *cda1Δ2Δ3Δ* cells conferred attenuated protective immunity to subsequent infection with wild-type R265 *C. gattii* cells. (A) Mice were immunized with either 10^7, 10^6, or 10^5 live CFU of *cda1Δ2Δ3Δ* cells through inhalation. PBS-inoculated mice served as control. Animals were left for 40 days to resolve the infection. Subsequently, both groups of mice were challenged with 50,000 CFU of virulent R265 cells. Virulence was recorded as mortality of mice. Mice that lost 20% of starting body weight at the time of inoculation were considered to be moribund and sacrificed. The percentage of mice that survived was plotted against the day postinfection. Each survival curve is the average of two independent experiments that had five mice per experimental group. (B) Mice were immunized with an inoculum of HK cells with a dose equivalent to 10^7 CFU of either the wild-type R265 or the *cda1Δ2Δ3Δ* strain. Control mice were inoculated with PBS. After 40 days, mice were challenged with 50,000 CFU of wild-type R265 cells. Survival of the animals was recorded as described above. The data shown are from a single experiment with five mice per experimental group. A second experiment showed similar results.

strain devoid of all three CDA genes (*cda1Δ2Δ3Δ* strain) was completely avirulent (Fig. 8A and B). For all mutants, the avirulent phenotype of the mutant strains was accompanied by the inability of the different CDA mutant strains to proliferate or maintain in the infected murine lung as revealed by their gradual clearance (data not shown). To further ascertain these results, we subjected a second independent isolate of each mutant to fungal virulence studies and obtained nearly identical results (Fig. S7).

Vaccination with the chitosan-deficient R265 cells confers partial protection to subsequent challenge with the virulent wild-type R265. For *C. neoformans*, we observed that mice infected with 10^7 CFU of the chitosan-deficient *cda1Δ2Δ3Δ* strain resulted in the clearance of the mutant. That clearance was accompanied by the induction of a robust protective response to a subsequent infection with wild-type, fully virulent KN99 (29). This protective response was observed only when mice were vaccinated with 10^7 CFU of the *cda1Δ2Δ3Δ* strain, while vaccination with either 10^6 or 10^5 CFU did not generate a protective response (29). Therefore, since the complete clearance of the chitosan-deficient *cda1Δ2Δ3Δ* strain is also observed for mutants generated in the R265 background, we wanted to determine whether this clearance of the mutant strain induces protective immunity to R265 infection. For this, we vaccinated naive mice (CBA/J) with either 10^5, 10^6, or 10^7 CFU of live preparations of the *cda1Δ2Δ3Δ* strain by intranasal inhalation. After 40 days PI, we challenged them with 10^5 CFU of strain R265. Two independent isolates of the *cda1Δ2Δ3Δ* strain (*cda1Δ2Δ3Δ-1* and *cda1Δ2Δ3Δ-2*) were used for this study. As shown in Fig. 9A, vaccination with either one of the chitosan-deficient *cda1Δ2Δ3Δ* isolates conferred only

partial protection to subsequent infection with R265. The control mice that received phosphate-buffered saline (PBS) alone succumbed to R265 infection with a median survival time of 20 days PI. However, the mice vaccinated with a live preparation of the *cda1Δ2Δ3Δ* strain had a median survival time of 30 to 40 days PI upon secondary challenge infection with strain R265. This protective immunity required a minimal dose of 10^7 CFU of the *cda1Δ2Δ3Δ* strain for vaccination (Fig. 9A), as observed for the *cda1Δ2Δ3Δ* mutant of strain KN99 (29). Next, we wanted to determine whether a heat-killed (HK) preparation of the *cda1Δ2Δ3Δ* strain induces protective immunity. We confirmed that incubating live R265 (either the wild-type strain or the corresponding *cda1Δ2Δ3Δ* mutant) at 70°C for 15 min was sufficient to kill both strains by plating them for CFU onto YPD agar. Then we vaccinated mice intranasally with 10^7 CFU of HK preparation of either strain R265 or the *cda1Δ2Δ3Δ* mutant, waited 40 days, and challenged them with 10^5 CFU of R265. The mice vaccinated with either PBS or with HK wild-type R265 succumbed to R265 infection with a median survival time of 20 days PI. However, the mice vaccinated with the HK *cda1Δ2Δ3Δ* strain had a median survival time of 33 days PI (Fig. 9B), similar to that observed for vaccination with the live *cda1Δ2Δ3Δ* strain (Fig. 9A). When the vaccination and protection experiment was done in C57BL/6 mice using the HK *cda1Δ2Δ3Δ* strain as a vaccine, mice had a median survival time of 41.5 days compared to 26 days for unvaccinated mice (Fig. S8).

DISCUSSION

Chitosan is one of the principal components of the *C. neoformans* cell wall. In its absence, yeast cells display a severe budding defect resulting in irregular shaped, often clumped cells with significant sensitivity to various cell wall-perturbing agents (28). More importantly, the absence of chitosan makes the yeast cells completely avirulent in a mammalian host, as the chitosan-deficient cells stimulate robust host immune responses, which in turn leads to rapid clearance of infection (26, 29). The outbreak strain of *C. gattii*, R265, is a hypervirulent strain that has been reported to possess several key features that enabled it to cause symptomatic infection, even in immuno-competent individuals (10, 32, 33). Its higher rate of intracellular proliferation with increased resistance to oxidative stress, its ability to dampen host immune response, and its potential to inhibit the maturation of specific immune cells all may contribute to its virulence (19, 22–25, 34, 35). A larger amount of chitosan in *C. gattii* R265 than in *C. neoformans* KN99 may efficiently shield surface-exposed PAMPs from being recognized by the host immune system, thereby limiting the intensity and complexity of the host immune response. *C. neoformans* and *C. gattii* share diverse ecological niches. Moreover, *C. gattii* R265 is predominantly detected in the environment and strictly associated with plants where they encounter chitinases from soil microbes and plant hosts, respectively (32). Extensive deacetylation of chitin to form chitosan may provide greater resistance to environmental chitinases, thereby increasing its fitness in the environment.

C. neoformans Cda1 plays an important role in the deacetylation of chitin especially during host infection (30). Among the individual *C. neoformans* CDA deletion strains, we did not observe phenotypic differences when the strains were cultured in YPD medium, suggesting the redundancy in their function (28). However, deletion of Cda1 alone caused a dramatic avirulent phenotype in virulence studies using CBA/J mice (30). Unlike *C. neoformans*, Cda1 of *C. gattii* seems to play an important role in deacetylation when grown in YPD medium, since its deletion caused a 34% reduction in the amount of cell wall chitosan (Fig. 3). The deletion of either *CDA2* or *CDA3* did not significantly reduce the chitosan amount when the strain was grown in YPD. However, when grown under host-mimicking conditions of RPMI 1640 medium with 10% FBS, 5% CO_2, and at 37°C, *C. gattii* Cda3 played an important role in the deacetylation of chitin, since its deletion in the *cda3Δ* strain caused a 76% reduction in the amount of chitosan. On the other hand, the decrease in the amount of chitosan in the *cda1Δ* strain when grown in RPMI 1640 conditions was around 32%, which is similar to what we observed for YPD culture conditions, suggesting a unique role of R265 Cda3 in deacetylating chitin in

host-mimicking conditions. For our initial animal virulence experiments, we chose two independent isolates of the *cda1*Δ mutant strain, expecting that they will mimic the avirulent phenotype observed in *C. neoformans* (30). However, we were surprised to see that for *C. gattii* R265, deacetylation of chitin during host infection was even more dependent on Cda3 than Cda1. The avirulent phenotype of the *cda3*Δ mutant was further confirmed by the *CDA3* complemented strain in CBA/J mice. In spite of its role in chitosan production in YPD and host-mimicking media, the decreased levels of chitosan in the *cda1*Δ strain did not affect its virulence (Fig. 4). It may be that the levels of chitosan present in the *cda1*Δ strain during infection are still sufficient to promote pathogenesis, or the pattern of deacetylation in the chitosan produced by Cda1 may be different from that of Cda3. This difference in the molecular structure of chitosan may have contributed to the observed differences in the virulence potential of different CDA deletion mutant strains.

All three CDAs contribute to chitosan production in *C. gattii* R265 while growing in YPD, since deletion of two *CDA* genes in combination significantly reduced cell wall chitosan (Fig. 7). This again was reflected in the virulence phenotype of the double and triple CDA deletion strains. When *CDA3* was deleted in combination with any one or both of the other CDA genes, the resulting strains had substantially reduced chitosan when grown in host-mimicking conditions and were avirulent. Any strain in which *CDA3* is deleted was avirulent in both CBA/J and C57BL/6 mouse strains and was cleared from the infected lung. Deletion of all three CDAs made the strain chitosan deficient similar to the *cda1*Δ*2*Δ*3*Δ strain of *C. neoformans*. The majority of the *in vitro* phenotypes of the *cda1*Δ*2*Δ*3*Δ strain of R265 were similar to the *cda1*Δ*2*Δ*3*Δ strain made in *C. neoformans*. However, *cda1*Δ*2*Δ*3*Δ of *C. gattii* R265 grew better in YPD (with markedly fewer morphological abnormalities) than the *cda1*Δ*2*Δ*3*Δ strain of *C. neoformans* (data not shown). *C. neoformans cda1*Δ*2*Δ*3* exhibited a leaky melanin phenotype when incubated in medium containing L--DOPA (28). However, the *cda1*Δ*2*Δ*3*Δ strain of R265 did not show this phenotype (Fig. S6) suggesting that there are differences in the cell wall architecture between *cda1*Δ*2*Δ*3*Δ cells of *C. neoformans* and *C. gattii*.

In *C. neoformans*, *CDA1* seems to be the most critical deacetylase gene during infection—the *CDA1* gene is much more highly upregulated during the growth of wild-type KN99 yeast cells in the infected lung than *CDA2* or *CDA3*, and a *cda1*Δ strain is avirulent, consistent with the hypothesis that differential regulation of *CDA1* is the driving force behind its importance during infection (30). Unlike *C. neoformans*, *CDA3* is the critical deacetylase gene in *C. gattii* R265 based on our genetic analysis, but the available data from genome-wide studies of transcriptional regulation are less clear, and may be different for different *C. gattii* strains. One study that looked only at transcript abundance of genes from strain R265 recovered through bronchoalveolar lavage, found that R265 *CDA3* was among the highly expressed genes during mammalian infection (36). Recent whole-genome transcriptome studies of different strains of *C. gattii* have shown multiple strain-specific differences in gene expression that include chitin and chitosan biosynthesis genes (37, 38). One of these studies (37) examined strains from the four lineages of *C. gattii*, and it showed that the *CDA2* gene of strain R265 was substantially downregulated after incubation of the R265 cells with bone marrow-derived macrophages. The results from these studies suggest that transcriptional regulation of chitin and chitosan biosynthesis in *C. gattii* is likely divergent from regulation in *C. neoformans*, is different among different *C. gattii* strains, may play a role in the virulence of the different strains, and could be a factor in the importance of *CDA3* in strain R265.

We have previously shown that vaccination with the chitosan-deficient *cda1*Δ*2*Δ*3*Δ strain of *C. neoformans* at the optimal concentration confers robust protective immunity to subsequent challenge infection with the wild-type virulent KN99 strain (29). Even though deletion of all three CDA genes of *C. gattii* produced a chitosan-deficient strain, vaccination with either the live strain or a heat-killed preparation of it induced only partial protection to subsequent infection with strain R265. This may be due to the

fact that R265 has the inherent ability to dampen host-induced immune responses (22–25).

In summary, the hypervirulent *C. gattii* strain R265 has evolved with a distinct transcriptional profile with altered expression of components of chitin deacetylation that may have enabled it to adapt more efficiently to various environmental conditions with ramifications on its virulence. Of the several reported differences in the virulence-related traits between *C. neoformans* and *C. gattii* R265, the difference in the regulation of chitosan biosynthesis as revealed from our studies may significantly contribute to the mechanisms of its unique and distinct nature of pathogenesis, since chitosan is one of the macromolecules that resides at the host-pathogen interface.

MATERIALS AND METHODS

Fungal strains and media. R265, *C. gattii* strain of VGII subtype linked to the 1999 outbreak in British Columbia, Canada (11), was used as the wild-type strain and as progenitor of mutant strains. This strain was kindly provided by Joseph Heitman (Duke University Medical Center, NC). All the strains used in this study are listed in Table 1. KN99α, a strain of *C. neoformans*, was used as the wild-type strain for serotype A (31). Strains were grown on YPD (1% yeast extract, 2% Bacto peptone, and 2% dextrose). Solid media contained 2% Bacto agar. Selective YPD medium contained 100 μg/ml nourseothricin (NAT) (Werner BioAgents, Germany) and/or 200 μg/ml G418 (Geneticin; Gibco Life Technologies, USA). RPMI 1640 medium (catalog no. 10-040-CM; Corning) contained 10% fetal bovine serum (FBS) (catalog no. 26140; Gibco-Thermo Fisher Technologies).

Generation of deletion constructs of *C. gattii*. Gene-specific deletion constructs of the chitin deacetylases were generated using overlap PCR gene technology described previously (39, 40) and included either the hygromycin resistance, Geneticin resistance cassette (41), or nourseothricin resistance cassette (42). The primers used to disrupt the genes are shown in Table S1 in the supplemental material. The Cda1 deletion cassette contained the Geneticin resistance cassette, resulting in a 1,539-bp replacement of the genomic sequence between regions of primers 3-Cda1 and 6-Cda1 shown in upper case in Table S1. The Cda2 deletion cassette contained the hygromycin resistance cassette, resulting in a 1,587-bp replacement of the genomic sequence, and the Cda3 deletion cassette contained the hygromycin resistance cassette, resulting in a 1,494-bp replacement of the genomic sequence. Constructs were introduced into the R265 strain using biolistic techniques (43).

Transformation and characterization of *C. gattii* mutants. Recipient strains of *C. gattii* were transformed biolistically following the protocol described earlier (41, 43). Drug-resistant transformants that formed colonies in 3 to 5 days were passaged four times in liquid YPD medium before reselection of drug resistance on agar. Transformants were further screened by diagnostic PCR of their genomic DNA using primers at the 5' and 3' junction of the integration site of the transforming DNA. Southern blot hybridizations were done to verify the absence of random DNA integrations, as described previously employing digoxigenin (DIG)-labeled DNA probes (44, 45).

Cellular chitosan measurement. As previously described, MBTH (3-methyl-2-benzothiazolinone hydrazone)-based chemical method was used to determine the chitin and chitosan content of *C. gattii* or *C. neoformans* (31). In brief, cells were collected after growing them in appropriate media and growth conditions by centrifugation. The cell pellets were washed two times with phosphate-buffered saline (PBS), pH 7.4, and lyophilized. The dried samples were resuspended in water first before adding KOH to a final concentration of 6% KOH (wt/vol). The alkali-suspended material was incubated at 80°C for 30 min with vortexing in between to eliminate nonspecific MBTH-reactive molecules from the cells. Alkali-treated material was then washed several times with PBS, pH 7.4, to make sure that the pH of the cell suspension was brought back to neutral pH. In the case of the cells grown in RPMI 1640 medium, alkali-treated material was sonicated as described previously to generate a uniform suspension (30). Finally, the cell material was resuspended in PBS, pH 7.4, to a concentration of 10 mg/ml in PBS (by dry weight), and a 0.1-ml aliquot of each sample was used in the MBTH assay (46).

Virulence and fungal burden assays. *C. gattii* strains were grown at 30°C and 300 rpm for 48 h in 50 ml YPD. The cells were centrifuged, washed in endotoxin-free 1$\times$ PBS, and suspended in 5 ml of the same PBS solution. The cells were counted with a hemocytometer and diluted to 2 $\times$ 10^6 cells/ml. CBA/J female mice (Jackson Laboratories) were anesthetized with an intraperitoneal injection (200 μl) of ketamine (8 mg/ml)-dexmedetomidine (0.05 mg/ml) mixture, which was reversed by an intraperitoneal injection of 200 μl of antipamezole (0.25 mg/ml). Mice were allowed to inhale 1 $\times$ 10^5 cells in 50 μl, which were dripped into the nares. For virulence assays, mice were weighed before and during the course of infection. Mice were euthanized by CO$_2$ asphyxiation if they reached 80% of their original body weight. At this point, the mice appeared morbidly ill, displaying a ruffled coat, lethargy, hunched posture, unstable gait, and loss of appetite. For the determination of CFU, lung or brain from each mouse was placed in 2.0 ml of 1$\times$ PBS (pH 7), homogenized, serially diluted, plated onto YPD agar supplemented with 100 μg/ml streptomycin and 100 μg/ml ampicillin, and incubated for 2 days at 30°C. Total CFU per organ were calculated. The infection protocol was reviewed and approved by the Washington University School of Medicine Animal Care and Use Committee (IACUC).

Evaluation of *C. gattii* to stress under *in vitro* conditions. Solid YPD medium was made with the desired amount of either SDS, NaCl, Calcofluor white, or Congo red. For plating, wild-type and mutant strains were grown in liquid YPD for 24 h at 30°C. Cells were diluted to an optical density at 650 nm (OD$_{650}$) of 1.0, and 10-fold serial dilutions were made. Five microliters of each dilution was spotted on the

plate, and the plates were incubated for 2 or 3 days at appropriate temperatures and photographed. Eosin Y staining was conducted as described earlier (29).

Analysis of melanin production. Strains were grown overnight in 2 ml YPD medium at 30°C with shaking to saturation. Cells were collected and washed in 1× PBS. Then, 1×10^8 (5×10^7/ml) of each mutant was added to 4 ml of glucose-free asparagine medium (1 g/liter L-asparagine, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 3 g/liter KH_2PO_4, and 1 mg/liter thiamine, plus 1 mM L-3,4-dihydroxyphenylalanine [L-DOPA]) for 7 days at 300 rpm and 30°C in the dark. Samples were then spun down at $>600 \times g$ for 10 min. The cells' ability to produce pigment was assessed visually.

Capsule analysis. Cells were grown in YPD at 30°C. After 48 h for growth, cells were collected, washed once with PBS, inoculated into RPMI 1640 medium containing 10% fetal bovine serum (FBS) at a concentration of 500 cells/μl, and incubated for 5 days at 37°C in the presence of 5% CO_2. The capsule-induced strains were resuspended in a 1:4 India ink-H_2O solution and photographed on an Olympus BX61 microscope. The capsule diameter was measured and averaged for a minimum of 100 cells per strain using SlideBook 5.0 (Intelligent Imaging Innovations, Inc. CO, USA).

Statistics. Data were analyzed using GraphPad Prism, version 7.0 (GraphPad Software, Inc., La Jolla, CA). The unpaired two-tailed t test with Welch's correction was used for comparisons of two groups. The one-way analysis of variance (ANOVA) with the Dunnett's multiple-correction test was used to compare more than two groups. Kaplan-Meier survival curves were compared using the Mantel-Cox log rank test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00644-19.

FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.04 MB.
FIG S3, PDF file, 0.03 MB.
FIG S4, PDF file, 0.1 MB.
FIG S5, PDF file, 0.1 MB.
FIG S6, PDF file, 0.04 MB.
FIG S7, PDF file, 0.04 MB.
FIG S8, PDF file, 0.04 MB.
TABLE S1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

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