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Cryptococcus gattii urease as a virulence factor and the relevance of enzymatic activity in cryptococcosis pathogenesis

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Ureases (EC 3.5.1.5) are Ni^{2+}-dependent metalloenzymes produced by plants, fungi and bacteria that hydrolyze urea to produce ammonia and CO_2. The insertion of nickel atoms into the apo-urease is better characterized in bacteria, and requires at least three accessory proteins: UreD, UreF, and UreG. Our group has demonstrated that ureases possess ureolytic activity-independent biological properties that could contribute to the pathogenicity of urease-producing microorganisms. The presence of urease in pathogenic bacteria strongly correlates with pathogenesis in some human diseases. Some medically important fungi also produce urease, including *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. gattii* is an etiological agent of cryptococcosis, most often affecting immunocompetent individuals. The cryptococcal urease might play an important role in pathogenesis. It has been proposed that ammonia produced via urease action might damage the host endothelium, which would enable yeast transmigration towards the central nervous system. To analyze the role of urease as a virulence factor in *C. gattii*, we constructed knockout mutants for the structural urease-coding gene *URE1* and for genes that code the accessory proteins Ure4 and Ure6. All knockout mutants showed reduced multiplication within macrophages. In intranasally infected mice, the *ure1*Δ (lacking urease protein) and *ure4*Δ (enzymatically inactive apo-urease) mutants caused reduced blood burdens and a delayed time of death, whereas the *ure6*Δ (enzymatically inactive apo-urease) mutant showed time and dose dependency with regard to fungal burden. Our results suggest that *C. gattii* urease plays an important role in virulence, in part possibly through enzyme activity-independent mechanism(s).

Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase) are Ni^{2+}-dependent metalloenzymes that catalyze the hydrolysis of urea to form ammonia and carbon dioxide [1]. Ureases are found in plants, fungi, and bacteria. Their high sequence similarities suggest that all ureases possess similar tertiary structures and catalytic

Abbreviations
AHA, acetohydroxamic acid; CFU, colony-forming unit; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; WT, wild-type.

mechanisms [2]. Accessory proteins are required to activate the urease apoprotein and to assemble its metallocenter [3]. Many fungi that are pathogenic to humans produce urease, including *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii* [4], and species of *Trichosporon* and *Aspergillus*. The coccidioidal urease gene has been shown to be expressed *in vivo* and to play a role in both sporulation and pathogenesis [5].

In microorganisms, urease enables the utilization of urea as a nitrogen source and, in some cases, contributes as a virulence factor to several human and animal diseases, such as gastroduodenal infections by *Helicobacter pylori*, and *Proteus mirabilis*-induced urinary stone formation [6]. However, urease might also play other important roles that are unrelated to its enzymatic activity [7], such as insecticidal [8,9], fungicidal [10] and exocytosis-inducing activities [11]. As a consequence of urease-induced ADP secretion from dense granules, platelets from different species, including humans, undergo aggregation upon exposure to nanomolar concentrations of plant (jack bean and soybean) or bacterial (*Bacillus pasteurii* and *H. pylori*) [12] ureases. The platelet-activating properties of these ureases were shown to be independent of urea hydrolysis [12–14]. Ureases also show potent proinflammatory activity. For instance, canatoxin, an isoform of *Canavalia ensiformis* urease [15], and *H. pylori* urease were shown to induce inflammation *in vivo* (mouse paw edema). Additionally, *H. pylori* urease at nanomolar concentrations activates human neutrophils to produce reactive oxygen species and inhibits their apoptosis, thus potentially contributing to the gastritis pathogenesis caused by this bacterium [16].

C. neoformans causes the most common central nervous system (CNS) fungal infection in HIV-1/AIDS populations, with high mortality and morbidity rates. Cryptococcosis has become one of the three most common opportunistic diseases associated with high-dose steroid therapies such as those administered to patients with transplants or immune disorders [17,18]. The risk of cryptococcosis has assumed global proportions over the years. An increasing number of AIDS cases since the early 1990s has led to a sharp increase in the number of reported cryptococcosis cases in the past decade [18]. The disease symptoms include severe pneumonia with the development of lung cryptococcal cysts, and tropism to the CNS, producing neurological symptoms of meningoencephalitis [19,20].

Several *C. neoformans* virulence factors are known, including a polysaccharide capsule, melanin production, enzymes (urease and phospholipase) [5,21], and the ability to grow at 37 °C. The majority of clinical isolates produce large amounts of urease. The primary role of urease in *C. neoformans* infections has been a matter of ongoing debate. *URE1*, which encodes *C. neoformans* urease, was first cloned in 2000, and used to disrupt native *URE1* in the serotype A strain H99. A comparison of the survival rates observed for the null mutant and the H99 urease-positive strains in murine models of intravenous and inhalational infections revealed significant differences. Mice infected with the *ure1*Δ mutant lived longer than those infected with H99 in both models [5]. Additional studies on the inoculation of H99, *ure1*Δ and reconstituted *ure1*Δ::*URE1* strains directly into the brain demonstrated that urease was not required for yeast growth in the brain. However, the dissemination patterns in the brain, spleen and other organs after intravenous inoculation suggested a contribution of cryptococcal urease to the process of CNS invasion by enhancing yeast sequestration within microcapillary beds (such as those within the brain) during hematogenous spread, thereby facilitating *C. neoformans* blood-to-brain invasion [20]. More recently, intravital microscopy analysis revealed that brain invasion by *C. neoformans* follows a capillary microembolic event. A major finding of this study was that fungal cells arrest suddenly at capillary sites without rolling or tethering, in a manner similar to that of polystyrene microspheres. The similarity of the arresting process to that shown by inert microspheres suggested that the initial mechanism leading to brain localization is mechanical and related to an inability of yeast cells to traverse narrow capillaries. In this scenario, the initial brain infection would result from a microembolic event. It was observed that, after arresting at capillary sites, *C. neoformans* cells cross the capillary wall in a process that requires viability and cell morphology alteration, but not replication. Moreover, this process was shown to be urease-dependent, because the deletion of *URE1* reduced the brain fungal burden in infected mice [22]. On the basis of these observations, pharmacological inhibition or urease-targeted antibody therapies have been proposed as potentially useful strategies to protect the brain against yeast invasion in cryptococcal meningitis [5,20–23]. Recently, Singh *et al.* [24] used *C. neoformans* mutants with *URE1*, *URE4*, *URE6* and *URE7* deletions to infect mice via intravenous injection. Yeast colony-forming units (CFUs) were determined in the brain tissues at 3 and 24 h postinfection, and the results demonstrated that the enzymatic activity, rather than the Ure1 protein, was crucial for brain invasion by *C. neoformans* [24].

It is well established that *C. neoformans* infects mainly immunosuppressed patients, although the majority of reports of *Cryptococcus gattii* infection are in non-HIV patients. Whereas cryptococcosis in patients infected with *C. gattii* is mainly characterized by pulmonary disease, meningoencephalitis is the main complication resulting from *C. neoformans* infections. In line with the epidemiological data, assays employing animal models of cryptococcosis revealed that, despite the fact that *C. gattii* has the molecular mechanisms needed to cross the blood–brain barrier; fatal lung infection is the main cause of death in mice infected with *C. gattii* [25].

In the present study, we aimed to contribute to the elucidation of the role(s) of *C. gattii* urease as a virulence factor in cryptococcosis. To that end, mutant yeast strains were constructed that lacked the urease protein or produced an enzymatically inactive apoprotein. Deletion mutants were phenotypically and molecularly characterized, and their virulence was tested in mice.

Results and Discussion

Identification of *URE* and accessory protein genes from *C. gattii*

A search of the *C. gattii* R265 genome Fungal Genome Initiative database at the Broad Institute of MIT and Harvard, available at http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html (5 August 2009), allowed the identification of *C. gattii URE1* (CNBG_4331.2), as well *URE7* (CNBG_1252.2 – ortholog of bacterial *ureG*), *URE4* (CNBG_0735.2 – ortholog of bacterial *ureD*), and *URE6* (CNBG_5207.2 – ortholog of bacterial *ureF*). Analysis of the predicted protein sequences in the Pfam database [26] confirmed their similarities to orthologous proteins in plants and fungi.

C. gattii urease appears to be a typical fungal urease, with a single subunit of 833 amino acids, a molecular mass of 90 kDa, and a theoretical pI of 5.5. Whereas plant and fungal ureases are oligomers of identical and typically ~ 90-kDa subunits, bacterial ureases are complex multimers with two or three subunits [6,26–28]. Remarkably, despite comprising different types of subunits, ureases from different sources, ranging from bacteria to plants and fungi, show high amino acid sequence similarity. For instance, *C. gattii* Ure1 shows 58% identity with jack bean urease. This suggests that all ureases are evolutionary variants of a single ancestral enzyme [27].

Ure accessory proteins are not as well conserved as Ure1 proteins across different species. A multiple sequence alignment of *C. gattii* Ure4 (Fig. 1) and Ure6 (Fig. 2) accessory proteins with those from different organisms allowed the identification of their characteristic domains, as detected by Pfam. As discussed below, Ure7 was not subjected to phylogenetic analysis, owing to its unique features. Regarding the phylogenetic inference of urease accessory proteins in *C. gattii*, Ure4 and Ure6 from this species did not cluster with the orthologous proteins in fungi from the Agaricomycetes (i.e. *Fomitiporia mediterranea* and *Trametes versicolor*) and Basidiomycetes (i.e. *Coniophora puteana* and *Stereum hirsutum*). These proteins also show clear divergence from those from members of the Ascomycota (i.e. *Arthroderma gypseum*, *Pyrenophora tritici-repentis*, *Schizosaccharomyces japonicus*, *Schizosaccharomyces pombe*, and *Verticillium dahliae*). This suggests that ancestors of cryptococcal Ure4 and Ure6 diverged from the ancestor of the corresponding proteins in the other fungal species analyzed. The Ure6 orthologs from plants seem to be the most distant from the clade that contains *C. gattii* proteins, whereas the orthologs from *Wallemia sebi* (Wallemiomycetes) are the closest to the referred clade. The topology is somewhat different in the Ure4 tree, as proteins from photosynthesizing organisms group with the Basidiomycota clade. It is noteworthy that Ure4 is one of the less-conserved accessory proteins, and less information is available for it [29].

In eukaryotes, the Ure7 accessory proteins also bind metals, possessing a dual activity that combines properties of the metallochaperone UreE in bacteria [30–32]. Ure7 was shown to be an intrinsically disordered protein [33], with structural fluctuations that respond to the cellular environment [29–35]. These facts led us to exclude Ure7 from our mutant analysis.

Construction of *C. gattii* mutant strains

We deleted the *URE* genes by employing inactivation cassettes harboring a hygromycin resistance construct. To evaluate the urease proficiency of the hygromycin-resistant yeast colonies that arose from transformation with *URE* inactivation cassettes, tests in urea media (YPD agar plates supplemented with urea, Ni^{2+}, and phenol red pH indicator) were conducted [36]. The wild-type (WT) *C. gattii* strain was used as a urease-positive control. After 24 or 48 h of incubation at 30 °C, colonies were selected on the basis of the absence of a reddish color, as this indicated deletion mutants lacking ureolytic activity. Urease-positive colonies were visible as a pink/magenta color, observed in WT and in com-

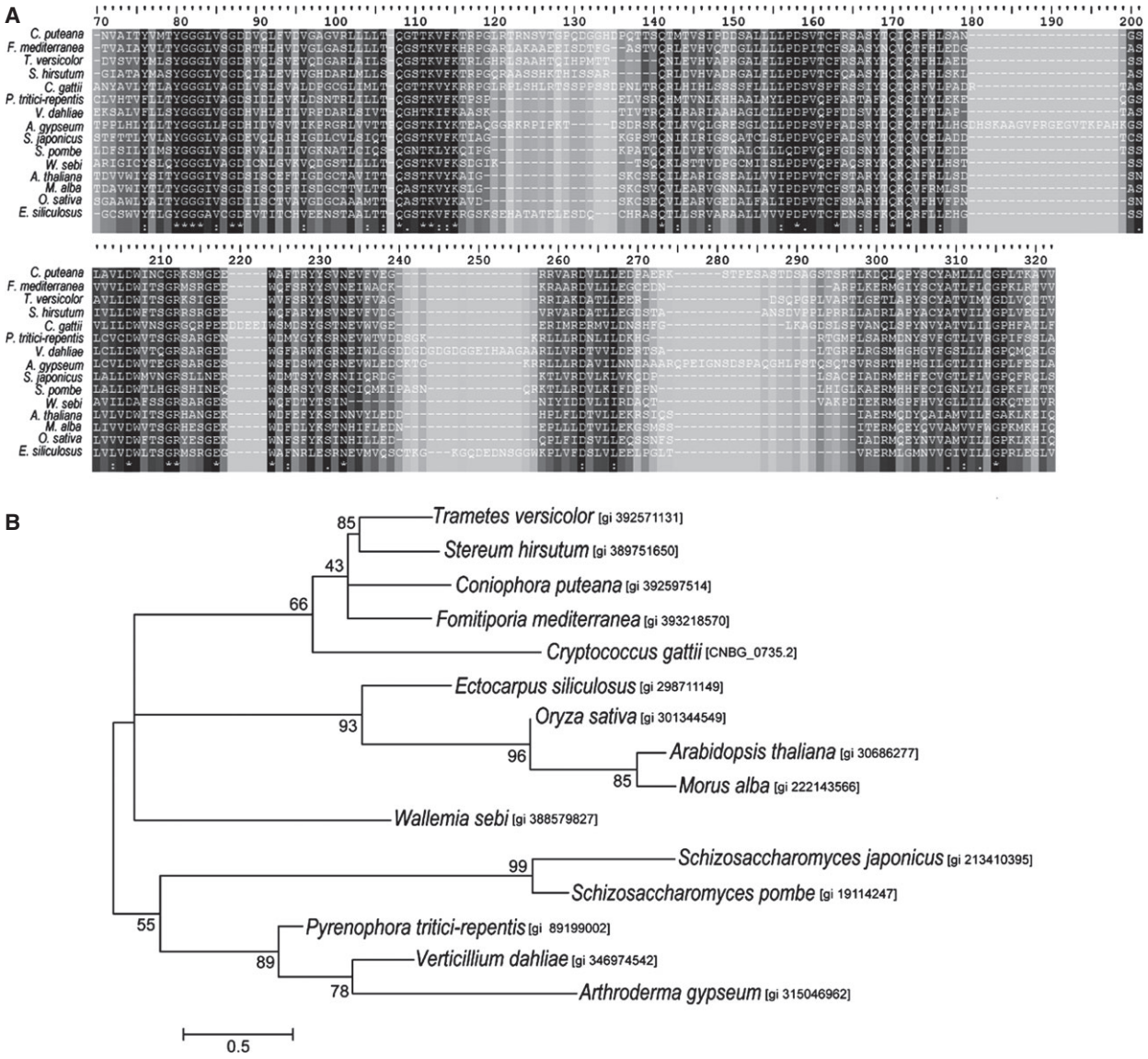

Fig. 1. Phylogeny of Ure4. (A) Multiple sequence alignment of the Ure4 characteristic domain in different proteins, as detected by Pfam. (B) Molecular phylogenetic analysis of Ure4 orthologs with the maximum likelihood method. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution and some evolutionarily invariable sites (WAG + G + I). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

plemented mutant *URE* strains (Fig. 3A). The ureolytic activities of the mutants and complemented clones were also determined in yeast cell extracts with the rapid urea test [37]. According to the results shown in Fig. 3B, all reconstituted mutants showed levels of ureolytic activity that were nearly equivalent to or even higher than those observed in WT *C. gattii*, whereas this activity could not be detected in mutant strains. The integration of the inactivation and complementation cassettes was confirmed (Fig. 4).

Virulence factors in *C. gattii URE* mutant strains

We evaluated the phagocytic activity of macrophages towards the *C. gattii* mutant strains, and the ability of the WT, mutant and complemented strain yeast cells to survive inside macrophages. Figure 2A shows that all yeast strains were equally phagocytosed by macrophages after 1 h of exposure, and survived within the phagocytes for at least 18 h. However, the *ure1*Δ mutant did not multiply within macro-

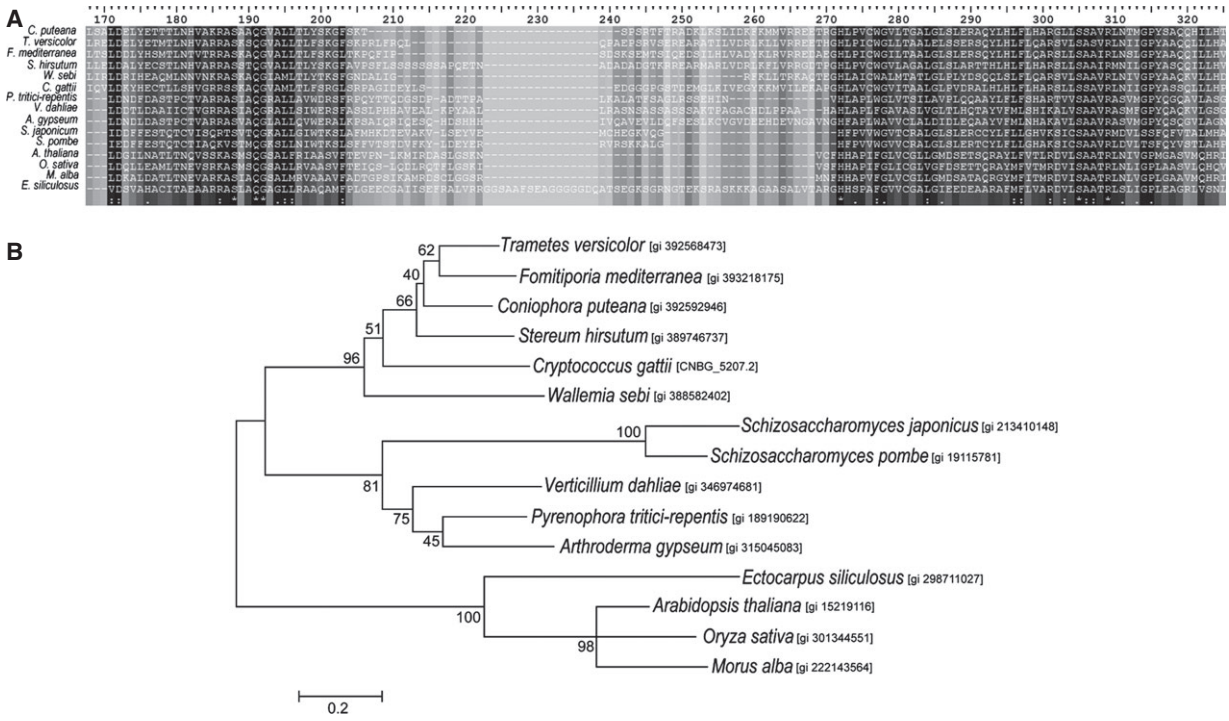

Fig. 2. Phylogeny of Ure6. (A) Multiple sequence alignment of the Ure6 characteristic domain in different proteins, as detected by Pfam. (B) Molecular phylogenetic analysis of Ure6 orthologs with the maximum likelihood method. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution (WAG + G). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

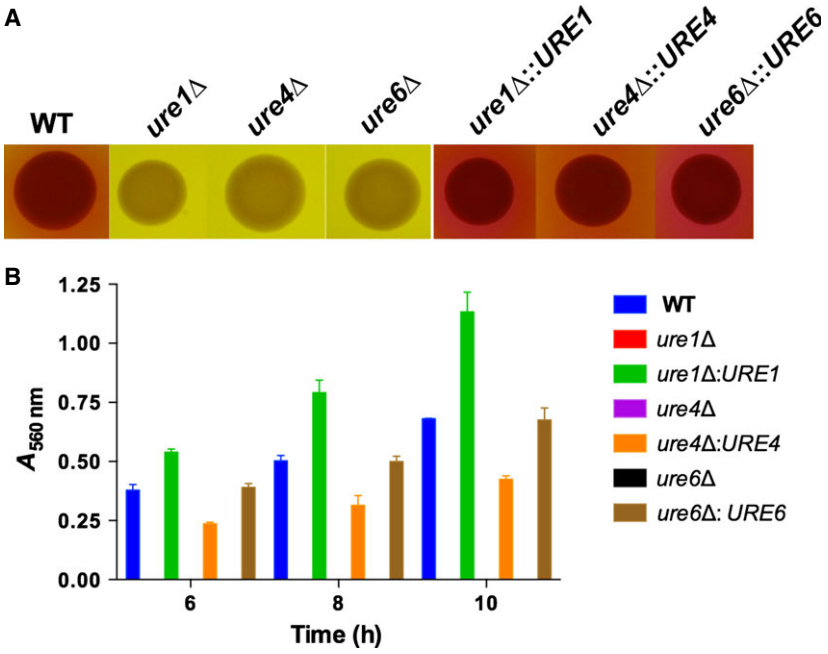

Fig. 3. Inactivation of *URE* genes abolishes *C. gattii* urease activity. (A) Urease activity-screening test on YPD agar with the WT, mutant and complemented strains. (B) Ureolytic activity in transformed *C. gattii* strains. Urease activity was measured in the extracts of cells that were incubated for up to 10 h in urea buffer. Data shown are means ± standard deviations of triplicates with endpoint readings at 6, 8, and 10 h.

phages (Fig. 5A). This contrasts with the WT *C. gattii* cells, which nearly quadrupled in number after 18 h inside the phagocytes. The ability to survive phagocytosis and multiply within macrophages was partially restored in the reconstituted mutants (Fig. 5B).

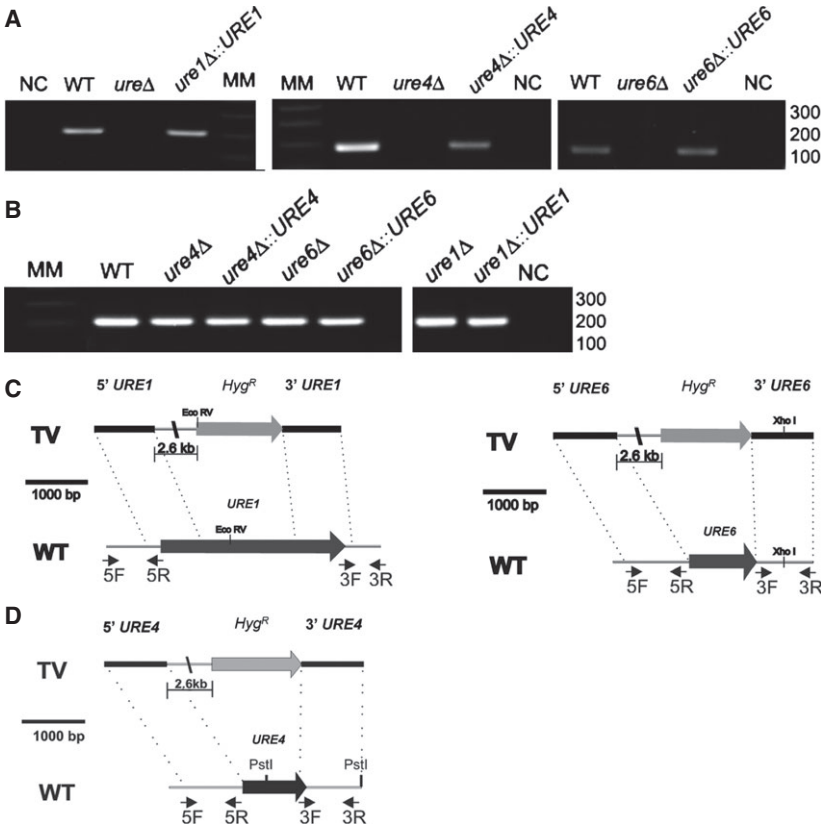

Fig. 4. Inactivation and complementation of *C. gattii URE* genes. (A) RT-PCR was performed to evaluate the expression of *URE1*, *URE4* and *URE6* in the respective mutants and complemented strains with *URE*-specific primers. (B) As a loading control, the expression of *ACT1* was also evaluated. NC, negative control; MM, molecular marker. (C–E) The cassettes for the generation of the null mutants for *URE1* (C), *URE4* (D) and *URE6* (E) are also illustrated. Target genes were replaced with hygromycin-resistant marker cassettes, as indicated by the light gray box (Hyg^R). The target gene 5′ and 3′ flanks were fused with the hygromycin cassette according to the Delsgate methodology. The WT locus of each gene and the primer positions for gene disruption are indicated. The constructed target vector (TV) was used for the *C. gattii* transformations. The restriction enzymes are also indicated for each TV.

Our data thus show that mutants lacking the urease protein (*ure1*Δ) or expressing enzymatically inactive apoureases (*ure4*Δ and *ure6*Δ) did not multiply within macrophages. Similarly, treatment of WT cells with acetohydroxamic acid, an Ni^{2+} chelant inhibitor of urease activity [38], did not affect phagocytosis by macrophages, but blocked the ability of the yeast to multiply within the cell in a dose-dependent fashion (Fig. 5C). Taken together, these data indicate a relevant role of the ureolytic activity in survival and/or multiplication of yeasts within the macrophages. Previously, it was proposed that urease-mediated alkalinization and phagosomal cathepsin inhibition, which are promoted by urease-producing microorganisms, are important factors allowing the survival and multiplication of such microorganisms within phagosomes [39].

Experimental infections in mice via intranasal inoculation were performed to follow the virulence of the WT, mutant and complemented strains. Fungal burdens were analyzed in lungs and blood with intranasal inoculation of 1×10^7 yeast cells of the WT, *ure1*Δ and *ure*6Δ strains, or the corresponding reconstituted strains, to simulate an acute infection. Mice were killed at an early stage of infection (day 7), as well as at a late stage (day 18). The mice had low yeast burdens in lungs after 1 week, regardless of the yeast strain (data not shown), but significant differences in lungs (Fig. 6A) and blood (Fig. 6B) burdens appeared at day 18 postinfection in these conditions. Another experimental infection was then performed to analyze the fungal burdens with a lower inoculum (5×10^4), and the fungal burden was analyzed at different stages of the infection process (days 17 and 24). This experiment confirmed that significant differences could be observed when *ure1*Δ mutant-infected mice were compared with the WT strain; specifically, lower lung or nearly absent blood yeast burdens were observed in the *ure1*Δ mutant-infected mice (Fig. 6C,D). This difference persisted until day 24 postinfection. On the other hand, the inactive apo-urease-producing mutants behaved similarly to the WT strain, at least in lungs and in the late stages of infection (Fig. 6C), suggesting that a ureolysis-independent contribution of *C. gattii* urease to pulmonary cryptococcosis could occur in specific stages of the infection. The same scenario could not be observed for fungal burdens in blood, where *URE4* and *URE6* are important for cryptococcal survival (Fig. 6D). The lower extent of spread of *ure1*Δ mutants from the lung (Fig. 6C) to the blood at

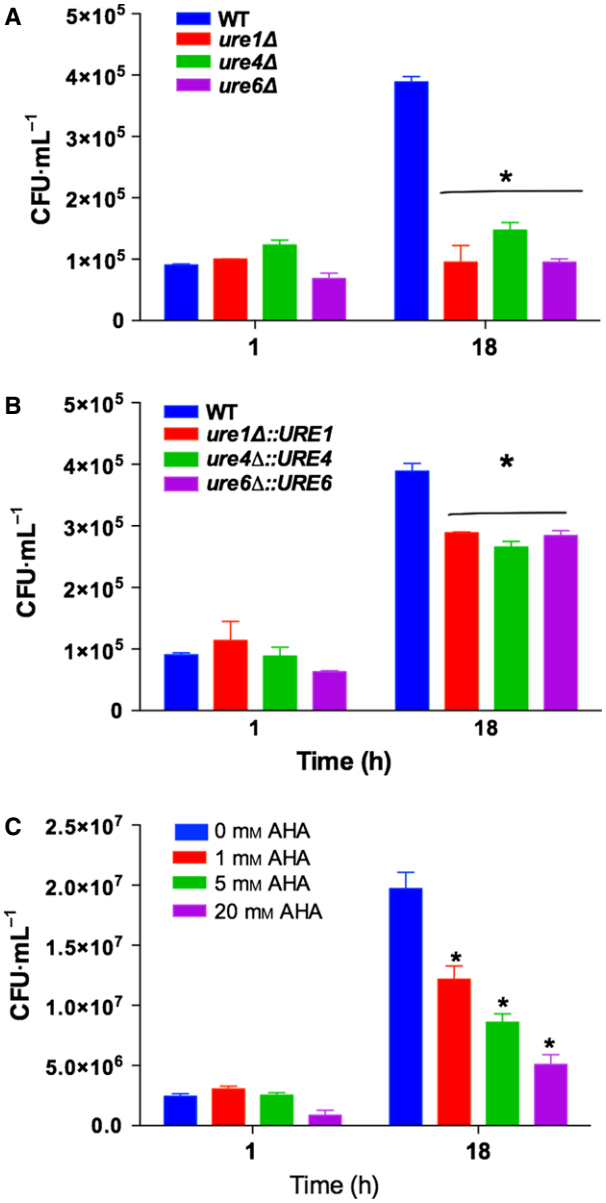

Fig. 5. Role of urease in cryptococcal survival and multiplication in macrophages. Macrophage-like RAW264.7 cells were incubated with *C. gattii* cells for 1 h (37 °C, 5% CO_2) to allow phagocytosis. The medium was then removed and replaced with DMEM/FBS. After a second incubation for 18 h (37 °C, 5% CO_2), yeast cells not associated with macrophages were removed with PBS washes. Fungal survival was evaluated after macrophage lysis with sterile ice-cold Milli-Q water and subsequent plating of the lysates on YPD agar plates for CFU determination. (A) Results obtained with deleted mutants, as compared with the WT strain. (B) Results obtained with reconstituted strains, as compared with the WT strain. (C) Results obtained with the WT strain pretreated with different concentrations of AHA, a urease inhibitor. The error bars represent standard deviation from technical triplicates of two independent biological replicates. The statistical analyses were conducted with the *t*-test. $*P < 0.05$ in relation to the WT or control.

this stage of infection and the impaired ability of these mutants to survive and multiply within phagocytes suggest that urease acts as a virulence factor immediately after lung infection. The *ure4*Δ and *ure6*Δ mutants appear to be similar to the WT strain in the lung at late stages of the infection process (24 days), indicating that, at this stage, ureolytic-independent activity of urease is important for virulence. On the other hand, such mutants showed CFUs in the blood that were very similar to those of the *ure1*Δ mutant, indicating that, in this case, urease activity is important. Mice from all strains showed low or no burdens in the kidneys, spleen and brain at all dilutions and and in all experiments (data not shown). A previous study using a lower inoculum by the intravenous route showed a low burden of *C. gattii* R265 in the blood, but detectable burdens in other organs (spleen, brain, and kidneys) [25]. Comparing our study with the results of Ngamskulrungroj *et al.* [25], we suggest that different mechanisms of infection or progression of the infection could be related to distinct inoculation routes of the pathogen. This corroborates with the statement by the same group implying that the paucity of meningoencephalitis upon inhalation of *C. gattii* may therefore be partly attributable to an unknown factor(s) in the host's blood coupled with immune protection that reduces dissemination to the brain and fosters lung infection.

Comparisons of *C. neoformans* (H99) and *C. gattii* (R265) infection patterns in mice revealed distinct primary organ targets [25]. Mice infected with H99 via the pulmonary route were killed by brain infection, whereas those infected with R265 were killed by lung infection. *C. gattii* cells have been reported to produce smaller capsules and to cross the blood–brain barrier more efficiently than *C. neoformans* [40] cells. Although *C. gattii* can cross the blood–brain barrier, the host immune system interferes more competently in infections caused by this yeast, and death is therefore frequently subsequent to severe lung infections before fatal meningoencephalitis can occur. A preliminary experiment with a high inoculum (10^7 cells) showed no significant differences in mortality among all mutant strains and WT yeast, with a mean time to death of 13 days (data not shown). In the present study, this infection model, which has been used in other studies with *C. neoformans* virulence factors [5,41,42], proved inconclusive in discriminating differences among the *C. gattii* strains with regard to the survival time after infection. We believe that this high inoculum favored an acute and severe lung infection that killed the animals before any yeast spread to other organs.

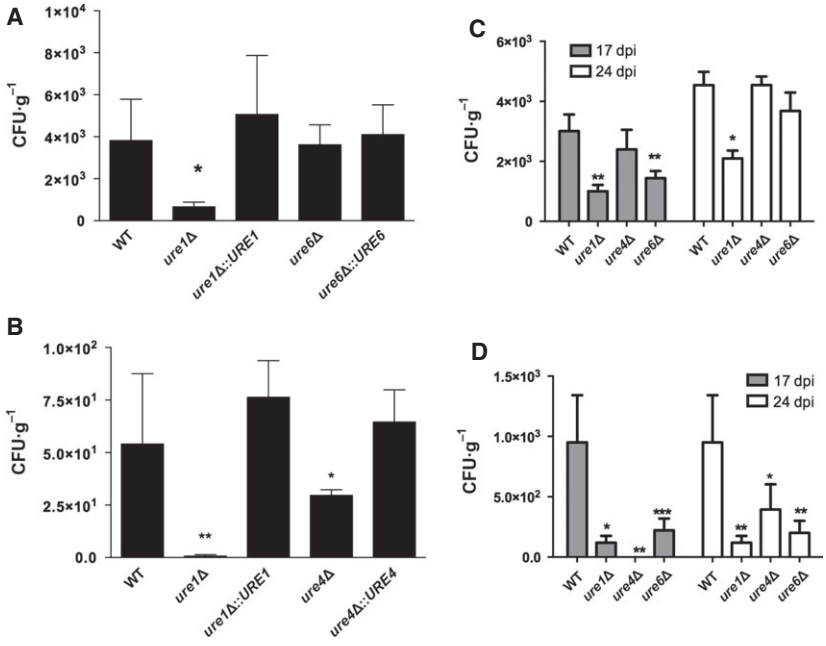

Fig. 6. Urease activity alters organ fungal burden in murine models of cryptococcosis. The fungal burden was assessed in lungs (A, C) and blood (B, D) of mice infected with WT, mutant and complemented *C. gattii* strains. BALB/c mice were inoculated via intranasal instillation of 1×10^7 (A, B) or 5×10^4 (C, D) *C. gattii* cells, and the CFUs were determined from organs collected from infected mice at 18 days postinfection (dpi) (A, B) or 17 and 24 dpi (C, D). The statistical analyses were conducted with the *t*-test. For samples that showed differences from the WT or control: $*P < 0.05$ or $**P < 0.01$.

Figure 7A shows that mice infected with the *ure1*Δ and *ure6*Δ mutants survived for longer periods (median survival times of 34 days and 29.5 days, respectively) than those infected with the WT strain (median survival of 20 days). The median survival times of mice infected with the complemented strains were not significantly different from that of mice infected with the WT strain (Fig. 7B). The mean survival time of mice infected with the *ure*4Δ mutant was not significantly different from that of mice infected with WT *C. gattii*. We also evaluated the pivotal cryptococcal virulence factors in the WT and mutant strains. No differences in the capsule size or growth at 37 °C could be found between the mutant strains and the WT strain (data not shown).

The results from the survival assay clearly indicated a role for urease as a virulence factor in *C. gattii*-induced cryptococcosis, in agreement with previous suggestions regarding *C. neoformans* infections [19,24]. The results showed that the *ure1*Δ and *ure6*Δ mutants present similar survival rates, higher than in the case of WT strains. However, our data suggested that cryptococcal urease-mediated ureolysis is not the only contributor to its role as a virulence factor. Although this activity is apparently necessary for initial lung colonization, the urease-mediated ureolysis is not fully necessary in the late stages of infection, as the enzymatically inactive apo-ureases present in the *ure4*Δ and *ure6*Δ mutants led to cryptococcal virulence comparable to that of the WT strain. However, this could not be observed for blood fungal burden. This suggests that Ure1 may be developing some urease-independent functions at specific stages of infection.

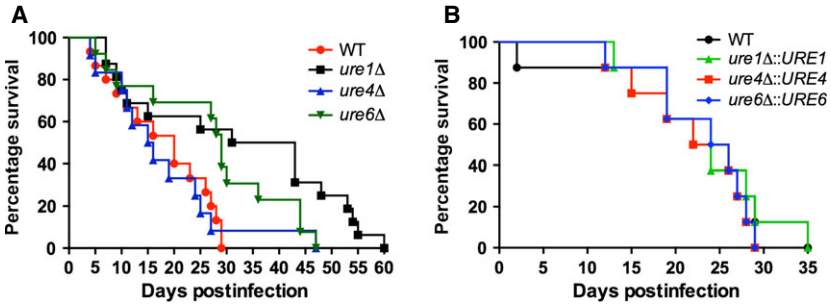

Fig. 7. Some *URE* genes are necessary for normal cryptococcal virulence. Survival analysis of the WT, null mutants or complemented mutants in a murine model. (A) Virulence assays of the WT, *ure1*Δ, *ure4*Δ and *ure6*Δ strains in an intranasal inhalation infection BALB/c mouse model. (B) Virulence assay performed with complemented strains.

Previous studies have demonstrated the importance of *C. neoformans* urease in CNS pathogenesis [20]. The use of artificial routes (injection versus inhalation) to deliver the yeast strains probably overrides most of the steps of the infection process that we have shown herein to involve ureolysis-independent cryptococcal urease activities. Unfortunately, several studies [20,22,24] did not show the survival rates of injected mice or assays to determine the interactions between mutated yeast strains and macrophages, which would have allowed a better comparison with our results.

Conclusions

Here, by using urease-deleted and urease-inactive yeast mutants and a simulated natural infection (inhalation) process in immunocompetent animals, we were able to distinguish the ureolysis-independent and ureolysis-dependent mechanisms by which urease might contribute to cryptococcosis. This study has clarified some matters regarding the contribution of urease to yeast invasion of the lung. Our data also highlight the importance of overall cryptococcal urease inhibition, rather than of its enzymatic activity alone, for the future development of therapeutic tools targeting cryptococcosis, either from *C. neoformans* or from *C. gattii* infections.

Experimental procedures

Ethics statement

All animal studies were reviewed and approved by the Ethics Committee for the Use of Animals of the Federal University of Rio Grande do Sul, concerning the housing and care of laboratory animals (CEUA – protocol number 17535). Mice were housed in groups of eight, and kept in filtered top-ventilated cages with constant temperature and humidity, and food and water *ad libitum*, according to the guidelines of the Brazilian National Council for Animal Experimentation (CONCEA) and the Brazilian College of Animal Experimentation (COBEA). Experiments were carried out in a Level 2 biosafety laboratory according to the rules of the National Technical Committee on Biosafety (CTNBio). The safety procedures that were applied to the experimental procedures as recommended by this committee are available at http://www.ufrgs.br/cbiot/CS/CS_CBiot01.htm.

Fungal strains, plasmids, and media

The *C. gattii* hypervirulent strain R265, serotype B, mating type α, molecular type VGII, from the cryptococcosis outbreak in Vancouver Island [43], was kindly provided by W. Meyer (Sydney University, Australia). Cells were grown at 30 °C with continuous shaking (200 r.p.m.) in YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v dextrose), or at 30 °C on YPD agar (YPD medium with 1.5% w/v agar). YPD agar plates with either hygromycin (200 $\mu g \cdot mL^{-1}$) or nourseothricin (100 $\mu g \cdot mL^{-1}$) were used to select the deletion and complementation transformants, respectively. The plasmid pJAF15 [43] was the source of the hygromycin resistance cassette, and pAI4 [44] was the source of the nourseothricin resistance cassette. The plasmids were maintained in *Escherichia coli* cells that were grown at 37 °C in Luria–Bertani broth or agar supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin.

Identification of target genes and sequences for *in silico* analysis

The putative *C. gattii URE1* (CNBG_4331.2) was identified from the Broad Institute *C. gattii* R265 genome database, available at http://www.broadinstitute.org. A BLAST search sequence alignment was performed to find the accessory protein-coding genes *URE4*, *URE6*, and *URE7*, with the sequences from the respective *C. neoformans* orthologs as queries, and then compared by the use of CLUSTALX2 [45]. Phylogenetic trees were developed with MEGA5 [46]. The evolutionary history was inferred by use of the maximum likelihood method based on the Whelan and Goldman model with discrete gamma distribution [47]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Disruption and complementation of the *URE* genes

The construction of vectors for transformations of *URE1* and the accessory protein-coding genes *URE4* and *URE6* are summarized in Fig. 4. These genes were individually inactivated in the *C. gattii* genome. Disruption was based on the DelsGate methodology [48]. The 5′ and 3′ target gene flanks were PCR-amplified (Table S1) and purified from agarose gels (Illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Uppsala, Sweden). The cloning reaction was performed with the BP clonase (Life Technologies, Carlsbad, CA, USA) and the 5′ and 3′ target gene flanks, as well the pDONRHYG vector [42,49,50]. The product of this reaction was transformed into the *E. coli* OmniMAX 2-T1 strain. After confirmation of the correct deletion construct, the plasmid was linearized with I-*Sce*I prior to *C. gattii* biolistic transformation [51]. The *C. gattii* mutants were subjected to a previously adapted screening test in YPD agar plates supplemented with urea (300 mM), $NiSO_4$ (1 μM), and phenol red as a pH indicator [36]. The urease activity would be expected to convert urea into ammonia, resulting in an increase in the medium pH

and causing a color change from yellow to bright pink. Urease activity negative-colonies (no color change) were further screened by colony PCR, and deletions were confirmed by Southern blotting and semiquantitative RT-PCR analyses.

For complementation of the *ure1*Δ, *ure4*Δ and *ure6*Δ mutants, fragments of approximately 5, 2.8 and 2.9 kb containing the respective WT genes, respectively, were cloned into the *Sma*I site of pAI4, which has a nourseothricin selection marker, and subjected to *in vivo* recombination, as previously described [52,53], in chemocompetent Omni-MAX 2-T1 *E. coli*, prepared as previously established [54]. The resulting plasmid was used for transformation into the respective mutant strains. The complemented mutants were subjected to a urease-screening test, as described above, to confirm the restored ureolytic activity. The primers used in these plasmid constructions are listed in Table S1.

Southern blotting and RT-PCR analysis

Southern blotting and RT-PCR analysis were used to evaluate the integration of the inactivation cassettes into the WT *URE1* or *URE4* and *URE6* locus. For Southern blotting, genomic DNA (10 μg) from strains was digested with the specific restriction enzyme for each mutant. The 3′ or 5′ flanking region was used as the Southern hybridization probe.

For RNA extraction, mutant and WT strain cultures were grown in YPD medium (18–20 h/30 °C) with shaking. RNA extraction and cDNA preparation were performed as previously described [49]. The Applied Biosystems 7500 real-time PCR system was used for the real-time PCR analysis, and the PCR cycling conditions, melting curves and relative expression determinations were as described previously [49]. The experiments were performed with two biological samples, and each cDNA sample was analyzed in triplicate with each primer pair. Actin cDNA levels were used to normalize each set of PCR experiments. The sequences of the primers used are listed in Table S1.

Phenotypic characterization assays

Urease activity

The rapid urea broth (RUH broth) developed by Roberts [55] and adapted by Kwon-Chung [37] was used to detect ureolytic activity in the WT and mutant yeast strains. *C. gattii* strains grown in solid YPD agar for 24 h at 30 °C were suspended in 2 mL of sterile PBS at pH 7.2. The $D_{600\ nm}$ of the cell suspensions was adjusted to 0.7. The cell suspensions were vortexed, mixed with an equal volume of 2× RUH broth mix, and maintained at 37 °C with shaking for 10 h. After 6, 8, and 10 h, the cultures were centrifuged (5000 ***g*** - 5 min), and $A_{560\ nm}$ values of the supernatants were determined. A sterile PBS blank was used as a control. The assay was performed in triplicate for each time point.

Urease inhibition assays

C. gattii WT cells were grown for 20 h in YPD medium, washed with PBS, and diluted to give an $D_{600\ nm}$ of 1.0 in 1 mL of cell suspension. The suspension were incubated with different concentrations (0.5–20 mM) of the urease inhibitor acetohydroxamic acid (AHA) [56], and incubated for 18 h, with shaking at 7 °C; a parallel control suspension of *C. gattii* with buffer and without AHA was used. After incubation, cells were washed three times with PBS, and macrophage interaction experiments were performed as described previously. Aliquots of all AHA inhibition experiments were plated on YPD agar for cell viability analysis. AHA-treated cells were monitored with the Roberts test for 18 h in parallel with the macrophage interaction assay to certify the absence of urease enzymatic activity throughout the whole assay.

Capsule induction and measurement

The capsule thicknesses and cell diameter ratios of the mutant and WT *C. gattii* strains were measured during a microscopic examination of India ink preparations that were grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA, USA) with 10% FBS (Life Technologies) at 30 °C in 5% CO_2 to stimulate capsule production. AXIOVISION LE microscope software (Carl Zeiss, Jena, Germany) was used to determine the capsule thicknesses of at least 100 cells per strain.

Sensitivity to temperature (37 °C)

To determine sensitivities of the mutant strains to the growth temperature, 5 μL of diluted cultures ($D_{600\ nm}$ values of 1.0, 0.1, and 0.01) from each yeast strain were spotted onto YPD agar plates and incubated at 30 or 37 °C. Yeast growth was estimated visually after 24 and 48 h of growth.

Macrophage infection assay

A phagocytosis assay was adapted from [57] and [49], with slight modifications, to evaluate whether the *C. gattii* WT and mutant strains were internalized by macrophages and whether the internalized yeast cells could multiply within macrophages. Macrophage-like RAW264.7 cells (1×10^5 cells/100 μL of DMEM supplemented with 10% FBS) were placed in 96-well culture plates. After 24 h of incubation at 37 °C in 5% CO_2, the medium was replaced with fresh medium containing 1×10^6 *C. gattii* cells (WT and mutant strains, as well the WT strain inhibited with

different concentrations of AHA) that had been previously incubated for 24 h in YPD and extensively washed in PBS. Macrophages were allowed to interact with *C. gattii* cells for 1 h (37 °C, 5% CO_2), after which the medium was removed and replaced with DMEM/FBS. After a second 18-h incubation (37 °C, 5% CO_2), yeast cells that were not associated with macrophages were removed by PBS washes. To evaluate fungal survival after phagocytosis, the macrophages were lysed with sterile ice-cold water, and the lysates were subsequently plated onto YPD plates for CFU determination, after 48 h of incubation at 30 °C. This assay was performed in triplicate for each strain.

Mouse survival assay and organ fungal burdens

Cryptococcal strains were grown for 24 h in YPD, washed three times with PBS, counted in a Neubauer chamber, and suspended in PBS. BALB/c female mice (5 weeks of age, ~20 g) were anesthetized with 100 $mg \cdot kg^{-1}$ ketamine and 16 $mg \cdot kg^{-1}$ xylazine administered by intraperitoneal injection. Anesthetized mice were suspended on a thread by their incisors, and 50 μL of the yeast suspension (5×10^4 or 2×10^6 cells) was slowly pipetted into the nostrils of each mouse. The mice were kept suspended for an additional 10 min, and then placed in a ventilated cage to recover. The survival rates were determined for groups of eight animals per strain. For the organ fungal burden assay, mice were killed by CO_2 inhalation on different days after inoculation, and their lungs, brains, spleens, kidneys and blood were obtained under aseptic conditions. The organs were weighed, and macerated in 1 mL of sterile PBS; appropriate dilutions of the homogenates were then plated on YPD agar plates for CFU determinations.

Statistical analyses and software

A Kaplan–Meier survival analysis was conducted with GRAPHPAD 5.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA, followed by Bonferroni's test, was used to evaluate the statistical parameters of capsule size, fungal burden, and macrophage interaction.

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Author contributions

V. Feder, L. Kmetzsch, C. C. Staats, C. R. Carlini, and M. H. Vainstein: conceived and designed the experiments. V. Feder, L. Kmetzsch, C. C. Staats, and N. Vidal-Figueiredo: performed the experiments. V. Feder, L. Kmetzsch, C. C. Staats, R. Ligabue-Braun, C. R. Carlini, and M. H. Vainstein: analyzed the data:. V. Feder, L. Kmetzsch, C. C. Staats, R. Ligabue-Braun, C. R. Carlini, and M. H. Vainstein: contributed reagents/materials/analysis tools. V. Feder: wrote the paper. V. Feder, L. Kmetzsch, R. Ligabue-Braun, C. C. Staats, C. R. Carlini, and M. H. Vainstein: reviewed the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. List of primers used during this work.