Transcription Factor STE12α Has Distinct Roles in Morphogenesis, Virulence, and Ecological Fitness of the Primary Pathogenic Yeast Cryptococcus gattii†

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Cryptococcus gattii is a primary pathogenic yeast, increasingly important in public health, but factors responsible for its host predilection and geographical distribution remain largely unknown. We have characterized C. gattii STE12α to probe its role in biology and pathogenesis because this transcription factor has been linked to virulence in many human and plant pathogenic fungi. A full-length STE12α gene was cloned by colony hybridization and sequenced using primer walk and 3’ rapid amplification of cDNA ends strategies, and a ste12Δ gene knockout mutant was created by URA5 insertion at the homologous site. A semiquantitative analysis revealed delayed and poor mating in ste12Δ mutant; this defect was not reversed by exogenous cyclic AMP. C. gattii parent and mutant strains showed robust haploid fruiting. Among putative virulence factors tested, the laccase transcript and enzymatic activity were down regulated in the ste12Δ mutant, with diminished production of melanin. However, capsule, superoxide dismutase, phospholipase, and urease were unaffected. Similarly, Ste12 deficiency did not cause any auxotrophy, assimilation defects, or sensitivity to a large panel of chemicals and antifungals. The ste12Δ mutant was markedly attenuated in virulence in both BALB/c and A/Jcr mice models of meningoencephalitis, and it also exhibited significant in vivo growth reduction and was highly susceptible to in vitro killing by human neutrophils (polymorphonuclear leukocytes). In tests designed to simulate the C. gattii natural habitat, the ste12Δ mutant was poorly pigmented on wood agar prepared from two tree species and showed poor survival and multiplication in wood blocks. Thus, STE12α plays distinct roles in C. gattii morphogenesis, virulence, and ecological fitness.

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Cryptococcus gattii is an encapsulated yeast that causes cryptococcal meningoencephalitis, a serious brain infection both in immunity-impaired and healthy individuals. A majority of cryptococcosis cases are diagnosed among immunocompromised individuals in whom the causal pathogen is Cryptococcus neoformans var. grubii; a much smaller number of infections are caused by C. neoformans var. neoformans. C. gattii and C. neoformans are closely related pathogenic species, but there are distinct differences in their biological, ecological, genetic, and disease-associated attributes (7, 13–16, 18, 26, 49, 81, 82). C. neoformans var. grubii and C. neoformans var. neoformans remain some of the most intensively studied human fungal pathogens, while experimental studies on C. gattii are still in their infancy. C. gattii was until recently considered a tropical and subtropical pathogen with a prominent association with Eucalyptus trees. Earlier reports from Australia indicated that C. gattii infections are less common among human immunodeficiency virus-AIDS patients than C. neoformans infections. However, as the AIDS epidemic spread around the world, reports started to appear on C. gattii cryptococcosis in human immunodeficiency virus-AIDS patients from Thailand, parts of Africa, and South and Central America (4, 37, 42, 71). Similarly, our analysis of a collection of Cryptococcus isolates from AIDS patients in Southern California revealed about 12% C. gattii strains (12). More recently, an ongoing C. gattii outbreak in healthy humans and animals on Vancouver Island (British Columbia, Canada) has caused multiple fatalities; this is the first documented outbreak in North America (43, 83). Thus, C. gattii continues to pose serious public health problems for immunocompromised and healthy individuals worldwide. Concomitant reports of severe and often fatal disease in animals also raise important concerns for the health of pets and wildlife. Along similar lines, a number of recent reports described the natural isolations of C. gattii from multiple tree species other than the Eucalyptus in Canada, Brazil, and India, thereby expanding the known geographical range and ecological niche of this pathogen around the globe (50, 51, 73). Still, the mechanisms behind host predilection and geographical distribution of C. gattii remain largely unknown, in part due to a paucity of systematic investigations.

STE12 was first identified among a group of Saccharomyces cerevisiae sterile mutants defective in sexual conjugation and related processes (30). Subsequent studies have shown that STE12 is a transcription factor downstream of the mitogen-activated protein kinase (MAPK) cascade that controls mating, filamentation, and cell wall integrity (25, 29). The STE12 is activated by two MAPKS, KSS1 and FUS1; has a transcription
partner, TEC1; and has two negative regulators, DIG1 and DIG2 (59). More recently, STE12 was reported to directly regulate expression of over 29 yeast genes that mediate a range of cellular processes, cell cycle, mating projections, cell fusion, polarized growth and budding, stress and/or starvation, and signal transduction (31, 75). The roles of STE12 homologues in various biological processes have thus so far been characterized in few fungi other than S. cerevisiae. In the filamentous model fungus Aspergillus nidulans, the steA mutant is sterile without ascosporogenous tissue and fruiting body, but there are no effects on either the sexual cycle-specific Hülle cells or a number of asexual developmental programs (87). Among the few pathogenic fungi studied, STE12 homologues have been implicated in the pathogenic process itself. In the rice blast pathogen Magnaporthe grisea, MST12 disruption caused a serious loss of virulence, as the mutant failed to infect rice leaves or onion epidermal cells, even through wound sites (68, 69). The STE12 homologue CHP1 from the common human yeast pathogen Candida albicans was found to be only partially involved in hyphal formation, when a mutant strain was tested on solid medium. However, a double mutation in CHP1 and another gene, PHD1, caused complete loss of filamentation as well as attenuation of virulence in mice (56, 58). In contrast, STE12 from the haploid yeast Candida glabrata, which is increasingly implicated in drug-resistant candidiasis, was found necessary for nitrogen starvation-induced filamentation and for a wild-type level of virulence in mice (6). In the opportunistic human pathogen Penicillium marneffei, stlA gene mutation caused no defects in growth, asexual division, or dimorphic switching, while CLS12, the STE12 homologue in Candida (Clavispora) lusitaniae, was required for mating but dispensable for filamentation (5, 94).

In attempts to understand hyphal formation, a C. neoformans var. neoformans STE12a homolog of the S. cerevisiae STE12 gene was identified. Its overexpression caused hyphal projections and induction of the MFA1 pheromone gene, important for mating reaction, and CnLAC1, the gene that encodes the important virulence factor laccase; the possibility is therefore raised that STE12a provides a bridge between C. neoformans mating type and virulence via melanin production (90). Subsequent study of C. neoformans var. grubii by gene knockout revealed that STE12a was essential for haploid fruiting but was not essential for mating and virulence (95). The results of an STE12a overexpression study by Chang and co-workers were subsequently verified by gene knockout in C. neoformans var. neoformans to show that STE12a was essential for haploid fruiting, melanin production, and virulence but was dispensable for mating (11). A second homolog of C. neoformans var. neoformans STE12 was subsequently identified as MATa specific; it was essential for mating and virulence (10). Interestingly, C. neoformans var. grubii STE12a was later shown to be involved in reversal of hypervirulence of a crg1 mutant, which encodes the regulator of G protein signaling (89). Taken together, these findings suggested that STE12 is co-opted to perform distinct regulatory control functions between closely related C. neoformans varieties. Therefore, we surmised that C. gattii STE12α is a potentially valuable target in studies aimed at unraveling the genetic basis for functional divergence among pathogenic Cryptococcus species.
(0.03 or 0.06 μg/ml), miconazole (0.03 or 0.06 μg/ml), terbinafine (1.0 or 5.0 μg/ml), voriconazole (0.015 or 0.03 μg/ml), and griseofulvin (2.0, 4.0, 8.0, or 16.0 μg/ml), followed by incubation at 30°C for 7 days.

Melanin production and laccase expression and regulation. Melanin production by the WT, ste12Δ mutant, and ste12Δ plus STE12α strains was assayed on Niger seed agar (46). Five microliters of cell suspension (10^7 cells/ml) from each strain was spotted on the agar surface, and cultures were incubated at 30°C for 3 to 6 days. Copper or cyclic AMP (cAMP)-mediated reversal of melanin pigmentation was tested on Niger seed agar supplemented with either 10 μM to 200 μM CuSO4 (97) or 2 mM to 50 mM cAMP (3). The laccase activity in the test strains was determined by a previously published method (95). In brief, equal numbers of glucose-starved cells of each strain were used to determine the oxidation of the diphenolic substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (IU of activity = 0.01 A420 absorbance unit in 30 min). The transcription of LAC1 was estimated with semiquantitative RT-PCR using the primer pair V1380 and V1381 (78).

Mating and haploid fruiting. The WT, ste12Δ, and ste12Δ plus STE12α strains were mixed with the compatible mating strain C. gattii NIH198 (MATα, serotype B) on V8 juice agar (pH 7.0) and were incubated at room temperature in the dark for up to 14 days (45, 48). Plates were periodically checked for the appearance of hyphal pinheads at the edge of the fungal growth. These edges were examined under the light microscope for the presence of characteristic basidia and basidiospores. Fifty nonoverlapping areas were selected to estimate the number of glucose-starved cells of each strain were used to determine the oxidation of the diphenolic substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (IU of activity = 0.01 A420 absorbance unit in 30 min). The transcription of LAC1 was estimated with semiquantitative RT-PCR using the primer pair V1380 and V1381 (78).

Virulence assays. The pathogenic potentials of the WT, ste12Δ, and ste12Δ plus STE12α strains were compared in mice models of meningococcal sepsis (16, 61). BALB/c or A/Jc mice (male, 6 to 8 weeks) were procured from Charles River Laboratories, Inc. All procedures for safe and pain-free handling of animals were followed, per the recommendations of the Institutional Animal Care and Use Committee. The log-phase cultures of test strains were resuspended in sterile phosphate-buffered saline, pH 7.4, at a concentration of 1 × 10^8 cells/ml. Groups of five or six mice were injected intravenously (i.v.) with 10^8 cells of each strain. The animals were given food and water ad libitum and were observed twice daily for any sign of distress. Mice that appeared moribund or in pain were sacrificed using CO2 inhalation and cervical dislocation. Survival data from the mouse experiments were analyzed by Kaplan-Meier survival curves using the SAS system (SAS Institute, Inc.).

Histological lesions were examined in groups of three mice infected with 1 × 10^6 cells (16). The mice were sacrificed on day 7 postinfection, and the whole brains were removed and fixed in Bouin’s fixative. After 24 h of fixation, tissues were dehydrating alcohols and xylenes into paraffin, for 15 min per station. Tissues were then embedded in paraffin blocks and sectioned at a 7-μm thickness. Sections were stained with hematoxylin and eosin and mucicarmine (Richard Allen Scientific).

To determine the in vivo capsule size of the test strains, 2 mice per strain were infected with 10^3 cells i.v. At 3 days postinfection, mice were sacrificed using CO2 inhalation, and the brain tissues were smeared on a glass slide, digested with KOH, and examined under a light microscope using Nomarski optics (Olympus).

Neutrophil (PMN) fungicidal activity. Polymorphonuclear leukocytes (PMNs) were isolated from the peripheral blood of human volunteers by Ficoll-Paque (Pharmacia LKB Biotechnology) centrifugation, as described previously (47). One-hundred-microliter aliquots of PMN, 100 μl of viable yeasts (5 × 10^4), and 1 μl of pooled human sera were added to the wells of a 96-well microculture plate and incubated at 37°C in 5% CO2-95% air. After incubation for 4 h, the plates were centrifuged at 2,000 rpm for 10 min, and the supernatants were carefully aspirated through 27-gauge needles. The PMN were lysed by the addition of 100 μl of 0.05% Triton X-100, and the yeasts were serially diluted and plated on YPD agar. The YPD agar plates were incubated at 30°C for 2 to 4 days for quantitation of CFU. The results were expressed as the percentage of C. gattii killed [1 − C (CFU of experiment × CFU of inoculum) × 100]. Values of zero indicated no killing. The study involving human subjects was performed under the guidance of a protocol approved by the Institutional Review Board.

Assessment of pigmentation and growth on wood-based media. The pigmentation and growth of the test strains were assessed on both wood chip and wood extract agar media. Black cherry (Prunus serotina Ehrh) chips were received courtesy of Roger Dziengaelski, Finch, Pruyon & Co., Inc., Glen Falls, NY. Chips were also prepared from Juniperus virginiana (red cedar), Tsuga canadensis (eastern hemlock), Populus tremuloides (trembling aspen), and Acer saccharum (sugar maple) collected from a local forest. These materials were not pretreated with any physical or chemical processes.

The wood agar was prepared by autoclaving 2.0 g of chips at 110°C for 30 min, followed by mixing with 100 ml of autoclaved 2% agar solution. Twenty-five-milliliter aliquots were dispensed in sterile petri plates and were designated wood agar.

Wood extract agar was prepared by mixing of 5.0 g chips in 250 ml of water. This mixture was stirred with magnetic stirrer for 3 h in the cold room, followed by centrifugation at 13,000 rpm for 30 min at 4°C. It was filter sterilized through a 0.22-mm membrane, mixed with 250 ml of autoclaved water containing 4% agar, and dispersed in 25-ml aliquots into sterile petri dishes.

FIG. 1. Maximum parsimony phylogram of STE12. The alignment of the predicted Ste12p from various fungi was performed using MacVector 7.1.1 (Accelrys) ClustalW multiple-sequence alignment, with the open gap penalty set at 15.0 and the extend gap penalty set at 0.3. The tree was arbitrarily rooted using Mucor racemosus Ste12p as an out-group. Organism sources and NCBI database accession numbers for sequences are as follows: C. gatti NIH444, AJY168185; C. gatti WM276, AY110430; C. gatti E566, AY10492; C. neoformans var. neoformans JEC21, AAN75715; C. neoformans var. neoformans JEC20 STE12a, AF545253; C. neoformans var. grubii H99, AAD44111; C. neoformans var. grubii 125.91 STE12a, AF542528; Candida glabrata, AJ51385; Emericella nidulans, AAC31206; Klyveromyces lactis, Q05400; Mucor racemosus, AJ400724; Neurospora crassa, AAK14814; S. cerevisiae, M2542; Candida albicans, A54767; Clavispora lusitaniae, AAD51741; Colletotrichum lagenarium, AB090340; Gibberella zeae, AAM34282; Magnaporthe grisea, AF432913; Penicillium marneffei, AK21854.
WT (α) X NIH198 (a) - 5 days

ste12αΔ (α) X NIH198 (a) - 5 days

ste12αΔ (α) X NIH198 (a) - 11 days

ste12αΔ+STE12α (α) X NIH198 (a) - 5 days
Both wood agar and wood chip agar plates were inoculated with a 5-μl suspension of yeast cell suspensions (10^8 cells/ml), were incubated for 10 days at 30°C, and were observed periodically for growth and pigmentation.

**Wood section microscopy.** Black cherry wood blocks (~1-cm cubes) were prepared from freshly pruned branches. The blocks were autoclaved at 110°C for 30 min. Sterile blocks were set on 2% sterile water agar in petri plates so that the vessel elements were vertically oriented. The blocks were inoculated with 5 μl of cell suspension (optical density at 600 nm [OD600] = 1.0), incubated at 30°C, and observed for up to 8 weeks. Adequate moisture was maintained throughout the incubation period. Finally, the blocks with fungal growth were removed, fixed in formalin for 24 h, and embedded in paraffin, and tangential sections (5 to 7 μm) were cut on a rotary microtome. Sections were deparaffinized, covered with glass coverslips, and examined without any staining under the light microscope.

**Nucleotide sequence accession number.** The complete sequence of STE12α was deposited in GenBank under accession number AY168185.

## RESULTS

**STE12α gene characterization.** The cloned C. gattii STE12α gene was a single copy with approximately 85% nucleotide identity to that of STE12α from C. neoformans var. grubii and C. neoformans var. neoformans (GenBank accession no. AAD 44111 and AAN 75715, respectively). The phylogenetic analysis of protein alignment of the C. gattii STE12α sequence with that of STE12α from C. neoformans var. neoformans, C. neoformans var. grubii, and 11 other fungal species revealed that this protein is segregated into distinct clades of ascomycetous yeasts, molds, and basidiomycetous yeasts, with good bootstrap support. Among basidiomycetes, C. neoformans var. grubii and C. neoformans var. neoformans formed one sister clade distinct from C. gattii. Interestingly, this clustering pattern was not evident for STE12α homologs (Fig. 1). Detailed characterization of the C. gattii STE12α protein revealed STE (amino acids 91 to 203) and C2H2-Zn2+ motifs (amino acids 543 to 565) (see Fig. S2 in the supplemental material). The STE motif is common in STE12 homologs reported from all fungi, while two C2H2-Zn2+ motifs have been reported only from STE12 homologs of ascomycetes (6, 30, 53, 66, 86). In silico analyses with deduced amino acid sequences using the PROSITE program (79) found a number of putative phosphorylation sites, including protein kinase C phosphorylation (11 sites), casein kinase II (19 sites), and cAMP- and cGMP-dependent protein kinase (3 sites). Transcriptional activation of STE12 by phosphorylation has been extensively studied in S. cerevisiae (64, 80, 93). The presence of phosphorylation sites has also been reported by bioinformatic analyses of STE12 of Magnaporthe grisea and Colletotrichum lagenarium (68, 69, 86).

**STE12α is required for efficient mating but not for haploid fruiting.** Mating among compatible strains is an important attribute of C. neoformans strains, with potential relevance in epidemiology and virulence (32, 33, 45, 47, 48). Although a good mating reaction between NIH444 (MATα) and NIH198 (MATa) is generally seen in 4 to 5 days, we observed all mating tests for up to 14 days, to include any poor or delayed reaction on V8 juice agar. Both WT and ste12αΔ plus STE12α strains showed mycelial elements at the edge of the colonies as early as 5 days postincubation (Fig. 2A, G). Under the SEM, these elements revealed well-formed basidia with chains of basidiospores (Fig. 2B, H). In contrast, mating reactions of ste12αΔ did not show any mycelial formation at the edges until 11 days postincubation under light microscopy (Fig. 2C). However, analysis under SEM revealed isolated formation of hyphae with basidia and basidiospores (Fig. 2D), which were clearly visible at 11 days postincubation (Fig. 2E and F). Semiquantitative analysis using random examination of edges revealed 2 of 50 edges with positive mating reactions in ste12αΔ strains compared to 42 to 45 of 50 edges positive for mating reactions in WT and ste12αΔ plus STE12α strains. C. neoformans var. grubii mating is regulated by both G-protein alpha subunit Gpa1-cAMP and Gpb1-MAPK (39). However, the addition of cAMP to V8 juice agar did not alter the mating reaction of the ste12αΔ mutant (Fig. 3). This indicated that either STE12α is required for efficient mating of C. gattii through the pathway downstream of cAMP and/or independent of cAMP.

C. gattii STE12 was not involved in the haploid fruiting, as all the strains were positive in the morphological assay (Fig. 4). The appearances of rough edges with aerial hyphae were visible in light microscopy of three strains on filament agar. Further examination of these structures with SEM showed hyphae, basidia, and basidiospores. This observation is in contrast to the reported essential role of STE12α in haploid fruiting of C. neoformans var. grubii and C. neoformans var. neoformans (10, 83).

**STE12α is not required for several cellular functions.** Capsule formation is an important C. neoformans virulence attribute, and the ste12αΔ mutants of C. neoformans var. grubii and C. neoformans var. neoformans revealed smaller capsules than did the corresponding WT strains (9, 11, 95). Interestingly, the capsule size examined by microscopic mounts of India ink preparations of the C. gattii ste12αΔ mutant was identical to that of WT and ste12αΔ plus STE12α strains when grown in YPD broth at 30°C or at 37°C or in capsule induction medium (LIM) at 30°C, indicating that C. gattii STE12α is dispensable for capsule growth. Similarly, C. gattii ste12αΔ did not exhibit any defect in the expression of other virulence factors, including urease, phospholipase, and Cu,Zn SOD (data not shown). In contrast, the C. neoformans var. neoforms ste12αΔ showed impairment of phospholipase and Cu,Zn SOD enzyme activities (11, 22, 23). To unmask any unexpected phenotype, we used the API 20C AUX, API ZYM, and YT MicroPlate systems to compare assimilation and enzymatic activities of the C. gattii WT, ste12αΔ, and ste12αΔ plus STE12α strains, but no differences were observed (data not shown). Similarly, the C. gattii ste12αΔ mutant did not show any defect in growth in the presence of chemicals used as C. neoformans cell wall or cell membrane inhibitors (44). Altogether, the ste12αΔ mutant did not show any defect in growth in the presence of chemicals used as C. neoformans cell wall or cell membrane inhibitors (44). Altogether, the ste12αΔ mutant did not show any defect in growth in the presence of chemicals used as C. neoformans cell wall or cell membrane inhibitors (44).
FIG. 3. Role of STE12 in *C. gattii* mating is independent of cAMP pathway. The light microscopic (magnification, ×100) analyses of mating reactions of the WT, *ste12αΔ*, and *ste12αΔ* plus *STE12α* strains crossed with the MATa (NIH198) with or without cAMP are shown after 5 days of incubation on V8 juice agar. cAMP did not reverse the poor and delayed mating observed in the *ste12αΔ* strain, while a higher concentration inhibited mating in the WT and *ste12αΔ* plus *STE12α* strains.
together, these results indicated that *C. gattii* STE12 did not regulate several of the putative virulence factors assayed in this fungal pathogen.

**STE12α is required for melanin pigmentation and laccase expression.** Melanization via *C. neoformans* laccase is an important virulence attribute, and it is down regulated in *C. neoformans* var. *neoformans* ste12αΔ mutants (10, 11, 76, 92). Interestingly, the *C. gattii* ste12αΔ mutant was also defective in melanization and showed weak yellowish pigmentation compared to the dark brown pigmentation formed by WT and ste12αΔ plus STE12α strains on Niger seed agar at 3 days postincubation (Fig. 5A). Supplementation of Niger seed agar with CuSO₄, a metal important in laccase induction, did not restore melanin pigmentation in the mutant strain (data not shown). However, supplementation of the medium with cAMP, important in transcriptional regulation of laccase via

FIG. 4. STE12α is not required for *C. gattii* haploid fruiting. The light microscopic (left panel; magnification, ×100) and SEM (right panel; magnification, ×6,000) analyses of haploid fruiting of the WT, ste12αΔ, and ste12αΔ plus STE12α strains are shown. The *C. gattii* strains were patched on dry, nitrogen-limited filament agar medium. The plates were incubated for 3 weeks at room temperature in the dark. The morphological assay showed characteristic filaments, basidia, and basidiospores.
parallel or multipath pathways (39), restored melanin pigmentation in the \( \text{C. gattii} \) \( \text{ste12} \)/\( H9251 \)/\( H9004 \) mutant (Fig. 5A). The reduced melanin pigmentation in the \( \text{C. gattii} \) \( \text{ste12} \)/\( H9251 \)/\( H9004 \) mutant was caused by defects in laccase enzyme activity and laccase transcript levels under glucose repression condition (Fig. 5B and C). These results indicated that \( \text{STE12} \)/\( H9251 \) regulates the expression of laccase in \( \text{C. gattii} \).

**STE12α is required for a wild-type level of virulence in mice.**

As \( \text{C. gattii} \) causes disease in both immunocompromised and healthy individuals, we utilized immunocompetent BALB/c and CS-deficient A/Jcr mice strains for comparison of pathogenicity. The Kaplan-Meyer survival curves for mice infected with three strains are shown in Fig. 6. A/Jcr mice infected with the wild-type strain survived for 4 days, while similarly infected BALB/c mice survived for 10 days. The survival pattern after infection by the reconstituted strain was 11 days for A/Jcr and 14 days for BALB/c mice. In contrast, mice of both strains infected with the \( \text{C. gattii} \) \( \text{ste12}α \) mutant strain survived as long as 29 and 30 days, respectively (\( P < 0.0005 \)). Thus, a significant reduction in the virulence of the \( \text{ste12}α \) mutant strain was observed for both complement-deficient A/Jcr mice and immunocompetent BALB/c mice.

The \( \text{ste12}α \) mutant produced much smaller cysts in the brains of both BALB/c and A/Jcr mice, and these cysts contained fewer yeast cells. On the contrary, WT and \( \text{ste12}α \) plus \( \text{STE12α} \) strains produced large cysts containing several to many yeasts. It was interesting to note that all strains tested divided relatively rapidly and produced more lesions in the brains of infected A/Jcr mice than in those of the infected BALB/c mice (Table 1), indicating that A/Jcr mice are relatively more susceptible than BALB/c to \( \text{C. gattii} \) infection. Light microscopy of brain smears revealed that all test strains

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**FIG. 5.** \( \text{STE12α} \) is required for melanin production, laccase enzyme activity, and \( LAC1 \) transcript expression. (A) \( \text{C. gattii} \) strains grown in YPD broth overnight were washed with phosphate-buffered saline, and a 5-μl suspension of \( 10^7 \) cells/ml was spotted on Niger seed agar with or without cAMP and incubated for 3 days at 30°C for melanin production. (B) Laccase enzyme activity from equal numbers of glucose-starved cells (5 h) was determined by measuring the oxidation of the diphenolic substrate ABTS (IU of activity = 0.01 absorbance unit in 30 min). Results are the means ± standard deviations from three individual experiments. The absence of laccase activity in the mutant is consistent with the initial loss of melanization in this strain. (C) Semiquantitative RT-PCR to examine the expression of the \( LAC1 \) gene in all of the test strains. Total RNA from each strain was isolated and reverse transcribed (cDNA) with 100-ng aliquots in 1:5 serial dilutions. Actin was used as a control.
produced large capsules in the infected mice (Fig. 6B). This observation was consistent with the large capsule size observed in vitro.

Interestingly, the ste12αΔ mutant was significantly more susceptible to in vitro PMN killing than the WT and ste12αΔ plus STE12α strains (Fig. 7). However, in vitro H2O2 sensitivity was similar in all three strains, which raised the possibility that highly reactive oxygen intermediates may be responsible for enhanced killing of the ste12αΔ mutant strain by human PMN. Collectively, these results indicated that functional STE12α is critical for C. gattii survival and multiplication against mammalian host defense mechanisms.

**STE12α is involved in wood utilization.** Many reports have described natural isolations of C. gattii from wood hollow and detritus of trees from around the world (28, 36, 50, 51, 73, 88). Additionally, experimental inoculations of almond tree seedlings with C. gattii showed that fungus remained viable and could be recovered from plant tissues 100 days postinfection, indicating its potential for survival in planta (38). It has been previously proposed that C. neoformans laccase plays an important role in lignin degradation in wood hollows (67, 72, 96). It was interesting that the ste12αΔ mutant was defective in pigmentation and appeared almost white, as opposed to the dark brown to red pigment produced by wild-type and reconstituted strains on water agar containing wood chips from black cherry and eastern hemlock trees (Fig. 8A). The loss of pigmentation in the mutant was not due to poor growth, as serial dilutions of all the strains on water agar containing either 2% wood chips or 2% wood chip extract exhibited similar growth patterns (Fig. 8B). It is important that the pigmentation ob-
TABLE 1. Comparative analysis of brain lesions induced by C. gattii strains in two mouse models

| Strain          | BALB/c mice |          |          | A/Jcr mice |          |          |
|-----------------|-------------|----------|----------|------------|----------|----------|
|                 | No. of foci | No. of yeast cells/focus | No. of foci | No. of yeast cells/focus |
| WT              | 26 ± 3b     | 66 ± 120b | 35 ± 4   | 62 ± 93    |
| ste12Δ          | 2 ± 2       | 6 ± 7    | 11 ± 6   | 15 ± 24    |
| ste12Δ + STE12α | 8 ± 3       | 50 ± 60  | 16 ± 7   | 31 ± 40    |

* Mean ± standard deviation of foci counted in 9 brain sections of 3 mice in each group.

** Mean ± standard deviation of yeast cells counted in all of the foci divided by the number of foci.

DISCUSSION

We have carried out a detailed characterization of the transcription factor STE12α in C. gattii biology. Currently, this pathogen is the cause of serious public health concerns due to its infectivity for AIDS patients and healthy individuals, its fatal outbreaks involving humans and animals, and its expanding geographical range, especially in North and South America (8, 12, 43). Experimental studies to determine what makes C. gattii such a potent pathogen are sparse. STE12α is among the very few transcription factors studied by the use of knockout mutants in both saprobic and pathogenic fungi. Therefore, STE12 is a valuable target for molecular pathogenesis studies to define C. gattii properties that are either distinctive or shared with other pathogens.

Previously, it was reported that STE12α was located within the MATα locus of the C. gattii genome, along with elements of the MAPK signaling cascade, pheromones, receptors involved in mating, housekeeping genes, and putative open reading frames of unknown function (31, 75). The overall organization of the mating locus in C. gattii is quite similar to that in C. neoformans var. grubii and in C. neoformans var. neoformans (31, 41, 75). However, the order and orientation of STE12α vis-à-vis other genes in the locus are not conserved in these fungi, raising the strong possibility that changes in STE12α expression can have occurred due to the positional effect of neighboring genes, as has been reported for other eukaryotic genomes (2, 20, 35, 85).

C. gattii STE12α was required for robust mating, as the ste12Δ mutant showed poor mating compared to the wild-type and reconstituted strains. Thus, unlike S. cerevisiae and other ascomycetes, the C. gattii ste12Δ mutant was not sterile but merely mating impaired; it shared this property with similar mutants from C. neoformans var. grubii and C. neoformans var. neoformans (11, 95). Taken together, our study and the two studies just cited provide a unique demonstration of a gene function conserved across all three pathogenic Cryptococcus forms (Table 2). Future studies with a congenic pair of C. gattii strains (MATα and MATα) and genetic epistasis experiments are clearly warranted to define the extent of the conserved role of STE12α in C. neoformans and C. gattii. Conventionally, mating studies in C. neoformans are carried out over a week, a length of time which, in our experience, proved insufficient for the knockout strain. However, when the observation period was extended to 2 weeks and when SEM was used for closer scrutiny of cells at edges of cocultures, a few spots with sparse mating reactions were found in mutant strain. Almost all fungal STE12 knockout mutants to date are reported to have mating or morphogenetic defects, a fact which affirms the central role of this transcriptional regulator in developmental programs. Our current data, taken together with the published literature from C. neoformans var. grubii and C. neoformans var. neoformans, do not identify the mechanism behind the observed partial defect in mating in the mutant strains. Perhaps other transcription factors play an important role in this process, either in association with or independent of STE12.

Another Cryptococcus developmental program, termed haploid or monokaryotic fruiting, involves filamentation and sporulation by MATα strains; it is reported to be defective in ste12Δ mutants of C. neoformans var. grubii and C. neoformans var. neoformans (11, 95). This observation is similar to
the complete or partial inhibition of filamentation seen in *S. cerevisiae* and *Candida albicans* ste12Δ mutants (55, 56). Interestingly, the *C. gattii* ste12Δ mutant showed normal haploid fruiting, suggesting either that STE12α is redundant or that there is some independent regulatory control of this pathway. Thus, unlike the role of STE12α in mating, the functional role of this gene in filamentation differs between *C. gattii* and *C. neoformans* and thus represents a gene function that has diverged in these closely related pathogens. The importance of this observation is difficult to assess at present, as we do not know how widespread and relevant the phenomenon of haploid fruiting is for *C. gattii* biology and virulence. Recently, it has been suggested that *C. neoformans* var. *grubii* haploid fruiting represents a self-fertilizing form of sexual reproduction in the absence of the out-crossing mode of reproduction due to the rarity of the occurrence of MATα mating partners (54).

The profound defects in laccase expression, enzyme activity, and melanin production in the *C. gattii* ste12Δ mutant strain are consistent with earlier reports from *C. neoformans* var. *neoformans* on down regulation or up regulation of laccase levels due to knockout or overexpression of *C. neoformans* var. *neoformans* STE12α (11, 90). In contrast, melanin production was reported to be unchanged in the *C. neoformans* var. *grubii* ste12Δ mutant (95). The latter observation highlights another instance in which gene regulation has been conserved between *C. gattii* and *C. neoformans* var. *neoformans* but not in *C. neoformans* var. *grubii*, even though *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are closely related phylogenetically. The results with ste12Δ mutants in *C. gattii* and *C. neoformans* var. *neoformans* raise the strong possibility that as-yet-undefined activators and/or suppressors of melanin participate in direct or indirect interactions with STE12α. Importantly, the addition of cAMP reversed the melanin defect in the *C. gattii* ste12Δ mutant strain; a possible interpretation is that cAMP acts to rescue laccase repression by STE12α via parallel or multipath pathways (39). An important role for the cAMP signaling pathway in regulation of *C. neoformans* melanin production has already been established by means of a number of gene knockout mutants (3, 72). Overall, there is a strong possibility of cross talk between elements of the MAPK and cAMP pathways, which are known to play important roles in mating. A recent discovery of *C. neoformans* DEAD box RNA helicase *VAD1* as a regulator of multiple virulence-associated genes is also highly relevant in this context (67). The *vad1Δ* mutant was melanin deficient due to up-regulation of *NOT1* (*CDC39*); the latter acted as an intermediary transcriptional repressor of

![FIG. 8. STE12α is involved in *C. gattii* ecological fitness. (A) *C. gattii* strains grown overnight in YPD broth were washed in phosphate-buffered saline and counted, and a 5-μl suspension of 10⁷ cells/ml was spotted on wood chip-based agar media; (B) 10-fold dilutions of each *C. gattii* strain, prepared similar to description for panel A, was spotted on wood chip extract agar media. YPD agar was used as a spotting control. All plates were incubated at 30°C for 10 days.](image-url)
laccase. *NOT1* is part of the Cr4-Not complex involved in global control of gene expression (21). The involvement of *NOT1* in the transcriptional regulation of laccase raised the possibility of direct or indirect interactions with *STE12* because some earlier studies have described involvement of *CDC36* and *CDC39* as negative regulators of the pheromone response in yeast (24, 63). Thus, multiple lines of evidence suggest that regulatory elements in the pheromone response pathway are involved in regulation of laccase expression. Further examinations will be imperative to define the regulatory network for *C. gattii* laccase. The evidence for other regulatory controls of melanization in fungi comes from the plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea*. Their respective *ste12* mutants have no defects in melanin, which is uniquely produced via polyketide synthesis (34). Melanin biosynthesis is controlled in these two organisms via novel transcription factors containing a Zn(II)2Cys6 binuclear cluster motif and a Cys2His2 zinc finger similar to motifs present in the *C. neoformans* Cys2His2 binuclear cluster motif and a *C. gattii* capsule size, all prominent characteristics of the less-virulent *C. gattii* strain. These blocks were placed on water agar in petri plates and incubated for 8 weeks at 30°C. Adequate moisture was maintained throughout the incubation period. Blocks with fungal growth were removed and processed for sectioning as illustrated in the accompanying schematics. The diagonal sectioning and selection of wood sections away from the inoculated surfaces allowed an estimation of fungal invasion (schematics). (a, b) WT-inoculated wood sections showing several pigmented yeast cells; (c, d) *ste12* mutant-inoculated wood section with few yeast cells with negligible brown pigment; (e, f) wood section inoculated with the *ste12* mutant-inoculated wood section showing abundant brown-pigmented yeast cells. V, vessels; P, parenchyma; F, fiber; R, rays. Both rays and vessels showed fungal growth.

**Table 2. Divergent roles of *STE12*α revealed by targeted gene disruptions in *Cryptococcus neoformans* and *Cryptococcus gattii***

| Feature                        | *C. neoformans* var. *grubii* (95) | *C. neoformans* var. *neoformans* (11) | *C. gattii* (present study) |
|--------------------------------|------------------------------------|---------------------------------------|---------------------------|
| Mating                         | Modest mating defects; ~10-fold reduction in quantitative assays | Reduced mating; frequency reduced by 95% in quantitative assay | Mating delayed and less frequent in semiquantitative assay |
| Haploid fruiting               | Absent                             | Absent                                | Unchanged                 |
| Auxotrophy                     | None                               | Not done                              | None                      |
| Melanin                        | Unchanged                          | Unchanged                             | Reduced                   |
| Laccase                        | Unchanged                          | Not tested                            | Reduced                   |
| *LAC1* expression              | Not done                           | Lower activity, GUS^*^ reporter construct | Lower laccase transcription level |
| Urease                         | Unchanged                          | Not tested                            | Unchanged                 |
| Phospholipase                  | Unchanged                          | Reduced                               | Unchanged                 |
| SOD activity                   | Not tested                         | Reduced                               | Unchanged                 |
| Capsule size                   | Reduced in vitro and in vivo       | Reduced only in vivo                   | Unchanged                 |
| Rabbit infection               | Similar cerebrospinal fluid yeast counts in parent and mutant strains | Not tested                          | Not tested                |
| Mice infection (inoculum)      | No difference in virulence (10^4–10^5 cells, intravenous) | Significantly reduced virulence (5 × 10^6 cells, intravenous) | Significantly reduced virulence (1 × 10^6 cells intravenous) |

*a* Escherichia coli β-glucuronidase.

**Figure 9.** *STE12*α is involved in *C. gattii* utilization of wood. The black cherry wood blocks (~1-cm cubes) were surface inoculated with a 5-μl suspension of 10^8 cells/ml of each *C. gattii* strain. These blocks were placed on water agar in petri plates and incubated for 8 weeks at 30°C. Adequate moisture was maintained throughout the incubation period. Blocks with fungal growth were removed and processed for sectioning as illustrated in the accompanying schematics. The diagonal sectioning and selection of wood sections away from the inoculated surfaces allowed an estimation of fungal invasion (schematics). (a, b) WT-inoculated wood sections showing several pigmented yeast cells; (c, d) *ste12* mutant-inoculated wood section with few yeast cells with negligible brown pigment; (e, f) wood section inoculated with the *ste12* mutant-inoculated wood section showing abundant brown-pigmented yeast cells. V, vessels; P, parenchyma; F, fiber; R, rays. Both rays and vessels showed fungal growth.
carried out extensive phenotypic tests to find additional fitness defects in the mutant strain, but these efforts were not successful, possibly due to reliance on laboratory media that are unlikely to reveal subtle STE12a regulatory control over functional genotypes in vivo. More refined studies are needed to identify diverse gene functions regulated by C. gattii STE12a, as was achieved for S. cerevisiae STE12 by means of an exquisite genome-wide location and expression profiling approach (74).

A remarkable observation in this study was the inability of the ste12aΔ mutant strain to produce robust pigmentation on wood chips simulating C. gattii’s environmental niche. To our knowledge, this is the first instance in which a gene function has been directly related to the specialized environmental niche of C. gattii or C. neoformans. Our wood chip agar model for testing environmental fitness was validated by the fact that the wild-type and reconstituted strains exhibited robust survival and multiplication on these substrates and produced copious amounts of melanin-like pigment. This observation is another manifestation of the ste12aΔ mutant strain to be due to its impaired ability to produce laccase. It may be relevant to recall that filamentous basidiomycetes are the major cause of wood damage in nature (white rot). This process involves breakdown of ligninocellulose by laccase working in concert with other fungal enzymes, mediators, and toxic radicals (52). The essential role of laccase in lignin degradation has also been confirmed by the use of laccase-deficient mutants of the white rot fungus Pycnoporus cinnabarinus (27). A corollary of these destructive fungal activities is represented by the industrial applications of purified laccase in bioremediation (40, 60). The results of our experiments suggest a model in which C. gattii STE12a senses appropriate nutrient/environment cues on the wood surface and responds by up regulation of laccase and melanization for cooperative utilization of substrate. This response was suboptimal in the mutant, due to the absence of Ste12p. Additionally, ste12aΔ mutant cells appeared defective in penetration and proliferation inside wood blocks compared to the WT and ste12aΔ plus STE12aΔ strains. We interpreted this observation as an under representation of the ste12a-associated fitness defect, paralleling the observed defect in colonization and proliferation inside mouse tissues (Fig. 9). Thus, STE12a appears to be an important regulatory gene for control of both the saprobic and the pathogenic aspects of the life cycle of Cryptococcus gattii.

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Cryptococcus neoformans, mating, sexual cycle, RNA polymerase, pathogenesis, virulence.

INTRODUCTION.

Cryptococcus neoformans is a dimorphic fungus that causes a potentially life-threatening systemic opportunistic infection in patients with impaired immune system. The sexual cycle is a crucial event in the development of C. neoformans virulence. Here we discuss the molecular events that lead to sexual development in this fungus.

MATERIALS AND METHODS.

C. neoformans strains were grown on YPD (1% yeast extract, 2% peptone, 2% dextrose) or SC (1% dextrose) media. Sexual development was induced by mating two different strains of the same mating type. The mating partners were grown on agar plates for 48 h, then washed with sterile water and added to each other in the same media. After 24 h of co-cultivation, the mixture was spread on YPD or SC agar plates and incubated at 28°C. After 24 h, the colonies were subcultured on the same media and incubated for 24 h. The colonies were then transferred to the same media and incubated for 24 h. The process was repeated until the colony reached the desired size. The colonies were then photographed and analyzed by microscopy.

RESULTS.

The sexual cycle of C. neoformans is a complex process that involves several key steps, including cell agglutination, cell conjugation, and the development of dikaryotic hyphae. The initial stage of the sexual cycle is cell agglutination, which occurs when two cells of the same mating type come into contact. This leads to the formation of a single cell that contains two nuclei, one from each parent cell. The next step is cell conjugation, which involves the fusion of the two nuclei to form a single nucleus that is capable of undergoing meiosis. This process occurs in the dikaryotic hyphae that develop from the conjugation nuclei.

DISCUSSION.

The sexual cycle of C. neoformans is a crucial event in the development of this fungus. The successful completion of this cycle is essential for the fungus to produce the sexual spores that are required for the survival of the fungus in the environmental niche. The sexual cycle is also an important target for antifungal drug development, as the性relationships that occur during this cycle are key steps in the development of drug-resistant strains. The understanding of the molecular events that lead to sexual development in C. neoformans is essential for the development of new antifungal drugs.

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