RESEARCH LETTER

A copper-responsive factor gene *CUF1* is required for copper induction of laccase in *Cryptococcus neoformans*

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Abstract

The multicopper laccase is a major virulence factor in *Cryptococcus neoformans*. Its expression regulation is complex. We presented molecular evidence to show that laccase expression was induced by high concentrations of exogenous copper. Melanin production and laccase enzymatic activity increased dramatically in response to the addition of copper to the media. Reverse transcription-PCR amplification of the laccase gene *LAC1* mRNA revealed that the induction occurred at the transcriptional level, which required the copper-responsive factor-encoding gene *CUF1*. Disruption of *CUF1* demolished the activation of *LAC1* transcription by copper, whereas the reconstituted strain restored the phenotypic defects. Furthermore, copper induction was shown to be independent of derepression by glucose starvation, a well-established activation factor for laccase expression. These results demonstrate a role of the copper-responsive factor gene *CUF1* in the expression of laccase in *C. neoformans*.

Introduction

Cryptococcus neoformans, an encapsulated basidiomycetous yeast, exists ubiquitously in the environment. It causes severe infections in populations with compromised immune systems, such as those who are HIV-positive (Idnurm *et al.*, 2005). *Cryptococcus neoformans* produces a copper-containing polyphenol oxidase, laccase, which plays a critical role in the infection (Salas *et al.*, 1996). In fact, laccase is widely distributed in higher fungi and plants with a variety of important biological functions (reviewed in Mayer & Staples, 2002). For instance, laccase is one of the key enzymes involved in lignin degradation by many white-rot fungi (Hassett *et al.*, 2008). Laccases have also been shown to be an important virulence factor for a number of plant pathogenic fungi (Pezet *et al.*, 1991; Adrian *et al.*, 1998). However, little is known about the molecular basis of laccase expression control in fungi.

Cryptococcus neoformans provides an excellent model for studying regulation of the expression of fungal laccases. Cryptococcal laccase oxidizes an appropriate substrate such as norepinephrine to form the dark high-molecular-weight pigment melanin, a useful characteristic, which has been widely applied in screening for mutants (Zhu & Williamson, 2003). Laccase is the only enzyme required for the pigmentation and catalyzes the first step of the reaction. When laccase activity is inhibited or the encoding gene *LAC1* is mutated, melanin production is impaired and the yeast exhibits an albino phenotype. In *C. neoformans*, laccase is encoded mainly by the *LAC1* gene despite there being two paralogs in the cryptococcal genome. Disruption of *LAC1* alone resulted in demolished enzymatic activity, melanin biosynthesis and attenuated virulence in a mouse cryptococcosis model (Salas *et al.*, 1996; Zhu & Williamson, 2004; Missall *et al.*, 2005). Previous studies have clearly demonstrated that various factors affect laccase expression. For example, 2% glucose is a strong inhibitor. Cells do not produce melanin and laccase in the presence of high concentrations of glucose (Nurudeen & Ahearn, 1979). Glucose depletion in the culture initiates laccase expression, which involves the Gα-cAMP-PKA signal pathway in *C. neoformans* (Alspaugh *et al.*, 1997). Earlier observations also indicated that laccase expression is linked to copper metabolism. Laccase-deficient mutants *mel1*, *mel5* and *mel7* could be restored by 100 μM $CuSO_4$ (Torres-Guererro & Edman, 1994). Lately, two genes, a vacuolar H^+-ATPase

VPH1 and CLC-type chloride channel *CLC-A*, were found to be essential for laccase activity. Likewise, copper ions restored the laccase-deficient phenotype in the disruption mutants *vph1* and *clc-a* (Zhu *et al.*, 2003; Zhu & Williamson, 2004). Considering that laccase is a copper-containing enzyme, these observations strongly suggest that copper is an elicitor for laccase expression in *C. neoformans*.

In this paper, we provide molecular evidence showing that copper is a strong inducer of *LAC1* transcription. Consequently, the enzymatic activity and melanin production were dramatically stimulated by the addition of exogenous $CuSO_4$ to the media. A formerly identified copper-responsive transcription factor gene, *CUF1*, was found to be required for the copper induction.

Materials and methods

Strains and media

Cryptococcus neoformans serotype A strain H99 (ATCC 208821) (Perfect *et al.*, 1993) was used as the wild type in this study. Δ*cuf1* (H99 *CUF1::URA5*) was a targeted knockout mutant of a copper-responsive factor Cuf1 in H99 and the complemented strain Δ*cuf1c* harbored a wild-type copy of *Cuf1* that was transformed back into Δ*cuf1* (Waterman *et al.*, 2007). Yeast cells were grown normally in liquid YPD (1% yeast extract, 2% peptone and 2% glucose) or on YPD agar (plus 2% agar) at 30 °C. Asparagine salt (0.1% asparagine, 0.3% KH_2PO_4, pH 5.2) was used for melanin production and laccase induction assays as described (Williamson, 1994). Norepinephrine-containing asparagine agar was supplemented with 100 mg L^{-1} norepinephrine.

Melanin production and laccase enzymatic activity assay

Assays to induce melanin biosynthesis and laccase activity with the yeast *C. neoformans* have been described previously (Zhu & Williamson, 2004). Briefly, for melanin biosynthesis, fresh cells grown in 10 mL of YPD overnight at 30 °C were harvested by centrifugation at 2000 ***g*** for 2 min and washed with sterile ddH_2O three times to remove residual glucose. Cells were then resuspended in 0.5 mL of water and spread on norepinephrine-containing asparagine agar supplemented with either 0.1% or 2% glucose plus copper ($CuSO_4$) at various concentrations. Cells were incubated at 30 °C for 24 h for melanin observation. For laccase induction, cells were grown in liquid YPD overnight at 30 °C, transferred into fresh liquid YPD again, and shaken at 30 °C for 3 h before induction. This step minimized the background expression of laccase due to glucose depletion in the overnight culture. Cells were washed three times with ddH_2O to remove remnant glucose and transferred to 100-mL liquid asparagine media supplemented with copper at desirable concentrations to obtain a final $OD_{600\,nm}$ reading of 1.0. Cells were then incubated for another 6 h for laccase expression. Then 1.0×10^7 cells were taken from individual cultures and incubated in 10 mM phosphate buffer (pH 5.8) for 30 min at 37 °C in the presence of 100 mg L^{-1} substrate epinephrine as described (Williamson, 1994). The enzymatic activity was defined as 1 U equal to 0.001 AU in 30 min at $A_{475\,nm}$. Assays were carried out in triplicate. Error was expressed as SD.

RNA preparation and reverse transcription-PCR (RT-PCR)

To determine the mRNA level of *LAC1*, total RNA was extracted from yeast cells after induction for 6 h in 25-mL asparagine liquid cultures in the presence of exogenous copper at appropriate concentrations. Cells were collected and washed with ice-cold diethylpyrocarbonate-treated ddH_2O twice. Cells were resuspended in 0.5-mL lysis buffer supplied with the BIOZOL Total RNA Extraction Kit (BIOER Technology Co. Ltd, Hangzhou, China) and broken with glass beads by vortexing for 3 min at maximal speed. RNA was extracted following the manufacturer's instruction. Residual contaminating DNA was removed by digestion with RNAse-free DNAse I (Promega Bioscience Inc., Beijing Division, China) according to the manufacturer's protocol. RNA was dissolved in 50 μL diethylpyrocarbonate-treated ddH_2O and stored at −80 °C immediately. Total RNA concentration was quantitated spectrometrically.

For reverse transcription, 1 μg of the total RNA solution was used as template to generate first-strand cDNA. The reaction mixture (total 20 μL) contained 1 μg of RNA, 2 μL oligo-$(dT)_{18}$ as the primer supplied with the TIANScript M-MLV Kit (Tiangen Co. Ltd, Beijing, China), 2 μL dNTP (10 mM total), 1 μL (200 U) of M-MLV reverse transcriptase. The reaction mixture was incubated at 25 °C for 10 min, and then at 42 °C for 50 min. The reaction was terminated by heating at 95 °C for 5 min. For the following PCR amplification, 2 μL of cDNA sample was used as template. A pair of primers, LAC-A/LAC-S, were 5′-TAC AACTTTCCCCGACCTC/5′-GATGGAGAAGGTGAGCGTC, designed to specifically amplify *LAC1* cDNA, which gave rise to a 400-bp product. The actin gene *ACT1* mRNA (GenBank accession number U10867) was used as an internal control and were amplified separately in parallel. The primers for *ACT1* cDNA amplification were: 5′-CGCTATCCTCCG TATCGATCTTGC/5′-CTGCTGGAAGGTAGACAAAGAGG, which generated a 500-bp PCR product. The PCR was performed using the TaKaRa BcaBEST^{TM} RNA PCR Kit [TaKaRa Biotechnology (Dalian) Co. Ltd, Dalian, China]. The PCR program for the amplification was denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at

72 °C for 1 min, for 35 cycles as determined beforehand. An aliquot of 5 μL of PCR reaction mixture was subjected to denaturing electrophoresis in 1.2% agarose gel.

Results

Upregulation of melanin production and laccase activity by exogenous copper

Formation of melanin has been used as an indication for laccase activity in *C. neoformans*. To examine copper regulation of melanin biosynthesis, yeast cells were spread on asparagine agar containing 100 mg L^{-1} substrate norepinephrine, 0.1% glucose and copper ($CuSO_4$) at 5, 20, 100 and 200 μM, separately, and incubated at 30 °C for 24 h. The melanin production was monitored and is shown in Fig. 1a. On the plates containing 5, 20 and 100 μM $CuSO_4$, melanin production was significantly stimulated, suggesting that laccase activity was activated by copper. In contrast, the cells remained a lighter color in the presence of 1 μM bathocuproinedisulfonic acid (a copper chelator), which created a copper-limiting condition (leftmost panel of Fig. 1a). In addition, cells exhibited an apparently darker color at higher concentrations of copper until the concentration reached 200 μM, above which the cells did not grow due to the toxicity of the copper (Fig. 1a).

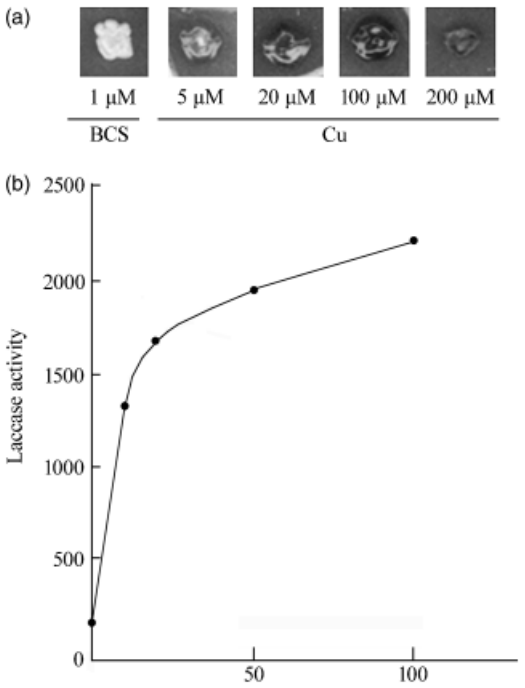

Fig. 1. (a) Induction of melanin production by copper (Cu). Cells were incubated separately on norepinephrine-containing asparagine agar (with 0.1% glucose) in the presence of exogenous Cu at 5, 20, 100 and 200 μM $CuSO_4$ at 30 °C for 24 h. The plate containing 1 μM bathocuproinedisulfonic acid (BCS) was used as control. Melanin was produced on the plates containing 5–100 μM $CuSO_4$. Little melanin was generated on the control plate. $CuSO_4$ 200 μM seemed to be highly toxic to the cells. (b) Upregulation of laccase enzymatic activity by Cu. Laccase activity was determined in 10 mM phosphate buffer by oxidizing 100 μg mL^{-1} epinephrine. A positive relationship was observed between laccase activity and Cu concentration. Cells were incubated in the presence of various concentrations of Cu for 6 h at 30 °C.

Copper induction of laccase expression was also confirmed by measuring the enzymatic activities by oxidizing the substrate epinephrine in 10 mM phosphate buffer (see Materials and methods). Cells were grown on asparagine agar supplemented separately with 0, 10, 20, 50 and 100 μM $CuSO_4$, for 24 h. In the assay, 10^7 cells were used. The enzymatic units obtained by the assay were 63.1 ± 3.2, 1377.5 ± 12.1, 1623.7 ± 8.3, 1986.2 ± 9.6 and 2235.4 ± 17.5, corresponding to cells grown in the presence of 0, 10, 20, 50 and 100 μM $CuSO_4$ (Fig. 1b) (the activity in 200 μM $CuSO_4$ dropped to 166.4 ± 6.6, data not shown). Laccase activity was activated dramatically by copper. The cells from the culture containing 100 μM copper produced *c.* 35-fold more laccase than did the cells from the culture to which copper was not added (0 μM in Fig 1b). This result clearly demonstrated that copper was a strong inducer of laccase expression in *C. neoformans*.

Induction of *LAC1* transcription by copper

To exclude the possibility that laccase activity increase was caused merely by copper incorporation to laccase apoenzyme present in the cells, an RT-PCR approach was used to amplify the mRNA of *LAC1* under induction conditions. Yeast cells were cultured in liquid asparagine media for 6 h in the presence of $CuSO_4$ at 0, 10, 20, 50 and 100 μM, separately. Total RNA preparation and RT-PCR procedure are described in Materials and methods. The actin gene *ACT1* mRNA served as the internal standard in the RT-PCR amplification. The PCR products of *LAC1* and *ACT1* mRNA were 400 and 500 bp in size, respectively. The PCR products were subject to 1.2% denaturing agarose gel separation (Fig. 2). The activation effect of copper on the transcription of *LAC1* was shown clearly by PCR amplification. In addition, a positive relationship between the copper concentration and the

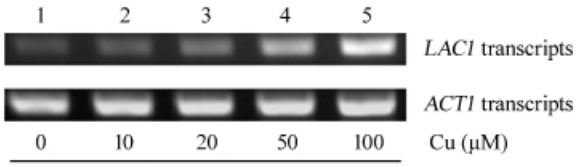

Fig. 2. Induction of *LAC1* transcription by copper (Cu). RT-PCR amplification confirmed that *LAC1* mRNA level was significantly boosted by Cu. An increasing *LAC1* mRNA level was observed with increased concentrations of Cu (lanes 2–5). Little mRNA was detected in the culture to which no exogenous Cu was added (lane 1). The *ACT1* mRNA level remained at approximately the same level (bottom panel).

LAC1 mRNA level was established (upper panel), whereas *ACT1* mRNA remained at approximately the same level regardless of copper concentration (bottom panel). Increasing the concentration of $CuSO_4$ resulted in an increased level of *LAC1* mRNA. This result suggested that copper activated *LAC1* expression at the transcriptional stage.

Copper induction of *LAC1* in glucose repletion condition

It should be noted that the results shown in Figs 1 and 2 were obtained under a glucose depletion condition (0.1% glucose used). Although the copper induction effect was obviously observed in the experiments, it may be questioned whether laccase induction by copper depends on glucose depletion. To address this, we examined copper induction in the media containing glucose at different concentrations, in particular at 2%, which is recognized to inhibit *LAC1* expression; thus, we were able to observe only the copper effect. We carried out the following analyses. First, melanin production was tested on asparagine/norepinephrine agar containing both 50 μM and 2% glucose to observe whether high concentrations of glucose inhibited the copper induction of melanin biosynthesis. Cells were spread on the plates and incubated at 30 °C for 24 h. As expected, laccase activity was inhibited and little melanin was produced on the control plate, indicating a deficiency of copper in the agar (Fig. 3a, left panel). On the plate containing 50 μM $CuSO_4$, melanin production by the cells increased dramatically, suggesting that melanin biosynthesis was activated by 50 μM $CuSO_4$ even in the presence of a high concentration of glucose (2%). In other words, copper induction of melanin biosynthesis did not require a glucose depletion condition.

RT-PCR was further performed to directly examine the effect of copper on the transcription of *LAC1* in the presence of both 50 μM $CuSO_4$ and glucose at three different concentrations. Yeast cells were grown in liquid YPD for 3 h before induction by copper. Cells were collected and treated as described in Materials and methods. Cells were then inoculated with liquid asparagine media supplemented with 50 μM $CuSO_4$ in combination with 0%, 0.1% and 2% glucose, respectively, and shaken at 200 r.p.m. for 6 h. Total RNA preparation and the performance of RT-PCR are described above. The RT-PCR result is shown in Fig. 3b. In terms of glucose concentration, in all three cases, 50 μM copper activated *LAC1* transcription regardless of the concentration of glucose provided in the cultures (lanes 2, 4 and 6). The *LAC1* mRNA level was much higher than that in the control lanes (1, 3 and 5), showing that copper induction was independent of glucose concentration. Particularly, in the culture containing 2% glucose, *LAC1* transcription (mRNA level) was still strongly stimulated by copper (lane 6). On the other hand, concentrations of glucose as low as 0.1% in the media strongly inhibited *LAC1* transcription (lanes 3 and 5). Glucose starvation alone turned on *LAC1* transcription (lane 1). As control, the *ACT1* mRNA level remained at approximately the same level in all cultures (Fig. 3b, bottom panel). Taken together, activation of *LAC1* transcription by copper does not require glucose starvation, which indicates that there are at least two distinct pathways leading to *LAC1* activation in *C. neoformans*. Interestingly, copper induction suppressed the repression of laccase by high concentrations of glucose in our experiment (Fig. 3b, lane 6).

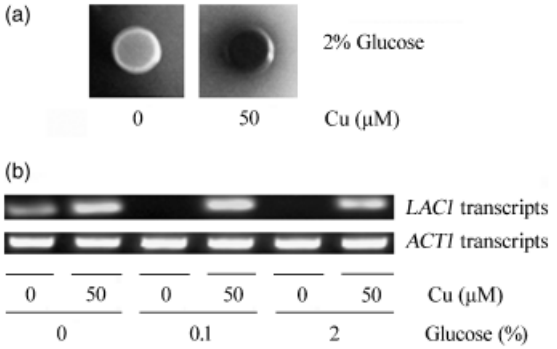

Fig. 3. (a) Induction of melanin production by copper (Cu) on norepinephrine-containing asparagine salt agar in the presence of 2% glucose (right panel). Melanin production was inhibited by 2% glucose without exogenous Cu in the media (left panel). Cells were incubated at 30 °C for 24 h. (b) Induction of *LAC1* transcription is independent of glucose in the media. RT-PCR amplification showed that *LAC1* mRNA level increased dramatically with the addition of 50 μM $CuSO_4$ regardless of the concentration of glucose (lanes 2, 4 and 6). Glucose inhibited *LAC1* transcription and little mRNA was detected when there was no supplementation with Cu (lanes 3 and 5). Glucose starvation alone turned on *LAC1* transcription (lane 1). $CuSO_4$ 50 μM activated *LAC1* transcription even with the strong inhibition of 2% glucose (lane 6). *ACT1* mRNA remained at a similar level in all cultures. Cells were incubated at 30 °C for 6 h.

Involvement of a copper-responsive transcription factor gene *CUF1* in the induction of *LAC1* by copper

A cryptococcal *CUF1* gene has been identified as a positive player in cryptococcal melanin biosynthesis (Waterman *et al.*, 2007). Based on its amino acid sequence, Cuf1 belongs to the copper-responsive transcription factor family found in other fungi, which was responsible for the activation of a number of copper-regulated genes. The best examples are Ace1/Mac1 from *Saccharomyces cerevisiae* and the Cuf1 factor from *Schizosaccharomyces pombe* (Beaudoin *et al.*, 2003; Keller *et al.*, 2005). Therefore, we speculate that cryptococcal Cuf1 may mediate *LAC1* induction by copper.

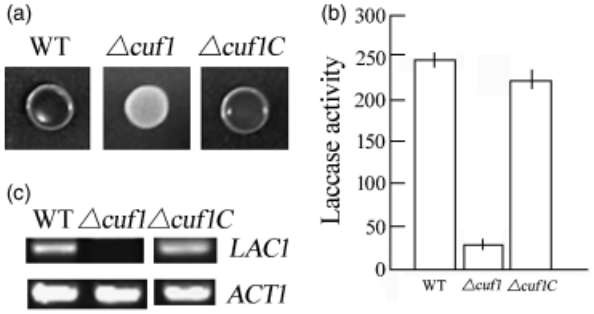

Fig. 4. *CUF1*-dependent induction of melanin biosynthesis, laccase activity and *LAC1* transcription. (a) Activation of melanin production by copper demolished in the mutant Δ*cuf1*. The wild type (H99) exhibited a normal induction of melanin biosynthesis by 10 μM $CuSO_4$. The reconstituted strain restored the induction. Cells were incubated in the presence of 2% glucose at 30 °C for 24 h. (b) Laccase activity was not induced by copper in Δ*cuf1*. H99 and the reconstituted strain exhibited normal induction. Cells were cultured in 2% glucose and 10 μM $CuSO_4$ at 30 °C for 6 h. (c) *LAC1* transcription in Δ*cuf1* was not induced by copper. *LAC1* mRNA was undetectable by RT-PCR amplification in the mutant Δ*cuf*. H99 and the reconstituted strain had the normal mRNA of *LAC1* (rightmost lane). Cells were incubated under the same condition as in (b). WT, wild type.

To address this issue, we examined melanin biosynthesis, laccase activity and *LAC1* transcription in the disruption mutant Δ*cuf1* using targeted homologous recombination (Waterman *et al.*, 2007). The wild-type strain H99 and a complement strain Δ*cuf1c* were used as control in the assays. Melanin biosynthesis was observed on norepinephrine salt plates containing 10 μM $CuSO_4$ and 2% glucose, which inhibited laccase expression via the glucose starvation route. Melanin was produced by H99 under the induction of 10 μM copper, whereas Δ*cuf1* only formed a light colony, indicating that melanin induction was impaired in this Δ*cuf1* mutant (Fig. 4a). In the reconstituted strain in which a wild-type copy of *CUF1* gene was reintroduced back into Δ*cuf1*, melanin biosynthesis was restored, suggesting that the *CUF1* gene was required for copper induction of laccase. The enzymatic activity assay consistently confirmed that copper induction required the *CUF1* gene. In the mutant Δ*cuf1*, laccase enzymatic activity did not show copper induction (10 μM $CuSO_4$), and the activity remained low (25.9 ± 4.2 U) due to the inhibition by 2% glucose in the culture, whereas the activity was 245.4 ± 3.3 for H99 and 220.5 ± 4.8 for the reconstituted strain Δ*cuf1c*, indicative of a strong induction by 10 μM copper (Fig. 4b). Likewise, RT-PCR amplification for the mRNA level of *LAC1* confirmed that Cuf1 was necessary for copper-induced *LAC1* transcription. In the mutant Δ*cuf1*, no *LAC1* mRNA was detected, whereas H99 and Δ*cuf1c* had strong bands of *LAC1* transcripts. The internal control *ACT1* mRNA maintained a similar level in all three strains (Fig. 4c). These results provide strong evidence that copper induction of *LAC1* in *C. neoformans* required the function of the copper-responsive factor gene *CUF1*. Disruption of *CUF1* impaired the induction of melanin biosynthesis, laccase enzymatic activity and the transcription of the laccase gene *LAC1*.

Discussion

The copper-containing oxidase laccase functions as a major virulence factor in the opportunistic pathogen expression of *C. neoformans*. It provides protection for the yeast cells from the attack of the host immune system by forming polymer melanin *in vivo* (Nosanchuk & Casadevall, 2003). The enzyme is encoded mainly by the *LAC1* gene. Regulation of *LAC1* is complex and remains elusive. In this work, we report for the first time that cryptococcal laccase activity is induced by high concentrations of exogenous copper ($CuSO_4$). Molecular analysis showed that mRNA of *LAC1*, the laccase-encoding gene, was intensely activated by copper, which is responsible for the great increase of the enzymatic activity of laccase and melanin production in the presence of copper ions. As we have shown, the yeast cells produced *c.* 35-fold more laccase in the cells induced by 100 μM copper than that produced by the cells without copper induction (indicated as 0 μM in Fig. 1b). A positive relationship between laccase activity/*LAC1* transcripts and the copper concentration was constantly observed within 100 μM copper as tested in this study (Figs 1 and 2). In addition, we found that laccase induction by copper and by glucose starvation are two distinct processes in *C. neoformans*, i.e. there are at least two distinct pathways leading to *LAC1* activation in the yeast. As shown by our analysis, copper induction of laccase expression was totally independent of the glucose supplemented in the media (Fig. 3) and did not require glucose depletion. Notwithstanding, a synergistic activation effect of the double induction by glucose starvation and copper was implicated in the assays (Figs 1b vs. 4b). For instance, when H99 cells were grown in the media containing 10 μM copper and 2% glucose, in which laccase was supposedly induced by copper alone, the laccase activity in the yeasts was only 245.4 ± 3.3 U 10^{-7} cells (Fig. 4b). In contrast, when H99 was cultured in 10 μM copper and glucose depletion (0.1% glucose), the activity increased to 1377.5 ± 12.1 U 10^{-7} cells (Fig. 1b).

Moreover, we demonstrated that a described copper-responsive transcription factor homolog, *Cuf1*, was required for the copper induction of the cryptococcal *LAC1*. The cryptococcal Cuf1 shares peptide sequence homology and similar biological functions in copper metabolism to the counterparts from a few other fungi (Waterman *et al.*, 2007). For example, cryptococcal Cuf1 harbors the canonical copper-fist DNA-binding region within its N terminus defined for the copper transcription factor family,

exemplified by Ace1 from *S. cerevisiae* and Cuf1 from *S. pombe*. Functionally, Cuf1 in *C. neoformans* is required for the yeast to grow under copper-limiting conditions (Waterman *et al.*, 2007). Lately, expression of another laccase gene, *lcs*, in the fungus *Ceriporiopsis subvermispora* was mediated by an Ace1-like copper transcription factor as well (Alvarez *et al.*, 2009). Given the fact that laccase contains four copper ions, it is comprehensible that *LAC1* is a copper-regulated gene.

Thus, the cryptococcal laccase gene *LAC1* is inducible in response to at least two stress conditions, glucose starvation and high concentration of copper. That multiple elements can activate the biosynthesis of a virulence factor may be significant for the pathogen, as it ensures that the pathogen can express the virulence factor in response to different environmental conditions. However, the biological implications for the copper induction of *LAC1* remain uncertain. It is unlikely that laccase is directly involved in copper detoxification as is the role of metallothioneins, which are involved in extra copper sequestration and are activated by high concentrations of copper (Thiele, 1988). A laccase mutant Δ*lac1* in *C. neoformans* did not show any difference in sensitivity to high concentrations of copper (data not shown). Rather, it is plausible that the activation may respond to the secondary stress caused by copper ions. Because of the redox nature of copper ions, free extra copper ions in the cells generate reactive oxygen species (Schmidt *et al.*, 2000). In *S. cerevisiae*, upregulation of *SOD1* by copper is believed to be initiated by secondary oxidative stress (Greco *et al.*, 1990). *FET3*, encoding a multicopper ferrous oxidase in the high-affinity iron transport complex, was also shown to be regulated by copper, but the gene has no role in copper sequestration (Schmidt *et al.*, 2000). In fact, Fet3 and laccase belong to the copper-containing oxidase family exhibiting considerable homology in amino acid sequence (Askwith & Kaplan, 1998). Given that *C. neoformans* is a saprophytic organism found ubiquitously in trees and soil (Mitchell & Perfect, 1995), laccase may be needed for detoxification of oxidants such as phenolic intermediates from the degradation of lignin, by oxidizing these chemicals into nontoxic substances (Kawai *et al.*, 1988; Thurston, 1994). Laccases have also been shown to detoxify phytoalexins produced by plants as a defense against fungal infections (Pezet *et al.*, 1991; Adrian *et al.*, 1998). Thus, copper induction of laccase in *C. neoformans* may reflect a conserved defensive function of the enzyme against oxidative stress caused by the toxic polyphenolic compounds or metal ions such as copper encountered in its natural niche.

Last but not least, the finding of copper induction of the virulence factor laccase may provide a clue to the sudden outbreak of AIDS-associated cryptococcosis in the 1980s (Mitchell & Perfect, 1995). Studies have shown that AIDS patients have a significant elevated blood copper level. In a prospective study, HIV-1 seropositive progressors were shown to have 115.6 $\mu g\,L^{-1}$ of the serum copper, whereas the value was 101.9 $\mu g\,L^{-1}$ in HIV-1 negative controls (Graham *et al.*, 1991). A cross-sectional study revealed a similar elevation in serum copper concentration (Moreno *et al.*, 1998). HIV-1-infected subjects in the study had up to 1.41 $mg\,L^{-1}$ (equal to 22.2 μM) serum copper compared with 1.12 $mg\,L^{-1}$ in the HIV-negative subjects. As shown by our study, laccase transcription will be activated at this range of concentrations (Fig. 1). As a result, activation of laccase by high levels of copper in the patients putatively favors survival of the pathogen and dissemination *in vivo*, which is significant for the pathogenesis of *C. neoformans*. The significance of this work also rests in the fact that Cuf1 may be a good target for antifungal drugs as no Cuf1 homologs have been reported in mammalian cells so far (Waterman *et al.*, 2007).

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