Laccase and Melanization in Clinically Important *Cryptococcus* Species Other than *Cryptococcus neoformans*

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The laccase enzyme and melanin synthesis have been implicated as contributors to virulence in *Cryptococcus neoformans*. Since isolations of *Cryptococcus* species other than *C. neoformans* from clinical specimens have been increasing, we examined the laccase activities of *C. albidus*, *C. laurentii*, *C. curvatus*, and *C. humicola*. Incubation of cells with epinephrine produced adrenochrome color in *C. albidus*, *C. laurentii*, and *C. curvatus* but not in *C. humicola*. Activity was always less than in *C. neoformans*. Laccase was detected in the soluble fractions of disrupted *C. albidus*, *C. laurentii*, and *C. curvatus* cells. Activity staining of partially purified enzyme after nondenaturing polyacrylamide gel electrophoresis revealed that laccases from *C. albidus*, *C. laurentii*, and *C. curvatus* migrated more slowly than that from *C. neoformans*. One strain of *C. curvatus* exhibited two melanin bands. Thus, several clinically emerging *Cryptococcus* species express laccase and can synthesize melanin.

Cryptococcosis is a life-threatening infection that complicates a variety of immunocompromising conditions (4). Although 34 species of the genus *Cryptococcus* are recognized (6), *C. neoformans* is generally considered to be the pathogenic species, while *C. albidus*, *C. laurentii*, and *C. curvatus* have been regarded as saprophytic species. However, species other than *C. neoformans* have, in fact, been isolated from normally sterile clinical specimens. We note isolations of *C. albidus* in septicemia and meningitis (15, 22, 23), *C. laurentii* in fungemia (14, 19) and meningitis (15), *C. curvatus* in myeloradiculitis (5), and *C. humicola* in meningitis (28), all in patients with AIDS or cancer. Thus, cryptococcosis due to species other than *C. neoformans* may be considered an emerging infection.

The virulence of *C. neoformans* has been ascribed to capsule formation, melanization due to laccase (phenoloxidase), proteinase secretion, and phospholipases (3). Although many pathogenic fungi synthesize precursors of melanin, melanization in *C. neoformans* occurs when exogenous or environmental catechols or aminophenols are oxidatively polymerized by the laccase enzyme and deposited as melanin in the cell wall (1, 26). Melanization has often been used for the identification of isolates, since growth of brown colonies on birdseed agar made from *Guizotia abyssinica*, or thistle seeds rich in caffeic acid, is characteristic of *C. neoformans* (29). However, we noted that several strains of species other than *C. neoformans* produced light-brown colonies on this medium or on defined medium containing synthetic substrates. In this study, we examined laccase enzymes and melanization in cryptococcal species other than *C. neoformans*.

MATERIALS AND METHODS

Strains used. The strains used in this study are listed in Table 1 as accessed in the culture collections Centraalbureau voor Schimmelcultures (CBS) and Japan Collection of Microorganisms (JCM). The strains were obtained from each institute directly and maintained on YM agar (Difco) slants. Some of the strains have recently been reclassified (30–34); therefore, current nomenclature is also given.

Laccase assay using permeabilized cells. The laccase activities of several *Cryptococcus* species were determined in suspensions of permeabilized cells by a modification of the method of Rhodes et al. (27). Each strain was inoculated into 5 ml of 2% glucose–2% yeast extract and incubated with agitation at 25°C for 24 h. The culture (0.1 ml) was inoculated into 50 ml of asparagine salts medium (per liter: glucose, 3 g; asparagine, 1 g; MgSO₄ heptahydrate, 0.5 g; KH₂PO₄, 3 g; thiamine, 1 mg) and again incubated at 25°C for 24 h with agitation. The cells were harvested by centrifugation at 1,000 × g for 10 min and washed once with asparagine salts medium without glucose. The cells were resuspended in 50 ml of the medium without glucose and incubated at 25°C for 12 h with agitation to induce laccase. The cells were harvested by centrifugation and washed once with 50 mM sodium phosphate buffer, pH 7.0. One milliliter of cell suspension adjusted to 10⁸ cells/ml and 0.1 ml of toluene-ethanol (1:4) were mixed for 90 s. The permeabilized cell suspension (0.1 ml) was mixed with 10 mM L-epinephrine (0.1 ml) and 50 mM sodium phosphate buffer (0.8 ml) and incubated at 30°C. After 0.5, 1, 2, 3, and 4 h, the reaction mixture was centrifuged at 1,000 × g for 5 min, and the optical absorbance of the supernatant at 480 nm was determined.

Preparation of laccase. Each strain was inoculated into 10 ml of 2% glucose and 2% yeast extract and incubated with agitation at 25°C for 24 h. The culture (0.3 ml) was inoculated into 12 bottles of 145 ml of asparagine salts medium and incubated for 24 h with agitation. The cells were harvested by centrifugation at 15,000 × g for 20 min at 4°C and washed once with asparagine salts medium without glucose. The cells were resuspended in medium without glucose and incubated at 25°C for 12 h. After centrifugation, the cells were washed once with 1 mM sodium phosphate buffer, pH 7.0. The cells were disrupted mechanically as follows (8). The cells were placed in a 50-ml ice-jacketed bead beater (Biospec Products) with 0.5-mm-diameter glass beads added almost up to the top, and phosphate buffer was added to displace all the air. Intervals of agitation (1 min) were interspersed with 1-min pauses for cooling. Five 1-min treatments generally provided good breakage. The suspension of disrupted cells was ultracentrifuged at 100,000 × g for 60 min. This crude extract was partially purified in a Phenyl Sepharose CL-4B column (9) and lyophilized.

Activity staining was performed after nondenaturing gel electrophoresis to compare laccases from several *Cryptococcus* species as described previously. Each partially purified laccase was dissolved in a small amount of water and applied to a 10% polyacrylamide gel without sodium dodecyl sulfate. The gel was run at a constant current of 18 mA. After electrophoresis, the gel was immersed in freshly prepared 1 mM L-dihydroxyphenylalanine dissolved in citrate phos-

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TABLE 1. Strains used

Previous species	Strain	Source	Current species	Reference
<i>C. neoformans</i>	B-3501			
<i>C. albidus</i>	CBS142	Atmosphere	<i>C. albidus</i>	32
	CBS945	Tubercular lung	<i>C. albidus</i>	
	CBS965	Sputum	<i>C. diffluens</i>	
<i>C. laurentii</i>	CBS2174	Tumor	<i>C. laurentii</i>	30
	CBS8645	Cerebrospinal fluid; AIDS with meningitis	<i>C. nodaensis</i>	30
	CBS8648	Lung	<i>C. laurentii</i>	30
	CBS2409	Surface of shrimp	<i>Cryptococcus</i> sp. strain	30
<i>C. curvatus</i>	CBS570	Sputum of tubercular patient	<i>C. curvatus</i>	31
	JCM1532 (CBS570)		<i>C. curvatus</i>	31
	JCM1605	Frozen food	<i>C. curvatus</i>	31
<i>C. humicola</i>	CBS571	Soil	<i>C. humicola</i>	31
	JCM1530	Strawberry	<i>C. fragicola</i> sp. nov.	34
	CBS1896	Bronchial secretion	<i>Trichosporon debeurmannianum</i> sp. nov.	33
	CBS5920	Radioactive solution at pH 2	<i>C. longus</i> sp. nov.	34
	CBS5123	Skin	<i>C. daszewskae</i> sp. nov.	34

phate buffer (pH 6.0) and incubated overnight at room temperature with gentle agitation. Laccase was visualized by melanization at the position to which the enzyme migrated.

RESULTS

Comparative laccase activities in various *Cryptococcus* species. Laccase activities in strains of *C. albidus*, *C. laurentii*, *C. curvatus*, and *C. humicola* were compared to that of *C. neoformans* B-3501 by observing the time course of color development in the presence of epinephrine after 0.5, 1, 2, 3, and 4 h. Initially, we performed many trials because the shapes of the plots for *C. neoformans* and for *C. albidus* were different: concave downward for *C. neoformans* and concave upward for *C. albidus*. We performed nine experiments on *C. neoformans*, seven on *C. albidus* CBS142, and five on *C. albidus* CBS945, each experiment in triplicate (Fig. 1). All other strains were studied once in triplicate. The precision of the first three plots is indicated. It seems clear that all preparations from *C. albidus* (Fig. 1), three of four from *C. laurentii* (Fig. 2), and all strains of *C. curvatus* (Fig. 3) oxidized the epinephrine substrate, while preparations from *C. humicola* (Fig. 3) contained little, if any, laccase activity. A strain of *Candida albicans* served as a negative control (Fig. 1). We quantitated laccase activities by calculating linear slopes, after 1 h in the case of *C. neoformans* and after 4 h in the other cases. These data are given in Table 2.

Cell-free laccases from *C. neoformans* and species other than *C. neoformans*. Laccase activities were detected in the crude soluble fractions of broken-cell extracts made from *C. albidus*, *C. laurentii*, and *C. curvatus*, although again, the activities were lower than in comparable extracts from *C. neoformans* (not shown). To test whether the laccases of species other than *C. neoformans* were similar to the *C. neoformans* protein, non-denaturing gel electrophoresis and activity staining were performed. As shown in Fig. 4, melanin bands were seen. Laccases from *C. albidus*, *C. laurentii*, and *C. curvatus* migrated more slowly than laccase from *C. neoformans*. We previously showed that laccases from *C. neoformans* var. *gattii* are slower than

those from *C. neoformans* var. *neoformans* (8). However, laccases from the *Cryptococcus* species other than *C. neoformans* studied here were even slower, although with mobilities similar among themselves. Interestingly, in *C. curvatus* JCM1532, two laccases with different mobilities were detected: one with mobility similar to those of *C. albidus* and *C. laurentii* and one with mobility similar to that of *C. neoformans* (Fig. 4, lane 6).

DISCUSSION

How do the virulence factors of *Cryptococcus* species other than *C. neoformans* compare to those of *C. neoformans*? The major virulence factors identified in *C. neoformans* are capsule formation and melanin synthesis (3). The polysaccharide cap-

FIG. 1. Laccase activities of strains of *C. albidus*. ●, *C. neoformans* B3501; ×, *C. albidus* CBS965; ■, *C. albidus* CBS142; ▲, *C. albidus* CBS945; △, *Candida albicans* CBS562. The error bars indicate standard deviations.

FIG. 2. Laccase activities of strains of *C. laurentii*. ●, *C. neoformans* B3501; ■, *C. laurentii* CBS2174; ▲, *C. laurentii* CBS8645; ×, *C. laurentii* CBS8648; ○, *C. laurentii* CBS2409.

sule aids pathogenicity through antiphagocytic activity (16, 18) and effects on the complement system (17), by preventing interaction with platelets (24), and by suppressing immune responses (3). Similarities in the chemical structures of polysaccharides from *C. neoformans* and several other *Cryptococcus* species have been suggested by chemical and serological studies. Thus, the serological reactivities of *C. albidus*, *C. curvatus*, and *C. humicola* strains were the same as those of *C. neoformans* serotypes A, AD, and D, respectively, indicating similarities in the chemical structures of the capsular polysaccharides (11). In fact, the chemical structures of polysaccharides from *C. albidus* CBS142 and *C. neoformans* serotype A

FIG. 3. Laccase activities of strains of *C. curvatus* and *C. humicola*. ●, *C. neoformans* B3501; ■, *C. curvatus* JCM1605; ▲, *C. curvatus* CBS570; ×, *C. curvatus* JCM1532; △, *C. humicola* JCM1530; ○, *C. humicola* CBS571; □, *C. humicola* CBS1896, CBS5920, and CBS5123.

TABLE 2. Laccase activities in *C. neoformans* and species other than *C. neoformans*

Species	Strain	Slope ^a
<i>C. neoformans</i>	B-3501	0.175
<i>C. albidus</i>	CBS142	0.036
	CBS945	0.029
	CBS965	0.043
<i>C. laurentii</i>	CBS2174	0.070
	CBS8645	0.061
	CBS8648	0.052
	CBS2409	0.015
<i>C. curvatus</i>	JCM1605	0.037
	CBS570	0.026
	JCM1532	0.025

^a Slopes were calculated after 1 h in *C. neoformans* and 4 h in other *Cryptococcus* species.

were highly similar and differed only in the molar ratios of component sugars and in the existence of unreplaced mannose residues in *C. neoformans* serotype A (10).

Melanin synthesis is a second virulence factor in *C. neoformans* (20). It is thought that melanin, made by a laccase from exogenous catecholamines, provides *C. neoformans* with some protection against physiological stress. For instance, melanin has been shown to act as a redox buffer, resulting in resistance to external oxidants and to intracellular killing by anions in phagocytosis (2, 12, 13). According to an alternative hypothesis, the laccase itself, rather than its product, may be the factor that protects the fungus from strong oxidants (21). In this study, we surveyed the laccases of clinically important *Cryptococcus* species other than *C. neoformans*. We observed (slight) browning of cultures on bird seed agar in some strains, suggesting melanization. We demonstrated oxidation of laccase substrate by suspensions of *C. albidus*, *C. laurentii*, and *C. curvatus*, although the activity of *C. neoformans* clearly was much greater. Because it was not possible to set a clear lower limit for this assay, we confirmed the production of laccase by other methods. First, we confirmed that laccase activity was present in the soluble fractions after cell disruption. Next, we showed that macromolecules could be electrophoresed in polyacrylamide gel and stained for laccase activity in the gel.

None of the laccase assays were perfectly linear with respect to time. The laccase assay in *C. neoformans* gave a curve which was concave downward. It is possible that the reaction ran out of substrate, although the concentration provided was 14 times the K_m (26) and was limited by the solubility of epinephrine. An alternative possibility is that the enzyme was inactivated by its extremely reactive intermediate products. We compensated for this curvature by calculating the activity in *C. neoformans* from the slope at 1 h. The time curves obtained for the species other than *C. neoformans* were repeatedly concave upward, which may be the result of slow production of chromogenic intermediates.

In the case of one strain of *C. curvatus*, we observed two bands of melanin on activity staining. It is possible that these represent isoenzymes, but the different mobilities may also represent an aggregation-disaggregation phenomenon.

FIG. 4. Activity-stained electropherograms of laccases produced by *C. neoformans* and *Cryptococcus* species other than *C. neoformans*. Lanes: 1, *C. albidus* CBS142; 2, *C. albidus* CBS945; 3, *C. laurentii* CBS8645; 4, *C. laurentii* CBS2174; 5, *C. curvatus* CBS570; 6, *C. curvatus* JCM1532; 7, *C. neoformans* B3501.

Finally, we consider the taxonomic consistency of the results (Table 1). With regard to *C. albidus*, all representative strains showed laccase activity. However, one of the three strains (CBS965) has been reclassified as *C. diffluens* on the basis of DNA sequencing of ribosomal and internal transcribed spacer regions (7, 32). Of the four representative strains of *C. laurentii*, strain CBS2409 showed little or no laccase activity; however, on the basis of recent DNA sequence data, the identification of that strain was incorrect (30). In *C. curvatus*, recently reclassified from the genus *Candida* into *Cryptococcus* (6), we found laccase activity in all three representative strains. Thus, all representative strains of *C. albidus*, *C. laurentii*, and *C. curvatus* which survived recent DNA-based reclassification are considered to express laccase. We attempted to obtain a fragment from the laccase gene of *C. curvatus* by PCR, using primers designed from a conserved region of the laccase genes from *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Although we obtained the expected products from five strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (35, 36), we obtained none from any of the strains of *C. curvatus* studied. This negative result is consistent with the qualitative differences seen on electrophoresis of laccases from species other than *C. neoformans* and suggests that the species other than *C. neoformans* produce laccases with molecular structures different from that of *C. neoformans* laccase.

Recently, Petter et al. (25) surveyed heterobasidiomycetous yeast genera for production of melanin-like pigment on medium containing L-dihydroxyphenylalanine or norepinephrine and for the CNLAC1 gene, which encodes the laccase of *C. neoformans*. Several species showed faint or delayed pigmentation. Interestingly, the cryptococcal species *C. podzolicus* alone showed positive production of pigment; it contained a DNA sequence homologous to the CNLAC1 gene. That report, with its focus on DNA homology, might be considered complementary to the present report, whose methodology was primarily enzymologic.

In conclusion, several clinically emerging *Cryptococcus* species have the potential to express laccase and to synthesize melanin, although the rates and amounts are less than in *C. neoformans*.

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