

## *Cryptococcus neoformans* Can Utilize the Bacterial Melanin Precursor Homogentisic Acid for Fungal Melanogenesis<sup>∇</sup>

Susana Frases,<sup>1†</sup> Angela Salazar,<sup>1†</sup> Ekaterina Dadachova,<sup>2</sup> and Arturo Casadevall<sup>1,3\*</sup>

Department of Microbiology and Immunology,<sup>1</sup> Department of Medicine,<sup>3</sup> and Department of Nuclear Medicine,<sup>2</sup> Albert Einstein College of Medicine, Bronx, New York 10461

Received 16 August 2006/Accepted 2 November 2006

*Cryptococcus neoformans* melanizes in the environment and in mammalian tissues, but the process of melanization in either venue is mysterious given that this microbe produces melanin only from exogenous substrates. Understanding the process of melanization is important because melanization is believed to protect against various stresses in the environment, including UV radiation, and pigment production is associated with virulence. Melanization in *C. neoformans* requires the availability of diphenolic precursors. In contrast, many bacteria synthesize melanin from homogentisic acid (HGA). We report that *C. neoformans* strains representing all four serotypes can produce a brown pigment from HGA. The brown pigment was acid resistant and had the electron paramagnetic resonance spectrum of a stable free radical, qualities that identified it as a melanin. Melanin “ghost”-like particles obtained from pigmented *C. neoformans* cells were hydrophobic, fluorescent under a variety of irradiation wavelengths, negatively charged, insoluble in organic solvents and alcohols, resistant to degradation by strong acids, and vulnerable to bleaching. HGA melanization was laccase dependent and repressed by high concentrations of glucose. The ability of *C. neoformans* to utilize a bacterial melanin precursor compound suggests a new substrate source for melanization in the environment.

*Cryptococcus neoformans* is a free-living, cosmopolitan organism that can survive in a variety of environmental niches. This fungus is a major pathogen in immunocompromised hosts. Cases of cryptococcosis have been reported from all regions of the world (1). *C. neoformans* strains have been divided into three varieties, known as *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, and *C. neoformans* var. *gattii*, based on biochemical and genetic differences, and recent evidence suggests that the phylogenetic distances between these varieties are sufficient to group them as distinct species (10). *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* strains are often recovered from soils contaminated with bird excreta, while the major environmental habitat of *C. neoformans* var. *gattii* is believed to be certain arboreal species (e.g., *Eucalyptus* species) (1, 14). *C. neoformans* has several virulence factors, such as capsule production, ability to grow at 37°C, and melanin synthesis. Unlike melanization in other melanotic fungi, melanization in *C. neoformans* requires exogenous substrates. Melanin synthesis in *C. neoformans* is catalyzed by a laccase, which is considered a phenol oxidase or diphenol oxidase because it produces pigment from phenolic compounds with two hydroxyl groups, but not tyrosine (2, 3, 16). Although *C. neoformans* cells in the environment are melanized, the environmental source of melanin precursors is unknown (11).

Melanins are negatively charged, hydrophobic macromolecules of high molecular weight formed by the oxidative polymerization of phenolic and/or indolic compounds (7). The resulting pigments are usually brown or black, but other colors

have also been observed, depending on the substrate used to induce melanization. Melanin polymers are remarkable in that they have a stable population of organic free radicals (4). Melanin pigments are multifunctional polymers found in all biological kingdoms. For *C. neoformans*, melanin synthesis confers reduced susceptibility to amoeba predators, UV light, temperature extremes, oxidative fluxes, and heavy metals. Although melanization may have developed in response to environmental pressures, the ability to produce melanin is associated with virulence in mammalian hosts (12).

Several types of melanins have been described in bacteria, plants, animals, and fungi: eumelanins, phaeomelanins, allomelanins, and pyomelanins. Eumelanins are formed from quinines and free radicals. Phaeomelanins are derived from tyrosine and cysteine. Allomelanins are synthesized from nitrogen-free precursors, and pyomelanins are derived from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate and homogentisic acid (HGA) (7, 9). HGA is a catabolite of phenolic metabolism in a wide variety of higher organisms, including mammals, fish, birds, amphibians, and plants. Pyomelanin formation is correlated with HGA production in three disparate marine bacterial species: *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana* (9).

*C. neoformans* produces eumelanin from catecholamine precursors, such as L-dopa, epinephrine, and norepinephrine (6). However, unlike other fungi, melanization in *C. neoformans* occurs only when the organism is exposed to precursor compounds. Since *C. neoformans* in the environment is melanized, initial human infection might occur with melanized organisms. However, practically nothing is known about the sources of melanin precursors in the environment. Recently, we showed that interaction between a bacterium, *Klebsiella aerogenes*, and *C. neoformans* resulted in fungal melanization (5). The mechanism for that process involved the production of dopamine by

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-2215. Fax: (718) 430-8968. E-mail: casadeva@aecom.yu.edu.

† Both authors contributed equally.

∇ Published ahead of print on 10 November 2006.

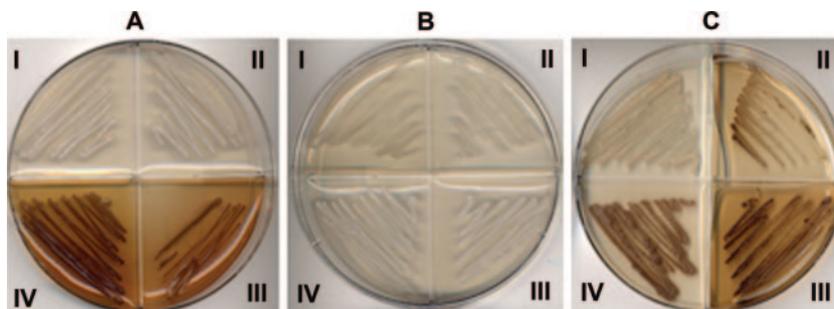


FIG. 1. *C. neoformans* cells grown in minimal medium supplemented with 1 mM HGA incubated at 30°C for 5 days. (A) B3501 parental strain. (B) 2E-TU Lac<sup>-</sup> mutant. (C) 2E-TUC mutant complemented with the laccase gene. I, minimal medium supplemented with 40 g/liter of glucose; II, minimal medium supplemented with 20 g/liter of glucose; III, minimal medium supplemented with 10 g/liter of glucose; IV, minimal medium supplemented with 3 g/liter of glucose.

the bacteria that was used as a melanin precursor by *C. neoformans*. That observation provided a precedent for microbial sources of substrates for *C. neoformans* melanization in the environment (5). Now we demonstrate that *C. neoformans* can make melanin from HGA, thus establishing that bacteria and fungi can use the same precursor in melanization.

#### MATERIALS AND METHODS

**Media.** SM medium is composed of 10 g/liter of glucose, 10 g/liter of proteose peptone, 1 g/liter of yeast extract, 1 g/liter of MgSO<sub>4</sub>, 1.9 g/liter of KH<sub>2</sub>PO<sub>4</sub>, and 0.6 g/liter of K<sub>2</sub>HPO<sub>4</sub> in water. Minimal medium consists of 15.0 mM glucose, 10.0 mM MgSO<sub>4</sub>, 29.4 mM K<sub>2</sub>HPO<sub>4</sub>, 13.0 mM glycine, and 3.0 mM thiamine, pH 5.5. Minimal medium agar was made by adding 20 g/liter to this medium.

**Fungal strains.** *C. neoformans* serotype A strain H99 was a gift from John Perfect (Durham, NC). Serotype D strain B3501 was purchased from the ATCC (Manassas, VA). Serotype AD strain MAS93-120 was provided by Mary Brandt (Atlanta, GA). Serotype B (NIH198) strain and serotype C strains 1343 and B 4546 were obtained from Thomas Mitchell (Durham, NC). In addition, we also used the laccase-negative mutant 2E-TU (URA5 Mel<sup>-</sup>) of strain B3501 and its laccase-complemented strain 2E-TUC (URA5 Mel<sup>+</sup>) (13).

**Agar assay for pigment induction.** To induce pigment production in *C. neoformans*, H99 cells were grown in SM agar supplemented with 1 mM HGA (Sigma Chemical Co., St. Louis, MO) at 22°C for 5 days. As a control for spontaneous pigment production, SM agar with HGA without cells was incubated in parallel for an equivalent time.

**Role of laccase enzyme in pigment production.** The laccase-negative mutant 2E-TU (URA5 Mel<sup>-</sup>) of strain B3501, its laccase-complemented strain 2E-TUC (URA5 Mel<sup>+</sup>), and the parental strain B3501 were grown in both liquid and agar minimal media. The media were supplemented with different concentrations of glucose (3, 10, 20, and 40 g/liter) and 1 mM of HGA. Cells were incubated at 30°C for 5 days.

**Isolation and study of biochemistry properties of melanin particles.** *C. neoformans* cells pigmented by growth in minimal medium supplemented with 1 mM of HGA at 22°C for 5 days were suspended in phosphate-buffered saline (PBS). Cells were collected by centrifugation at 3,000 rpm for 10 min and suspended in 1.0 M sorbitol-0.1 M sodium citrate (pH 5.5). Protoplasts were generated by incubating cells at 30°C in 10 mg/ml of cell wall-lysing enzymes (*Trichoderma harzianum*; Sigma Chemical Co., St. Louis, MO) overnight. The protoplasts were then collected by centrifugation, washed with PBS, and incubated in 4.0 M guanidine thiocyanate for 12 h at room temperature with frequent vortexing. The resulting brown material was collected by centrifugation, washed with PBS, and digested with 1.0 mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN). The resulting material was washed again in PBS and boiled in 6.0 M HCl for 1 h to hydrolyze cellular contaminants associated with melanin. The particles were collected by centrifugation and washed with PBS. Aliquots from each step of the sample preparation were visualized by microscopy for size measurements after heat fixation on glass slides using an Olympus AX 70 microscope (Melville, NY) equipped with fluorescence filters. The resultant particles were then analyzed for their solubility in bleach and various organic solvents (1-butanol, methanol, chloroform, 1-butanol:chloroform [1:1, vol/vol], methanol:chloroform [1:1, vol/vol], acetone, carbon tetrachloride, *N,N*-dimethyl formam-

ide, and toluene). Additionally, the particles recovered from brown cells after treatment with enzymes, denaturant, and hot acid were visualized using fluorescence microscopy. Melanin particles obtained from *C. neoformans* cells grown with L-dopa were used as controls.

**HPLC of oxidized melanins and culture supernatants.** The melanin particles derived from H99 cells grown with 1 mM of HGA at 22°C for 5 days and those grown with L-dopa were oxidized with permanganate and analyzed by high-performance liquid chromatography (HPLC). Samples were dried at 50°C and then oxidized with acidic permanganate. Pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid, 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA), and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) were used as standard compounds of melanin degradation products (gifts from K. Wakamatsu, Toyooka, Japan). Chromatograms of pyrrole-2,3-dicarboxylic acid, TDCA, TTCA, and PTCA standards yielded peaks at 5.1, 6.1, 7.1, and 11.0 min, respectively. The oxidation products were analyzed by HPLC using a Shimadzu LC-600 liquid chromatograph (New York, NY), a Hamilton PRP-1 C<sub>18</sub> column (dimensions, 250 by 4.1 mm; particle size, 7 μm), and a Shimadzu SPD-6AV UV detector. The mobile phase was 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). At 1.0 ml/min, the elution gradient (at the indicated times in min, in percent solvent B) was as follows: 0, 0; 1, 0; 12, 25; 14, 25; 16, 0. The UV detector was set at a 255-nm absorbance. A filtered supernatant from a *C. neoformans* culture was analyzed for 7 days by HPLC using a Waters 600 liquid chromatograph, an Alltech C<sub>18</sub> column (dimensions, 250 by 4.6 mm; particle size, 5 μm), and a Waters 486 detector measuring absorbance at 270 nm. The mobile phase was 25 mM ammonium bicarbonate buffer, pH 7.9. The flow rate was 1.0 ml/min. The standards consisted of 1 to 50 mM solutions of HGA.

**Zeta potential.** A suspension of 10<sup>7</sup> cells/ml from *C. neoformans* H99 grown in SM medium supplemented with 1 mM HGA for 5 days at 22°C was suspended in 10 mM KCl and the cells' zeta potential was measured with a Zeta Plus machine (Brookhaven Instruments Corporation, Moltville, NY). The zeta potential, or surface charge, of the particles was determined by applying an electric field to the particles in suspension and then determining the direction and velocity of the particles' movements by measuring the light scattering from a laser beam passed through the sample. Each value reported represents the average from 20 measurements.

**EPR.** Particles recovered from pigmented cells grown in medium supplemented with HGA were analyzed by electron paramagnetic resonance (EPR). EPR spectra were obtained with a Varian E112X-Band model spectrometer. The parameters for EPR were as follows: modulation frequency, 9.07 GHz; modulation amplitude, 1,633.0 G; center field, 3,240.0 G; sweep width, 80.0 G; microwave frequency, 9.2995 GHz; microwave power, 0.20 mW; and temperature, 77 K. As controls, we used cells grown in medium without HGA (nonpigmented cells) and L-dopa melanin particles.

**Transmission electron microscopy.** Particles from pigmented cells were fixed in 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate at room temperature for 2 h, followed by overnight incubation in 4% (vol/vol) formaldehyde, 1% (vol/vol) glutaraldehyde, and 0.1% (vol/vol) PBS. The samples were subjected to 90 min of postfixation treatment in 2% (wt/vol) osmium tetroxide, serially dehydrated in ethanol, and embedded in Spuris epoxy resin. Sections (70 to 80 nm) were cut on a Reichert Ultracut UCT and stained with 0.5% (wt/vol) uranyl acetate and 0.5% (wt/vol) lead citrate. Samples were viewed in a JEOL 1200EX transmission electron microscope at 80 kV. The diameters and thicknesses of "ghost" shells

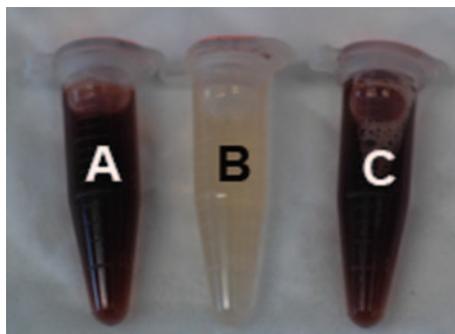


FIG. 2. Supernatants obtained from cultures of *C. neoformans* in SM medium supplemented with 1 mM HGA. (A) B3501 parental strain. (B) 2E-TU Lac<sup>-</sup> mutant. (C) 2E-TUC mutant complemented with the laccase gene.

from 200 particles recovered from cells pigmented with L-dopa or HGA were measured. The respective averages, standard deviations, and *P* values were calculated.

**Susceptibility of L-dopa- or HGA-melanized and nonmelanized *C. neoformans* cells to UV radiation.** Eight-day-old melanized (HGA or L-dopa) and nonmelanized *C. neoformans* cells were plated in equal numbers ( $1 \times 10^4$ ) on Sabouraud dextrose agar (Difco, Detroit, MI). Plates were exposed to UV light (254 nm) generated in a Stratalink 1800 (Stratagene, La Jolla, CA) at  $10,000 \mu\text{J}/\text{cm}^2$ . The percentages of cells surviving were measured. The respective averages, standard deviations, and *P* values were calculated from five measurements each for melanized (HGA or L-dopa) and nonmelanized *C. neoformans* cells.

**Phagocytosis experiments.** Macrophage assays were done with *C. neoformans* cells grown in minimal medium for 6 days in the presence (melanized) or absence (nonmelanized) of 1 mM of L-dopa or 1 mM of HGA. The ratio of macrophages (J774) to *C. neoformans* cells was 1:1. The assays were done in the presence or absence of antibody 18B7. The results were expressed as the phagocytic index, which was defined as the number of attached and internalized yeast cells per number of macrophages times 100. The respective averages from four experiments, standard deviations, and *P* values were calculated.

**Statistical analysis.** Statistical analysis was performed using Prism version 4 (GraphPad Software). The results were compared by *t* test.

## RESULTS

***C. neoformans* brown pigment production from homogentisic acid is laccase dependent.** *C. neoformans* yeast cells (wild-type B3501) grown in medium supplemented with homogentisic acid were brown (Fig. 1A). Since melanization in *C. neoformans*

is catalyzed by laccase, we investigated whether this enzyme was involved in HGA-derived pigment production. No pigment production was observed after incubation of the *C. neoformans* 2E-TU Lac<sup>-</sup> mutant in minimal medium supplemented with HGA (Fig. 1B). In contrast, incubation of the *C. neoformans* 2E-TU mutant complemented with laccase (2E-TUC) or of the wild-type strain resulted in brown pigment production (Fig. 1A and C). Hence, brown pigment production was dependent on fungal laccase, and since laccase expression is repressed by glucose, we hypothesized that the glucose concentration in the medium would affect pigment induction. Consistent with this premise, pigment production was inhibited at glucose concentrations above 20 g/liter (Fig. 1). The same results were observed irrespective of whether the experiment was done in liquid or on agar plates.

Pigment production required live *C. neoformans* cells (data not shown). No differences in pigment induction were observed between different serotypes of *C. neoformans* (data not shown). Finally, pigment was observed not only in the yeast cells but also in culture medium (Fig. 2). To test if the HGA precursor *p*-hydroxyphenylpyruvate induced brown pigmentation in *C. neoformans*, yeast cells were grown in minimal medium supplemented with either 1, 10, or 50 mM *p*-hydroxyphenylpyruvate at 22°C for 5 days. No pigment induction was observed when *C. neoformans* cells were grown with this compound (data not shown).

**The brown pigment is a melanin.** Characteristics of melanin are acid resistance and insolubility in aqueous solvents. Since melanin particles can be recovered from melanized cells with L-dopa by a method that involves digestion with cell wall-lytic enzymes, denaturant extraction, and hot acid treatment, we evaluated whether similar particles could be recovered from brown cells induced by growth in minimal medium supplemented with HGA. Treatment of brown cells sequentially with enzymes, guanidinium isothiocyanate, and boiling 6 M HCl led to the recovery of spherical particles similar to those recovered from L-dopa-melanized cells, although the particles from the brown cells were significantly smaller (Fig. 3).

Treatment of brown-pigmented *C. neoformans* cells with 100% and 10% bleach led to the complete disappearance of color. However, *C. neoformans* cells melanized with HGA were

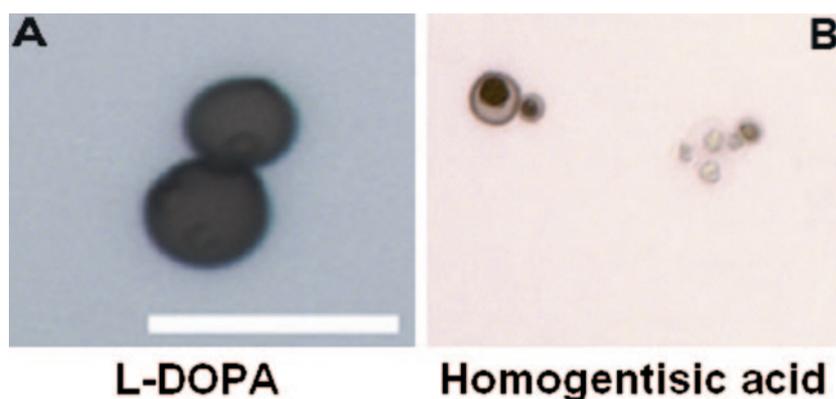


FIG. 3. Melanin particles from *C. neoformans* H99 cells after treatment with HCl. (A) Positive control. Particles were recovered from H99 cells grown in SM medium with L-dopa. (B) Particles recovered from H99 cells grown in SM medium supplemented with 1 mM of HGA. Scale bar represents 10  $\mu\text{m}$  for both panels.

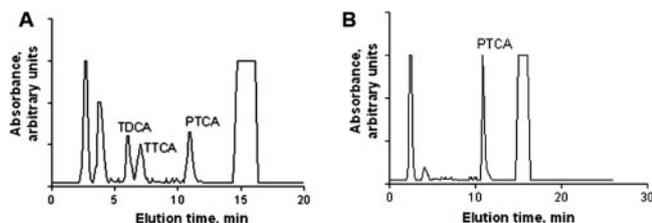


FIG. 4. HPLC chromatograms of melanin particles of HGA- (A) and L-dopa-grown (B) *C. neoformans* after treatment with HCl. The results show the presence of peaks with retention times matching those of TDCA, TTCA, and PTCA in HGA melanin particles and that of an exclusive PTCA product in L-dopa melanin.

resistant to degradation by organic solvents and alcohols, such as 1-butanol, methanol, chloroform, 1-butanol:chloroform (1:1, vol/vol), methanol:chloroform (1:1, vol/vol), acetone, carbon tetrachloride, *N,N*-dimethyl formamide, and toluene. On the basis of the susceptibility of the brown pigment to bleaching, its resistance to acid, and its insolubility in aqueous and organic solvents, we tentatively identified the HGA-derived brown pigment as a melanin.

To explore the biochemical composition of the brown pigment, the material was oxidized with acidic permanganate and the products were analyzed by HPLC. The chromatogram of oxidized products from brown cells revealed three peaks, eluting at 6.1, 7.1, and 11.0 min with retention times that matched those of TDCA, TTCA, and PTCA, respectively (Fig. 4A). The chromatographic results are consistent with the presence of melanin degradation products in permanganate-oxidized particles. In contrast, oxidation of melanin particles derived from L-dopa-melanized *C. neoformans* cells revealed the presence only of a PTCA component characteristic of eumelanin (Fig. 4B).

Since melanization is known to impart an additional negative charge to *C. neoformans* cells, we evaluated the zeta potential of brown-pigmented cryptococcal cells and particles. Melanin particles from cells grown in minimal medium supplemented with HGA manifested a significantly higher negative charge ( $-48.90 \pm 1.63$  mV) than melanin particles from cells grown in minimal medium supplemented with L-dopa ( $-35.93 \pm 0.93$  mV) ( $P < 0.05$ ).

**Fluorescence of HGA-derived melanins.** Melanin particles recovered from brown cells were evaluated for their fluorescence properties under different wavelengths. Fluorescence was observed when the particles were irradiated with light of 420, 535, and 610 nm (Fig. 5). In contrast, melanin particles from cells melanized by growth in L-dopa did not fluoresce (data not shown). Additionally, intact pigmented *C. neoformans* cells grown in minimal medium with HGA did not fluoresce (data not shown).

***C. neoformans* consumes HGA to produce melanin.** HPLC analysis of *C. neoformans* culture supernatants in minimal medium supplemented with HGA was used to establish whether there was a correlation between the metabolism of HGA in solution and the appearance of brown pigmentation in *C. neoformans*. The chromatograms of the HGA standard revealed a main peak eluting at 9.76 min. As culture incubation times increased, the HPLC peak corresponding to HGA diminished

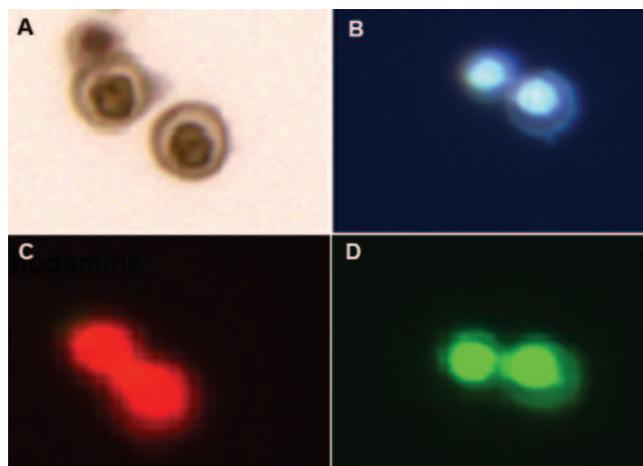


FIG. 5. Fluorescence of particles recovered after HCl treatment from *C. neoformans* H99 cells grown in SM medium supplemented with 1 mM of HGA, shown by (A) light microscopy, (B) fluorescence under a DAPI (4',6'-diamidino-2-phenylindole) filter, (C) fluorescence under a rhodamine filter, and (D) fluorescence under a fluorescein isothiocyanate filter.

in size, which was consistent with utilization of the compound by the melanizing fungal cells. (Fig. 6).

***C. neoformans* melanin particles induced by HGA showed the presence of stable free radicals.** The acid-resistant melanin particles isolated from *C. neoformans* cells grown in minimal medium supplemented with HGA produced an EPR signal indicative of a stable free radical population (Fig. 7). L-Dopa melanin particles used as positive controls showed the same signal as the melanin particles induced by HGA. Nonmelanized cells used as a negative control did not produce signal (data not shown).

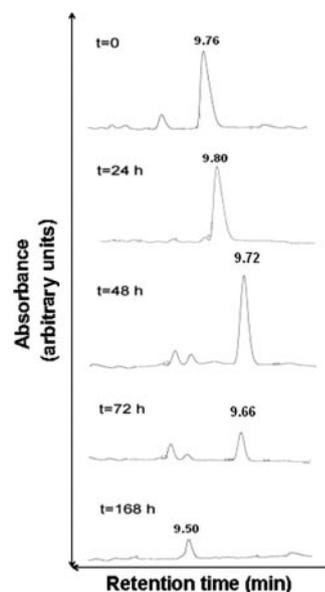


FIG. 6. Analysis by HPLC of *C. neoformans* supernatant grown in SM medium supplemented with 1 mM of HGA. The results show the presence of HGA at 9.76 min and its consumption with the passage of time. The substrate is consumed by *C. neoformans* to produce melanin.

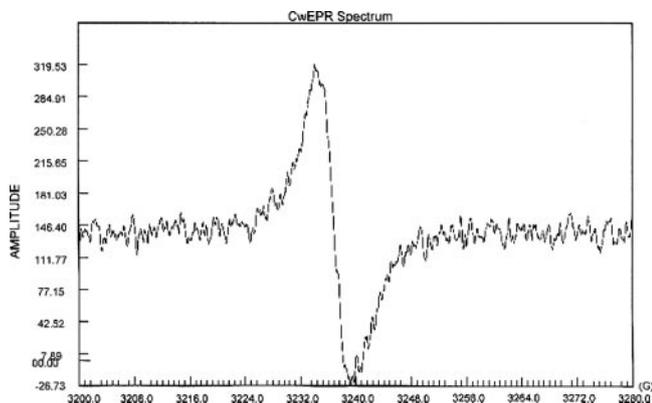


FIG. 7. Electron paramagnetic resonance spectrum reveals a signal at 3,240 G, showing the presence of a stable free radical population. CwEPR, continuous-wave EPR.

**Visualization, measurement, and statistical analysis of melanin particles.** Melanin particles made from L-dopa or HGA were sectioned and viewed by transmission electron microscopy (Fig. 8A and B). The particle diameters and thicknesses of the ghost shells were measured. Ghost particles from L-dopa had diameters similar to those of the parental cells, but the HGA particles showed a significant reduction in size ( $P < 0.05$ ) (Fig. 8C). The thicknesses of the shells in the HGA particles were significantly less than those of L-dopa ghost particles ( $P < 0.05$ ) (Fig. 8D).

**Susceptibilities of melanized and nonmelanized cells to UV radiation.** To study whether cells melanized with L-dopa or

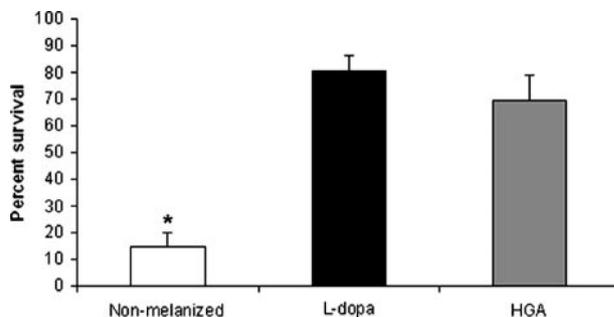


FIG. 9. Percent survival after UV radiation of *C. neoformans* cells melanized with HGA or L-dopa and nonmelanized cells. Bars represent the averages from five measurements each. Error bars indicate standard deviations. \*,  $P < 0.05$  compared with melanized cells.

HGA were susceptible to UV light, 8-day-old melanized cells were exposed to UV radiation (Fig. 9). *C. neoformans* cells grown in the presence of HGA were significantly less susceptible to the fungicidal effect of UV radiation than were non-melanized cells ( $P < 0.05$ ). The differences in susceptibilities between L-dopa- and HGA-melanized cells were not significant ( $P = 0.06$ ).

**Effect of melanin on macrophage phagocytosis of *C. neoformans*.** To study the effect of melanin on phagocytosis, J744 macrophages were incubated with melanized (HGA or L-dopa) and nonmelanized *C. neoformans* cells in the presence and absence of capsule-binding monoclonal antibody 18B7. In the absence of 18B7, there was no phagocytosis or difference in the phagocytic indexes (data not shown). Phagocytosis of HGA-

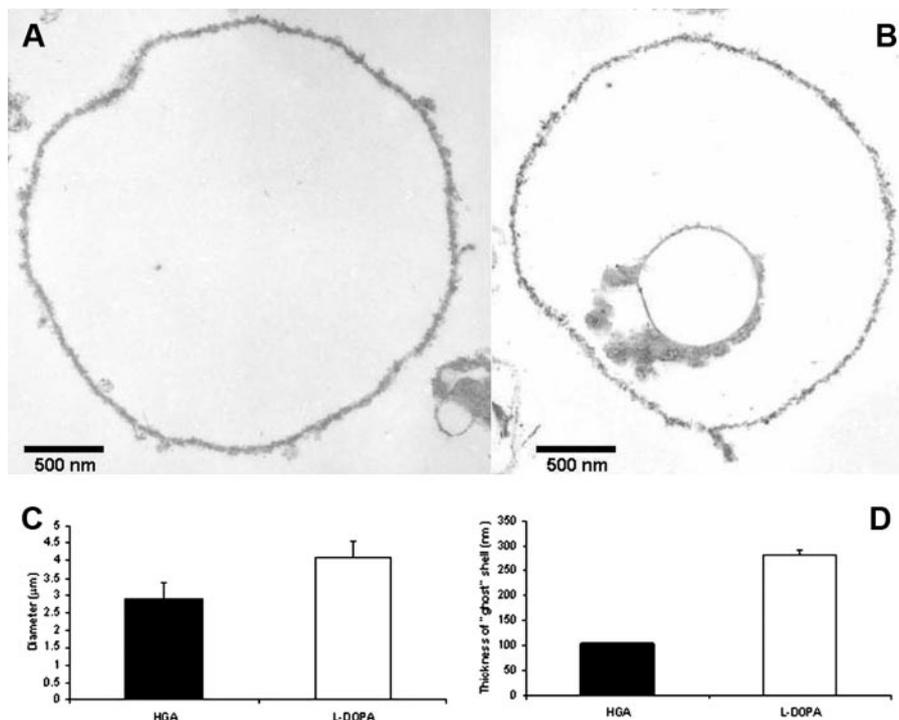


FIG. 8. (A and B) Transmission electron microscopy of melanin particles obtained from H99 cells of *C. neoformans* grown in minimal medium supplemented with 1 mM of HGA. The diameters (C) and thicknesses (D) of the shells of melanin particles were averaged from 200 particles (from each substrate) measured. Error bars indicate standard deviations.

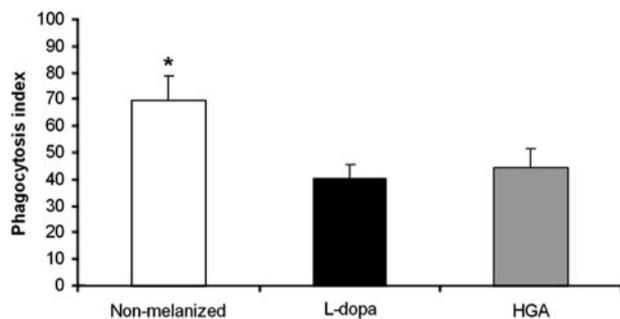


FIG. 10. Index of phagocytosis by J774.16 cells of *C. neoformans* cells melanized with HGA or L-dopa and nonmelanized cells. Bars represent the averages of the results from four experiments for each. Error bars indicate standard deviations. \*,  $P < 0.05$  compared with melanized cells.

melanized cells in the presence of 18B7 was significantly less than that of nonmelanized cells ( $P < 0.05$ ) (Fig. 10). There were no significant differences in the phagocytosis indexes of L-dopa- and HGA-melanized cells ( $P = 0.3$ ).

## DISCUSSION

In this study, we analyzed the effect of homogentisic acid on pigment production in *C. neoformans*, since this substrate produces melanin pigment in a wide variety of organisms (9). Growth of *C. neoformans* in medium supplemented with HGA resulted in brown pigmentation of fungal colonies. This phenomenon was observed with *C. neoformans* strains representing all serotypes and required viability of fungal cells. Pigment production required expression of *C. neoformans* laccase and was inhibited by glucose concentrations higher than 20 g/liter, consistent with the fact that laccase expression is glucose repressible (8). Since *C. neoformans* can produce melanin from diverse compounds, including catecholamines such as L-dopa, dopamine, epinephrine, and norepinephrine, but not tyrosine, we investigated whether or not *C. neoformans* could produce pigment from an HGA precursor (*p*-hydroxyphenylpyruvate). Like the situation for the catecholamines, the precursor compound for HGA is unable to sustain melanogenesis in *C. neoformans*.

HGA is a precursor of melanin for many types of melanogenic bacteria. Several lines of evidence indicate that the brown pigment synthesized by *C. neoformans* from HGA is a melanin. First, brown pigment particles were insoluble in aqueous or acidic solutions yet were susceptible to oxidation by permanganate. Second, HPLC analysis of permanganate oxidation products revealed that the compounds with chromatographic migration matched the migrations of the melanin degradation products TDCA, TTCA, and PTCA. Third, pigment production required the presence of laccase, which catalyzes the first step of melanin production in *C. neoformans*. Fourth, the EPR spectrum revealed that the brown pigment showed a signal indicative of a stable free radical population that is characteristic of melanins. Hence, we conclude that HGA can also serve as a precursor for melanin synthesis in fungi.

Nevertheless, there were significant differences between melanin produced from HGA and the classic black pigments produced from L-dopa and other catecholamines. The finding

of compounds with chromatographic properties similar to those of TDCA, TTCA, and PTCA among HGA oxidative-degradation products contrasts with the solitary presence of PTCA in L-dopa-derived melanin. Although one cannot infer the presence of these compounds in HGA oxidative-generation products on the basis of chromatographic migration alone, the presence of additional oxidative products implies different molecular structures for HGA and L-dopa-derived melanins. The diameters and the thicknesses of the shells of the particles recovered from the HGA-pigmented cells were less and the particles were more negatively charged than L-dopa-derived particles. Furthermore, HGA-derived particles were fluorescent when illuminated with several wavelengths, while no fluorescence is produced by similarly irradiated L-dopa particles. Since melanization in *C. neoformans* is dependent on the exogenous substrate and the type of melanin formed reflects the chemical structure of the substrate (6), the differences between the brown-pigmented particles derived from cells grown with HGA and the black particles derived from cells grown in L-dopa implied significant differences in the chemical structures. These differences probably reflect variances in the precursor compounds, but they do not result in different effects on phagocytosis or susceptibility to UV radiation.

Despite the biochemical and biophysical differences apparent from the comparison of the HGA- and L-dopa-derived pigment particles, HGA- and L-dopa-derived pigments conferred remarkably similar photoresistant and antiphagocytic properties to *C. neoformans* cells. Hence, the HGA- and L-dopa-derived pigments appeared to be functionally similar and suggest that precursor compounds for melanogenesis are relatively interchangeable with regard to the function of the final product.

The fact that *C. neoformans* can utilize HGA for melanin synthesis indicates that this compound can be used as a precursor of melanization in both eukaryotes and prokaryotes. Given that HGA is involved in bacterial, animal, and plant metabolism, this result implies a new source for *C. neoformans* melanization substrate both in the environment and during animal infection. From the vantage point of *C. neoformans* biology, the finding that HGA is a precursor for melanization could help significantly to explain the paradox that this organism has the machinery for melanization and for remodeling its cell wall in the melanized state yet is unable to make melanin unless provided with certain substrates. Although some *Aspergillus* spp. can synthesize HGA (15), there is no evidence that *C. neoformans* can use endogenous compounds for melanization, even if they are available, since the fungus makes no pigments without exogenous substrates. For *C. neoformans*, the capacity for scavenging chemical compounds made by other microbes suggests that this organism gains the benefit of melanization by having the machinery for melanin synthesis and remodeling without having to invest in the biosynthetic pathways required to generate precursor compounds.

In summary, we report that HGA can induce melanization of *C. neoformans* by a fungal laccase. Although the effect was observed only in laboratory conditions, its demonstration establishes a precedent for a new substrate for melanization in *C. neoformans* that could provide a new source for melanin substrates in the environment, since HGA is a ubiquitous microbial product that is also found in arboreal species and animals.

## ACKNOWLEDGMENTS

Funding for this project was provided by NIH awards R21GM071421 and AI052733-03.

We thank Gary Gerfen, Marta Feldmesser, and Joshua Nosanchuk for discussions and assistance with various aspects of this work.

## REFERENCES

1. Casadevall, A., and J. R. Perfect. 1998. *Cryptococcus neoformans*. ASM Press, Washington, DC.
2. Chaskes, S., and R. L. Tyndall. 1978. Pigment production by *Cryptococcus neoformans* and other *Cryptococcus* species from aminophenols and diamino-benzenes. *J. Clin. Microbiol.* **7**:146–152.
3. Chaskes, S., and R. L. Tyndall. 1975. Pigment production by *Cryptococcus neoformans* from *para*- and *ortho*-diphenols: effect of the nitrogen source. *J. Clin. Microbiol.* **1**:509–514.
4. Enochs, W. S., M. J. Nilges, and H. M. Swartz. 1993. A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. *Pigm. Cell Res.* **6**:91–99.
5. Frases, S., S. Chaskes, E. Dadachova, and A. Casadevall. 2005. Induction by *Klebsiella aerogenes* of a melanin-like pigment in *Cryptococcus neoformans*. *Appl. Environ. Microbiol.* **72**:1542–1550.
6. Garcia-Rivera, J., H. C. Eisenman, J. D. Nosanchuk, P. Aisen, O. Zaragoza, T. Moadel, E. Dadachova, and A. Casadevall. 2005. Comparative analysis of *Cryptococcus neoformans* acid-resistant particles generated from pigmented cells grown in different laccase substrates. *Fungal Genet. Biol.* **42**:989–998.
7. Hamilton, A. J., and B. L. Gomez. 2002. Melanins in fungal pathogens. *J. Med. Microbiol.* **51**:189–191.
8. Jacobson, E. S., and H. S. Emery. 1991. Temperature regulation of the cryptococcal phenoloxidase. *J. Med. Vet. Mycol.* **29**:121–124.
9. Kotob, S. I., S. L. Coon, E. J. Quintero, and R. M. Weiner. 1995. Homogenitic acid is the primary precursor of melanin synthesis in *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. *Appl. Environ. Microbiol.* **61**:1620–1622.
10. Kwon-Chung, K. J., T. Boekhout, J. W. Fell, and M. Diaz. 2002. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). *Taxon* **51**: 804–806.
11. Nosanchuk, J. D., J. Rudolph, A. L. Rosas, and A. Casadevall. 1999. Evidence that *Cryptococcus neoformans* is melanized in pigeon excreta: implications for pathogenesis. *Infect. Immun.* **67**:5477–5479.
12. Nosanchuk, J. D., and A. Casadevall. 2003. The contribution of melanin to microbial pathogenesis. *Cell. Microbiol.* **5**:203–223.
13. Salas, S. D., J. E. Bennett, K. J. Kwon-Chung, J. R. Perfect, and P. R. Williamson. 1996. Effect of the laccase gene CNLAC1, on virulence of *Cryptococcus neoformans*. *J. Exp. Med.* **184**:377–386.
14. Sorell, T. C., and D. H. Ellis. 1997. Ecology of *Cryptococcus neoformans*. *Rev. Iberoam. Micol.* **14**:42–43.
15. Ueno, T., F. Yoshizako, and A. Nishimura. 1973. The formation of homogenitic acid from phenylacetic acid by an *Aspergillus* sp. *Can. J. Microbiol.* **19**:393–395.
16. Williamson, P. R. 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J. Bacteriol.* **176**:656–664.