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Induction by *Klebsiella aerogenes* of a Melanin-Like Pigment in *Cryptococcus neoformans*

Susana Frases,[1] Stuart Chaskes,[2] Ekaterina Dadachova,[3] and Arturo Casadevall[1,4]*

Department of Microbiology and Immunology,[1] Department of Medicine,[4] and Department of Nuclear Medicine,[3] Albert Einstein College of Medicine, Bronx, New York 10461, and Department of Biology, Farmingdale State University of New York, Farmingdale, New York 11735[2]

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While studying the interaction of *Cryptococcus neoformans* with *Dictyostelium discoideum*, we noticed that yeast colonies in agar with a feeder lawn of *Klebsiella aerogenes* were brown. This finding was intriguing because *C. neoformans* colonies are not pigmented unless they are provided with precursors for melanization. Strains of all *C. neoformans* serotypes produced brown pigment in response to *K. aerogenes* at 22, 30, and 37°C. Pigment production required fungal laccase and was suppressed by high concentrations of glucose. Treatment of brown cells with guanidinium isothiocyanate and hot concentrated HCl yielded particulate material that had the physical and chemical characteristics of melanins. No pigment formation was observed when *C. neoformans* was exposed to live *Escherichia coli* or heat-killed *K. aerogenes*. Analysis of *K. aerogenes* supernatants revealed the presence of dopamine, which can be a substrate for melanin synthesis by *C. neoformans*. Our findings illustrate a remarkable interaction between a pathogenic fungus and a gram-negative bacterium, in which the bacterium produces a substrate that promotes fungal melanization. This observation provides a precedent that could explain the source of a substrate for *C. neoformans* melanization in the environment.

The realization that the virulence of certain fungi for mammalian hosts may originate and be maintained by interactions with other microbes has stimulated interest in the study of fungus-protozoan, fungus-slime mold, and fungus-bacterium interactions (1, 8, 19). *Cryptococcus neoformans* is a free-living cosmopolitan organism that can survive in a variety of environmental niches. This fungus causes cryptococcosis, a disease that is relatively common in individuals with immune suppression. *C. neoformans* strains have been divided into three varieties based on biochemical and genetic differences, which are designated *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, and *C. neoformans* var. *gattii* (5), and recent evidence suggests that the phylogenetic distance between these varieties is sufficient to identify them as distinct species (12). *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 tree species (2, 5).

Like many fungi, *C. neoformans* can produce melanins, but pigment synthesis 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 from tyrosine (3, 4, 22). The cryptococcal enzyme exhibits high substrate activity with catecholamines, which may contribute to the neurotropism of *C. neoformans* (23). The three types of melanins that have been described in fungal pathogens are eumelanins formed from quinines and free radicals, phaeomelanins derived from tyrosine and cysteine, and allomelanins formed from nitrogen-free precursors (7). *C. neoformans* produces eumelanin. *C. neoformans* cells in the environment, particularly in pigeon excreta, are melanized, but the environmental source of melanin precursors is unknown (16). Melanin synthesis is associated with reduced fungal susceptibility to a variety of insults that this organism may encounter in the environment, including predation by amoebae, UV light, extremes of temperature, and heavy metals (15). Although melanization may have developed in response to environmental pressures, the ability to make melanin pigments is associated with virulence in mammalian hosts (15).

C. neoformans colonies are normally white or creamy on routine laboratory agar. While carrying out studies of the interaction of *Dictyostellium discoideum* and *C. neoformans* in a lawn of feeder *Klebsiella aerogenes*, we noticed that fungal colonies in the proximity of bacterial colonies were brown. Since pigment production by *C. neoformans* requires the presence of a substrate for laccase, we found this phenomenon intriguing and studied it further. Here we report that *K. aerogenes* can metabolize different substrates into products that can subsequently be used for pigment production by *C. neoformans. K. aerogenes* has a membrane-bound tyramine oxidase encoded by the gene *tynA* that is induced by specific monoamine compounds, such as tyramine and catecholamines (19). This enzyme catalyzes the oxidation of tyramide and catecholamines to hydroxyphenylacetaldehyde compounds and ammonium ions and is implicated in the regulation of arylsulfatase synthesis. These observations suggest that certain bacterial species may serve as sources of substrates for melanization of *C. neoformans* in the environment.

* 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.

MATERIALS AND METHODS

Media. SM medium is composed of 10 g/liter glucose, 10 g/liter proteose peptone, 1 g/liter yeast extract, 1 g/liter $MgSO_4$, 1.9 g/liter KH_2PO_4, and 0.6

g/liter K_2HPO_4. SM agar is made by adding 20 g/liter of agar to SM medium. Sabouraud dextrose broth (Difco, Sparks, Md.) is composed of 10 g/liter enzymatic digest of casein and 20 g/liter dextrose. Sabouraud dextrose agar (Difco) is composed of 10 g/liter casein and 40 g/liter of dextrose with 20 g/liter agar. Luria broth (LB) (Difco) contains 10 g/liter casein, 5 g/liter yeast extract, and 10 g/liter NaCl. Minimal medium consists of 13.6 g/liter KH_2PO_4, 0.02 g/liter $CaCl_2$, 0.001 g/liter $Fe(NH_4)_2(SO_4)_2$, 0.02 g/liter $MgCl_2$, and 1 g/liter glucose.

Bacterial strains and growth conditions. *K. aerogenes* was obtained from Richard Kessin (New York, NY), *Escherichia coli* ATCC 25928 and *Enterobacter cloacae* ATCC 13047 were purchased from ATCC (Manassas, VA). *Klebsiella pneumoniae*, *Enterobater aerogenes*, and *Serratia marcescens* were obtained from John Slattery (Garden City, NY). Bacterial strains were grown in LB at 37°C with rotary shaking at 150 rpm.

Fungal strains and growth conditions. *C. neoformans* serotype A strain H99 was a gift from John Perfect (Durham, NC). Serotype D strain B3501 was purchased form ATCC (Manassas, VA). Serotype AD strain MAS93-120 was provided by Mary Brandt (Atlanta, GA). A serotype B strain (NIH198) and serotype C strains 1343 and B 4546 were obtained from Thomas Mitchell (Durham, NC). In addition, we also used laccase-negative mutant 2E-TU (URA5 Mel^-) of strain B3501 and its laccase-complemented derivative strain 2E-TUC (URA5 Mel^+) (18). Other fungal species evaluated for pigment production in response to bacteria were *Candida albicans* (SC 5314), *Sporothrix schenckii* (ATCC 14285), and *Histoplasma capsulatum* (CIB 1980) obtained from Joshua D. Nosanchuk (Bronx, NY). In the presence of L-3,4-dihydroxyphenylalanine (L-DOPA), *H. capsulatum* and *S. schenckii* are pigmented due to the production of melanin in their cell walls (13), whereas the cell walls of *C. albicans* lack melanin (14). Most fungal strains were grown in Sabouraud dextrose broth at 30°C with rotary shaking at 150 rpm; the exception was *H. capsulatum*, which was grown at 37°C to maintain a yeast form.

Agar assay for pigment induction. To induce pigment production in *C. neoformans*, H99 cells were grown in SM medium at 22°C for 5 days adjacent to a lawn of *K. aerogenes* cells. As a control, *K. aerogenes* and *C. neoformans* were each grown alone in SM medium plates. In a similar manner, *C. neoformans* was plated with killed bacteria. Bacteria were grown at 22°C for 5 days and then killed by incubation at 65°C for 1 h. In another experiment, the yeast cells were killed by incubation at 65°C for 1 h and then exposed to bacteria in liquid SM medium to determine if pigment formation required fungal viability.

To analyze whether pigment induction was due to a diffusible component produced by bacteria, 10^4 cells/ml of both organisms were grown in 25 ml of SM medium. Bacterial cells (25 ml of a culture containing 10^4 cells/ml) were inoculated into a dialysis bag (12,000- to 14,000-molecular-weight cutoff), and fungal cells (100 μl of a culture containing 10^4 cells/ml) were inoculated on top of a moist 0.45-μm membrane filter (Millipore, Billerica, MA) that was placed over the dialysis bag; the filter was able remain moist by wicking fluid from inside the dialysis bag. Dialysis bags containing L-DOPA, dopamine, and SM medium were used as controls. The samples were incubated for 5 days at 22°C in a sterile humid chamber and inspected for color changes.

Measurement of pigment accumulation in supernatants of liquid cultures. To measure fungus-derived pigment accumulation in liquid cultures, 10^4 cells/ml of *C. neoformans* and *K. aerogenes* were incubated in liquid SM medium at 22°C for 5 days, and the medium was collected by centrifugation and filtered. The absorbance spectrum was determined, and the maximal signal was observed at a wavelength of 405 nm. The absorbance was compared with the signals obtained with media from cultures containing only fungal cells or only bacterial cells.

Isolation of melanin particles. *C. neoformans* cells pigmented by exposure to *K. aerogenes* in liquid SM medium grown 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), and protoplasts were generated by overnight incubation at 30°C in a solution containing 10 mg/ml of cell wall-lysing enzymes (*Trichoderma harzianum*; Sigma Chemical Co., St. Louis, MO). The protoplasts were 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 and washed with PBS, and then it was treated with 1.0 mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN). The material was washed 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 procedure were visualized by microscopy for size measurement after heat fixation on glass slides using an Olympus (Melville, NY) AX 70 microscope equipped with fluorescence filters.

Additionally, the particles obtained from brown cells after treatment with enzymes and hot acid denaturant were visualized using fluorescence microscopy. Melanin particles obtained from *C. neoformans* cells grown with L-DOPA were used as a positive control.

TLC. Supernatants from cultures in which pigment was produced were concentrated with acetonitrile and analyzed by thin-layer chromatography (TLC). TLC was carried out in flexible plates (20 by 20 cm) containing Silica Gel 60 (F254; 200 μm; Selecto Scientific, Georgia). Different aliquots of samples were treated with KH_2PO_4 (pH 4.4) at a concentration of 0.4 g/liter and organic solvents, such as ethanol–1-butanol–water (4:1:1) and 1-butanol–acetid acid–water (60:30:10). The flow rate (R_f) was measured.

HPLC of oxidized melanins. The melanin ghosts derived from H99 cells melanized by growth with *K. aerogenes*, L-DOPA, and dopamine were oxidized with permanganate and analyzed by high-performance liquid chromatography (HPLC). The supernatants of these cultures were also analyzed for the presence of compounds produced during microbial growth. Samples were dried at 50°C and oxidized with acidic permanganate. Pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA), 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA), and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) were used as standard compounds for melanin degradation products (gifts from K. Wakamatsu, Toyoake, Japan). Chromatograms of PDCA, 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_{18} column (250 by 4.1 mm; particle size, 7 μm), and a Shimadzu SPD-6AV UV detector. The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). At a flow rate of 1.0 ml/min, the elution gradient was as follows (min, %B): for zero time and 1 min, 100% solvent A; for 12 min and 14 min, 75% solvent A and 25% solvent B; and for 16 min, 100% solvent A. The UV detector was set to absorbance at 255 nm.

HPLC of *K. aerogenes* cell culture supernatants. A *K. aerogenes* culture in LB was incubated at 37°C with shaking for 5 days. Supernatants from bacterial cultures were analyzed at 24, 48, 72, 96, and 120 h by HPLC using a Waters 600 liquid chromatograph (New York, NY), an Alltech C_{18} column (Deerfield, IL) (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 potassium phosphate buffer (pH 3.1) at a flow rate of 1.0 ml/min. The standards consisted of 50 mM solutions of catecholamines (L-DOPA, norepinephrine, and epinephrine; Sigma) and a 2.4 mM solution of L-tyrosine (Sigma).

Mass spectrometry. Supernatants from bacterial cultures were examined by HPLC using a Waters 600 liquid chromatograph as described above, except that the mobile phase was 25 mM ammonium bicarbonate (pH 7.4) and the flow rate was 1.0 ml/min. The fractions that eluted from the HPLC were dried and reconstituted in aqueous formic acid (0.1%) containing 50% acetonitrile. Samples were analyzed using an Applied Biosystems QSTAR XL mass spectrometer (Foster City, CA). Mass spectrometry-mass spectrometry was performed to confirm the results. Fractions that eluted from the HPLC were added to SM agar plates in the presence of *C. neoformans*.

Zeta potential. A suspension of 10^7 cells/ml was prepared in 10 mM KCl, and the zeta potential of pigmented cells was determined with a zeta potential analyzer (Brookhaven Instrument Corporation, Moltsville, NY).

Effects of amino acids on pigment induction. The effects of various amino acids on pigment formation at 22, 30, and 37°C were evaluated in minimal medium. The amino acids evaluated were L-tyrosine, L-proline, L-glutamic acid, and L-asparagine at a concentration of 0.2%. In addition, the effect of 2% urea was evaluated. In a second experiment, the effects of 10, 5, 1, and 0.5 mM L-tyrosine on pigment formation were evaluated.

RESULTS

Induction by *K. aerogenes* of a brown pigment in *C. neoformans*. *C. neoformans* yeast cells growing adjacent to *K. aerogenes* lawns on SM agar developed brown pigmentation, and the intensity of the color was directly proportional to the proximity to the bacterial cells (Fig. 1A). Induction of the brown color in *C. neoformans* did not require physical contact between the bacterial and fungal cells. Growth of *C. neoformans* on SM agar plates without bacteria produced white colonies. The fact that contact was not needed for brown pigmentation of *C. neoformans* in SM agar plates implied that fungal cell pigment synthesis was caused by a diffusible and soluble bacterial product. In support of this, pigment production also

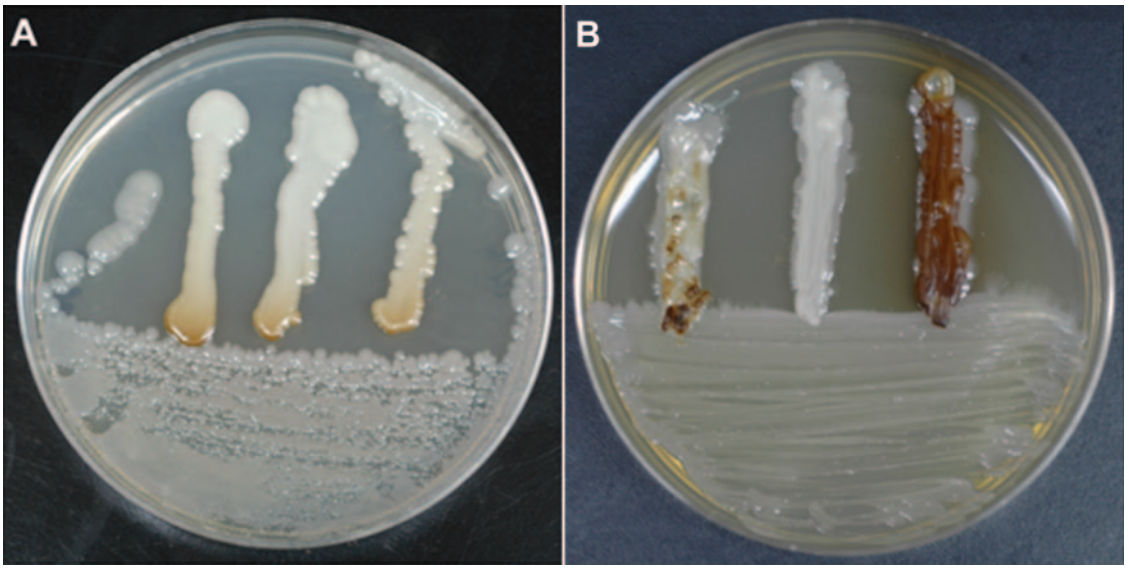

FIG. 1. (A) Plate containing *C. neoformans* strain H99 streaked perpendicular to a *K. aerogenes* lawn in SM medium. Pigmentation occurred without contact between the different organisms and was greatest in the *C. neoformans* area closest to *K. aerogenes*. (B) Plate containing (from left to right) *C. neoformans* streaks of laccase-complemented mutant strain 2E-TUC, laccase-negative mutant strain 2E-TU, and parental strain B3501 with a bacterial lawn on SM medium. The plates were incubated at 22°C for 5 days.

occurred in fungal cells when the fungi grew separated from the bacteria by a dialysis membrane and a filter (0.45 μm) (Fig. 2). Growth of *C. neoformans* on SM agar plates coated with dead bacteria did not induce pigment production. Similarly, pigment production occurred only in live *C. neoformans* cells. No differences in pigment induction were observed between different serotypes of *C. neoformans*. Finally, pigmentation not only was observed in the yeast cells but also occurred in the culture medium (Fig. 3).

Conditions for pigment induction. Pigment induction was dependent on the medium used. When *C. neoformans* and *K. aerogenes* were grown in the same culture, pigmentation of *C. neoformans* was observed in LB and SM medium but not in Sabouraud dextrose broth or agar. These media have different

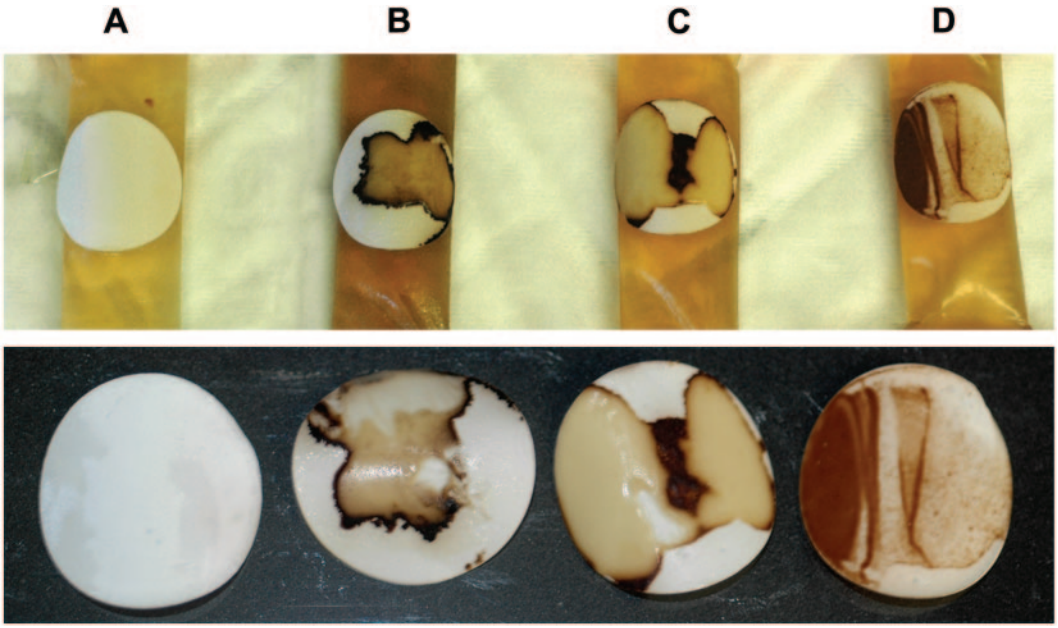

FIG. 2. Pigment production in filters containing H99 cells induced by placement over dialysis bags containing *K. aerogenes* or different substrates. (A) Filter over a dialysis bag containing SM medium (negative control). (B) Filter over a dialysis bag containing L-DOPA. (C) Filter over a dialysis bag containing dopamine in SM medium. (D) Filter over a dialysis bag containing *K. aerogenes* in SM medium. The photographs were taken after 5 days of coculture at 22°C.

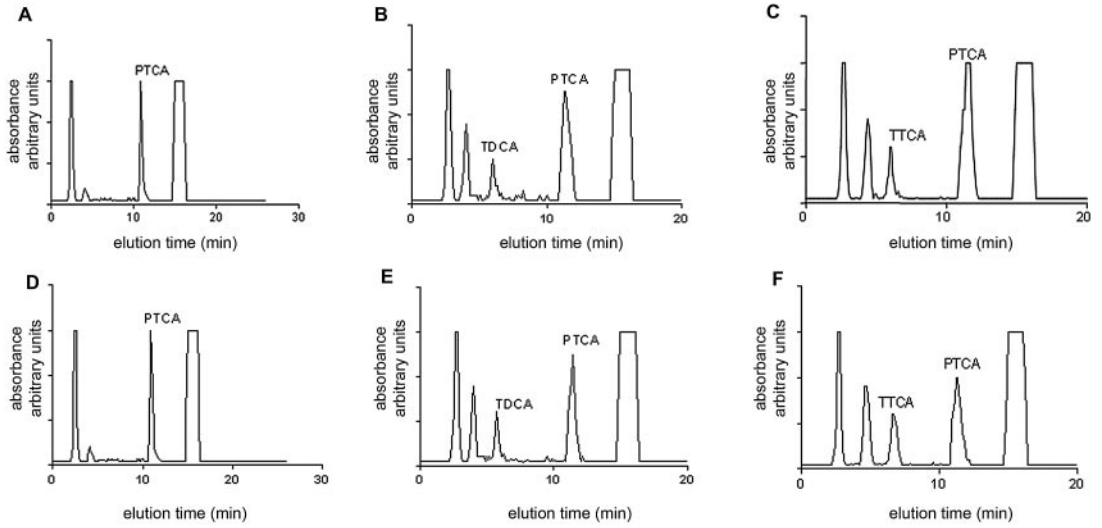

FIG. 3. Chromatograms of products from oxidized melanin particles (A, B, and C) and lyophilized pigmented supernatants (D, E, and F). (A and D) Chromatograms for oxidized brown particles recovered from *C. neoformans* cells grown in a bacterial culture after enzyme and hot HCl treatment and bacterium-fungus culture supernatant-associated pigment, respectively. (B and E) Chromatograms for oxidized black particles recovered from *C. neoformans* cells grown in the presence of L-DOPA after enzyme and hot HCl treatment and supernatant-associated pigment, respectively. (C and F) Chromatograms for oxidized particles recovered from *C. neoformans* grown with dopamine after enzyme and hot HCl treatment and supernatant-associated pigment, respectively. The elution time was measured in minutes, and the absorbance was measured in arbitrary units. All elution profiles contained a peak that eluted at the same time as PTCA, which is a component of eumelanins.

glucose concentrations (0, 10, and 40 g/liter, respectively). Because we suspected that laccase was involved in this process (see below) and since laccase expression is repressed by glucose, we hypothesized that the glucose concentration in the medium affects pigment induction. Consequently, we prepared LB with different glucose concentrations (0, 10, 20, 30, and 40 g/liter) and observed that when the yeast and bacteria grew together in these media, glucose concentrations greater than 20 g/liter inhibited induction of the *C. neoformans* pigment (data not shown). The same results were observed when the experiment was done in liquid and agar plates.

Induction by other bacteria and fungi of pigment in *C. neoformans*. To investigate whether other bacteria promoted pigment production by *C. neoformans*, the fungus was grown in close association with *K. pneumoniae*, *E. coli*, *E. cloacae*, *E. aerogenes*, and *S. marcescens* on SM agar. None of these bacteria stimulated pigment formation by *C. neoformans* (data not shown). *C. albicans*, *S. schenckii*, and *H. capsulatum* were grown in SM medium in the presence of *K. aerogenes*, but these organisms did not produce pigmented colonies (data not shown).

Effect of L-tyrosine on pigment induction. *C. neoformans* can produce a melanin-like pigment from L-DOPA and dopamine, compounds that can be synthesized by *K. aerogenes* from L-tyrosine. Thus, we wanted to investigate if addition of this amino acid to fungal cells growing with *K. aerogenes* enhanced pigment production by the fungus. Consistent with this hypothesis, pigment formation was enhanced in the presence of L-tyrosine at concentrations higher than 0.5 mM. More pigmentation occurred at high concentrations of L-tyrosine (Fig. 4). No pigmentation occurred at concentrations less than 0.5 mM. Addition of L-tyrosine to *C. neoformans* in the absence of bacteria did not result in pigment production (data not shown). In these conditions pigment production was dependent on the temperature, and greater pigmentation was observed at the lower temperatures. In most comparisons the *P* value was <0.05; the exceptions were when the concentration of L-tyrosine was 0.5 mM at temperatures between 30 and 37°C (Fig. 4).

Induction of the brown pigment requires *C. neoformans* laccase. Since melanization in *C. neoformans* is catalyzed by laccase, we investigated the role of this enzyme in *K. aerogenes*-induced pigment production by comparing wild-type and laccase-deficient *C. neoformans* strains. No pigment production was observed after incubation of the 2E-TU Lac$^-$ mutant of *C. neoformans* with *K. aerogenes* (Fig. 1B). In contrast, incubation of the 2E-TU mutant complemented with laccase (2E-TUC) and *K. aerogenes* resulted in production of a brown pigment, albeit to a lesser extent than in parental strain B3501.

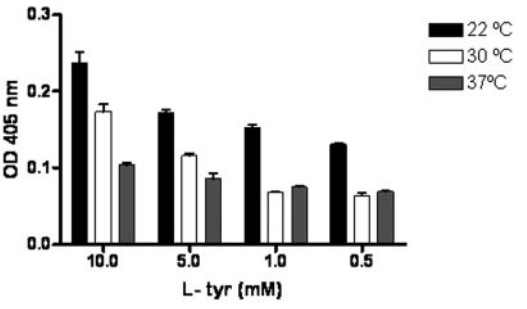

FIG. 4. Optical densities at 405 nm (OD 405 nm) of bacterial and *C. neoformans* H99 supernatants from cultures grown at different temperatures (22, 30, and 37°C) with 10, 5, 1, or 0.5 mM L-tyrosine. The initial number of yeast cells was 10^4 cells in 3 ml of culture, and the culture was incubated for 5 days. The *P* value was <0.05 in all cases except for the values for 30 and 37°C with 0.5 mM L-tyrosine.

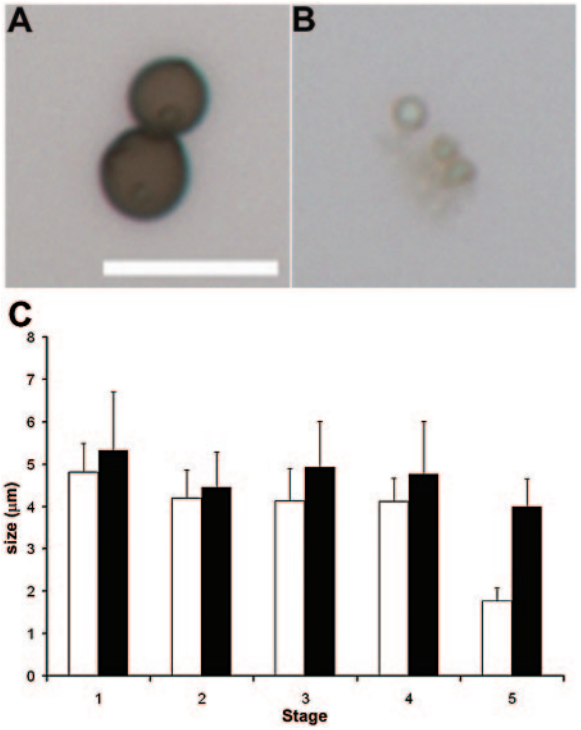

FIG. 5. Melanin particles isolated after serial enzyme and denaturing hot acid treatment from *C. neoformans* cells grown with L-DOPA (A) and brown-pigmented cells induced with *K. aerogenes* (B). Scale bar = 10 μm (for both panels). (C) Sizes of particles recovered from cells grown with L-DOPA (solid bars) and *K. aerogenes* (open bars) as a function of the step in the preparation protocol. Stage 1, cell size before the extraction procedure was started; stage 2, cell size after treatment with sorbitol-sodium citrate solution; stage 3, particle size after enzyme lysing treatment; stage 4, particle size after treatment with guanidinium; stage 5, particle size after incubation in HCl. The bars indicate the averages for 100 size measurements, and the error bars indicate the standard deviations.

Hence, production of the brown pigment was dependent on fungal laccase.

Brown pigment is a melanin. The characteristics of melanin include acid resistance and insolubility in aqueous solvents. Since melanin particles can be recovered from melanized cells, we evaluated whether similar particles could be recovered from brown cells induced by exposure to *K. aerogenes.* Brown cells were treated sequentially with enzymes, guanidinium isothiocyanate, and boiling 6 M HCl. This protocol yielded debris similar to the melanin particles typically isolated from *C. neoformans* grown with L-DOPA, which were derived from melanin deposited in the cell wall and retained the shape and size of the parent cells (21). However, compared to the melanin spheres derived from L-DOPA-melanized cells, the spheres isolated from brown cells were significantly smaller. To investigate the cause of this apparent disparity, we evaluated the particle size after each step of the melanin extraction protocol. The reduction in the size of the particles derived from brown cells occurred after the boiling in concentrated HCl (Fig. 5).

TABLE 1. Zeta potentials of melanin particles and cells of *C. neoformans* H99

Sample	Zeta potential (mV)[a]
Dopamine (melanin particles)	−40.32 ± 1.35
L-DOPA (melanin particles)	−35.93 ± 0.93
Brown particles (melanin particles)	−42.66 ± 2.31
H99 from Sabouraud	−26.14 ± 4.68
H99 brown colony	−31.80 ± 5.45
H99 with L-DOPA	−27.27 ± 3.45
H99 with dopamine	−30.30 ± 3.24

[a] The values are averages ± standard deviations for 10 measurements.

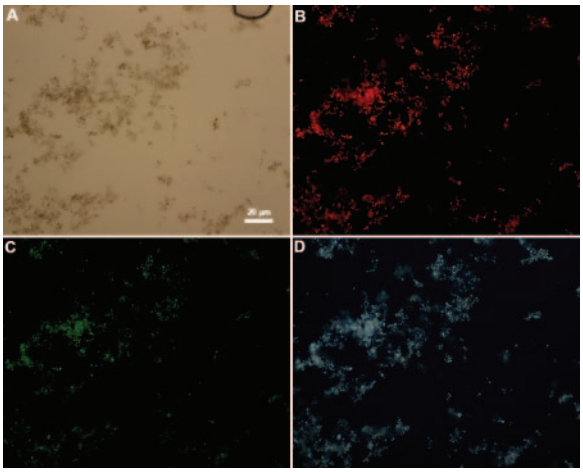

FIG. 6. Fluorescence of melanin particles obtained from brown colonies of *C. neoformans*. Scale bar = 20 μm. (A) Light microscopy. (B) Fluorescence at 610 nm. (C) Fluorescence at 535 nm. (D) Fluorescence at 420 nm.

Particles recovered from brown cells induced by *K. aerogenes* and lyophilized debris recovered from filtered pigmented supernatants were subjected to acidic permanganate oxidation and analyzed by HPLC for the presence of melanin degradation products. The chromatogram of oxidized products from brown cells had a peak eluting at 11.0 min, which matched the retention time of PTCA (Fig. 3), indicating that a major component of the particles was 5,6-dihydroxyindole-2-carboxylic acid, which is consistent with identification of the pigment as a eumelanin (11, 20). Melanin particles from H99 cells grown with L-DOPA and dopamine and supernatant-associated pigment were treated in the same way, and HPLC revealed the presence of a PTCA component (Fig. 3). HPLC of cells and supernatants from cultures grown with L-DOPA and dopamine substrates revealed the presence of TDCA and TTCA components characteristics of melanin polymers.

The charges of H99 yeast cells grown with *K. aerogenes*, L-DOPA, and dopamine were determined. The results showed that particles from cells grown in different media had different charges (Table 1). For the dopamine- and *K. aerogenes*-derived particles from *C. neoformans* the charges were significantly more negative than the charge of the L-DOPA-derived particles ($P < 0.05$). The charge of the dopamine particles was not significantly different from the charge of the *K. aerogenes* particles. The charges of *C. neoformans* cells grown with *K. aerogenes* or dopamine were more negative than the charges of cells grown in medium alone or with L-DOPA.

Fluorescence analysis of melanins. Melanin particles recovered from brown cells were evaluated to determine their fluo-

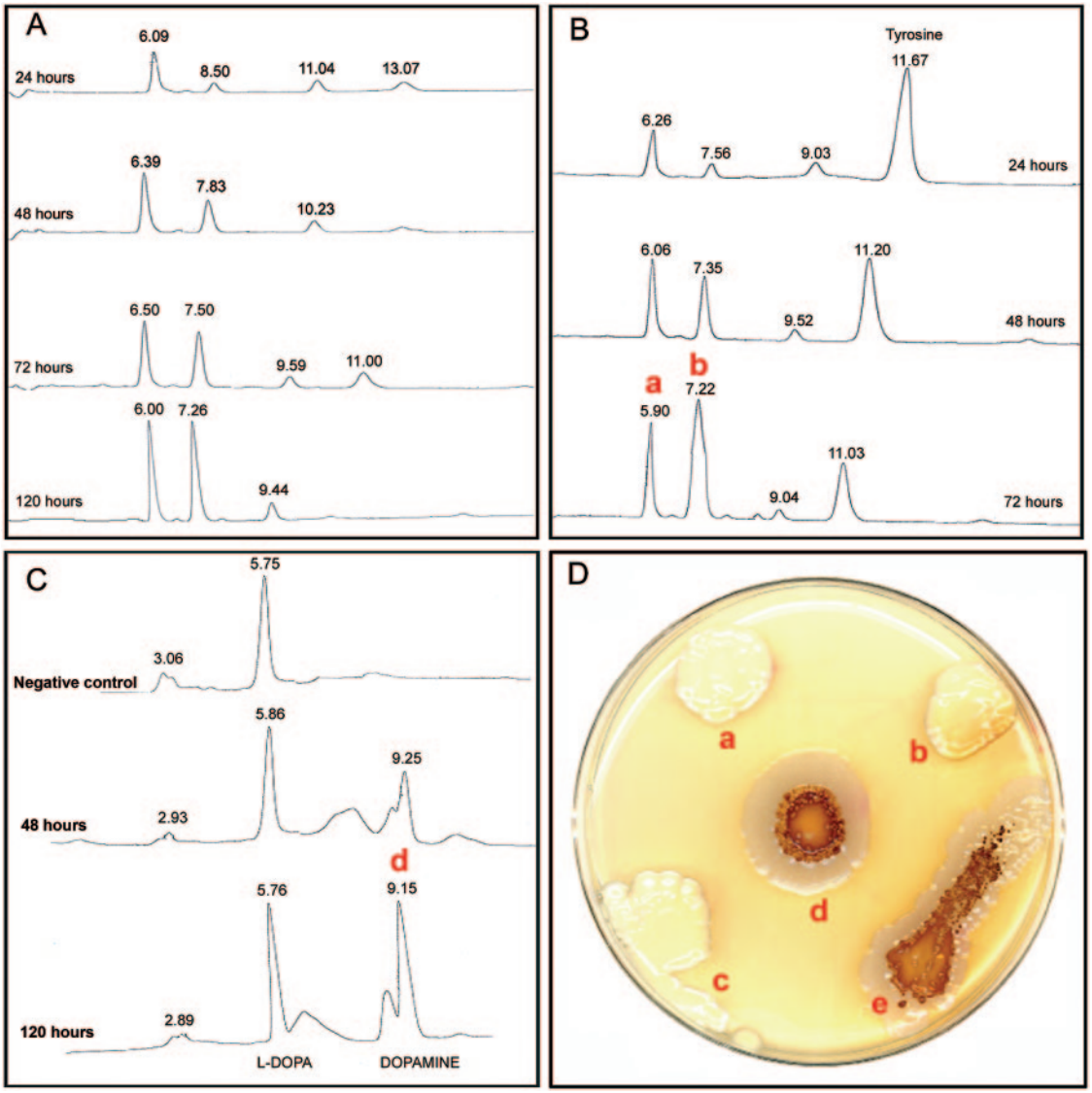

FIG. 7. (A) HPLC chromatograms of *K. aerogenes* culture supernatants in LB. (B) HPLC chromatograms of *K. aerogenes* culture supernatants in LB supplemented with L-tyrosine. (C) HPLC chromatograms of *K. aerogenes* culture supernatants in LB supplemented with L-DOPA. For all chromatograms the elution time was measured in minutes, and the absorbance was measured in arbitrary units. (D) Effects of different HPLC elution conditions on *C. neoformans* pigment induction. One-hundred-microliter portions of the different eluant solutions from HPLC were spotted in different areas of an SM agar plate, and after the fluid was allowed to dry, 100 μl of a *C. neoformans* suspension in minimal medium (10^4 cells/ml) was placed on the agar surface where the different elution solutions had been placed. Colony a, 5.9-min elution component; colony b, 7.22-min elution component from HPLC of LB supernatants with L-tyrosine; colony c negative control; colony d, 9.15-min elution component from HPLC of LB with L-DOPA; colony e, dopamine positive control.

rescence properties under different wavelengths. Fluorescence was observed when the particles were irradiated with 420-, 535-, and 610-nm light (Fig. 6). 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 with *K. aerogenes* did not fluoresce (data not shown).

TLC. Pigments in supernatants of fungus-bacterium cultures were analyzed by TLC using a methanol-water (50:50) solvent. TLC resulted in two spots at R_f 0.575 and 0.65 in the culture supernatants when fungal and bacterial cells were grown together. In contrast, TLC of supernatants from bacterial cultures resulted in two spots at R_f 0.750 and 0.875, and the spot at R_f 0.750 originated from the LB growth medium. The TLC spot at R_f 0.875 fluoresced under UV light (254 or 365 nm).

HPLC of *K. aerogenes* supernatant-associated pigment. HPLC of *K. aerogenes* culture supernatant from cells grown in LB was used to investigate the nature of the bacterial product(s) responsible for brown pigmentation in *C. neoformans*. The chromatogram contained two main peaks eluting at 6 and 7 min. As the incubation time increased, larger peaks were observed. These peaks eluted at the same time as compounds found in supernatant from a *K. aerogenes* culture grown in LB supple-

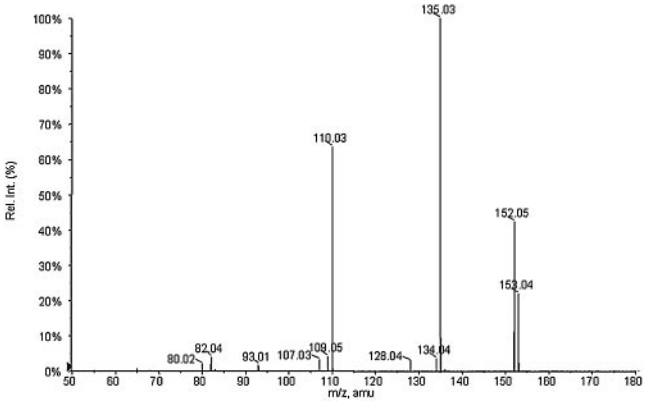

FIG. 8. Mass spectrum for HPLC fraction that induced production of the brown pigment in *C. neoformans*. *m*/*z* 152.05 corresponds to oxidized dopamine with a reduction of 17 Da from loss of NH_3 and $CH_2CH_2NH_2$ groups. Rel. Int., relative intensity; amu, arbitrary mass units.

mented with L-tyrosine. The material that eluted at 11 min matched the HPLC profile of L-tyrosine, and the peak was found to decrease with time of bacterial culture, consistent with metabolic exhaustion (Fig. 7).

Mass spectrometry identified a bacterially synthesized precursor as dopamine. The bacterial supernatant compound eluting at 9 min from the HPLC was analyzed by mass spectrometry. The results showed that a molecule at *m*/*z* 152.05 corresponded to dopamine (Fig. 8). *C. neoformans* was grown on an SM medium plate supplemented with eluted fractions from HPLC of *K. aerogenes* supernatants to confirm that the eluted fractions recovered induced pigmentation in yeast cells. Pigmentation occurred when *C. neoformans* was incubated with the HPLC fraction that eluted at 9 min, identified by mass spectrometry as dopamine (Fig. 7C). We also observed that with these fractions there was a white halo around the pigmented colonies, suggesting that the local concentration of dopamine in the areas was significantly lower than the concentration in the center of the spot. To confirm this result, we added dopamine to a *C. neoformans* culture on SM agar, and as expected, a brown pigment was induced. By comparing the size of the dopamine HPLC peak in bacterial supernatants to the sizes of the peaks for dopamine solutions having known concentrations, we estimated that concentration of dopamine in a culture of *K. aerogenes* in LB after 48 h was 135 mM.

Scheme for *C. neoformans* melanization. On the basis of the results obtained in this study, we developed a scheme for *C. neoformans* melanization in the presence of *K. aerogenes*, in which the bacteria synthesize a precursor that is used by fungal cells for melanin production (Fig. 9).

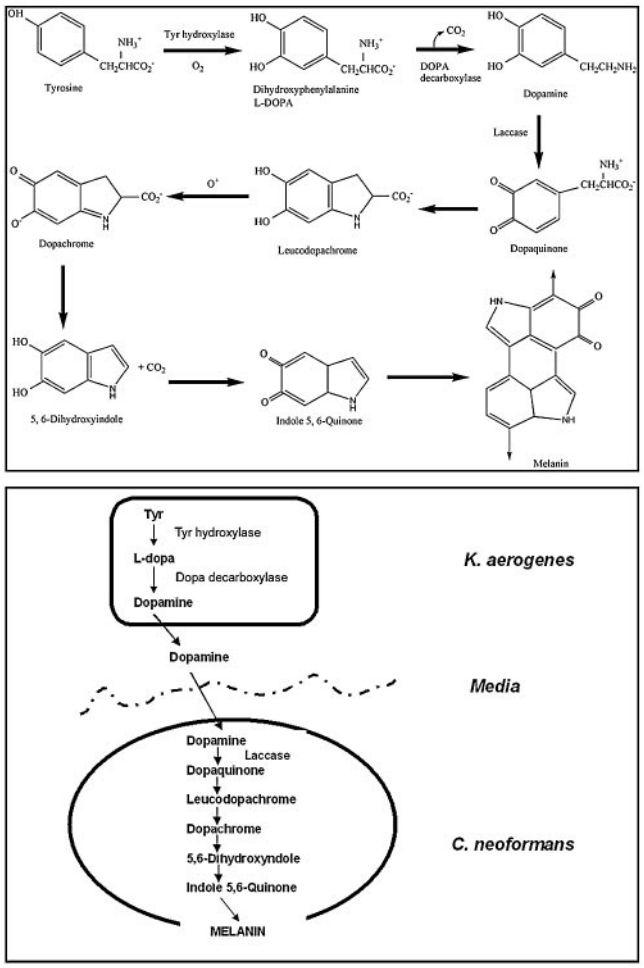

FIG. 9. Schematic representation of the *K. aerogenes*-*C. neoformans* interaction resulting in the production of melanin by *C. neoformans*.

DISCUSSION

Growth of *C. neoformans* in close proximity to *K. aerogenes* resulted in brown pigmentation of fungal colonies. This phenomenon was observed with *C. neoformans* strains representing all serotypes and required that both fungal and bacterial cells were viable. No pigmentation was observed when *C. neoformans* was grown with the other bacterial species examined or when other fungi were grown with *K. aerogenes*. Pigment production required expression of *C. neoformans* laccase but did not require contact between bacterial and fungal cells in agar plates. In addition, we observed that *C. neoformans* cells developed pigmentation even when they were separated by a dialysis membrane and a filter. Hence, formation of a yeast cell pigment was the result of a diffusible compound produced by the bacteria. This phenomenon was of great interest because it suggested that certain bacteria might stimulate pigment production in a pathogenic fungus. Since pigment production in *C. neoformans* required laccase and brown pigments could be melanins, we investigated the possibility that certain types of fungus-bacterium interactions could lead to fungal cell melanization.

Digestion of brown-pigmented cells with glycolytic and proteolytic enzymes followed by extraction with guanidinium isothiocyanate and hot concentrated acid produced dark particles that were suggestive of the types of melanin spheres recovered from L-DOPA-melanized cells (20). This material was a eumelanin as determined by HPLC analysis, which revealed the presence of PTCA after acid permanagate oxidation. However, unlike the melanin particles recovered from cells grown with L-DOPA, the particles recovered from the brown-pigmented

cells were more negatively charged and had dimensions that were significantly smaller than the cells from which they originated. The reduction in size occurred during digestion with hot concentrated acid, which can result in the collapse of cells (17). Hence, brown-pigmented cells yielded particles that contained melanin but had different physical properties than melanin ghosts derived from L-DOPA-melanized cells. Since melanization in *C. neoformans* is dependent on exogenous substrate and the type of melanin formed reflects the chemical structure of the substrate, the differences between brown-pigmented particles derived from cells grown with bacteria and the black particles derived from cells grown in L-DOPA implied that there were differences in the chemical structures of the precursor compounds. The color of the pigment observed in *C. neoformans* grown in the presence of *K. aerogenes* (brown) was similar to the melanization observed when *C. neoformans* was incubated with dopamine and not L-DOPA (black pigment). The zeta potential of the melanin particles recovered from brown cells grown with bacteria or dopamine was different from the charge of black cells grown with L-DOPA. We found that the HPLC analysis results for melanin particles derived from *C. neoformans* grown with *K. aerogenes* were more complex than the results for particles generated with dopamine alone and suspected that this reflected the additional incorporation of unidentified bacterially derived products. It this regard, it is noteworthy that many types of compounds, including steroids, can be incorporated into melanins during their synthesis, (10). TLC revealed the presence of soluble pigment, suggesting that some fraction of the melanin precursor did not polymerize after laccase oxidation.

Bacterium-induced melanization in *C. neoformans* required proximity of fungal and bacterial cells but not contact. This strongly suggested that pigment formation in cryptococcal colonies resulted from a diffusible substance produced by *K. aerogenes* that was metabolized by *C. neoformans* laccase into a form of melanin. The fact that *K. aerogenes* has a tyrosine hydroxylase (tyrosinase) that can catalyze the formation of catecholamines from tyrosine suggested a potential mechanism for *C. neoformans* melanization, in which the bacteria produce substrates, such as dopamine, for the fungal laccase. Consistent with this mechanism, pigmentation of *C. neoformans* cells was not observed when the fungus was grown with bacteria lacking this enzyme, such as *E. coli.* Also, addition of L-tyrosine to the medium resulted in a significant increase in brown pigment production. Brown pigmentation of *C. neoformans* was enhanced by low glucose concentrations and low temperatures, which is consistent with the fact that laccase expression is repressed by high concentrations of glucose and elevated temperatures (11, 22). For identification of the bacterial product we combined HPLC analysis and mass spectrometry of HPLC fractions. HPLC analysis revealed that there was accumulation of compounds with chromatographic characteristics of catecholamines in bacterial supernatants, which increased with culture age. Growth of bacteria in medium supplemented with L-tyrosine, which promotes brown pigment formation, revealed that there was a marked increase in catecholamine-like products in bacterial supernatants and a concomitant decrease in the L-tyrosine concentration. Growth of *C. neoformans* on medium supplemented with the material eluting at 9 min, dopamine, resulted in the characteristic brown pigmentation observed in the presence of bacteria. Since *K. aerogenes* is known to have a tyrosine hydroxylase that converts tyrosine to L-DOPA, we surmised that the bacterial cells must also encode a DOPA decarboxylase for dopamine to be formed. Adding L-DOPA to bacterial medium and demonstrating recovery of dopamine in the HPLC chromatograms established the existence of this enzyme. Hence, *K. aerogenes* promotes melanization in *C. neoformans* by catalyzing the oxidation of tyrosine to L-DOPA and dopamine, and the latter serves as a substrate for the *C. neoformans* laccase. The step that catalyzes the transformation of L-DOPA to dopamine presumably is fast, so only dopamine is detected in bacterial supernatants.

In *K. pneumoniae*, the expression of certain genes encoding a 4-hydroxyphenylacetic acid hydroxylase is associated with production of a dark brown pigment in the cultures (6). This pigment was identified as polymer characteristic of microbial melanins and resulted from the oxidative activity of 4-hydroxyphenylacetic acid hydroxylase on dihydroxylated compounds to form *o*-quinones. The study of this process revealed that the hydroxylase activity, other than tyrosinase, was associated with the synthesis of a bacterial melanin (6). Our results are qualitatively different from this phenomenon since they involve production of substrates by bacteria and utilization of these compounds by fungi rather than induction of laccase activity that results in melanin synthesis.

In summary, we describe an interaction between a gram-negative bacterium and a human-pathogenic fungus that results in fungal melanization. Our results suggest that melanization of *C. neoformans* in the presence of *K. aerogenes* involves conversion of tyrosine to L-DOPA by a bacterial enzyme and subsequent conversion of dopamine to a cell wall-associated melanin by a fungal laccase. Although the effect was observed only in laboratory conditions, it establishes a precedent for the notion that fungi expressing laccases could salvage dopamine-like compounds produced by bacteria for melanin synthesis. A similar phenomenon could explain the mechanism of *C. neoformans* melanization in soils, where melanin synthesis can be expected to confer a significant survival advantage to the fungal cells. These results parallel recent findings which showed that certain gram-negative bacteria can promote changes in human-pathogenic fungi that may be associated with virulence (8, 9). To our knowledge, this is the first example of bacterium-dependent fungal melanization and suggests a potential mechanism by which fungi expressing laccases can synthesize melanin in microbial communities.

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