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Synthesis of Melanin Pigment by *Candida albicans* In Vitro and during Infection

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Melanins are implicated in the pathogenesis of several important human diseases. This study confirmed the presence of melanin particles in *Candida albicans* in vitro and during infection. Dark particles were isolated from the digestion of *C. albicans* cultures and from infected tissue, as established by electron microscopy and immunofluorescence techniques.

The polymorphic fungus *Candida albicans* is the causative agent of candidiasis, which is the most commonly encountered human fungal disease (3). Despite its global importance, there is limited information relating to the factors that play a role in the pathogenesis of candidiasis; however, there is mounting evidence to suggest that virulence in this organism is multifactorial (11). Melanins make up a heterogeneous class of natural pigments that have a myriad of biological functions (7). Melanization has gained increasing importance as a putative virulence factor in several fungal pathogens and may therefore be a virulence trait conserved across many fungal species (7). In this study, we aimed to determine whether *C. albicans* could synthesize melanin in yeast cells, utilizing techniques developed to study and isolate melanin from other fungal pathogens. *C. albicans* strains used were as follows: F28370, F14985, W90572, W91405, T31119, F10646, and M9958 (University College Hospital, London, United Kingdom); 3179 (National Collection of Pathogenic Fungi [NCPF], Bristol, United Kingdom); and CIB 1276 and ATCC 24433 (Corporación para Investigaciones Biológicas [CIB], Medellin, Colombia). *Cryptococcus neoformans* JEC21 (Mel^+) and its albino mutant HMC6 (Mel^-) were used as positive and negative controls. Melanin particles were isolated from yeast cells of all strains of *C. albicans* and from infected murine kidney and human skin tissue, as previously described (4), by sequential digestion steps and boiling in hot concentrated acid. The resultant particles were a quarter of the size of the yeast cells, as demonstrated by scanning electron microscopy (Fig. 1A and B). No particles were isolated from the *C. neoformans* albino mutant. Transmission electron microscopy of the melanin particles from *C. albicans* revealed small thin layers of electron-dense material surrounding a void (Fig. 1C and D). Electron spin resonance spectroscopy of the dark particles isolated was performed at Albert Einstein College of Medicine, Bronx, N.Y., by documented methodology (8, 13). The spectra were identical to the signals generated from *C. neoformans*-derived melanin (reference 14 and data not shown).

Enzyme-linked immunosorbent assay plates coated with melanin (from *C. albicans*, *Sporothrix schenckii*, and *C. neoformans*) were prepared as previously described (6) and incubated with anti-melanin monoclonal antibodies (MAbs) 8B5 generated against *S. schenckii* yeast melanin (6), 6D2 generated against melanin from *C. neoformans* (10), and 8F5 generated against melanin from *Aspergillus fumigatus* (15). Negative controls included wells with no melanin and peroxidase-conjugated goat anti-mouse (GAM) alone. The anti-melanin MAbs reacted against the melanin particles derived from *C. albicans* (Fig. 2), and the reactivities were equal in intensity to those observed with melanin particles from the positive controls.

C. albicans 3179 yeast cells were embedded in optimal cutting temperature compound (BDH), and the frozen blocks were sectioned (cryostat Figocut 2700) and stored at −20°C. Fungal sections were fixed in cold acetone and air dried. Slides were blocked with Superblock (Roche, Sussex, United Kingdom) overnight at 4°C and incubated for 2 h at 37°C either with 10 μg of MAb 8B5, 8F5, or 6D2 with a 1:100 dilution of fluorescein isothyiocyanate (FITC) GAM immunoglobulin M (IgM) for 2 h at 37°C. Negative controls consisted of 5C11 with FITC-labeled antibody or FITC-labeled antibody alone. Microscopy showed anti-melanin MAb bound to small structures within the cryosectioned *C. albicans* yeast cells (Fig. 3A to C). Uncut yeast did not bind to the anti-melanin MAbs. Melanin extracted from *C. albicans* yeast was also reactive; these particles typically formed aggregates (Fig. 3D and E). Melanin particles were unreactive with the negative control, MAb 5C11.

Immunocompetent mice were immunized with *C. albicans* F28370. The infection protocol was a modified version of that previously described (2). At the CIB in Colombia, isogeneic 6-week-old male BALB/c mice were immunized intravenously via the lateral tail vein with 100 μl of *C. albicans* (4×10^6 CFU/mouse) in sterile phosphate-buffered saline and sacrificed at 21 days. Target organs (heart, lungs, liver, spleen, and kidneys) were fixed and embedded in paraffin, and sera were stored at −70°C. Paraffin-embedded human skin samples from patients infected with cutaneous *C. albicans* (a gift from A.

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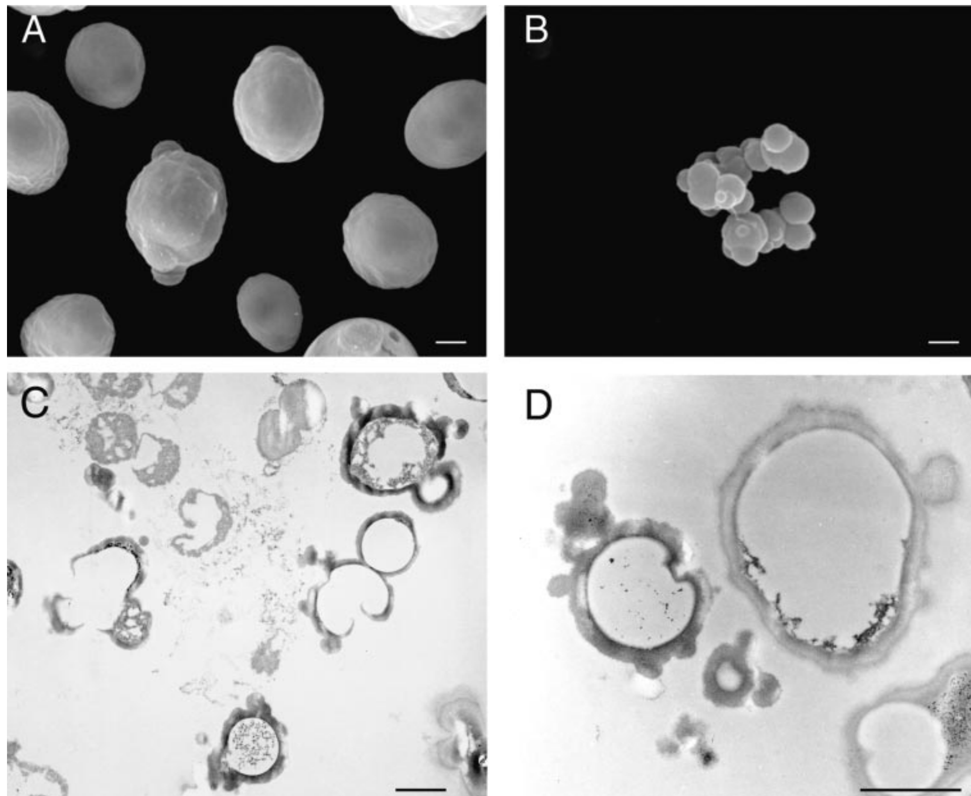


FIG. 1. Scanning electron microscopy of *Candida* yeast cells, before and after treatment with enzyme denaturant and hot acid. *C. albicans* F14985 yeast cells (A) and melanin particles (B); bars, 1 μm. Transmission electron microscopy of *C. albicans* F14985 melanin particles at low (C) and high (D) magnification, respectively; bars, 2 μm.

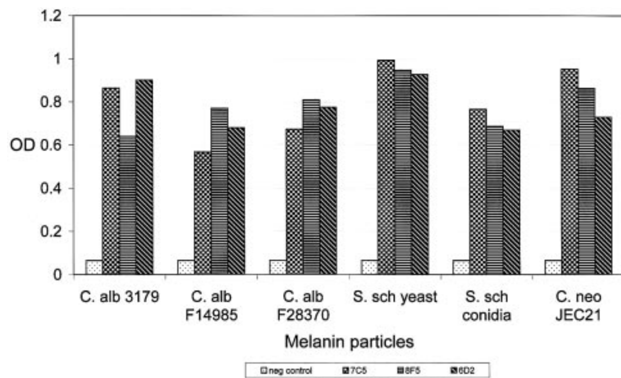
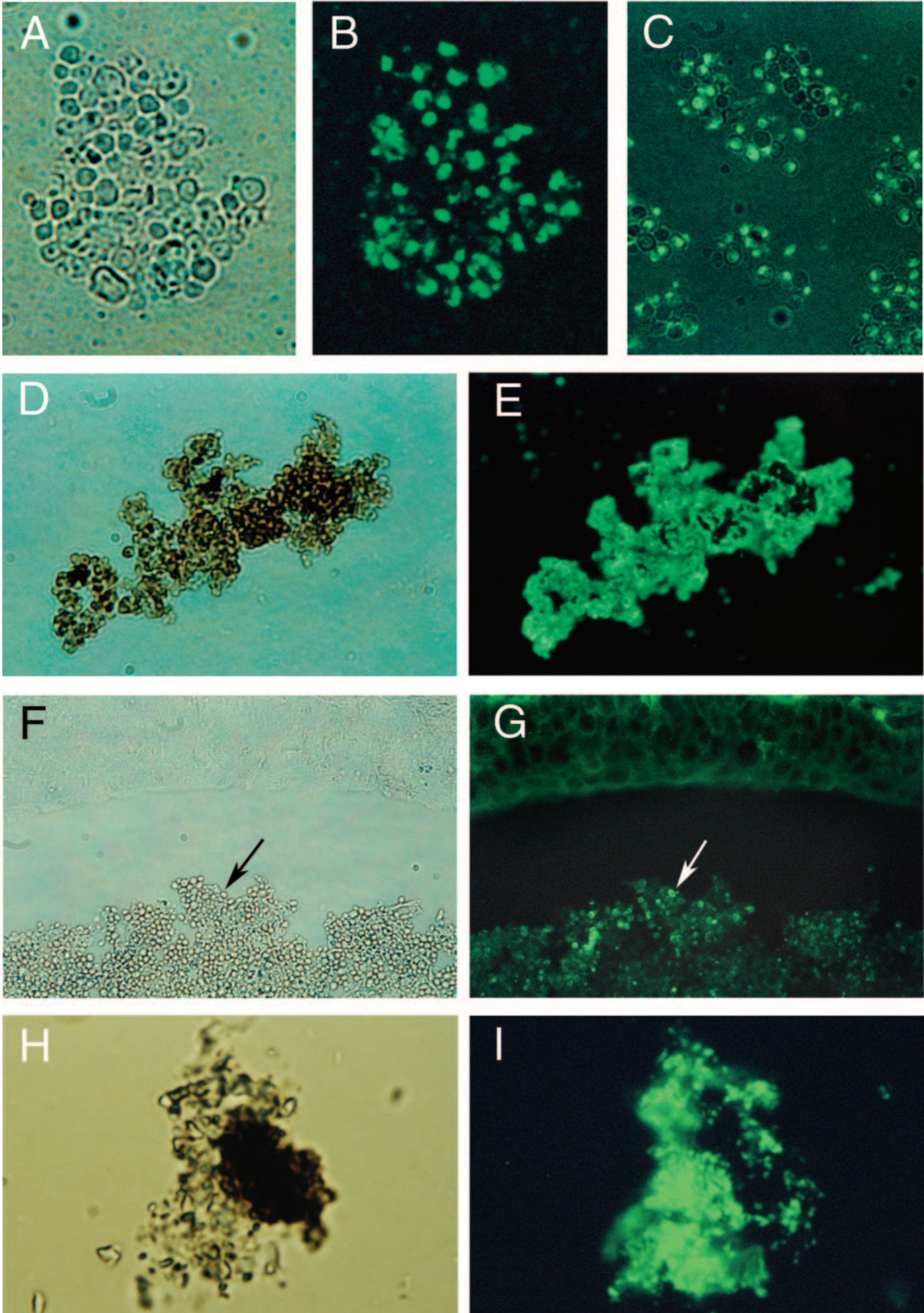


FIG. 2. Enzyme-linked immunosorbent assay reactivities of anti-melanin MAbs 7C5 (*S. schenckii* melanin), 8F5 (*A. fumigatus* melanin) and 6D2 (*C. neoformans* melanin) with melanin particles extracted from *C. albicans* isolates (3179 NCPF, F14985, and F28370). Positive control melanins, *S. schenckii* (16127) and *C. neoformans* (JEC21). The secondary antibody was a 1:1,000 dilution of peroxidase-labeled GAM (IgM isotype). Negative controls (i.e., wells with no melanin, peroxidase-conjugated GAM alone, and the irrelevant MAb 5C11) are shown as a combined mean optical density value. Bars are negative control, 7C5, 8F5, and 6D2, respectively.

Restrepo, CIB) were also used. Tissue was sectioned and stained with periodic acid-Schiff stain and methenamine silver (Grocott modification); blocks positive for fungi were processed for immunohistochemical staining with MAbs, as previously described (6). Briefly, tissue was incubated with melanin-binding MAbs at a dilution of 1:100 at 37°C and then with 1:100 FITC-conjugated GAM IgM. Negative controls were described above. *C. albicans* melanin particles were isolated from the tissues and from cultures and air dried onto 3-aminopropyltriethoxysilane slides. Tissues were then probed with anti-melanin MAbs and FITC GAM IgM, as described above. *C. albicans* yeast and hyphae were seen in significant numbers in the kidneys alone. Reactivity with anti-melanin MAb 8B5 was observed as small fluorescent particles within yeast cells (Fig. 3F and G) but not in the hyphal forms. Digestion of infected murine kidneys resulted in isolation of melanin particles, which reacted with anti-melanin MAb (Fig. 3H and I). Sera (diluted 1:100) from *C. albicans*-infected mice showed positive recognition of the melanin from cultures and tissue compared to normal mice sera (data not shown). Melanin

FIG. 3. *C. albicans* (3179 NCPF) yeast cells cryosectioned and stained with anti-melanin MAb 8B5. Bright-field (A) and corresponding immunofluorescence (B) images of the yeast culture (magnification, ×100) are shown. Bright-field imagery superimposed on immunofluorescence (C) to show the spatial relationship between the yeast cells and the fluorescent particles (magnification, ×50). Corresponding bright-field (D) and immunofluorescence (E) microscopy images of *C. albicans* 3179 NCPF melanin particles isolated from yeast cells reacted with anti-*S. schenckii* MAb 8B5 (magnification, ×100) are shown. (F and G) Murine kidney infected with *C. albicans* F28370, showing fungal hyphae and yeast cells within the glomerulus. Corresponding bright-field (F) and immunofluorescence (G) images show small fluorescent particles within the yeast cells (arrows) but not the hyphae when the preparation was stained with anti-*S. schenckii* melanin MAb 8B5 (magnification, ×40). (H and I) Corresponding bright-field (H) and immunofluorescence (I) microscopy images showing labeling of *C. albicans* F28370 aggregated melanin particles recovered from infected murine kidney with anti-melanin MAb 8B5 (magnification, ×100).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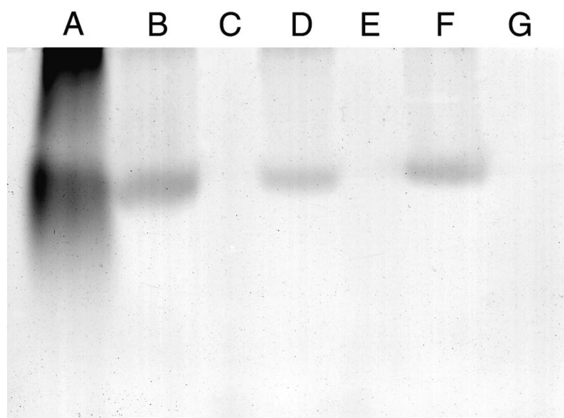

FIG. 4. Nonreducing sodium dodecyl sulfate-polyacrylamide gel of cytoplasmic antigen extract of *C. albicans* developed with L-DOPA. Lanes: A, commercial laccase (50 U equivalent); B, 300 μg of yeast antigen of *C. albicans* F28370; C, the same as for lane B but boiled for 5 min; D, 300 μg of yeast antigen of *C. albicans* F14985; E, the same as for lane D but boiled for 5 min; F, 300 μg of yeast antigen *C. albicans* NCPF 3179; G, the same as for lane F but boiled for 5 min.

particles were also isolated from infected human skin tissue and reacted with the anti-melanin MAbs (data not shown).

Laccase enzymes are utilized in the synthesis of melanin; hence, their activity was anticipated in *C. albicans* cytoplasmic yeast extracts (CYEs). These were harvested as previously described (13), and protease inhibitor cocktail was added (Sigma). The suspension was frozen using liquid nitrogen and smashed until >90% of the cells were broken. The CYE was concentrated using Amicon tubes (molecular mass cutoff, 1 kDa), and the protein content was determined by Coomassie blue methodology. Commercially prepared laccase (*Rhus vernificera*) (Sigma) and CYEs from *C. albicans* strains were separated by 10% polyacrylamide gel electrophoresis (30 mA overnight) under nondenaturing conditions. Duplicate samples were loaded onto the gels, one of which had been boiled for 5 min. Gels were incubated with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) buffer overnight. Positive laccase activity was revealed by *C. albicans* CYEs, as shown by dark bands (Fig. 4), which confirmed L-DOPA had polymerized to form melanin. Boiling of the samples prior to loading into the gel eliminated the laccase activity.

This study provides the first definitive evidence that melanization occurs in *C. albicans* and may represent a new virulence factor. The biosynthesis of melanin in most fungal pathogens leads to the accumulation of pigment beneath the cell wall, resulting in so-called melanin ghosts which retain the shape and size of the original propagules (9). However, the small spheres of melanin derived from *C. albicans* are more akin to the sclerotic bodies (comparable to melanosomes) found in *Fonsecaea pedrosoi*, which causes chromoblastomycosis (1). *C. albicans* yeast cells secrete complex polymers into biofilm structures (5) whose composition is not fully understood; one hypothesis therefore might be that melanin particles are secreted into these resistant extracellular structures. Melanization may be an attractive target for novel antimicrobial drugs; therefore, the investigation of melanin-inhibiting compounds should be pursued in the future. The genome of *C. albicans* has recently been unraveled (12), so evidence from genetic studies is likely to shed further light on these findings.

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REFERENCES

1. **Alviano, C. S., S. R. Farbiarz, W. D. De Souza, J. Angluster, and L. R. Travassos.** 1991. Characterization of *Fonsecaea pedrosoi* melanin. J. Gen. Microbiol. **137:**837–844.
2. **Brieland, J., D. Essig, C. Jackson, D. Frank, D. Loebenberg, F. Menzel, B. Arnold, B. DiDomenico, and R. Hare.** 2001. Comparison of pathogenesis and host immune responses to *Candida glabrata* and *Candida albicans* in systemically infected immunocompetent mice. Infect. Immun. **69:**5046–5055.
3. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans.* Annu. Rev. Microbiol. **45:**187–218.
4. **Gomez, B. L., J. D. Nosanchuk, S. Diez, S. Youngchim, P. Aisen, L. E. Cano, A. Restrepo, A. Casadevall, and A. J. Hamilton.** 2001. Detection of melanin like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis* in vitro and during infection. Infect. Immun. **69:**5760–5767.
5. **Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother. **46:**1773–1780.
6. **Morris-Jones, R., S. Youngchim, B. L. Gomez, P. Aisen, R. J. Hay, J. D. Nosanchuk, A. Casadevall, and A. J. Hamilton.** 2003. Synthesis of melanin-like pigments by *Sporothrix schenckii* in vitro and during mammalian infection. Infect. Immun. **71:**4026–4033.
7. **Nosanchuk, J. D., and A. Casadevall.** 2003. The contribution of melanin to microbial pathogenesis. Cell. Microbiol. **5:**203–223.
8. **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.
9. **Rosas, A. L., J. D. Nosanchuk, B. L. Gomez, W. A. Henson, and A. Casadevall.** 2000. Isolation and serological analyses of fungal melanins. J. Immunol. Methods **244:**69–80.
10. **Rosas, A. L., J. D. Nosanchuk, M. Feldmesser, G. M. Cox, H. C. McDade, and A. Casadevall.** 2000. Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. Infect. Immun. **68:**2845–2853.
11. **Ruechal, R.** 1990. Virulence of *Candida* species, p. 47–65. *In* L. P. Samaranayake and T. W. MacFarlane (ed.), Oral candidosis. Wright, London, United Kingdom.
12. **Tait, E., M. C. Simon, S. King, A. J. Brown, N. A. R. Gow, and D. J. Shaw.** 1997. A *Candida albicans* genome project: cosmid contigs, physical mapping, and gene isolation. Fungal Genet. Biol. **21:**308–314.
13. **Wang, Y., P. Aisen, and A. Casadevall.** 1995. *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect. Immun. **63:**3131–3136.
14. **Wang, Y., P. Aisen, and A. Casadevall.** 1996. Melanin, melanin "ghosts," and melanin composition in *Cryptococcus neoformans.* Infect. Immun. **64:**2420–2424.
15. **Youngchim, S., R. Morris-Jones, R. J. Hay, and A. J. Hamilton.** 2004. Production of melanin by *Aspergillus fumigatus.* J. Med. Microbiol. **53:**175–181.

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