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NOTES

Novel Application of the Masson-Fontana Stain for Demonstrating *Malassezia* Species Melanin-Like Pigment Production In Vitro and in Clinical Specimens

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Melanin-like pigment produced in vitro and in vivo by *Malassezia* yeasts has not been described before. Masson-Fontana staining confirmed accumulation of black pigment on the cell walls of L-dihydroxyphenylalaline (L-DOPA)-cultured *Malassezia* species. Black pigment was also observed in cells and hyphae from hyperpigmented patient lesions with culture-confirmed pityriasis versicolor and seborrheic dermatitis.

Melanin production, mediated by a copper-containing phenoloxidase enzyme using L-dihydroxyphenylalaline (L-DOPA) as a substrate, is a well-studied pathogenetic mechanism in fungi (9, 24), especially in species of the human pathogen *Cryptococcus*. Many properties related to the evasion of the host immune system (9) and antifungal drug resistance (23) have been attributed to melanin or melanin-like pigments, assessed in vitro by growth in L-DOPA agar (18) and in vivo by staining with Masson-Fontana stain, which detects melanin deposited on the cells of this basidiomycetous yeast (10).

The aims of this study were (i) to assess the in vitro abilities of *Malassezia* yeasts to oxidize L-DOPA and produce a melanin-like pigment and (ii) to demonstrate that this pigment can be detected in yeast cells and hyphae by Masson-Fontana silver staining of skin scales from pityriasis versicolor (PV) and seborrheic dermatitis (SD) patients.

We studied 53 type, reference, and clinical isolates of 11 *Malassezia* species (Table 1) (7, 8, 20–22). The type and reference strains for this study were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The clinical isolates were strains kept in the Mycology Reference Laboratory of the Hellenic National Collection of Pathogenic Fungi (HNCPF; World Data Centre on Microorganisms member type ID 2023). All *Malassezia* strains, the control *Cryptococcus grubii* strains (HNCPF 6417a and 6417b) demonstrating maximum and no melanization, respectively, and the autochthonous clinical *Cryptococcus gattii* serotype B (HNCPF 55/CBS10090, HNCPF 54W/CBS10088) strains with various degrees of melanization, as well as nonmelanized *Cryptococcus laurentii* (HNCPF 02/NCPF 3832 [National Collection for Pathogenic Fungi, Bristol, United Kingdom]) were semiquantitatively tested for their abilities to produce pigment when grown in lipid-supplemented and lipid-depleted L-DOPA and tyrosine agars. Briefly, 1 liter of medium comprised 200 ml filter-sterilized (0.2 μm; catalog no. 14831; Corning, Germany) water solution at pH 5.5 (adjusted with 1 M potassium dihydrogen phosphate [KH_2PO_4]) supplemented with 0.04 g L-DOPA or tyrosine, 1 g asparagine, 1 g L-glutamine, and 1 g glycine (all from Sigma, Saint Louis, Mo.). This solution was mixed with 800 ml of a sterile solution, cooled to approximately 50°C, of 4 g KH_2PO_4, 2.5 g hydrated magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 10 mg thiamine HCl, 20 μg biotin, 0.5 g glucose, 25 g agar, 4 g OxBile, 1 ml glycerol, 0.5 g glycerol monostearate, and 0.4 ml Tween 20, readjusted at pH 5.5 with KH_2PO_4 (all from Sigma).

Phenoloxidase activity in the *Cryptococcus* control and the *Malassezia* strains was corroborated by modifying the semiquantitative method of Cooper and Christine-Brown (2). Briefly, half the agar plate contained L-DOPA medium (0.005% L-DOPA [wt/vol], 2% Bacto Agar in 0.1 M phosphate buffer solution [all from Sigma]) and the other half contained a control medium without the addition of L-DOPA. The solidified agar surface was punctured with a standard sterile 5-mm-diameter cork borer, producing wells. In each well, 100 μl of whole-cell suspensions or cells that had been aseptically mechanically disrupted by an orbital homogenizer at 100 rpm and suspended in sterile water was inoculated and incubated at 35°C for 3 and 7 days for the *Cryptococcus* and *Malassezia* strains, respectively (Fig. 1A and 2B). All plates were inspected daily for pigment production. Absence of a lipid source in the culture medium did not allow growth of *Malassezia* yeasts.

The Masson-Fontana silver stain was employed to demonstrate melanin deposition (10) on the walls of *Malassezia* cells harvested from tyrosine and L-DOPA media, respectively. In order to ascertain the necessity for L-DOPA in the synthesis of melanin, cells grown in the L-DOPA-depleted medium were also tested for melanin-like pigment detection by Masson-

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TABLE 1. Intensity of melanization displayed by 11 *Malassezia* species in L-DOPA agar

Species	Strain(s)[a] with the following score[b]:		
	+3	+2	+1
M. furfur		CBS6001, CBS7984, CBS7985	ATCC 14521, CBS7019, CBS5333, CBS7983, CBS8735, CBS8736, CBS8737, CBS9580, CBS9584, CBS9585, CBS9574, CBS9579, CBS9589, CBS9583, CS
M. sympodialis	CBS7866, CBS8740, CBS8741, pityriasis versicolor CS	CBS7859, CBS7978, CBS7979	ATCC 96803
M. globosa	CBS9590, CBS9591	ATCC 96807, CBS9570, CBS9576, CBS9578, CBS9581, CBS9557	CBS9574, CBS9579, CBS9589, CBS9583
M. restricta		Seborrheic dermatitis CS	
M. slooffiae	CBS8738	CBS7956	CBS9575, CBS9582
M. obtusa	CBS7876		
M. dermatis	CBS9145, CBS9169, CBS9170		
M. pachydermatis	ED206		CBS1879
M. nana		CBS9557	
M. japonica		CBS9431, CBS9432	
M. yamatoensis		CBS9725	

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CS, clinical strains.
[b] Scores indicate intense (+3), moderate (+2), and subtle (+1) melanization scored relative to the semiquantitative melanization intensity of the control *Cryptococcus* species and strains.

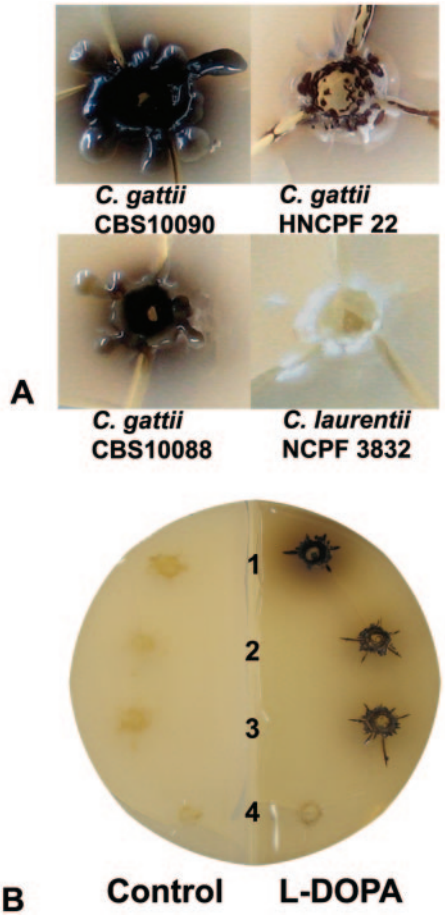

FIG. 1. (A) Various degrees of melanization in L-DOPA agar displayed by reference control cultures of *Cryptococcus gattii* and *Cryptococcus laurentii*. (B) Melanization of *Cryptococcus neoformans* in Bacto (control) and L-DOPA (test) agars in wells 1 to 4. Wells: 1, *C. grubii* (HNCPF 6417a); 2, *C. neoformans* serotype D (HNCPF 17); 3, *C. neoformans* serotype AD (HNCPF 34); 4, *C. grubii* (HNCPF 6417b).

Fontana staining. To examine whether melanin-like pigment can be directly detected in *Malassezia* yeasts parasitizing the skin of patients, multiple skin scale specimens from 15 Caucasian patients with hyperpigmented PV lesions, 15 patients with hypopigmented PV lesions, and 7 SD patients were tested for deposition of dark-brown to black pigment in vivo by Masson-Fontana staining. At least six different lesions from each patient were sampled. The presence of *Malassezia* yeasts was culture confirmed, and the yeasts were identified to species level (6, 7). Informed consent was obtained at all times. Specimens from six hypopigmented and six hyperpigmented lesions were obtained from one Caucasian female patient who presented with both types of macula.

No oxidation of tyrosine was detected when *Malassezia* yeasts were grown on tyrosine agar, indicating that melanogenesis may occur via a tyrosinase-independent pathway. By contrast, *Malassezia* strains tested on L-DOPA agar produced a pigment with various melanization intensities. *Malassezia dermatis* strains demonstrated maximum, and *M. furfur* demonstrated minimum, pigment production (Table 1; Fig. 2A). In the *Cryptococcus* melanin production model, *C. gattii* CBS10090 demonstrated the maximum melanization and *C. grubii* HNCPF 6417b demonstrated the minimum (Fig. 1B). The L-DOPA substrate was oxidized only after *Malassezia* membrane disruption (Fig. 2A), suggesting that phenoloxidase, the enzyme mediating melanin production, may not be secreted, but either attached to the cell wall or bound to the membrane as in *Cryptococcus neoformans* (14). Variable oxidation of the medium was also noted with the disrupted cryptococcal cells (Fig. 1).

Masson-Fontana staining showed a melanin-like pigment deposited in the walls of L-DOPA-grown mature yeast cells, while differential melanization intensity was observed in mother and daughter cells (data not shown). The intensity of fungal wall pigmentation was proportional to the intensity of melanization displayed in the L-DOPA medium (Table 1). Therefore, the *M. furfur* strains that produced minimum or no pigment in L-DOPA agar counterstained pink, indicating lack of melanin-like pigment. Similarly, the *C. grubii* and *C. gattii* strains demonstrated various degrees of melanization in L-DOPA agar and upon Masson-Fontana staining, whereas no pigment was detected in the nonmelanized *C. laurentii* and *C. grubii* strains.

The results confirmed that melanization takes place in vivo, as evidenced by the fact that skin scales originating from hyperpigmented PV and SD lesions, even those from the patient displaying both types of lesions, showed Masson-Fontana-positive (dark-brown to black) *Malassezia* cells and hyphae (Fig. 2C). Furthermore, the positive Masson-Fontana staining of *Malassezia* yeasts from hyperpigmented lesions of the epidermal keratin layer demonstrated melanin-like pigment accumulation (Fig. 2C). All isolates from hyperpigmented PV lesions were *M. sympodialis*, and all those from SD lesions were *M. restricta* (Table 1). *M. sympodialis* was isolated from the hyperpigmented and *M. furfur* from the hypopigmented lesions of the same patient. No melanin-like pigment was detected in yeast cells and hyphae in skin scales from the hypopigmented PV lesions (Fig. 2D). The statistical importance of this finding was not assessed, because the clinical implications of melanin production were not examined in this study.

The Gomori-Grocott silver stain has been used twice in the past to demonstrate the implication of *Malassezia* yeasts in systemic infections (16, 19) and was found superior (19) to periodic acid-Schiff in demonstrating fungal elements in tissue, but no explanation was provided for that finding. In earlier studies, hypertrophy of melanocytes had been noted in postinflammatory hyperpigmentation (12), whereas almost a decade later, controversy existed as to whether the distribution pattern and size of melanosomes were associated with hyperpigmentation (1, 3). However, the various degrees of *Malassezia* species and strain melanization observed in culture and in hyperpigmented-lesion material (Table 1; Fig. 2C) correlate with previous findings on hyperpigmented, nonvitiliginous tinea versicolor skin sections examined by periodic acid-Schiff staining, Fontana staining, and electron microscopy (1, 3). Furthermore, the isolation of pityriacitrin (11), a UV-absorbing indole alkaloid, from *M. furfur* sensu stricto correlates with the recorded reduced capacity of *M. furfur* sensu stricto for melanization in vivo and in vitro (Table 1; Fig. 2D) and with previous observations that epidermal melanin is absent from vitiliginous skin specimens affected by tinea versicolor (3), al-

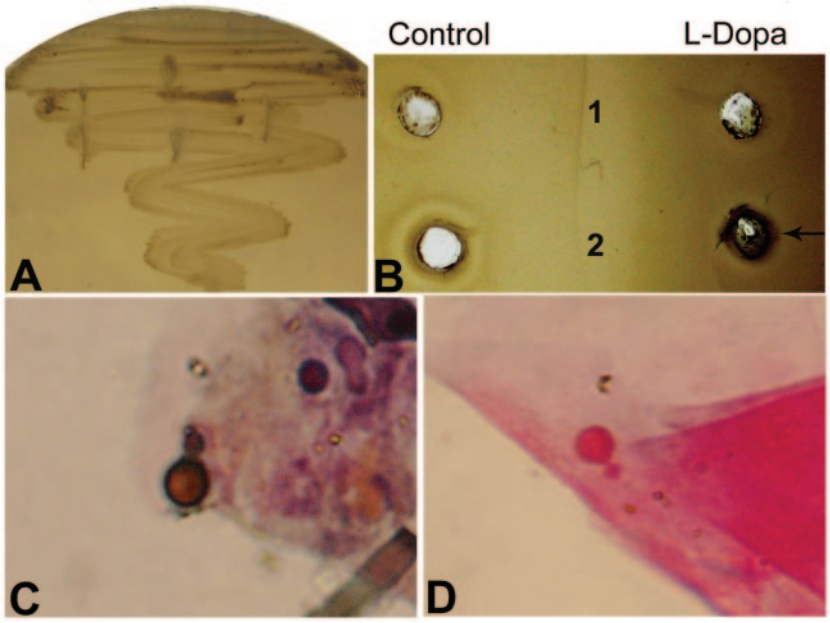

FIG. 2. (A) Intense melanization of *M. dermatis* CBS9169, grown in L-DOPA medium at 35°C for 7 days. (B) Demonstration of melanization by *M. sympodialis* CBS8741 tested by the modified method of Cooper and Christine-Brown after incubation at 35°C for 12 days. Wells: 1, whole cells; 2, mechanically disrupted cells inoculated into the wells. Arrow indicates precipitation of melanin-like pigment. (C) Intensely melanized Masson-Fontana silver-stained *Malassezia* cells from hyperpigmented PV lesions (original magnification, ×1,000). (D) Pink (nonmelanized) appearance of *Malassezia* cells from hypopigmented PV lesions (original magnification, ×1,000).

though the latter was attributed to *M. furfur* sensu lato. Coevaluation of these observations might contribute to elucidating the causative mechanism involved in hypopigmentation and variable fluorescence phenomena documented in PV.

Melanization, as visualized by the positive Masson-Fontana staining of *Malassezia* yeast cells and hyphae in vivo, indicates the presence of L-DOPA in the epidermal cells. However, the occurrence of L-DOPA in the epidermis is thus far supported by indirect evidence, based on the detection of a specialized L-DOPA transport system in the Langerhans cells, while L-DOPA uptake takes place in the epidermis (5). Whether this phenomenon takes place in deeper layers of the epidermis and within the sebaceous gland or whether pigmentary changes in PV also require the involvement of factors besides those involved in normal skin pigmentation, such as melanosomes (1), has not been fully clarified.

Melanins are important biologically active compounds with recognized virulence properties (9). For *Cryptoccoccus*, a basidiomycetous yeast phylogenetically close to *Malassezia*, intensive research during the past 30 years was required to elucidate the significance of melanization in virulence, immunomodulation, and neurotropism. Also, the demonstration of diphenoloxidase activity in the neurotropic *Mycobacterium leprae* further supported the importance of this pathway in human pathogens that can use melanin as an immunomodulator (15, 17).

The proposed Masson-Fontana staining for evaluation of the production of melanin-like pigment by *Malassezia* species has potential applications in clinical and laboratory studies. Melanin in *Cryptococcus* has been shown to have the ability to activate the complement pathway (17). A key element in the pathogenesis of SD (4) is the activation of the complement pathway. Thus, studies of complement activation by melanin-producing *Malassezia* strains, coupled with assessment of melanin production in SD lesions by Masson-Fontana staining of skin scales and biopsy material, could highlight aspects of SD pathogenesis. Intensely melanized *Cryptococcus* strains demonstrate reduced amphotericin B susceptibility (23). Whether the relapses of SD episodes after maintenance topical therapy with amphotericin B (13) are correlated with melanized *Malassezia* yeasts is an issue for clinical investigation. Clinical observations concerning reinstatement of normal skin pigment following successful treatment, (13) suggest that the melanin-like pigment production reported here is elicited by *Malassezia* yeasts.

In conclusion, the presence of melanin-like pigment in *Malassezia* yeasts, as confirmed following growth on modified L-DOPA agar and demonstrated in lesion material (skin scales) by Masson-Fontana staining, offers a novel, simple, and cost-effective method for the assessment of this important biological function in the pathogenesis of *Malassezia*-induced dermatoses.

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