Malassezia of deposited on the cells of this basidiomycetous yeast (10).

Melanin, a dark-colored pigment, plays a crucial role in the survival of many 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, which are detected in yeast cells and hyphae by Masson-Fontana silver staining. This technique allows researchers to assess the presence of melanin-like pigments, which are essential for the pathogenesis and survival of fungi.

In this study, we aimed to evaluate the in vitro abilities of Malassezia yeasts to oxidize L-DOPA and produce melanin-like pigment and 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). All strains were grown in agar media containing L-DOPA or tyrosine, 1 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 KH2PO4 (all from Sigma).

Phenoloxidase activity in the Cryptococcus control and the Malassezia strains was corroborated by modifying the semi-quantitative 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 strains and Malassezia species, 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-Fontana silver staining.
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain(s)* with the following scoreª:</th>
<th>Strain(s)* with the following scoreª:</th>
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<tbody>
<tr>
<td>M. furfur</td>
<td>CBS6001, CBS7984, CBS7985</td>
<td>ATCC 14521, CBS7019, CBS5333, CBS7983, CBS8735, CBS8736, CBS8737, CBS9580, CBS9584, CBS9585, CBS9574, CBS9579, CBS9589, CBS9583, CS</td>
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<td>M. globosa</td>
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<td>ATCC 96807, CBS9570, CBS9576, CBS9578, CBS9581, CBS9557</td>
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<td>M. restricta</td>
<td>Seborrheic dermatitis CS</td>
<td>CBS9574, CBS9579, CBS9589, CBS9583</td>
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<td>CBS956</td>
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<td>M. dermatis</td>
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<td>M. pachydermatis</td>
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<td>CBS9431, CBS9432</td>
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<td>M. yamaeensis</td>
<td>CBS9725</td>
<td></td>
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</tbody>
</table>

ª CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CS, clinical strains.

ª 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 (Table 1). 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-
though the latter was attributed to *M. furfur* sensu lato. Co-evaluation 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 *Cryptococcus*, 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 dopamine oxidase 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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REFERENCES


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).