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Carotenogenesis Associated with Arthrosporulation of *Trichophyton mentagrophytes*

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Carotenoid pigments were demonstrated in arthrospores of the dermatophyte *Trichophyton mentagrophytes* but were absent from hyphae and microconidia of this fungus. Incubation at higher temperatures (39°C) allowed arthrosporulation to occur, but essentially no carotenoid was detected in such arthrospores. The carotenoid formation in arthrosporulating *T. mentagrophytes* did not appear to be either induced or stimulated by light illumination. Mature arthrospores contained the carotenoids phytoene, phytofluene, ζ-carotene, neurosporene, lycopene, and γ-carotene and a few minor unidentified carotenoids. These carotenoids were localized within intracellular granules consisting of osmiophilic matrices and complex membranous elements. This is the first demonstration of carotenoid pigments in dermatophytic fungi.

Dermatophytes are a group of fungi responsible for ringworm infections in humans and animals. Many strains of dermatophytes produce characteristic pigments which are of some diagnostic significance (1, 15). It is now generally believed that the major class of pigments produced by dermatophytes is naphthoquinone or its derivatives, represented by xanthomegnin (2, 6, 11, 14, 21, 23). No carotenoid pigments have been reported in any strain of dermatophytes, although these pigments are known to occur widely in other fungi (3, 7). During the course of our work on the factors affecting arthrospore formation and germination (8, 9), we observed that the dermatophyte *Trichophyton mentagrophytes* accumulated a large quantity of carotenoid pigments intracellularly during arthrosporogenesis (T. Hashimoto, J. H. Pollack, and H. J. Blumenthal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, J21, p. 80). Because this was the first report of carotenoid pigments in this important group of fungi, and the carotenogenesis appeared to occur only in association with spore formation, we further investigated the nature and location of carotenoid pigments occurring during arthrosporulation of *T. mentagrophytes*.

MATERIALS AND METHODS

Fungus strain. *T. mentagrophytes* ATCC 26323 was used throughout this investigation.

Preparation of arthrospores. Arthrospores of *T. mentagrophytes* were produced by a slight modification of the method described earlier (9). Approximately 0.15 to 0.2 ml of a microconidial suspension (2 $\times 10^6$ spores per ml of distilled water) was inoculated on a cellulose dialysis membrane (Union Carbide Corp., New York, N.Y.), which had been placed on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) containing 0.1% sodium acetate. The dialysis membrane was previously boiled in 1% sodium bicarbonate solution for 10 min, rinsed with distilled water, and subsequently sterilized by autoclaving. After the inoculated plates were incubated at 37°C for 36 h, 2 ml of Sabouraud dextrose broth (Difco) was added aseptically over the dialysis membrane. By this time, hyphae emerging from microconidia adhered to the membrane, and the addition of the broth caused no separation of hyphae from the membrane. The plates were placed in a large covered glass jar (21-cm diameter, 25 cm high) and further incubated under saturated humidity at 37°C for an additional week. By the end of the incubation period, essentially all hyphae were transformed into arthrospores.

The arthrospores were readily removed from the cellulose membrane by gently scraping with a spatula. The harvested arthrospores were washed in distilled water three times by means of centrifugation (1,500 $\times g$, 15 min). In some experiments, young hyphae or arthrosporulating hyphae were collected similarly from the cellulose surface after specified periods of incubation, washed, and stored as described above.

Quantitation of arthrospore formation. The degree of arthrospore formation was determined according to the method of Timberlake and Turian (20). Cells removed from the membrane culture were placed on a glass microscope slide and examined microscopically using an oil immersion objective (×100; numerical aperture 1.25; Nikon). The percentage of hyphal tips containing arthrospores was determined after examining 200 tips.

Extraction and quantitation of pigments. The pigments were repeatedly extracted from cells with

methyl alcohol at 25°C. The extracts containing pigments were pooled and saponified in methanol at 25°C for 15 h. The pigmented fraction was collected in hexane by means of a phase separation technique (5, 22). The epiphase fraction containing pigments was concentrated under vacuum. When the pigments were not used immediately, they were stored under vacuum in the dark at −20°C.

The relative amounts of carotenoid pigments contained in cells undergoing arthrosporulation were determined by measuring absorbance at 460 nm of the pigments collected in hexane, and was expressed as percentage relative to the amount of pigments obtained from mature arthrospores (8 days old).

Thin-layer chromatography. The pigment solution in hexane was applied on alumina thin-layer sheets (no. 6063, Eastman Organic Chemicals, Rochester, N.Y.), or on silica gel thin-layer sheets (no. 6061, Eastman Organic Chemicals). Development of thin-layer plates was carried out at 25°C in the following solvent systems: hexane–benzene–*n*-butanol (10:1:1) or hexane–benzene (10:1.5). Colorless spots were visualized either by illumination of chromatograms with UV light (Mineral light, model R52, equipped with filter no. 20119 for short wavelength and no. 20118 for long wavelength; Ultraviolet Products, Inc., San Gabriel, Calif.) or spraying with the reagents specified below. To ascertain the nature of the pigments, chromatograms were sprayed with various reagents as specified in Table 2. Authentic carotenoids, such as α- and β-carotene, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lycopene was isolated from tomato according to the method described by Weedon (22). Phytoene and phytofluene were similarly prepared from *Rhodotorula rubra*.

Carr-Price test for carotenoids. The Carr-Price test for carotenoids (3, 5) was performed by spraying chromatograms with 20% antimony trichloride (J. T. Baker Chemical Co., Phillipsburg, N.J.) in chloroform.

Spectroscopy. For spectroscopic study of individual carotenoids, the extract was streaked on thin-layer plates, and distinctly separated lines of pigments after development were eluted in hexane. Absorption spectra of individual carotenoids in hexane were examined in a Cary 15 spectrophotometer.

Reagents. All chemicals used in the present investigation were of reagent quality. All solvents were either of chromatographic or spectroscopic quality, depending on the purpose of the experiments, and were used without further purification.

Dry-weight determination. Dry weight of cells on extracted materials was determined by desiccating samples in preweighed aluminum planchets over P_2O_5 placed in vacuum at 25°C for 72 h.

Cytological techniques. The light-microscope appearance of the wet-mounted cells was examined with either an ordinary or phase-contrast microscope using oil immersion objectives. Photomicroscopy was made on panchromatic film (Kodak Plus X) with a Nikon camera equipped with an automatic exposure system. Thin sections and freeze-etched replicas of arthrospores were prepared by the methods described earlier (17). Specimens were examined with a Hitachi HU-11A electron microscope operating at 50 kV.

RESULTS

Identification and characterization of pigments. The thin-layer chromatographic and spectroscopic properties of the pigments extracted from mature (8 days old) arthrospores of *T. mentagrophytes* are shown in Table 1. The results of various color reactions performed directly on thin-layer chromatograms are summarized in Table 2. The characteristic color of these pigments on thin-layer chromatogram sheets tended to fade when the pigments were dessiccated in air or exposed to intensive light. Although it was not possible to firmly establish the identification of a few minor pigments because of the lack of certain authentic carotenoids, the data presented in Tables 1 and 2 are compatible with our conclusion that the majority of the pigments occurring in arthrosporulating *T. mentagrophytes* are indeed carotenoid in nature. It is clear from Table 3 that the pigments isolated from arthrospores of *T. mentagrophytes* were not xanthomegnin. To our knowledge, this is the first demonstration of carotenoid pigments in the group of dermatophytic fungi.

Kinetics of growth, arthrosporulation and pigment formation. Under our experimental conditions, arthrosporulation began as soon as the maximum hyphal growth had been attained, and intracellular pigment granules became evident upon the completion of multiple segmentation of hyphae (Fig. 1). During the maturation of arthrospores, cells transformed from rectangular to oval shape and formed short chains or were released as single cells as a result of disarticulation of connecting septa (Fig. 1). During this maturation process, arthrospores continuously accumulated intracellular pigments, reaching a maximum in about 8 days.

Although carotenoid pigments could not be demonstrated in any states of growth (hyphae or microconidia) other than during arthrosporulation, carotenogenesis does not seem to be an essential event for either the induction or the completion of arthrosporulation. Complete arthrosporulation took place when this fungus was grown on Sabouraud-sodium acetate medium at 39°C (data not shown). Downy strains, naturally occurring asporogenic variants of *T. mentagrophytes*, were able neither to arthrosporulate nor to form carotenoid pigments under all conditions we tested. Light was not required for the induction of carotenoid biosynthesis in arthrosporulating *T. mentagrophytes*.

Cytology of pigment granules. Pigment granules usually emerged in the subterminal portion of sporulating cells. As spores matured, they tended to translocate to the center of the

TABLE 1. *Thin-layer chromatographic and spectroscopic characteristics of epiphase carotenoids found in T. mentagrophytes arthrospores*

Identification	Rate of flow (R_f)				Absorption maxima in hexane[a] (nm)
	Alumina		Silica gel		
	Solvent A[b]	Solvent B[c]	Solvent A	Solvent B	
Ergosterol (non-carotenoid)	0.28	0	0.48	0	271, 282, 294
Unidentifiable spot	0.32	0	0.51	0	403, 425, 450
Lycopene	0.40	0.02	0.77	0.33	443, 469, 500
					(443, 469, 500)
Unidentifiable spot	0.47				440, 465, 496
Neurosporene	0.57	0.09	0.80	0.43	414, 438, 467
					(414, 437, 466)
γ-Carotene	0.57	0.06	0.80	0.49	431, 458, 487
					(434, 459, 490)
ζ-Carotene	0.61	0.14	0.84	0.53	379, 399, 424
					(379, 400, 424)
Unidentifiable spot	0.67	0.14	0.84	0.59	426, 449, 475
β-Carotene	0.67	0.20	0.84	0.64	428, 450, 478
					(430, 450, 477)
Phytofluene	0.73	0.33	0.87	0.60	330, 348, 367
					(331, 348, 367)
Phytoene	0.78	0.59	ND[d]	0.83	277, 286, 298
					(275, 286, 298)

[a] Figures in parentheses represent values reported by others (3, 5). Major peaks are underlined.
[b] Solvent A: hexane–benzene–butanol (10:1:1).
[c] Solvent B: hexane–benzene (10:1.5).
[d] ND, Not detectable.

TABLE 2. *Color reaction of epiphase carotenoids of T. mentagrophytes arthrospores with various reagents*

Identification	Color	Carr-Price test for carotenoids[a]	2 N NaOH[a,b]	70% $HClO_4$	0.5% Mg acetate[a,c] (methanolic)
Ergosterol (non-carotenoid)	Colorless	–	–	Red	–
Unidentifiable spot	Yellow	+	–	Blue	–
Lycopene	Pink	+	–	Blue	–
Unidentifiable spot	Light pink	±	–	Blue	–
Neurosporene	Yellow	+	–	Blue	–
γ-Carotene	Orange	+	–	Blue	–
ζ-Carotene	Yellow	+	–	Blue	–
Unidentifiable spot	Orange	+	–	Blue	–
β-Carotene	Light yellow	+	–	Blue	–
Phytofluene[d]	Colorless	±	–	Yellow	–
Phytoene[d]	Colorless	±	–		–

[a] –, No change in color; ±, weakly positive; +, positive.
[b] Xanthomegnin became purple when sprayed with 2 N NaOH.
[c] Xanthomegnin and quinone derivatives turned to purple to violet.
[d] Phytoene strongly absorbed UV light. Phytofluene fluoresced under long-wavelength UV light.

cells (Fig. 2A). Pigment granules were readily stained with Sudan black B. In thin sections, the pigment granules consisted of osmiophilic matrices embedding membranous substructures (L in Fig. 2B). The lamellar nature of the substructure within pigment granules was more clearly seen when arthrospores were freeze-fractured and etched for electron microscopy (Fig. 2C). It is not clear at this time whether the lamellar organelle within lipid granules is directly involved in the synthesis of the pigments. Neither pigment granules nor intragranular lamellar

TABLE 3. *Some major differences between carotenoid and quinone pigments*[a]

Pigment	Absorption spectra (visible light)	Carr-Price test	Alkali solubility (2 N NaOH)	Color change in:		R_f (TLC)[b] in polar solvent benzene-acetone (3:1)
				2 N NaOH	0.5% Mg-acetate	
Carotenoids (α- or β-carotene)	Usually three characteristic peaks	+	−	−	−	1.0
Xanthomegnin[c]	One broad peak	−	+	+	+	<1.0

[a] Results expressed as −, no change in color; +, positive.
[b] Rate of flow on thin-layer chromatographic (TLC) sheet (silica).
[c] A representative quinone pigment from *Microsporum cookei* (provided by Y. Nozawa).

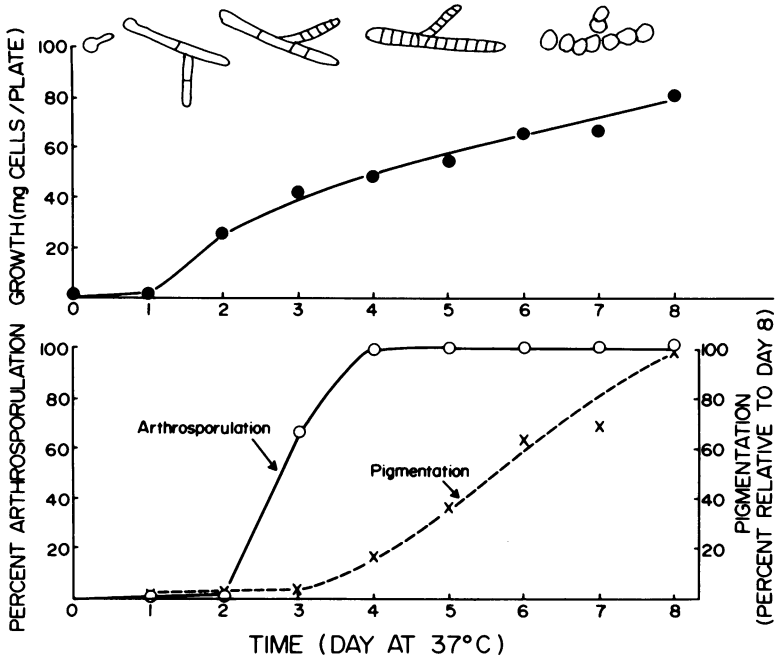

FIG. 1. *Carotenoid pigment formation during arthrosporulation of T. mentagrophytes. The typical appearance of cells is depicted in the upper graph, and the relationship of carotenoid pigment formation to arthrosporulation is shown in the lower graph.*

structures were found in vegetative hyphae or in microconidia of this fungus.

DISCUSSION

The data presented in Table 1 and 2 clearly indicate that the pigments associated with arthrosporogenesis of *T. mentagrophytes* are carotenoids and not quinone derivatives. In addition to the positive test for carotenoids (Carr-Price test) and the typical carotenoid three-peak light absorption spectra (Table 1), the insolubility of the pigments in 2 N NaOH and their failure to change color in alkali or in magnesium acetate solution (18) render it highly unlikely that these pigments from *T. mentagrophytes* arthrospores are quinone derivatives as generally reported for dermatophytes. The authentic fungal quinone pigment xanthomegnin, (−)3,3'-bis-[2-methoxy-5-hydroxy-7-(2-hydroxypropyl)-8-carboxy-1,4-naphthoquinone lactone], reacted quite differently from the *T. mentagrophytes* pigments in both the physical and chemical tests (Table 3).

Considering the rather ubiquitous occurrence of carotenoid pigments in fungi (7), it is not surprising to find carotenoids in a dermatophyte. However, the unusual finding that carotenogenesis in this fungus occurs only during arthrosporulation suggests a reason why carotenoid pigments have not been previously detected in *T.*

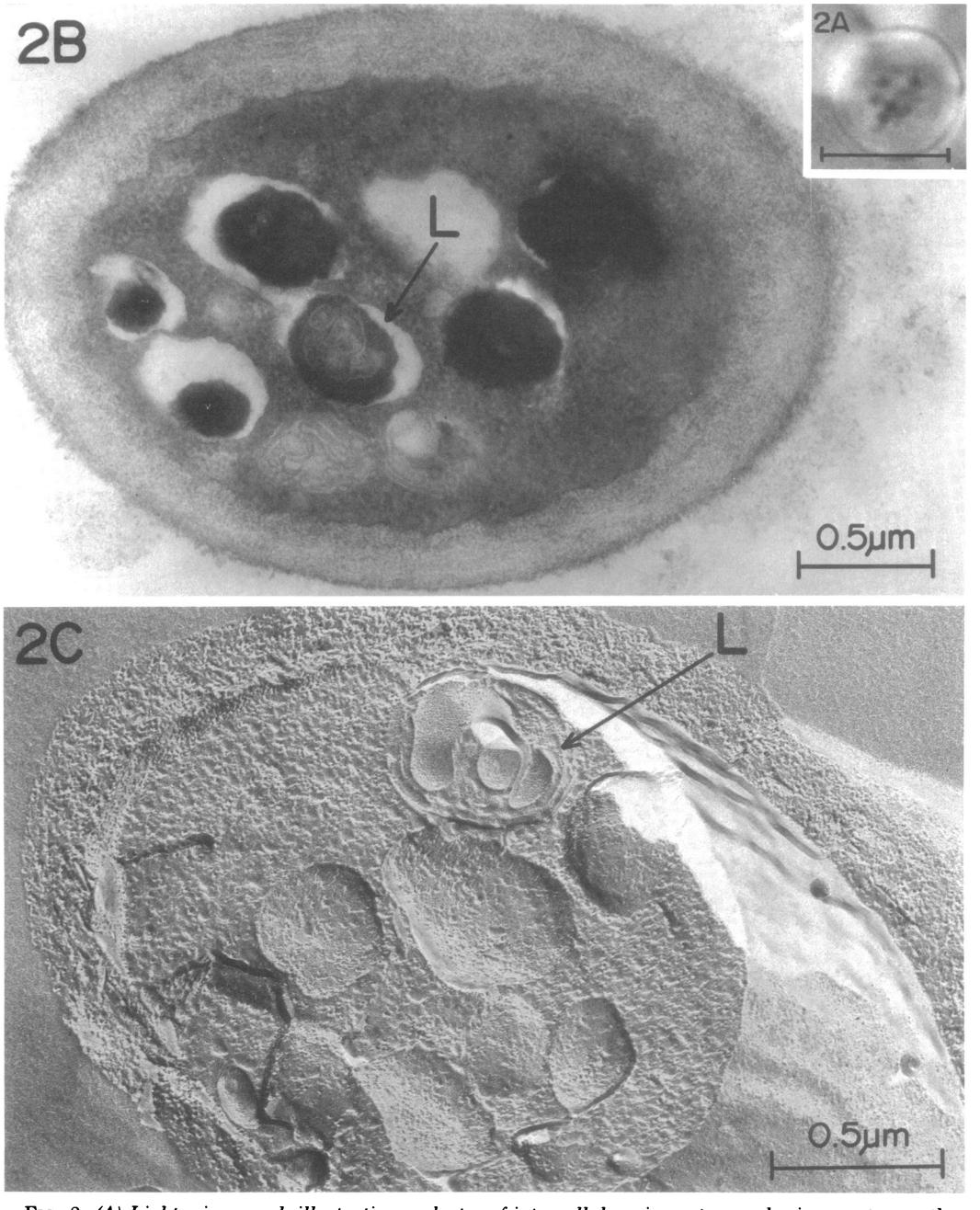

FIG. 2. *(A) Light micrograph illustrating a cluster of intracellular pigment granules in a mature arthrospore of T. mentagrophytes. Bar in (A) represents 5 μm. (B) and (C) Electron micrographs of thin-sectioned and freeze-etched arthrospores of T. mentagrophytes, respectively. Note the osmiophilic nature of pigment granules and the presence of complex membranous structures (L) within pigmented granules.*

mentagrophytes. Recent studies (R. Emyanitoff, Ph.D. dissertation, Loyola University, 1978) have shown that the induction of arthrosporulation in this dermatophyte is possible only under specific nutritional and physiological conditions and that arthrospores would not be formed under most routine growth conditions. Mares et al. (12) recently observed either

yellow or orange substance in *T. mentagrophytes* growing under a condition which, in our judgment, was conducive to arthrosporulation. Without attempting chemical analysis, they assumed the pigment to be a "compound of quinone structure since similar substances are common in the pigmented strains of dermatophytes." In fact, many strains of dermatophytes are known to produce pigments which are of quinone derivatives. Among these, the most common pigment is xanthomegnin, the major pigment produced by *T. megnini* (2). Xanthomegnin was subsequently found in many other strains of dermatophytes (6, 11, 14, 21, 23) as well as in other fungi (19). It appears that the unique association of carotenogenesis with arthrosporulation and an a priori assumption by some workers that dermatophytes produce only quinone type pigments are jointly responsible for undue delay in demonstrating carotenoids in dermatophytes. It is anticipated that with the elucidation of specific conditions for arthrosporulation, similar carotenoids may be found in other dermatophytic fungi.

Despite the intimate association of carotenogenesis and arthrosporulation in this fungus, the former does not seem to be an essential event for the completion of arthrospore formation, although the properties of arthrospores with or without carotenoids need further examination. Morphologically normal arthrospores could be produced when the fungus was arthrosporulated at 39°C instead of 37°C. Such arthrospores were almost completely devoid of carotenoid pigments (Hashimoto and Mock, unpublished data).

The exact localization of carotenoid pigments has not been firmly established in many fungi. They are reported to be located in mitochondria of *Blakeslea trispora* (4), *Neurospora crassa* (13), and *Phycomyces* (10), or in lipid globules and the particulate fraction of *Phycomyces blakesleeanus* (16). In *T. mentagrophytes* arthrospores, we found the carotenoid pigments to be mostly, if not completely, localized in microscopically discernible intracellular granules (Fig. 2). The presence of membranous structures within the pigmented granules (Fig. 2B and 2C) suggests their involvement in carotenoid synthesis during arthrosporulation and may provide a useful system to investigate the physiological and ultrastructural aspects of carotenoid formation in microbial cells.

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