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Oxygen as a possible tropic factor in hyphal growth of *Candida albicans*

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Hyphae of *Candida albicans* elongated towards the oxygen-rich direction when exposed to gradients of oxygen concentration in thin-layer and capillary-tube cultures with corn meal (CM) agar. The thin-layer culture was prepared by covering a drop of molten CM agar containing *C. albicans* cells with a cover slip in Petri dishes. Cells located in the central region of the thin-layered medium neither grew nor produced hyphae. Cells in the marginal regions at first directed their hyphae in arbitrary directions after forming a small colony. Hyphae then gradually changed their direction of elongation and eventually oriented towards the nearest margin. Under anaerobiosis, cells seeded in the thin-layered medium did not grow even in the marginal regions. When exposed to air, the cells in the marginal regions rapidly began to form hyphae which elongated towards the nearest margin. To prepare an oxygen gradient in capillary-tube cultures, CM agar, and dilute and dense cell suspensions in CM agar were introduced sequentially into the capillary tubes, and the end closest to the dense cell suspension was sealed with paraffin. Among cells in the dilute layer, only that located closest to the meniscus grew well and extended hyphae towards the meniscus, where oxygen concentrations were highest. These studies suggest a positive aerotropic response in the hyphal growth of *C. albicans*.

Key Words——aerotropism; *Candida albicans*; hyphal growth; oxygen.

The opportunistic fungal pathogen *Candida albicans* (Robin) Berkhout can grow as an oval yeast cell or an elongated hyphal cell, depending on nutritional and environmental conditions. Many studies have focused on the dimorphism of *C. albicans*, because its morphological conversion is of interest in respect not only to cellular differentiation but also to pathogenesis (Odds, 1988, 1993). A number of factors that control the morphological conversion of *C. albicans* have been reported, including the partial pressure of oxygen. McClary (1952) reported the inability of *C. albicans* to grow anaerobically. Others, however, reported limited but discernible anaerobic growth (Szawatkowski and Hamilton-Miller, 1978; Kennedy, 1981; Eklund and Jarmund, 1983; Samaranayake et al., 1983; Webster and Odds, 1986). The effects of anaerobiosis on morphogenesis include a lack of germ tube formation (Pollack and Hashimoto, 1985), a predominance of pseudohyphal growth (Preusser and Rostek, 1983), and production of spiral hyphae (Kaminishi et al., 1994). Thus, it was of interest to examine the modes of hyphal growth of *C. albicans* when exposed to an oxygen gradient in culture.

Morphological and biochemical events associated with the hyphal growth of fungi, including *C. albicans*, are currently a major topic in mycology (Gow, 1994, 1995a, b; Carlile, 1995; Gooday, 1995). Hyphae of *C. albicans* exhibit galvanotropism in response to applied electrical fields (Crombie et al., 1990) and thigmotropism due to contact sensing (Sherwood et al., 1992; Gow et al., 1994). To date there has been little evidence for chemotropism in the Fungi Imperfecti (Gooday, 1975), except perhaps towards oxygen, if this is regarded as a nutrient. To our knowledge, there is no report of an aerotropic response in *C. albicans*. Here, we report that *C. albicans* extended its hyphae in response to oxygen gradients in thin-layer and capillary-tube cultures in corn meal (CM) agar medium.

Materials and Methods

Fungal strain The K strain of *C. albicans* used in the present study was isolated from a patient with oral candidosis (Aoki and Ito-Kuwa, 1982). It was maintained on slants of Sabouraud glucose agar (Nissui) held at 4°C.

Culture conditions Yeast-form cells grown on Sabouraud glucose agar at 30°C for 3–4 d were washed in sterile distilled water and transferred into CM agar (Nissui), which had been autoclaved and kept molten at

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50°C in a water bath. The cell concentration of the suspension was adjusted to about 2.5×10^4/ml. For thin-layer cultures, 20 μl of the suspension was dropped onto the center of a glass Petri dish (9 cm in diam) warmed on a hot plate at 55°C, then immediately covered with a cover slip (24 × 32 mm), producing an agar layer of about 26 μm in thickness that was calculated to contain about 500 yeast cells. The margins of the cover slips were sealed with molten 1.5% (w/v) Bact agar (Difco) to prevent drying of the thin agar layer. The dishes were turned upside down and pieces of moist filter paper were placed in the dishes. The cultures were incubated aerobically at 25°C. An anaerobic culture system, Anaeromate-P (Nissui), was used for the anaerobic studies. Anaerobiosis was achieved within a few hours after the onset of incubation of the dishes with a deoxidizer in the Anaeromate-P bag.

Capillary tubes (1–5 μl micropipettes of 0.34 mm in inner diam and 12.6 cm in length; Drummond Sci.) were used. Molten CM agar was introduced first into the tubes by capillary action, followed by a dilute cell suspension (2.5×10^4 cells/ml) and finally by a dense one (10^6 cells/ml) in molten CM agar. The first column of CM agar in the tubes was 0.5–1 cm in length, and the dilute and dense cell suspensions were 2–3 cm each. The lower end, proximal to the dense cell suspension of the tubes, was sealed with paraffin to prevent contact with the atmosphere, while the opposite end was left open to the air. These tubes were incubated aerobically at 25°C in a plastic box with pieces of moist filter paper. Growth of the hyphae in the thin-layer and capillary-tube cultures was observed using a light microscope (Olympus BHS) or a low power microscope with dark field illumination (Wild M400), and photographed on Fuji Neopan SS film.

Results

Hyphal growth types in thin-layer cultures When *C. albicans* was grown in thin-layered CM agar, cells in the central region of the thin agar medium stopped growth after forming minute colonies that could not produce hyphae. In contrast, the cells distributed in the marginal regions of the thin agar medium (less than 3–4 mm from the cover slip edges) produced hyphae after forming minute colo-

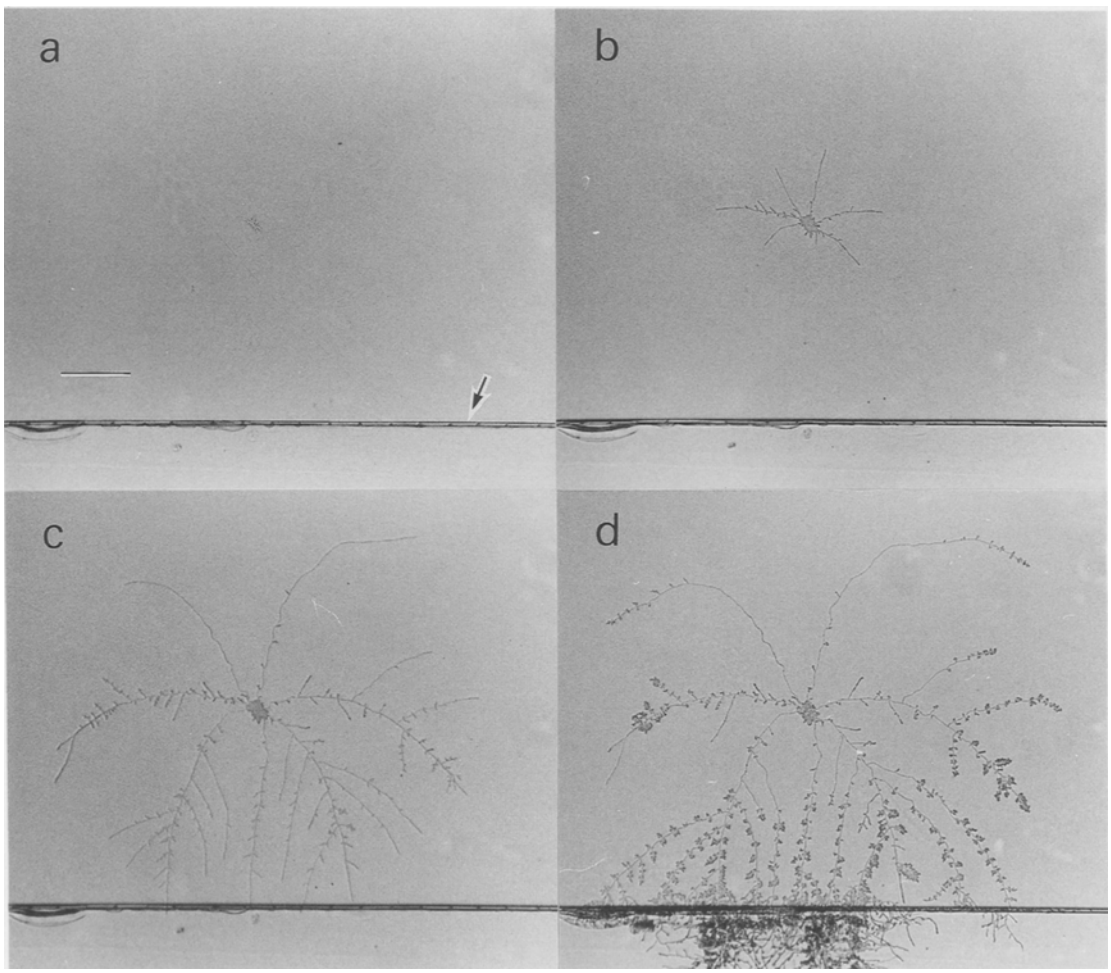

Fig. 1. Time course of hyphal extension from a colony located in the marginal region of the thin-layer culture. Times (h) of incubation are: a, 25; b, 40; c, 64; and d, 92. Arrow indicates the edge of the cover slip. Bar = 200 μm.

nies, as shown in Fig. 1. The minute colonies had the potential to direct their hyphae in arbitrary directions. However, the hyphae gradually changed the direction of their extension towards the nearest margin of the thin-layered agar medium. The primary hyphae repeatedly extended and branched towards the margin (Fig. 2). Similar patterns of hyphal extension were observed when CM agar was used to seal the cover slips. On CM agar, larger colonies produced hyphae which had more yeast cells at locations near the edges of the thin-layered medium (Fig. 3).

The mode of hyphal extension in the marginal regions of the thin-layer cultures was influenced strongly by the density of the seeded cells. When the density was about 500–1,000 cells/20 μl, hyphae elongated vigorously, as shown in Figs. 1 and 2. In contrast, when the density was over about 2,000 cells/20 μl, hyphae were produced from minute colonies, but their elongation was interrupted by the formation of chlamydospores at the hyphal tips (Fig. 4). Thus, we chose a density of about 500 cells/20 μl in order to study their tropic behaviour in gradients of oxygen.

A sparse distribution of seeded cells in the thin-layer culture was also necessary to avoid hyphal orientation caused by contact with adjacent hyphae. As shown in Fig. 5, the hyphae of three close colonies extended and oriented themselves as if they sensed their mutual growth and wished to keep some space between each other, although the hyphae eventually extended towards the nearest margin.

Effects of conversion from anaerobiosis to aerobiosis on hyphal extension in thin-layer culture are shown in Fig. 6. When a thin-layer plate was incubated anaerobically, the *C. albicans* cells could neither grow in their yeast form nor produce hyphae even in the marginal regions of the thin-layered medium. When released from anaerobiosis to aerobic conditions, the cells located in the marginal regions rapidly began to grow and formed minute colonies. The hyphae produced from these colonies extended towards the nearest margin, demonstrating that the hyphal growth was strictly dependent on oxygen.

The above results suggested that the tropic hyphal extension in the thin-layer cultures on CM agar was due to a response to oxygen. To demonstrate the presence of oxygen in the thin-layer cultures, a photogenic bacterium isolated from a marine fish was used. The luminescence was generated by the bacterium's cells only at the edges of the thin-layered medium, suggesting a very slow diffusion of oxygen into the thin-layered agar medium (data not shown). Thus, the oxygen concentration was likely to be highest at the margins of the thin-layered plates and lower as the distance from the margins in-

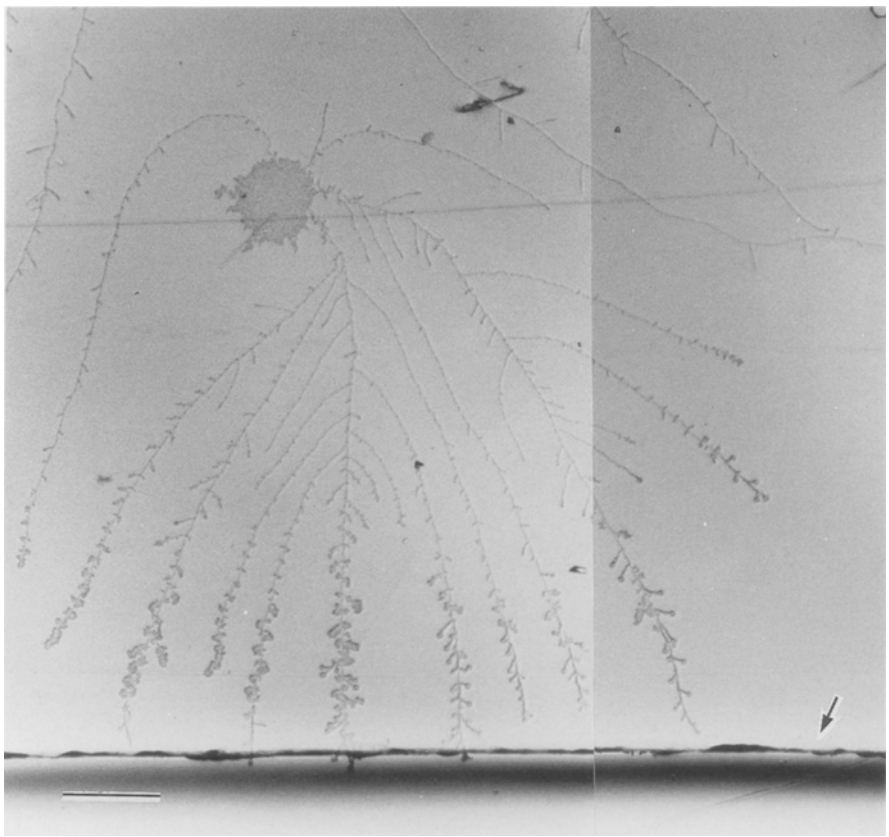

Fig. 2. Hyphal extension from a colony located in the marginal region of a 4 d thin-layer culture. Arrow indicates the edge of the cover slip. Bar = 200 μm.

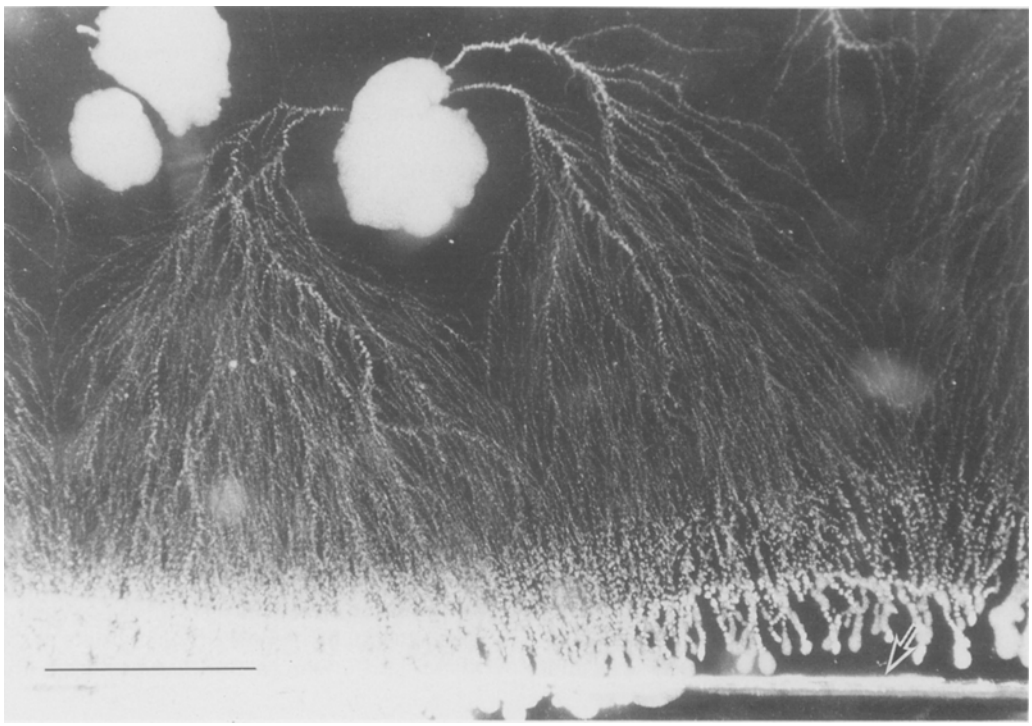

Fig. 3. Dark-field photomicrograph of aerotropic hyphal growth from a colony located in the marginal region of a 29 d thin-layer culture.
Note the larger size of the colony and extensive blastospore production by the hyphae as a result of the cover slip being sealed with CM agar, instead of plain agar, and the prolonged culture period. Arrow indicates the edge of the cover slip. Bar = 1 mm.

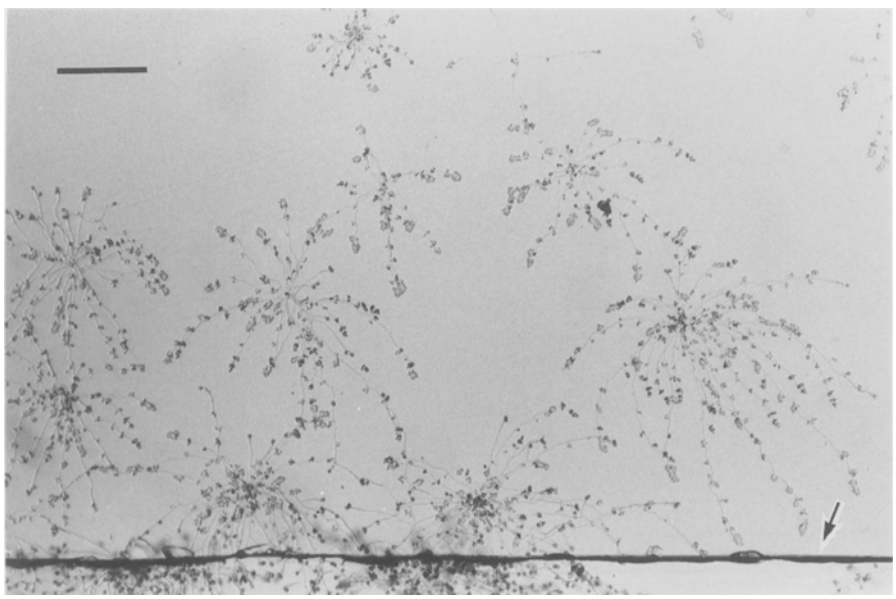

Fig. 4. Hyphal growth by densely seeded cells in a 4 d thin-layer culture.
Hyphal extension ceased after formation of chlamydospores at the hyphal tips. Arrow indicates the edge of the cover slip. Bar = 200 μm.

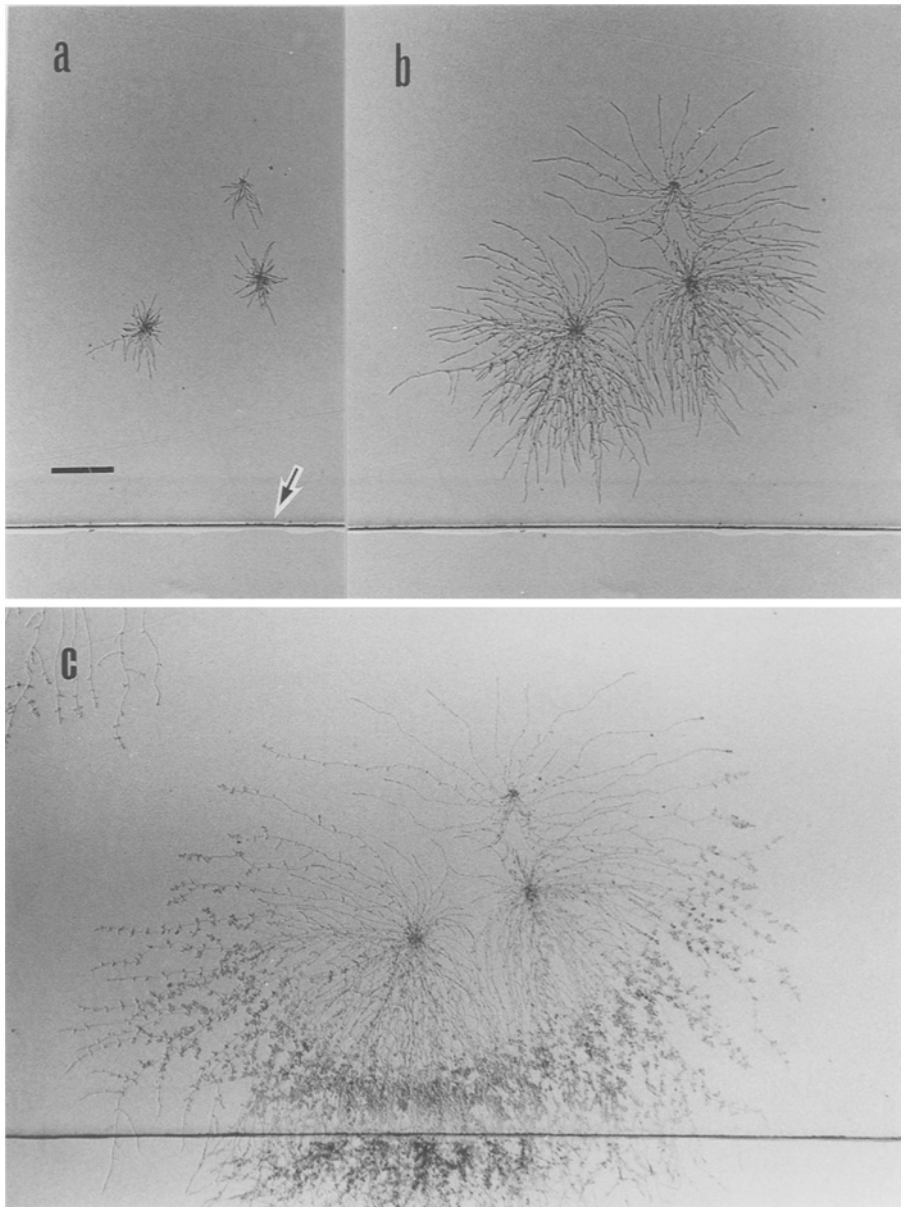

Fig. 5. Time course of hyphal growth from closely located colonies in thin-layer culture.
The direction of hyphal extension was probably affected by the "Langeron effect", although the hyphae eventually extended towards the margin. Times (h) of incubation are: a, 25; b, 40; c, 64. Arrow indicates the edge of the cover slip. Bar = 200 μm.

creased.

To confirm the prevalence of the tropic hyphal growth among other *C. albicans* strains, five oral isolates from normal subjects were subjected to thin-layer culture. All the tested strains, like strain K, exhibited tropic hyphal elongation in the culture, demonstrating that the tropic response was not restricted only to strain K (data not shown).

Hyphal extension in capillary-tube cultures To make an oxygen gradient in an agar medium, capillary-tube cultures were devised by successively introducing CM agar, dilute and dense yeast cell suspensions into the tubes. The lower ends of the tubes were sealed with paraffin, while the upper ends were left open to the atmosphere. In the three-layered capillary medium, the oxygen concentration was expected to be highest at the meniscus of the first CM agar layer and lowest at the sealed tip of the third layer with a dense cell suspension, because of the rapid consumption of oxygen by the densely seeded cells and absence of oxygen diffusing from the sealed tip. Thus, cells located in the middle, dilute cell suspension layer were expected to be exposed to an oxygen gradient.

Cells located in the dilute layer of the capillary-tube culture grew to form minute colonies. Among the colonies, only that located nearest to the meniscus produced

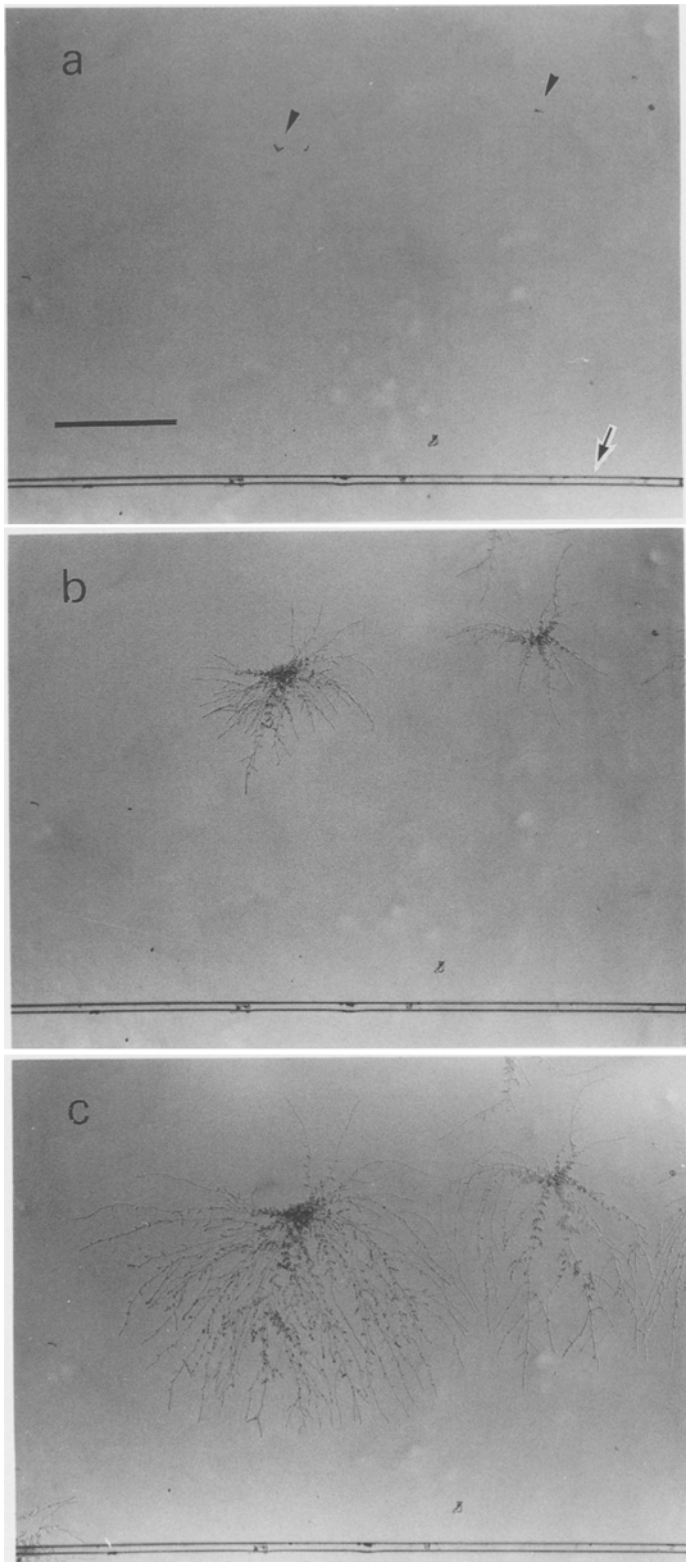

Fig. 6. Effects of conversion from anaerobiosis to aerobiosis on hyphal elongation in thin-layer culture.
a, Minute colonies formed after 8 d under anaerobic conditions (arrow heads); b and c, hyphal growth 1 and 2 d after exposure to aerobic conditions, respectively. Arrow indicates the edge of the cover slip. Bar = 400 μm.

hyphae extensively. The hyphae extended towards the meniscus, but not in the opposite direction, where the capillary had been sealed with paraffin (Fig. 7). The other colonies in the dilute layer did not develop any hyphal growth towards the meniscus.

Experiments were devised to determine whether the absence of hyphal extension towards the sealed capillary tip by the colony located closest to the meniscus was due to depletion of nutrients or inhibition by accumulation of metabolites. When a capillary tube cultured for 7 d was cut at a site in the dense cell suspension layer and exposed to the atmosphere, the cells in the dense cell layer began to grow rapidly (data not shown). This result showed that the limited growth of the cells in the dense cell layer before cutting was due to anaerobiosis and excluded the possibility of hyphal growth inhibition due to the depletion of nutrients or the accumulation of metabolic products in the capillary-tube cultures.

Discussion

In the present study, the CM agar medium was used to observe the manner of hyphal extension of *C. albicans* in thin-layer and capillary-tube cultures. We reported previously the inability of respiration-deficient mutants of *C. albicans* to grow on CM agar (Ito-Kuwa et al., 1990). This indicated that CM agar contains little or no amounts of easily fermentable carbon sources and, thus, growth of *C. albicans* on CM agar depends on respiration. Therefore, oxygen is most likely to be the sole factor controlling the growth of *C. albicans* in the thin-layer and capillary-tube cultures.

Robinson (1973) demonstrated positive aerotropism in *Geotrichum candidum* Link: Persoon using a perforated plate with a central hole. When arthrospores of the fungus were seeded densely on the perforated plate, germination and germ tube production occurred in response to the oxygen which had diffused from the central hole of the perforated plate. On the basis of these observations, he hypothesized that a tropic response to oxygen occurred when the oxygen concentration began to limit respiration of the spores and their germ tubes. In the micro-environment of the perforated plate assay, a steep oxygen gradient caused an imbalance in respiratory activity between the opposite sides of the hyphae. This imbalance gave rise to production of fewer apical vesicles on the side of the hypha nearest to the lower oxygen concentration. As a consequence, the hypha turned towards the hole from which the oxygen had diffused (Robinson, 1973). In *C. albicans*, the apical cells of the hyphae are rich in cytoplasm (Gow and Gooday, 1984) and large mitochondria (Aoki et al., 1989), and they extend leaving behind highly vacuolated intercalary compartments. These morphological features demonstrate the crucial role of respiration in the production of energy for cell wall synthesis and hyphal tip extension. Incorporating the hypothesis of Robinson (1973) into these results, the tropic hyphal growth of *C. albicans* described above can be explained as follows. The concentration of oxygen in thin-layered CM agar is highest in the margin of the medium, and decreases gradually as the distance from the margin increases. Thus, the zone of the respiration-limiting oxygen concentration moves from the central region to the marginal region of the medium as the fungal cells consume oxygen. Thus, the apical cells of the hyphae direct their extension towards the oxygen-rich margin. This is also the case in the hyphal growth towards the meniscus in the capillary-tube cultures.

Recently, Bartnicki-Garcia et al. (1995) demonstrated that the shape of the hypha of *Rhizoctonia solani* Kühn was determined by movement of the Spitzenkörper in the hyphal tips. Thus, it would be of interest to examine whether such an intracellular structure functions in the aerotropic hyphal growth of *C. albicans*, although a Spitzenkörper or an equivalent structure has yet to be demonstrated in this species.

Two tropic responses, galvanotropism (Crombie et al., 1990) and thigmotropism (Sherwood et al., 1992; Gow et al., 1994), have been reported in the hyphal growth of *C. albicans*. It has been suggested that the

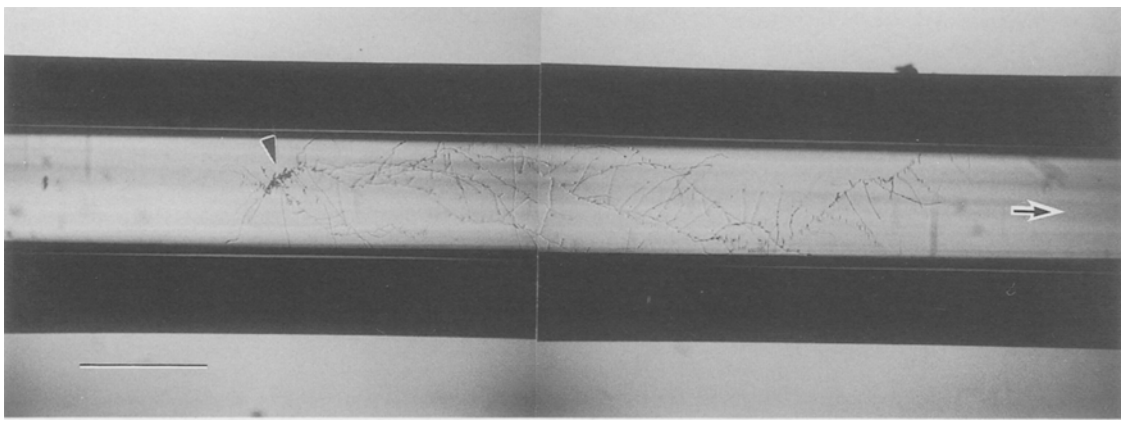

Fig. 7. Hyphal growth 14 d after inoculation in capillary-tube culture.
The cell located nearest to the meniscus grew to form a minute colony (arrow head) and hyphae extended from the colony in the direction of the meniscus (arrow). Bar = 500 μm.

latter tropic response plays a role in the primary invasion of epithelia during superficial *Candida* infections. In the present study the presence of positive aerotropism was strongly suggested as the third tropic response in the hyphal growth of *C. albicans*. We reported previously the importance of respiration in both yeast and hyphal growth of *C. albicans* (Aoki and Ito-Kuwa, 1982, 1987; Ito-Kuwa et al., 1990; Aoki et al., 1993), and that the morphological studies of mitochondrial behaviour supported this view (Ito-Kuwa et al., 1988; Aoki et al., 1989). Considering the results obtained in the present study together with those reported previously, it should be stressed that the growth of *C. albicans* is predominantly dependent on respiration. Thus, aerotropism seems to play an important role in the hyphal extension of *C. albicans* when subjected to oxygen-limited conditions. The operation and role of the aerotropism of *C. albicans* in vivo or in pathogenesis remains to be resolved in future studies.

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Literature cited

Aoki, S. and Ito-Kuwa, S. 1982. Respiration of *Candida albicans* in relation to its morphogenesis. Plant Cell Physiol. **23**: 721–726.

Aoki, S. and Ito-Kuwa, S. 1987. Induction of petite mutation with acriflavine and elevated temperature in *Candida albicans*. J. Med. Vet. Mycol. **25**: 269–277.

Aoki, S., Ito-Kuwa, S., Nakamura, Y. and Masuhara, T. 1989. Mitochondrial behaviours during the yeast-hypha transition of *Candida albicans*. Microbios **60**: 79–86.

Aoki, S., Ito-Kuwa, S., Nakamura, K., Osafune, T., Ehara, T. and Takeo, K. 1993. Growth responses of a wild-type parent and a respiratory mutant of *Candida albicans* to respiratory inhibitors. Jpn. J. Oral Biol. **35**: 520–524.

Bartnicki-Garcia, S., Bartnicki, D. D., Gierz, G., Loez-Franco, R. and Bracker, C. E. 1995. Evidence that Spitzenkörper behavior determines the shape of a fungal hypha: a test of the hyphoid model. Exp. Mycol. **19**: 153–159.

Carlile, M. J. 1995. The success of the hypha and mycelium. In: The growing fungus, (ed. by Gow, N. A. R. and Gadd, G. M.), pp. 1–19. Chapman & Hall, London.

Crombie, T., Gow, N. A. R. and Gooday, G. W. 1990. Influence of applied electrical fields on yeast and hyphal growth of *Candida albicans*. J. Gen. Microbiol. **136**: 311–317.

Eklund, T. and Jarmund, T. 1983. Microculture model studies on the effect of various gas atmospheres on microbial growth at different temperatures. J. Appl. Bacteriol. **55**: 119–125.

Gooday, G. W. 1975. Chemotaxis and chemotropism in fungi and algae. In: Primitive sensory and communication systems, (ed. by Carlile, M. J.), pp. 155–204. Academic Press, London.

Gooday, G. W. 1995. The dynamics of hyphal growth. Mycol. Res. **99**: 385–394.

Gow, N. A. R. 1994. Growth and guidance of the fungal hypha. Microbiology **140**: 3193–3205.

Gow, N. A. R. 1995a. Tip growth and polarity. In: The growing fungus, (ed. by Gow, N. A. R. and Gadd, G. M.), pp. 278–299. Chapman & Hall, London.

Gow, N. A. R. 1995b. Yeast-hyphal dimorphism. In: The growing fungus, (ed. by Gow, N. A. R. and Gadd, G. M.), pp. 403–422. Chapman & Hall, London.

Gow, N. A. R. and Gooday, G. M. 1984. A model for the germ tube formation and mycelial growth form of *Candida albicans*. J. Med. Vet. Mycol. **22**: 137–143.

Gow, N. A. R., Perera, T. H. S., Sherwood-Higham, J., Gooday, G. W., Gregory, D. W. and Marshall, D. 1994. Investigation of touch-sensitive responses by hyphae of the human pathogenic fungus *Candida albicans*. Scan. Microsc. **8**: 705–710.

Ito-Kuwa, S., Aoki, S., Nakamura, Y. and Masuhara, T. 1990. Further characterization of respiratory mutants of *Candida albicans*. Jpn. J. Oral Biol. **32**: 193–197.

Ito-Kuwa, S., Aoki, S., Watanabe, T., Ehara, T. and Osafune, T. 1988. Fluorescence microscopic studies on mitochondria and mitochondrial nucleoids in a wild-type strain and respiratory mutants of *Candida albicans*. J. Med. Vet. Mycol. **26**: 207–217.

Kaminishi, H., Iwata, A., Tamaki, T., Cho, T. and Hagihara, Y. 1994. Spiral hyphae of *Candida albicans* formed in anaerobic culture. Mycoses **37**: 349–352.

Kennedy, M. J. 1981. Inhibition of *Candida albicans* by the anaerobic oral flora of mice in vitro. Sabouraudia **19**: 205–208.

McClary, D. O. 1952. Factors affecting the morphology of *Candida albicans*. Ann. Missouri Bot. Gard. **39**: 137–164.

Odds, F. C. 1988. *Candida* and candidosis, pp. 42–59. Bailliere Tindall, London.

Odds, F. C. 1993. Morphological change in *Candida albicans*. Jpn. J. Med. Mycol. **34**: 99–111.

Pollack, J. H. and Hashimoto, T. 1985. Ethanol-induced germ tube formation in *Candida albicans*. J. Gen. Microbiol. **131**: 3303–3310.

Preusser, H. J. and Rostek, H. 1983. Der Einfluss von Nahrmedium und Sauerstoff-Partialdruck auf Wachstum, Morphologie und Cytologie von *Candida albicans* in vitro. Mykosen **26**: 501–512.

Robinson, P. M. 1973. Oxygen – positive chemotropic factor for fungi? New Phytol. **72**: 1349–1356.

Samaranayake, L. P., Geddes, D. A. M., Weetman, D. A. and McFarlane, T. W. 1983. Growth and acid production of *Candida albicans* in carbohydrate supplemented media. Microbios **37**: 105–115.

Sherwood, J., Gow, N. A. R., Gooday, G. W., Gregory, D. W. and Marshall, D. 1992. Contact sensing in *Candida albicans*: a possible aid to epithelial penetration. J. Med. Vet. Mycol. **30**: 461–469.

Szawatkowski, M. and Hamilton-Miller, J. M. T. 1978. Anaerobic growth and sensitivity of *Candida albicans*. Microbios Lett. **5**: 61–66.

Webster, C. E. and Odds, F. C. 1986. Growth of pathogenic *Candida* isolates anaerobically and under elevated concentrations of CO_2 in air. J. Med. Vet. Mycol. **25**: 47–53.