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Ultrastructural Studies of the Mycelium-to-Yeast Transformation of *Sporothrix schenckii*

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Fine details of the internal and external morphology of the in vitro mycelial phase (MP) to yeastlike phase (YP) transition of the dimorphic fungal pathogen *Sporothrix schenckii* are shown in electron micrographs of ultrathin sections. Morphological transformation at the ultrastructural level was observed to occur by direct formation of budlike structures at the tips and along the hyphae and by oidial cell formation. Direct budding of yeast from conidiospores was not observed. Early transitional forms arising by direct blastic action from the MP possessed conspicuous electron-dense microfibrillar material at the outer limits of the cell wall. The electron density of this microfibrillar material was enhanced by staining with acidified dialyzed iron. It is believed that this extracellular material may be composed in part of an acid mucosubstance. No acid phosphatase activity was associated with this microfibrillar material. This substance was found to be a characteristic of the outer limits of the cell wall of the YP of *S. schenckii*. Oidial YP cell formation occurred later during the transition. The cell wall of the developing oidial YP transitional form arose from an inner layer of the converting hyphae. No conspicuous alterations of the cytoplasmic content of the parent MP cell was observed during MP-to-YP transition. It is suggested that the MP-to-YP transition of *S. schenckii* may be regulated by at least two mechanisms involving alterations of the biochemical and/or biophysical nature of the cell wall of the MP cell in response to the conversional stimuli.

Sporothrix schenckii Hektoen and Perkins, the etiological agent of the chronic mycotic infection sporotrichosis, is one of those pathogenic fungi characterized by the ability to undergo phase transformation depending upon the environmental conditions under which the fungus is grown. The ability of the dimorphic fungi to invade the deep tissues of animals is related in part to the potential of a morphological transformation to a parasitic stage, which may be yeastlike (1).

In recent years, several studies on the fine structure of the yeastlike phases (YP) and mycelial phases (MP) of *S. schenckii* have appeared in the literature (14, 15, 18), whereas aspects of the ultrastructural reorganization during in vitro YP-to-MP conversion have been studied by Lane and Garrison (16). To date, no comparable information is available on sequential ultrastructural events of the reverse MP-to-YP conversion of this dimorphic fungus. Using methods of conventional light microscopy, Howard (9) described the ontogenic relationship of the YP of *S. schenckii* to its MP after induction of MP-to-YP conversion. Two different morphological transformations were observed during MP-to-YP transition in tissue culture systems: (i) the formation of budding, club-shaped structures at the tips of the hyphae or on the lateral hyphal branches, and (ii) the formation of chains of oidia and subsequent fragmentation of the chains into their constituent YP elements. A third type of transition has been reported in which YP cells arose by direct budding from conidiospores (25).

The present study was undertaken to examine the morphological changes which occur at the ultrastructural level during in vitro MP-to-YP conversion of *S. schenckii*, and perhaps to gain added insight of the phenomenon of dimorphism as characterized by this important fungal pathogen.

MATERIALS AND METHODS

Preparation of cultures. Pasteur Institut strain no. 29 (ATCC 28388) and strain UL, a recent clinical isolate, were used throughout this study. Finely divided hyphal suspensions were prepared by inocu-

lating small pieces of hyphal mat into flasks containing 300 ml of sterile modified Sabouraud glucose broth (Difco) followed by incubation at 25 C for 72 to 96 h under continuous aeration on a rotary shaking apparatus. The hyphae were harvested by centrifugation, washed twice in sterile 0.9% saline, and inoculated into flasks containing 200 ml of sterile brain heart infusion broth (Difco) containing 0.01% cysteine-hydrochloride. The flasks were incubated at 37 C in a controlled environment incubator shaker (New Brunswick Scientific, New Brunswick, N.J.). Alternately, large pieces of hyphal mat containing masses of conidia were inoculated directly into the above medium contained in 250-ml spinner flasks (Bellco Glass Inc., Vineland, N.J.) followed by incubation at 37 C. Specimens undergoing MP-to-YP conversion were taken at 24-h intervals and washed by centrifugation in 0.9% saline. YP cells were maintained in serial transfer on brain heart infusion agar slants after previous conversion from stock MP cultures.

Preparation for electron microscopy. Primarily, cells were fixed at 4 C with 3% glutaraldehyde in 0.1 M *s*-collidine buffer (pH 6.8) for 20 h. After exhaustive washing in 0.9% saline, they were post-fixed overnight at 4 C with 1% osmium tetroxide in 0.1 M *s*-collidine buffer (pH 7.4). Other portions of the cells were fixed for 30 min at 25 C in a mixture containing 1% acrolein, 1% glutaraldehyde, and 1% tris(hydroxymethyl)aminomethane - (1 - aziridinyl) phosphine oxide (TAPO) (Polysciences Inc., Warrington, Pa.) according to Djaczenko and Cassone (5). Before osmium fixation, portions of the glutaraldehyde- and TAPO-fixed cells were stained for 1 h at 25 C in the acidified dialyzed iron solution described by Rinehart and Abul-Haj (22), while acid phosphatase activity was determined by a modified (8) Gomori technique. Alternately, cells were fixed for 10 min at 4 C in 0.6% aqueous potassium permanganate.

After fixation, all specimens were embedded in 2% Noble agar to facilitate handling. Small agar cubes containing the cells were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultrotome III with a diamond knife and placed on uncoated copper grids of 300 mesh. Staining was 5 min with lead citrate followed by 10 min with 3% uranyl acetate in absolute ethanol. Electron micrographs were made on a Hitachi electron microscope model HU-11B-1.

RESULTS

The YP of *S. schenckii* is characterized by ovoid to fusiform cells which may show either single or multiple budding (6). The use of glutaraldehyde-osmium in agar fixation techniques suggests the presence of an electron-transparent capsular or slime layer with associated electron-dense microfibrils to be present external to the cell wall of the YP cell but not the MP cell (15). Figure 1 shows an ultrathin section through a budding ovoid YP cell from a control YP culture after fixation in glutaraldehyde and staining with dialyzed iron. Scattered throughout the cytoplasm are organelles described previously for the ovoid YP cell of *S. schenckii* (15). Conspicuous material binding dialyzed iron is present along the outer limits of the cell wall, and appears to consist of lighter inner and a more electron-dense outer layer measuring about 60 and 20 nm, respectively. A thin layer of material binding dialyzed iron was demonstrable at the surface of the hyphae (see Fig. 4, 5, and 10), but never to the extent observed for yeast cells. Electron cytochemical determinations for acid phosphatase indicated that such enzyme activity was localized in discrete areas of the cytoplasm of the YP cell. However, no acid phosphatase activity was demonstrable in the material comprising the electron-dense microfibrillar substance associated with the cell wall.

After 48 h of incubation under cultural conditions conducive for phase conversion, numerous hyphae were observed with lateral, budlike protrusions that frequently occurred at or near septa. Figure 2 shows a lateral budlike structure arising immediately adjacent to the septum of a glutaraldehyde-fixed hyphal segment. The cell wall of the bud appears to arise from the innermost part of the hyphal wall. Although the outer surface of the hyphal wall appears finely roughened, the cell wall of the developing bud shows prominent, electron-dense microfibrillar material over the entire surface. This extracellular microfibrillar material was demonstrable by permanganate fixation (Fig. 3), on staining of glutaraldehyde-fixed cells with dialyzed iron (Fig. 4), and was especially conspicuous when the developing buds were observed to be slightly out of plane of section. Occasionally, budlike structures having an iron-binding substance along the outer limits of the cell wall were observed being pinched off at the hyphal tips (Fig. 5). Figure 6 illustrates a maturing bud cell with a completed septum. The outer surface of the bud cell shows a marked binding of dialyzed iron when compared to the cell wall of the parent hyphal cell. Except for perhaps increased numbers of mitochondria, no unusual alterations in the fine structure of the cytoplasmic content of the hyphal cell were seen in those areas immediately associated with the developing buds. At this time (48 h), many free ovoid to fusiform budding cells resembling that shown in Fig. 1 were evident. The presence of conspicuous microfibrillar substance at the outer limits of the cell wall is regarded as a characteristic of the YP but not the MP cell of *S. schenckii*. Thus we

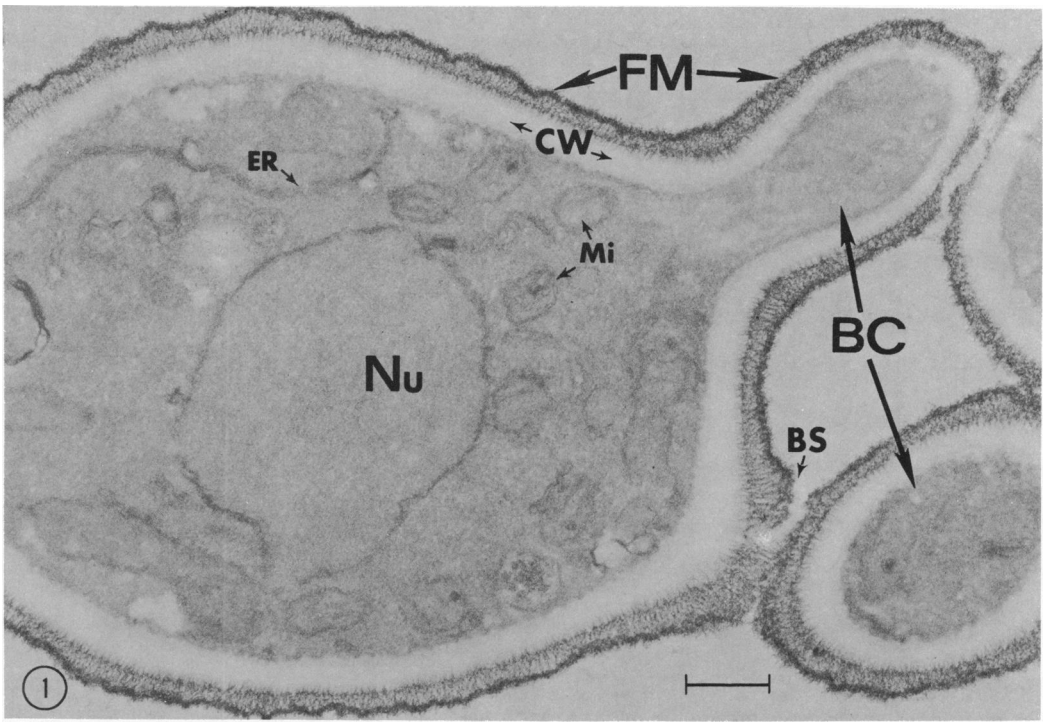

FIG. 1. *Three-day-old ovoid yeastlike cell showing multiple budding (BC), bud scar (BS), nucleus (Nu), mitochondria (Mi), and endoplasmic reticulum (ER). Note the electron-dense microfibrillar material (FM) at the outer limits of the cell wall (CW). Glutaraldehyde-dialyzed iron-osmium. Bar indicates 0.25 μm.*

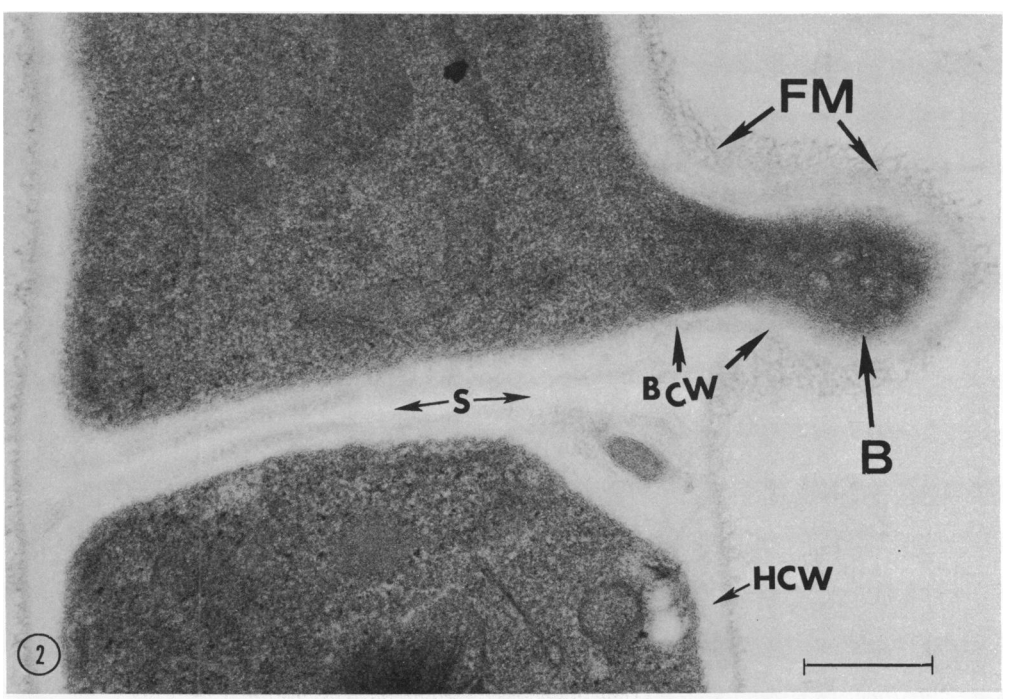

FIG. 2. *Portion of a mycelial element showing budlike protrusion (B) immediately adjacent to the septum (S). Note the electron-dense microfibrillar material (FM) at the surface of the bud cell wall (CS) and the roughened appearance of the hyphal cell wall (HCW). Glutaraldehyde-osmium. Bar indicates 0.20 μm.*

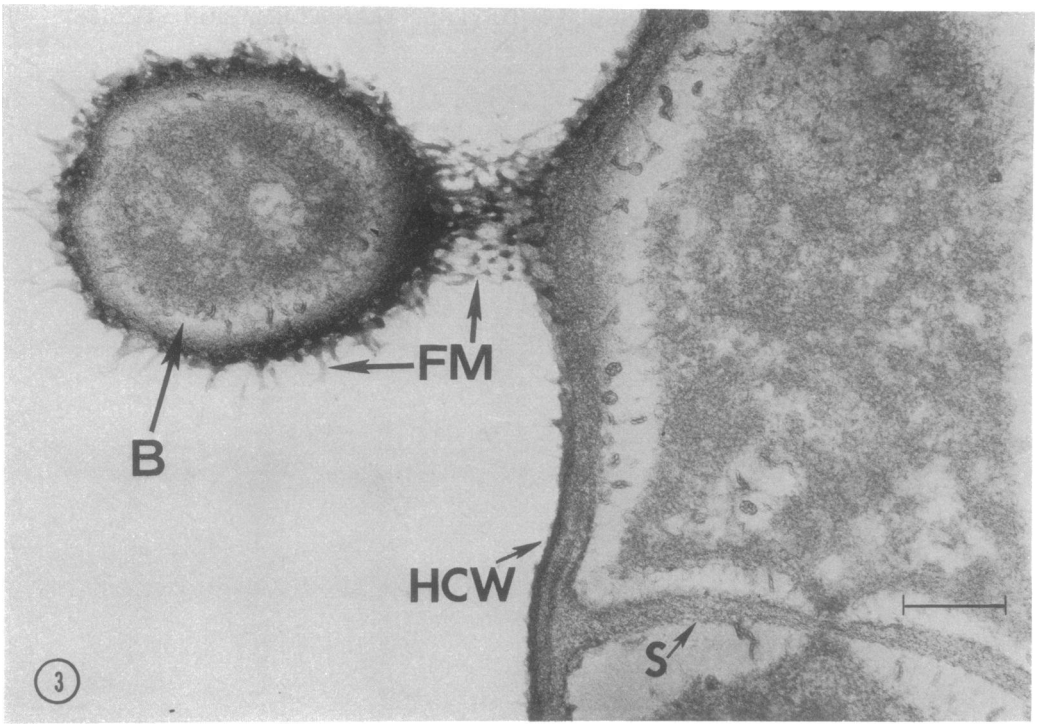

FIG. 3. *Portion of a mycelial element showing budlike protrusion (B) near the septum (S). Note the communication of the microfibrillar material (FM) between the bud and the hyphal cell wall (HCW). Permanganate. Bar indicates 0.25 μm.*

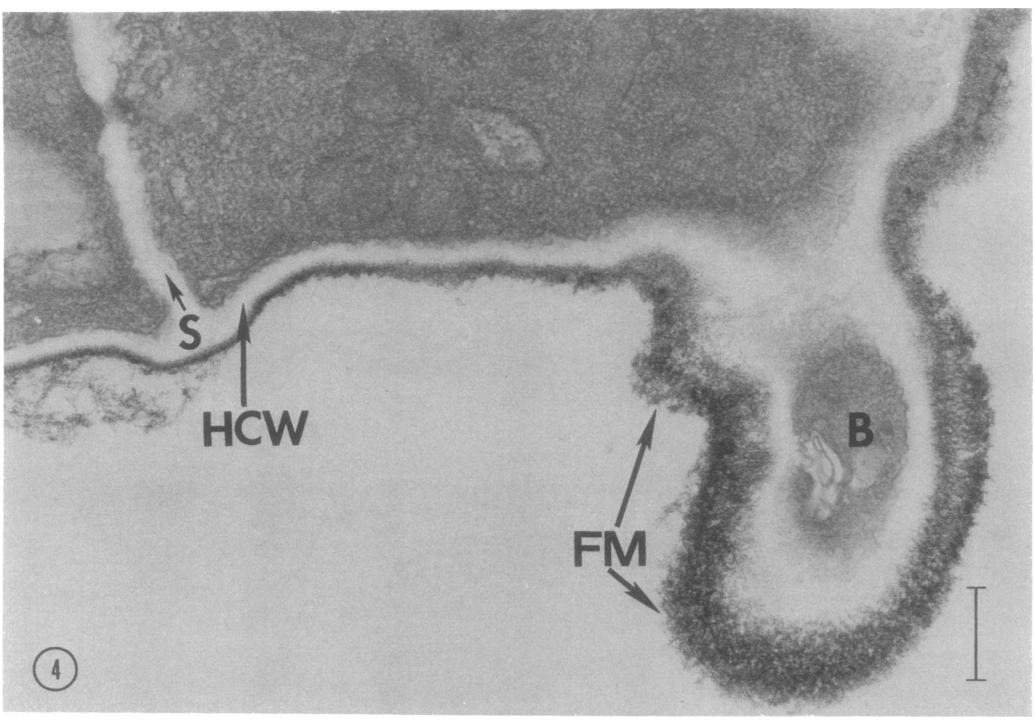

FIG. 4. *Portion of a mycelial element showing marked enhancement of electron density of the microfibrillar material (FM) at the surface of a budlike protrusion (B) located near the septum (S). Note the roughening of the hyphal cell wall (HCW). Glutaraldehyde-dialyzed iron-osmium. Bar indicates 0.25 μm.*

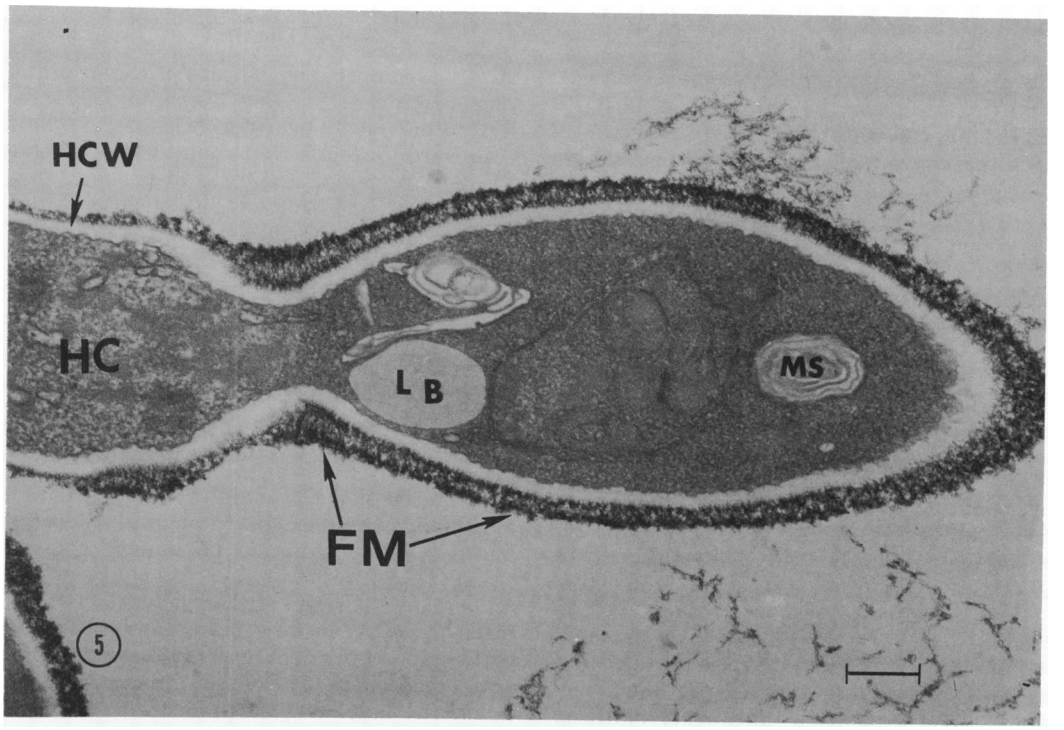

FIG. 5. *Terminal portion of a hyphal cell (HC) showing increased amounts of microfibrillar material (FM), lipid body (LB) and membrane system (MS). Note the roughening of the hyphal cell wall (HCW). Glutaraldehyde-dialyzed iron-osmium. Bar indicates 0.50 μm.*

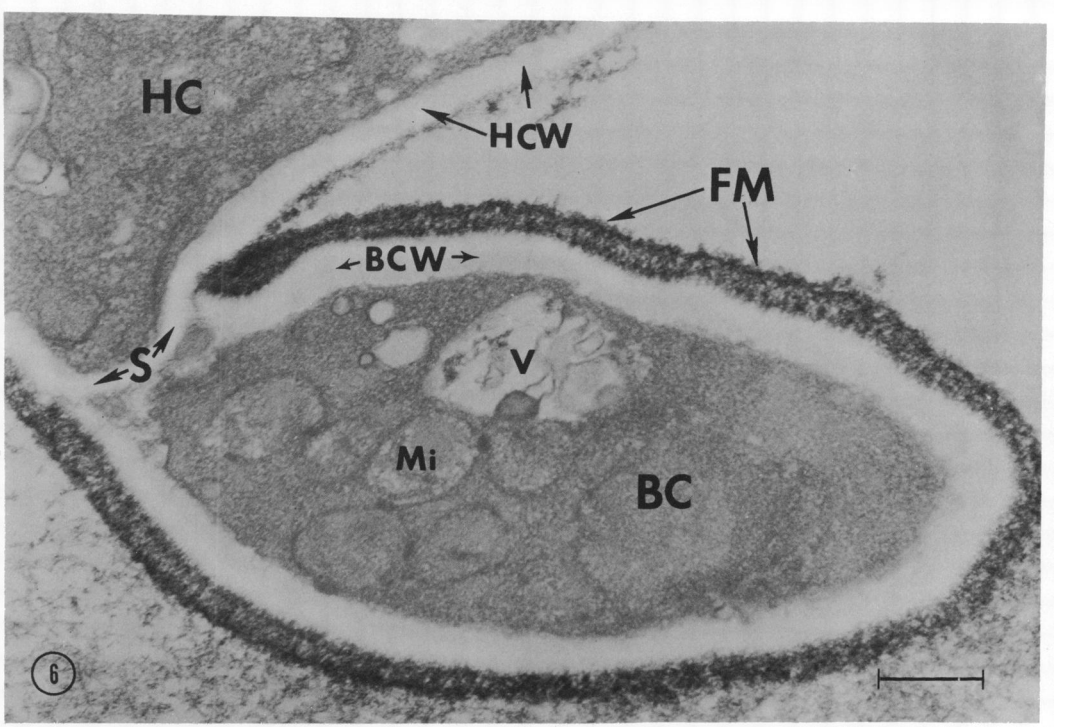

FIG. 6. *Hyphal cell (HC) and bud cell (BC) after septation. Note the septum (S), mitochondria (Mi), vacuoles (V), and the difference in thickness of the microfibrillar material of the bud cell wall (BCW) and hyphal cell wall (HCW). Glutaraldehyde-dialyzed iron-osmium. Bar indicates 0.25 μm.*

interpret the budlike structures shown in Fig. 2 to 6 as representing the formation of early YP transitional cells after the in vitro induction of MP to YP conversion. Such ontogenic relationship of the YP to MP is similar to that observed by Howard (9) on light microscopy of MP-to-YP conversion of *S. schenckii* in tissue culture systems.

In static cultures, the conidiospores of *S. schenckii* are borne at the tips and along the sides of conidiophores and eventually on undifferentiated hyphae of the mycelium. The conidia are elliptical to elongate and measure 3 to 6 × 2 to 3 μm in their greater and lesser dimensions (6, 20). On thin section, mature conidia were observed to contain typically from one to several large lipid storage bodies which may occupy up to 50% or more of the internal cell area. Figure 7 shows a thin section of a conidium after 48 h of incubation at 37 C. Two budlike structures are seen in slight oblique section. The outer cell wall surfaces of the conidium and buds are finely roughened after staining with dialyzed iron. We interpret these elongate structures as germ tubes since multiseptate hyphae arising from conidial germinants were observed frequently at 72 h of incubation. No conclusive ultrastructural evidence was obtained to support the concept of the origin of yeasts by direct budding from conidia as described previously by Weise (25).

Howard (9) found that many longer hyphal elements remained unaltered in tissue culture systems until 96 h of incubation, after which they too showed evidence of changes associated with MP-to-YP transition. The hyphal elements underwent a process of cytoplasmic condensation with the formation of chains of oidia and subsequent fragmentation of the chains into their constituent elements. The fragmented oidial YP cells then multiplied by budding. Figures 8 and 9 show longitudinal thin sections of glutaraldehyde-fixed hyphal cells after 72 h of incubation at 37 C which illustrate what is believed to be stages of oidial YP cell formation during the MP-to-YP transition of *S. schenckii*. Figure 8 shows what may be an early stage of oidial YP cell formation at a hyphal tip. The cell wall of the hypha appears split into at least three layers. A pinching of the terminal portion of the hypha is evident with the cell wall of the developing oidial YP cell continuous with the innermost layer of the hyphal cell wall. The maturing oidial YP cell shown in Fig. 9 is bounded by an outer layer of the hyphal cell wall, whereas the cytoplasm of the parent hy-

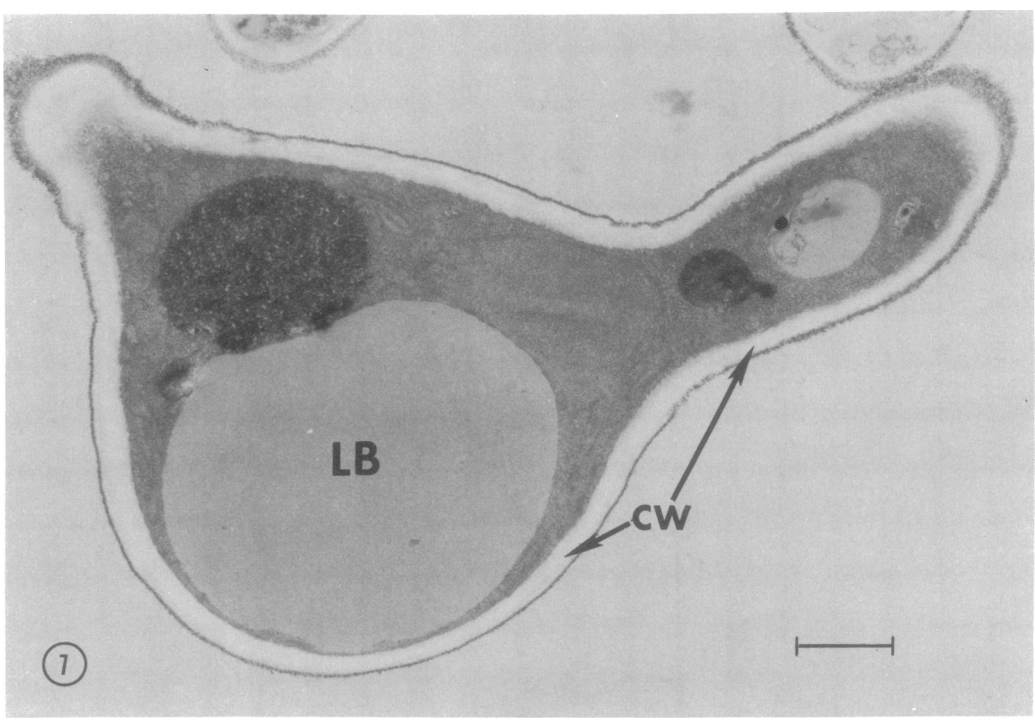

FIG. 7. *Conidiospore showing large lipid body (LB) and two germ tubes. Note the roughened appearance of the cell wall (CW). Glutaraldehyde-dialyzed iron-osmium. Bar indicates 0.5 μm.*

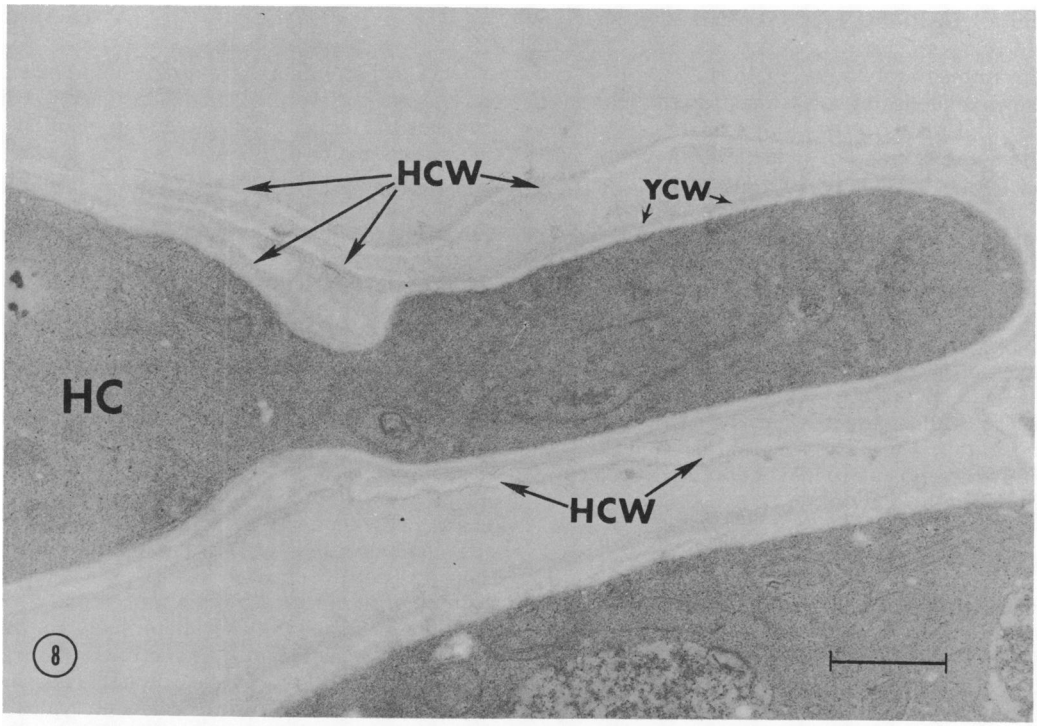

FIG. 8. *Oidial cell formation. Note the pinching off of the hyphal cell (HC), the splitting of the hyphal cell wall (HCW) and the continuous yeast cell wall (YCW) with the innermost layer of the HCW. Glutaraldehyde-osmium. Bar indicates 0.50 μm.*

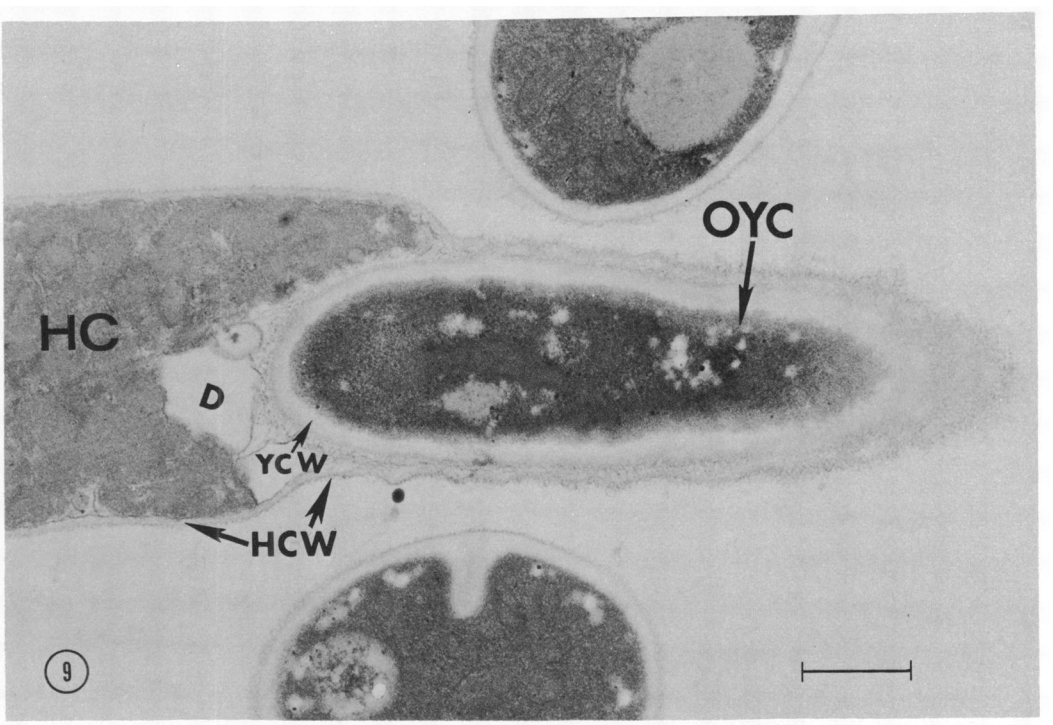

FIG. 9. *Oidial cell formation (OYC) at the terminal portion of a hypha (HC). Note the relationship of the oidial cell wall (YCW) to the hyphal cell wall (HCW) and the early degeneration of the hyphal cytoplasm (D). Glutaraldehyde-osmium. Bar indicates 0.50 μm.*

pha immediately adjacent to the oidial YP cell shows evidence of early degenerative changes. Figure 10 illustrates what is believed to be a late stage of oidial yeast cell development after fixation in TAPO and subsequent staining with dialyzed iron. The cytoplasm of the parent hyphal cell is degenerate. The oidial YP cell in plane of section shows evidence of electron-dense microfibrillar material at its cell wall. Another oidial YP cell, in acute oblique section, shows the origin of its cell wall from an inner layer of the degenerate parent hypha. Oidial YP cell formation was not observed to occur before 72 h of incubation at 37 C. At 96 h of incubation, the cultures examined consisted entirely of fusiform to ovoid budding cells typical of the YP of *S. schenckii*.

DISCUSSION

The results of the present study of the electron microscopy of in vitro MP-to-YP conversion of *S. schenckii* were quite similar to the findings obtained by Howard (9) when the conversion process was studied in tissue culture systems by conventional light microscopy. In vitro MP-to-YP conversion of this dimorphic fungus appears to be temperature-nutritional dependent (7), and the cultural conditions used in this study for the induction of conversion resulted in the demonstration on ultrathin section of transitional forms which arose from the hyphae by direct budding and by oidial cell formation. Yeast cells arising by direct budding from conidiospores of *S. schenckii* were not observed.

The criterion for identifying fine structural changes after induction of MP-to-YP conversion was the appearance of a thickened, electron-dense layer of microfibrillar material seen characteristically at the outer limits of the yeast cell wall. The hyphal wall appeared finely roughened with what may be a similar substance, but its relative thickness was always much less conspicuous than that observed for the yeast-like cell. Although this microfibrillar material was demonstrable by the usual heavy-metal staining used in electron microscopy (14, 15), staining with dialyzed iron enhanced markedly the electron density of the microfibrillar material. This selective ultrastructural staining characteristic with dialyzed iron suggests that this microfibrillar material associated typically with the yeast cell wall of *S. schenckii* is composed in part of an acid mucosubstance similar

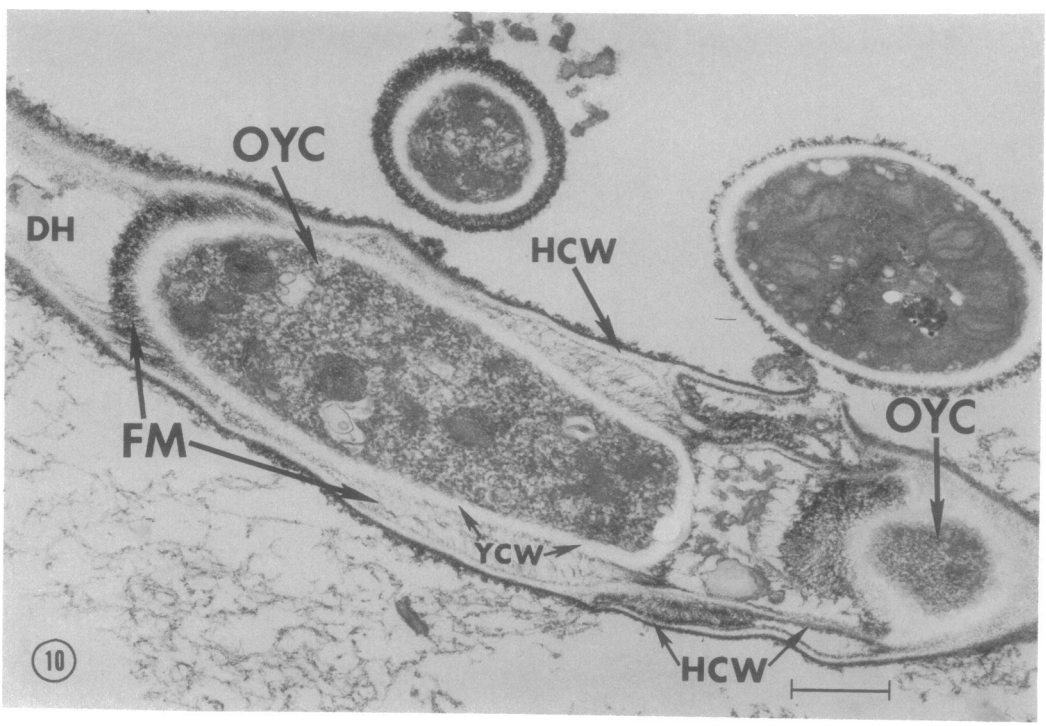

FIG. 10. *Late oidial cell (OYC) formation within a degenerate hypha (DH). Note the splitting of the hyphal cell wall (HCW) and the microfibrillar material (FM) at the outer limits of the yeast cell wall (YCW). TAPO-dialyzed iron-osmium. Bar indicates 0.50 μm.*

to that described by Mahvi et al. (19) for the mucoid capsule of *Cryptococcus neoformans*. These workers found acid phosphatase activity at the ultrastructural level of this fungus to be confined to the mucoid capsule, cell wall, and internal extensions presumed to be plasmalemmasomes. No similar acid phosphatase activity was found associated with such structures in *S. schenckii*.

Direct budding of the yeast from the hyphae of *S. schenckii* by blastic action resembles that reported for the MP-to-YP conversion of *Phialophora dermatitidis* (21) and *Histoplasma farciminosum* (R. G. Garrison and K. S. Boyd, Sabouraudia, in press), although prior changes in ultrastructural organization of the cytoplasm of the parent hyphal cell were noted with the latter fungus. At the same time, oidial cell formation by *S. schenckii* resembles the type of yeast cell ontogeny reported for *Paracoccidioides brasiliensis*. Carbonell (3) found that the yeast of this dimorphic fungus was produced by "self-transformation" of a hypha by an enlargement of the septal spaces and cracking of the outer electron-dense layer of the hyphal cell wall. The cell wall of the yeast form appeared to arise from an inner layer of the hyphal cell wall.

Lateral or terminal bud formation by blastic action and by oidial yeast cell formation suggests that yeast cell ontogeny may be regulated by two different mechanisms. Direct budding of yeastlike elements from the hyphae always preceded oidial YP cell formation. The age of the hyphal cell, changing nutritional composition of the supporting medium, or other regulatory factors may influence the mode of conversion during the period of transition. However, our studies suggest that both modes of conversion may be regulated by mechanisms specifically involving biochemical and/or biophysical alterations of the hyphal cell wall after induction of the conversion stimuli. Carbonell and co-workers have reported several studies on the ultrastructure and comparative biochemistry of the cell wall of the two phases of *P. brasiliensis* (2, 4, 10, 11–13). They found that the main polysaccharide of hexoses of the YP form was α-glucan, whereas the polysaccharides of the MP cell wall were β-glucans and galactomannan. Cell-free extracts of the whole cell of the YP had five times more protein disulfide reductase activity than the MP cell, whereas extracts of the MP contained five to eight times more β-glucanase activity than the YP. They concluded that change of cell wall glucans may play an important role in the dimorphism of *P. brasiliensis*. It was postulated that the information necessary for the synthesis of those enzymes regulating alterations of cell wall configuration during MP-to-YP conversion of *P. brasiliensis* may not be evenly distributed throughout the fungus. Although the chemical structures of certain polysaccharides and glycoproteins of the two phases *S. schenckii* have been studied (17, 23, 24), no information is now available on the comparative cell wall biochemistry of this dimorphic fungal pathogen. Thus the role of changes in cell wall glucans or other cell wall constituents may play in the dimorphism of *S. schenckii* remains to be determined.

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