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Morphological and Structural Changes During the Yeast-to-Mold Conversion of *Phialophora dermatitidis*

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The details of the morphological and structural events occurring during yeast-to-mold conversion of the human pathogenic fungus *Phialophora dermatitidis* as seen by phase-contrast microscopy and electron microscopy are described and illustrated. Budding yeasts growing exponentially were observed to have thin walls and a cytoplasm exhibiting the characteristics of rapidly growing cells including numerous mitochondria, abundant ribosomes, few vacuoles, and little accumulation of storage material. In contrast, thick-walled yeasts were characterized by less apparent or significantly fewer mitochondria and ribosomes and the presence of considerable amounts of storage materials. Microscope observations of yeast-to-mold conversion revealed that only thick-walled yeasts having prominent lipid bodies in their cytoplasm converted to hyphal forms. Typically, the thick-walled yeast formed two to a number of moniliform hyphal cells which in turn often produced true hyphae. The results indicated that yeasts of *P. dermatitidis* must acquire spore-like characteristics by becoming thick-walled and by accumulating considerable endogenous substrate reserves before they convert and produce hyphae.

Phialophora dermatitidis is an etiological agent of cutaneous and generalized chromomycosis and may be the only yeast-like dematiaceous human pathogen with neurotropic properties (11). In vivo or in vitro, this fungus exhibits conspicuous polymorphism. Lesions produced by *P. dermatitidis* usually contain large numbers of brown, elongated septate cells, whereas brain tissue infected with disseminated *P. dermatitidis* may contain narrow hyphae as well as numerous spherical and septate cells (4). Recently, five distinct cell types were observed in in vitro cultures of *P. dermatitidis* (11). Newly formed yeasts were always hyaline and thin-walled, whereas older yeasts were pigmented, relatively thick-walled, and supported one or occasionally more than one bud scar protuberance. Older cultures were reported to contain both moniliform and true hyphae surrounded by clusters of yeast-like spores. The relationship of the thin-walled and thick-walled yeasts to the hyphal forms was not established.

During preliminary studies of the yeast-to-mold conversion of *P. dermatitidis* in submerged culture, it became increasingly evident that our results were dependent upon the characteristic of the yeast inoculum. If the inoculum represented yeasts or yeast-like spores from media solidified with agar, the resulting broth subcultures contained both yeasts and moniliform and true hyphae. However, only yeasts were obtained when broth subcultures were inoculated with logarithmically growing yeasts. Phase-contrast microscope observations revealed that the yeasts from agar media often represented heterogeneous populations of thin-walled and thick-walled yeasts. In contrast, logarithmically growing yeasts from broth cultures were entirely thin-walled.

The purpose of this report is to confirm that five distinct cell types exist in in vitro cultures of *P. dermatitidis*. Additionally, we describe for the first time the growth kinetics of *P. dermatitidis* in shaken broth cultures and the morphological and ultrastructural changes associated with the transformation of thin-walled yeasts to thick-walled yeasts and their subsequent conversion to hyphal forms.

MATERIALS AND METHODS

Organism and yeast-phase growth conditions. *P. dermatitidis* strain 8656 (originally received from

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C. W. Emmons, National Institutes of Health, Bethesda, Md.) was obtained from B. H. Cooper (Temple University School of Medicine, Philadelphia, Pa.). The organism was grown as a thin-walled budding yeast in 100 ml of shaken modified Czapek Dox broth (MCDB) contained in 250-ml conical flasks stoppered with foam plugs. The MCDB was prepared by supplementing Czapek Dox broth (Difco) with 1.0% yeast extract (Difco). The temperature during growth was maintained at 25 ± 1 C. The inoculum consisted of yeast cells (1 ml of about 1×10^8 cells) that were derived from a 2-day-old shaken MCDB culture. Growth was measured by the use of a Klett-Summerson colorimeter with a no. 42 filter. Viable counts were conducted by plating serial dilutions on Sabouraud dextrose agar (SDA). A late exponential-phase reading of 200 Klett units (0.4 optical density units) corresponded to about 2.5×10^7 viable units/ml.

Thick-walled yeast production. Numerous slants of SDA were inoculated with exponential-phase, thin-walled yeasts (about 2×10^4 cells) to obtain populations of thick-walled yeasts for microscope observations and culture. Groups of the subcultures were incubated at 37 C for different periods of time (1–10, 15, 20, 25, and 30 days). At the end of each incubation period, the surface growth was washed from the individual slants of SDA with 10 ml of MCDB and observed with the phase-contrast microscope for thick-walled yeast formation.

Yeast-to-mold conversion conditions. Portions (1 ml) of the yeast suspension washed from each SDA slant were used to inoculate 50 ml of MCDB contained in 250-ml conical flasks. Incubation of these cultures was for 3 days at 25 ± 1 C using stationary conditions. At the end of the 3-day period, the broth cultures were observed by phase-contrast microscopy for yeast-to-mold conversion.

Electron microscope investigations. Logarithmically growing yeasts, yeasts washed from the SDA slants, and yeasts and hyphae from the stationary MCDB were fixed overnight at 25 C with a mixture of 2% gluteraldehyde-2% formaldehyde in 0.1 M sodium cacodylate buffer (*p*H 6.8). The fungal materials were collected subsequently on membrane filters (Millipore Corp.) and covered with 2% agar (Difco). The membrane filters were then suspended in several changes of sodium cacodylate buffer (*p*H 6.8) over a period of 30 to 60 min, treated for 4 to 6 hr with 1% OsO_4 in the cacodylate buffer, washed a second time with the cacodylate buffer, rinsed in water, and suspended overnight in 0.5% aqueous uranyl acetate. After dehydrating in a series of increasingly concentrated acetone solutions, the fungal materials were passed through two changes of anhydrous acetone and embedded in Spurr's low-viscosity embedding medium (15). Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 ultramicrotome. The sections were mounted on collodion-carbon-coated grids and stained with uranyl acetate and lead citrate. Electron micrographs were obtained in Hitachi HS7 and HU11E electron microscopes.

RESULTS

Growth kinetics of yeast phase. To become acquainted with the morphological and ultrastructural characteristics of rapidly growing *P. dermatitidis* yeasts, we cultured the fungus in MCDB by using shake-culture growth conditions. The time course of growth of *P. dermatitidis* yeast phase in MCDB at 25 C is shown in Fig. 1. Since pigmentation was evident early in the growth cycle, viable counts were determined each time that Klett readings were recorded. The absence of lag when viable units were determined reflects the exponential-phase characteristics of the inoculum and also the large inoculum size. The generation time for the yeast phase of *P. dermatitidis* during exponential growth in MCDB at 25 C was 5 hr.

Thin-walled to thick-walled yeast transformation. During exponential growth of *P. dermatitidis* in shaken broth, the cell populations are totally homogeneous, consisting of single yeasts in various stages of bud formation (Fig. 2). Cultures inoculated with stationary-phase yeasts instead of exponentially growing yeasts did not always show this total homogeneity of cell form. For this reason, only exponentially growing yeasts were used to inoculate the SDA slants. Ultrastructurally, the exponential-phase yeasts are thin-walled and contain abundant ribosomes (Fig. 3). Electron-transparent areas representing polysaccharide storage areas are frequently observed next to the plasma membrane. Vacuoles are either few in number or absent. Only single nuclei are observed in the yeast sections. Mitochondria are

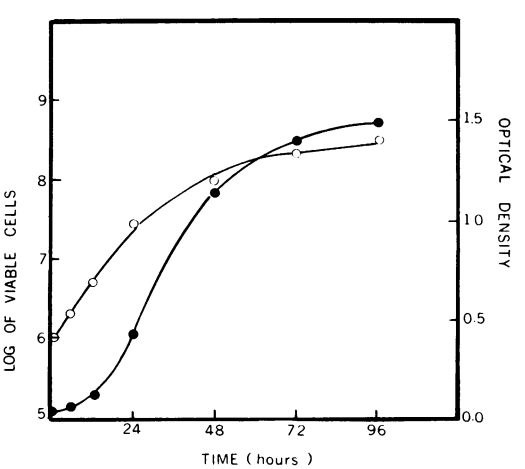

FIG. 1. *Kinetics of yeast-phase growth of P. dermatitidis cultures at 25 ± 1 C in shaken MCDB. Relationship between viable units and optical density. Symbols: ○, log of viable cells determined as colony-forming units; ●, optical density (measured as Klett units using a no. 42 filter and subsequently converted to optical density units).*

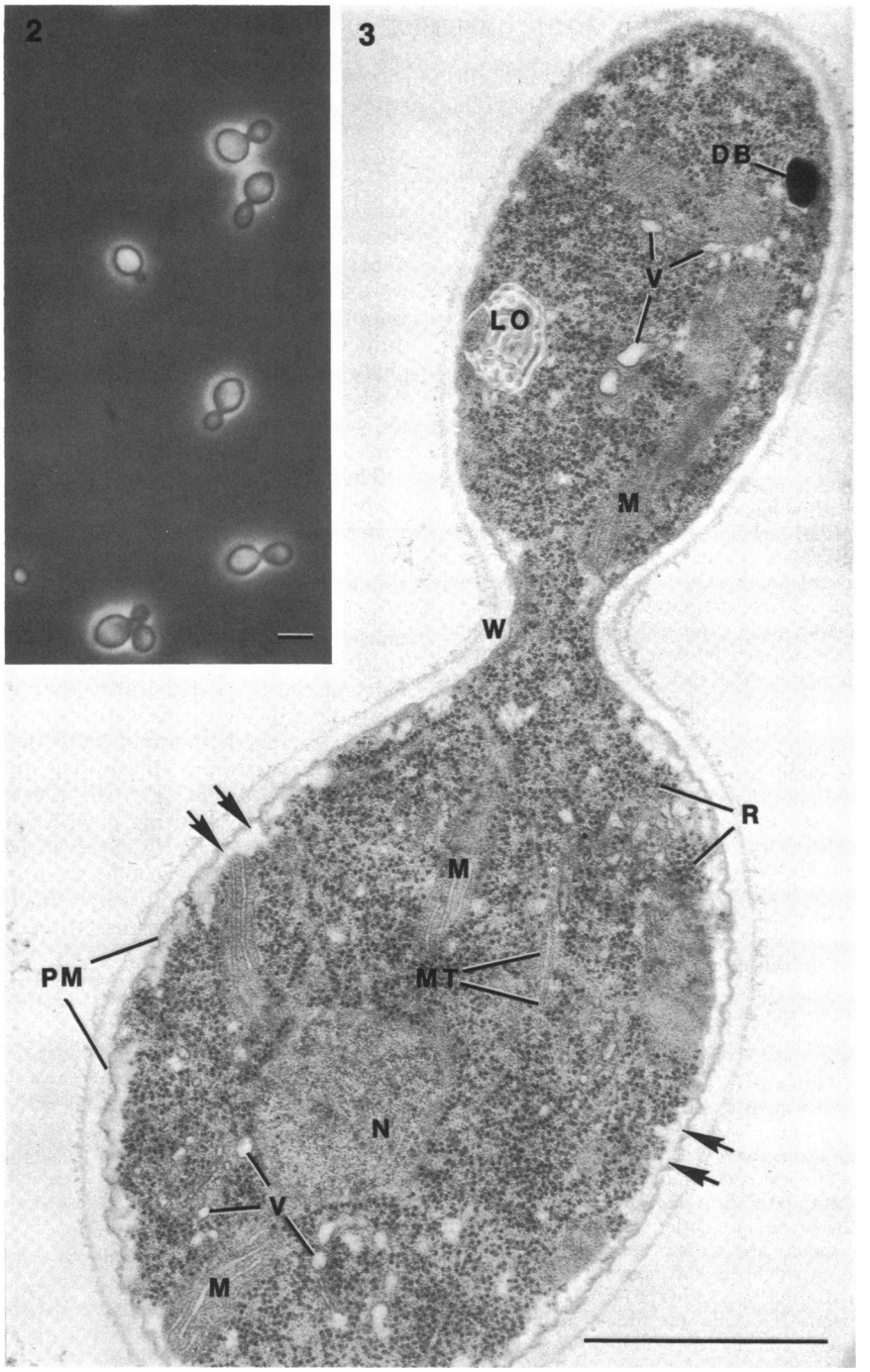

FIG. 2. *Phase-contrast micrograph of cell population from 2-day-old shaken P. dermatitidis yeast-phase culture showing total yeast homogeneity. Marker bar indicates 5 μm.*

FIG. 3. *Electron micrographs of sectioned yeasts from 2-day-old shaken yeast-phase culture showing late stage of budding. The walls are thin and electron transparent and have an outer electron-opaque fringe. Polysaccharide storage regions (double arrows) are limited and localized near the cell periphery. Small groups of vesicles are scattered throughout the cytoplasm and are often found in ribosome-free regions where they are associated with smooth membranes which may represent Golgi membranes. DB, Dense body; LO, plasmalemmosome; M, mitochondria; MT, microtubules; N, nucleus; PM, plasma membrane; R, ribosomes; V, cytoplasmic vesicles; W, wall. Marker bar indicates 1 μm.*

numerous and scattered throughout the cytoplasm. Endomembranes delimiting the nuclei, endoplasmic reticulum, and vacuoles are not always clearly defined. In contrast, plasma membrane is distinct and easily resolved into a dark-light-dark substructural pattern. Lomosomes or plasmalemmasomes are observed frequently in the yeast profiles. Cytoplasmic vesicles are found scattered throughout the cell and are sometimes associated with a Golgi apparatus residing in certain ribosome-free regions of the cytoplasm.

Yeasts washed from SDA after 1 to 7 days of incubation exhibit few perceptible morphological or cytological changes when observed with the phase-contrast microscope. However, thin sections reveal that ultrastructural changes begin occurring in some of these yeasts very soon after the exponential-phase yeasts were transferred to SDA (Fig. 4). The yeasts exhibiting changes have somewhat thicker walls and fewer ribosomes and mitochondria. An increase in size of the polysaccharide storage areas is characteristic of the thick-walled yeasts. Material of low electron density, representing lipid bodies, are present in many of the cells. No membrane appears to delimit the lipid bodies from the cytoplasm. Yeasts still in the process of budding and new yeast progeny retain the general characteristics of the exponential-phase yeasts used as inoculum.

Yeasts washed from SDA slants 20 to 30 days after inoculation have undergone dramatic changes when compared with the inoculum. When observed with the phase-contrast microscope, most of the yeasts have a definite thick wall and one to four or five refractile inclusions (Fig. 5). Ultrastructural observations confirm their thick-walled characteristics (Fig. 6). The wall is composed of at least two layers, with the outer layer having a fragile quality exemplified by missing outer wall portions. A great proportion of the cytoplasmic volume is occupied by large lipid bodies. The extensive polysaccharide storage areas observed in the younger thick-walled yeasts (Fig. 4) are noticeably reduced in size in these older thick-walled yeasts (Fig. 6).

Yeast-to-mold conversion. Exponential-phase yeasts and yeasts washed from SDA slants up to 15 days after inoculation grow only by the budding process after inoculation into stationary MCDB. However, the thick-walled yeasts washed from slants after 20 or 30 days and inoculated into stationary MCDB either bud or, more frequently, form moniliform and true hyphae (Fig. 7). Invariably, the thick-walled yeast converts by forming a moniliform hypha consisting of two to a number of attached moniliform daughter cells. These moniliform hyphal cells act as transition cells and often give rise to true hyphae which extend by tip growth. When the thick-walled yeasts produce the first cell of a moniliform hypha, they exhibit large populations of ribosomes, lipid bodies, electron-dense bodies, and numerous mitochondria (Fig. 8a, b, c). The wall of the first moniliform hyphal cell is thin and continuous with an inner layer of the thick-walled parental yeast. As the moniliform hypha elongates, prominent septa form between the hyphal cells, and in near median sections these septa are observed to be simple septa having the characteristic single pore (Fig. 8b and c, 9a). Woronin bodies are usually seen near each septal pore, and often the septal pores are closed by conspicuous septal pore plugs.

True hyphal cells are elongate, thin-walled, and contain numerous ribosomes (Fig. 9b, 10a and b). Prominent, electron-dense bodies are characteristic of the true hyphae. Lateral hyphal branch formation and lateral hyphal bud formation by main hyphal elements are common (Fig. 10a, b). Both branches and buds form distal to the mother thick-walled yeast. The wall of a lateral branch is continuous with the wall of the main hypha (Fig. 10a). This same continuity is not observed during lateral hyphal bud formation (Fig. 10b). Instead, a lateral bud forms by blastic budding action so that only the innermost part of the hyphal wall is continuous with the spore wall. The young lateral bud has the cytoplasmic characteristics of a newly budded yeast and when cultured in standing MCDB is observed to grow only as a budding yeast. The lateral hyphal buds described in this work are often termed spores by other investigators (4, 5, 11).

DISCUSSION

The experiments reported establish the relationships which exist among the five cell types observed in in vitro cultures of *P. dermatitidis*. With the light and electron microscope, it was shown that thin-walled yeasts can become thick-walled yeasts and then undergo a yeast-to-mold conversion. In no instance were rapidly growing, thin-walled yeasts observed to convert directly into hyphal forms even though a number of different environmental factors were manipulated in efforts to induce such a conversion (Oujezdsky and Szaniszlo, *unpublished data*). Thus, the yeast-to-mold conversion of *P. dermatitidis* does not represent a short-term response of the yeast phase to alterations in the external environment so characteristic of other

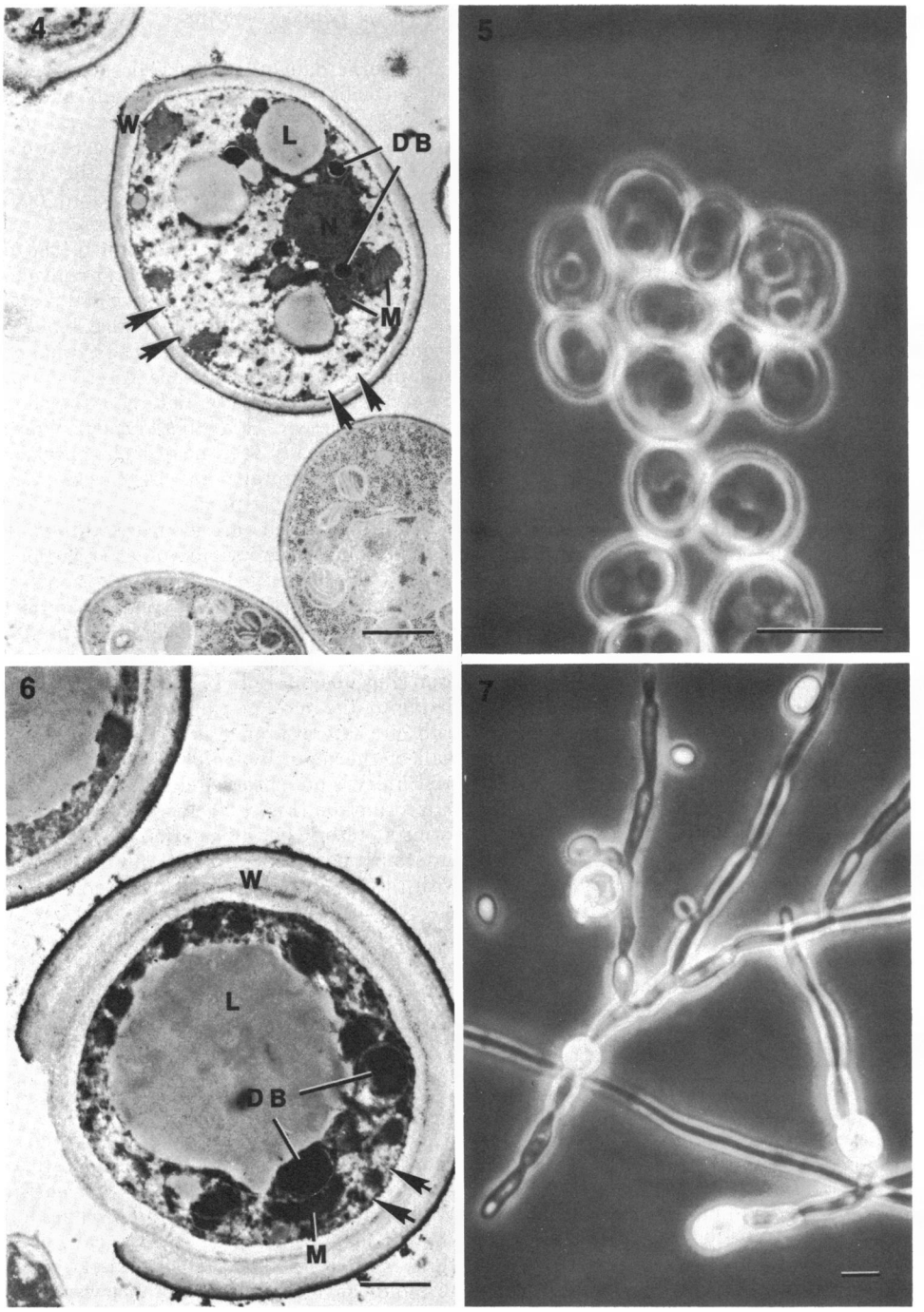

FIG. 4. *Electron micrograph of sectioned yeast washed from SDA after 4 days of incubation showing thickened wall, extensive polysaccharide storage areas (double arrows), and several lipid bodies. Mitochondria are small or few in number, or both, and ribosomes are difficult to detect. DB, Dense bodies; L, lipid bodies; M, mitochondria; W, wall. Marker bar indicates 1 μm.*

FIG. 5. *Phase-contrast micrograph of yeasts washed from SDA after 30 days of incubation showing thickened walls and prominent lipid inclusions. Marker bar indicates 5 μm.*

FIG. 6. *Electron micrograph of sectioned yeasts washed from SDA after 30 days of incubation showing very thick walls composed of at least two layers, large lipid bodies, few mitochondria, dense bodies which may represent pigment pools, and limited polysaccharide storage areas (double arrows). The two wall layers are*

dimorphic fungi such as *Mucor rouxii*, *Candida albicans*, *Blastomyces dermatitidis*, and *Paracoccodioides brasiliensis* (10, 14). Instead, the yeast-to-mold conversion in *P. dermatitidis* appears to be a long-term consequence of cytoplasmic changes which occur in the yeast as it becomes transformed into a thick-walled cell.

Changes in the cytoplasm are very apparent when the ultrastructural characteristics of rapidly growing, thin-walled yeasts are compared with thick-walled yeasts produced by prolonged incubation of thin-walled yeasts on SDA. The thin-walled yeasts possess all the ultrastructural characteristics usually associated with rapidly growing cells, such as numerous ribosomes, few vacuoles, and very little storage polysaccharide or lipid. As yeasts become thick-walled, however, they appear to pass through at least two stages of development. In the first stage, the yeast wall thickens, ribosomes and mitochondria become less visible, and there is a pronounced accumulation in the cytoplasm of electron-transparent regions. These regions are presumed to represent areas from which storage polysaccharides such as glycogen have been extracted during the preparations of the material for electron microscopy (1, 3, 6). In the second stage of development, the wall of the yeast appears to become composed of at least two layers. The outer layer of the wall exhibits a fragile quality, and portions are often missing. Additionally, there is an obvious reduction in storage polysaccharide and a concomitant increase in the cytoplasm of large lipid bodies. These bodies exhibit a refractile quality when observed with the phase-contrast microscope. The staining properties of the lipid bodies and the absence of boundary membranes of the unit membrane type around the bodies indicate they are similar to lipid bodies observed in other organisms (3, 8, 9, 13, 16, 17). Only thick-walled yeasts which have matured to the second stage of development are observed to undergo a yeast-to-mold conversion.

Thus, it appears from the electron micrographs that the ability of *P. dermatitidis* to convert from a yeast to a mold may be dependent upon the accumulation in the yeast of a suitable endogenous substrate. Most likely this endogenous substrate is the lipid which has accumulated in the thick-walled yeast during their prolonged incubation on SDA. As the thick-walled yeasts begin to grow, some of the stored endogenous substrates may be utilized and so significantly influence growth that yeast-to-mold conversion occurs. Evidence for this supposition resides in the fact that *P. dermatitidis* yeasts which have not accumulated the lipid, but have become thick-walled and contain large electron-transparent areas representing accumulated storage polysaccharide, do not form hyphae. When these particular cells resume growth, they are observed to grow only by the budding process. Additionally, the presence of simple septa and associated Woronin bodies located between the thick-walled yeast cell and the first moniliform hyphal cell, and the initiation of the hyphal wall from only the inner wall layer of the thick-walled yeast indicate that growth processes are significantly altered during conversion. These alterations can only be attributable to endogenous cellular conditions of the thick-walled yeast and not to external factors; all yeasts from SDA were cultured identically to ascertain their conversion potential. Other investigators working with *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Paracoccidioides brisiliensis* have suggested previously that simple septa and Woronin bodies characterize hyphae and that their presence between the conversion cell and the first hyphal cell indicates that major metabolic alterations are occurring during conversion (2, 6, 7, 12). In fact, conversion at the ultrastructural level in most, if not all, dimorphic human pathogenic fungi may be characterized by the presence of simple septa and associated Woronin bodies between the converting yeast and the first hyphal cell.

In *P. dermatitidis* (strain 8656), all thin-walled yeasts are derived from terminal or lateral hyphal buds (spores of some investigators) or their yeast progeny. Moreover, both lateral hyphal buds and their yeast progeny can attain resistant spore characteristics. Thus, it appears we have demonstrated an asexual life cycle phenomenon as well as a yeast-to-mold conversion sequence. In *P. dermatitidis*, yeast-like cells grow by budding or attain resistant spore characteristics including thick walls, endogenous substrate reserves, dormancy qualities, and considerable longevity. Once in con-

especially evident adjacent to the regions where the outer wall portion is missing. DB, Dense bodies; L, lipid bodies; M, mitochondria; W, wall. Marker bar indicates 1 μm.

FIG. 7. *Phase-contrast micrograph of yeast-to-mold conversion showing refractile thick-walled yeasts giving rise to one to three or more moniliform hyphal cells which in turn often give rise to true hyphae. Marker bar indicates 5 μm.*

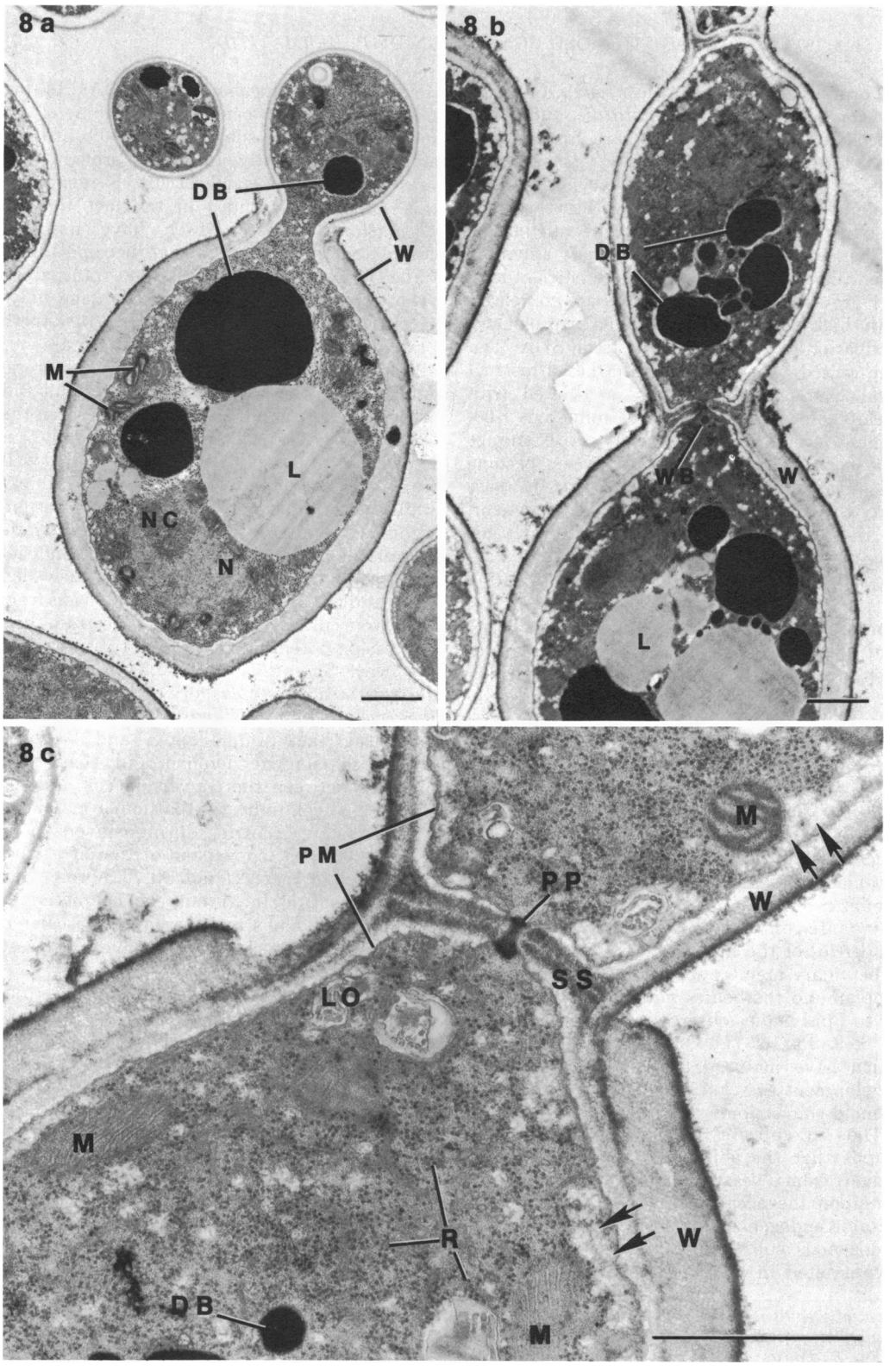

FIG. 8. *Electron micrographs of sectioned thick-walled yeasts during conversion (a) and after conversion (b, c) showing reappearance in the cytoplasm of numerous mitochondria and abundant ribosomes characteristic of actively growing cells. The wall of the first moniliform hyphal cell is always continuous with an inner wall layer of the thick-walled yeast. A simple septum (b, c), which is near media section exhibits a single pore, and*

tact with a suitable substrate, the spore-like thick-walled yeasts essentially germinate, producing yeasts or hyphae. In a short time, the hyphae again begin producing lateral hyphal buds which in turn bud yeast progeny. An asexual life cycle of *P. dermatitidis* based on our findings is summarized diagrammatically in Fig. 11.

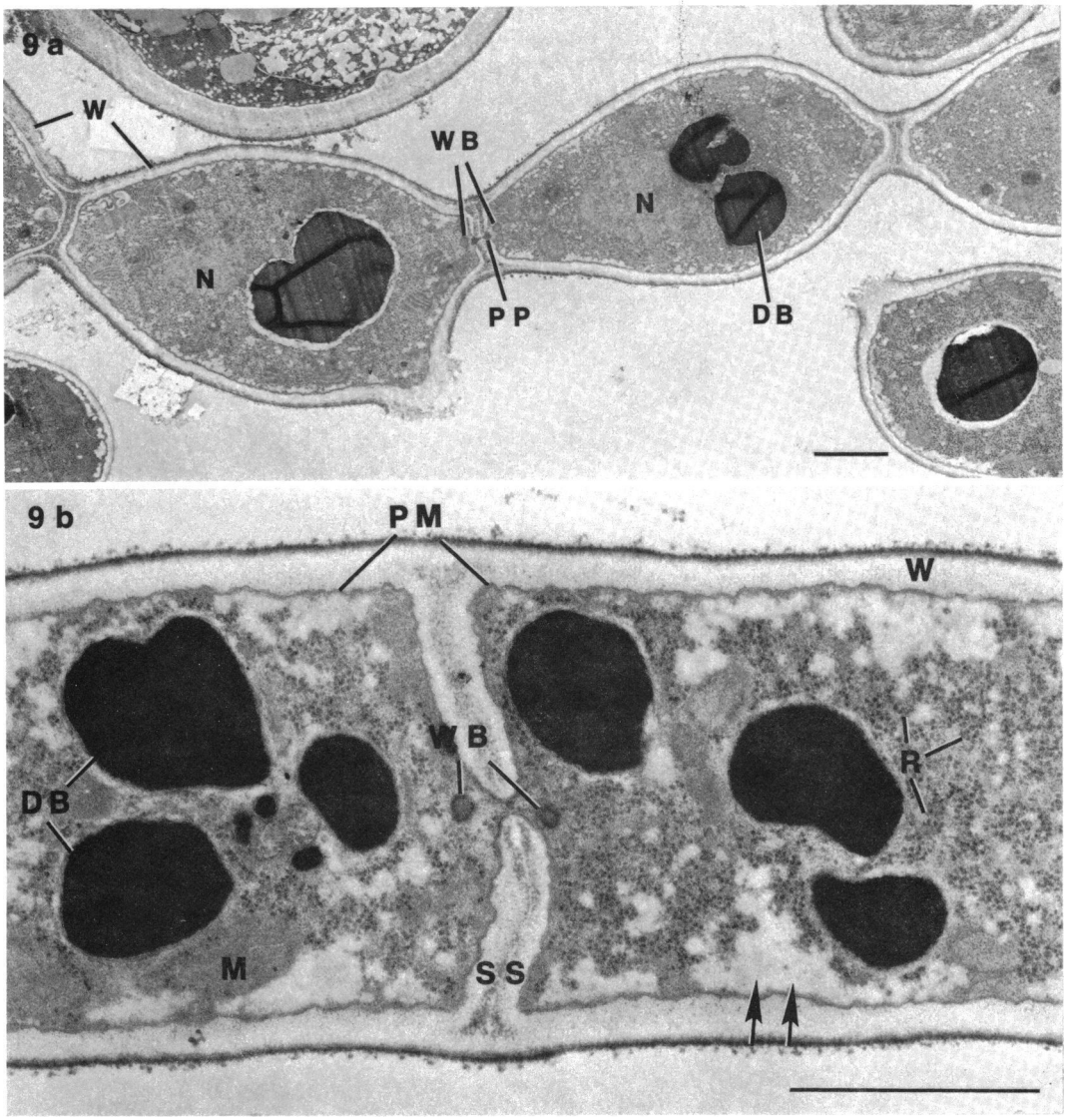

FIG. 9. *Electron micrographs of sectioned hyphal elements showing moniliform hyphal cells (a) and true hyphal cells (b) separated by simple septa which in near median section exhibit a single pore, usually occluded with a pore plug, and associated Woronin bodies. DB, Dense bodies; M, mitochondria; N, nucleus; PM, plasma membrane; PP, pore plug; R, ribosomes; SS, simple septum; W, wall; WB, Woronin bodies. Marker bar indicates 1 μm.*

associated Woronin bodies form between the thick-walled yeast and the first moniliform hyphal cell. Double arrows, Polysaccharide storage areas; DB, dense bodies; LO, plasmalemmosome; L, lipid bodies; M, mitochondria; N, nucleus; NC, nucleolus; PM, plasma membrane; PP, pore plug; SS, simple septum; W, wall; WB, Woronin bodies. Marker bars indicate 1 μm.

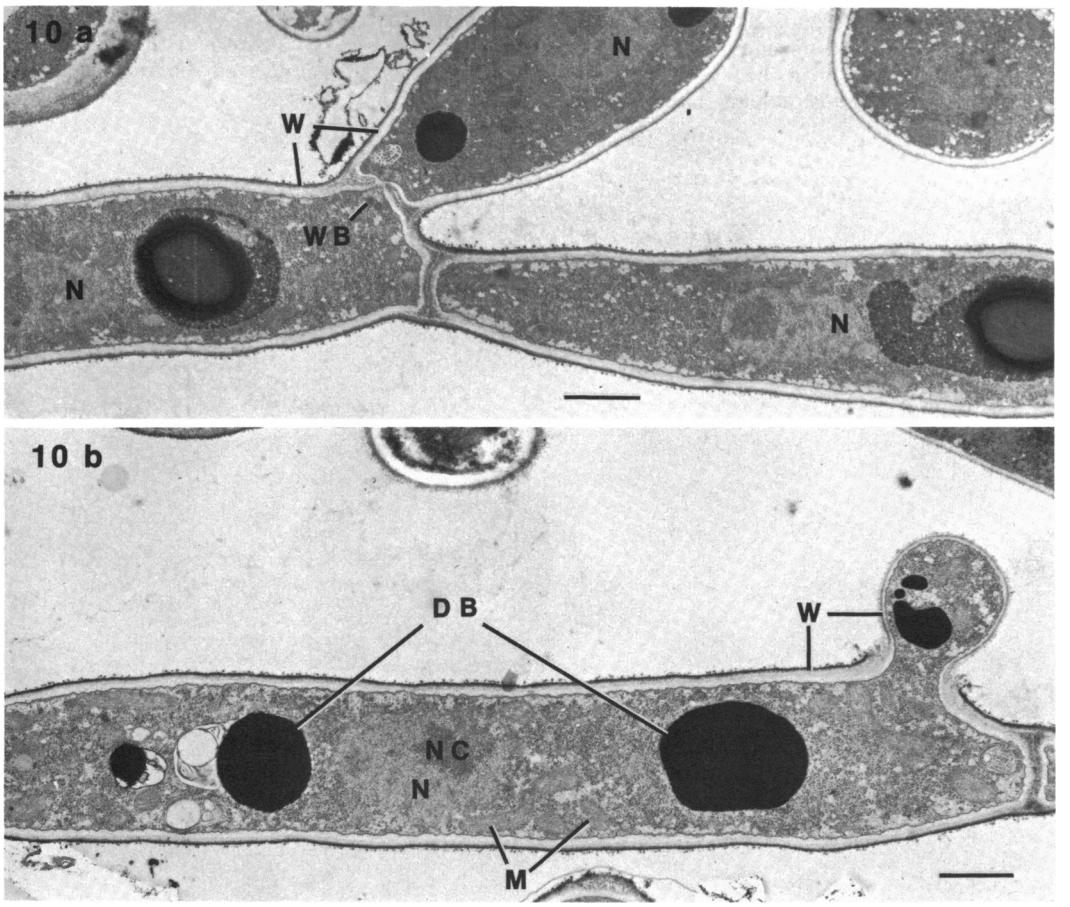

FIG. 10. *Electron micrographs of main hyphal cells showing lateral hyphal branch formation (a) and lateral hyphal bud formation (b). The wall of the lateral hyphal branch (a) is continuous with both the outer and inner wall surface of the main hyphal wall, whereas the wall of the lateral hyphal bud (b) is continuous with only the inner surface of the main hyphal wall. DB, Dense bodies; M, mitochondria; N, nucleus; NC, nucleolus; W, wall; WB, Woronin bodies. Marker bar indicates 1 μm.*

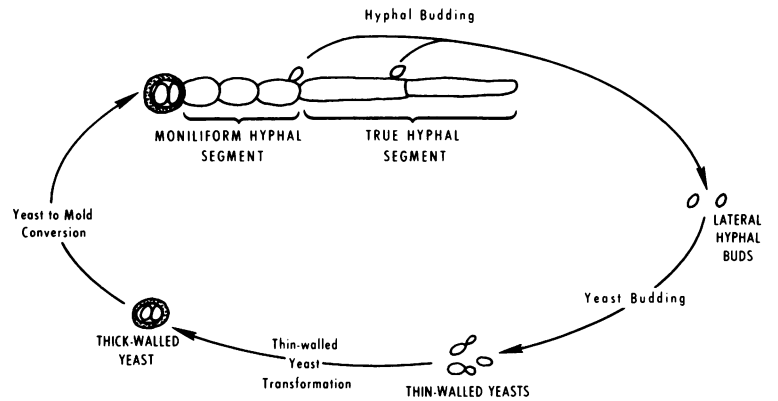

FIG. 11. *Proposed partial asexual life cycle and yeast-to-mold conversion sequence for Phialophora dermatitidis. Explanation is provided in the text.*

The concept that any thin-walled yeast cell or lateral hyphal bud can become essentially a resistant spore after accumulating quantities of storage products may explain the in vitro polymorphism exhibited by *P. dermatitidis*. Polymorphism may simply reflect, in part, the characteristics of the inoculum. Young thin-walled yeasts transferred to suitable media remain yeasts. In contrast, older, matured, thick-walled yeasts transferred to the same media produce hyphae. Inocula consisting of mixtures of these two cell types give rise to subcultures of mixed character which are more yeast-like to mold-like depending upon the ratio of thin-walled to thick-walled yeasts in the inoculum. Results reflecting these conditions have been obtained in our laboratory and have also been indicated by others (5, 11). However, until the present report, no explanation for polymorphism in *P. dermatitidis* has been available.

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