VISUALIZATION OF NEW ULTRASTRUCTURAL COMPONENTS IN THE CELL WALL OF *CANDIDA ALBICANS* WITH FIXATIVES CONTAINING TAPO

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INTRODUCTION

Electron microscope studies of yeasts have revealed the laminated structure of the cell wall (1, 9, 10, 19), its major chemical constituents being distributed in separate layers (13, 15). The number of layers and their morphology are, however, a matter of controversy mainly because the methods of chemical fixation of yeast cells for electron microscopy employed to date have been inadequate (11, 16).

In the search for an effective fixative for the cell wall in *Candida albicans*, we tested several mixtures of Tris-(l-aziridinyl) phosphine oxide (TAPO) (21) and aldehydes (8, 18). The ultrastructural aspect of the cell wall fixed with aldehydes and TAPO was compared with that observed after application of conventional methods of fixation of yeasts for electron microscopy (7, 11).

MATERIALS AND METHODS

Yeast-phase cells of strain 44 of *C. albicans* (kindly furnished by Professor Ortali, Istituto Superiore

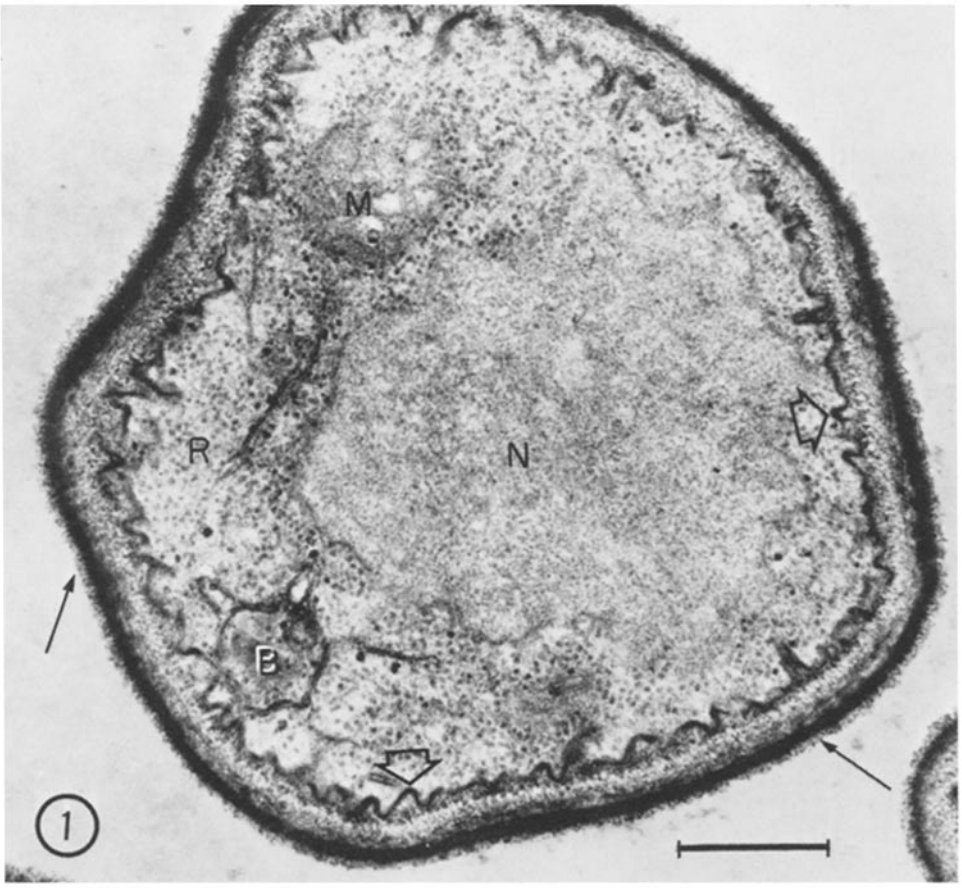

FIGURE 1 Low magnification of young *C. albicans* cell. The section was understained to permit better differentiation of the plasma membrane (empty arrows). The contrast of the cytoplasmic membranes is low, but that of the cytoplasmic granules is appreciable. A major portion of the cell periphery is covered by floccular material, and only occasionally are well-formed segments of the outermost layer of the cell wall visible (small arrows). Cell fixed by the acrolein-TAPO-OsO_4 method. *B*, body of unknown origin; *M*, mitochondrion; *N*, nucleus; *R*, cytoplasmic granules, presumably ribosomes. Scale marker, 0.5 μ. × 40,000.

della Sanitá, Rome) were cultivated in Sabouraud broth (No. 2000) with 2% agar "Bios C" (Biolife Italiana, Milan, Italy) at 37°C until the stationary phase of growth was reached. The layer of grown cells was scraped off the nutrient medium and washed several times with a sterile 0.9% solution of sodium chloride. After centrifugation, the pellet was fixed at 4°C in one of the following prefixatives dissolved in phosphate buffer (pH 7.2; 0.1 M): (*a*) 1% acrolein, 1% TAPO (K & K Laboratories Inc., Plainview, N. Y.); (*b*) 1% glutaraldehyde, 1% acrolein, 1% TAPO; (*c*) 1% glutaraldehyde, 1% TAPO; (*d*) 3% glutaraldehyde; (*e*) 1% acrolein; (*f*) 1% TAPO. The times of fixation were 10 min except for mixture (*d*) which reacted for 3 hr. Prefixatives with TAPO were left for at least 50 min at room temperature before use. If this step was omitted, no improvement in the quality of fixation could be observed. Cells prefixed with the mixtures specified above were postfixed in 4% unbuffered OsO_4 for 20 hr, or in 2% aqueous $KMnO_4$ for 2 hr. A small portion of *C. albicans* cells was fixed exclusively in 2% aqueous $KMnO_4$. All fixed cells were subsequently stained overnight with uranyl acetate (0.5%) in Michaelis buffer (pH 5.8), then dehydrated and embedded in plastic mixtures according to conventional methods. Ultrathin sections were stained with lead and uranyl ions (5, 17), and observed in a Siemens Elmiskop I A electron microscope.

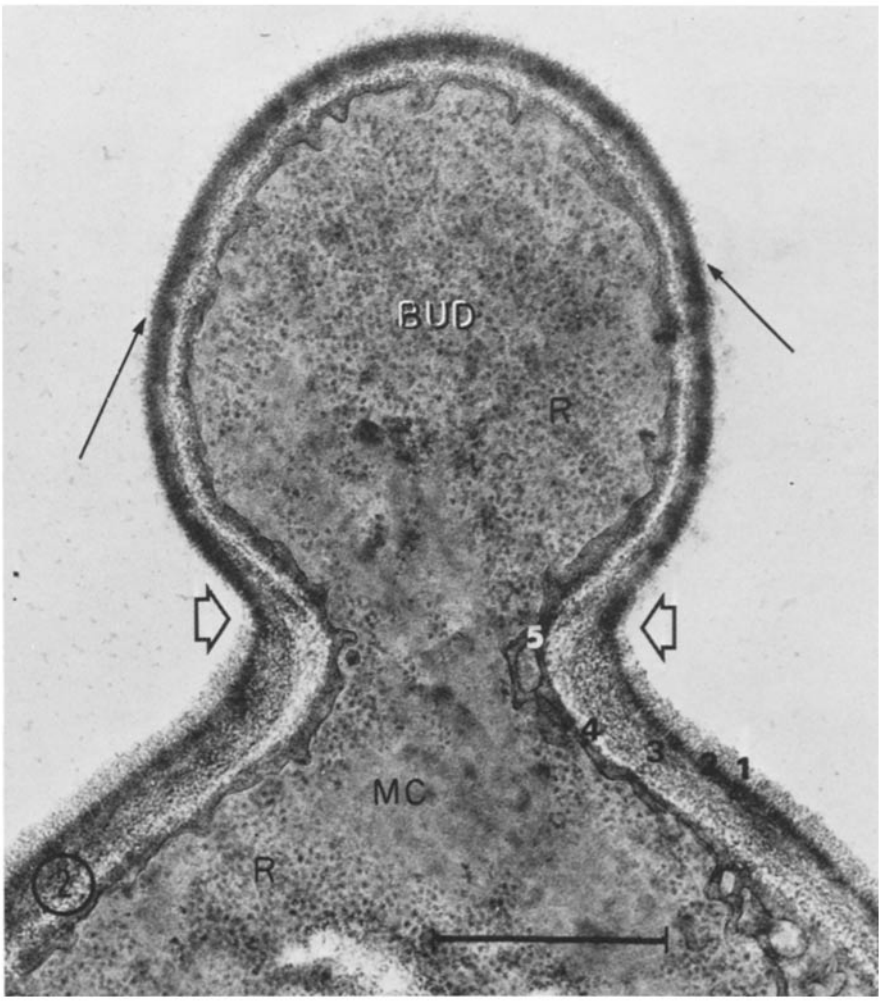

FIGURE 2 Five layers of the cell wall of mother cell of *C. albicans* (consecutively numbered: 1, 2, 3, 4, 5) are distinctly seen. The outermost layer of the cell wall of the mother cell does not continue into the bud; its limits are indicated by empty arrows. The periphery of the bud is covered only by floccular material (small arrows). Lead- and uranyl-stained section. Cell fixed by the acrolein-TAPO–OsO_4 method. *BUD*, the bud; *MC*, mother cells; *R*, cytoplasmic granules, presumably ribosomes. Scale marker, 0.5 μ. × 60,000.

RESULTS

The contrast of the cytoplasmic membranes, except the plasma membrane, in cells fixed with acrolein-TAPO–osmium tetroxide was low, and only cytoplasmic granules, presumably ribosomes, had appreciable electron opacity (Fig. 1). The cell wall had a laminated structure with five distinguishable layers. The interface between the cell wall and the cytoplasm had a complicated shape because of the presence of many irregular invaginations into the interior of the cell (Figs. 1–3). Details of the five wall layers are shown in Fig. 3.

The thickness of the outermost layer of the cell wall, for convenience called the first, ranged from 550 to 800 A, depending on the developmental stage. This layer was composed of anastomosing filaments of medium electron opacity and of variable dimensions. Granules, 25–30 A thick, were embedded with a certain regularity in the network of filaments. The electron opacity of the granules was higher than that of the fila-

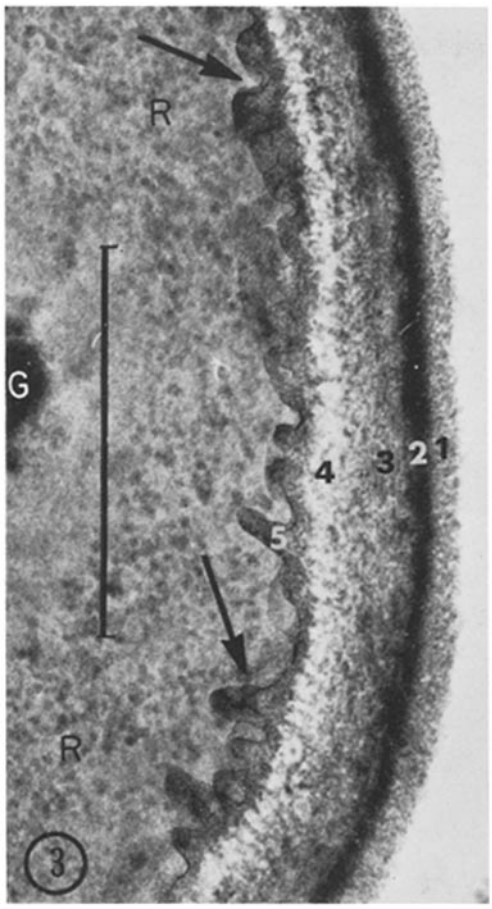

FIGURE 3 Higher magnification of the mature blastospore of *C. albicans*. Five distinguishable layers are consecutively numbered: 1, 2, 3, 4, 5. Large arrows indicate the plasma membrane. Lead- and uranyl-stained section. Cell fixed by the acrolein-TAPO-OsO_4 method. *G*, granules of unknown origin; *R*, cytoplasmic granules, presumably ribosomes. Scale marker, 0.5 μ. × 100,000.

ments. In some sections, in the deeper part of the first layer the granules formed linear arrays situated perpendicular to the cell surface.

An amorphous matrix of high electron opacity was a major component of the second layer, 350–450 A thick, which was the most electron-opaque layer of the cell wall in *C. albicans*.

The third layer, 500–700 A thick, was composed of electron-opaque filaments, 30–40 A thick, which formed bundles oriented parallel to the cell surface. A small number of electron-transparent spaces of variable form was seen between the filaments.

The fourth layer, 400–550 A thick, was composed predominantly of narrow, about 50 A wide, electron-transparent spaces oriented perpendicular to the cell surface. Amorphous material of medium electron opacity was interspersed between the spaces.

The cell wall's innermost layer was found to be closely appressed to the plasma membrane, which was mostly asymmetrical in type and had a tortuous course (Figs. 1–3). In a majority of cells studied, this layer was composed of homogeneous material, the electron opacity of which was slightly lower than that of the second layer (Figs. 1–3).

Cell walls prefixed with glutaraldehyde alone or mixed with acrolein and/or TAPO were structureless, but the cytoplasm of *C. albicans* cells prefixed with a mixture of 3% glutaraldehyde, 1% acrolein, and 1% TAPO was rich in ultrastructural details. Acrolein or TAPO alone gave poor visualization of both the cell wall and the cytoplasm of *C. albicans* cells. Acrolein-TAPO-$KMnO_4$ provided visualization of only one distinguishable layer of the cell wall with floccular material located peripherally. The same aspect of the cell wall was observed in cells fixed exclusively with $KMnO_4$.

DISCUSSION

Prefixation of *C. albicans* cells with a mixture of acrolein and TAPO and postfixation with OsO_4 gave better visualization of the structure of the cell wall than any other known method of preparation of yeast cells for electron microscopy. The term "visualization" is more proper than the term "preservation," because we have no proof that the major chemical components responsible for the full appearance of the cell wall are lost in cells in which the cell wall appears structureless.

The mechanism of action of the acrolein-TAPO mixture is not known. A simple mixture of acrolein and TAPO did not give any appreciable visualization of the ultrastructure of the cell wall. It seems that some reaction must occur between the compounds in the mixture to produce a new "compound" that is responsible for the visualization of the components of the cell wall. In a compound formed by acrolein and TAPO, free active ethylenimine groups of TAPO may bind to biological structures, while the osmiophilic end of the acrolein could be responsible for the contrast of structures cross-linked with TAPO. Even if a

cross-linking of polysaccharides by TAPO is highly probable in view of the affinity of alkylating agents toward polysaccharides, we have no decisive evidence in favor of a specific binding of TAPO to mannan or glucan, which are known to be the main polysaccharide constituents of the cell wall in yeasts (3, 14).

We demonstrate the presence of five distinguishable layers in the cell wall of mature blastospores and of four layers in the cell wall in buds and young cells of *C. albicans*. A majority of reports on the ultrastructure of *C. albicans* mention the presence of two layers in the cell wall (2, 4, 12, 20). The external layer may correspond to our second layer because of its high electron opacity. The internal electron-transparent layer described by most authors probably corresponds to our third and fourth layers. It is not possible to find a definite correspondence between our layers and the three layers of the cell wall in *C. utilis* described by Sentandreu and Villanueva (19) because of the lack of sufficient structural details in their electron micrographs.

There is no net separation of elementary structures in the layers of the cell wall. If each elementary structure corresponds to only one major chemical component of the cell wall, the conclusion is that each layer of the cell wall has a complex chemical composition. We assume that our first and second layers contain mainly mannan-protein and perhaps chitin, whereas our third and fourth layers contain predominantly glucan-protein. This assumption is supported in part by the fact that in chlamydospores of *C. albicans*, which do not contain mannan (6), the first and the second layers of the cell wall are undetectable or greatly reduced in size (unpublished results).

It may be said, in conclusion, that the acrolein-TAPO–osmium tetroxide method offers new technical possibilities in the study of the synthesis and morphological behavior of the constituents of the cell wall in various physiological and drug-induced states of *C. albicans*.

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