

DEVELOPMENT OF THE SPORE WALL DURING ASCOSPORE FORMATION IN *SACCHAROMYCES CEREVISIAE*

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The events involved in ascospore formation in *Saccharomyces cerevisiae* have been studied both physiologically and cytologically. The physiological studies have been focused on nutritional conditions required for sporulation (1, 2) and on the chemical composition of spores (3); cytological investigations have focused upon nuclear events, with the use of light microscopy to study the development of daughter nuclei (4), and upon those constituents of the spores which are easily identifiable, such as fat globules. Investigations of the ultrastructure of the developing ascus of *S. cerevisiae* have been undertaken with permanganate fixation (5, 6) and freeze etching (7). Hashimoto et al. (5) observed the early laying down of the cytoplasmic membrane or plasmalemma of the future spore, but they were unable to suggest its origin. They also observed formation first of the outer coat of the spores and later of the inner layer. Marquardt (6) showed that the outer coat was formed, in part, from material in the cytoplasm. He also noted a section of endoplasmic reticulum parallel to the plasmalemma of the immature spores.

Using the technique of permanganate fixation followed by postfixation with OsO_4 (8), we have been able to confirm and extend these observations on spore wall formation. The present study casts light on the origin of two of the three major portions of the spore envelope: the inner coat and the outer coat.

MATERIALS AND METHODS

Isolation and Growth of Diploid S. cerevisiae

Diploid cells were obtained by selection of prototrophs from cross-streaks of auxotrophic haploids from the collection of this laboratory. For sporulation, the cells were inoculated from a slant into 10 ml of presporulation medium (yeast extract, 0.5%; $(\text{NH}_4)_2\text{SO}_4$, 0.2%; KH_2PO_4 , 0.2%; glucose, 2.0%) and grown for 24–48 hr. They were then diluted 1:100 into sporulation medium (yeast extract, 0.25%; anhydrous potassium acetate, 0.98%; glucose, 0.1%; final pH 6.9). The sporulated cells were harvested at various times by centrifugation.

Electron Microscopy

Washed cultures of cells were fixed and stained by the method of Conti and Brock (8), except that the cells were dehydrated in a graded series of washes of from 50–100% ethanol. After dehydration, they were allowed to stand overnight in a mixture of propylene oxide and embedding mixture, which consisted of 20 ml of Araldite 502, 25 ml of Epon 812, 60 ml of dodecylsuccinic anhydride and 1.5 ml of Tri (dimethylamino methyl) phenol. They were then embedded, sectioned on an LKB microtome with a glass knife, and examined in an RCA G3 electron microscope.

RESULTS

Fig. 1 shows a diploid cell with mitochondria, extensive endoplasmic reticulum, and numerous granules corresponding to the vacuoles previously identified as lipid (5).

An ascus containing four nuclei in the early stages of the formation of the spore plasmalemma is shown in Fig. 2. The lipid granules, which become increasingly poorly preserved in pictures of cells fixed later in spore formation, can be seen to be aligned along the newly formed plasmalemma of the spores. There are regions (arrows) in which

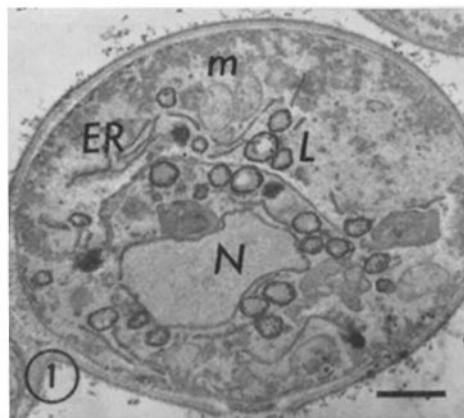


FIGURE 1 A typical diploid cell after 24 hr in sporulation medium. Meiosis has apparently not yet occurred. Visible in addition to the nucleus (N) are mitochondria (m), endoplasmic reticulum (ER), and lipid granules (L). $\times 9,000$. Mark: 1μ .

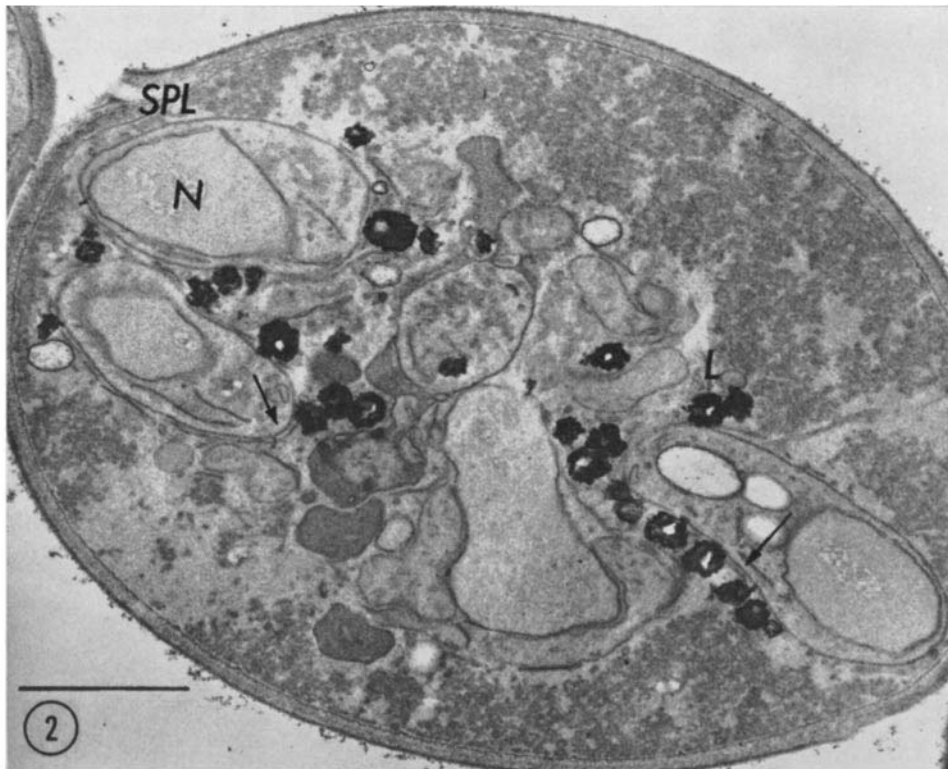


FIGURE 2 An early stage in formation of the plasmalemma (*SPL*) of the spores. The lipid granules (*L*) are now aligned along the newly formed plasmalemma. The latter seems to arise from the inner of two unit membranes (arrows). $\times 22,000$. Mark: 1μ .

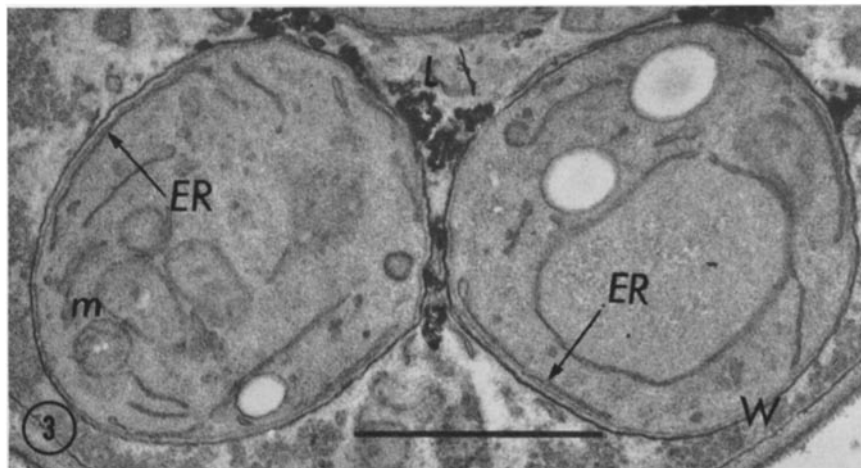


FIGURE 3 Two spores which have achieved mature size and shape. The outer wall (*W*) of the spores has begun to darken, and the beginnings of the inner coat are evident opposite the section of endoplasmic reticulum (*ER*) parallel and adjacent to the plasmalemma. The lipid granules (*L*) are much more amorphous, but remain adjacent to the spore outer wall. Mitochondria (*m*) can be seen in the spore cytoplasm. $\times 33,000$. Mark: 1μ .

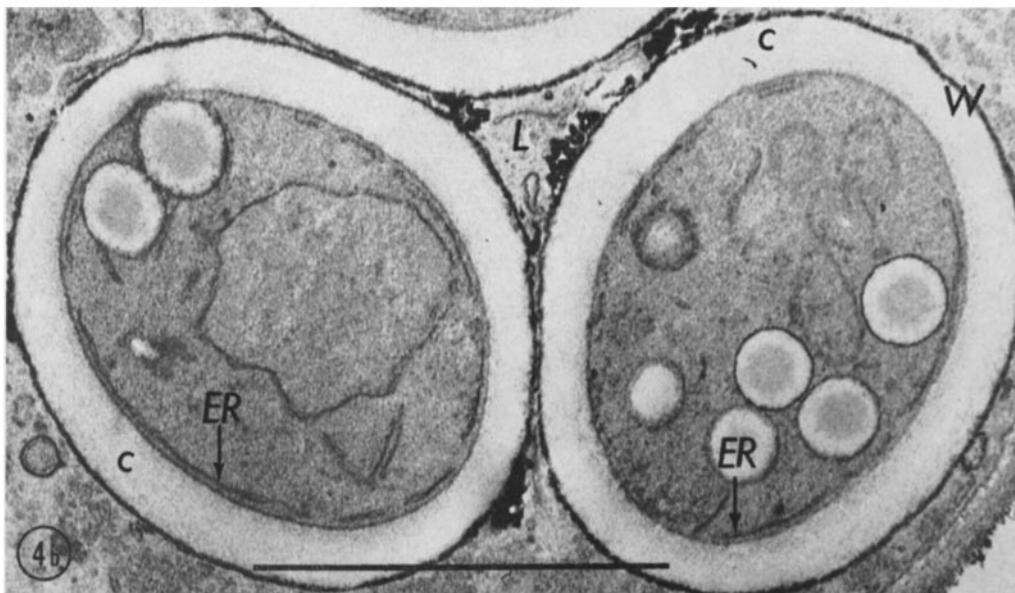
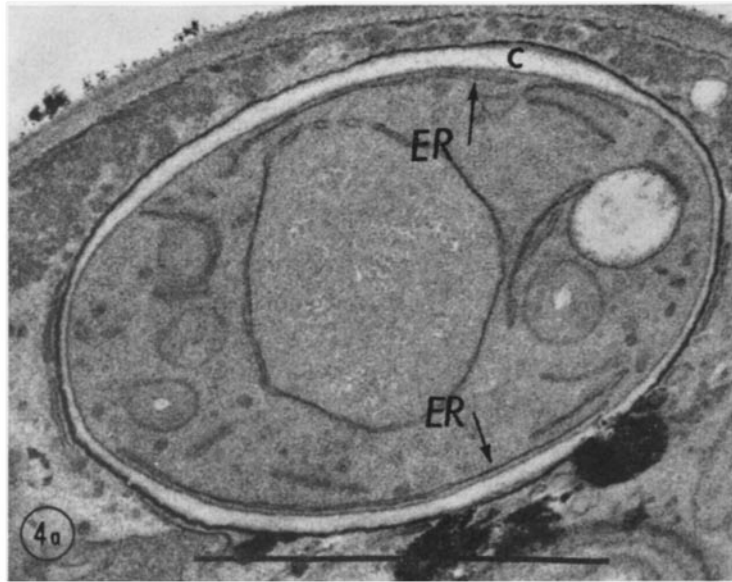


FIGURE 4 *a*, A spore in the early stages of inner coat (*c*) synthesis. The inner coat is first elaborated opposite the endoplasmic reticulum (*ER*). *b*, Two spores with inner coat (*c*) nearly complete. Note that both show endoplasmic reticulum (*ER*) virtually throughout the circumference of the spore. In both *a* and *b*, the lipid granules (*L*) are very amorphous and much decreased in number as compared to Fig. 2, while the outer wall (*W*) is very electron opaque. 4 *a* and *b*, $\times 55,000$. Mark: 1μ .

a second, incomplete membrane runs parallel to the plasmalemma. Near the nucleus at upper left, two concentric membranes, one of which is continuous with the plasma membrane partially sur-

round one of the dark lipid granules. While we have no pictures showing intermediate stages, it seems quite likely from this section that one of the membranes of the spore envelope arises from the

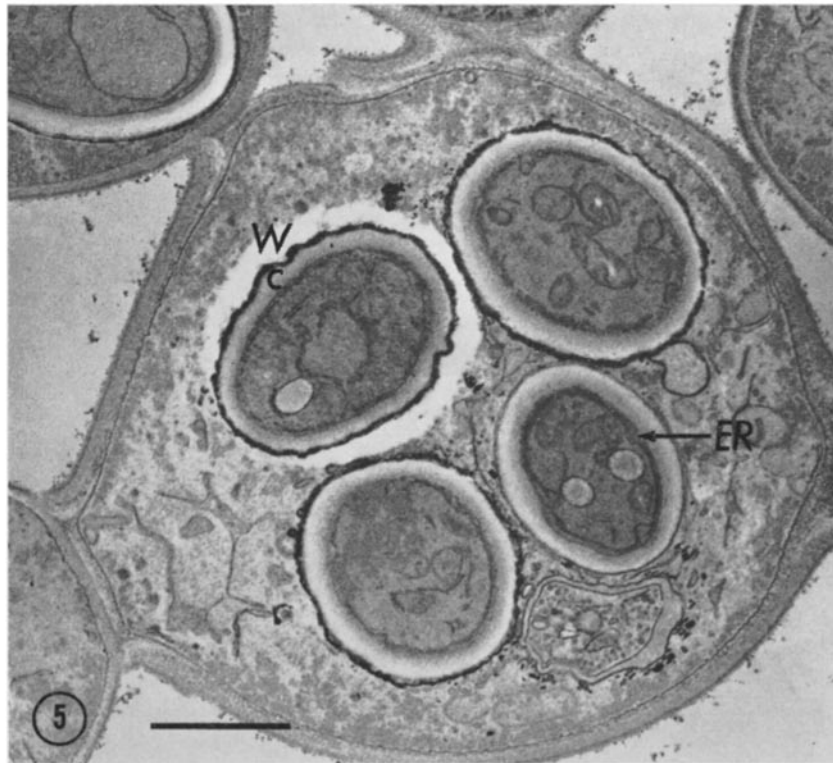


FIGURE 5 An ascus late in development. The spores are in varying stages of maturity. The most mature shows a very dark outer wall (*W*), together with an electron-transparent area in the surrounding ascus. The inner coat (*c*) has darkened somewhat. The least mature spore (lower right) has a complete endoplasmic reticulum (*ER*) parallel to the plasmalemma. $\times 18,000$. Mark: 1μ .

endoplasmic reticulum of the mother cell. The strikingly ordered arrangement of lipid granules suggests that they play a role in envelope formation.

Fig. 3 shows two spores from an ascus later in sporulation. The spores have assumed a rounded shape, and the cytoplasm has increased nearly to the mature size. The two unit membranes of the envelope laid down around the nucleus have separated slightly in places. The outer unit is considerably darkened, particularly in the area adjacent to the lipid granules. Regions of the endoplasmic reticulum of the spores lie adjacent and parallel to the plasmalemma (*ER*, arrow). The separation of the unit membranes is greater opposite those regions than at other places. Other endoplasmic reticulum is also visible.

Soon after the spores round up, they begin to synthesize the material called the "inner coat" by Hashimoto et al. In Fig. 4 *a* is shown a spore in the early stages of inner coat synthesis. The

greatest amount of synthesis takes place opposite the regions of adjacent endoplasmic reticulum, leading us to the hypothesis that the endoplasmic reticulum somehow aids in elaborating this material. The lipid granules show up only as very dark blobs, while the outer coat continues to darken. A spore in a later stage of inner coat synthesis (Fig. 4 *b*) shows parallel endoplasmic reticulum along most of its circumference. The lipid granules are much reduced in size, and the outer coat is uniformly dark.

In a relatively mature spore (Fig. 5), the cortex is complete and the outer coat has darkened and thickened. Owing either to the resistance of the mature spore to the sectioning knife or to a shrinkage of the spore, the spore is often surrounded by an electron-transparent area in the ascus. Mature spores are resistant to the fixation process, so that detail is difficult to make out, but one can see that the parallel endoplasmic reticulum remains close

to the plasmalemma, and that the inner coat now appears denser next to the cytoplasm of the spore.

DISCUSSION

The use of permanganate fixation and OsO₄ postfixation has revealed certain events which take place during the elaboration of ascospores in *S. cerevisiae*. The origin of one of the membranes of the spore envelope seems likely to be the endoplasmic reticulum of the ascus. Although this has not been proved definitely, the concentric layers of membranes surrounding the newly formed plasmalemma certainly support the idea. It is not clear whether these layers are precursors of the outer membrane or are involved in the formation of the plasmalemma.

The ascospore inner coat is elaborated almost simultaneously with the darkening of the outer wall, beginning soon after the spores have assumed their mature size and shape. This layer is presumed to be synthesized through the mediation of that section of the endoplasmic reticulum located parallel and adjacent to the spore plasmalemma. This suggestion is based upon two reciprocal pieces of evidence: the inner coat arises first opposite the endoplasmic reticulum; and when most of the inner coat is synthesized, there is endoplasmic reticulum parallel to most of the circumference of the spore plasma membrane.

Marquardt (6) first suggested that cytoplasmic material from the ascus was incorporated into the spore outer wall. Use of OsO₄ postfixation has allowed us to show that this material is lipid and that it is present from the very early stages of sporulation. It undergoes what appears to be breakdown of its structures and a decrease in its amount, and correlated with its decrease is the increase in electron opacity of the outer walls.

The more mature spores are very difficult to fix satisfactorily, but one can see that there is no alteration in previously observed structures. The darkening of the inner coat near the cytoplasm may be

due to the general state of the spores which are probably somewhat dehydrated, or it may be another artifact of the difficult fixation.

There are many questions about the cytology of yeast sporulation which have not yet been answered. The most interesting have to do with meiosis. The present lack of a good fixative for nuclear structures has prevented an advance along these lines. Other problems include the origin of the membranes of the spore envelope, the mechanism causing the increase in cytoplasm of the spores after the plasmalemma appears complete, the exact chemical nature of the inner wall, and the details of the role of the endoplasmic reticulum in envelope formation.

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