MORPHOLOGICAL CHANGES IN ASCOSPORES OF SACCHAROMYCES CEREVISIAE DURING AEROBIC AND ANAEROBIC GERMINATION

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The stainability of ascospores and vegetative cells of Saccharomyces cerevisiae to acid-fast staining, using hot Ziehl's carbolic fuchsin solution, 5% sulfuric acid, and diluted Löfller's methylene blue, was examined. Resting spores and growing haploid cells (a type strain 24428 and α type 3626) retained much fuchsin dye in the cells. Only mature spores of diploid G2-2 resisted methylene blue staining. The stainability of Mycobacterium phlei IFO 3158 also examined.

The kinetics of germination were examined. The loss of the stainability with acid-fuchsin and of the resistance to methylene blue was used as a criterion of germination. The ascospores germinated anaerobically as well as aerobically. Especially in the early stage of germination, there was found no difference in the germination rates under both conditions. Ultrastructure of germinating ascospores cultured in aerobic and anaerobic conditions was examined by ultrathin sectioning and electron microscopy. At the first stage of germination, the ascospores swelled in aerobic as well as anaerobic cultures. The outer spore coat and the outer zone of inner spore wall disappeared during the germination process, the inner zone of the spore wall then giving rise to a germinated spore cell wall (= extruded germ tube wall). The vacuole became granular. The mitochondria showed no change in shape and number in aerobic cultures, but seemed to swell and disintegrate in the later stages of anaerobic germination.

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The germination of yeast ascospores might be considered as a model system for a study of differentiation or initiation of the active metabolic state from the dormant one in eucaryotic cells. Ascospores of *Saccharomyces cerevisiae*, being formed only under aerobic conditions, contain intact mitochondria and germinate aerobically or anaerobically. The functions of mitochondria during the sporulation or germination processes have been elucidated by Halvorson and his collaborators (1, 2). In addition, the germination process has been analysed from many other standpoints, especially physiologically and biochemically, e.g., the effects of carbon sources (3–5), gaseous conditions (4, 6, 7), respiration and changes in the spore components and enzymatic activities (6, 8, 9), and also the stainability and morphology with the freeze-etching method or scanning electron microscopy, *etc* (9–11).

In the present paper, the electron microscopical changes were examined in ultrathin sections mainly on the spore wall structures, in relation to changes in the stainability of intact spores under a light microscope.

**MATERIALS AND METHODS**

**Organisms and culture conditions.** *Saccharomyces cerevisiae* diploid strain G2-2, and the haploid strains 24428 (mating type a) and 3626 (mating type a) were used. Culture media and conditions for growth and sporulation were the same as those described previously (12). The sporulation culture including 60–70 percent asci were harvested, washed twice with sterilized deionized water and preserved at 0–4°C. The composition of the germination medium was the same as that of the growth medium with the omission of tomato juice. The ascus suspension was inoculated into the germination medium to give a concentration of OD<sub>660</sub> 0.3, using a Hitachi photometer EPO-8. The culture was incubated in flasks with reciprocal shaking at 30°C either aerobically or anaerobically. For anaerobic cultivation, Millipore-sterilized nitrogen gas was supplied to the flasks at a constant flow rate.

Aliquots were sampled hourly. Cells were centrifuged, washed with deionized water, and submitted to bacterial spore staining by the method modified by Möller, as described below, or to fixation for preparation for examination by electron microscopy.

For a control test of acid-fast staining (the modified Möller's method), *Mycobacterium phlei* IFO 3158 was used. The cells of one-day culture on bouillon agar at 37°C were smeared, washed with water, and stained by the same procedure as that for yeast cells described below.

**Staining method for germination and stainability tests.** Washed asci and cells smeared on a slide glass were fixed by heating over a flame, stained with hot Ziehl's carbol fuchsin solution, decolorized with 5% sulfuric acid and washed with water. The stained asci and cells were counterstained with a solution of
Löffler’s methylene blue diluted four times with water, washed with water and finally dried under a stream of air. The preparation was observed in anisol with light microscopy. The resting spores were stained red, and the germinated ones blue. Incidence of germination was shown as the ratio of the number of all spores stained blue to the total number of spores in all asci observed. For testing the stainability of the haploid and diploid vegetative yeast cells, the mature asci and Mycobacterium cells, these were fixed and stained either with hot-carbol fuchsins only, followed by washing with 5% sulfuric acid, or with methylene blue only, then washed with water and examined as described above.

**Methods for electron microscopy.** In order to observe ultrastructure of yeast protoplast, the cells were preferentially treated with a β-1,3-glucanase, Zymolyase (13) to remove the cell wall which resists the permeation of osmium tetroxide (OsO₄), and then fixed with glutaraldehyde-OsO₄ (14). In this study, the fixation method using glutaraldehyde-potassium permanganate was performed especially for observation of fine structure of the yeast spore or cell walls and intracellular membrane systems.

Washed cells were fixed with 6% glutaraldehyde in 0.1 M K, Na-phosphate buffer (pH 7.2) for 1.5 hr, washed twice with the same buffer at 4° for 15–18 hr to remove the aldehyde, and fixed again with 2% potassium permanganate for 3 hr at 4°. After several washings with water, the specimens were stained with 1.5% uranyl acetate for 1 hr, dehydrated with an acetone series and embedded in the mixture of N-butyl methacrylate and methyl methacrylate (7:3). The specimens were then sectioned at 60 nm thickness or less with a glass knife, stained successively with uranyl acetate and lead citrate, and examined in a JEM-100B electron microscope operating at 80 kV.

**RESULTS**

**Changes in stainability during germination**

*Stainability to acid-fast strain.* Comparison of stainabilities between various cell types of the yeast and the acid-fast bacterium was performed. As criteria for germination of the yeast ascospores, changes in optical density, dry weight (5, 6) and stainability have been adopted besides the morphological changes (7). Usually the staining is performed by the same method as that for bacterial spores with carbol fuchsin-sulfuric acid. And one might often consider the reaction of yeast ascospores to the acid-fast stain to be due to the acid-fastness, similar to the reaction of acid-fast bacteria using the original method of acid-fast staining (4, 7). However, the stainability in *Bacillus* spores has been found not to be due to the acid-fastness but to the resistance to permeation of the methylene blue into the spore (15). Thus, in yeast spores the stainability to the acid-fast stain was examined preliminarily before germination experiments with light and electron microscopies.

Growing cells of an acid-fast bacterium *Mycobacterium phlei* IFO 3158 were
stained with each of the solutions for Möller’s test, in order for comparisons to be made with the stainability of yeast cells and spores. The bacterial cells were easily stained either with hot carbol-fuchsin solution, followed by sulfuric acid rinse, or with alkaline methylene blue solution. Though the stained figures are not shown, the stainability in yeast was the same in the case of small daughter cells extruded from germinated ascospores or new bud cells in exponentially proliferating haploid cells of either strains 24428 or 3626 as in the young immature or germinated ascospores, that is, these kinds of cells were stained with either methylene blue or carbol-fuchsin-H$_2$SO$_4$, when treated independently. Thus only the mature spores were not stained with the methylene blue but with the carbol-fuchsin-H$_2$SO$_4$. During germination, the spores seemed to change to allow permeation of the methylene blue inside the spore wall. Of course, proliferating cells in the diploid phase, G2-2, and the resting ones in the stationary phase were stained blue with the methylene blue but not with the carbol-fuchsin-H$_2$SO$_4$.

![Fig. 1. Kinetics of germination in spores in asci of Saccharomyces cerevisiae.](image)

\[\text{\textbullet, aerobic; \textcircled{O}, anaerobic.}\]

![Fig. 2. Mature and resting spores in the ascus from 60-hr sporulation culture in acetate medium.](image)

a, a whole ascus. Ascospores show spore wall, cytoplasmic membrane, nucleus, vacuoles with electron-dense areas, mitochondria, lipid granule and endoplasmic reticulum. The ascospore wall is composed of two layers, outer spore coat and inner spore wall, both of which comprises two zones with different electron densities. Bud scars are found on ascus wall. b, a part of spore wall.

Key to abbreviations: AW, ascus wall; BS, bud scar; SW, spore wall; CM, cell membrane; N, nucleus; V, vacuole; M, mitochondrion; ER, endoplasmic reticulum; L, lipid granule; 1, outer zone on outside of outer spore coat (OS); 2, inner zone on inside of OS; 3, outer zone on outside of inner spore wall (IS); 4, inner zone on inside of IS. In all figures the length of the black line represents 1 µm, unless otherwise indicated.
Fig. 3. Germinated ascospores incubated aerobically for 1 hr. Ascospores swell and elongate. In the spore wall OS and outer zone of IS are lost partly (arrow). Granules in a vacuole show an aggregated state.

Fig. 4. Germinated ascospores incubated aerobically for 2 hr. OS in spore wall disappears over a fairly wide range. An ascospore is shown just before extrusion of a germ tube. Double arrows in the figure indicate the rupture of spore wall.
Fig. 5. A germ tube of germinated ascospore incubated aerobically for 3 hr. The tube wall is extruded continuously with the inner zone of IS in ascus wall. Nucleus
Kinetics of germination under aerobic or anaerobic conditions. Spores in asci germinated as shown kinetically in Fig. 1. Under either aerobic or anaerobic conditions, spores germinated very similarly, though the final percentage of anaerobically germinated spores was slightly less than that under the aerobic condition. In the absence of exogenous glucose, however, dye-permeability did not change, though the data are not shown. Thus glucose may be considered essential for the germination of yeast spores.

Change in ultrastructure during germination

Structure of resting ascospores. As shown in Fig. 2a and 2b, the wall of ascospores consisted mainly of four layers, i.e., an outer spore coat (OS) with higher electron density, consisting of an outer zone of OS (darker layer) and an inner one of OS (less dark layer), and an inner spore wall (IS), consisting of a thick layer of electron-less density (outer zone of IS) and an inner one with more or less higher electron density (inner one of IS). The endoplasmic reticulum was observed inside the spore cell membrane. The mitochondria had ellipsoidal shape and a few cristae. Three to five mitochondria were found in a section of the spore. Vacuoles were observed as entities denser than parts of the cytosol and surrounded with a single unit membrane.

Germination under aerobic conditions. As germination proceeded, the spores swelled up to fill the ascus within the ascus wall and the zone of IS became thinner or lost with OS at the swollen part of the spore wall, especially near the ascus wall (Fig. 3). Similar figures showing changes in the coat and wall substances might be observed abundantly in 2-hr germination culture (Fig. 4). Also, these changes in ultrastructure might be supported by the kinetical observation with light microscopy as shown in Fig. 1. In the electron micrographs from 2- and 3-hr germination culture, a newly extruded spore cell wall (germ tube wall) was observed discretely connected with the inner zone of IS in the periphery of the protoplast of some germinated spores (Figs. 4 and 5).

The cristae in mitochondria became visible more clearly in spores from 1-hr germination culture (Fig. 3). Thereafter, no remarkable change was observed in the number or morphology of the mitochondria. Distinct DNA fibrils, which appeared in the mitochondria during sporogenesis (16), could not be observed even during the aerobic germination (Figs. 2–5).

Some morphological changes were observed in vacuoles. As germination moves near the front of tube. ER and small vesicles are observed at the front of the tube.

Fig. 6. Anaerobically germinated ascospores (2 hr). The same coat structures in the wall are observed as in the aerobically germinated spore wall. Mitochondria are normal.

Fig. 7. Anaerobically germinated ascospores (3 hr). Nucleus moves near the front of germ tube. ER and small vesicles are observed at the front of the tube. Mitochondria disintegrated.
proceeded, the granular structure inside the vacuolar membrane formed aggregates (Figs. 3-5). However, the aggregates were observed in vacuoles in the vegetative cells. The nucleus showed no remarkable change during germination but moved near the front of the germ tube at the later stage of germination (Fig. 5).

**Germination under anaerobic conditions.** Figures 6 and 7 show electron micrographs of spores germinated anaerobically. The structural changes were very similar to those found with aerobic germination in many respects, e.g. the initial swelling of the spore and the changes in the spore wall, vacuoles and nuclei.

Only mitochondria in the germinating spores were observed to be more or less abnormal in anaerobic germination. Until 2-hr germination culture the spores contain normal mitochondria similar to those germinated aerobically (Fig. 6), but some irregularly elongated or swollen and disintegrated ones were found in the later stages of anaerobic germination (Fig. 7). The nucleus moved near the front of the germ tube.

**DISCUSSION**

Stainability to basic dyes in the ascospore of yeast seems to be different from the acid-fast character of mycobacterial cells. The similar "acid-fastness" was confined rather to haploid vegetative cells of the yeast. The principal characteristic of the yeast ascospore stained with basic dyes may derive from the incapability of permeation of the dye into spores. Therefore the authors recommend use of "dye resistance" or "impermeability to basic dyes" in the place of "acid-fastness" in *Saccharomyces* ascospores.

Under either aerobic or anaerobic conditions, a similar pattern in morphological alteration in germination was observed in ascospores of *Saccharomyces cerevisiae* with light and electron microscopies. As in other previous reports (1, 2, 7), however, the mitochondria degenerated in the later stage of anaerobic germination. The degeneration may occur due to the inhibition of mitochondrial activity and repression of mitochondriogenesis, *i.e.*, a kind of catabolite inactivation and repression by glucose in the germination medium (17-19).

Also, the following facts might coincide well with the morphological changes in mitochondria in that mitochondrial DNA fibrils could not be observed in spores under aerobic or anaerobic germination possibly because of the lack of multiplication of mitochondria at least at the initial stage of germination. Halvorson described that spores of *S. cerevisiae*, formed in presence of ethidium bromide, germinated without respiratory activity and active mitochondria (1, 2), and that DNA was synthesized at the later stage of germination (8).

On the other hand, changes in the spore wall were noted aerobically or anaerobically during germination. The electron-dense layer, the outer spore coat (OS), disappeared at the germination site, and the electron-less dense one, the inner zone of inner spore wall (IS), elongated and later formed a germ tube,
a discrete new cell wall. This type of germ tube wall connected with the spore wall was observed similarly in *Aspergillus* conidia, with the same method of fixation and staining (20, 21). According to Bartnicki-Garcia (22), this pattern of fungal spore germination is classified as type I, in which the inner layer of the spore wall elongated and was synthesized under the spore wall and formed a germ tube wall connected with it. Also, Marchant (23) described that the germ tube, which is produced as the asexual or sexual fungal spore germinates, may be continuous with existing layers (holoblastic) or a layer of the spore wall (enteroblastic), citing the diagram of conidial formation by Hughes (24). On the germination of ascospore of *S. cerevisiae*, Kreger-Van Rij (11) suggested the extension of IS to germinated tube wall being formed as a new layer. Also in our results, no new layer could be found in the IS or outside of the spore cell membrane, but a discrete cell wall was newly formed around the germinating area connected with the inner zone of IS. These results suggest this type of spore germination to be of type I (22) and enteroblastic as with many other fungal spores (23).

It is a well-known fact in electron microscopical studies on the ascospore of *Saccharomyces* yeast that it is surrounded with a wall composed of two or three layers, i.e., outer electron-dense, middle less-dense and inner dense (11, 16). This result shows four zones included in two main layers, OS and IS, in the spore wall. Loss of electron density in the outer zone in OS might occur at the early phase of germination and probably in accordance with an appearance of methylene blue stainability as shown kinetically in Fig. 1.

Lysis of wall components by enzyme was suggested to occur on the germination of *Fusarium* spores (25) and also in our biochemical experiments on the germination of ascospores of this *S. cerevisiae* (unpublished data). Electron microscopical figures presented above show rather a possible lysis of wall components over a wide range of OS and outer zone of IS than a simple rupture and wrinkling of the rigid outer zone of OS (Figs. 4 and 5).

The outer zone in OS in the spore wall with the higher density for electron might be mostly composed of chitin-like substance or chitin-bound protein(s), as suggested in the biochemical and electron microscopical observations by Whelan and Ballou (26, 27), using glucosamine auxotrophic mutants of yeast. The spore wall of the mutants lack the electron-dense outer zone in OS. It has not been ascertained further that the outer layer of the ascospores was composed of substance(s) including chitin. On the other hand, surface substances of isolated *Saccharomyces* spores, possibly being composed of proteins (28), might be discriminated electrophoretically at least from those of a-type haploid and diploid vegetative cells and asci (29). And the outer surface of the chitin layer in the spore wall might be covered with an other substance preventing the chitin from binding with an added chitin-specific fluorescent dye (unpublished data).
REFERENCES