Sporulation in Protoplasts of the Yeast, *Saccharomyces cerevisiae*

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(Received 7 January 1974; revised 19 February 1974)

SUMMARY

Protoplasts of *Saccharomyces cerevisiae* cultivated in sporulation medium with an osmotic stabilizer at pH 7.0 were studied by means of light and electron microscopy. After 15 h, mature ascospores with complete cell walls were formed in the protoplasts. During sporulation, protoplasts regenerated only the fibrillar wall component and were osmotically sensitive. By using snail enzymes to block fibril regeneration on the protoplast surface, it was proved that sporulation was quite independent of such regeneration. Most of the ascospores produced by protoplasts were viable, osmotically resistant and gave rise to new yeast cells. Meiotic division and sporulation in the yeasts are processes quite independent of the presence of the ascus wall.

INTRODUCTION

Protoplasts have been prepared from bacteria, blue-green and green algae, moulds, yeasts and from a number of meristematic cells of higher plants. For all the investigated species except blue-green algae, conditions facilitating regeneration of the cell wall have been found (for review see Gabriel, 1973).

Protoplasts of bacteria, yeasts, moulds and green algae with incomplete walls cannot undergo cell division, even though nuclear division occurs, and consequently they die (for review see Nečas, 1971). However, another way in which protoplasts of yeasts and bacteria can survive is by sporulation (Salton, 1955; Eddy & Williamson, 1959; Fitz-James, 1964; Stárka & Čáslavská, 1964).

This study was conducted in an attempt to investigate: (i) the process of sporulation in yeast protoplasts, both qualitatively and quantitatively, by means of light and electron microscopy; (ii) whether meiotic division and sporulation of the protoplasts are dependent on ascus wall regeneration; (iii) whether spores formed by protoplasts are viable and can give rise to new yeasts.

METHODS

Organism. *Saccharomyces cerevisiae*, laboratory strain No. 7, was used throughout the experiments.

Preparation of protoplasts. Yeasts from the stock culture were inoculated into 10 ml malt-extract-wort medium, pH 5.5, and incubated at 28 °C without shaking. After approximately 30 h, yeast cultures were near the end of the log phase of growth and were ready for the preparation of protoplasts by the use of snail enzymes as described by Eddy & Williamson (1957). Fresh protoplasts were washed three times with McIlvaine citrate-phosphate buffer, pH 5.5, with 0.6 M-KCl and 0.01 M-MgSO₄ added to the buffer.

Sporulation of protoplasts. Fresh protoplasts were transferred to the sporulation medium

with an osmotic stabilizer (0.5 %, w/v, potassium acetate and 4.5 %, w/v, potassium chloride in Sørensen phosphate buffer, pH 7.0) to give a concentration of about 1.0×10^7 to 2.0×10^7 /ml medium (Fowell, 1969). Incubation was carried out either in liquid sporulation medium shaken (100 rev./min) at 28 °C or on the surface of 1.5 % (w/v) agar sporulation medium with a given concentration of protoplasts spread over the agar surface. Sporulating protoplasts were observed by phase-contrast microscopy.

To determine the ability of growing protoplasts to sporulate, fresh protoplasts were grown either in malt extract wort at pH 5.5 or in acetate pre-sporulation growth medium (Roth & Halvorson, 1969) to which osmotic stabilizer (4.5 %, w/v, potassium chloride) was added. After growth for about 30 h, protoplasts were washed three times with fresh sporulation medium and the washings were cultivated on a shaker, as mentioned above.

In order to determine whether sporulation and wall regeneration were independent processes protoplasts were cultivated in the sporulation medium supplemented by snail digestive juice: fresh crude juice from snail (*Helix pomatia*) stomach was evaporated and frozen in a refrigerator at 0 °C and used at 1.0 % (w/v), a concentration which is known to block wall regeneration in yeast protoplasts (Kopecká, Čtvrtníček & Nečas, 1965) but not to affect ascospores (Johnson & Mortimer, 1959).

Bacterial contamination was prevented by the addition of 10 µg Tetracyklin Spofa (United Pharmacological Works, Prague) per ml sporulation medium.

Electron microscopy. The ultrastructure of sporulating protoplasts was studied by means of a freeze-etching technique. The sporulating protoplasts were frozen either directly in the sporulation medium or after 12 h incubation in 20 % (w/v) glycerol at 4 °C, and freeze-etched in an apparatus (Balzer) by Moor & Mühlethaler's (1963) method. The replicas were washed with sulphuric acid and Eau de Javelle as described by Moor & Mühlethaler (1963) and then subjected to a 40 % chromic acid treatment (R. Janisch, personal communication).

Synthesis of the fibrillar wall component in sporulating protoplasts was observed on preparations shadowed with platinum and carbon. Living protoplasts collected at various times of cultivation in sporulation medium were placed on Formvar-coated grids and allowed to dry. The dried preparations were washed two or three times with distilled water and, after drying, shadowed with platinum and carbon. The preparations were examined with a Tesla BS 242 electron microscope.

Viability of ascospores produced by protoplasts. Protoplasts cultivated in sporulation medium were collected by centrifugation and washed three times with distilled water. Distilled water was added to the pellet to achieve immediate lysis of protoplasts and release of ascospores, which were then inoculated at 1.0×10^7 /ml on to the surface of 2.0 % malt-extract-wort agar or into liquid malt extract wort without an osmotic stabilizer, and incubated on a shaker. They were examined by means of phase-contrast microscopy and freeze-etching techniques.

RESULTS

Sporulation of protoplasts. The rate of ascospore formation in the protoplasts was the same as that in normal yeasts. In the fresh protoplasts, or those cultivated in liquid sporulation medium for 10 h, no ascospores could be found (Figs. 1, 2 and 6). After incubation of 20 h about 45 % of the protoplasts formed ascospores (Fig. 3). Such a culture consisted of a mixture of ascospores (either liberated from or still inside protoplasts) and vacuolated protoplasts in different stages of ascospore formation, but without visible ascospores. After 30 h incubation the number of sporulating protoplasts had increased to 73 %.

Following sporulation, the ascospores can be liberated from protoplasts by osmotic stress with distilled water and separated from protoplast ghosts by centrifugation (Fig. 4). The liberated ascospores were osmotically resistant and remained associated in groups of four (Fig. 4). Figure 5 shows the arrangement of ascospores in normal sporulating yeast. The maximum yield of four-spored clusters in the protoplast culture approached 60 %; the remaining protoplasts produced only 1, 2 or 3 ascospores per protoplast. These results corresponded to those in normal yeasts of our strain.

Protoplasts sporulated much better in liquid sporulation medium than on agar, where sporulation was slower and resulted in production of fewer ascospores. This observation agrees with the findings of other authors working with intact organisms (for review see Fowell, 1969).

Neither regeneration of rigid wall on the protoplast surface nor reversion of protoplasts was observed during sporulation.

Electron microscopy of sporulating protoplasts. In contrast to fresh protoplasts, protoplasts after 10 h of incubation in sporulation medium contained numerous mitochondria, lipid vesicles and smaller vacuoles, and were rich in the cisternae of endoplasmic reticulum, often arranged in a parallel array (Fig. 6). Occasionally, meiotic division of nuclei was observed. After 20 h ascospores were found in protoplasts (Fig. 7). These ascospores contained the structures characteristic of normal yeast ascospores (Guth, Hashimoto & Conti, 1972). During sporulation the fibrillar component of the wall regenerated on the surface of the protoplasts (Fig. 8). The mature ascospores liberated from protoplasts could be seen inside fibrillar nets (Fig. 9).

Sporulation in growing protoplasts. Protoplasts grown previously for about 30 h in liquid nutrient medium with glucose, and which showed nuclear division and regeneration of fibrillar wall components on the protoplast surfaces (Nečas, 1956; Kopecká *et al.* 1965; Kopecká, Nečas & Svoboda, 1970), were subsequently transferred to sporulation medium. After transfer, even such pre-grown multinucleate protoplasts were able to sporulate and the dynamics of ascospore formation was similar to that of the fresh protoplasts. Liberated ascospores remained caught within clearly visible dense fibrillar nets (Fig. 10).

Sporulation of protoplasts in the presence of snail enzymes. It is apparent from the above that protoplasts can regenerate the fibrillar component of the wall in sporulation medium. To determine whether protoplast wall-synthesis and sporulation were processes dependent upon each other, we studied sporulation in protoplasts in which regeneration of the fibrillar wall component was blocked by snail enzymes. Even under these conditions the protoplasts formed ascospores.

Germination of ascospores produced by protoplasts. When liberated ascospores were inoculated onto the surface of malt-extract-wort agar about 90 % of ascospores formed microcolonies of yeast. When inoculated into liquid malt extract the ascospores increased in size, but for the first 2 to 3 h their contents remained homogeneous. Afterwards, vacuoles appeared and buds were formed (Fig. 11). When cultivated on a shaker, germinating ascospores formed zygotes which gave rise to new yeast cells by budding (Fig. 12).

Electron microscopy of germinating ascospores showed the same organelles as in mature yeast cells (Moor & Mühlethaler, 1963) and the process of budding was analogous to that found in the budding yeast (Moor, 1967).

Fig. 1. Protoplasts of *S. cerevisiae* prepared by 2 h digestion with snail enzymes in 0.6 M-KCl (pH 5.5) at 28 °C on a shaker. Vacuoles are visible in the protoplasts.

Fig. 2. Protoplasts of *S. cerevisiae* cultivated in sporulation medium for 10 h. Vacuoles have disappeared in most protoplasts but no ascospores are yet present.

Fig. 3. Protoplasts of *S. cerevisiae* cultivated in sporulation medium for 20 h, showing vacuolated spherical sporulating protoplasts and mature liberated ascospores in four-spore clusters.

Fig. 4. Free ascospores, without the ascus wall, of *S. cerevisiae*, separated from protoplasts by osmotic stress with distilled water and by centrifugation.

Fig. 5. Ascospores produced by normal sporulating cells of *S. cerevisiae*. Ascospores are enclosed in the ascus.

Fig. 6. Electron micrograph of freeze-etched sporulating protoplast of *S. cerevisiae* after 10 h in sporulation medium shows many mitochondria, lipid granules, smaller vacuoles and parallel cisternae of endoplasmic reticulum. In the plasma membrane deep invaginations were found (arrowed) caused by the treatment of protoplasts with 20 % glycerol (Kopecká, Svoboda & Brichá, 1973). ER, Endoplasmic reticulum; M, mitochondrion; L, lipid granules; V, vacuole.

Fig. 7. Electron micrograph of a freeze-etched sporulating protoplast of *S. cerevisiae* after 20 h incubation in sporulation medium. Two ascospores are visible inside the protoplast. Treated with 20 % glycerol before freeze-etching. A, Ascospore.

DISCUSSION

The above results show that protoplasts of the yeast *Saccharomyces cerevisiae* sporulated when cultivated in 0.5 % (w/v) potassium acetate with 4.5 % (w/v) potassium chloride at pH 7.0. This finding is in agreement with the results of Eddy & Williamson (1959) who observed the sporulation of protoplasts of the yeasts *Saccharomyces carlsbergensis* in a complex synthetic medium without any source of nitrogen.

Conclusive evidence was obtained that the ascospores occurring among protoplasts in sporulation medium arose from the protoplasts and not from the normal cells occasionally present among protoplasts: (i) the number of living cells among protoplasts in our experiments did not exceed 1.0 %; (ii) the fresh sporulating protoplasts remained spherical during the whole sporulation process, while the normal cells were oval; (iii) ascospores inside the protoplasts were observed by both phase-contrast and electron microscopy; (iv) the ascospores formed by protoplasts were free of the ascus wall, while ascospores produced by normal cells were enclosed by the ascus (see Figs. 4 and 5); (iv) the most conclusive evidence of protoplast sporogenesis was provided by observations with the electron microscope of the ascospores enclosed in the fibrillar wall component, a structure formed exclusively by protoplasts in liquid media (Kopecká *et al.* 1970).

In all the cultures tested, sporulation occurred more frequently with protoplasts harvested at the end of the exponential phase of growth than with those from the early exponential and stationary growth phases, with which lysis often occurred. The osmotic stabilizers tested were 0.6 M-potassium chloride, 0.6 M-sodium chloride, 0.7 M-mannitol and 0.7 M-rhamnose, of which potassium chloride stabilizer was the most suitable. For sporulation of protoplasts pH 7.0 was used, because between pH 8.0 and 10.0 immediate lysis of protoplasts occurred. About 70 % of the protoplasts sporulated. This finding corresponds to the number of cells sporulating in normal cultures of our strain. Most of the spores produced by protoplasts were viable.

The fact that in liquid medium protoplasts cannot produce rigid cell walls (except for the fibrillar component) yet can build the complete wall of the ascospore inside the protoplast, is apparently connected with the removal of wall components and wall-synthesizing enzymes from the protoplast surface by the liquid medium (for reviews see Nečas, 1971; Kreger & Kopecká, 1973).

The ability of sporulating protoplasts to regenerate the fibrillar wall component raised the question of whether the two processes were independent of each other. By blocking the regeneration of the fibrillar wall component with snail enzymes during sporulation it was proved that sporulation occurred quite independently of wall regeneration. The two pro-

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Fig. 8. Electron micrograph of freeze-etched *S. cerevisiae* protoplast after 30 h on sporulation medium, showing fibrillar wall component (arrowed) on the spore surface.

Fig. 9. Electron micrograph of three freeze-etched spores formed by one *S. cerevisiae* protoplast and enclosed inside the same fibrillar component (shown by arrow).

Fig. 10. Two spores which were produced by one growing yeast protoplast of *S. cerevisiae*. Spores are enclosed in the fibrillar component of the wall (arrowed).

Fig. 11. Germinating ascospores of *S. cerevisiae* which were produced by a growing protoplast, and which are enclosed in a fibrillar net (arrowed).

Fig. 12. Shake culture of germinating ascospores of *S. cerevisiae* which have formed zygotes in malt-extract-wort medium. Germinating spores and zygotes (enclosed in a fibrillar net - arrowed) have given rise to new yeast cells by budding.

cesses are probably controlled by different mechanisms. This is unlike the sporulation in bacterial protoplasts, in which the sporogenesis needs to be induced before the bacterial cell is converted to a protoplast (Salton, 1955; Fitz-James, 1964; Stárka & Čáslavská, 1964).

Concerning the relationship between nuclear and cell division in yeast protoplasts, it seems that the triggering of both the mitotic (Nečas, 1956) and meiotic nuclear divisions is not dependent on the wall present on the protoplast surface. However, vegetative division of the whole protoplast cannot occur in the absence of the complete cell wall around the protoplast. In contrast, division of the protoplast after meiosis is triggered without a wall (ascus) on the protoplast surface. It can therefore be presumed that the two types of cytokinesis are controlled by different mechanisms.

The author thanks Dr M. Gabriel and Dr N. J. W. Kreger-van Rij for useful discussions, and Professor O. Nečas for critically reading the manuscript.

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