The Fine Structure of Yeast Regenerating Protoplasts

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SUMMARY

The process of regeneration of *Candida utilis* and *Pichia polymorpha* protoplasts obtained by means of an enzyme preparation from *Streptomyces violaceus* MR and an enzyme preparation from the snail (*Helix pomatia*) was followed with the electron microscope. A reticular network of fibrils making up a tube or surrounding the regenerating protoplasts was observed. The fibrils are glucan fibrils, and in *C. utilis* they are packed in groups of 5 or 6. *P. polymorpha* fibrils occurred singly. The fibrils are the framework for building the new cell wall. The relation between fibrils and plasmalemma particles is discussed.

INTRODUCTION

Regeneration occurs when yeast protoplasts are kept for a time in a suitable isotonic medium. Necas (1956, 1961) studied this process with *Saccharomyces* strains, and Rost & Venner (1965) with strains of *Saccharomyces fragilis*, *S. willianus* and *Schizosaccharomyces pombe*. Eddy & Williamson (1959) first found that growing protoplasts are covered with a reticular network. Necas (1965) proved that this fibrous network builds the new cell wall. Villanueva (1966) has reviewed this problem. The aim of the present paper is to determine whether the regenerating protoplasts of *Candida utilis* and *Pichia polymorpha* show, in the electron microscope, fibrils or a network which represents the beginning of cell-wall synthesis.

METHODS

Organisms. The strains of *Candida utilis* 1016 and *Pichia polymorpha* 1132 used in these experiments were obtained from the Colección Española de Cultivos Tipo (CECT).

Culture media. *Candida utilis* was grown in 300 ml. flasks containing 100 ml. of Hansen medium (30 g. sucrose; 10 g. bactopectone; 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g. K_2HPO_4 ; 1000 ml. distilled water). For *Pichia polymorpha* cultures, 100 ml. Winge medium (3 g. yeast extract Difco; 20 g. glucose; 1000 ml. tap water) was used in 300 ml. flasks. Cultures were incubated by shaking at 29°.

Preparation of protoplasts. *Candida utilis* was harvested in the early logarithmic phase of growth and the organisms washed with distilled water. For protoplast formation the organisms were suspended in an enzyme solution from *Streptomyces violaceus* MR (Elorza, Muñoz-Ruiz & Villanueva, 1966), containing m- MgSO_4 as a stabilizer; the final concentration of organism was 0.075% (w/v) dry wt. The mixture was shaken at 30° and the extent of lysis and protoplast formation was measured with a Zeiss phase-contrast microscope. After 2 hr the protoplasts had been free.

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Pichia polymorpha organisms were similarly harvested and washed. Protoplasts were liberated by the snail enzyme preparation (*Helix pomatia*) obtained from the Industrie Biologique Française (Gennevilliers, France) by the Eddy & Williamson (1957) method.

Regeneration of protoplasts. *Candida utilis* protoplasts were washed three times with M-MgSO₄ solution, resuspended in M-MgSO₄ solution containing 0.0017% (w/v) of sucrose and incubated at 30°.

Pichia polymorpha protoplasts were washed with M-MgSO₄ solution, resuspended in Winge medium containing 20% (w/v) MgSO₄ as stabilizer and incubated at 30°. The regeneration process was studied by taking samples of protoplasts and examining them with the Zeiss phase-contrast microscope.

Electron microscopy. Embedding. Samples of regenerating protoplasts taken at 6 and 8 hr were centrifuged down, the deposit washed with distilled water, suspended in 5% (w/v) aqueous potassium permanganate and kept at room temperature for 2 hr. The fixed protoplasts were dehydrated through 25%, 50% and 75% (v/v) acetone in water, then 100% acetone. During dehydration the material was stained overnight in 2% (w/v) uranyl acetate dissolved in 75% (w/v) acetone. The fixed and stained material was embedded in Durcupan ACM from Fluka AG, Buchs, Switzerland, cut with a LKB Ultratome Microscope 4804 Nife (glass knives) and picked up on Formvar-coated grids.

Shadow-casting. Drops of 6 hr and 8 hr suspensions of regenerating protoplasts were applied directly to Formvar-coated grids (after having been washed with water to free them of salts), allowed to dry and shadowed at an angle of 45° with gold + palladium in a Siemens VB 6500 evaporator.

Sections and shadowed preparations were examined with a Siemens Elmiskop I electron microscope and in a EM 9 Zeiss electron microscope.

RESULTS

The regeneration process as seen with the phase-contrast microscope

After incubation for 1 hr the protoplasts showed signs of growth. Budding protoplasts were seen in the medium. After 2 hr the initially spherical protoplasts changed to tubular forms, which grew to form large cytoplasmic masses, from which new yeast cells originated (Pl. 1, fig. 1). These large tubular forms were similar to those obtained by Necas (1956, 1961, 1965), but we produced them in a liquid medium instead of on a solid medium. These forms were not sensitive to osmotic shock, and for this reason could be handled in distilled water.

Thin sections of regenerating protoplasts

Plate 1, fig. 2 and 3, shows sections through regenerating protoplasts of *Candida utilis* and *Pichia polymorpha*. While these protoplasts were not sensitive to osmotic shock, they were very sensitive to the embedding process and the protoplasm is affected (Monreal, Uruburu & Villanueva, 1967). No clear structures were seen in the cytoplasm, but around it was a fairly wide porous layer, more manifest in *C. utilis* than in *P. polymorpha*.

Shadow-casting of regenerating protoplasts

Plate 2, fig. 4-7, and Plate 3, fig. 8, show shadow-casting of regenerating protoplasts. A network of fibrils can be seen building a tube, or surrounding the protoplasts.

In regenerating protoplasts of *Candida utilis* the fibrils are packed in groups of 5 or 6. Each fibril is 250-300 Å wide (Pl. 2, fig. 5, 6). The bundles of fibrils are 3000-4000 Å wide (Pl. 2, fig. 5). Scattered along the tubes are large cytoplasmic bodies which represent the protoplasts (Pl. 2, fig. 7; Pl. 3, fig. 8). Sometimes protoplasts which have not put out a fibrous tube are also surrounded with a fibrous network (Pl. 3, fig. 8).

Regenerating protoplasts of *Pichia polymorpha* show a network of fibrils (Pl. 2, fig. 4). These do not join to form a bundle, however, but remain single, and form a disorderly framework. Each fibril is 200-250 Å wide. Protoplasts can be seen in the fibrous frame-work.

DISCUSSION

The fibrils found in the shadow-casting preparations denote the beginning of new cell walls. Previously, shadow-casting of *Candida utilis* protoplasts showed no fibrils around them (Garcia-Mendoza & Villanueva, 1967). From the chemical composition of the *C. utilis* cell walls (Novaes & Villanueva, 1963) we assume these fibrils are made of glucan. The fact that we were not able to find the fibrils in thin sections is because they do not stain with potassium permanganate. Reports of the fine structure of yeast cell walls have always shown a lack of contrast of the inner glucan layer (Northcote, 1963; Vitols, North & Linnane, 1961). On the other hand, the stained layer in sectioned *C. utilis* regenerating protoplasts is made up of protein, which does react with potassium permanganate. This layer represents the stainable part of the inner layer of the yeast cell wall. While it is believed that protein is located only in the outer cell-wall layer (Mundkur, 1960, 1964), probably it is scattered throughout the glucan framework. Besides, polysaccharides combine with the protein in the form of a glucan-protein (Falcone & Nickerson, 1956). Since protein is not fibrous, one cannot see it in shadowed preparations. Protoplasts have not yet begun to build the outer protein layer.

Moor & Mühlethaler (1963) reported, with frozen-etched yeast cells, that the cytoplasmic membrane was covered with particles which sometimes were concentrated in hexagonal arrangements. From these arrangements grew fibrils which extended into the cell wall. When particles were scattered on the cytoplasmic membrane, no fibrils were seen. The fact that in our pictures the fibrils are sometimes packed in bundles of about six units leads us to assume that perhaps there is a relation between hexagonal arrangements and packed fibrils, and between unarranged particles and single fibrils. This discussion also applies to regenerating protoplasts of *Pichia polymorpha*.

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REFERENCES

- EDDY, A. A. & WILLIAMSON, D. H. (1957). A method of isolating protoplasts from yeast. *Nature, Lond.* **179**, 1252.
- EDDY, A. A. & WILLIAMSON, D. H. (1959). Formation of aberrant cell walls and of spores by the growing yeast protoplasts. *Nature, Lond.* **183**, 1101.
- ELORZA, V., MUÑOZ-RUIZ, E. & VILLANUEVA, J. R. (1966). Production of yeast cell wall lytic enzymes on a semi-defined medium by a *Streptomyces*. *Nature, Lond.* **210**, 442.
- FALCONE, G. & NICKERSON, W. J. (1956). Cell-wall mannan-protein of baker's yeast. *Science, N.Y.* **124**, 272.
- GARCIA-MENDOZA, C. & VILLANUEVA, J. R. (1967). Preparation and composition of the protoplast membrane of *Candida utilis*. *Biochim. biophys. Acta* **135**, 189.
- MONREAL, J., URUBURU, F. & VILLANUEVA, J. R. (1967). Lytic action of (1-3) glucanase on yeast cells. *J. Bact.* **94**, 241.
- MOOR, H. & MUHLETHALER, K. (1963). The fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**, 609.
- MUNDKUR, B. (1960). Electron microscopical studies of frozen-dried yeast. I. Localization of polysaccharides. *Expl. Cell Res.* **20**, 28.
- MUNDKUR, B. (1964). Electron microscopical studies of frozen-dried yeast. V. Localization of protein-bound sulphhydryl. *Expl. Cell Res.* **34**, 155.
- NECAS, O. (1956). Regeneration of yeast cells from naked protoplasts. *Nature, Lond.* **177**, 898.
- NECAS, O. (1961). Physical conditions as important factor for regeneration of naked yeast protoplasts. *Nature, Lond.* **192**, 580-581.
- NECAS, O. (1965). The mechanism of regeneration of yeast protoplasts. II. Formation of the cell wall de novo. *Folia biol. Praha* **11**, 97.
- NORTHCOTE, D. H. (1963). The structure and organization of polysaccharides of yeast. *Proc. of the Symposium on the Chemistry and Biochemistry of Fungi and Yeast. International Union of Pure and Applied Chemistry*, Dublin, p. 669.
- NOVAES, M. & VILLANUEVA, J. R. (1963). Quelques données sur la composition chimique des parois de *Candida utilis*. *Biochim. biophys. Acta* **78**, 797.
- ROST, K. & VENNER, H. (1965). Wachstum von Hefeprotoplasten und ihre Reversion zu intakten Hefezellen. *Arch. Mikrobiol.* **51**, 130.
- VILLANUEVA, J. R. (1966). Protoplasts of fungi: in *The Fungi: an Advanced Treatise*. Ed. by G. C. Ainsworth and A. S. Sussman, vol. 2, p. 3. New York and London: Academic Press.
- VITOLS, E., NORTH, R. J. & LINNANE, A. W. (1961). Studies on the oxidative metabolism of *Saccharomyces cerevisiae*. I. Observations on the fine structure of the yeast cell. *J. biophys. biochem. Cytol.* **9**, 689.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Regeneration process of regenerating protoplasts of *Candida utilis* and *Pichia polymorpha*. Regeneration in *Pichia polymorpha*: a, b, c. Regeneration process in *Candida utilis*: d, e, f. Phase-contrast microscope. $\times 500$.

Fig. 2. Thin section through a regenerating protoplast of *Candida utilis*. $\times 22,000$.

Fig. 3. Thin section through a regenerating protoplast of *Pichia polymorpha*. $\times 20,000$.

PLATE 2

Fig. 4. Shadow-casting of regenerating protoplast of *Pichia polymorpha*. $\times 13,200$.

Fig. 5. Detail of a bundle of fibrils of regenerating protoplasts of *Candida utilis*. Shadow-casting. $\times 190,000$.

Fig. 6. Fibrils of regenerating protoplasts of *Candida utilis*. Shadow-casting. $\times 25,000$.

Fig. 7. Shadow-casting of regenerating protoplasts of *Candida utilis*. $\times 17,000$.

PLATE 3

Fig. 8. Shadow-casting of regenerating protoplasts of *Candida utilis*. $\times 21,000$.