Fusion of Yeast Protoplasts^{*}

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Recently, we found the papers described the fusion of spheroplasts of *Saccharomyces cerevisiae*.^{1,2)} However, independently, we have also studied the asexual cell fusion of the microorganism by applying protoplast-system. In this paper, we present a technique for fusion of the protoplasts prepared from the same mating type cells of auxotrophs; *Saccharomyces cerevisiae* 156-5C (*a gal5 his1*) and G4-a (*a gal3 met1 ura1 ade6*), these were obtained from the Yeast Genetic Stock Center, University of California, U. S. A.

The cells of the auxotrophs were separately cultured in 100 ml of YPG medium (0.4% yeast extracts, 0.5% peptone, 5% glucose, 0.5% KH_2PO_4 and 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) without shaking at 30°C and harvested at an early log-growth phase (approximately 1×10^8 cells/ml) by centrifugation. The cells were suspended in 2 ml of SET medium (1.3 M sorbitol, 0.1 mM EDTA and 10 mM Tris-HCl buffer, pH 7.4). To the cell suspension were added 0.1 ml of β -mercaptoethanol and 0.1 ml of 0.1 M EDTA solution, then incubation was performed at 30°C for 30 min. The cells were collected and resuspended in 2 ml of SET medium. The cell suspensions of both strains were mixed, and immediate addition of Zymolyase (High Grade 60,000, Kirin Brewery, Japan) was conducted to the mixed suspension (final enzyme concentration, 250 $\mu\text{g}/\text{ml}$), followed by incubation at 30°C for 3 hr. The protoplasts thus formed (Micrograph 1) were collected by centrifugation ($10,000 \times g$) and suspended in fusion medium (40% polyethylene

MICROGRAPH 1. Protoplasts before Fusion Process, Mixture of 156-5C and G4-a.

MICROGRAPH 2. Protoplasts after Fusion Process (overnight), Mixture of 156-5C and G4-a.

MICROGRAPH 3. Nuclei in 156-5C Cells. Similar micrograph was obtained with G4-a cells.

MICROGRAPH 4. Nuclei in "Hybrid" Cells (F-3). Bars in micrographs are 10 μm . Nuclei were stained with Giemsa reagent.⁵⁾

glycol 6,000 (PEG), 0.75 M sorbitol, 5% glucose, 50 mM CaCl_2 and 50 mM Tris-HCl buffer, pH 7.4) to bring to a protoplast concentration of approximately $2 \times 10^9/\text{ml}$. Incubation was carried out at 37°C for 30 min (Fusion Process). Then, the protoplasts were collected by centrifugation ($10,000 \times g$), and resuspended in 2 ml of fusion medium with omission of PEG. For regeneration of the protoplasts to vegetative cells,³⁾ 0.1 ml of the suspension was mixed with 10 ml of a hypertonic YPG medium (0.75 M sorbitol and 30% gelatin (Difco) in YPG medium) and immediately poured into petri dish (at 45°C), then incubation was performed at 30°C for one day. To the petri dish was added 1.0 ml of gelatinase solution (Tokyo Kasei, 10 mg/ml) to solubilize gelatin in the medium. The regenerated cells in the solubilized medium were collected, diluted with saline and plated onto both minimal medium (5% glucose, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01%

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TABLE I. FREQUENCIES OF "HYBRID" FORMATION BY FUSION-REGENERATION PROCESS FROM PROTOPLASTS OF *Saccharomyces cerevisiae*

Strains	Frequencies ^{a)}
G4-a+156-5C	3.0×10^{-7}
G4-a	less than 10^{-9}
156-5C	less than 10^{-9}
G4-a+156-5C ^{b)}	less than 10^{-9}

^{a)} colony number on minimal medium/colony number on supplemented medium (see text)

^{b)} PEG- or/and Ca^{2+} -free fusion media were used.

NaCl and vitamins*) and supplemented one (minimal medium supplemented with L-methionine 20 μg , L-histidine 20 μg , uracil 2 μg and adenine 2 μg per ml). After 2-day incubation at 30°C, number of colonies formed was counted. The regenerated cells capable of growth on minimal medium were found to gain the ability to metabolize galactose. Thus, we would like to designate these cells as "hybrid" cells or the "asexually induced hybrid" cells in this paper. A frequency of the "hybridization" is approximately 3.0×10^{-7} (Table I). When Ca^{2+} ions and/or PEG were removed from fusion medium, the frequencies decrease to negligible level. Deoxyribonucleic acid (DNA) content in a cell of the "hybrid" was higher than that in a cell of haploid strains used here (Table II). Morphological properties of the "hybrid" cells were somewhat different from those of the haploid cells; the "hybrid" cells are larger

in size and less-aggregative in mass behavior in liquid culture.

During fusion process (30 min) it was very hard to obtain clear evidence supporting occurrence of protoplast fusion as morphological alteration. However, formation of monster protoplasts could be observed by prolongation of fusion process, such as overnight incubation (Micrograph 2). Both Ca^{2+} ions and PEG were essentially required for formation of monster protoplasts. In Micrographs 3 and 4, nuclear staining was carried out with haploid (156-5C) and "hybrid" (F-3, see Table II) cells, respectively. From these micrographs, however, the determinative information about ploidy of the "hybrid" cell was not available. Genetical properties of the "hybrid" cells are under investigation.

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TABLE II. DEOXYRIBONUCLEIC ACID CONTENT IN YEAST CELL

Diploid strain was obtained from haploid strains of 156-5C and G4- α . F-1 to F-7 were randomly picked-up strains from the hybrids isolated in this paper.

Strain	156-5C	G4-a	Diploid	F-1	F-2	F-3	F-4	F-5	F-6	F-7
Relative content of DNA	1.0 ^{a)}	0.9	1.9	3.1	1.9	1.8	1.4	1.8	1.2	1.6

^{a)} net content of DNA is 4.5 ng/10⁴ cells. This value was used as standard in this table. An amount of DNA was determined by the method of Schneider.⁴⁾

* Vitamines: biotin 20 μg , calcium pantothenate 2 μg , folic acid 2 ng, inositol 10 μg , nicotinic acid 0.4 μg , p-aminobenzoic acid 0.2 μg , pyridoxine-HCl 0.4 μg , riboflavin 0.2 μg and thiamine-HCl 0.4 μg per ml.