TRANSFER OF MITOCHONDRIA INTO PROTOPLASTS OF *SACCHAROMYCES CEREVISIAE* BY MINI-PROTOPLAST FUSION

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1. Introduction

Protoplast fusion has become a valuable method in the study of yeast genetics and biochemistry. Intraspecific, interspecific and intergeneric fusion of yeast protoplasts has been accomplished [1–8].

Isolated mitochondria have also been transferred into yeast protoplasts [9,10], although with very low efficiency of transformation ($\sim 10^{-8}$). To raise this efficiency, the mini-protoplast method was devised, by which mitochondria contained in mini-protoplasts (protoplasts lacking nuclei) were transferred into protoplasts of a respiratory-deficient (ρ°) strain to yield respiratory-sufficient cells (A. Maráz, personal communication). Mini-protoplasts can be obtained by treating log-phase cells with lytic enzyme [11]. In the log phase, yeast cells are dividing, and the migration of mitochondria into buds or daughter cells precedes that of the nuclei.

We have conducted intraspecific mitochondrial transfer experiments as the first step in investigating through intergeneric transfer the inhibitory effect of mitochondria on expression of the hexokinase isozyme gene in *Hansenula jadinii*, which we found in the analysis of fermentative production of CDP-choline [12–14].

2. Materials and methods

2.1. *Strains*

Heterothallic haploid strains of *Saccharomyces cerevisiae* were employed: A cytoplasmic petite mutant BO60AF-1 (*a ade2 arg4 leu2* $C^{\circ}E^{\circ}O^{\circ}$ ρ°) was used as mitochondrial recipient and respiratory-sufficient haploid $AN^{R}OR12D$ (a *leu2 his4 thr4* $C^{S}E^{S}O^{R}$ ρ^{+}) was used as mitochondrial donor. These were kindly provided by Dr N. Gunge.

2.2. *Preparation of protoplasts*

Cells of BO60AF-1 were cultured in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) to the late log phase, and harvested cells were washed with water and suspended in protoplasting buffer, consisting of 0.8 M sorbitol in 0.05 M potassium phosphate buffer (pH 7.5). To the cell suspension, 2-mercaptoethanol (final conc. 10 mM) and zymolyase 60000 (final conc. 10 μg/ml) were added, and the mixture was incubated with gentle shaking for 30 min at 30°C. Protoplasts were collected by centrifugation at 1000 × *g* for 5 min, washed twice and resuspended in the protoplasting buffer. By the same procedure, protoplasts were prepared from the respiratory-sufficient (ρ^{+}) haploid $AN^{R}OR12D$, aerobically grown in YPG medium (1% yeast extract, 2% peptone and 3% glycerol) to the middle log phase. This procedure gave a mixture of the usual large nucleated protoplasts and small anucleated ones, (mini-protoplasts), which contained mitochondria but not nuclei. The large protoplasts were removed by centrifugation at 1950 × *g* for 5 min, and the mini-protoplasts were collected by centrifugation at 3100 × *g* for 15 min, then washed twice with protoplasting buffer.

2.3. *Protoplast fusion*

Mini-protoplasts prepared from 2 g (wet) cells of $AN^{R}OR12D$ (mitochondrial donor) and protoplasts prepared from 0.2 g (wet) cells of BO60AF-1 (mitochondrial recipient) were mixed and carefully suspended in 5 ml 35% (w/v) polyethylene glycol (PEG)-

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4000 containing 50 mM $CaCl_2$, then incubated for 30 min at 30°C. This fusion mixture was centrifuged for 15 min at 3100 × *g*, and the precipitate was resuspended in 1 ml protoplasting buffer. To regenerate the fused protoplasts, 0.1 ml of the resuspension was spread onto several plates containing a selective medium, the surface of the plates was covered with the same medium, and the plates were incubated at 30°C. The selective medium contained 0.67% Difco yeast nitrogen base without amino acids, 50 μg/ml each of adenine sulfate, arginine and leucine, 3% glycerol and 0.1% glucose as carbon source, and 2.5% agar, and was osmotically stabilized with 0.8 M sorbitol.

2.4. *Determination of DNA content*

DNA was extracted by method in [15] and estimated according to [16] as modified [17].

3. Results and discussion

The ρ° petite mutant BO60AF-1 could not grow on the selective medium since it was unable to assimilate glycerol for growth. The mitochondrial donor cells of AN^ROR12D could not grow on the selective medium either, because of their requirements of histidine, leucine and threonine for growth. When mini-protoplasts from AN^ROR12D were fused with protoplasts from BO60AF-1 in the presence of PEG and $CaCl_2$, several respiratory-sufficient colonies appeared on the selective medium after incubation for 5–7 days. Two conceivable kinds of fusion could account for these colonies. Fusion between recipient protoplasts and the anucleate mini-protoplasts of the mitochondrial donor would give rise to cells retaining the auxotrophic requirement for adenine, arginine and leucine of the recipient. Alternatively, fusion between recipient protoplasts and nucleate donor protoplasts contaminating the mini-protoplasts preparation would give rise to cells having an auxotrophic requirement only for leucine, the common requirement of donor and recipient cells.

Seven respiratory-sufficient colonies were obtained in two series of experiments, and tested for their genetic markers shown in table 1. Four of them (strains 3–6 in table 1) required adenine, arginine and leucine for growth, showing the same nutritional requirement as the recipient strain BO60AF-1. They also showed the same mating type as BO60AF-1. These 4 strains were thus concluded to be transformants made by the transfer of mitochondria in the mini-protoplasts. The remaining 3 colonies (strains 7–9 in table 1) required only leucine for growth. They were also of a mating type, but their DNA content indicated they were diploid cells. These strains were thus considered to be the fusion products of the two strains used.

In tests of mitochondrial drug resistance, all the fusion isolates were sensitive to chloramphenicol and erythromycin but resistant to oligomycin, matching the donor strain AN^ROR12D.

Table 1
Characteristics of mitochondrial donor and recipient strains and fusion products

Strains	Mating -type	ρ	Auxotrophic markers					DNA content (μg/10^8 cells)	Ploidy
			ade2	*arg4*	*leu2*	*his4*	*thr4*		
(1) AN^ROR12D[a]	a	+	+	+	–	–	–	2.45	*n*
(2) BO60AF-1[b]	a	○	–	–	–	+	+	2.16	*n*
(3) F 1-1	a	+	–	–	–	+	+	2.24	*n*
(4) F 1-11	a	+	–	–	–	+	+	2.26	*n*
(5) F 2-12	a	+	–	–	–	+	+	2.55	*n*
(6) F 2-13	a	+	–	–	–	+	+	1.68	*n*
(7) F 2-2	a	+	+	+	–	+	+	4.44	2*n*
(8) F 2-4	a	+	+	+	–	+	+	4.50	2*n*
(9) F 2-11	a	+	+	+	–	+	+	4.10	2*n*

[a] mitochondrial donor
[b] mitochondrial recipient

The frequency of mitochondrial transfer by the mini-protoplast method was $\sim 10^{-7}$ (calculated from the protoplast number regenerated on the complete agar). This is higher than that reported for transformation by use of isolated mitochondria [9]. Before we succeeded with the mini-protoplast method, we had failed to obtain transformants by using isolated mitochondria. We can therefore recommend this mini-protoplast method, since it is simple and effective, and carries less risk of damaging mitochondria than the isolation procedure. This technique will facilitate mitochondrial transfer both interspecifically and intergenerically, and contribute to mitochondrial biochemistry and yeast breeding. We are now planning to transfer the mitochondria of *Hansenula jadinii* into protoplasts of *Saccharomyces cerevisiae* to analyze the fermentative mechanism of CDP-choline formation.

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