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Use of protoplast fusion to introduce methionine overproduction into *Saccharomyces cerevisiae*

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Summary. DL-Ethionine-resistant mutants of *Saccharomyces uvarum* ATCC 26602 were found to overproduce exogenous L-methionine. DL-Ethionine-resistant mutant ER 108, carrying a mutation to chloramphenicol resistance, was converted to *petite* form, and protoplasts obtained from it were fused with protoplasts from antibiotic-sensitive *S. cerevisiae* X2928 carrying six auxotrophies. The resulting fusants maintained four auxotrophies and were capable of overproducing L-methionine. These fusants were stable after ten passages on complete medium.

Introduction

Several yeasts, and especially *Saccharomyces cerevisiae,* are currently used as a source of protein for animal and human nutrition. In relation to the amino acid requirements of mammals, yeast proteins are low in methionine content, and for this reason the use of mutants that have increased proportions of methionine in their proteins has been suggested (Dunyak and Cook 1985; Morzycka et al. 1976; Komatsu et al. 1974; Okanishi and Gregory 1970).

Mutants resistant to ethionine, an analogue of methionine, have generally been found to be the best methionine overproducers. Most industrial strains of yeasts are polyploid, sporulate with great reluctance, and no mating type is expressed. Therefore, they are more stable or less susceptible to classical genetic methods. Most of these difficulties can be surmounted with two techniques, i.e. protoplast fusion and transformation. Recently several papers concerning transfers of industrial characters by protoplast fusion such as killer factor (Bortol et al. 1986), osmotolerance (Legmann and Margalith 1986), dextrin (Barney et al. 1980), cellobiose (Pina et al. 1986) and lactose utilization (Farahnak et al. 1986) have been published. The fusants obtained by these authors were stable, suggesting that modified yeasts for industrial application can be obtained by this method. The aim of this research was to obtain methionine-overproducer mutants of *S. uvarum* ATCC 26602 and to transfer this character to a methionine-auxotrophic strain of *S. cerevisiae* by protoplast fusion.

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Materials and methods

Strains. The yeast strains used in this study were: *S. uvarum* ATCC 26602, *S. cerevisiae* X2928 *a* adel gall leu2 his2 ura3 trp1 met14 (Yeast Genetic Stock Center, University of California, Berkeley, Calif., USA), *S. saké* ATCC 26422, *S. cerevisiae* L8 from a distillery, *Candida utilis* ATCC 9950, *C. utilis* var. *thermophilus* CCY 29383, *C. tropicalis* ATCC 15114, and *C. lipolytica* CBS 6124.

Media. The complete medium used was YEPD, which contained 1% yeast extract, 2% peptone and 2% glucose. Yeast Nitrogen Base without amino acids (Difco, Detroit, USA) was used as a synthetic medium and glucose was used as the carbon source. Fermentation ability was determined in medium containing 0.5% yeast extract and 2% of the sugar tested, with Durham tubes (Kreger-van Rij 1984).

Mutagenesis. Cell suspensions (10^7 cells/ml) of the strains more sensitive to DL-ethionine were treated with 200 µg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and plated on Yeast Nitrogen Base without amino acids (Difco) supplemented with 2% glucose and 2% DL-ethionine. Colonies appearing on the plates within 72 h (Et^r) were picked up and assayed for methionine content.

Assay of methionine. L-Methionine was estimated in both the supernatant (exogenous methionine, µg/ml) and cells washed

twice with distilled water. The pellets were suspended in distilled water and boiled in a water bath for 1 h. The supernatants obtained by centrifugation were assayed for L-methionine, determined as pool methionine (mg/g dry weight). L-Methionine content was determined by bioassay with *Leuconostoc mesenteroides* P 60 (ATCC 8052) in Methionine Assay Medium (Difco).

Antibiotic-resistant and respiratory-deficient mutants. Chloramphenicol-resistant mutants of *S. uvarum* Et^r, obtained after treatment in complete medium containing 8 mM $MnCl_2$ according to the method of Putrament et al. (1973), were treated with ethidium bromide, following the method of Slonimski et al. (1968), to obtain respiratory-deficient mutants *(petite).* Cytochrome spectra of wild-type and *petite* mutants were assayed in the following way. Differential spectra of a suspension of 60 mg wet mass of cells/ml were recorded at room temperature in 1 ml 0.1 M phthalate-NaOH, pH 5.0, using a Cary 219 spectrophotometer. The bandwidth was 1 nm and the scan speed was 0.5 nm/s. The cell suspension reduced by sodium dithionite corresponded to 200 mg wet mass.

Protoplast formation. Strains *S. uvarum* Et$^r\rho^-$ ER 108 and *S. cerevisiae* X2928 were grown at 30° C on a rotatory shaker to early stationary phase in 250-ml flasks containing 50 ml YEPD medium. Portions (10 ml) of each culture were collected and centrifuged at 500*g* for 5 min. Cells were then washed twice with sterile distilled water and were suspended in the protoplasting buffer containing 0.6 M KCl in 50 mM phosphate buffer (pH 7.5). A pretreatment with 10 mM 2-mercaptoethanol was used, and the suspensions were incubated for 20 min at 30° C with gentle agitation. Then 5 mg/ml Novozym SP 234 (Novo Industri, Copenhagen, Denmark) were added, and the suspensions were incubated again at 30° C with gentle agitation and checked periodically under the microscope for the formation of protoplasts. Practically all of the cells were converted into protoplasts within 30 min. These protoplasts were lysed when exposed to a hypotonic environment, as confirmed by microscopic examination. Protoplasts were collected by centrifugation at 3000*g* for 10 min and washed four times with protoplasting buffer.

Protoplast fusion. The protoplasts from both strains of yeast were mixed and suspended carefully in polyethylene glycol (PEG) solution (Tris buffer 10 mM, pH 7.5, 0.8 M KCl, 30% PEG 4000 and 10 mM $CaCl_2 \cdot 2H_2O$), and the suspension was incubated for 25 min at 30° C with gentle agitation. The fused cells were mixed with melted minimal regeneration medium containing 3% agar, 0.8 M KCl, Yeast Nitrogen Base without amino acids (Difco), 1% glycerol and 1% sodium lactate as carbon source and amino acid pools such as leucine, uracil, tryptophan, adenine, or leucine, uracil, tryptophan, histidine. This medium was poured onto plates containing a thin bottom layer of agar with the same medium. Plates were incubated for 7—10 days at 30° C. Possible revertants of the parent strain *S. cerevisiae* X2928 were evaluated by spreading 10^8 cells on the regeneration medium in triplicate.

Results and discussion

Isolation of mutants

Preliminary tests with the above-indicated yeast strains showed that the strains more sensitive to DL-ethionine were *S. saké* ATCC 26422 (0.007% DL-ethionine) and *S. uvarum* ATCC 26602 (0.05% DL-ethionine). These strains were treated with NTG and plated on minimal agar medium containing 2% DL-ethionine. The survivors on these plates were isolated, and their abilities to accumulate exogenxous and free L-methionine were examined. The best results were obtained with *S. uvarum* ATCC 26602. Seventy-eight ethionine-resistant mutants were assayed for L-methionine content; 12 (15%) showed that free L-methionine was enhanced 5—7 times, whereas L-methionine content in the supernatants was 15—25 times

Table 1. L-Methionine concentration in the wild-type and in some DL-ethionine-resistant mutants of *Saccharomyces uvarum* ATCC 26602

Strain	24 h		48 h	
	A	B	A	B
S. uvarum ATCC 26602 (wild)	0.113	—	0.9	0.3
S. uvarum ER 85	6.4	2.0	4.9	20.2
S. uvarum ER 95	7.6	4.1	5.0	18.0
S. uvarum ER 108	6.2	5.2	4.7	33.0

A: Free cell L-methionine (mg/g dry weight)
B: L-methionine in supernatant (μg/ml)

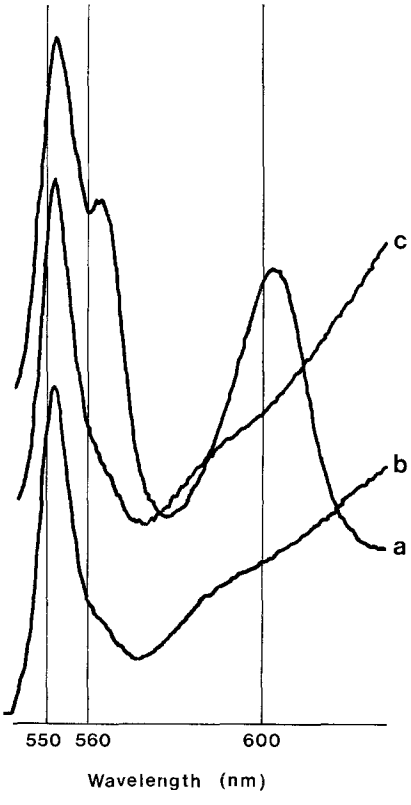

Fig. 1. Ethidium bromide effects on the cytochrome spectra of *Saccharomyces uvarum* ATCC 26602 **(a)** and its respiratory-deficient mutants **(b, c)**

Table 2. Results of fusing *S. uvarum* $Et^r\rho^-$ and *S. cerevisiae* X2928

Experiment number	*S. uvarum* $Et^r\rho^-$ protoplasts plated/0.1 ml	*S. cerevisiae* X2928 protoplasts plated/0.1 ml	Total protoplasts recovered/0.1 ml from fusion mixture on complete medium	Protoplast recovery (%)	Frequency of successful fusions (complementation)
1	9.5×10^5	8.8×10^5	7.3×10^5	39.8	2.9×10^{-4}
2	5.3×10^6	4.9×10^6	4.1×10^6	40.2	1.1×10^{-5}

higher than in the parent strain. The best producer mutant was repeatedly treated with NTG and reselected on minimal medium containing 2.3—2.6% DL-ethionine.

Of 189 DL-ethionine-resistant mutants examined, 24 (13%) produced considerable amounts of both endogenous and exogenous L-methionine. The best mutants, ER 95 and ER 108 (Table 1), produced 67 and 110 times, respectively, free and exogenous L-methionine.

Cytochrome spectra of the wild-type *S. uvarum* ATCC 26602 and some respiratory-deficient mutants are reported in Fig. 1. It shows that *petite* cells contain cytochrome c (550 nm peak), but the bands of cytochrome b (560 nm) and cytochromes a a_3 (605 nm) are missing. Since these *petite* mutants arise as a result of gross deletion of the mit DNA, they are consequently unable to revert to the respiratory-competent phenotype.

Protoplast fusion products

The colonies grown in minimal regeneration medium were isolated as the fused hybrids between *S. uvarum* $Et^r\rho^-$ (overproducer, *petite*) and *S. cerevisiae* (ade^-, ura^-, leu^-, met^-, his^-, trp^-). Complementation must have occurred in these hybridized fusants, since the parent strains were incapable of growing on this medium. The controls of each fusion experiment showed that no spontaneous revertants of either fusion parents grew on minimal regeneration medium.

Protoplast and fusion yields of some fusion experiments are reported in Table 2. The complementation frequency was found to be in the range 2.9×10^{-4}—1.1×10^{-5} according to results obtained by others (Barney et al. 1980; Pina et al. 1986). This value is the ratio of the number of hybridized protoplasts capable of growing on the minimal regeneration medium to the number of colonies growing on complete medium. The highest percentage (83%) of the fusants obtained consisted of prototrophic respiratory-competent strains, many of which were CAF^r (45%). It may be supposed that *petites* derived from the mitochondrial CAF^r strain will retain that sequence of the mit DNA in which the ant^r mutation resides. Since *petites* do not possess a functional mitochondrial protein synthesis apparatus, the CAF^r mutation cannot be deleted unless the mutant is crossed with a *grande* strain. Mitochondrial recombination will generate ant^r hybrid progeny. All of these strains presented the same fermentative capability as *S. uvarum* $Et^r\rho^-$ and grew on glycerol. The most likely explanation for this higher incidence of respiratory competent fusants is that generally fusion is followed by the cyto-

Table 3. Analysis of fusion products

Organism	Auxotrophic markers	Growth on Glycerol	Carbohydrate fermentation		L-Methionine content	
			Galactose	Maltose	(mg/g dry weight)	(μg/ml supernatant)
Parents:						
S. uvarum ER 108ρ^-	–	–	+	+	6.2	33
S. cerevisiae X2928	ade, ura, leu his, met, trp	+	–	–	0	0
Hybrids:						
F1	ura, leu, trp, his	+	–	–	4.1	21
F2	ura, leu, trp, ade	+	–	–	3.6	18
F3	ura, leu, trp, ade	+	–	–	3.5	20

plasmic mixing of the two protoplasts and by the exclusion of one parental nucleus. The most promising strains were considered to be those not growing on plates of minimal medium, not needing any more methionine addition, and presenting the same fermentative pattern as *S. cerevisiae* X2928.

The analysis of some fusion hybrids is reported in Table 3. The hybrids thus examined present, with respect to the parent *S. cerevisiae* X2928, the same fermentative pattern and different auxotrophies. No fusants required methionine while several produced methionine levels similar to those of the parent *S. uvarum* Et^r.

The genetic stability of fusants recovered on minimal regeneration medium was examined by a series of replications on sets of YEPD and minimal agar plates enriched with the required amino acids. Fusants were stable after ten passages on complete medium.

It may be concluded from our work that genetic information for methionine overproduction can be transferred from *S. uvarum* to *S. cerevisiae* by interspecific protoplast fusion and that the fusants obtained were stable.

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