SOMATIC HYBRIDIZATION IN SACCHAROMYCES CEREVISIAE: ANALYSIS OF PRODUCTS OF PROTOPLAST FUSION

by

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Yeast strains of identical mating type were converted into protoplasts with snail gut enzymes. Fusion induced with polyethylene glycol gave products in which nutritional requirements had complemented each other. The somatic hybrids were invariably maters and non-sporulators. When the strains differed in non-mendelian markers not used in the selection, these markers were found in the expected parental and recombinant combinations in the fusion products. Fusion products of two strains of mating type α were mated with fusion products of two strains of mating type a . Two cycles of sporulation and tetrad analysis of the presumed tetraploid gave a regular distribution of all markers showing that the somatic hybrids were indeed diploids homozygous for mating type. A single exception to the expected segregation was found not to be due to an abnormal chromosome number but is attributed to a gene conversion or a mitotic crossing over.

1. INTRODUCTION

In the life cycle of *Saccharomyces* mating plays an important role. Mating and sporulation are the normal processes for the exchange and redistribution of genetic material. Since most brewing strains of *Saccharomyces* are impaired in these processes, they are not easily subjected to cross-breeding. Mutation breeding is likewise met with difficulties, as most brewing strains are diploid or polyploid.

One possibility for circumventing the sexual inabilities of brewing strains is offered by somatic hybridization through protoplast fusion

(23). Protoplasts (2) or spheroplasts are cells which are completely or partially devoid of their cell wall. When such naked cells are mixed with the surface active agent polyethylene glycol (9), coalescence between the plasmalemmas of two or more cells may take place. Such fusion products are regenerated to normal yeast cells in the same way as protoplasts of single cells (12, 13, 14, 15, 22). If karyogamy takes place diploid or polyploid cells are obtained. Finally sporulation or treatment with a haploidising agent (3, 8, 10) may restore the original chromosome number.

Table I

Yeast strains used

Strain	Genotype	Source
C77-D2	<i>a his3 leu2 trp</i> [ERY ^R CAP ^R]	spore from cross 4116E × 3725C
D273-11A	<i>α ade1 his1</i>	Course manual on yeast genetics, Cold Spring Harbor Laboratory
D286-2A	<i>a ade1 his1</i>	—
D517-4B	<i>a ade2 lys9</i>	—
D585-11C	<i>a lys1</i>	—
D587-4B	<i>α his1</i>	—
3725C	<i>α his3 trp</i> [ERY ^S CAP ^R]	—
4116E	<i>a leu2</i> [ERY ^R CAP ^S]	—
XXa-I-9	<i>a his4-864 ade2-119 trp5a leu2 ilv1</i>	T. NILSSON-TILLGREN
XXa-III-7	<i>α his4-864 ade2-119 trp5a leu2</i>	—
XXI-I-5	<i>α his4-594 ade2-40 trp5b cyh2 lys1</i>	—
XXI-I-49	<i>a his4-594 ade2-40 trp5b cyh2 lys1</i>	—

Somatic hybridization by protoplast fusion in yeast is best explored by the use of genetically well marked strains of *Saccharomyces cerevisiae* permitting the selection of products and their unequivocal characterisation.

Intraspecific fusion of protoplasts of *S. cerevisiae* of identical mating type has recently been reported by VAN SOLINGEN and VAN DER PLAAT (20), FERENCZY and MARÁZ (5) and YAMAMOTO and FUKUI (25), whereas STEWART (21) reports on fusion of *S. cerevisiae* with *S. carlsbergensis*. With the aid of a detailed progeny analysis GUNGE and TAMARU (7) demonstrated that protoplast fusion of two *a* strains of *S. cerevisiae* gave rise to a regular diploid strain homozygous for the *a* mating type gene. After mating the fusion product with an $\alpha\alpha$ diploid strain the tetrasomic inheritance of suitable markers was followed by tetrad analysis.

The present study has taken an analogous approach. One series of experiments involves cytoplasmic markers, the other involves fusion of two *a* strains, fusion of two α strains and subsequent mating and tetrad analysis of the mating product.

2. MATERIALS AND METHODS

2.1. Growth and medium

Yeast cells to be converted into protoplasts were grown on a rotary shaker at 30 °C in liquid YPD: 1 % Difco Bacto Yeast Extract, 2 % Difco Bacto Peptone, 2 % glucose. Cells were har-

vested in the logarithmic phase ($1-3 \times 10^7$ cells·ml⁻¹) by centrifugation.

2.2. Strains, crosses and genetic characterisation

The strains used in this study are shown in Table I. In the strains XXI-I-49, XXI-I-5, XXa-III-7 and XXa-I-9 the *his4-594* and *his4-864* alleles complement as do *ade2-40* and *ade2-119*. Strains carrying *ade2-40* and *ade2-119* are red and pink, respectively. There is no complementation between *trp5* and *trp5b*. Strains D585-11C, D587-4B, D273-11A and D286-2A were used as mating type testers.

Crosses and tetrad analysis were carried out according to HAWTHORNE and MORTIMER (11). Sporulation medium was 1 % potassium acetate, 0.1 % Difco Bacto Yeast Extract, 0.05 % glucose, solidified with 2 % Difco Bacto Agar (1). Staining with malachite green – safranin (18) was used to detect sporulation.

Tests for resistance to erythromycin and chloramphenicol were performed on YPG (3 % glycerol substituted for glucose in YPD) plates supplemented with 4 mg·ml⁻¹ erythromycin (Sigma) and 4 mg·ml⁻¹ chloramphenicol (Sigma), respectively (1).

Synthetic complete media and media for detection of nutritional requirements were 0.67 % Difco Yeast Nitrogen Base without amino acids, 2 % glucose, 2 % Difco Bacto Agar, appropriately supplemented with nutrients as described by ZIMMERMANN (26).

2.3. Protoplast formation

Protoplasts were made essentially according to the procedures of SCHWENCKE et al. (19) and NEWLON and FANGMAN (16). Cells were harvested, washed twice in cold water and suspended in pretreatment buffer. In experiment 3.2 the buffer consisted of 0.2 M-Tris, 0.02 M-EDTA, 1.2 M-sorbitol, 0.1 M- β -mercaptoethanol, pH 9.1 and in experiment 3.3 the pretreatment was carried out in 5 mM-EDTA, 50 mM-dithiothreitol (DTT), 0.1 M-Tris-HCl and 1 mg·ml⁻¹ pronase (Sigma, type VI), pH 8.9. The pretreatment lasted for 15 min at 30 °C and was followed by 3 washes with 1 M-sorbitol, 0.1 M-sodiumcitrate, pH unadjusted.

The cells were then treated at 30 °C with snail gut enzymes in the same medium. In experiment 3.2 *Suc d'Helix Pomatia* (Industrie Biologique Française) was used at a concentration of 10% for 90–100 min with a cell density of 3×10^9 cells·ml⁻¹. In experiment 3.3 5% of a corresponding enzyme preparation (β -glucuronidase/arylsulfatase Boehringer) was used at a cell density of 5×10^8 cells·ml⁻¹ for 30 min. The procedure for experiment 3.2 was initially also

applied to the strains used in experiment 3.3, but the protoplast formation was very poor and slow (200–300 min). Protoplasts were collected by centrifugation and washed three times with sorbitol stabilizing buffer: 0.67% Difco Yeast Nitrogen Base, 1 M-sorbitol, 10 mM-CaCl₂.

2.4. Fusion procedure

Fusion of the washed protoplasts was performed according to VAN SOLINGEN and VAN DER PLAAT (20). Gently and slowly the protoplast pellet was suspended in 40% w/v polyethylene glycol 4000 (Merck), 10 mM-CaCl₂, 10 mM-Tris-HCl, pH 7.5, to a density of $5 \times 10^7 - 2 \times 10^8$ cells·ml⁻¹. The suspension was incubated with gentle shaking at 30 °C for 30 min. At this stage several forms of fusion products could be observed in the phase contrast microscope including large aggregations of protoplasts, huge heterocaryons of fused protoplasts and single giant cells made up of a few protoplasts. Polyethylene glycol was removed by washing 3 times with sorbitol stabilizing buffer, and appropriate dilutions were made.

Figure 1. Flow diagram of the fusion experiments.

2.5. Regeneration of the fused protoplasts

0.1 ml of fusion products in sorbitol stabilizing buffer was placed on a supplemented or non-supplemented regeneration plate (22): 0.67% Yeast Nitrogen Base without amino acids, 1 M-sorbitol, 3% Difco Bacto Agar, 2% glucose. 1.5 ml hot 3.2% Difco Bacto Agar, 1 M-sorbitol from a boiling waterbath was placed in a ring around the droplet, and with a Drigalski spreader, agar and fused protoplasts were mixed quickly, in such a way that solidification took place in a few seconds (6). A flow diagram of the actual fusion experiments and the three controls is presented in Figure 1.

3. RESULTS

3.1. Protoplasting efficiency and regeneration frequency

Protoplasts were suspended in and washed two times with water and then plated on YPD plates. Only cells osmotically stable will turn into colonies, which gives a measure of the efficiency of the protoplasting treatment. The results are shown in Table II. Protoplasting efficiency is better than or equal to 10^6 protoplasts per intact cell.

Good regeneration is required for the efficient recovery of somatic hybrids. Table II includes the regeneration frequencies of the protoplasts plated on a complete regeneration medium after treatment with polyethylene glycol. The regeneration frequency ranges from 0.03% to 20%, the variation probably being due to strain

differences as well as differences in the procedure. Especially the extent of snail gut enzyme treatment can be critical.

3.2. Fusion involving cytoplasmic markers

Protoplasts of the auxotrophic strains D517-4B *a ade2 lys9* and C77-D2 *a his3 leu2 trp* ERY^R CAP^R were mixed, treated with polyethylene glycol 4000 and plated for regeneration on minimal medium. After two to three days prototrophic colonies appeared (Table III). When parent strains were treated separately with polyethylene glycol and plated on minimal medium no prototrophic colonies were observed indicating that the polyethylene glycol treatment does not cause the appearance of prototrophic colonies. Likewise when the mixture of D517-4B and C77-D2 protoplasts was plated without polyethylene glycol treatment prototrophic colonies were absent. Normal mating was thus not the cause for the occurrence of the prototrophic colonies. None of the fusion products sporulated (149 tested), and all had the expected *a* mating type (71 tested). As one of the parents, C77-D2, carried the cytoplasmic genes for resistance to erythromycin and chloramphenicol, the behaviour of the fusion products towards these drugs was tested. As seen in Table IV the tested progenies comprised the four possible combinations of the two markers. About 10% of the progeny were recombinant for the cytoplasmic markers. This reveals that the cytoplasmic markers segregated and recombined in the same way as is observed after mating (24).

Table II

Regeneration frequencies of yeast protoplasts from the different strains used in fusion experiments

Strain	Washing of protoplasts in water and plating on YPD		Embedding of protoplasts for regeneration in complete medium		
	Number of protoplasts	Number of colonies	Number of protoplasts	Number of colonies	Regeneration frequency (per cent)
C77-D2	4×10^7	0	2.5×10^3	5×10^2	20
D517-4B	4×10^6	1	8×10^2	84	10.5
XXI-I-49	1×10^6	0	3×10^7	10^4	0.03
XXI-I-5	1×10^6	0	2×10^7	10^4	0.05
XXa-III-7	1×10^6	0	3×10^5	300	0.1
XXa-I-9	1×10^6	0	2×10^7	10^4	0.05

Table III

Fusion experiment involving cytoplasmic markers

D517-4B:	<u>+</u> <u>a</u>	<u>lys9</u>	<u>+</u>	<u>ade2</u> <u>+</u>	
C77-D2:	<u>leu2</u> <u>a</u>	<u>+</u>	<u>trp</u>	<u>+</u> <u>his3</u>	[ERYRCAP ^R]
		D517-4B	C77-D2	D517-4B + C77-D2	
Polyethylene glycol treatment		+	+	-	+
Protoplasts plated on minimal medium		4.7×10^7	1.7×10^7	1.2×10^7 + 1.2×10^7	2×10^8 + 2×10^8
Number of colonies		0	0	0	156

3.3. Fusion of *his4* and *ade2* complementing strains

Protoplasts of the two *a* mating type strains XXI-I-49 *a his4-594 ade2-40 trp5b cyh2 lys1* and XXa-I-9 *a his4-864 ade2-119 trp5a leu2 ilv1* were fused. As the two *his4* mutant alleles and the two *ade2* mutant alleles complement somatic hybrids were selected by plating on minimal medium supplemented with tryptophane.

Likewise the two α mating type strains XXI-I-5 $\alpha his4-594 ade2-40 trp5b cyh2 lys1$ and XXa-III-7 $\alpha his4-864 ade2-119 trp5a leu2$ were fused.

Table V shows that several hundred colonies were obtained in both experiments with none of the controls giving rise to colonies. Of 244 tested fusion products all were found to be homozygous for the mating type gene, since they could only sporulate after mating with the opposite mating type.

3.4. Genetic analysis of fusion products from the *his4* and *ade2* complementing strains

The genetic analysis of the somatic hybrids was carried out according to Figure 2: After mating of two fusion products the tetraploid was

propagated and sporulated. Individual asci were dissected and the individual diploid spores grown and tested for their capability to sporulate. Only spores of the $\alpha\alpha$ genotype yield cultures capable of sporulating without previous mating.

In this way a tetrad was selected which had yielded four $\alpha\alpha$ clones. Asci from these clones contain haploid spores, which were dissected out and analysed for the auxotrophic and cycloheximide resistant markers. From these tetrad analyses the genotypes of the diploid spores could be determined and these are listed in Table VI for two pairs of fusion products.

In the first case the expected segregation for all testable markers is obtained which proves that the mating product indeed was a tetraploid containing all chromosomes of the parents XXI-I-5, XXI-I-49, XXa-I-9 and XXa-III-7, and that the fusion products were diploid.

In the second case also a perfect segregation is observed for all markers with the exception of the leucine2 gene where a 6:2 instead of a 4:4 segregation is observed. This exceptional segregation must be due to a gene conversion or a mitotic crossing over and not due to aneuploidy of chromosome III since the mating type locus on the same chromosome segregates regularly.

Table IV

Resistance to erythromycin and chloramphenicol of 152 fusion products

	ERYSCAPS	ERYRCAPS	ERYSCAP ^R	ERYRCAP ^R
Number of colonies	72	7	10	63

Table V

Somatic hybridization by protoplast fusion of yeast strains carrying complementing auxotrophic markers

XXI-I-49	:	<u>his4-594</u>	+	<u>a</u>	<u>trp5b</u>	<u>cyh2</u>	<u>lys1</u>	<u>ade2-40</u>	+
		↑						↑	
		complementing						complementing	
		↓						↓	
XXa-I-9	:	<u>his4-864</u>	<u>leu2</u>	<u>a</u>	<u>trp5a</u>	+	+	<u>ade2-119</u>	<u>ilv1</u>
XXI-I-5	:	<u>his4-594</u>	+	<u>α</u>	<u>trp5b</u>	<u>cyh2</u>	<u>lys1</u>	<u>ade2-40</u>	+
		↑						↑	
		complementing						complementing	
		↓						↓	
XXa-III-7	:	<u>his4-864</u>	<u>leu2</u>	<u>α</u>	<u>trp5a</u>	+	+	<u>ade2-119</u>	+
<hr/>									
		XXI-I-49	XXI-I-5	XXa-III-7	XXa-I-9	XXI-I-49 + XXa-I-9	XXI-I-5 + XXa-III-7		
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Polyethylene glycol treatment		+	+	+	+	-	+	-	+
<hr/>									
Number plated on minimal regeneration medium supplemented with tryptophan		4 × 10 ⁷	2 × 10 ⁷	3 × 10 ⁷	7 × 10 ⁷	2 × 10 ⁷ + 3 × 10 ⁷	5 × 10 ⁷ + 8 × 10 ⁷	1 × 10 ⁷ + 2 × 10 ⁷	3 × 10 ⁷ + 5 × 10 ⁷
<hr/>									
Number of colonies		0	0	0	0	0	~ 800	0	714

4. DISCUSSION

Intact yeast cells of like mating type do not exchange genetic material, but when a mixture of protoplasts of two different auxotrophic strains of identical mating type were treated with polyethylene glycol, recombinant cells arose in which the fusion partners had complemented each others deficiencies.

The data in Tables III and V give a statistical argument that the prototrophs were indeed somatic hybrids: The appearance of wild type colonies was not due to reversion of markers in the parent strains; since in that case single polyethylene glycol treated parents would have given colonies on selective regeneration medium. Likewise, mating may be ruled out because of absence of colonies on regeneration plates inoculated with mixed parents which had not been in contact with polyethylene glycol. More

important than the statistical argument however, is the genetic analysis of the products. The possibility that substances present in the polyethylene glycol might have acted mutagenically on the mating type locus and thereby enabled cells to mate, would imply sporulation of the products, but this was not found. Furthermore segregation of mating type alleles in the tetrad analysis shows the expected 4:4 ratio.

If the fusion products are assumed to be diploid, mating between the fusion products of opposite mating type will lead to a tetraploid. When these $a/a/\alpha/\alpha$ cells are induced to sporulate, three types of diploid spores are expected to arise, a/a and α/α spores which do not sporulate and a/α which can be induced to go through meiosis. Tetrads should be frequent in which all four spores are a/α (17). Such two tetrads were subjected to further analysis by

Figure 2. Diagram of the steps involved in the genetic analysis of the fusion products presented in Tables V and VI.

sporulation and tetrad analysis. All but one of the markers segregated in the expected ratios 2:6 for *ilv1* and 4:4 for the others. The one odd segregation of *leu2* in the second tetrad must be due to a gene conversion or mitotic crossing over, and not aneuploidy, since the mating type alleles on the same chromosome segregated regularly.

Products from the fusion of C77-D2 carrying [ERY^RCAP^R] and D517-4B will be expected to show resistance to erythromycin and chloram-

phenicol. Indeed when tested against these drugs recombinants [ERY^RCAP^R] and [ERY^RCAP^S] were found thus confirming the assumption of fusion.

Neither in the study of GUNGE and TAMARU (7) nor in the present study is there reason to believe that aneuploidy occurred for any of the unmarked chromosomes. I conclude that not only *a* + *a* fusions (7) but also α + α fusions can give rise to regular diploids homozygous for the mating type locus.

Table VI

Genetic analysis of two pairs of protoplast products by the procedure outlined in Figure 2

Expected genotypes of the fusion products:

XXI-I-5 : his4-594 + α trp5b cyh2 lys1 ade2-40 +
 +

XXa-III-7 : his4-864 leu2 α trp5a + + ade2-119 +

X (MATING)

XXI-I-49 : his4-594 + a trp5b cyh2 lys1 ade2-40 +
 +

XXa-I-9 : his4-864 leu2 a trp5a + + ade2-119 ilv1

Genotypes of the diploid spores in the 2 experiments:

Progeny
tetrads

Spore 1 : his4 + a trp5 cyh2 lys1 ade2-40 + 12
his4 leu2 α trp5 + + ade2-119 +

Spore 2 : his4 + a trp5 cyh2 lys1 ade2-40 ilv1 3
his4 leu2 α trp5 + lys1 ade2-119 +

Spore 3 : his4 leu2 a trp5 cyh2 lys1 ade2-40 ilv1 6
his4 + α trp5 + + ade2-119 +

Spore 4 : his4 leu2 a trp5 cyh2 + ade2-40 + 18
his4 + α trp5 + + ade2-119 +

Segregation: 4:4 4:4 4:4 4:4 4:4 6:2

Spore 1 : his4 + a trp5 cyh2 lys1 ade2-40 + 5
his4 + α trp5 cyh2 + ade2-119 +

Spore 2 : his4 + a trp5 + + ade2-40 + 6
his4 + α trp5 + + ade2-119 +

Spore 3 : his4 leu2 a trp5 + lys1 ade2-40 ilv1 7
his4 + α trp5 + lys1 ade2-119 +

Spore 4 : his4 leu2 a trp5 cyh2 lys1 ade2-40 ilv1 7
his4 + α trp5 cyh2 + ade2-119 +

Segregation: 6:2 4:4 4:4 4:4 4:4 6:2

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