Cytoplasmic Transfer of Oligomycin Resistance During Protoplast Fusion of *Saccharomycopsis lipolytica*

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Mitotic segregation of oligomycin resistance and oligomycin sensitivity was observed among the prototrophic progeny of protoplast fusion between drug-resistant and drug-sensitive complementary auxotrophs of *Saccharomycopsis lipolytica*. The transfer of oligomycin resistance by protoplast fusion without karyogamy suggests a cytoplasmic inheritance of this drug resistance determinant.

The development of genetic techniques in analyzing *Saccharomycopsis lipolytica* (formerly *Candida lipolytica*) has made it possible to analyze this yeast by its sexual cycle (12). A limited chromosomal linkage map has been constructed, but the genetics of cytoplasmic or mitochondrial inheritance has not yet been studied. Since extensive inbreeding is necessary for the genetic analysis of *S. lipolytica* by its sexual cycle, we used the method of protoplast fusion mediated by polyethylene glycol (10, 13).

All the mutants employed here were derivatives from a wild-type strain, *S. lipolytica* ATCC 44601, previously designated strain MT4 (11). Compositions of minimal and YEPD media used were the same as those of Fink (7). Auxotrophic mutants were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (11), except that the final concentration of the mutagen was reduced to 40 μ g/ml and the mutagenesis was followed by enrichment with nystatin (Sigma Chemical Co., St. Louis, Mo.) as described by Snow (14). Double auxotrophic mutants were derived from single auxotrophs. Oligomycin-resistant mutants were isolated spontaneously from auxotrophs spread on YEPG plates (glucose in YEPD medium was replaced by 2% glycerol) containing 10 μ g of oligomycin (Sigma) per ml dissolved in methanol. Mutants were observed at a frequency of 10^{-8} to 10^{-7} .

Protoplasts were prepared by the method of Domanski and Miller (5) and were used for fusion as described by Stahl (15). Logarithmic-phase cells grown in supplemented minimal medium were pretreated with 0.4% (vol/vol) 2-mercaptoethanol-1 mM EDTA in 0.05 M potassium phosphate buffer (pH 7.5) for 30 min at 30°C. Cells were collected by centrifugation and were suspended in 0.05 M sodium maleate

buffer (pH 5.8) containing 0.6 M KCl. Cell walls were digested with 3 U of chitinase complex from *Streptomyces griseus* (Sigma) per ml and 30 U of Zymolyase 60000 (Seikagaku Kogyo, Tokyo, Japan) per ml. The conversion of *S. lipolytica* cells into protoplasts was complete within 60 min of incubation only in the presence of both enzyme preparations, as judged by using an optical microscope. Protoplasts were collected by centrifugation (800 $\times$ g, 5 min) and were washed twice with 0.6 M KCl. The yield of protoplast formation was more than 99.9%.

For protoplast fusion, approximately equal amounts of the protoplasts in question were mixed and centrifuged. The pellet was overlaid with 30% polyethylene glycol (molecular weight, 6,000) in 0.1 M CaCl_2 and then immediately was mixed vigorously and allowed to stand at 30°C for 20 min. The mixture was then diluted and washed with 0.6 M KCl. Protoplasts were regenerated on selective agar plates containing 0.6 M KCl. The protoplast suspension was placed on agar plates in 0.1-ml portions, and the plates were overlaid with the same agar that had been melted at 48°C.

Protoplasts of *S. lipolytica* could be regenerated at frequencies of 0.5 to 50%, depending on the auxotrophic markers used. Fusion frequencies assessed from the ratio of regenerated colonies on minimal medium to those on YEPD agar plates ranged from 10^{-5} to 10^{-3} . These values for fusion frequency were significantly higher than values for the reversion of auxotrophic mutants (10^{-8} to 10^{-7}), indicating that these regenerants were true fusion products. Genetic evidence that prototrophic colonies selected on minimal medium have resulted from nutritional complementation was obtained by following haploidization with *p*-fluoro-DL-phenylalanine (13, 15).

TABLE 1. Recovery of auxotrophic markers by haploidization from fusion products of *S. lipolytica*

Parental strains (phenotypes)	Regenerant	Auxotrophic segregant		Total colonies examined	% Auxotrophs
		Phenotype	No.		
MO4 (Ade ⁻ Met ⁻), MU4 (His ⁻)	FP2-1	Ade ⁻	3	2,020	1.1
		Met ⁻	14		
		His ⁻	6		
	FP2-2	Ade ⁻	1	1,880	0.2
		Met ⁻	2		
MK3 (Lys ⁻ Arg ⁻), MK5 (His ⁻ Leu ⁻)	FP4-1	Leu ⁻	51	463	15
		Lys ⁻ Leu ⁻	3		
		Arg ⁻ His ⁻	2		
		Arg ⁻ His ⁻ Leu ⁻	11		
	FP4-2	Leu ⁻	22	545	4.2

Table 1 shows the recovery of parental auxotrophic markers in haploidized segregants. Parental-type as well as recombinant-type auxotrophs were obtained at frequencies of 0.2 to 15%. When these fusion products were grown in YEPD medium, auxotrophic segregants were rarely recovered by replica plating ($<10^{-4}$). The stable prototrophy for fusion products of *S. lipolytica* here is in sharp contrast with *Candida tropicalis* (8), in which heterokaryotic fusion products frequently give rise to auxotrophic segregants with parental-type auxotrophic requirements after growth on complete medium.

In fact, the Giemsa staining (8) of the fusion products revealed exclusively uninucleate cells, indicating that karyogamy had taken place in fused cells (diploid or polyploid). However, estimates of DNA contents failed to demonstrate an exact euploidy; i.e., DNA contents per fused cell ranged from 1.2 to 1.5 times those of the haploid parents when DNA was extracted from

logarithmic-phase cells by hot perchloric acid (3, 4).

When a specific oligomycin-resistant mutant which had been derived spontaneously from an auxotroph was fused with the drug-sensitive complementary auxotroph, prototrophic regenerants selected on minimal plates were either drug resistant or drug sensitive (Table 2). Transmission frequencies of oligomycin resistance in two separate experiments averaged 18%. This phenomenon might well indicate the mitotic segregation of drug resistance and sensitivity, a criterion often used to determine the cytoplasmic inheritance of drug resistance in *Saccharomyces cerevisiae* through zygote formation between drug-resistant and drug-sensitive parents (2). If the determinant of oligomycin resistance were chromosomal, fusion products would have been either totally resistant or totally sensitive to the drug, depending on the dominance or recessiveness of the mutation.

TABLE 2. Mitotic segregation of resistance and sensitivity to oligomycin in protoplast fusions of *S. lipolytica*

Parental strains (phenotypes ^a)	Selective medium ^b	Fusion frequency	Regenerant		% Regenerants
			Phenotype	No.	
MK6 (His ⁻ Trp ⁻ Oli ^a), MK2-01 (Lys ⁻ Met ⁻ Oli ^r)	MMKA	8.5×10^{-5}	Prototrophic Oli ^a	172	77
			Prototrophic Oli ^r	52	23
		4.0×10^{-6}	Prototrophic Oli ^r	14	100
		4.8×10^{-4}	His ⁻ Trp ⁻ Oli ^r	250	100
MK5 (His ⁻ Leu ⁻ Oli ^a), MK2-01 (Lys ⁻ Met ⁻ Oli ^r)	MMKA	1.9×10^{-4}	Prototrophic Oli ^a	173	87
			Prototrophic Oli ^r	27	13
		4.0×10^{-5}	Prototrophic Oli ^r	50	100
		1.3×10^{-3}	His ⁻ Leu ⁻ Oli ^r	200	100

^a Oligomycin-resistant and oligomycin-sensitive phenotypes are designated Oli^r and Oli^a, respectively.

^b MMKA denotes minimal medium agar containing 0.6 M KCl. Leucine (Leu, 1 mM), histidine (His, 0.5 mM), tryptophan (Trp, 0.5 mM), and oligomycin (Oli, 10 µg/ml) were added as indicated.

Other workers have reported aneuploid formation in protoplast fusion (6). If the rearrangement or loss of chromosomes in the first step of the regeneration should occur, the segregation of phenotypes for drug resistance and drug sensitivity like that shown in Table 2 could be accounted for, even if the resistance to oligomycin were chromosomal. To check the possibility of the chromosomal origin of drug resistance, prototrophic fusion products were extensively haploidized, and the auxotrophic segregants were tested for their drug resistance or drug sensitivity. Oligomycin-resistant segregants did not appear from the drug-sensitive regenerants, and no drug-sensitive segregant was recovered from the drug-resistant regenerants (data not shown). The fact that parentage on drug resistance and sensitivity was preserved despite segregation of auxotrophic (nuclear) markers in the mitotic haploidization is most unlikely to support the conclusion that the resistance to oligomycin was chromosomal.

Intraclonal distribution of resistance and sensitivity to the drug was examined by resspreading fusion products on minimal plates and replicating them onto drug plates. No oligomycin-resistant clone was recovered from the drug-sensitive regenerants; oligomycin-sensitive segregants never occurred from drug-resistant regenerants. This observation suggests that the regenerants were not heteroplasmic. The rapid segregation of the cytoplasmic determinant was in accord with findings for another budding yeast, *S. cerevisiae* (2).

Selection of fusion products on minimal medium containing the drug produced only prototrophic oligomycin-resistant regenerants at significantly lower frequencies than those selected on minimal plates (Table 2). On the other hand, Table 2 shows that regenerants obtained on drug-containing plates supplemented with requirements for the sensitive partner were all drug-resistant clones with the nuclear phenotype of the sensitive partner. It was noted that the appearance of clones on the supplemented drug plates was rapid (about 4 days) in comparison with the 7 to 10 days required for prototrophic clones regenerated on minimal plates. These clones could not be spontaneous drug-resistant mutants from the sensitive partner since no regenerants were obtained on the same plates inoculated with protoplasts from the sensitive partner. These observations suggest that the cytoplasmic oligomycin-resistant determinant was transferred from the drug-resistant partner to the sensitive one without concomitant karyogamy. /

Wright and Lederberg (16) pointed out earlier that karyogamy did not occur at low frequency even in zygotes of *S. cerevisiae*. Hence, we

studied the problem of whether protoplast fusion of *S. cerevisiae* was always accompanied by karyogamy, especially if the cytoplasmic mutation (for respiratory deficiency) was used as the selective marker.

When the products of fusion between respiratory-deficient and respiratory-sufficient complementary auxotrophic mutants of *S. cerevisiae* with identical mating types were selected on minimal medium containing a nonfermentable carbon source (3% sodium lactate and 0.1% glucose) and supplemented with requirements of the respiratory-deficient partner, about 20% of the regenerants were prototrophic, indicating that the protoplast fusion proceeded to karyogamy. Most of the regenerants (about 80%), however, were respiratory-sufficient auxotrophs with markers of the respiratory-deficient partner, suggesting the absence of karyogamy (data not shown). Consequently, it might be plausible that protoplast fusion proceeding to karyogamy is more likely to occur in *S. cerevisiae* than in *S. lipolytica*.

Avner and Griffiths have identified both nuclear and mitochondrial heredity of oligomycin-resistant mutants in *S. cerevisiae* (2). Most probably, the resistance to this drug in *S. lipolytica* reported in this work is inherited by the mitochondrial genome. In petite-positive yeasts, the mitochondria or mitochondrial genome can be transferred to a respiratory-deficient strain either by protoplast-protoplast fusion (6) or by protoplast-mitochondria fusion (9). In petite-negative yeasts, such as *Kluyveromyces lactis*, protoplast fusion can be used to identify the cytoplasmic inheritance of respiratory deficiency (1). Protoplast fusion unaccompanied by karyogamy in *S. lipolytica* offers the possibility of analyzing the mitochondrial genome of this petite-negative yeast.

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