Generation of genetic recombinants in *Trichosporon cutaneum* by spontaneous segregation of protoplast fusants

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Auxotrophic mutants were isolated in two strains of *Trichosporon cutaneum*. Complementing auxotrophs were hybridized by protoplast fusion. Some of the fusants were apparently transient diploids and segregated to give recombinant marker combinations.

Introduction

The soil yeast *Trichosporon cutaneum* is very versatile in metabolizing various organic components. It has been shown to grow on various disaccharides (Mörtberg & Neujahr, 1986), xylans, cellulose (Hrmova *et al.*, 1984) and starch (De Mot *et al.*, 1984). In addition, *T. cutaneum* is able to metabolize phenol and other aromatic compounds (Gaal & Neujahr, 1979; Anderson & Dagley, 1980), and it gives high biomass yield (Petkov *et al.*, 1984; Fiechter *et al.*, 1987). These properties make *T. cutaneum* an interesting candidate for conversion of various carbon sources to biomass (Glumoff *et al.*, 1989).

Genetical studies depend on the availability of suitable mutants. Leucine-requiring mutants were isolated in *T. cutaneum* by Stępień *et al.* (1988), and successful genetic transformation and gene cloning have been described recently (Glumoff *et al.*, 1989; Ochsner *et al.*, 1991). Isolation of a variety of auxotrophic mutants in *Trichosporon adeninovorans* has also been reported (Samsonova *et al.*, 1989).

Here we describe the isolation of auxotrophic mutants and protoplast fusion in two strains of *T. cutaneum*. The data show the formation of transitional diploids which segregate spontaneously, often producing progeny with a nonparental combination of markers.

Methods

Strains, media and culture conditions. Two prototrophic strains of *Trichosporon cutaneum*, both isolated from nature, were used in this study: Tr1, from the collection of Łódź Technical University (Poland), and UCD 54-169, from the Industrial Yeast Collection (Perugia, Italy).

Cells were grown in a rotary shaker (150 r.p.m.) at 30 °C. For standard cultures, yeast extract (1%), Bacto-peptone (2%) and glucose (2%) (YPG) was used as a complete medium and the glucose-mineral salt medium of Galzy & Slonimski (1957) as minimal medium (Go). The latter was supplemented when necessary with amino acids at a concentration of 30 µg ml⁻¹, and with adenine and uracil at a concentration of 20 µg ml⁻¹.

Mutagenesis. Cells from the exponential phase of growth (2×10^7 cells ml⁻¹) were centrifuged at 3000 g for 10 min at 20 °C, washed with 0.9% NaCl and resuspended in 5 ml sodium citrate buffer, pH 5.5. Mutants were obtained after mutagenesis with MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) at a concentration of 0.5 mg ml⁻¹ for 30 min. The mutagen was removed by washing twice with 0.9% NaCl. The survival rate was 10–20% and 1–10% for strains Tr1 and UCD 54-169, respectively. After plating on complete medium and screening on minimal medium auxotrophs were isolated and their requirements determined.

Protoplast formation. The formation of protoplasts was achieved using Novozym 234 (Hamlyn *et al.*, 1981). The cells were grown to mid-exponential phase (1×10^7 cells ml⁻¹) in YPG medium. They were collected by centrifugation at 3000 g for 10 min at 20 °C and resuspended in 5 ml buffer A (0.01 M-potassium phosphate pH 6.0 containing 0.6 M-KCl). Novozym was added (5 mg ml⁻¹) and the cell suspension was incubated at 30 °C for 1 h with gentle shaking. Protoplast formation was monitored microscopically and confirmed by measuring the OD₆₀₀ of the cell suspension before and after the addition of SDS to a final concentration of 1%. The protoplasts were centrifuged at 1000 g for 5 min at 20 °C and washed twice with 5 ml buffer B (0.05 M-Tris/HCl pH 7.5, 0.01 M-CaCl₂, 0.6 M-KCl).

Protoplast fusion. For fusion, equal numbers of protoplasts from each strain were suspended in the same volume of buffer B. Portions (0.1 ml) of each suspension were mixed (10^7 cells ml⁻¹) and 1 ml PEG solution (40% PEG 4000, 0.1 M-CaCl₂) was added. The mixture was incubated at 30 °C for 30 min, diluted with 3 ml buffer A, mixed with 4 ml top agar (Go medium, 0.6 M-KCl, 2%, w/v, agar) warmed to 50 °C, supplemented with amino acids when necessary, and poured onto minimal plates stabilized with 0.6 M-KCl. The plates were incubated at 30 °C for 4–6 d and the fusion hybrid colonies were replicated on Go and YPG. Those growing only on YPG were suspended in 0.9% NaCl

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and plated on YPG for single-cell colonies. Their phenotype was determined by replica-plating on selective medium.

The regeneration rate was determined by plating protoplasts into top agar supplemented appropriately with nutritional requirements.

Results and Discussion

Isolation of auxotrophic mutants

Only leucine-requiring auxotrophic mutants (11 among approximately 20000 colonies tested) were obtained in strain Tr1. This confirms our previous results (Stępień *et al.*, 1988), when after many rounds of mutagenesis only leucine auxotrophs were obtained. The appearance of only one class of auxotrophic mutants, which were recessive, as found in fusants (see below), indicates that this strain is not haploid; it may be aneuploid or, more probably, have one mutated or nonfunctional copy of a gene involved in leucine biosynthesis. In contrast, in strain UCD 54-169 a variety of auxotrophic mutants was readily selected, suggesting that this strain is haploid. Among approximately 500 colonies tested which survived mutagenesis, 16 auxotrophs with the following requirements were found: histidine (5), arginine (4),

lysine (2), adenine (1), methionine (1), tryptophan (1), isoleucine + valine (1) and uracil (1).

The frequency of mutants among surviving cells was 2×10^{-4} and 320×10^{-4} for strains Tr1 and UCD 54-169, respectively. All the mutations had a reversion frequency of $<10^{-7}$. Several auxotrophs were further mutagenized and double auxotrophic mutants were obtained. These mutants and their derivatives are listed in Table 1. They were used for fusion experiments.

Fusion of protoplasts of auxotrophic strains

In all the experiments the frequency of fusion resulting in colonies growing on selective medium was about $0.5-1 \times 10^{-3}$, which is similar to the results described for *Candida* sp. N-16 (Fujii *et al.*, 1988). No colonies appeared on the control plates. Regeneration was 40-60%.

All colonies selected on minimal medium (or minimal medium with supplements required by both strains) were stable prototrophs (or carried common markers) even after four passages through the non-selective (YPG) medium.

Fusion between strains Tr1 and UCD 54-169

When mutants derived from strains Tr1 and UCD 54-169 were fused, the resulting prototrophic colonies showed the pattern of carbon source utilization characteristic of Tr1 or a new phenotype: i.e. no growth on rhamnose, sucrose and sorbose (Table 2). It appears, therefore, that the majority of selected colonies simply represent strain Tr1 which has acquired the wild-type copy of the *leu* gene. The fact that these two strains fuse readily indicates their compatibility in spite of the differences in growth on disaccharides, response to

Table 1. *Trichosporon cutaneum* strains used for fusion

Strain	Origin	Phenotype
M20	Tr1	Leu ⁻
U6	UCD 54-169	Trp ⁻
U7	UCD 54-169	His ⁻
U22	UCD 54-169	His ⁻
U25	UCD 54-169	Arg ⁻
U7-1	U7	His ⁻ Ura ⁻
U25-2	U25	Arg ⁻ Met ⁻
U25-11	U25	Arg ⁻ Ade ⁻

Table 2. *Fusion of auxotrophic mutants*

Strains	No. of hybrids from 10^7 protoplasts	No. of colonies examined	Growth of the fusants on different disaccharides*			
			Lactose	Rhamnose	Sucrose	Sorbose
Parental						
UCD 54-169	—	—	+	—	+	+
Tr1	—	—	+	+	+	—
Fusants						
U22 × U25	42	20	+	—	+	+
U6 × U25	120	50	+	—	+	+
U25-11 × U25-2	166	50	+	—	+	+
M20 × U25	87	26	+	+	+	—
		4	+	—	—	—
M20 × U25-2	79	23	+	+	+	—
		7	+	—	—	—

* +, Growth; —, no growth.

Table 3. Marker segregation in hybrids

Parental strains	No. of hybrids from 10 ⁷ protoplasts	Selection medium	No. of colonies examined	No. of colonies with segregants	Phenotype of segregants	Frequency of segregants (%)
U25-11 (Arg ⁻ Ade ⁻) × U7-1 (His ⁻ Ura ⁻)	254	Go	50	0	—	—
	190	Go + Arg + His	50	4	Arg ⁻	100
	130	Go + Arg + Ura	50	1	Arg ⁻	4
					Ura ⁻	1
					Arg ⁻ Ura ⁻	71
					Arg ⁻ Ade ⁻	24
	180	Go + Ade + His	50	0	—	—
	114	Go + Ade + Ura	50	1	Prototrophs	2
					Arg ⁻	47
					Arg ⁻ His ⁻	3
					Arg ⁻ Ura ⁻	2
					Arg ⁻ Ade ⁻	45
					Arg ⁻ His ⁻ Ura ⁻	1
U25-2 (Arg ⁻ Met ⁻) × U7-1 (His ⁻ Ura ⁻)	24	Go	24	0	—	—
	20	Go + His + Met	20	0	—	—
	13	Go + Met + Ura	13	1	Prototrophs	62
					Arg ⁻ Met ⁻	34
					Arg ⁻ Met ⁻ Ura ⁻	4
	100	Go + Arg + Ura	50	17	Arg ⁻	100
				1	Prototrophs	1
					Arg ⁻	95
					Arg ⁻ Met ⁻	4
	110	Go + Arg + His	50	22	Arg ⁻	100
				1	Prototrophs	99
					His ⁻	1
				1	Prototrophs	84
					Arg ⁻ Met ⁻	16

mutagenic treatment and, most probably, differences in chromosome sets. Precise determination of DNA content per cell was not feasible, as the strains of *T. cutaneum* we used produce a large proportion of biomass in the form of filaments of variable lengths.

Fusion between mutants derived from strain UCD 54-169

When fusion within UCD-derived mutants was carried out, all colonies selected on minimal medium were also stable prototrophs.

Different results were observed, however, when the preliminary selection was done on partially selective medium which lacked one of the supplements necessary for growth of parental strains. Colonies which were grown on such plates after fusion appeared homogeneous (no sectors were observed). They were then replicated on Go and YPG medium. Those colonies which after passage grew on YPG medium only were suspended in 0.9% NaCl and plated on YPG medium for single-cell colonies. The resulting colonies were replicated on selective media to determine their phenotype.

It is clear from the results shown in Table 3 that the primary colonies were composed of cells showing both parental and recombinant phenotypes. Phenotypes of segregants were stable during four successive passages through the non-selective medium. A few auxotrophic segregants were further mutagenized and new auxotrophic mutations were obtained in them, which strongly suggests that these segregants were haploid or aneuploid.

It is worth noting that only one parental type (Arg⁻ Ade⁻ and Arg⁻ Met⁻) was observed in the progeny of fusants in each combination tested and that segregation of auxotrophic markers showed various patterns for different fusants. In addition, many segregants with non-supplemented requirements were obtained in these experiments, with a strong preponderance of arginine auxotrophs. These segregants survived the whole screening procedure, possibly due to the accumulation of arginine in cells. It is known, for instance, that *Saccharomyces cerevisiae* and *Neurospora crassa* accumulate large amounts of arginine in vacuoles (Messenguy *et al.*, 1980; Subramanian *et al.*, 1973).

The results presented here indicate that naturally

occurring *T. cutaneum* strains differ in ploidy but in spite of this they fuse readily. At least in a proportion of fusants karyogamy takes place and the resulting nuclei are unstable, spontaneously segregating out both parental and recombinant progeny (due to chromosome segregation and possibly mitotic crossing-over). The fact that nuclear fusion occurs readily provides an opportunity of obtaining strains with desired marker combinations. Together with transformation and cloning techniques (Glumoff *et al.*, 1990; Ochsner *et al.*, 1991) it creates a basis for efficient genetic manipulation in *T. cutaneum*, in spite of the lack of sexual reproduction in this organism.

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