

POLYMORPHISM OF TREHALOSE ACCUMULATION IN SIBLING SPECIES OF *Saccharomyces* SENSU STRICTO

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ABSTRACT

Trehalose accumulation (Tac⁺) was studied in three sibling species *Saccharomyces cerevisiae*, *S. paradoxus* and *S. bayanus*. In the former two species, a new type of Tac⁺ was found in non-maltose fermenting strains, which was not controlled by known maltose regulatory genes. Strains with active Tac⁺ patterns were selected. In strain N25 of *S. paradoxus* the Tac⁺ property is controlled by two complementary genes while in *S. cerevisiae*, strain UFRJ 50613, several polymeric cumulative genes are probably responsible for the high level of trehalose accumulation. This new Tac⁺ phenotype apparently is characteristic of wild strains of *Saccharomyces* s.str.

INTRODUCTION

As a storage carbohydrate and as a protectant of proteins and membranes, trehalose plays an important role in germination, viability and acquisition of thermotolerance of yeast cells (Panek and Panek, 1990). The pathway of endogenous trehalose accumulation is well studied in some genetic lines and industrial strains. However, they represent only a limited part of the gene pool of *Saccharomyces*. Natural strains sensu stricto have practically not been studied for this characteristic.

Three biological species of *Saccharomyces* s.str. can be differentiated from each other by genetic hybridization analysis of DNA/DNA reassociation homology (Naumov, 1987; Naumov *et al.*, 1992b; Vaughan Martini, 1989).

It has been shown that trehalose accumulation (Tac⁺) is linked to maltose fermentation in *S. cerevisiae* and that it is determined by special mutations (*MAL*^C) of constitutive α -glucosidase synthesis (Panek *et al.*, 1980; Oliveira *et al.*, 1981). Maltose fermentation in *S. cerevisiae* is known to be controlled by a family of polymeric loci

MAL1-MAL4, *MAL6* each of which contains three closely linked complementary genes: a maltose permease GENE1 (*MAL11*, *MAL21* ...), a maltase (α -glucosidase) GENE2 (*MAL12*, *MAL22*, ...) and a regulator GENE3 (*MAL13*, *MAL23*, ...). The first digit indicates the locus of the gene and the second one indicates the gene function. For example, *MAL11* encodes maltose permease at the *MAL1* locus. Strains having only one or two of these complementary genes are unable to ferment maltose (Charron *et al.*, 1989; Needleman, 1991). Natural *Mal*⁻ strains of *S. cerevisiae* are *mal*⁰ mutants on one or several maltose genes (Naumov, 1977; Naumov *et al.*, 1991).

In the present study we compared trehalose accumulation in three sibling species *S. cerevisiae*, *S. paradoxus* and *S. bayanus* and tried to find a relationship between this phenomenon and α -glucosidase synthesis in natural strains.

To discover new systems controlling Tac⁺ we chose non-maltose fermenting (*Mal*⁻) strains of *S. cerevisiae* and *S. paradoxus*.

MATERIAL AND METHODS

Strains

Monosporic cultures of different natural strains of *Saccharomyces* s.str. and some mutants were used (Figure 1A,B,C). The origin of the strains has been described previously (Naumov, 1987; Naumov *et al.*, 1983a,b,

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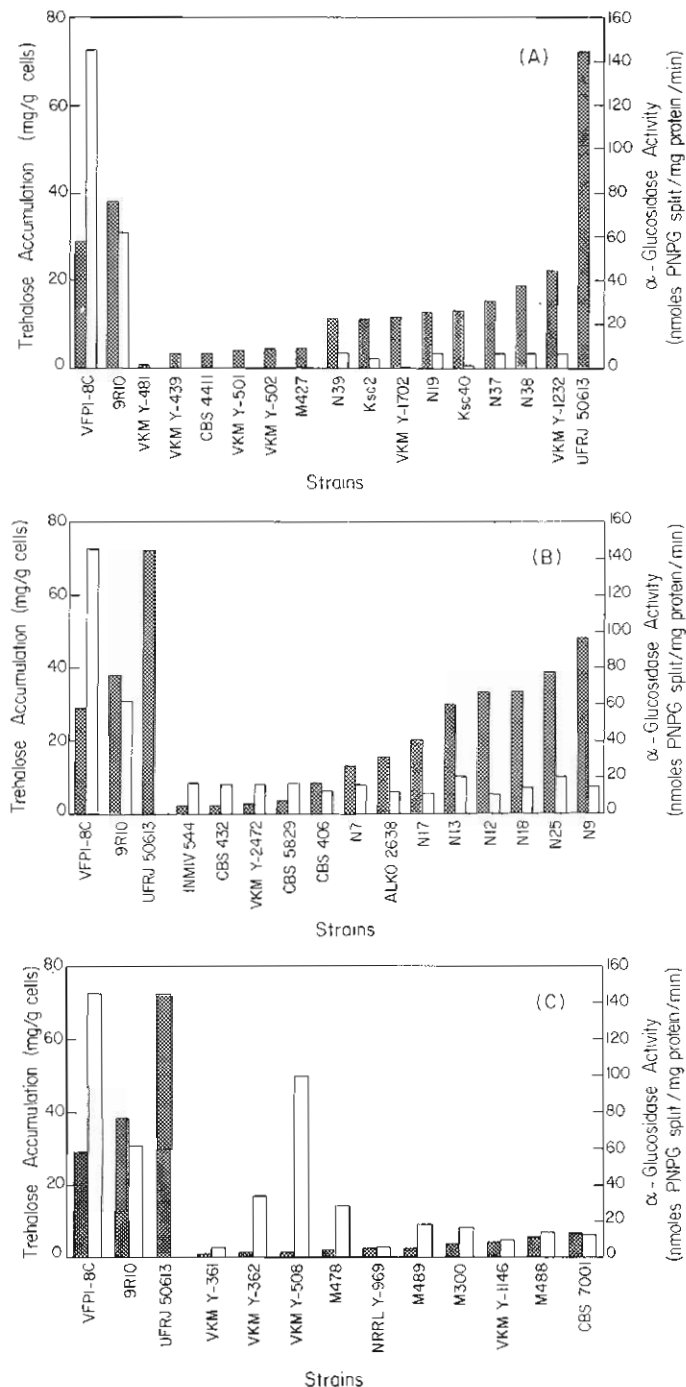


Figure 1 - Natural polymorphism of trehalose accumulation and constitutive α -glucosidase activity in the sibling species *Saccharomyces cerevisiae* (A), *S. paradoxus* (B) and *S. bayanus* (C). *S. cerevisiae* strains VFPI-8C and 9R10 are presented as controls. ■, Trehalose accumulation; □, α -glucosidase activity.

1992a,b; Naumov and Naumova, 1991; Naumova and Nikonenko, 1988, 1989; Naumova *et al.*, 1991). The UFRJ 50613 strain was isolated in 1983 by J.B. da Silva Filho from a river in Rio de Janeiro. As controls we used two constitutive mutants VFPI-8C (*MAL4^C*) (Paschoalin *et al.*, 1987) and 9R10 (*MAL6^C*) (from C.A. Michels, New York).

In Figure 1A, B and C we used the following acronyms of Culture Collections: VKM = National Collection of Microorganisms, Moscow, Russia; CBS = Centraalbureau voor Schimmelcultures, Delft, The Netherlands; UFRJ = Coleção de Culturas, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brazil; ALKO = Research Laboratories of the Finnish State Alcohol Company, Helsinki, Finland; INMIV = Institute of Microbiology and Virology, Kiev, Ukraine; M = Institute of Viticulture and Wine Making, Yalta, Ukraine; NRRL = Northern Region Research Center, Peoria, Ill., USA. Strains without acronyms, viz. *S. cerevisiae* NN 19, 37, 38, 39, and *S. paradoxus* NN 7, 9, 12, 13, 17, 18, 25, are from the collection of G.I. Naumov. Ksc strains were received from the Institute for Fermentation, Osaka, Japan.

Growth conditions

Strains were grown at 28°C in YED medium (1% yeast extract, 2% glucose, 0.2% (NH₄)₂SO₄, 0.2% KH₂PO₄) on a rotary shaker at 160 rpm. The strains were grown on YPD medium (1% yeast extract, 1% peptone, 2% glucose and 2% agar) at 30°C. Sporulation was induced for 2 days on acetate medium (1% CH₃COONa, 0.5% KCl, 2% agar).

Analytical methods

Trehalose accumulation and α -glucosidase activity were tested during the transition phase of growth on glucose (Boucherie, 1985). Trehalose was extracted with 0.5 M trichloroacetic acid (Trevelyan and Harrison, 1952) and determined by the anthrone method (Brin, 1966). Alpha-glucosidase activity was determined by the hydrolysis of p-nitrophenol- α -D-glucoside (PNPG) by permeabilized cells. For each strain, proportionality between the time of incubation of permeabilized cells with 5 mM PNPG at 30°C and enzymatic release of p-nitrophenol was determined (Oliveira *et al.*, 1981).

Genetic analysis

The methods used for hybridization of yeasts were as described before (Naumov *et al.*, 1986). Hybrids of homothallic yeasts were obtained by the spore-to-spore mating method, using a micromanipulator. Crosses between heterothallic and homothallic strains were performed by the haploid cell-to-spore mating method, using a micromanipulator.

Maltose fermentation

The ability of strains to ferment 2% (w/v) maltose was tested on pH indicator agar medium with

eosin-methylene blue (EMB) and in Durham fermentation tubes (Naumov and Gudkova, 1979). *Mal*⁻ strains did not ferment maltose during a test period of 10 days.

RESULTS AND DISCUSSION

S. cerevisiae

All natural *Mal*⁻ strains studied have known *MAL* gene genotypes (Naumov, 1977; Naumov *et al.*, 1991). Five strains (VKM Y-439, CBS 4411, M427, VKM Y-1232 and UFRJ 50613) harbor active GENE1, GENE2 and mutant, inactive GENE3; eight strains (VKM Y-481, Ksc2, Ksc40, VKM Y-1702, N19, N37, N38 and N39) have active GENE2 and mutant GENE1, GENE3; two strains (VKM Y-501 and VKM Y-502) possess active GENE3 and mutant GENE1, GENE2. Figure 1A presents the data on trehalose accumulation and α -glucosidase activity of these strains. In some of the *Mal*⁻ strains tested the levels of trehalose accumulation were comparable to those of control strains VFPI-8C and 9R10. One strain UFRJ 50613 showed superproduction of trehalose (72.2 mg/g cells). Trehalose accumulating *Mal*⁻ strains had either very low α -glucosidase activity or no activity at all, as expected in accordance with their *MAL* genotypes. Variation of this activity is probably due to different genetic background of strains from various sources (Naumov, 1987; Naumov *et al.*, 1983a,b). None of the strains tested showed a constitutive synthesis of α -glucosidase, including strain UFRJ 50613 which had a high level of trehalose accumulation (Figure 1A). The α -glucosidase activity we observed is likely not to be connected with maltase but rather with α -methylglucosidase, which can also hydrolyze PNPG. Strains N38, VKM Y-1232 and UFRJ 50613 which accumulate more trehalose than the other strains do not have active maltose regulatory GENE3 or its dominant constitutive homologues. In these strains trehalose accumulation is not controlled by known regulatory maltose genes.

S. paradoxus

The 13 strains studied showed widely varying levels of trehalose accumulation, ranging from 2.5 to 48.5 mg/g cells (Figure 1B). Eight strains with high levels of trehalose accumulation (N7, N9, N12, N13, N17, N18, N25 and ALKO 2638) were isolated from nature in 1980-1989 (Naumov, 1987; Naumov *et al.*, 1992a). Some of them accumulated more trehalose than control constitutive mutants VFPI-8C and 9R1. But, none of the *S. paradoxus* strains accumulated more trehalose than *S. cerevisiae* strain UFRJ 50613 (Figure 1B). Strains of *S.*

paradoxus isolated more than 20 years ago (INMIV 544, CBS 5829 and VKM Y-2472) or earlier (CBS 432, CBS 406) formed the group of *Tac*⁻ yeasts. It is possible that these strains lost the *Tac*⁺ property after a long period of storage in culture collections.

All strains had low α -glucosidase activity, which most probably corresponds to α -methylglucosidase since most *S. paradoxus* strains can ferment α -methylglucosidase. As many natural *Mal*⁻ mutants of *S. cerevisiae*, *S. paradoxus* strains (N7, N9, N12, N13, N17, N18, N25 and INMIV 544) have active maltase GENE2 and mutant GENE1 and GENE3 (Naumov *et al.*, 1991). Our data indicate that the wild type *S. paradoxus* is characterized by active trehalose accumulation, also independent of known maltose regulatory genes.

S. bayanus

Unlike *S. cerevisiae* and *S. paradoxus*, all the *S. bayanus* strains studied had a *Mal*⁺ phenotype, with the exception of strain VKM Y-1146. All strains displayed *Tac*⁻ phenotypes (Figure 1C). The levels of trehalose accumulation in *S. bayanus* strains (1.3-6.7 mg/g cells) were much lower than in most *S. cerevisiae* (Figure 1A) and *S. paradoxus* ones (Figure 1B). Despite the *Mal*⁺ phenotype, the *S. bayanus* strains showed, as a rule, a non-constitutive synthesis of α -glucosidase (Figure 1C). The only constitutive strain, VKM Y-508, also did not accumulate trehalose.

Tetrad analysis of the *Tac*⁺ property

In order to determine the genetic basis of the *Tac*⁺ phenotype two intraspecies hybrids *Tac*⁺ x *Tac*⁻ of *S. cerevisiae* and *S. paradoxus* were analysed.

Strain N25 of *S. paradoxus*, with a high level of trehalose accumulation (38.7 mg/g cells), was crossed with a non-accumulating strain, CBS 5829 (3.6 mg/g cells). As a control marker we used auxotrophy for adenine (red colonies). Segregants were very different in their trehalose accumulation patterns (Table I). Most of the segregants can be classified into two groups based on their *Tac* levels: lower than 10 mg/g cells (29 strains) and higher than 20 mg/g cells (11 strains). Only four segregants (2A, 2B, 6A and 8A) showed intermediate values. We considered the segregants having 15 mg/g cells or lower levels of trehalose as *Tac*⁻ yeasts. Segregants which accumulated more than 20 mg/g cells of trehalose were designated as *Tac*⁺. Eleven complete tetrads were analysed (Table I). Tetrads gave digenic complementary segregation on trehalose accumulation: 2⁻:2⁺ (nos. 3, 8 and 9), 4⁻:0⁺ (nos. 4 and 5), 3⁻:1⁺ (nos. 1, 2, 6, 7, 10 and 11). It means that two complementary genes are responsible for the *Tac*⁺ property in strain N25. The hybrid N25 x CBS 5829 was *Tac*⁻ and

Table I - Segregation of Tac and Ade phenotypes in *Saccharomyces paradoxus* hybrid N25 x CBS 5829.

Tetrads	Tac (mg/g cells)	Ade	Tetrads	Tac (mg/g cells)	Ade
1A	51.1 (+)	-	7A	3.3 (-)	+
1B	8.9 (-)	-	7B	31.3 (+)	+
1C	6.7 (-)	+	7C	7.1 (-)	-
1D	1.1 (-)	+	7D	9.8 (-)	-
2A	13.8 (-)	-	8A	16.7 (+)	+
2B	11.6 (+)	-	8B	77.8 (+)	+
2C	2.2 (+)	+	8C	2.7 (-)	-
2D	53.3 (+)	+	8D	1.3 (-)	-
3A	27.8 (+)	+	9A	6.2 (-)	-
3B	20.4 (+)	-	9B	27.8 (+)	+
3C	6.2 (-)	-	9C	1.1 (-)	+
3D	8.9 (-)	+	9D	37.3 (+)	-
4A	2.8 (-)	+	10A	7.1 (-)	-
4B	8.9 (-)	-	10B	23.3 (+)	+
4C	1.7 (-)	+	10C	5.3 (-)	-
4D	7.1 (-)	-	10D	1.1 (-)	+
5A	2.7 (-)	-	11A	21.1 (+)	+
5B	8.9 (-)	+	11B	4.4 (-)	+
5C	6.2 (-)	-	11C	1.8 (-)	-
5D	2.2 (-)	+	11D	5.3 (-)	-
6A	13.3 (-)	+			
6B	2.2 (-)	-			
6C	22.2 (+)	+			
6D	0.9 (-)	-			

Note: Tac = trehalose accumulation. Tac phenotype of the segregants is given in parentheses. Ade⁺ = prototrophy for adenine. Strain N5829 was marked by *ade* mutation.

accumulated even more trehalose than both parents: 55.6 mg/g cells. Therefore, the two *TAC* genes found are dominant.

For the *S. cerevisiae* hybrid UFRJ 50613 x VKM Y-502 it was difficult to estimate how many genes were involved in the inheritance of the Tac⁺ property (Table II). Segregants showed practically all intermediate levels of trehalose accumulation. The hybrid was Tac⁺ and accumulated 66.7 mg/g cells. Strain UFRJ 50613 is likely to have several polymeric dominant genes which are responsible for the cumulative nature of the Tac⁺ phenotype. The control marker *ade/ADE* showed monogenic segregation of 2⁻:2⁺ in 12 tetrads (Table II).

The hybrid UFRJ 50613 x VKM Y-502 had a Mal⁺ phenotype while both parents were Mal⁻. Strain VKM

Y-502 possesses only active GENE3. Therefore, the second parent UFRJ 50613 had active GENE1 and GENE2. These genes are probably closely linked as the hybrid gave digenic complementary segregation for maltose fermentation: 2⁻:2⁺ (5), 4⁻:0⁺ (3), 3⁻:1⁺ (10) in tetrads (Table II). However, strain UFRJ 50613 could have two unlinked clusters of genes GENE1 and GENE2, one of which could be located at the *MAL1* locus as GENE3 of strain VKM Y-502. From Table II it is clear that there is no correlation between an active pattern of trehalose accumulation and maltose fermentation. These two properties segregated independently.

To determine whether there is a relationship between high level of trehalose accumulation and constitutive α -glucosidase synthesis, all Mal⁺ segregants

Table II - Segregation of Tac, Mal and Ade phenotypes in *Saccharomyces cerevisiae* hybrid UFRJ 50613 x VKM Y-502.

Tetrads	Tac (mg/g cells)	Mal	Ade	Glu	Tetrads	Tac (mg/g cells)	Mal	Ade	Glu
1A	22.2	-	-	*	7A	8.0	-	-	*
1B	2.2	-	+	*	7B	49.8	-	-	*
1C	37.3	+	-	29.4	7C	50.0	+	+	20.0
1D	77.8	+	+	61.6	7D	32.2	+	+	24.5
2A	62.2	-	-	*	8A	69.4	-	+	*
2B	4.4	-	+	*	8B	34.4	-	+	*
2C	38.3	-	+	*	8C	5.3	-	-	*
2D	26.7	-	-	*	8D	12.4	+	-	15.9
3A	62.2	+	-	12.2	9A	83.3	-	+	*
3B	20.0	+	+	55.1	9B	3.6	+	-	22.9
3C	38.9	-	+	*	9C	6.1	-	-	*
3D	24.0	-	-	*	9D	61.1	-	+	*
4A	4.4	+	+	45.4	10A	16.9	-	-	*
4B	16.0	-	-	*	10B	55.6	-	+	*
4C	19.6	-	-	*	10C	16.0	-	-	*
4D	54.2	-	+	*	10D	7.2	-	+	*
5A	12.2	+	-	48.6	11A	6.1	-	+	*
5B	75.6	+	-	9.6	11B	10.2	-	-	*
5C	44.4	-	+	*	11C	6.9	+	+	56.8
5D	7.8	-	+	*	11D	26.7	-	-	*
6A	13.3	-	-	*	12A	12.4	-	-	*
6B	50.0	-	+	*	12B	7.1	+	-	12.8
6C	6.7	-	+	*	12C	80.6	+	+	16.8
6D	25.8	+	-	0.8	12D	83.3	-	+	*

Note: Tac, trehalose accumulation. Mal⁺, maltose fermentation. Ade⁺, prototrophy for adenine. Strain VKMY-N502 was marked by *ade* mutation. Glu - α -glucosidase activity (nmoles PNPG split/mg protein/min).

* α -glucosidase activity was not analysed.

were tested for α -glucosidase activity (Table II). Three segregants (1D, 3B and 11C) demonstrated constitutive synthesis of α -glucosidase. The levels of their α -glucosidase activity were close to that of a constitutive mutant 9R10 (62.1 nmoles PNPG split/mg protein/min). However, only one segregant, 1D, also showed an active Tac⁺ pattern.

Thus, we found a new mechanism for trehalose accumulation in yeast cells, independent of the constitutive synthesis of α -glucosidase. Among natural strains of *S. paradoxus* and *S. cerevisiae* we found strains with superproduction of trehalose. Strain UFRJ 50613 accumulating 72.2 mg trehalose /g cells would be a good starting point for breeding work.

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RESUMO

O acúmulo de trehalose (Tac⁺) foi estudado em três espécies irmãs, *Saccharomyces cerevisiae*, *S. paradoxus* e *S. bayanus*. Entre as cepas de *S. cerevisiae* que não fermentam maltose, um novo tipo de

fenótipo Tac⁺, não regulado por genes *MAL*, foi encontrado. Cepas com fenótipo Tac⁺ foram selecionadas entre as espécies *S. cerevisiae* e *S. paradoxus*. Na cepa N25 de *S. paradoxus* a característica Tac⁺ é controlada por dois genes complementares enquanto que em *S. cerevisiae* cepa UFRJ 50613, vários genes poliméricos, cumulativos são provavelmente responsáveis pelo elevado teor de trealose acumulado. Este novo fenótipo Tac⁺ parece ser característico de cepas selvagens de *Saccharomyces* s.str.

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