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EXPERIMENTAL ARTICLES

Peculiarities of Hybrid Formation and Copulation Activity in the Yeast *Pachysolen tannophilus*

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Abstract—The copulation activity and hybrid formation efficiency have been studied in the xylose-assimilating yeast *Pachysolen tannophilus.* It was shown that the presence of 2% D-glucose, 0.5% yeast extract, and 2% agarose in the growth medium provided for the highest frequencies of hybrid formation. Atypical hybrid cultures similar in morphophysiological characteristics to native haploid strains of *P. tannophilus* were revealed in the course of hybridization. The genesis mechanism of such cultures and the reasons for the restricted applicability of hybridological analysis to genetic studies of *P. tannophilus* are discussed.

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Key words: yeast *Pachysolen tannophilus*, copulation, conjugated asci, prototrophic hybrids.

The use of *Pachysolen tannophilus* in the processes of xylite and ethanol production would be impossible without the development of techniques based on the knowledge of the biological peculiarities of the species. Previously, the main stages of the life cycle of this yeast were established during the study of native strains [1], and the conditions favoring the stabilization of a vegetative haploid culture and providing for the transition of cells to sexual process and spore formation were determined [2]. *P. tannophilus* has been reported to exhibit a tendency toward aneuploidy [3]; this fact prevents us from stating that the copulation of native haploid strains always yields valid heterozygotes. In order to avoid the emergence of artifacts in the course of hybridological analysis, it is necessary to study the peculiarities of the sexual process in yeasts. Therefore, the goal of this work was not only to study the copulation activity of haploid strains on different media but also to analyze the nature of hybrid cultures formed in the course of hybridization of auxotrophic mutants of *P. tannophilus*.

MATERIALS AND METHODS

Mutants of the haploid strain *P. tannophilus* 22-Y-1532 [2] that were obtained upon exposure to 1-methyl-3-nitro-1-nitrosoguanidine according to [4] and characterized by noncomplementary requirements for methionine (genotypes *met1-76* and *met2-83*) and adenine (genotype *ade1-79*) [5] were used in this work. Yeasts were grown on standard and modified solid media, the full composition of which is presented in Table 1, at a temperature of $30 \pm 2°C$. The frequency of occurrence of revertants in the population was determined as follows. An aqueous suspension of the mutants, 1×10^8 cells/ml, prepared from a two-day culture grown on medium 2, was plated as a lawn onto Petri dishes with minimal medium 5. On day 4 of cultivation under the above conditions, the ratio of the quantity of prototrophic clones to the total number of cells in the initial suspension was calculated. The intensity of the sexual process (copulation activity) on different media was assessed by morphophysiological criteria. The result was recorded on day 3 of incubation by counting of copulation figures, conjugant-type asci, and yeast cell number in a Goryayev chamber [10]. The parent strain *P. tannophilus* 22-Y-1532 was used as the control.

For hybridization, the mutants were cloned for 48 h on medium 2, and then typical clones were selected on the basis of their morphological characteristics as described in [1]. Then, haploid strains were mixed by thick streaks on media of different compositions, grown for three days, and transferred to minimal medium 5, where the appearance of prototrophic clones was registered. The ploidy of each clone was analyzed according to the morphological criteria developed in [2] and confirmed by spore progeny generation analysis. The frequency of formation of *P. tannophilus* prototrophs was

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Table 1. Nutrient media used in this work

No.	Composition, g/l	Reference
1	*D*-glucose, 100; peptone, 3; yeast extract, 8; agar–agar, 20	[6]
2	*D*-glucose, 20; peptone, 20; yeast extract, 5; agar–agar, 20	[7]
3	*D*-glucose, 20; yeast extract, 5; agarose, 20	[2]
4	*D*-glucose, 20; KH_2PO_4, 0.9; K_2HPO_4, 0.1; $(NH_4)_2SO_4$, 3.5; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 5; agar–agar, 20; vitamins and trace elements according to [8], μg/l: biotin, 20; calcium pantothenate, 2; folic acid, 2; inositol, 10; nicotinic acid, 400; *p*-amino-benzoic acid, 200; pyridoxine hydrochloride, 400; riboflavin, 200; thiamine hydrochloride, 400; boric acid, 500; $CuSO_4$, 40; KJ, 100; $FeCl_3$, 200; $MnSO_4$, 400; $NaMoO_4$, 200; $ZnSO_4$, 400	[2]
5	*D*-glucose, 20; KH_2PO_4, 0.9; K_2HPO_4, 0.1; $(NH_4)_2SO_4$, 3.5; $MgSO_4$, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; agar–agar, 20; vitamins and trace elements, as in medium 4	[9]
6	*D*-glucose, 10; agarose, 20; other components, as in medium 4	[2]
7	*D*-glucose, 4; yeast extract, 4; malt extract, 10; agarose, 20	[3]

Table 2. Diploidization of different strains of *P. tannophilus*

Medium composition				Prototroph (phenotype)	Mutants (genotype)		
Sugar, %		Other components		Ade^+Met^+	*met1-76*	*met2-83*	*ade1-79*
10.0	Yeast extract	agar–agar	peptone	–	+	+	–
2.0				–	–	–	+
			$(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, K_2HPO_4, trace elements and vitamins	+	+	+	+
		agarose		+	+	+	+
0.4			malt extract	–	–	–	+

Note: Diploidization was tested by the presence of copulation figures and conjugated asci in mutant cultures. "+" means that the quantity of diploids in the suspension was more than 10^{-3} per cell, and "–" means absence of diploidization. The conditions most favorable for diploidization of mutants are marked with gray background color.

determined by micromanipulation, calculating their quantity in relation to the number of cells that entered into copulation. Culture morphology was studied using a Jenamed variant microscope (Germany) at 18- and 40-fold magnifications of the eyepiece and objective, respectively.

RESULTS

Our previous analysis of the induction of sexual process in the native strains of *P. tannophilus* on media with different concentrations of the carbon, nitrogen, sulfur, phosphorus, and potassium sources, trace elements, and vitamins has revealed that the maximal number of diploid subclones was 1×10^{-3} to 1×10^{-2} per cell [2]. For studying the nature of the hybrids formed during hybridization, it is convenient to use biochemical markers that permit visual monitoring of the formation of heterozygotes on selective media. Different auxotrophic mutants of yeasts are commonly used for this purpose. Before isolation of prototrophic hybrids, the frequency of occurrence of revertants in the population was assessed. This frequency was no more than 1×10^{-8} revertants per cell for all of the mutant

strains. In addition, the mutants were characterized by a stable haploid state: they produced mucous colonies and formed only conjugated asci [1, 2]. The intensity of generation of copulation figures and conjugated asci in the marked derivatives of strain 22-Y-1532 on nutrient media of different composition is shown in Table 2. The conditions favoring the induction of the sexual reproduction phase proved to be considerably different in auxotrophic mutants and in the parent haploid strain. Low carbon source concentrations (medium 7) and the presence of peptone and agar–agar (media 1 and 2) stabilized the vegetative growth of the initial strain *P. tannophilus* 22-Y-1532 [2] but caused copulation of cells, followed by appearance of conjugated asci, in the mutants. It was shown that the deficiency of reduced forms of nitrogen and sulfur upon replacement of the above nutrient components with agarose (medium 3) and additional enrichment of the medium with vitamins and trace elements (medium 4) provided for the appearance of diploid subclones in all the auxotrophs under study, with frequencies of more than 1×10^{-3} per cell. As the frequency of formation of diploid subclones was much higher than the frequency of occurrence of revertants in the population, the yeast mutants were considered suitable for hybridization.

The copulation of auxotrophs was realized on media 3, 4, and 6, which induce sexual process in native haploids [2], and on medium 7, recommended by James and Zahab for the genetic analysis of the yeast *P. tannophilus* [3] (Table 3). Analysis of the presence of copulating cells and copulation figures and estimation of the formation frequency of prototrophic hybrids by the technique described in Materials and Methods revealed the following regularities:

(1) the maximal frequencies of hybrid formation (5×10^{-6} to 2×10^{-7}) in all cases were obtained on medium with 2% D-glucose, yeast extract, and agarose; however, even these values were comparable with the frequencies of revertant occurrence;

(2) hybridization was not induced if the D-glucose concentration in the medium was below 1%;

(3) the efficiency of hybrid formation was different for different pairs of parents;

(4) copulation intensity in all cases correlated with the frequency of hybrid formation.

Taking into account the instability of the diploid vegetative culture of *P. tannophilus*, each prototrophic clone was assessed morphologically to confirm its ploidy according to [1]. Some prototrophs incubated for 3 days on medium 2 produced small budding cells (1.3–6.3 × 2.1–4.2 μm), while prototrophs incubated on medium 4 formed copulation figures; such regularities had been previously noted in the haploid culture of this yeast [2]. The percentage of such prototrophs, conventionally termed "atypical," was 3.8 to 86.9%, depending on the phenotypic peculiarities of the mutants and the nutrient medium used for hybridization (Table 3). The minimal number of clones of this type was observed in the presence of 2% D-glucose, yeast extract, and agarose (medium 3). However, even in the latter case, the frequency of occurrence of atypical prototrophs in the populations of most hybrids was higher than the frequency of occurrence of revertants.

DISCUSSION

Our experiments showed the possibility of regulating the copulation activity not only in native cultures but also in mutants of the xylose-assimilating yeast *P. tannophilus*. However, even the conditions most strongly inducing the copulation of haploid cells of *P. tannophilus* 22-Y-1532 (1–2% D-glucose, $(NH_4)_2SO_4$, yeast extract, a complex of vitamins and trace elements) [2] did not provide for active formation of prototrophic hybrids in the course of hybridization of auxotrophic mutants. A similar phenomenon was observed on the medium recommended by James and Zahab for the genetic analysis of *P. tannophilus* (0.4% D-glucose, yeast and malt extracts) [3]; on this medium, the frequency of hybrid occurrence was not higher than 0.6×10^{-7} per cell. The nutrient composition most suitable for hybridization of mutant strains was the combination of 2% D-glucose, 0.5% yeast extract, and 2% agarose. This composition may be recommended for obtaining heterozygous hybrids of *P. tannophilus*.

Nevertheless, even under these selective conditions, a considerable part of the prototrophs formed resembled the initial haploids in morphophysiological characteristics and type of sporulation and can be classified as atypical prototrophs. The number of such hybrids was in each case determined by the genotype of *P. tannophilus* 22-Y-1532 derivatives involved in hybridization. The multiplicity of atypical prototrophs apparently reflects the biological features of this species, namely its tendency toward aneuploidy. Aneuploidy, along with the predominance of the haplophase in the life cycle and the easiness of induction of the sexual process [1, 2, 7], may provide adaptive advantages to *P. tannophilus* by promoting rapid exchange of hereditary characters without maintenance of the diploid state of cells.

However, these peculiarities considerably complicate the application of the traditional genetic analysis of sporogenous yeasts to the study of *P. tannophilus*. Nevertheless, the convenience of using this species as a model for studying the regulation of D-xylose catabolism [11] makes it necessary to search for ways of overcoming these difficulties. It is known that a series of inbred hybridizations often provides for the elimination of the genetic defects of cells that result in aneuploidy [6]. The use in such experiments of biochemical markers permits quantitative assessment of the content of

Table 3. Efficiency of hybrid formation in *P. tannophilus* on different media

Medium composition			Hybrids								
			met1⁻met2⁺/met1⁺met2⁻			ade1⁺met1⁻/ade1⁻met1⁺			ade1⁺met2⁻/ade1⁻met2⁺		
Sugar, %		Other components	Copulation activity	Prototrophs		Copulation activity	Prototrophs		Copulation activity	Prototrophs	
				frequency of formation (10^{-7})	atypical, % of total quantity		frequency of formation (10^{-7})	atypical, % of total quantity		frequency of formation (10^{-7})	atypical, % of total quantity
2.0	Yeast extract	agar–agar, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, K_2HPO_4, trace elements, vitamins	–	≤0.6	50.0	+	4.3	69.7	–	≤0.6	50.0
		agarose	++	69.0	27.5	++	26.0	3.8	++	3.0	33.3
1.0		agarose, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, K_2HPO_4, trace elements, vitamins	–	2.3	86.9	–	≤0.6	50.0	+	9.5	73.8
0.4		agarose, malt extract	–	≤0.6	50.0	–	≤0.6	50.0	_	≤0.6	50.0

Note: "–", no copulating cells; "+", 10^{-3} to 10^{-2} copulating cells in the culture; "++", over 5% copulating cells. Frequency of prototroph formation was determined as the ratio of their quantity to the number of cells involved in copulation. The medium providing for maximal efficiency of hybrid formation is marked with gray background color.

atypical prototrophs in the population. Therefore, the goal of our further studies will be to develop methods for increasing the efficiency of hybrid formation and the share of valid diploid heterozygotes upon hybridization of auxotrophic strains of *P. tannophilus*; the development of such methods will make it possible to apply hybridological analysis to this species.

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