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EXPERIMENTAL ARTICLES

Saccharomyces bayanus var. *uvarum* comb. nov., a New Variety Established by Genetic Analysis

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Abstract—Partial genetic isolation of two *Saccharomyces bayanus* varieties, *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum* comb. nov., was established by hybridological analysis. The hybrids of these two varieties were semisterile: their ascospores were characterized by low survival. Earlier, the new variety was described as a group of cryophilic wine yeast cultivars capable of fermenting melibiose.

Key words: *Saccharomyces bayanus*, *Saccharomyces uvarum*, hybrid, fertility, variety

There is substantial genetic, molecular genetic, and physiological evidence [1, 2] that cryophilic, usually melibiose-fermenting wine yeasts of the genus *Saccharomyces* belong to *S. bayanus* (syn. *S. uvarum*), which is a sibling species of *S. cerevisiae* [3]. The type strain *S. bayanus* CBS 380 is a low-fertility aneuploid wild-type brewer's yeast presumably containing nucleotide sequences typical of the *S. cerevisiae* genes [4]. Strain CBS 380 is unable to ferment melibiose, but it contains the silent sequence MEL^0 (pseudogene) and can revert to the Mel^+ phenotype [5, 6].

Based on the results of PCR analysis, the species *S. bayanus* has recently been differentiated into two groups [7], one comprising the type cultures *S. bayanus*, *S. globosus*, *S. heterogenicus*, *S. intermedius* var. *valdensis*, and *S. inustitatus* and the other comprising the type cultures *S. uvarum* and *S. abuliensis* and multiple wine *S. bayanus* (*S. uvarum*) strains. In spite of the high DNA–DNA homology level (86–100%) of these two groups with the type culture *S. bayanus* [8], the homogeneity of this species is doubtful. Moreover, some authors believe that the species *S. uvarum* should be reestablished [9].

The present work was undertaken to reidentify the type strains *S. bayanus* CBS 380, *S. globosus* CBS 424, *S. heterogenicus* CBS 425, and *S. bayanus* strains NCAIM Y.00676, NCAIM Y.00677, and NCAIM Y.00789, whose affiliation to the species *S. bayanus* has recently been established by the DNA–DNA reassociation method [8, 10].

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MATERIALS AND METHODS

The cultures used in this work were as follows: the type strain *S. bayanus* CBS[2] 380 isolated from beer [4, 7, 8]; Sb5 and B19-13C derived from CBS 380 [11–14]; the type strain *S. abuliensis* MCYC[3] 623 = CBS 7001 (isolated from *Mesophylax adopersus*) [1, 4, 7, 8]; *S. cerevisiae* strains NCAIM[4] Y.00676 and NCAIM Y.00677 isolated from beverage, and NCAIM Y.00789 isolated from *Carpinus betulus* sap [10]; *S. coreanus* VKM[5] Y-1446(Y-6B), or CBS 8687, isolated from grapes [1, 4]; the type strain *S. globosus* CBS 424 isolated from pear juice [7, 8]; strains 424-3A and AR1 derived from CBS 424 [14]; and the type strain *S. heterogenicus* CBS 225 isolated from apple juice [7, 8].

Yeasts were cultivated and hybridized at 25°C on complete YPD agar medium. Spore formation was induced on acetate medium. Hybridization was carried out by the "spore-to-spore" or bulk method on complete agar medium with the subsequent selection of hybrids on minimal selective media. The asci envelopes were disrupted enzymatically, and ascospores were isolated using a micromanipulator. The monosporous homothallic strains *S. bayanus* MCYC 623 and *S. cerevisiae* VKM Y-502 [1] with the *ura3* (or *lys*) and *ade1* mutations, respectively, were used as high-fertility test cultures. The monosporous strains NCAIM Y.00676, NCAIM Y.00677, and NCAIM Y.00789 used for

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[3]MCYC, Departamento de Microbiologia, Escuela Tecnica Superior de Ingenieros Agronomos, Universidad Politecnica de Madrid, Spain.

[4]NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary.

[5]VKM, All-Russia Collection of Microorganisms, Moscow, Russia.

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Table 1. Survival rates of ascospores of the *Saccharomyces* strains studied

Original species name and strain number	Number of isolated tetrads	Survival rate of ascospores, %	Original species name and strain number	Number of isolated tetrads	Survival rate of ascospores, %
S. bayanus			*S. heterogenicus*		
CBS 380	12	48	CBS 425	65	7
Sb5	11	77	425-4A	11	98
B19-3C	13	65	*S. cerevisiae*		
Sb5-3A	5	95	NCAIM Y.00676	8	84
B19-3C-5A	20	75	NCAIM Y.00677	8	69
S. globosus			NCAIM Y.00789	8	100
CBS 424	11	77	00676-3-4	5	95
424-3A	10	60	00677-5-2	5	100
AR1	11	64			

hybridization were marked by the spontaneous auxotrophic mutation *lys*. The *lys* mutants were selected on agar media with DL-α aminoadipic acid as the selective agent (Sigma, the United States).

RESULTS

For the genetic analysis of yeasts, it was necessary to have high-fertility monosporous cultures. The analysis of ascospores showed that all of the yeasts chosen for analysis were homothallic; however, the ascospores of some yeast strains, first of all CBS 380, CBS 424, and CBS 425, were characterized by low survival rates (Table 1). For this reason, instead of strains CBS 380 and CBS 424, the respective inbred yeast lines marked by auxotrophic mutations were used for genetic analysis: Sb5 (*mel gal*4) and B19-3C (*mel gal*4 *ura*3 *his*6) derived from CBS 380 and 424-3A (*mel suc*) and AR1 (*ade mel suc*) derived from CBS 424 (parenthesized are the spore genotypes) [11–14]. The fertility of strains Sb5, CBS 425, NCAIM Y.00676, and NCAIM Y.00677 was increased by monosporous cloning (Table 1). However, the diploid segregants of the cloned strains 424-3A and AR1 turned out to be asporogenous in the second generation. Furthermore, the growth rates of the monosporous strains CBS 380, CBS 424, NCAIM Y.00676, and NCAIM Y.00677 were low, so that they produced colonies one day later than the ordinary wine strains of *S. bayanus* and *S. cerevisiae*. As a result, the attempts to hybridize strains 424-1A, AR1, Sb5, and B19-3C with the genetic test strain *S. bayanus* MCYC 623 by the "spore-to-spore" method were unsuccessful: no zygotes were obtained during the hybridization of 30–35 parent spore pairs. This explains why Kaneko and Banno hybridized the sporogenous auxotrophic derivatives of CBS 380 on YPD agar using a micromanipulator but selected prototrophic hybrids on minimal selective media [12]. With allowance for this, further attempts to obtain hybrids were undertaken by means of the bulk hybridization of spores of auxotrophic parents with the subsequent selection of their hybrids on minimal selective media.

Hybrids HB1, HB3, HB4, and HB7, whose parents were marked by the complementary markers of auxotrophy, were selected on a minimal medium with glucose (Table 2). Hybrids HB2, HB5, and HB6, one of whose parents was marked by induced auxotrophy, the other of whose parent was marked by the natural recessive mutation leading to the inability to utilize some sugars, were selected on media with melibiose, sucrose, or galactose.

The spores of one of the hybrids obtained, CBS 380 × CBS 424 (HB1), were characterized by a high survival rate (64%). The fertility of the other hybrids, MCYC 623 × CBS 380 (HB2 and HB3) and MCYC 623 × CBS 424 (HB4, HB5, and HB6), was low (from 9 to 39%). The low-fertility hybrids HB2–HB6, as well as the high-fertility hybrid HB1, exhibited a digene meiotic segregation of control markers, thus indicating a normal recombination of parent genomes. Presumably, only two hybrids, HB5(a) and HB5(b), segregated anomalously; however, the number of spores of these two hybrids was insufficient for thorough genetic analysis. The highly different survival rates of the ascospores of hybrids HB2–HB6 implied a mitotic instability in their genomes. As expected, the control interspecies hybrid CBS 380 × VKM Y-502 (HB7) was sterile.

Strains CBS 425 and NCAIM Y.00676 were hybridized with test strains by the "spore-to-spore" method, whereas the hybrids of strains NCAIM Y.00677 and NCAIM Y.00789 were selected on a minimal medium with glucose. The genetic analysis of hybrids HB8–HB15 (Table 3) confirmed the DNA–DNA reassociation data [8] that all four strains belong to the species *S. bayanus*. Indeed, the hybridization of these strains with the test culture *S. bayanus* MCYC 623 gave fertile hybrids (HB8–HB11) with the normal meiotic segregation of control markers, whereas their hybrids with *S. cerevisiae* VKM Y-502 (HB12–HB15) were sterile.

Table 2. Genetic identification of the type cultures *Saccharomyces bayanus* CBS 380 (Sb5, B19-3C), *S. globosus* CBS 424 (AR1, 424-1A), and *S. abuliensis* MCYC 623 assigned to the species *S. bayanus*

Hybrid	Parents	Sugar in selective medium	Number of isolated tetrads	Survival rate of ascospores, %	Number of encountered spores of the genotype:			
					aB	Ab	AB	ab
			S. bayanus × *S. bayanus*					
HB1	AR1 (ade) × B19-3C (his)	Glucose	41	64	2P : 2N : 8T			
HB2	623 (ura) × Sb5 (gal)							
	a)	Galactose	53	32	15	17	21	13
	b)	Galactose	23	32	7	9	4	8
	c)	Melibiose	29	37	9	10	13	8
HB3	623 (lys) × B19-3C (his)	Glucose	27	27	5	7	8	8
HB4	623 (lys) × AR1 (ade)	Glucose	29	16	5	3	4	6
HB5	623 (ura) × AR1 (ade)							
	a)	Sucrose	14	9	0	5	0	0
	b)	Sucrose	11	14	0	2	4	0
	c)	Melibiose	41	23	8	7	8	13
	d)	Melibiose	13	39	5	5	4	5
HB6	623 (ura) × 424-1A (mel)	Melibiose	27	15	1	4	3	8
			S. bayanus × *S. cerevisiae*					
HB7	B19-3C (his) × 502 (ade)							
	a)	Glucose	15	0	–	–	–	–
	b)	Glucose	16	0	–	–	–	–

Note: In the second column, a, b, c, and d indicate independently obtained hybrids. In the last column, a and b indicate the auxotrophy of the first and second parents, respectively; A and B indicate their prototrophy; the formula 2P : 2N : 8T represents the proportion of the tetrads of parent (P) and nonparent (N) ditypes and tetratype (T).

Noteworthy was the low fertility (26%) of hybrid CBS 425 × MCYC 623 (HB10).

DISCUSSION

The high survival rate (64%) of the ascospores of hybrid CBS 380 × CBS 424, which is close to that of the parent strains, suggests the close relatedness of these strains and their affiliation to one species, *S. bayanus*. Earlier, the same inference was drawn based on the DNA–DNA hybridization data [8]. On the other hand, the relatively low fertility (9–39%) of the hybrids of strains CBS 380, CBS 424, and CBS 425 with the test culture MCYC 623 is indicative of the partial genetic isolation of these strains.

Earlier, the test cultures *S. bayanus* MCYC 623 and VKM Y-1146 were used for the identification of fifty wine and five wild-type strains of *S. bayanus*. According to fertility values, intraspecies *S. bayanus* hybrids can be divided into two distinct groups characterized by the 50 and 100% survival of spores (only one wine strain, CECT 1884, had a fertility of 33%). The genetic relatedness of yeasts producing semisterile hybrids with the test cultures MCYC 623 and VKM Y-1146 must be established more exactly.

As for the type strains *S. bayanus* CBS 380, *S. globosus* CBS 424, and *S. heterogenicus* CBS 425, they essentially differed from the other 55 *S. bayanus* strains studied, as was proven by hybridological analysis. This might be due to introgressive hybridization [4] or chromosomal rearrangements [14].

These findings agree well with the data of Nguyen and Gaillardin [7], who, based on the results of PCR amplification and the restriction polymorphism of the second nontranscribed spacer in ribosomal DNA, revealed two subgroups within the *S. bayanus* species. The first subgroup contained five strains (including CBS 380, CBS 424, and CBS 425), whereas the second subgroup comprised 28 strains, including the type strains *S. uvarum* CBS 395 and *S. abuliensis* MCYC 623 and 19 wine strains of the species *S. bayanus*. A more complete genetic characterization of the *S. bayanus* species requires a further search for high- fertility strains genetically close to the type strain CBS 380. However, the experimental data presented in this study and those available in the literature [7, 15] are sufficient to establish a new *S. bayanus* variety: *Saccharomyces bayanus* Saccardo var. *uvarum* (Hansen) Naumov G. comb. nov. Basionym *Saccharomyces uvarum* Beijerinck, Arch. Néerl. Sci. Exactes Nat., Ser. II. 2: 269–289

Table 3. Genetic identification of the type strain *Saccharomyces heterogenicus* CBS 425 and *Saccharomyces* strains NCAIM Y.00676, NCAIM Y.00677, and NCAIM Y.00789 assigned to the species *S. bayanus*

Hybrid	Parents	Number of hybridized spore pairs	Number of zygotes obtained	Total number of isolated tetrads	Survival rate of ascospores, %	Number of tetrads of the type:		
						P	N	T
S. bayanus × *S. bayanus*								
HB8	623 × 00677	ND	ND	13	96	2	1	10
HB9	623 × 00789	ND	ND	13	90	1	4	7
HB10	623 × 425	39	3	23	26	*2URA* : *2ura* (3)*		
HB11	623 × 00676	42	7	11	89	*2URA* : *2ura* (9)		
S. cerevisiae × *S. bayanus*								
HB12	502 × 00677	ND	ND	24	0	–	–	–
HB13	502 × 00789	ND	ND	24	0	–	–	–
HB14	502 × 425	69	4	27	0**	–	–	–
HB15	502 × 00676	46	7	26	0	–	–	–

Notes: P, N, and T stand for parent and nonparent ditypes and tetratype, respectively. ND indicates that no data are available, since hybrids were selected on minimal selective media. Hybrids HB8–HB11 had the genotypes *ura3 LYS/URA3 lys* and *ura3/URA3*, respectively.

* Parenthesized is the number of tetrads with the indicated segregation genotype.

** Two spores produced microcolonies.

(1898). The type strain is CBS 395. Synonyms *Saccharomyces tubiformis* Osterwalder (1924) [16] and *Saccharomyces abuliensis* Santa Maria (1978) [17].

The formal description of the taxon *Saccharomyces bayanus* Saccardo var. *bayanus* [18]: The type strain CBS 380. Synonyms *Saccharomyces globosus* Osterwalder (1924) [16], *Saccharomyces heterogenicus* Osterwalder (1924) [16], *Saccharomyces intermedius* Hansen var. *valdensis* Osterwalder (1924) [16], and *Saccharomyces inusitatus* van der Walt (1965) [19].

The two varieties described here were also differentiated by molecular karyotyping [7, 15]. Unlike *S. bayanus* var. *bayanus*, which has two chromosomes in the region 245–370 kb, *S. bayanus* var. *uvarum* has three or four chromosomes in this region. According to the karyotyping data [15], the type strain *S. tubiformis* CBS 431 and strain "*S. carlsbergensis*" CBS 1604 are assigned to *S. bayanus* var. *uvarum*, whereas the strain "*S. uvarum*" CBS 378 is assigned to *S. bayanus* var. *bayanus*. Earlier, these three strains were reidentified as *S. bayanus* based on the DNA–DNA reassociation data [20].

According to the earlier classification of *Saccharomyces* cultivars [1, 21], cryophilic wine yeasts *Saccharomyces bayanus* 'Uvarum' g. comprise one of the eight cultivar groups. The genetic and molecular genetic data presented here suggest a partial genetic isolation of this group from other yeasts of the species *S. bayanus*.

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