Antonie van Leeuwenhoek 53: 77–84 (1987)

Three newly delimited species of *Saccharomyces sensu stricto*

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Key words: *Saccharomyces sensu stricto*, taxonomy, DNA reassociation

Abstract: Deoxyribonucleic acid reassociation studies of 24 different wine and beer-associated strains of *Saccharomyces* confirmed the presence of three separate species. *S. cerevisiae* and *S. bayanus* strains had only 22% of their genomes in common. *S. pastorianus*, with intermediate hybridization values between *S. cerevisiae* and *S. bayanus* (52 and 72%, respectively) could possibly be a natural hybrid of the two species. *S. pastorianus* replaces *S. carlsbergensis* with which it is homologous for 93% of its genome, since the former species was described first by Hansen in 1904. These data do not agree with the results of traditional physiological tests.

Introduction

In the course of a study of the nDNA-nDNA relationships between some wine-associated species of the genus *Saccharomyces* [Rosini et al., 1982], it was seen that the type strains of *S. cerevisiae* and *S. bayanus*, previously considered synonymous [Yarrow & Nakase, 1975], were related only for 29.4% of their genomes. This was confirmed in an enlarged study [Vaughan Martini & Kurtzman 1985] of 24 different species of *Saccharomyces sensu stricto* [van der Walt 1970]. These authors also found that the type strain of *S. carlsbergensis* was characterized by intermediate homology values with both *S. cerevisiae* and *S. bayanus*. This led to the hypothesis that the large group of so-called synonyms of *S. cerevisiae* [Barnett et al. 1983; Yarrow 1984] actually included three or even more separate species. In the present study a greater number of strains of *S. carlsbergensis* from various habitats, as well as some commonly used strains from wine were investigated for nDNA-nDNA relatedness.

Materials and methods

Strains.

Twenty-four strains (Table 1), all obtained from the Centraalbureau voor Schimmelcultures (CBS) of Delft, Holland were studied.

Physiological studies.

Routine methods, as described by van der Walt and Yarrow (1984), were employed for studies of some fermentative and assimilative abilities.

Cell lysis.

Two different methods were alternatively employed for cell lysis. The autolytic method of Smith and Halvorson [1967] as modified by Meyer and Phaff [1969] was employed for most strains. Washed cells, resuspended in saline EDTA containing 2% sodium lauryl sulfate and 1% 2-mercaptoethanol to make a thick paste, were incubated under chloroform fumes for 16–18 hours. When the autolytic method failed to yield appreciable amounts of DNA, (as in the case of CBS 1503, CBS 429, and CBS 1538), an alternative mechanical method described by Martini [1973] and modified by Vaughan Martini [1983] was employed. This involved the agitation of lyophilized cells in round-bottom flasks together with stainless steel beads (0.5–1.0 cm diameter), at 150 rpm for 16–18 hours until 50–100% breakage was obtained. The powder was then rehydrated with ice-cold saline EDTA in order to prevent DNase activity.

DNA purification.

The purification of DNA was accomplished by a combination of the procedures of Marmur [1961] and Bernardi et al. [1970] as described by Price et al [1978].

DNA reassociation.

The extent of DNA reassociation was determined spectrophotometrically in a Gilford 250 spectrophotometer equipped with a Gilford 2527 Thermoprogrammer, using the method described by Kurtzman et al. [1980a].

Results

The strains studied are grouped in Table 1 under the species designation according to van der Walt [1970], i.e. '*S. cerevisiae*', '*S. uvarum*', and '*S. bayanus*'. This was done in order to avoid the confusion possibly originating from placing them under a single epithet [Yarrow 1984] as well as to correlate our results with the designations based on the discriminating criteria of conventional taxonomy. As expected the majority of the '*S. uvarum*' strains were positive on all sugars tested, even though they included strains of three different species (*S. cerevisiae*, *S. bayanus* or *S. pastorianus*), as revealed by nDNA-nDNA reassocia-

Table 1. Salient physiological reactions and homologus nDNA-nDNA relatedness within *Saccharomyces sensu stricto*

Organism (Original epithet)	CBS[1] Strain Designation	Isolation Source	Physiology[2]			Percentage nDNA-nDNA homology		
			GA[3]	RA[3]	ME[4]	1171[T5,6]	380[T7]	1538[T8]
'S. cerevisiae'								
S. willianus[T]	381	unknown	–	+	+w	–	98	–
S. lindneri[T]	403	ginger wine	+	+w	+w	87	–	–
S. steineri[T]	423	wine	+	–	–	95	–	–
S. tubiformis[T]	431	perry	+vw	+w	+	–	95	–
S. cerevisiae[T]	1171	beer	+	+	–	100	–	–
S. ellipsoideus[T]	1395	wine	+	+w	+w	91	–	–
S. intermedius var. *valdensis*[T]	1505	grape fruit	+	+	+	–	94	–
C. robusta[T]	1907	unknown	+	+w	+s	86	–	–
S. cerevisiae	6503	brewery	+	+	–	93	–	–
'S. uvarum'								
S. uvarum	378	beer	+	+w	+w	–	98	–
S. logos[T]	382	brewery	+	+	+	96	–	–
S. carlsbergensis	1260	unknown	+	+	+	–	–	99
S. carlsbergensis	1486	beer	+	+	–	–	–	100
S. monacensis[T]	1503	beer	+	+w	+ors	–	–	87
S. carlsbergensis[T]	1513	beer	+	+w	+ors	–	–	93
S. carlsbergensis	1604	perry	+	+	+	–	90	–
S. carlsbergensis	2354	grape must	+	+w	+	90	–	–
S. carlsbergensis	2444	distillery	+	+w	+	96	–	–
S. carlsbergensis	4734	sugar cane	+vw	+	+	97	–	–
'S. bayanus'								
S. bayanus[T]	380	beer	–	+w	–	–	100	–
S. odessa[T]	422	beer	–	+vw	–	96	–	–
S. oviformis[T]	429	grape must	+vw	+	–	87	–	–
S. cheresiensis[T]	1250	sherry	–	+w	+w	89	–	–
S. pastorianus[T]	1538	beer	–	+w	+w	–	–	100

[1] CBS = Centraalbureau voor Schimmelcultures; Delft, Holland
[2] Symbols: +w = weak; +s = slow; +vw = very weak; +ors = positive or slow
[3] Fermentation: GA = galactose; RA = raffinose
[4] Assimilation: ME = melibiose
[5] T = Type strain
[6] Type strain of *S. cerevisiae*
[7] Type strain of *S. bayanus*
[8] Type strain of *S. pastorianus*

tion results (Table 1). The strain CBS 1486, originally designated as *S. carlsbergensis*, showed the only negative value of the group in melibiose assimilation.

Galactose fermentation followed essentially the old species designations with

Table 2. Intermediate and low DNA-reassociation reactions within *Saccharomyces sensu stricto*

	S. cerevisiae									
CBS:	403	422	429	1171	1250	2354	2444	4734	6503	
Reassociation										Mean
CBS 380[Ta]	30	30	28	5	10	21	27	18	32	22
CBS 1538[Tb]	56	52	58	37	56	55	33	62	66	53
	S. bayanus									
CBS:	378		380	381		431	1505		1604	
Reassociation										Mean
CBS 1171[Tc]	3		5	28		28	26		17	18
CBS 1538[T]	78		76	80		68	77		61	73
	S. pastorianus									
CBS:	1260		1486	1503		1513	1538			
Reassociation										Mean
CBS 1171[T]	71		54	19		57	37			48
CBS 380[T]	67		74	72		72	78			71

[a] CBS 380 = type strain of *S. bayanus*
[b] CBS 1538 = type strain of *S. pastorianus*
[c] CBS 1171 = type strain of *S. cerevisiae*

the *'S. bayanus'* strains for the greater part negative, and *'S. cerevisiae'* and *'S. uvarum'* strains positive. Again, as in the case of *'S. uvarum'*, uniformity in these physiological tests did not agree with whole genome similarity, since strains of *'S. cerevisiae'* were highly homologous with the type strains of *S. cerevisiae* and *S. bayanus*; while *'S. bayanus'* included strains from all three species tested.

Our studies demonstrated the presence of three distinct DNA hybridization groups, each including highly related species (Table 1): *S. cerevisiae* (13 strains); *S. bayanus* (6 strains); and *S. pastorianus* (5 strains). Results of DNA reassociation experiments between the strains of the newly delimited species are reported separately in Table 2. Very low DNA-reassociation values were found between all strains of *S. cerevisiae* and *S. bayanus* (mean value: 20). Intermediate values characterized the DNA-relatedness between strains of the *S. pastorianus* group and the other two groups: *S. pastorianus* to *S. cerevisiae* mean value 55%; and *S. pastorianus* to *S. bayanus*, mean value 72%.

Discussion

The variability found in this study in fermentative and assimilative activity confirms the premise already expressed by previous authors [Price et al. 1978] that these tests, although perhaps useful for routine laboratory procedures, are not reliable enough for designation of yeasts at the species level. In addition, others have demonstrated the tendency of yeast strains to modify assimilative patterns after extended periods of storage in culture collection [Scheda & Yarrow 1968; Rosini et al. 1982]. Finally, it is widely recognized that a positive reaction in two different strains is not necessarily the result of identical biochemical pathways [Winge & Roberts 1958] and consequently of identical nucleotide sequences in nuclear DNA. The results of this study further emphasize the fact that most of the species of *Saccharomyces sensu stricto* can only be differentiated by the determination of DNA/DNA homologies and not by conventional physiological tests.

Consistently low nDNA-nDNA hybridizations between strains of the *S. cerevisiae* and *S. bayanus* groups, indicate that the two species are unrelated genetically. This agrees with the findings of Pedersen [1983] who separated the two species on the basis of results of a study on some brewing yeasts by restriction analysis and molecular hybridization of radioactively labeled yeast genes. Finally, two previous DNA-reassociation studies limited to type strains, also suggested this separation [Rosini *et al.*; 1982 Vaughan Martini & Kurtzman 1985]. The results of our study, based on 19 different strains of the two species, provide a larger body of evidence for the separation of the above species which have been classified as synonymous in recent years [Yarrow & Nakase 1975; Barnett et al. 1983; Yarrow 1984].

Intermediate values previously reported by Vaughan Martini and Kurtzman [1985] between the type strain of *S. carlsbergensis* CBS 1513 and those of *S. cerevisiae* and *S. bayanus* are largely confirmed in the present study (57 and 72% respectively, Table 2). *S. bayanus* shows somewhat higher affinity to *S. pastorianus* (which replaces *S. carlsbergensis* as the epithet) than does *S. cerevisiae:* 72 versus 52%. Phaff [1984] suggested that intermediate values such as those observed here could indicate more differentiated strains within a single species. Due to its intermediate position between two non-related groups, however, it seems difficult to extend this hypothesis to *S. pastorianus* which cannot concurrently belong to two different species.

These results find support in a series of studies on the differences in nucleotide sequences of the functionally undistinguishable chromosomes III and V of the S288C strain classified as *S. cerevisiae* and of a Carlsberg laboratory strain classified as *S. carlsbergensis*. Both significant deviations as well as significant similarities were found between the above strains [Kielland-Brandt et al. 1981; Holmberg 1982]. This appears in agreement with our results in two ways. Firstly,

the divergence observed between *S. cerevisiae* and *S. pastorianus* (ca. 48% of their genome), can possibly be accounted for by the demonstration by Kleiland-Brandt et al. [1981] and Holmberg [1982] that identical functions can be present in two organisms even when their nucleotide sequences differ significantly. It has long been known, in fact, that the degeneracy of the genetic code permits the coding of identical proteins even by somewhat different nucleotide sequences. Secondly, the intermediate relationships seen in this work between the *S. cerevisiae* and *S. pastorianus* groups, (52% homology) can be explained by the conservation of sequential similarities [Kielland-Brandt et al. 1981; Holmberg 1982]. Further studies could prove if this is also the case for the intermediate relationships observed between the *S. pastorianus* and *S. bayanus* groups. It seems probable that the differences and similarities found in just two chromosomes are likely to be present in other chromosomes as well.

In the light of the above observations, as well as from evidence of interspecific mating experiments within *Saccharomyces sensu stricto* [Winge & Laustsen 1938], it seems that *S. pastorianus* could be the result of a rare sexual interaction between *S. cerevisiae* and *S. bayanus* resulting in the formation of a stable hybrid genome different from both of the original parents. The possibility of interspecific recombination was in fact demonstrated by Johannsen and van der Walt [1978] using auxotrophic strains of *Kluyveromyces marxianus* and *K. thermotolerans*. Phaff [1984] postulated that in this case a conventional meiotic process does not occur at all, but that a process resembling protoplast fusion may take place, with subsequent degeneration of some genetic material, possibly resulting in an aneuploid nucleus. This does not seem to be the case here, however, since Spencer et al. [1985] demonstrated that the genome of interspecific hybrids always contains 90% or more of one parental DNA and very little (perhaps only a few genes or single chromosome) of the other. *S. pastorianus,* on the contrary, shows a more balanced distribution of both parental genomes (about 52 and 72% respectively).

An alternative explanation could involve the actual homologous sequences themselves between *S. cerevisiae* and *S. bayanus* (ca. 20%). Perhaps they are sufficiently distributed throughout the entire genome so as to permit a rare synapsis of nuclear DNA followed by a mismatch repair in favor of the *S. bayanus* parent, resulting in a hybrid which contains about 72% of one predominant parental genome and 52% of the other. An analogous situation was reported in a study by Kurtzman *et al.* [1980b] correlating DNA complementary and mating reactions between *Issatchenkia scutulata* var. *scutulata* and *I. scutulata* var. *exigua*. Even with a DNA relatedness of only 25%, crosses yielded 3–6% viable ascospores in the F1 generation and 17% in F2. From these results it was concluded that the lower limits of DNA-DNA homology values separating species are perhaps not yet well defined and that base sequences can diverge as much as 75% before genetic exchange can no longer occur.

Taxonomists generally agree, however, on the postulates of common ancestral origin and differentiation due to either chromosomal changes or progressive substitution in nucleotide sequences of the nuclear genome. Therefore, two organisms may be considered conspecific only when they have conserved a major portion of their genomes directly descending from a common ancestor. As a consequence, it seems necessary to confirm the re-establishment of *S. bayanus* [Saccardo 1895] as a separate secies as already suggested by Rosini et al. [1982], Pedersen [1983] and Vaughan Martini and Kurtzman [1985]. *S. pastorianus* [Hansen 1904] must also be reinstated because of its intermediate position between two distantly related species. S. *pastorianus* replaces *S. carlsbergensis,* recently reinstated by Vaughan Martini and Kurtzman [1985], since the two strains are homologous for 93% of their nucleotide sequences. According to the international Code of Botanical Nomenclature, the species described first (in this case *S. pastorianus*, described by Hansen in 1904 versus *S. carlsbergensis* [Hansen 1908], has precedence as the epithet. Mating studies, if successful, should help to confirm the possibility that *S. pastorianus* is truly a rare natural hybrid of *S. cerevisiae* and *S. bayanus.*

Acknowledgements

The authors thank Massimo Bellini for valuable technical assistance and Drs. L. Rodrigues de Miranda for kindly reviewing the manuscript.

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Received 20 November 1986; accepted in revised form 8 December 1986