Genetic Variability in the *Saccharomyces* Sensu Stricto Complex Revealed by Multilocus Enzyme Electrophoresis

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Fifty-two strains of the genus *Saccharomyces*, including 50 yeasts of the *Saccharomyces* sensu stricto complex (sensu Vaughan Martini 1989 [A. Vaughan Martini, Syst. Appl. Microbiol. 12:119–122, 1989]), were analyzed for nine isozyme loci with starch gel electrophoresis. The genetic analysis of isozyme data provided further evidence that the *Saccharomyces* sensu stricto complex is a genetically heterogeneous group. Twenty-two different electrophoretic types were identified and could be grouped into four clusters. However, no correlation between these clusters and the currently defined species *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, and *Saccharomyces paradoxus* was detected. Several strains could not be assigned to their putative cluster.

The criteria used in traditional yeast taxonomy are not always satisfactory for differentiation of species, in part because many separations are based on the presence or absence of assimilative abilities, which are often controlled by single mutable genes (15). Vaughan Martini (45) differentiated several species within the *Saccharomyces* sensu stricto group, while Yarrow (52) considered these as a single species (*S. cerevisiae*). This is a good example of this problem of strain variability in standard carbon assimilation and fermentation tests (3, 52). Other traditional methods also lead to ambiguous results (5).

Recently there have been several reports on the use of molecular techniques to distinguish between strains of the *Saccharomyces* sensu stricto group. These criteria include levels of rRNA and DNA relatedness (16, 36, 46), electrophoretic whole-cell protein patterns (44), restriction endonuclease patterns (17, 22, 28, 30), and electrophoretic karyotyping (23, 31, 49). Using DNA-DNA reassociation, Vaughan Martini et al. (46, 48) found that at least four species exist in the *Saccharomyces* sensu stricto complex: *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, and *S. pastorianus*. This technique has been widely used to define species limits and relationships. However, it lacks the precision required for the analysis of genetic relationships among closely related strains (37). Analysis of whole-cell protein patterns and restriction enzyme analysis of genomic DNA or genes coding for rRNA were largely limited to differentiation of strains (12). Restriction enzyme analysis of mitochondrial DNA has given good results in the past (6, 22, 34) but requires a large number of screenings in order to define a suitable restriction enzyme. Recently, Guillaumon et al. (9) have differentiated among the four species with four restriction enzymes. However, their results were not statistically checked. Previous studies of karyotypes of *Saccharomyces* strains were not always able to differentiate between *S. cerevisiae* and *S. bayanus* (13, 23). In other cases, chromosome fingerprinting was able to provide a definitive fingerprint for every strain employed (5). This method is reliable but relatively complex and time-consuming and requires sophisticated and expensive equipment. Moreover, it does not permit analysis of a large number of samples. Thus, chromosome fingerprinting would be inappropriate for rapid identification of yeasts.

None of these techniques is fast and specific enough to

differentiate strains. Multilocus enzyme electrophoresis (MLEE), which has long been a standard method in eucaryotic population genetics (1, 18, 24, 33, 38) and systematics (11, 25), could be used in large-scale studies to estimate the genetic diversity in natural populations of a variety of yeast species. In the case of the systematics of *Saccharomyces* species, some promising studies have been done. These studies differentiated between several strains of the same species, but the strains of the *Saccharomyces* sensu stricto and/or enzymatic systems investigated were too limited (10, 41, 51).

The present paper deals with the electrophoretic comparison of seven enzymes of *Saccharomyces* sensu stricto strains and discusses their relationships.

MATERIALS AND METHODS

Yeast strains. We studied 52 strains belonging to the genus *Saccharomyces* (Table 1). For clarity, species will be discussed according to the group divisions established in a recent study with partial rRNA sequence analysis of type strains (16). Accordingly, the genus can be divided into three groups: (i) *Saccharomyces* sensu stricto spp. (sensu Vaughan Martini 1989 [45]), which include *S. bayanus*, *S. cerevisiae*, *S. paradoxus*, and *S. pastorianus*; (ii) *Saccharomyces* sensu lato spp. (sensu Vaughan Martini 1988 [47]), comprising *S. dairensis*, *S. castellii*, *S. exiguus*, *S. servazzii*, and *S. unisporus*; and (iii) *S. kluyveri*.

Fifty strains were representatives of *Saccharomyces* sensu stricto spp. (52). Since it was important to include representatives of each species or group in a screening experiment, we used two genetically different species, *S. kluyveri* (group 3) and *S. servazzii* (group 2), as controls. Moreover, at least one strain of each subgroup of *Saccharomyces* sensu stricto spp. was used.

The identity of the strains was confirmed by numerical analysis of sugar assimilation patterns with the commercial kit ID32C (BioMérieux, Marcy l'Etoile, France).

Enzyme extracts. All yeast cells were cultured on Sabouraud medium at 27°C for 48 h. Cells were harvested by centrifugation at 1,000 × g and 4°C for 10 min, and then 1.6 g (wet weight) was suspended in approximately 1 ml of distilled water. Yeast cells were disrupted in a Braun cell homogenizer with 0.25-mm-diameter glass beads. The mixture was then centrifuged at 12,000 × g and 4°C for 5 min. The supernatant was distributed into 150-μl aliquots and stored at –20°C.

Electrophoresis and staining procedures. The enzymes (abbreviations in parentheses) studied were aspartate aminotransferase (*Aat*; EC 2.6.1.1), NADP⁺-glutamate dehydrogenase (*Gdh*; EC 1.4.1.4 [coenzyme NADP]), glucose-6-phosphate dehydrogenase (*G6pd*; EC 1.1.1.49), glucose phosphate isomerase (*Gpi*; EC 5.3.1.9), isocitrate dehydrogenase (*Iah*; EC 1.1.1.42), malate dehydrogenase (*Mdh*; EC 1.1.1.37), and peptidase 1 (*Pep 1*; EC 3.4.11.13; [substrate, L-valine-L-leucine]).

Enzymes were separated by electrophoresis in a horizontal starch gel. Starch gel electrophoresis is relatively inexpensive and can be used to screen a large number of strains and many different enzyme activities simultaneously. The resolution by this technique is adequate for the detection of isozymes coded by different loci. Starch gels were prepared as described by Pasteur et al. (27), with 40 g of hydrolyzed starch (Sigma, S4501) suspended in 400 ml of diluted electrophoresis buffer (4, 27). Staining was performed according to the methods of

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TABLE 1. *Saccharomyces* strains examined

Strain (UM1 no.)	Original epithet ^a	Other designation	Obtained from ^b	Isolation source	ET
386	<i>S. carlsbergensis</i>		ENSAM	Unknown	1
1298	<i>S. carlsbergensis</i>	ATCC 10596	UM1	Unknown	1
1642	<i>S. carlsbergensis</i>		IP	Unknown	1
4051	<i>S. cerevisiae</i>	CBS 2354	CBS	Grape must	1
7005	<i>S. carlsbergensis</i>		IP	Unknown	1
7023	<i>S. carlsbergensis</i>		ENSAM	Unknown	1
7015	<i>S. cerevisiae</i>		UL1	Unknown	2
7018	<i>S. uvarum</i>		ENSAM	Unknown	3
7019	<i>S. uvarum</i>		ENSAM	Unknown	3
7022	<i>S. carlsbergensis</i>		ENSAM	Unknown	3
389	<i>S. cerevisiae</i> var. <i>ellipsoideus</i>		ENSAM	Unknown	4
842	<i>S. cerevisiae</i>	CBS 2978	UM1	Distillery	4
1196	<i>S. cerevisiae</i>		ENSAM	Unknown	4
1197	<i>S. cerevisiae</i>		ENSAM	Unknown	4
1198	<i>S. cerevisiae</i>		ENSAM	Unknown	4
1295	<i>S. cerevisiae</i>	CBS 2978	ENSAM	Distillery	4
4056	<i>S. pastorianus</i>	CBS 1539	CBS	Grapes	4
7006	<i>S. cerevisiae</i>	CBS 2978	IP	Distillery	4
7007	<i>S. cerevisiae</i>		IP	Unknown	4
7010	<i>S. uvarum</i>	CBS 395 ^T	UL1	Berries	4
7017	<i>S. cerevisiae</i>	CBS 1512	ENSAM	Unknown	4
840	<i>S. cerevisiae</i>	CBS 1544	UM1	Fruit juice	5
7020	<i>S. cerevisiae</i>		ENSAM	Unknown	5
997	<i>S. cerevisiae</i>	CBS 1171 ^T	UM1	Beer (top yeast)	6
1000	<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	ATCC 560	UM1	Distillery	6
7014	<i>S. cerevisiae</i>	CBS 1171 ^T	UL1	Beer (top yeast)	6
1152	<i>S. cerevisiae</i>		UM1	Unknown	7
1153	<i>S. cerevisiae</i>		UM1	Unknown	7
7009	<i>S. cerevisiae</i>		UL1	Unknown	7
7012	<i>S. uvarum</i>		UL1	Unknown	8
998	<i>S. cerevisiae</i>		UM1	Unknown	9
1154	<i>S. cerevisiae</i>		UM1	Unknown	9
1155	<i>S. cerevisiae</i>		UM1	Unknown	9
7004	<i>S. cerevisiae</i>		IP	Unknown	10
7008	<i>S. cerevisiae</i>	CBS 679	IP	Unknown	10
4054	<i>S. pastorianus</i>	CBS 1260	CBS	Unknown	11
7021	<i>S. carlsbergensis</i>		ENSAM	Unknown	12
841	<i>S. cerevisiae</i>	CBS 2980	UM1	Unknown	13
1195	<i>S. cerevisiae</i>		ENSAM	Unknown	14
999	<i>S. cerevisiae</i>	ATCC 287	UM1	Lager beer	15
1755	<i>S. pastorianus</i>	CBS 1513 ^T	CBS	Beer	16
4055	<i>S. pastorianus</i>	CBS 1538 ^T	CBS	Beer	17
1753	<i>S. bayanus</i>	CBS 380 ^T	CBS	Beer	18
7013	<i>S. bayanus</i>	CBS 380 ^T	UL1	Beer	18
4049	<i>S. bayanus</i>	CBS 1604	CBS	Berries	19
4050	<i>S. bayanus</i>	CBS 7001	CBS	<i>Mesophylax adoperus</i>	19
7011	<i>S. bayanus</i>		UL1	Unknown	19
1754	<i>S. paradoxus</i>	CBS 432 ^T	CBS	Unknown	20
4052	<i>S. paradoxus</i>	CBS 406	CBS	Oak exudate	20
4053	<i>S. paradoxus</i>	CBS 5829	CBS	Soil	20
1640	<i>S. servazzii</i>	CBS 4311 ^T	UM1	Soil	21
1641	<i>S. kluyveri</i>	CBS 4798	UM1	Unknown	22

^a *S. cerevisiae* var. *ellipsoideus* is actually synonymous of *S. cerevisiae*, *S. uvarum* is synonymous of *S. cerevisiae* (2) or *S. bayanus* (22, 36), and *S. carlsbergensis* is actually a synonym of *S. pastorianus*.

^b ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Delft, Holland; ENSAM, Ecole Nationale Supérieure d'Agronomie de Montpellier, P. Galzy, Laboratoire de Génétique Microbienne, ENSAM, France; IP, Institut Pasteur, Paris, France; UL1, J. Villard, Laboratoire de Mycologie Fondamentale et Appliquée aux Biotechnologies Industrielles, Université Lyon 1, France; UM1, J.-M. Bastide, Laboratoire d'Immunologie et de Parasitologie, Université Montpellier 1, France.

Pasteur et al. (27), Ben Abderrazak et al. (4), Shaw and Prasad (39), and Richardson et al. (35), with slight modifications.

Analysis of data. Comparison of the mobilities of the enzymes from different isolates was made visually against one another on the same gel slice. Because the net electrostatic charge, and hence the rate of migration of a protein during electrophoresis, is determined by its amino acid sequence, each band can be directly equated with an allele of a specific locus. Bands are numbered in order of decreasing anodal mobility.

Isolates were grouped into electrophoretic types (ETs) with identical multilocus genotypes (i.e., a combination of alleles at all the loci). Allelic frequencies

were used to calculate genetic distances between pairs of ETs with the index proposed by Thorpe (42). Gregorius (8) and Katz and Goux (14) consider this method of calculating genetic distances to be the most appropriate method for analysis of electrophoretic data because it is the only one which satisfies the axioms characteristic of a metric distance. Furthermore, because the distribution of its estimator fits the normal distribution, we were able to statistically test the significance of distance between pairs of ETs. Dendrograms were derived from the distance matrices by using the unweighted pair-group method with arithmetic averages algorithm of Sneath and Sokal (40) as computed with the RESTSITE version 1.1 package (21a).

FIG. 1. Dendrogram of the 52 *Saccharomyces* strains, constructed by the unweighted pair-group method with arithmetic averages from a Gregorius distance matrix, obtained by MLEET. 1 to 22, ETs (see Table 1). ETs represent the following species (asterisks indicate type strains): *S. bayanus*, ETs *18 and 19; *S. carlsbergensis*, ETs 1, 3, and 12; *S. cerevisiae*, ETs 1, 2, 4, 5, *6, 7, 9, 10, 13, 14, and 15; *S. kluyveri*, ET 22; *S. paradoxus*, ET *20; *S. pastorianus*, ETs 4, 11, 16, and *17; *S. servazzii*, ET *21; *S. uvarum*, ETs 3, 4, and 8.

In addition, several measures of intraspecific diversity were calculated. They included the percentage of polymorphic loci (i.e., number of loci having more than one variant divided by the total number of loci [7]) and the average number of alleles per locus. Genotypic diversity was calculated as the number of distinct ETs found in a species or population (37).

RESULTS

Overall genetic diversity. Isocitrate dehydrogenase and malate dehydrogenase activities were each expressed at two loci: *Idh-1* and *Idh-2* and *Mdh-1* and *Mdh-2*. Locus 1 had the fastest electrophoretic migration, while the slower one was coded 2. Data were obtained for nine loci, all of which were polymorphic (polymorphic rate, 1.0), yielding 22 ETs: 12 of these were represented by only one strain. The overall genotypic diversity was 0.42. The number of alleles per polymorphic locus ranged from 4 to 8, with a mean of 5.1 per locus.

The genetic relationships among the *Saccharomyces* strains surveyed are represented in a dendrogram (Fig. 1). As expected, *S. servazzii* and *S. kluyveri* were separated from the *Saccharomyces sensu stricto* strains, with genetic distances of 0.896 and 0.985, respectively. The remaining 50 strains fell into four distinct clusters. The distances separating the clusters were greater than 0.6.

The first cluster is very heterogeneous. It includes all but one of the *S. cerevisiae* strains and also eight *S. carlsbergensis* strains (UM1 386, 1298, 1642, 4051, 7005, 7021, 7022, and 7023), two *S. cerevisiae* var. *ellipsoideus* strains (UM1 389, and 1000), four *S. uvarum* strains (UM1 7010, 7012, 7018, and 7019), and two *S. pastorianus* strains (UM1 4054 and 4056). Because this cluster contains the type strain of *S. cerevisiae* (UM1 997 = CBS 1171^T), it is designated *S. cerevisiae*. It includes two distinct subclusters (ETs 1, 2, and 3 and ETs 4 to 9). The overall isozyme patterns in each subcluster were similar: they differed by only one or two alleles. Two other *S. pastorianus* strains (UM1 1755 and 4055) and one *S. cerevisiae* strain (UM1 999) belong in the second cluster.

The five strains belonging to the third cluster (UM1 1753,

TABLE 2. Significance of the genetic distances between strains

Location	ET(s) involved	% of ^a :	
		S/T	NS/T
Inside <i>S. cerevisiae</i> cluster	1, 6, 11, 13	13.3	86.7
Between <i>S. pastorianus</i> and <i>S. cerevisiae</i> cluster	15, 17 1, 6, 11, 13	21.9	78.1
Between <i>S. bayanus</i> and <i>S. cerevisiae</i> cluster	19 1, 6, 11, 13	15.5	84.5
Between <i>S. paradoxus</i> and <i>S. cerevisiae</i> cluster	20 1, 6, 11, 13	24.4	75.6

^a S, number of significant combinations in which given ETs were involved (i.e., when the two distances compared were significantly different from each other); NS, number of nonsignificant combinations in which given ETs were involved; T, total number of combinations in which given ETs were involved.

4049, 4050, 7011, and 7013) were all defined as *S. bayanus*. The fourth and last cluster included all of the *S. paradoxus* strains studied (UM1 1754, 4052, and 4053).

These three clusters are designated, respectively, *S. pastorianus*, *S. bayanus*, and *S. paradoxus* with regard to the type strains they include.

We have calculated the statistical significance of the genetic distances obtained among the eight strains selected in order to have one or more examples from each cluster. The comparisons were made on the one hand within the *S. cerevisiae* cluster and on the other hand between each cluster (as defined below) and the *S. cerevisiae* cluster. The results are regrouped in Table 2. The data show that genetic distances between the four clusters are not statistically different. Therefore, these clusters or groups were just a nonrigorous representation of observed variances at the allelic level.

Genetic diversity in each group. For the four groups (*S. cerevisiae*, *S. bayanus*, *S. pastorianus*, and *S. paradoxus*) the percentage of polymorphic loci, the mean number of alleles per locus, and the genotypic diversity were determined to estimate the relative diversity for each of them (Table 3). Results were not significantly different between them, except for the *S. pastorianus* group, but the number of strains studied was too small to be significant.

TABLE 3. Comparison of diversity values between *Saccharomyces* strains

Species or group	No. of strains	ET	Polymorphic rate ^a	Mean no. of alleles/locus	Genotypic diversity ^b
<i>Saccharomyces</i> spp.	52	22	1.00	5.10	0.42
<i>Saccharomyces sensu stricto</i> group	50	20	1.00	3.44	0.40
<i>S. cerevisiae</i>	39	14	1.00	2.67	0.36
<i>S. bayanus</i>	5	2	0.22	1.22	0.40
<i>S. paradoxus</i>	3	1	0.00	1.00	0.33
<i>S. pastorianus</i>	3	3	0.44	1.78	1.00

^a Polymorphic rate, number of loci having more than one variant divided by the total number of loci (7).

^b Genotypic diversity, number of distinct ETs found in a species or population (37).

FIG. 2. Isozyme variation in various *Saccharomyces* strains for the enzyme glucose phosphate isomerase. Lanes: 1, 10, and 17, *S. uvarum* 7012 (ET 8), 7010 (ET 4), and 7018 (ET 3), respectively; 2 and 13, *S. carlsbergensis* 386 (ET 1) and 7021 (ET 12); 3, 5, 6, 7, 8, and 9, *S. cerevisiae* 1000 (ET 6), 998 (ET 9), 1152 (ET 7), 1154 (ET 9), and 7004 (ET 10); 4, *S. paradoxus* 1754 (ET 20); 11 and 16, *S. pastorianus* 4054 (ET 11) and 4056 (ET 4); 12 and 15, *S. bayanus* 1753 (ET 18) and 7011 (ET 19); and 14, *S. servazzi* 1640 (ET 21). Several three-banded patterns are seen, which suggests that this enzyme has a dimeric structure and that the corresponding strains are heterozygous for this locus.

DISCUSSION

The genetic interpretation of banding patterns was consistent with that of diploid organisms. The commonly accepted notion of *S. cerevisiae* as a diploid is supported by the observation of typical heterozygous patterns in the enzyme assays (Fig. 2). However, some strains should be aneuploids (31). In particular, the UM1 1753^T and 7013^T (CBS 380^T) strains have been claimed to be trisomic aneuploids (13). However, this aneuploidy didn't appear in the isoenzymatic patterns studied and probably involves loci other than the ones studied by us.

The results obtained by MLEE were reproducible: enzyme extracts obtained at different times from separate cultures of the same strain showed the same profiles.

Our analysis of isozyme polymorphism provided further evidence that *S. cerevisiae* Meyen ex. Hansen (also referred to as *Saccharomyces sensu stricto* spp. [20]) represents a genetically heterogeneous group that is different from two other species of the genus *Saccharomyces*, *S. kluyveri* and *S. servazzi*.

The four clusters observed on the dendrogram (Fig. 1) were referred to as *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, and *S. paradoxus* in relation to the type strains that were included. In the first cluster (*S. cerevisiae* cluster), no correlation was observed between the different subclusters and denominations of the strains (*S. cerevisiae*, *S. cerevisiae* var. *ellipsoideus*, *S. uvarum*, and *S. carlsbergensis*), described previously as different species (before their inclusion in the new circumscribed *S. cerevisiae* species [3]). Moreover, some strains previously described as belonging to other groups turned up in the *S. cerevisiae* cluster. These were *S. uvarum* strains (UM1 7010, 7012, 7018, and 7019), which were previously described as synonymous with *S. bayanus* (22, 36), although Barnett et al. (3) placed them in synonymy with *S. cerevisiae*. Brewing lager strains (5) were also described under different epithets: *S. cerevisiae* (*uvarum*) (20), *S. cerevisiae* var. *uvarum* (Casey), or *S. uvarum* (*carlsbergensis*) (26). Thus, the designation of *S. uvarum* strains was not precisely defined. Another possibility is that the designation of these strains was based on the assimilation of melibiose. *S. uvarum* strains are considered to be able

TABLE 4. Multilocus genotypes of the 52 *Saccharomyces* strains identified with nine polymorphic loci

ET ^a	Allele(s) shared								
	<i>Aat</i>	<i>G6pd</i>	<i>Gdh</i>	<i>Gpi</i>	<i>Idh-1</i>	<i>Idh-2</i>	<i>Mdh-1</i>	<i>Mdh-2</i>	<i>Pep 1</i>
1	5	5	2	4	2	3	3	4	5
2	5	7	2	4	2	3	3	4	5
3	5	5	2	5	2	3	3	4	5
4	5	6	2	4	2	3	3	4	5
5	5	6	2	4	2	3	3	4	4/5 ^c
6	5	6	2	4/5	2	3	3	4	5
7	5	6	2	3/4	2	3	3	4	5
8	5	6	2	2/4	2	3	3	4	4/5
9	5	6	2	3	2	3	3	4	5
10	5	6/8	3	2/4	2	3	3	4	3/5
11	5	6/8	2	2/4	4	3	3	3	5
12	5	6/8	2	2/4	3	3	3	4	5
13	2	6	3	4	2	4	3	4	5
14	5	2	3	4	2	3	3	4	5
15	4	6/8	2	2/4	3	3	3	2	4
16	4	6/8	3	2/4	3	3	3	2	4
17	4	6/8	2	2	0 ^b	3	3	3	4
18	4	8	3	2	4	3	2	4	3/4
19	4	4	3	2	4	3	2	4	3
20	3	7	3/4	4	2	4	3	5	4
21	3	4/6	4	1	5	1	4	6	2
22	1	1/3	1	2	1	2	1	1	1

^a ETs are ordered as in Fig. 1.

^b Note that 0 means the lack of activity for enzyme under study, but it has been interpreted as an allele quite different from the alleles observed.

^c Homozygotes a/a are noted as a; heterozygotes are noted as a/b.

to assimilate this sugar, although some reports have shown that other *Saccharomyces sensu stricto* species can be Mel⁺ (23, 43).

The other strains that turned up in the *S. cerevisiae* cluster were *S. carlsbergensis* strains (UM1 386, 1298, 1642, 4051, 7005, 7021, 7022, and 7023) and *S. pastorianus* strains (UM1 4054 and 4056). The UM1 4056 strain was described as belonging to the *S. cerevisiae* cluster (12), while UM1 4054, previously described as *S. carlsbergensis*, was later included in the *S. pastorianus* group (43). However, in earlier studies (52), *S. carlsbergensis* and *S. pastorianus* were both considered to be synonyms of *S. cerevisiae*.

The *S. pastorianus* cluster includes one *S. cerevisiae* strain (UM1 999 = ATCC 287), described as a lager beer yeast strain by the American Type Culture Collection. It could be related to *S. carlsbergensis* and *S. pastorianus* strains, according to Pedersen (29). However, Pedersen (30, 31) later demonstrated that lager beer strains (*S. carlsbergensis*) were genetically distant from *S. uvarum*. Furthermore, other lager beer strains were included in the *S. cerevisiae* cluster (described above). The taxonomic status of *S. uvarum* strains remains unresolved.

The genetic distances observed between the four clusters were considerable (>0.6). However, they were not statistically different, and the observed clusters could not be regarded as distinct species on the basis of these results. This is partly because the enzymes studied here involve only a small fraction of the genome (approximately 30% [21]). Moreover, it could be caused by the low number of loci analyzed, particularly in view of the fact that five of nine loci (*Gdh*, *Idh-1*, *Idh-2*, *Mdh-1*, and *Mdh-2*) shared one or two of the same alleles. Nevertheless, the observed groups remain quite different with regard to their multilocus genotypes (Table 4).

The confrontation between strain designations and observed clusters showed that many of the original strain designations

were not exactly correct, as observed by other authors (12, 30, 31, 43). This is due to the fact that the genus *Saccharomyces* has been frequently revised with respect to the composition of its members, and numerous species have been reclassified as synonyms (2). The realization that a single gene can confer on a yeast the ability to ferment or assimilate a sugar (19, 50) raises the possibility that species might be inadvertently and artificially established simply on the basis of the function of one or a few genes (32).

Because of the difficulty in increasing the number of loci analyzed, MLEE may not be a useful tool for the taxonomy of the *Saccharomyces sensu stricto* group. However, this method has an advantage compared with other methods in that it could be used in large-scale studies for the estimation of the genetic diversity and structure within natural populations or species of the *Saccharomyces sensu stricto* group and other species. A second goal of MLEE study is to identify a number of isozymes and their coding alleles unique to each group of isolates that can be used for identification. It seems that allele 2 of *Mdh-1* is characteristic of the *S. bayanus* group, but further analysis with a larger number of strains and loci is needed.

ACKNOWLEDGMENTS

We thank P. Galzy, S. Jouvert, J. Villard, and Institut Pasteur (Paris) for kindly providing yeast strains and D. Castel for technical assistance. Special thanks go to C. Pujol and F. Renaud for helpful discussions.

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