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MEL Gene Polymorphism in the Genus *Saccharomyces*

HILKKA TURAKAINEN,[1] SIRPA AHO,[2] AND MATTI KORHOLA[2]*

Department of Genetics, University of Helsinki, SF-00100 Helsinki,[1] and Research Laboratories, Alko Ltd., P.O. Box 350, SF-00101 Helsinki,[2] Finland

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In *Saccharomyces* spp. the ability to use melibiose depends on the presence of a *MEL* gene encoding α-galactosidase. We used two cloned *MEL* genes as probes to characterize the physical structure and chromosomal location of the *MEL* genes in several industrial and natural Mel$^+$ strains of *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, and *Saccharomyces bayanus*. Electrokaryotyping showed that all of the *S. pastorianus* strains and most of the *S. bayanus* strains studied had one *MEL* locus. The *MEL* gene in *S. bayanus* strains was similar but not identical to the *S. pastorianus MEL* gene. Mel$^+$ *S. cerevisiae* strains had one to seven loci containing *MEL* sequences. The *MEL* genes of these strains could be divided into two categories on the basis of hybridization to *MEL1*, one group exhibiting strong hybridization to *MEL1* and the other group exhibiting weak hybridization to *MEL1*. In *S. pastorianus* and *S. bayanus* strains, the *MEL* gene was expressed as a single 1.5-kb transcript, and the expression was galactose inducible. In some *S. cerevisiae* strains, the *MEL* genes were expressed even without induction at fairly high levels. Expression was usually further induced by galactose. In two strains, CBS 5378 and CBS 4903, expression of the *MEL* genes was at the same level without induction as it was in most other strains with induction. In all *S. cerevisiae* strains, irrespective of the number of *MEL* genes, mRNA of only one size (1.6 kb) was observed.

Most strains of *Saccharomyces* spp. are not able to ferment melibiose. Some strains produce α-galactosidase and are able to utilize melibiose for growth and fermentation (5, 10). Previous genetic studies (6, 13, 24, 28, 39) suggested that a *MEL* gene family is present in *Saccharomyces* spp. Preliminary studies whose results suggest that the *MEL* gene is polymorphic have been reported for brewing and distilling yeast strains (31, 32). Recently, we described nine new *MEL* loci, five of which were found in one strain (19) and seven of which were found in another strain (18), putting the total number of defined *MEL* loci in *Saccharomyces cerevisiae* at 10. Gene families have also been shown to control the fermentation of maltose, methyl α-glucoside, sucrose, and starch (for a review, see reference 2).

So far, two *MEL* genes from yeasts have been cloned and sequenced, a *MEL1* gene from a nonbrewing strain (11, 23, 25, 29) and a *MEL* gene from the type strain of *Saccharomyces carlsbergensis*, NCYC 396 (33). *MEL* genes from the bacterium *Escherichia coli* (12), the mold *Aspergillus* sp. (4), the plant *Cyamopsis tetragonolobe* (20), and humans (1) have also been cloned and sequenced.

The ability to utilize melibiose has been used in yeast taxonomy to differentiate *Saccharomyces* species (34), but this classification technique has been shown to be questionable (40). In this study cloned yeast *MEL* genes were used as molecular probes to study the number, physical structure, and chromosomal location of the different *MEL* genes in Mel$^+$ strains belonging to the genus *Saccharomyces* and closely related genera. This study was undertaken to elucidate the polymorphism of the *MEL* gene families in the genus *Saccharomyces*. We also wanted to determine whether knowledge of the *MEL* gene families could help us understand the taxonomic relationships within the *Saccharomyces* sensu stricto species *Saccharomyces bayanus*, *S. cerevisiae*, and *Saccharomyces pastorianus* (synonym, *S. carlsbergensis*) (35, 36).

* Corresponding author.

Since α-galactosidase is a useful enzyme with applications in the food industry, we studied its expression levels in several Mel$^+$ strains of different origins in order to determine whether some *MEL* genes were more suitable for industrial utilization than other *MEL* genes.

MATERIALS AND METHODS

Yeast strains and growth media. The yeast strains studied are shown in Table 1. The yeasts were grown in YPD medium (1% Bacto yeast extract [Difco], 2% Bacto peptone [Difco], 2% glucose). Nutrient agar plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal) (Sigma Chemical Co., St. Louis, Mo.) were used to differentiate Mel$^+$ colonies from Mel$^-$ colonies (30).

Restriction analysis of DNA. Total DNAs were prepared from the yeast strains by the method described by Sherman et al. (27). Restriction endonucleases obtained from Boehringer, Mannheim, Germany, or New England Biolabs, Beverly, Mass., were used as described by Maniatis et al. (14). DNA fragments were separated by gel electrophoresis in 0.5% agarose gels.

Preparation of chromosomes. The basic procedure used to prepare chromosomes was that of Schwartz and Cantor (26). Yeast cells were grown in YPD medium at 30°C overnight, harvested, and washed with 50 mM EDTA (pH 7.5). The final pellet was suspended in 0.5 ml of a solution containing 1.2 M sorbitol, 0.1 M sodium citrate, 0.06 M EDTA, 25 μg of β-mercaptoethanol per ml, and 20 mg of Zymolyase (Seikagaku Kogyo, Tokyo, Japan) per ml. A 1-ml portion of a 1% low-melting-point agarose preparation (prepared in 0.125 M EDTA [pH 7.5] and cooled to 42°C) was added to the solution, and the mixture was allowed to gel at 4°C, overlaid with a suspension solution, and incubated at 37°C overnight. The overlay was then replaced with 1 ml of a solution containing 0.5 M EDTA, 0.01 M Tris (pH 7.5), 1% *N*-lauroyl sarcosine, and 2 mg of proteinase K (Sigma) per ml, and the tubes were incubated at 37°C for 7 h. The overlay then was

TABLE 1. Yeast strains studied

Taxon[a]	Strain[b]	Mel phenotype[c]	Isolation source
S. cerevisiae species			
S. logos	CBS 382^T	+	Brewery
S. logos	CBS 2444	+	Distillery
S. logos	CBS 4734	+	Sugar cane
S. coreanus	CBS 5635	+	Grape must
S. hienipiensis	CBS 4903^T	+	Alpechin
S. hispalensis	CBS 7002^T	+	Alpechin
S. oleaceous	NRRL Y-12056	+	Olives
S. oleaceous	CBS 3093^T	+	Olives
S. oleaginosus	NRRL Y-12057^T	+	
S. oleaginosus	CBS 3081^T	+	Alpechin
S. norbensis	CBS 5378^T	+	Alpechin
S. carlsbergensis	CECT 1323	+	
S. carlsbergensis	ALKO 1228	+	Distillery
S. uvarum	ATCC 9080	+	
S. cerevisiae	CBS 4411	+	Pig intestine
S. cerevisiae	VKM Y-1830	+	Grapes
S. lindneri	CBS 403	−	Ginger wine
S. ellipsoides	CBS 1395	−	Wine
S. robusta	CBS 1907	−	
S. cheresiensis	CBS 1250	−	Sherry
S. bayanus species			
S. bayanus	CBS 380^T	+	Beer
S. uvarum	CBS 395^T	+	Berries
S. heterogenicus	CBS 425^T	+	Fermenting apple juice
S. intermedius var. *valdenis*	CBS 1505	+	Grape juice
S. inusitatus	CBS 1546	+	Beer
S. carlsbergensis	CBS 1604	+	Berries
S. abuliensis	CBS 7001	+	*Mesophylax adoperus*
S. uvarum	CBS 378	−	
S. willianus	CBS 381^T	−	Beer
S. globosus	CBS 424	−	Pear juice
S. globosus	CBS 3008	−	Must
S. tubiformis	CBS 431^T	−	Fermenting pear juice
S. pastorianus species			
S. carlsbergensis	CBS 1260	+	
S. carlsbergensis	CBS 1486	+	Beer
S. carlsbergensis	CBS 1513^T (= NCYC 396^T)	+	Beer
S. pastorianus	CBS 1538^T	+	Beer
S. monacensis	CBS 1503^T	+	Beer
Unclassified *Saccharomyces* sp.			
Lager strain	NCYC 1146	+	Brewery
Lager strain	NCYC 1324	+	Brewery
Lager strain	ALKO 1350	+	Brewery
Lager strain	ALKO 1353	+	Brewery
Lager strain	ALKO 1356	+	Brewery
Other taxa			
S. kluyveri	CBS 4569	+	
Zygosaccharomyces cidri	CBS 5666	+	Rotting bark, *Araucaria* sp.
Z. cidri	CBS 4575^T	+	Cider
Schwanniomyces occidentalis	ATCC 26074	+	Soil

[a] The strains were grouped under the species *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* on the basis of DNA-DNA reassociation data (16a, 35, 36) but listed with their original epithets.

[b] ALKO, Collection of Alko, Ltd., Helsinki, Finland; ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; CECT, Coleccion Espanola de Cultivos Tipo, Valencia, Spain; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom; NRRL, Northern Regional Research Center, Peoria, Ill.; VKM, All-Union Collection of Microorganisms, Moscow, Russia. T, type strain.

[c] Mel phenotype was determined on YPD medium plates in the presence of X-α-gal (30).

replaced with 0.5 M EDTA (pH 9), and the tubes were stored at 4°C. Agarose slices were cut, washed in 0.05 M EDTA, and placed into a well in a 1% agarose gel for separation of chromosomes by contour-clamped homogeneous electric field gel electrophoresis (CHEF-GE).

CHEF-GE. A model CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) was used to separate the chromosomal DNAs by CHEF-GE. Electrophoresis was carried out in 1% agarose according to the instructions of the manufacturer. The electrophoresis buffer (0.5× TBE [1× TBE is 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA; pH 8.0]) was circulated around the gel and cooled to 11°C by passing it through a coil of silicone tubing placed in a thermostat-controlled water bath. Electrophoresis was performed at 200 V for 15 h with a switching time of 60 s and then for 9 h with a switching time of 90 s.

Southern blot analysis and hybridization. A Southern blot analysis of yeast DNA was carried out essentially as de-

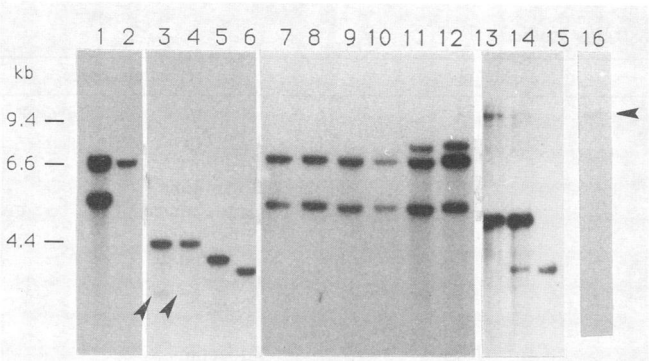

FIG. 1. Southern hybridization analysis of DNAs from 15 *S. cerevisiae* strains. Genomic DNAs from strains CBS 7002^T (lane 1), CBS 5635 (lane 2), ALKO 1228 (lane 3), CBS 2444 (lane 4), CBS 382^T (lane 5), CBS 4734 (lane 6), NRRL Y-12056 (lane 7), NRRL Y-12057 (lane 8), CBS 3081^T (lane 9), CBS 3093^T (lane 10), CBS 4903^T (lane 11), CBS 5378^T (lane 12), ATCC 9080 (lane 13), CECT 1323 (lane 14), CBS 403 (lane 15), and NCYC 396^T (*S. pastorianus*) (lane 16) were digested with *Eco*RI, subjected to electrophoresis in an agarose gel, transferred to nitrocellulose filters, and hybridized with a *MEL1* probe. Lanes 3 through 6 and 13 through 16 were exposed longer (6 days) than the other lanes (1 day). The fragment sizes of an M_r standard are indicated on the left. The arrowheads in lanes 3, 4, and 16 indicate DNA fragments that exhibited weak hybridization.

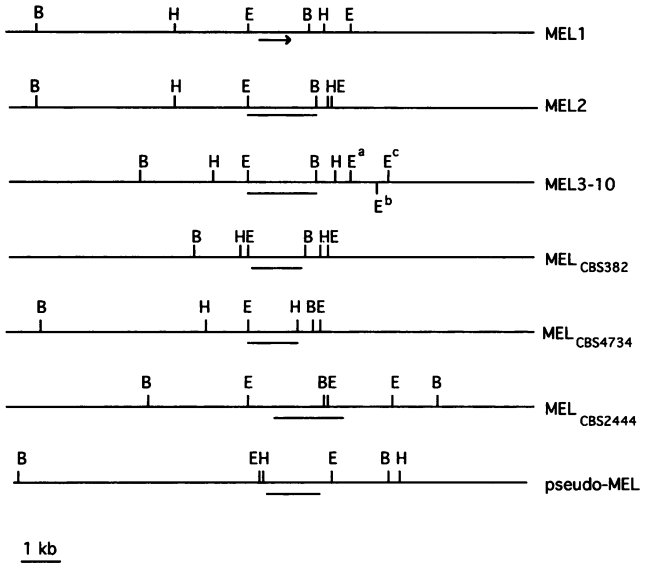

FIG. 2. Restriction maps of the *S. cerevisiae MEL* loci. The maps are based on data obtained from hybridization to the *MEL1* probe. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The arrow indicates the location and direction of transcription of the *MEL1* gene. The horizontal lines below the maps indicate the locations of other *MEL* genes. Strains CBS 3081^T, NRRL Y-12056 (*S. oleaceous*), CBS 3093^T, NRRL Y-12057 (*S. oleaginosus*), CBS 4039^T (*S. hienipiensis*), CBS 7002^T (*S. hispalensis*), and CBS 5635 (*S. coreanus*) have the same restriction map as *MEL3-10*. The polymorphic *Eco*RI sites E^a, E^b, and E^c were distributed as follows: E^a, CBS 4411 (*MEL5*), CBS 5378 (*MEL5*, *MEL7*, *MEL9*), CBS 3081, NRRL Y-12056, CBS 3093, NRRL Y-12057, CBS 4903, and CBS 7002; E^b, CBS 4411 (*MEL3*, *MEL6*), CBS 5378 (*MEL3*, *MEL4*, *MEL6*, *MEL10*), CBS 3081, NRRL Y-12056, CBS 3093, NRRL Y-12057, CBS 4903, CBS 7002, and CBS 5635; E^c, CBS 4411 (*MEL4*), CBS 5378 (*MEL8*), and CBS 4903. Pseudo-*MEL* was found in strains CECT 1323 and CBS 403.

scribed by Maniatis et al. (14). After the agarose gels were soaked in 0.25 M HCl for 30 min, the chromosomal DNA separated by CHEF-GE was denatured, neutralized, and transferred to nitrocellulose filters. The probes used for hybridizations were prepared by using in vitro transcription (16) with an [α-^{32}P]UTP (Amersham International, Amersham, United Kingdom) label. The *MEL1* probe was prepared from the 2.8-kb *Bam*HI-*Sal*I insert (25) in plasmid pGEM3, and the NCYC 396 *MEL* probe was transcribed from a 2.5-kb template containing the *MEL* coding region, 0.3 kb of the 5′ flanking region, and 0.8 kb of the 3′ flanking region in plasmid pIBI76 (33).

Northern blot analysis. Total RNA was isolated from the yeast cells as described by McAlister and Finkelstein (15). For Northern (RNA) blot analysis the RNA was denatured with 48% formamide and 6% formaldehyde, subjected to electrophoresis in a gel containing 1% agarose and 2% formaldehyde, and transferred to nitrocellulose (3).

Determination of α-galactosidase activity. Nutrient agar plates containing X-α-gal were used to differentiate Mel$^+$ strains from Mel$^-$ strains (30). To measure α-galactosidase activity, yeast cells were grown on YPD medium which contained 3% glycerol and 2% ethanol instead of glucose in the absence (noninducing conditions) or presence (inducing conditions) of 2% galactose or on YPD medium (repressing conditions). The enzyme activity was measured in the culture medium and in the cells as described by Kew and Douglas (9).

RESULTS

***S. cerevisiae MEL* genes.** Total DNAs isolated from yeast strains were digested with restriction endonucleases and subjected to a Southern hybridization analysis with ^{32}P-labelled *MEL* probes. An autoradiograph of the DNA fragments obtained after *Eco*RI digestion and hybridization with the *MEL1* probe is shown in Fig. 1. The Mel$^+$ *S. cerevisiae* strains were clearly divided into two categories, one exhibiting strong hybridization to the *MEL1* probe and one exhibiting weak hybridization to the *MEL1* probe. *S. pastorianus* NCYC 396^T exhibited very weak hybridization under the conditions used (Fig. 1, lane 16, arrowhead). The following strains were members of the *MEL1* gene family (i.e., strains whose DNAs hybridized strongly to the *MEL1* probe): strains ATCC 9080 (*MEL1* donor) (Fig. 1, lane 13), CBS 7002^T (*Saccharomyces hispalensis*) (lane 1), CBS 5635 (*Saccharomyces coreanus*) (lane 2), NRRL Y-12056 and CBS 3093^T (*Saccharomyces oleaceous*) (lanes 7 and 10), NRRL Y-12057 and CBS 3081 (*Saccharomyces oleaginosus*) (lanes 8 and 9), CBS 4903^T (*Saccharomyces hienipiensis*) (lane 11), CBS 5378^T (*Saccharomyces norbensis*) (lane 12), and CECT 1323 (*S. carlsbergensis*) (lane 14). The DNAs of three of the four Mel$^-$ *S. cerevisiae* strains (CBS 1250, CBS 1395, CBS 1907) did not hybridize to the *MEL1* probe (data not shown), whereas one strain (CBS 403) exhibited weak hybridization (Fig. 1, lane 15). The lack of hybridization in several Mel$^-$ strains suggested that the sequences flanking the *MEL1* gene present in our probe did not interfere in the analysis of *MEL* genes. Hybridization of the *S. cerevisiae* DNA fragments to the NCYC 396^T *MEL* probe was very weak (data not shown). The results of the restriction enzyme-generated DNA fragment analysis are summarized in Fig. 2.

The *MEL* genes of the *S. oleaceous* strains (Fig. 1, lanes 7

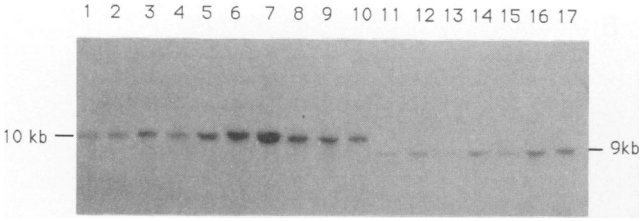

FIG. 3. Southern hybridization analysis of DNAs from *S. pastorianus* (lanes 1, 2, and 8 through 10), *S. bayanus* (lanes 11 through 17), and unclassified *Saccharomyces* strains (lanes 3 through 7). Genomic DNAs from strains NCYC 396^T (lane 1), CBS 1503^T (lane 2), NCYC 1146 (lane 3), NCYC 1324 (lane 4), ALKO 1350 (lane 5), ALKO 1353 (lane 6), ALKO 1356 (lane 7), CBS 1260 (lane 8), CBS 1486 (lane 9), CBS 1538^T (lane 10), CBS 380^T (lane 11), CBS 395^T (lane 12), CBS 425^T (lane 13), CBS 1505 (lane 14), CBS 1546 (lane 15), CBS 1604 (lane 16), and CBS 7001 (lane 17) were digested with *Eco*RI, subjected to electrophoresis in an agarose gel, transferred to nitrocellulose filters, and hybridized with a NCYC 396^T *MEL* probe. Fragment sizes are indicated on the left and right.

and 10) and the *S. oleaginosus* strains (lanes 8 and 9) were located in two *Eco*RI fragments, as are the *MEL3*, *MEL6*, and *MEL10* genes (6.7 kb) and the *MEL5*, *MEL7*, and *MEL9* genes (5.2 kb) of strains CBS 4411 and CBS 5378^T (18, 19). Strain CBS 7002^T (*S. hispalensis*) (Fig. 1, lane 1) also exhibited hybridization to *MEL1* in a 5.2-kb *Eco*RI fragment and in a doublet band (visible after short exposures) at 6.7 to 6.8 kb. Strain CBS 4903^T (*S. hienipiensis*) (Fig. 1, lane 11) produced a pattern of *Eco*RI fragments hybridizing to *MEL1* similar to the pattern of CBS 5378^T (*S. norbensis*) (Fig. 1, lane 12). Strain CBS 5635 (*S. coreanus*) (Fig. 1, lane 2) exhibited hybridization to *MEL1* in one *Eco*RI fragment (6.7 kb).

The variation in the relative hybridization intensities among different *Eco*RI fragments suggested that there was a variable number of *MEL* genes in each fragment, as has been shown with strains CBS 4411 and CBS 5378^T (18, 19). For example, the 5.2-kb fragment of strain NRRL Y-12056 (Fig. 1, lane 7) exhibited less intense hybridization than the 6.7-kb fragment, whereas in strain NRRL Y-12057 (Fig. 1, lane 8) the 5.2-kb fragment hybridized at least as strongly as the 6.7-kb fragment.

Previously (32), the *MEL1* gene has been found not only in ATCC 9080 (the source of the cloned *MEL1* gene) but also in CECT 1323 (originally classified as *S. carlsbergensis* by Winge et al. [38, 39]). In addition to *MEL1*, CECT 1323 carried a 3.8-kb *Eco*RI fragment which hybridized to some extent to the *MEL1* probe (Fig. 1, lane 14). CECT 1323 shows single-gene segregation for the Mel$^+$ phenotype (19)

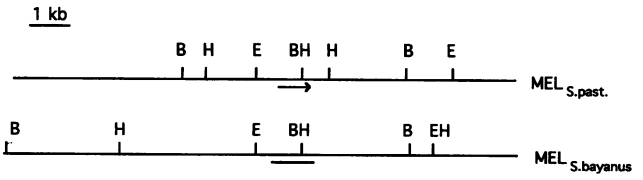

FIG. 4. Restriction maps of the *S. pastorianus* and *S. bayanus* *MEL* genes. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The arrow indicates the location and direction of transcription of the NCYC 396^T *MEL* gene. The horizontal line below the *S. bayanus* map indicates the location of the *S. bayanus* *MEL* gene.

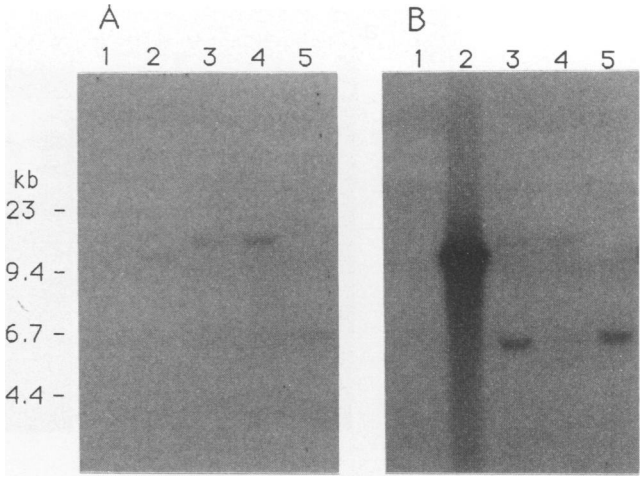

FIG. 5. Southern hybridization analysis of *Eco*RI-digested DNAs from *Schwanniomyces occidentalis* ATCC 26074 (lanes 1), *Z. cidri* CBS 4575^T (lanes 3) and CBS 5666 (lanes 4), and *S. kluyveri* CBS 4569 (lanes 5). *S. pastorianus* NCYC 396^T DNA (lanes 2) was used for comparison. The Southern filters were hybridized with a *MEL1* (A) or NCYC 396^T *MEL* (B) probe. The fragment sizes of an M_r standard are indicated on the left.

(strain 303-49), which suggests that the weakly hybridizing band may have been due to an inactive gene or to close linkage of the genes. In this study, an *Eco*RI fragment of the same size (3.8 kb) and hybridization intensity was found in strain CBS 403 (*Saccharomyces lindneri*) (Fig. 1, lane 15), which had the Mel$^-$ phenotype, supporting the hypothesis that there is an inactive gene. A restriction map of this locus is shown in Fig. 2 (pseudo-*MEL*).

A Mel$^+$ American whiskey yeast strain (ALKO 1228) (Fig. 1, lane 3) hybridized to both probes but yielded a unique pattern of restriction fragments. In this strain the *MEL* gene was found in a 4.4-kb *Eco*RI fragment, as was the *MEL* gene of strain CBS 2444 (*Saccharomyces logos*) (Fig. 1, lane 4). Both strains exhibited very weak hybridization with a 3.3-kb *Eco*RI fragment (Fig. 1, arrowheads), suggesting that a pseudo-*MEL* gene or an *Eco*RI site is present in the hybridizing *MEL* gene region. The two other strains of *S. logos*, CBS 382^T and CBS 4734, hybridized to *MEL1* in *Eco*RI fragments of 4.2 and 3.9 kb, respectively (Fig. 1, lanes 5 and 6).

***S. pastorianus* *MEL* gene.** Brewing lager strains (Fig. 3, lanes 3 through 7) formed a unique group in which the Mel$^+$ characteristic was conferred by a *MEL* gene identical to the NCYC 396^T *MEL* gene (lane 1). A strain of *Saccharomyces monacensis* (CBS 1503^T) (lane 2) also produced an identical hybridization pattern of endonuclease fragments. A restriction map is shown in Fig. 4.

***S. bayanus* *MEL* gene.** We also investigated 12 *S. bayanus* strains for the presence of a *MEL* gene(s). Seven of these strains yielded similar patterns of restriction fragments containing *MEL* DNA sequences (Fig. 3). The hybridization of the NCYC 396^T *MEL* probe to *S. bayanus* restriction fragments was not as strong as the hybridization to NCYC 396^T restriction fragments. This may have been due to the probe's 3′ end 800-bp noncoding sequence, which, according to restriction analysis data, is different in the *S. bayanus* and NCYC 396^T genomes (data not shown). A restriction map is shown in Fig. 4. Five of the *S. bayanus* strains studied (CBS 378, CBS 381^T, CBS 424, CBS 431^T, and CBS 3008) were

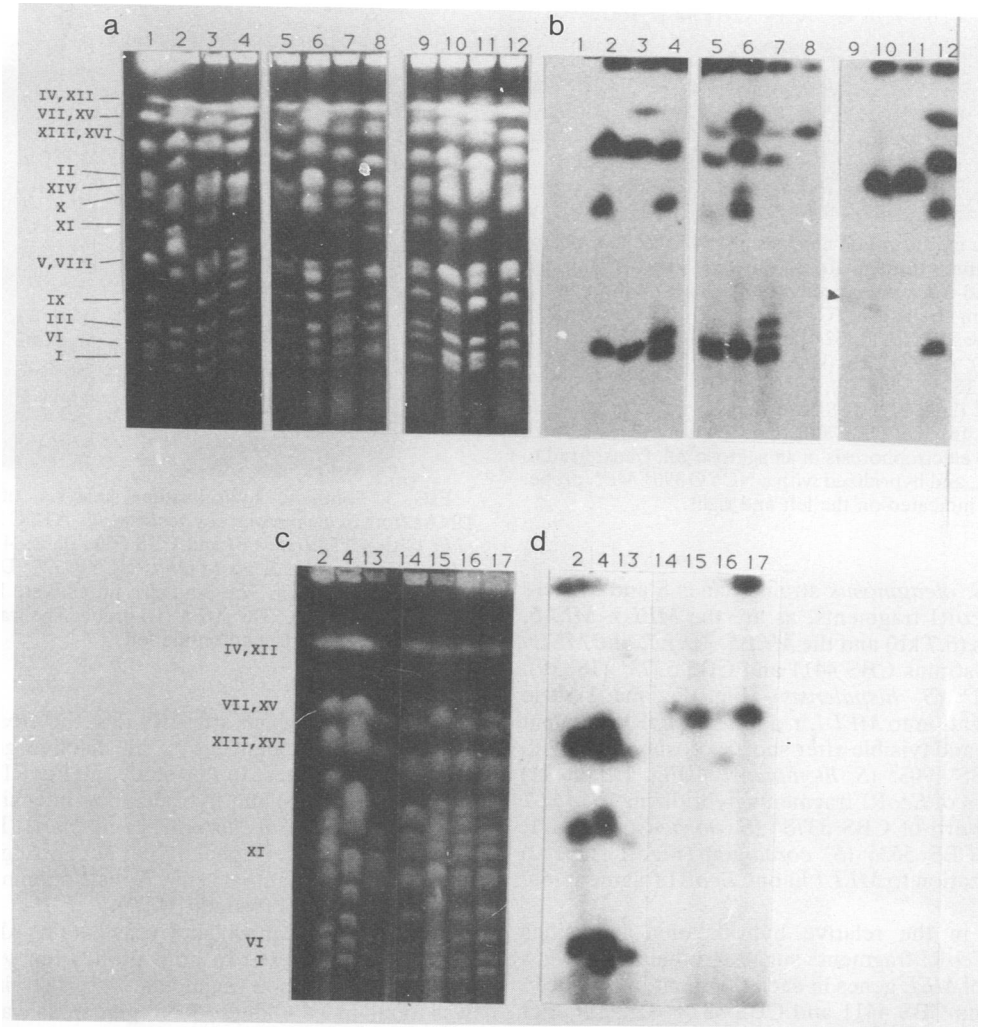

FIG. 6. Analysis of chromosomal DNAs of *S. cerevisiae* strains by CHEF-GE. After electrophoresis the gels were stained with ethidium bromide to visualize the chromosomes (a and c). Autoradiographs (b and d) revealed hybridization with the *MEL1* probe. The strains used were X 2180-1A (reference strain) (lanes 1), NRRL Y-12056 (lanes 2), NRRL Y-12057 (lanes 3), CBS 3081^T (lanes 4), CBS 4903^T (lanes 5), CBS 5378^T (lanes 6), CBS 7002^T (lanes 7), CBS 5635 (lanes 8), CBS 403 (lanes 9), CECT 1323 (lanes 10), ATCC 9080 (lanes 11), CBS 4411 (lanes 12), CBS 3093^T (lanes 13), ALKO 1228 (lanes 14), CBS 382^T (lanes 15), CBS 2444 (lanes 16), and VKM Y-1830 (lanes 17). The arrowhead indicates the position of weakly hybridizing chromosome IX in lanes 9 and 10.

Mel$^-$ and did not exhibit any hybridization to the *MEL* probes.

Other *MEL* genes. We also studied some Mel$^+$ strains belonging to other genera. A strain of *Schwanniomyces occidentalis* (ATCC 26074) hybridized very weakly to the NCYC 396^T *MEL* probe in a 6.7-kb *Eco*RI fragment (Fig. 5A and B, lanes 1). Two strains of *Zygosaccharomyces cidri* (CBS 4575^T and CBS 5666) (Fig. 5A and B, lanes 3 and 4) hybridized weakly to both probes in a 12-kb *Eco*RI fragment and variably to the NCYC 396^T *MEL* probe in a 6.7-kb fragment. A strain of *Saccharomyces kluyveri* (CBS 4569) generated a 6.7-kb *Eco*RI fragment which exhibited weak hybridization to both probes (Fig. 5A and B, lanes 5). The hybridization of the *MEL* genes of two strains of *Z. cidri* and of one strain of *S. kluyveri* to *MEL* probes was weaker than the hybridization of the *MEL* probes to each other. This suggests that the level of homology between the *MEL* genes of *Z. cidri* or *S. kluyveri* strains and the cloned *MEL* genes is less than 77% (which is the degree of homology between the two cloned *MEL* genes) (33). The *MEL* gene of *Schwanniomyces occidentalis* is even more divergent since weak hybridization to the probes was observed only after prolonged exposure. *S. pastorianus* NCYC 396^T DNA (Fig. 5A and B, lanes 2) was used for comparison.

Chromosomal mapping of *S. cerevisiae*. We used pulsed-field gel electrophoresis and Southern hybridization for chromosomal mapping of the *MEL* genes in different yeast strains. Figure 6 shows the chromosomal patterns of the *S. cerevisiae* strains and their patterns of hybridization to the *MEL1* probe. Previously, we have shown that the *MEL1* through *MEL10* genes are located on different chromosomes, as follows: *MEL1* on chromosome II (as shown by Vollrath et al. [37]), *MEL2* on chromosome VII, *MEL3* on chromosome XVI, *MEL4* on chromosome XI, *MEL5* on chromosome IV, *MEL6* on chromosome XIII, *MEL7* on chromosome VI, *MEL8* on chromosome XV, *MEL9* on chromosome X or XIV, and *MEL10* on chromosome XII (18, 19).

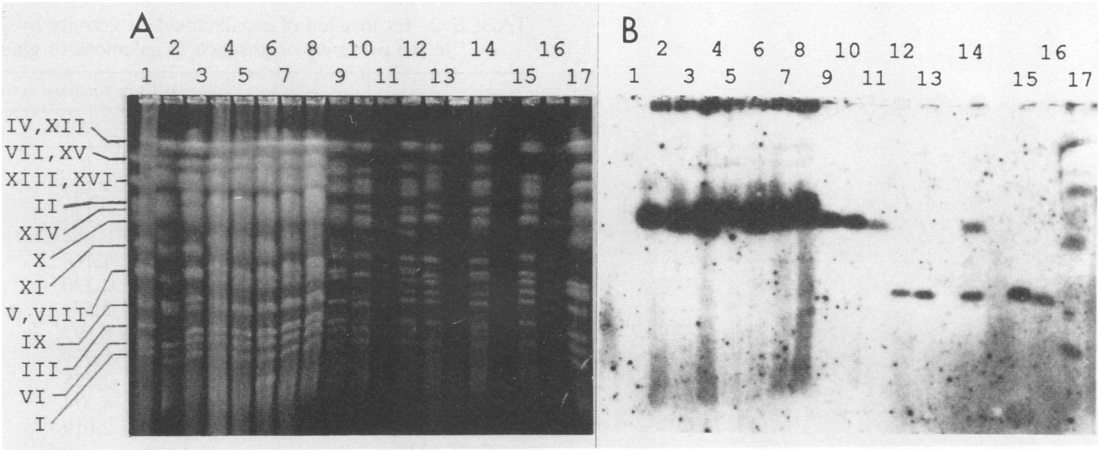

FIG. 7. Analysis of chromosomal DNAs of *S. pastorianus* (lanes 2, 3, and 9 through 11), unclassified strains (lanes 4 through 8), and *S. bayanus* (lanes 12 through 16) strains by CHEF-GE. After electrophoresis the gel was stained with ethidium bromide (A). An autoradiograph (B) revealed hybridization with the NCYC 396^T *MEL* probe. The strains used were X 2180-1A (reference strain) (lanes 1), NCYC 396^T (lanes 2), CBS 1503^T (lanes 3), NCYC 1146 (lanes 4), NCYC 1324 (lanes 5), ALKO 1350 (lanes 6), ALKO 1353 (lanes 7), ALKO 1356 (lanes 8), CBS 1260 (lanes 9), CBS 1486 (lanes 10), CBS 1538^T (lanes 11), CBS 380^T (lanes 12), CBS 395^T (lanes 13), CBS 1505 (lanes 14), CBS 1604 (lanes 15), CBS 7001 (lanes 16), and CBS 5378^T (*S. cerevisiae*) (lanes 17).

In two *S. oleaceous* strains (NRRL Y-12056 [Fig. 6, lanes 2] and CBS 3093^T [lanes 13]) and two *S. oleaginosus* strains (NRRL Y-12057^T [lanes 3] and CBS 3081^T [lanes 4]) the *MEL1* probe hybridized to chromosome VI (*MEL7*) and to the doublet containing chromosomes XIII and XVI (*MEL6* and/or *MEL3*). In addition, strains CBS 3081^T, CBS 3093^T, and NRRL Y-12056 exhibited hybridization on chromosome XI (*MEL4*), strain NRRL Y-12057^T exhibited hybridization on chromosome IV or XII (*MEL5* or *MEL10*), and strain CBS 3081^T exhibited hybridization on chromosome I.

In *S. hienipiensis* CBS 4903^T (Fig. 6, lanes 5), the *MEL1* probe hybridized to chromosomes I, VI, XI, XIII and/or XVI, and VII and/or XV. In *S. hispalensis* CBS 7002^T (Fig. 6, lanes 7), the *MEL1* probe hybridized to six chromosomal bands. This strain had a different pattern of chromosomes, presumably because of the presence of homologous or homeologous chromosomes. Hybridization to chromosome VII or XV (comigrating; *MEL2* and/or *MEL8*) and to chromosome XIII and/or XVI (comigrating; *MEL6* and/or *MEL3*) was observed. There was hybridization to four bands in the region of the three smallest chromosomes of *S. cerevisiae* (chromosomes I, VI, and III). Only *MEL7* is known to be located on these chromosomes (chromosome VI) (19).

In strains CBS 5635 (*S. coreanus*) (Fig. 6, lanes 8), CBS 382^T (*S. logos*) (lanes 15), and ALKO 1228 (lanes 14), *MEL1* hybridized to only one chromosomal band (containing chromosomes VII and XV). In another strain of *S. logos* (CBS 2444) (Fig. 6, lanes 16), *MEL1* also hybridized to the chromosomal band containing chromosomes XIII and XVI.

As expected, chromosome II of strain CECT 1323 (Fig. 6, lanes 10) exhibited strong hybridization to the *MEL1* probe, whereas chromosome IX exhibited weaker hybridization (Fig. 6, arrowhead). CBS 403 (Mel$^-$) (Fig. 6, lanes 9) also exhibited hybridization to chromosome IX. The separate chromosomal locations of two loci containing *MEL* gene information in strain CECT 1323 suggests that the hybridizing sequence in chromosome IX may represent an inactive locus (pseudo-*MEL*) (Fig. 2).

Chromosomal mapping of *S. pastorianus*. In the brewing lager strains (NCYC 1146, NCYC 1324, ALKO 1350, ALKO 1353, and ALKO 1356) the *MEL* gene was found on chromosome X (Fig. 7, lanes 4 through 8), as was the *MEL* gene of *S. pastorianus* strains (lanes 2, 3, and 9 through 11). In several strains, chromosome X occupied an extended band, indicating that homologous or homeologous chromosomes were present. *S. monacensis* CBS 1503^T produced a similar chromosomal hybridization pattern (Fig. 7, lanes 3).

Chromosomal mapping of *S. bayanus*. In most strains, the chromosome migrating between chromosomes IX and VIII of *S. cerevisiae* exhibited hybridization to the NCYC 396^T *MEL* probe (Fig. 7, lanes 12 through 16). An additional chromosome of strain CBS 1505 (*Saccharomyces intermedius* var. *valdensis*) (Fig. 7, lanes 14) exhibited hybridization at the location of *S. cerevisiae* chromosome X. This strain produced some additional chromosomal bands compared with other *S. bayanus* strains (e.g., in the location of chromosome VI of *S. cerevisiae*).

Northern blotting. An analysis of total RNAs from the Mel$^+$ *S. cerevisiae*, *S. pastorianus*, and *S. bayanus* strains studied revealed one band which hybridized to the *MEL1* or NCYC 396^T *MEL* probe (Fig. 8). On the basis of the migration of the rRNA subunits, the α-galactosidase mRNA was estimated to be about 1.6 kb long in *S. cerevisiae* strains and about 1.5 kb long in *S. bayanus* and *S. pastorianus* strains. Even *S. cerevisiae* strains with multiple *MEL* loci (NRRL Y-12056, NRRL Y-12057, CBS 3081^T, CBS 3093^T, CBS 4903^T, CBS 5378^T, and CBS 7002^T) expressed a single size of α-galactosidase mRNA. The hybridization of the larger ribosomal subunit to the NCYC 396^T probe shown in Fig. 8B was independent of the presence of galactose in the growth medium (33).

α-Galactosidase activity. *MEL* expression in the yeast strains was studied in yeast extract-peptone-glycerol-ethanol medium in the absence or presence of galactose or glucose (Table 2). All Mel$^+$ strains of *S. cerevisiae* produced an easily detectable basal level of α-galactosidase without induction. Enzyme activity was induced in the presence of galactose and repressed in the presence of glucose. In strains CBS 5378^T (*S. norbensis*), CECT 1323 (*S. carlsbergensis*), and CBS 4903^T (*S. hienipiensis*) the α-galactosidase activity remained unchanged at the basal level even after the addition of galactose. Galactose uninducibility was monogenic be-

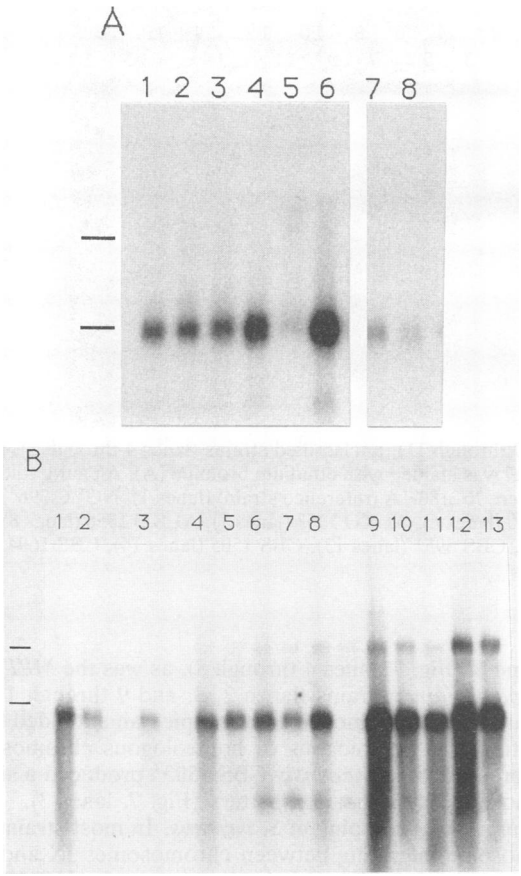

FIG. 8. Northern hybridization analysis of total RNAs isolated from *S. cerevisiae* (A), *S. bayanus* (B, lanes 1 through 4), and *S. pastorianus* (B, lanes 5 through 13) yeast strains cultured in the presence of 2% galactose. (A) The strains used were NRRL Y-12056 (lane 1), NRRL Y-12057 (lane 2), CBS 3081^T (lane 3), CBS 3093^T (lane 4), CBS 4903^T (lane 5), CBS 5378^T (lane 6), CBS 7002^T (lane 7), and CBS 5635 (lane 8). (B) The strains used were CBS 395^T (lane 1), CBS 1505 (lane 2), CBS 1604 (lane 3), CBS 7001 (lane 4), CBS 1260 (lane 5), CBS 1486 (lane 6), CBS 1503^T (lane 7), CBS 1513^T (lane 8), NCYC 1146 (lane 9), NCYC 1324 (lane 10), ALKO 1350 (lane 11), ALKO 1353 (lane 12), and ALKO 1356 (lane 13). For hybridization, ^{32}P-labelled anti-sense RNA probes from *MEL1* (A) and NCYC 396^T *MEL* (B) genes were used. The migration of rRNA subunits is shown.

cause a hybrid between a monosporic segregant of CBS 5378^T and a *GAL4* strain (X 2180-1A) exhibited ordinary tetrad segregation with a ratio of inducibility to uninducibility of 2/2 (data not shown). In *S. cerevisiae* strains, the production of enzyme activity was dose dependent: i.e., the strains containing several *MEL* genes produced more α-galactosidase than the strains containing only one gene. The difference was more marked under uninduced conditions than under induced conditions. The presence of glucose repressed α-galactosidase activity independent of galactose inducibility.

As the restriction mapping and chromosomal analysis indicated that the *MEL1* gene was present in strain CECT 1323, this strain was expected to express the gene as efficiently as ATCC 9080 did (ATCC 9080 was the donor of the cloned *MEL1*). However, the basal level of α-galactosidase activity produced by strain CECT 1323 was low, and the activity was not induced by galactose (Table 2).

TABLE 2. Expression of α-galactosidase activity by yeast strains in the presence or absence of galactose or glucose

Strain	α-Galactosidase activity[a]		
	−Gal	+Gal	+Glucose
S. cerevisiae strains			
CBS 382^T	23	2,442	1.9
CBS 2444	30	114	0
CBS 4734	30	964	2.9
CBS 5635	200	1,168	7.0
CBS 4903^T	2,194	2,130	83
CBS 7002^T	1,835	2,561	85
NRRL Y-12056	2,053	8,597	83
CBS 3093^T	2,341	8,979	92
NRRL Y-12057	1,695	3,525	58
CBS 3081^T	2,638	4,374	82
CBS 5378^T	2,262	2,019	97
CECT 1323	4.2	6.5	2.8
ALKO 1228	59	112	0.2
ATCC 9080	106	2,133	17
CBS 4411	2,113	5,170	59
VKM Y-1830	89	375	7.1
S. bayanus strains			
CBS 1505	0.3	3.9	0.3
CBS 1604	0.3	19	0.1
S. pastorianus strains			
NCYC 1146	2.5	14	2.2
NCYC 1324	0.4	0.5	0.1
ALKO 1350	0.9	0.3	0.5
ALKO 1353	0.7	3.4	0.4
CBS 1260	1.9	2.3	0.5
CBS 1486	0.5	12	0.2
NCYC 396^T	1.2	2.7	0.5

[a] Yeast cells were grown for 24 h in YPD medium which contained 3% glycerol and 2% ethanol instead of glucose in the presence (+Gal) or absence (−Gal) of 2% galactose or in YPD medium (+Glucose). Enzyme activity was measured as described by Kew and Douglas (9). Enzyme activity is expressed in nanomoles of substrate consumed per minute per 1.0 ml of culture at a cell density of 10 A_{540} units.

S. pastorianus and *S. bayanus* strains produced very low levels of α-galactosidase activity without induction. Although galactose markedly increased the production of α-galactosidase, even the induced activity was lower than the activity in *S. cerevisiae* strains. Glucose repressed the enzyme activity also in these strains.

DISCUSSION

Classification of yeast strains. Numerous criteria have been used in traditional yeast taxonomy to delimit species. These criteria include cell and spore morphology, fermentation characteristics (34), ability to form spores, ability to hybridize (7), and differences in DNA base composition (41). The yeast strains used in this study (Table 1) were placed in the species *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* on the basis of DNA-DNA reassociation data (35, 36). This method is based on genome differences and is also more specific than the DNA base composition method. Most of the strains which we used in this study have been characterized by DNA-DNA reassociation previously (35, 36). Differentiation of *S. cerevisiae* and *S. bayanus* into distinct species has been supported by the results of genetic crossing and segregation, electrokaryotyping (17), and restriction analysis and molecular hybridization (21). *S. pastorianus* is considered to be a hybrid between *S. cerevisiae* and *S. bayanus* (22, 35), but more studies are needed to confirm the hybrid nature and origin of this species.

The karyotypes of the *S. bayanus* strains were very similar to one another but different from those of *S. cerevisiae*. Strain CBS 1505 produced some additional bands compared with other *S. bayanus* strains. The variability in band intensity and the fact that the strain was not cloned from one spore suggested that strain CBS 1505 could be heterogeneous on some chromosomes.

Most *S. bayanus* strains carried only one *MEL* locus, but the presence of two *MEL* loci in one strain suggested the existence of a *MEL* gene family also in *S. bayanus*. The results of a restriction fragment analysis indicated that the two *MEL* loci are identical and obviously represent a polymeric *MEL* gene family in *S. bayanus* similar to the *MEL* gene families in *S. cerevisiae*. The comigration of one CBS 1505 chromosome containing a *MEL* gene with a *S. pastorianus* chromosome also containing a *MEL* gene supports the hypothesis that *S. bayanus* is one parent of the hybrid species *S. pastorianus* and that this *MEL* gene originates from this parent. Some of the *S. bayanus* chromosomes have been identified by hybridization with *S. cerevisiae* probes, but this has not been done for the chromosomes exhibiting *MEL* hybridization (17).

The unclassified lager strains isolated in breweries belonged to the species *S. pastorianus* on the basis of the results of a Southern hybridization analysis of separated chromosomes or restriction fragments. They also differed from *S. cerevisiae* strains in the level of α-galactosidase activity produced. All of the *S. pastorianus* strains which we studied carried only one *MEL* locus. The lack of restriction site polymorphism at the *MEL* gene prevented differentiation between the brewing lager strains. On the basis of this evidence, all of these strains carried an identical *MEL* gene.

The Southern hybridization analysis of separated chromosomes performed with *MEL* probes showed that a Mel^+ strain of *S. cerevisiae* may contain up to seven distinct *MEL* loci. In the strains studied so far, *MEL* genes have been observed on 11 separate chromosomes (chromosomes I, II, IV, VI, VII, X or XIV, XI, XII, XIII, XV, and XVI).

The *MEL* genes of other genera, including the genera *Schwanniomyces* and *Zygosaccharomyces*, and of another *Saccharomyces* species, *S. kluyveri*, are so distantly related to *S. cerevisiae* or *S. pastorianus MEL* genes that a detailed study would require cloning of homologous *MEL* genes.

Expression of the *MEL* genes was studied in different growth media. Measurements of α-galactosidase activity showed that *S. cerevisiae* strains are more efficient in α-galactosidase production than *S. bayanus* or *S. pastorianus* strains. Under basal conditions, the *S. cerevisiae* strains carrying one *MEL* locus produced low levels of enzyme activity, whereas 10- to 100-fold-greater enzyme activity was produced by the strains carrying several *MEL* loci. α-Galactosidase activities were induced 2- to 100-fold by galactose except in strains CBS 5378^T and CBS 4903^T. CBS 5378^T is known to be (and CBS 4903^T might be) a *gal4* mutant (18), which may explain the absence of galactose induction (*GAL4* encodes a positive regulator required for activation of the transcription of *MEL* genes [8]). It is possible that both of these strains have developed multiple *MEL* genes to overcome their inability to increase their α-galactosidase production by galactose induction in a high-melibiose or high-raffinose environment. Glucose repression was very tight in strains carrying one *MEL* locus but less tight in strains with several *MEL* loci. The reason for this is not clear, but some of the multiple *MEL* genes could have escaped from a negative regulatory protein involved in glucose repression.

Strain CECT 1323 has a slower rate of galactose fermentation and a three- to four-times-longer lag phase in α-galactosidase production than strain ATCC 9080 (10). Thus, it is possible that the time for galactose induction used in these experiments (24 h) was not sufficient for this strain. These results suggest that the positive regulatory genes *GAL3* and/or *GAL4* needed for efficient induction may not be functional in strain CECT 1323.

The most effective *S. cerevisiae* strains in α-galactosidase production were the two *S. oleaceous* strains, NRRL Y-12056 and CBS 3093^T. They produced high levels of enzyme activity without induction, the production was further induced by galactose, and even the presence of glucose did not fully repress *MEL* gene expression.

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