Characterization of Genetically Transformed *Saccharomyces cerevisiae* Baker's Yeasts Able To Metabolize Melibiose

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Three transformant (Mel^+) *Saccharomyces cerevisiae* baker's yeast strains, CT-Mel, VS-Mel, and DADI-Mel, have been characterized. The strains, which originally lacked α -galactosidase activity (Mel^-), had been transformed with a DNA fragment which possessed an *ILV2-SMR1* allele of the *ILV2* gene and a *MEL1* gene. The three transformed strains showed growth rates similar to those of the untransformed controls in both minimal and semi-industrial (molasses) media. The α -galactosidase specific activity of strain CT-Mel was twice that of VS-Mel and DADI-Mel. The yield, $Y_{X/S}$ (milligrams of protein per milligram of substrate), in minimal medium with raffinose as the carbon source was 2.5 times higher in the transformed strains than in the controls and was 1.5 times higher in CT-Mel than in VS-Mel and DADI-Mel. When molasses was used, $Y_{X/S}$ (milligrams of protein per milliliter of culture) increased 8% when the transformed strains CT-Mel and DADI-Mel were used instead of the controls. Whereas no viable spores were recovered from either DADI-Mel or VS-Mel tetrads, genetic analysis carried out with CT-Mel indicated that the *MEL1* gene has been integrated in two of three homologous loci. Analysis of the DNA content by flow cytometry indicated that strain CT-Mel was $3n$, whereas VS-Mel was $2n$ and DADI-Mel was $1.5n$. Electrophoretic karyotype and Southern blot analyses of the transformed strains showed that the *MEL1* gene has been integrated in the same chromosomal band, probably chromosome XIII, in the three strains. Tetrads of CT-Mel strain segregated $4+:-0-$, $3+:-1-$ and $2+:-2-$, for both positive *MEL1* Southern blots and α -galactosidase activity, indicating, as expected, integration of the *MEL1* gene in two of three homologous chromosomes.

Baker's yeast production is carried out as fed-batch fermentation. The fermentable sugar for yeast growth is derived from beet molasses (8, 18). In addition to sucrose, which is predominant, beet molasses contains the sugar raffinose in the 1 to 8% (wt/vol) range. Only one-third of the molecule is utilized by baker's yeasts (8). The β -fructosidase (invertase) enzyme present in baker's yeasts yields fructose and melibiose from raffinose (13). The fructose formed is readily assimilated by the yeast. Baker's yeasts are unable to utilize the disaccharide melibiose because they lack the α -galactosidase enzyme (melibiase) (5, 8, 14) required to break the α -galactosidic bond in the melibiose moiety between glucose and galactose. Thus, the complete hydrolysis of raffinose requires the action of both β -fructofuranosidase (invertase) and α -galactosidase (melibiase). Clearly, the productivity and the process economics could be markedly improved by the use of baker's yeast strains that have the added capability of utilizing melibiose. Synthesis of yeast α -galactosidase is encoded by a *MEL* gene carried by *Saccharomyces cerevisiae* var. *uvarum*, *carlsbergensis* and *oleaginosus* (1). Normal baker's yeast lacking α -galactosidase is phenotypically Mel^- and genetically *mel⁰* (5).

The protoplast fusion technique has been used to develop MEL^+ industrial hybrids capable of improving the molasses yield in yeast propagation (13). However, fusants between a laboratory and an industrial baker's yeast consistently showed higher molasses yield but performed poorly in baking applications. The use of rDNA technology for the construction of MEL^+ baker's yeasts has been reported previously (5, 6); the *MEL1* gene from *S. cerevisiae* var. *uvarum* was cloned into a shuttle vector. The isolated *MEL1* gene, encoding secreted

α -galactosidase from *S. cerevisiae* var. *uvarum*, was either integrated into chromosomal DNA or introduced via an autonomously replicating 2 μ m-based plasmid vector into the cells of commercial *mel⁰* baker's yeast strains (5). When the two strains were tested for their ability to utilize raffinose in molasses, α -galactosidase was produced, more carbohydrate was available, and a reduction in biological oxygen demand load was obtained. However, marketing considerations have resulted in a reluctance to introduce bacterial genes into industrial strains.

A one-step dominant-selection procedure has been developed (19) to construct recombinant baker's yeasts devoid of plasmid sequences. It consisted of a region of nonessential DNA from chromosome XIII in which *SMR1* (sulfometuron resistance) and *MEL1* dominant-selection markers replaced internal sequences. Such stable integrations of *MEL1* devoid of bacterial plasmid DNA facilitates the commercial application of recombinant DNA technology in the food and beverage industries. In this work we describe the characterization of Mel^+ transformed baker's yeast strains obtained by this procedure. These strains were stable, increased the biomass /sub-

TABLE 1. Growth rate of transformed and untransformed baker's yeasts in different media

Medium	Growth rate, μ (h^{-1}), of strain ^a :					
	CT	CT-Mel	VS	VS-Mel	DADI	DADI-Mel
SD	0.45	0.45	0.48	0.45	0.42	0.44
SS	0.40	0.39	0.41	0.40	0.39	0.39
SM	0.36	0.36	0.36	0.35	0.33	0.31
SR	0.29	0.30	0.10	0.12	0.11	0.11
SMeg	0	0.25	0	0.15	0	0.24

^a Results are the average of four experiments. Standard deviations were less than 4%.

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FIG. 1. Growth (measured as increase in optical density [O.D.] at 660 nm) of the transformed baker's yeast strains CT-Mel, VS-Mel, and DADI-Mel (solid circles) and the untransformed controls CT, VS, and DADI (open circles) in minimal medium plus 0.1% raffinose and 0.01% galactose (SMeg) as the carbon source. The biomass/substrate yields ($Y_{X/S}$) (micrograms of protein per milligram of raffinose), after 3 days of incubation, of the transformed baker's yeast strains CT-Mel, VS-Mel and DADI-Mel were 38, 21, and 27, respectively; and those of the untransformed controls CT, VS, and DADI were 15, 9, and 11, respectively. Results are the average of five experiments.

strate yield in beet molasses about 8%, and had growth rates similar to those of the untransformed control strains.

MATERIALS AND METHODS

Strains. The following *S. cerevisiae* strains were used: baker's strains DADI, VS, and CT (La Cruz del Campo, S.A., Seville, Spain); Mel⁺ transformants DADI-Mel, CT-Mel, and VS-Mel (G. H. Rank, University of Saskatchewan, Saskatchewan, Canada); and laboratory yeasts MMY1, MMY2 (Solar Energy Research Institute, Golden, Colo.), S288C (Yeast Genetic Stock Center, Berkeley, Calif.), ATCC9080 (ALKO LTD., Helsinki, Finland), and DS81 (a diploid formed between S288C, possessing the a mating type, and its isogenic a strain X2180-1A).

Enzymes and chemicals. Proteinase K and sucrose were obtained from E. Merck AG, Darmstadt, Germany; Zymolyase 20000 was obtained from Seikagako Kosyo Co., Ltd., Tokyo, Japan; and *N*-laurylsarcosine, melibiose, and all the other chemicals used were purchased from Sigma Chemical Co., St. Louis, Mo. The herbicide sulfometuron was generously provided by G. H. Rank.

Media. Yeast strains were grown in complete YP medium (0.5% yeast extract [Difco], 1% Bacto Peptone) supplemented with either 2% glucose (YPD) or 3% glycerol (YPG) or in minimal medium (0.17% yeast nitrogen base [Difco] without amino acids but with 0.5% ammonium sulfate) supplemented with either 2% glucose (SD), 2% sucrose (SS), 0.8% galactose (SGal), 2% maltose (SMA), 2% melibiose (SMe), or 2% raffinose (SR); for melibiase induction, SMe and SR were supplemented with 0.01% galactose (SMeg and SRg, respectively). Occasionally, 0.1% instead of 2% raffinose was used. This has been indicated in the text. Beet molasses (72% sucrose) obtained from Unión Alcohólica Española, S.A., Granada, Spain, diluted 20-fold (3.6% sucrose), filtered, and supplemented with 0.018% galactose (M medium) was also used. The presporulation (PRE) and sporulation (SPO) media used were PRE1 (0.8% yeast extract [Difco], 0.3% Bacto Peptone, 10% glucose) and SPO1 (0.5% potassium acetate) (2). When necessary, media were solidified by the addition of 2% agar.

Culture conditions. Precultures were obtained by incubating the cells in YPD

at 30°C until the stationary phase. Flasks (250 ml to 1 liter) containing 100 to 500 ml of medium were inoculated with the stationary-phase culture to an initial A_{660} of 0.05 and incubated at 30°C with shaking. When minimal media were used, the precultures were centrifuged and the cells were washed twice with distilled water before inoculation.

The growth rate (μ , expressed in reciprocal hours) was determined by measuring the A_{660} in any laboratory media and the A_{690} in molasses.

Yield and productivity. The final yield ($Y_{X/S}$) (final amount of biomass obtained per amount of a given substrate) was determined by measuring either final dry weight or protein content and calculating the ratio micrograms of biomass (dry weight or protein) per milligram of sugar or per milliliter of culture. Productivity (biomass increase per unit of time) was calculated by periodically measuring the biomass increase during incubation.

Construction of the recombinant baker's yeasts. The yeast *SMR1* gene (allelic to the *ILV2* gene), which confers resistance to sulfometuron (19), was used as the dominant resistance-selectable marker for the baker's yeast transformation as well as for targeting integration of the *MEL1* gene at the *ILV2* locus. The one-step replacement that generated a stable integration of *MEL1* devoid of bacterial plasmid DNA (19) was used by G. H. Rank to construct a Mel⁺ strain by using as receptors the Mel⁺ baker's yeasts DADI, VS, and CT.

Transformants from strains DADI, VS, and CT grew on solid SD minimal medium supplemented with 40 mg of sulfometuron per ml. Control strains did not grow at sulfometuron concentrations greater than 10 to 20 mg/ml. The transformants were further tested for α -galactosidase activity (Mel⁺ phenotype).

Analytical procedures. Yeast cells were grown at 30°C in flasks with 20 ml of SGal for α -galactosidase induction. When the culture reached an A_{660} of 0.5 to 0.7, the cells were harvested by centrifugation, washed, and resuspended in 20 ml of 0.1 M phosphate buffer (pH 6.5). To permeabilize the cells, 2 ml of a toluene-ethanol (1:1) solution was added, the mixture was agitated with vigorous stirring for 5 min and centrifuged, and the pellet was resuspended in 0.5 ml of the same buffer.

α -Galactosidase (melibiase; EC 3.2.1.22) activity was routinely assayed by monitoring glucose production in a YSI27 glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). The standard assay mixture contained 200

FIG. 2. Biomass/substrate yield ($Y_{X/S}$) (milligrams of protein per milliliter of culture) of the transformed baker's yeast strains CT-Mel, VS-Mel, and DADI-Mel (solid bars) and the untransformed controls CT, VS, and DADI (open bars) grown in semi-industrial medium (beet molasses). Results are the average of three experiments.

TABLE 2. Segregation displayed by complete and incomplete tetrads of the transformed Mel⁺ strain CT-Mel

Characteristic	Segregation ^a					Total no. (%) of spores ^a	
	4+:0-	3+:1-	2+:2-	1+:3-	0+:4-	(+)	(-)
Complete tetrads							
Viability	4					16 (100)	0 (0)
Prototrophy ^b	2	2				14 (87)	2 (13)
Petite			2	1	1	5 (31)	11 (69)
Growth in galactose (SGal)	1	1	1	1		10 (63)	6 (37)
Sulfometuron resistance	2	2				14 (87)	2 (13)
Sporulation			1 ^c	3 ^d		5 (31)	11 (69)
Incomplete tetrads							
Viability		11	10	6	5	59 (46)	69 (54)
Prototrophy						48 (81)	11 (19)
Petite						15 (26)	44 (74)
Growth in galactose (SGal)						41 (69)	18 (31)
Sulfometuron resistance						51 (86)	8 (14)

^a (+) and (-) indicate the presence and absence, respectively, of the characteristic.

^b All the nonprototrophic meiotic products were auxotrophs for tryptophan (Trp⁻).

^c The two spores unable to sporulate mated with α cells.

^d Two of the three spores unable to sporulate mated with α cells, and the third mated with α cells.

ml of permeabilized cells obtained from 240 ml of SGal medium and 200 ml of a 5% (wt/vol) melibiose solution in 0.1 M phosphate buffer (pH 6.5). A 25-ml portion of the mixture was injected into the glucose analyzer, and the amount of glucose liberated was measured for 30 min.

Sporulation procedures and tetrad analysis. Cells were sporulated by a previously described procedure (2). Tetrad analysis was also performed as described previously (15). Tetrads were printed via a velvet replicator system onto various diagnostic media to determine their phenotypes: minimal medium SD to determine auxotrophies; SD plus 40 mg of sulfometuron per ml to establish resistance or sensitivity to the herbicide; YPG for the petite phenotype; SGal to measure α -galactosidase activity (Mel⁺ phenotypes); and PRE1 and SPO1 to establish ability to sporulate. Finally, the meiotic products were mixed with either MMY1 or MMY2 as testers to determine the capacity to conjugate when zygotes were visualized under the microscope.

Flow cytometry. Yeast cells were grown with vigorous stirring at 30°C in flasks containing 20 ml of YPD. When the culture reached an A_{660} of 0.5 to 0.7, the cells were harvested by centrifugation, washed, resuspended in 70% ethanol, maintained at -20°C for 30 min, and further transferred to 4°C for 4 days.

About 5×10^5 cells were then resuspended in 1 ml of 50 mM sodium citrate, centrifuged, and then resuspended in 0.5 ml of fresh 50 mM sodium citrate containing 50 mg of RNase. The preparation was incubated for 2 h at 37°C, and then 0.5 ml of 50 mM sodium citrate containing 2 mg of propidium iodide was added. The samples were incubated for 30 min at room temperature and sonicated for 30 s, and the DNA content was measured in a flow cytometer (Becton-Dickinson FACSscan analyzer) by previously described procedures (3).

Electrophoretic karyotype. The basic procedure used for chromosomal DNA preparation was that described in reference 9. The system used was a CHEF-DRII gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.). Electrophoresis was carried out at 14°C and 200 V for 15 h with a switching time of 60 s and then for 8 h with a switching time of 90 s. A standard set of *S. cerevisiae* YNN295 chromosomes was obtained commercially (Bio-Rad).

Southern blot analysis. The chromosomal DNA separated by the CHEF-DRII apparatus (see above) was treated with depurination solution (0.25 N HCl) for 4 min, denatured for 3 min, neutralized for another 3 min, and transferred to nitrocellulose filters. The basic procedure used was that of Naumov et al. (9). The MEL probes used in the hybridizations (6, 14, 17) were prepared as described in reference 6.

Protein determination. Total protein in permeabilized whole cells was determined by the procedure described in reference 7.

RESULTS

Growth rate of baker's yeast strains CT, VS and DADI and the transformed CT-Mel, VS-Mel, and DADI-Mel yeasts in minimal medium with different carbon sources. Plasmid pWX813 contains a chimeric *MEL1-SMRI-30* cassette in which *MEL1* is flanked by *SMRI-30* upstream sequences. The cassette, released upon cleavage, has recombinogenic ends that are homologous to yeast chromosome XIII DNA at the *ILV2*

locus, which targeted the DNA replacement at the recipient *ILV2* locus (19).

Strains DADI, CT, and VS transformed with plasmid pWX813 were selected in minimal medium with 40 mg of sulfometuron per ml. The untransformed controls were unable to grow in over 20 mg of the herbicide per ml. The frequency of spontaneous sulfometuron-resistant mutants was less than 10^{-7} . One colony of each transformed strain was then selected for further characterization. Once selected, the strains named DADI-Mel, CT-Mel, and VS-Mel were checked after several cycles of growth in nonselective medium (YPD) for sulfometuron resistance. The persistence of vigorous growth in SD with sulfometuron indicated the stability of the resistance marker.

Transformants were then checked for their ability to grow in the laboratory minimal media SD, SS, SMa, SR, and SMeg (Table 1). When the media used were SD, SS, SMa, and SR, growth rates were similar for the transformed strains and the untransformed controls. However, in SMeg, only the transformed strains were able to grow, with growth rates that varied from 0.25 h^{-1} for strain CT-Mel to 0.15 h^{-1} for VS-Mel. Results therefore indicated that the transformed strains possessed a functional α -galactosidase (melibiase) gene that allowed the hydrolysis of melibiose into glucose and galactose. The results also showed that the transformation process did not negatively affect the strains, since their growth rates were almost identical to those of the untransformed controls.

Growth and biomass/substrate yields ($Y_{X/S}$) of the transformed baker's yeast strains and the untransformed controls in laboratory minimal medium SR. To establish more precisely the growth rates and biomass/substrate yields ($Y_{X/S}$) of the transformed baker's yeasts compared with the untransformed controls, strains were inoculated into minimal medium with 0.1% raffinose as the carbon source (supplemented with 0.01% galactose) and the increase in turbidity was measured periodically for 3 days. The reason for using 0.1% raffinose instead of 2% is that all the strains can hydrolyze raffinose into fructose and melibiose. Thus, in any case, they can metabolize one-third of the sugar added. This carbon source could be enough to keep the cultures growing to the point that a different nutrient would become limiting. In this case, it would be impossible to

FIG. 3. DNA content (arbitrary units) measured by flow-cytometric analysis of the laboratory yeasts MMY1 (haploid) (panel 1) and DS81 (diploid) (panel 2); the baker's yeast strains CT (panel 3), VS (panel 5), and DADI (panel 7); the transformed baker's yeast strains CT-Mel (panel 4), VS-Mel (panel 6), and DADI-Mel (panel 8); and two complete tetrads (panels 9 through 12 and panels 13 through 16) obtained from strain CT-Mel. The two other complete tetrads displayed identical results. The figure represents the number of cells (ordinate) which had an absorbance (arbitrary units) indicative of the DNA content (abscissa). Experiments were repeated twice, and standard deviations were less than 5%.

establish differences between transformed and untransformed yeasts.

In fact, Fig. 1 shows that the untransformed strains were able to grow up to about 0.4 to 0.5 optical density units when 0.1% raffinose was used. The transformed strains, however, grew until they reached an optical density greater than 0.8 and at growth rates either similar to (strains VS-Mel and DADI-Mel versus VS and DADI) or higher than (CT-Mel versus CT) those of the untransformed controls.

The biomass substrate yield ($Y_{X/S}$) reached by the transformants CT-Mel, VS-Mel, and DADI-Mel (38, 21, and 27 μg of protein per mg of raffinose, respectively) and the controls CT, VS, and DADI (15, 9, and 11 μg of protein per mg of raffinose, respectively) after 3 days of maintenance in the laboratory minimal medium with 0.1% raffinose is up to 2.5 times greater in each of the transformants than in the controls and about 1.5 times higher in strain CT-Mel than in the other two strains.

Growth and biomass/substrate yields ($Y_{X/S}$) of the transformed and untransformed baker's yeasts in beet molasses (semi-industrial conditions). Beet molasses has variable concentrations of raffinose (0.5 to 8%, wt/vol) in addition to the major sugar component, sucrose (13). When growth and biomass/substrate yields of transformed and control baker's yeasts were measured at different times during incubation in beet molasses (Fig. 2), it was observed that the transformed strains grew at almost identical growth rates to those of the controls. Furthermore, strains CT-Mel and DADI-Mel increased their yield, measured as micrograms of protein per milliliter culture, when this was determined after 24, 48, 72, and 96 h of incubation. This increase was up to 8% compared with the control. Strain VS-Mel, however, had a lower yield than that of the control at all times during the incubation. Great variations in biomass yields were found in the different fermentations. However, differences in the yield of the trans-

TABLE 3. DNA content obtained by flow cytometry of baker's yeast strains and tetrads^a

Strain or tetrad	DNA content (arbitrary units)	Ploidy (approx)
Laboratory strains ^b		
MMY1	7	1 <i>n</i>
DS81	13	2 <i>n</i>
Baker's yeasts		
CT	18	3 <i>n</i>
VS	14	2 <i>n</i>
DADI	10	1.5 <i>n</i>
Transformants		
CT-Mel	18	3 <i>n</i>
VS-Mel	14	2 <i>n</i>
DADI-Mel	10	1.5 <i>n</i>
Tetrads		
1a	9	1.4 <i>n</i>
1b	10	1.5 <i>n</i>
1c	9	1.4 <i>n</i>
1d	5	1 <i>n</i>
2a	9	1.4 <i>n</i>
2b	9	1.4 <i>n</i>
2c	10	1.5 <i>n</i>
2d	11	1.7 <i>n</i>
3a	10	1.5 <i>n</i>
3b	9	1.4 <i>n</i>
3c	11	1.7 <i>n</i>
3d	9	1.4 <i>n</i>
4a	9	1.4 <i>n</i>
4b	10	1.5 <i>n</i>
4c	10	1.5 <i>n</i>
4d	9	1.4 <i>n</i>

^a Results are the average of two experiments. Standard deviations were less than 5%.

^b The laboratory yeasts MMY1 and DS81 were used as haploid and diploid controls, respectively.

formed and the control strains were systematically maintained in each experiment.

Meiotic analysis of the transformed baker's yeasts CT-Mel, VS-Mel, and DADI-Mel. Most baker's and other industrial yeasts are polyploid or aneuploid. The presence of several potential *ILV2* homologous genes in the recipient strain opened the possibility of the cassette being inserted in one or more copies of the *ILV2* genes or even in other regions in different chromosomes which might have certain partial homology with the *ILV2* gene.

To test how many copies of the cassette had been integrated in the different baker's yeasts and whether this integration had always occurred in the homologous *ILV2* gene or at other loci, meiotic analysis was carried out with the transformed strains CT-Mel, VS-Mel, and DADI-Mel. This analysis was completed by comparing the results with results for the untransformed strains. Not a single viable spore from 20 micromanipulated tetrads was obtained from strain DADI-Mel. Similarly, no viable meiotic products were isolated after micromanipulation of 15 tetrads from the untransformed DADI strain. With regard to the VS strain, after micromanipulating 15 tetrads the viability of the spores was around 10%; this viability was even lower when 20 tetrads of strain VS-Mel were dissected, since only two spores survived. These results make the meiotic analysis of VS-Mel and DADI-Mel strains impossible.

The viability of strain CT-Mel, however, was considerably higher: from 36 micromanipulated tetrads of the transformed

TABLE 4. Ability to grow in SGal, resistance to sulfometuron, and α -galactosidase (melibiase) activity baker's yeasts and tetrads^a

Strain or tetrad	Growth in SGal	Resistance to sulfometuron	α -Galactosidase activity (nmol liberated glucose/min/mg of protein)
Strains			
VS	+	—	—
VS-Mel	+	+	15
DADI	+	—	—
DADI-Mel	+	+	14
CT	+	—	—
CT-Mel	+	+	27
Tetrads			
1a	+	+	17
1b	—	+	—
1c	+	+	17
1d	—	+	—
2a	+	+	15
2b	+	+	17
2c	—	+	—
2d	+	+	27
3a	+	+	—
3b	+	+	28
3c	+	+	19
3d	+	—	—
4a	—	+	—
4b	+	+	12
4c	—	+	—
4d	—	—	—

^a Results are the average of at least two experiments. Standard deviations were less than 10%.

CT-Mel strain, 4 were viable in all four products and viability was variable in the rest. In total, 52% of the meiotic products survived, allowing genetic analysis of the strain. The untransformed CT strain also had viable meiotic products, approximately 50% of the 15 tetrads that were micromanipulated.

Several features were studied in the meiotic products: presence of auxotrophic markers, petite phenotype, ability to grow in galactose (since galactose is necessary to induce the α -galactosidase activity), resistance to sulfometuron, and capacity to sporulate and/or conjugate.

The segregations observed for tryptophan auxotrophy (17% of the meiotic products), petite (27%), lack of growth in galactose (32%), and sulfometuron sensitivity (13%) are consistent with the presence of three homologous chromosomes for each of the markers studied (Table 2), so that two functional copies of one allele and a nonfunctional copy of the other allele exist. Results of sporulation and conjugation also indicated that there are three copies of chromosome III, where the mating type is located, so that the CT-Mel strain is $\alpha/a/a$. The segregation of these alleles in the meiotic products produces almost double the number of **a** strains (those which are either **a** or **a/a**) as compared with either α strains or strains which do not conjugate but are able to sporulate (and which have inherited the α/a genotype).

When data obtained from complete and incomplete tetrads are compared (Table 2), the results are seen to be very similar. In fact, when a statistical test is applied, there are no differences in the segregation observed. This indicates that the lack of viability is not associated with any of the markers studied. Also, segregation of sulfometuron resistance (10 sensitive mei-

FIG. 4. Expected segregation of sulfometuron resistance (R) and α -galactosidase activity (M) in the complete tetrads of the strain CT-Mel.

otic products versus 65 resistant ones) points to the insertion of the cassette in two of the three homologous chromosomes. Meiotic analysis therefore indicated that CT-Mel is trisomic for the chromosomes where the markers tested are located, with two functional copies and one nonfunctional copy of several markers. These markers segregate randomly, and their segregation does not correlate with the lack of viability that affects almost half of the meiotic products (48%).

DNA content of the baker's yeast strains CT, VS, and DADI, the Mel⁺ transformant strains CT-Mel, VS-Mel, and DADI-Mel, and four complete tetrads of CT-Mel. When the DNA content in the baker's yeast strains was determined, the laboratory yeasts MMY1 and DS81 were used as haploid and

diploid controls, respectively. The results obtained are expressed in Fig. 3 and summarized in Table 3. When compared with MMY1 and DS81 laboratory yeasts, strain CT seemed to be $3n$; DADI had a DNA content of about $1.5n$, which explains the lack of viability of the meiotic products; also, strain VS was $2n$, but the lack of viability of the meiotic products indicated the strain to be strongly unbalanced, having several copies of some homologous chromosomes and probably a single copy of some others. The Mel⁺ transformant strains CT-Mel, VS-Mel, and DADI-Mel possessed the same DNA content as the untransformed controls. CT-Mel meiotic products had a DNA content that varied between 1.4 and 1.7. Results indicate that the transformation process has not resulted in major DNA

FIG. 5. Electrophoretic profiles of the laboratory yeast ATCC 9080 (lanes 1, 8, 18, and 28); the baker's yeast strains CT (lane 2), VS (lane 4), and DADI (lane 6); the transformed baker's yeast strains CT-Mel (lanes 3, 9, and 19), VS-Mel (lane 5), and DADI-Mel (lane 7); and four tetrads of the CT-Mel strain (tetrad 1 [lanes 10 through 13], tetrad 2 [lanes 14 through 17], tetrad 3 [lanes 20 through 23], and tetrad 4 [lanes 24 through 27]).

reorganizations in the recipient strains and that meiotic products of complete tetrads isolated from CT-Mel have received an almost identical DNA content. The DNA content (1.4 to 1.7) of the CT-Mel meiotic products would explain the complete lack of viability of the spores of those meiotic products able to form asci. Finally, results also indicated that strain CT is heterothallic; otherwise, the DNA of the meiotic products, after duplication, should have been almost equal to that of their parental CT-Mel strain.

Meiotic analysis of strain CT-Mel and its four complete tetrads with regard to its ability to grow in SGal, resistance to sulfometuron, and α -galactosidase activity. Untransformed VS, DADI, and CT controls were sensitive to sulfometuron and lacked α -galactosidase activity (genetically *mel⁰* [see Fig. 6]), but the strains grew in SGal. Transformed CT-Mel could also grow in SGal and in 40 mg of sulfometuron per ml, and its specific α -galactosidase activity was 27 (Table 4). When meiotic products from CT-Mel strains were analyzed, about one-third of them were unable to grow in SGal, and most of these were petite (Table 2). Growth in the presence of galactose was absolutely necessary to induce α -galactosidase activity. When those meiotic products unable to grow in SGal were grown in SD, washed, and transferred to SGal or SMeg for several hours, no α -galactosidase activity was detected in any case (Table 4). This result explains the lack of both growth in galactose and melibiase activity of the meiotic products 1b, 1d, 2c, 4a, 4c, and 4d (of which 1b, 1d, 2c, 4a, and 4d are petite), in spite of their being resistant to sulfometuron.

Table 4 shows that whereas meiotic products 1b, 1d, and 2c do not have activity for the reasons given above, 1a, 1c, 2a, and 2b have specific activities of 17, 17, 15, and 17, respectively, very close to the expected data. The activity of 2d, equal to that of the parent, is difficult to interpret.

Tetrads 3 and 4 segregate 3+:1- with regard to sulfometuron resistance, consistent with a 3+:1- segregation for α -galactosidase activity so that one meiotic product does not receive a *MEL1* copy, two of them receive one *MEL1* copy, and a fourth receives two copies (Fig. 4). Meiotic product 3b seems in fact to have received two *MEL1* copies (specific activity, 28) and 3c and 4b have received one copy (specific activity, 19 and 12, respectively). Again, the lack of growth in SGal leads to lack of activity in other meiotic products. The result for 3a seems to indicate recombination just inside the *ILV2* gene, so that it has inherited sulfometuron resistance but not the *MEL1* gene.

VS-Mel and DADI-Mel grew in SGal and in 40 mg of sulfometuron per ml and seemed to possess just one copy of the

MEL1 gene, since their activity was about half of that shown by the strain CT-Mel (Table 4).

Electrophoretic karyotype. The baker's yeast strains CT, VS, and DADI, the transformants CT-Mel, VS-Mel, and DADI-Mel, and the four complete tetrads of the transformed strain CT-Mel were subjected to karyotype analysis by the pulsed-field technique, using contour-clamped homogeneous electric field electrophoresis (Fig. 5). No strain yielded a chromosome-banding pattern similar to any other. Rather, the karyotype patterns were readily distinguishable for each strain. Besides, the transformed CT-Mel, VS-Mel, and DADI-Mel strains had a chromosome-banding pattern very similar to that of their untransformed controls. Even so, some minor changes seem to have occurred in the larger chromosomes, since changes in electrophoretic mobility are observed, mostly in strains DADI-Mel and VS-Mel. Tetrads of strain CT-Mel displayed different patterns both among themselves and from their parental strain (Fig. 5). The main feature was the segregation of bands among the meiotic products, which might indicate the presence in the parental strain of homologous chromosomes of different sizes and their unequal distribution among the meiotic products.

Presence of *MEL1* genes. Southern blot hybridizations with a *MEL1* probe from a strain of *S. cerevisiae* (14) were carried out with, as above, the untransformed baker's yeast controls CT, VS, and DADI; the transformed strains CT-Mel, VS-Mel, and DADI-Mel; and the four complete tetrads of strain CT-Mel. As a positive control, *S. cerevisiae* ATCC 9080 was included. Figure 6 shows that only the positive control and the transformed CT-Mel, VS-Mel, and DADI-Mel were able to hybridize with the *MEL1* probe. Also, results seem to indicate that integration had taken place in just one chromosome band and that it was the same in all the three strains, presumably in chromosome XIII (where the *ILV2* gene is located) (19), as judged by the chromosome-banding pattern (9), where the chromosome XIII band is probably located in these strains.

Figure 6 shows that in tetrads 1 and 2, all four meiotic products possessed at least one copy of the *MEL1* gene. This 4+:0- segregation of tetrads 1 and 2 confirms the results of the genetic analysis (Table 4). Tetrads 3 and 4 (Fig. 6) seemed to possess *MEL1* copies in two and three meiotic products, respectively. Again, this result confirms the genetic analysis of sulfometuron resistance and α -galactosidase activity expressed in Table 4. As indicated, both tetrads segregated 3+:1- with regard to sulfometuron resistance. In spore 3a, a recombination event seems to have occurred within the *ILV2-SMR30-MEL1* cassette, so that the spore was resistant to sulfometuron but lacked α -galactosidase activity.

FIG. 6. Southern hybridization analysis of chromosomal DNAs with the cloned gene *MEL1*. Strains are as in Fig. 5.

DISCUSSION

In this study α -galactosidase-positive (Mel^+) transformant baker's yeast strains were characterized. The absence of bacterial DNA in the integrant structure will facilitate the commercial application of these procedures in the food industry. Most commercial yeasts are polyploid or aneuploid strains, and in this study it was demonstrated that baker's yeast CT is $3n$, VS is $2n$ and DADI is $1.5n$ (Fig. 3; Table 3). Therefore, transformation of these yeasts requires a good dominant selectable marker (6). Sulfometuron resistance is an appropriate marker, since transformed yeasts grew in 40 mg of the herbicide per ml whereas the untransformed strains stopped growing at 10 mg/ml. Also, the marker has proved useful in baking, brewing (ale and lager), distillery, wine, and sake yeasts (19).

The transformed strains grew vigorously in minimal laboratory media (Table 1; Fig. 1) and in molasses (Fig. 2), with growth rates similar to or even higher than those of the control, indicating that the transformation process had not negatively affected the strains and that the transformation markers were stable even under nonselective conditions. Other authors (6) have reported that the Mel^+ integrant strains segregate to Mel^- at a rate of only 0.02% per generation so that even when there is no selective advantage for the Mel^+ segregants, the culture would be expected to be Mel^+ at the end of commercial propagation.

On the basis of a commercial yield of over 47% of yeast dry matter per unit of glucose, an increase in yeast content should be expected when using a Mel^+ strain. This increase would depend on the raffinose content of the molasses, which varies from 1 to 8% (wt/vol) (13). However, because of the complexity of this medium and the large number of unknown compounds that masked the raffinose peak in the high-pressure liquid chromatography, it was not possible to establish the raffinose content of the beet molasses used in this study.

Even so, the $Y_{X/S}$ obtained was up to 8% percent higher than that of the untransformed controls (Fig. 2). Similar results have been observed when molasses fermentation medium with different amounts of added raffinose was used (6). Furthermore, these authors found great variations in biomass yields in different strains or even different fermentations, which supports the results of this study. Some authors found that when using Mel^+ derivatives of baker's yeasts, another advantage, as well as the increase in yield, was the avoidance of problems and costs associated with the contribution of melibiose to biological oxygen demand in factory effluent (6, 5). In addition, the hydrolysis of raffinose aids in the crystallization of sucrose during the refining of sugar beets (6).

Several polymorphic genes for the fermentation of melibiose in different Mel^+ strains of *S. cerevisiae* have been identified

(12). Whereas some strains contained only one *MEL* gene, some others contained five or more of these genes located on different chromosomes (11, 12, 16) even in Mel^- strains. This suggests that the *MEL* loci have evolved by transposition of an ancestral gene to specific locations within the genome (4, 12, 17) in a way similar to that of many other gene families such as polymeric *SUC* genes or *MAL* loci (10, 16). As a consequence, the transformed strains used in this work could have integrated cassettes in several loci that presented total or partial homology with the cassette sequence (*ILV2* or *MEL1*). However, when analyses were carried out, the results seemed to indicate that the cassette had been integrated in two of three homologous chromosomes of the CT-Mel strain (Tables 2 and 4). These results seem to be confirmed by Fig. 5 and 6, because when *MEL1* probes were hybridized to the chromosomal bands of these strains, only a single band appeared in transformed CT-Mel, VS-Mel, and DADI-Mel strains. Results therefore indicate that the cassette had integrated in a single chromosome, probably chromosome XIII (19).

When *MEL1* probes were hybridized to the meiotic products of the transformed CT-Mel strain, the observed segregation ($4+ : 0-$, $3+ : 1-$, and $2+ : 2-$) once again confirmed the data of Tables 2 and 4 and the suggestion that *MEL1* is integrated in two of three homologous chromosomes (Fig. 4). Because of the low viability of the VS-Mel and DADI-Mel meiotic products, it was impossible to carry out genetic analysis with these strains. However, it has been demonstrated (6) that when the copy number of the *MEL1* gene is increased, the amount of α -galactosidase produced also increases. Assuming this gene dosage effect, the results in Table 4 and Fig. 6 would indicate that whereas CT-Mel had received two copies of the *MEL1* gene, both VS-Mel and DADI-Mel had received only a single copy. This gene dosage effect also seems to be indicated by the meiotic products of strain CT-Mel (Table 4; Fig. 6).

Attempts to map the *MEL1* gene in the meiotic products of the CT-Mel strain able to mate were all unsuccessful. Hybrids between these meiotic products and the laboratory strains (MMY1 and MMY2) gave rise, after sporulation, to inviable tetrads. In no case was viable product obtained after meiotic products of the baker's yeasts and laboratory strains were crossed.

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