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New *Saccharomyces cerevisiae* Baker's Yeast Displaying Enhanced Resistance to Freezing

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Three procedures were used to obtain new *Saccharomyces cerevisiae* baker's yeasts with increased storage stability at −20, 4, 22, and 30 °C. The first used mitochondria from highly ethanol-tolerant wine yeast, which were transferred to baker's strains. Viability of the heteroplasmons was improved shortly after freezing. However, after prolonged storage, viability dramatically decreased and was accompanied by an increase in the frequency of respiratory-deficient (petite) mutant formation. This indicated that mitochondria were not stable and were incompatible with the nucleus. The strains tested regained their original resistance to freezing after recovering their own mitochondria. The second procedure used hybrid formation after protoplast fusion and isolation on selective media of fusants from baker's yeast meiotic products resistant to parafluorphenylalanine and cycloheximide, respectively. No hybrids were obtained when using the parentals, probably due to the high ploidy of the baker's strains. Hybrids obtained from nonisogenic strains manifested in all cases a resistance to freezing intermediate between those of their parental strains. Hybrids from crosses between meiotic products of the same strain were always more sensitive than their parentals. The third method was used to develop baker's yeast mutants resistant to 2-deoxy-D-glucose (DOG) and deregulated for maltose and sucrose metabolism. Mutant DOG21 displayed a slight increase in trehalose content and viability both in frozen doughs and during storage at 4 and 22 °C. This mutant also displayed a capacity to ferment, under laboratory conditions, both lean and sweet fresh and frozen doughs. For industrial uses, fermented lean and sweet bakery products, both from fresh and frozen doughs obtained with mutant DOG21, were of better quality with regard to volume, texture, and organoleptic properties than those produced by the wild type.

KEYWORDS: Freezing resistance improvement; heteroplasmon formation; hybrid formation; invertase; maltase and trehalose deregulated mutants; baker's yeast; bakery products

INTRODUCTION

Baker's yeasts are used to ferment doughs made with flour from mostly wheat and barley (*1*). The paramount consideration in baker's yeast production has been its quality, as expressed by its fermentation characteristics in dough substrates and its storage stability, including that in frozen doughs (*2*−*4*). The production of frozen doughs is increasing considerably in response to the challenge of supplying wide-ranging distribution networks and an increase in the consumption of homemade products, which are prepared rapidly (*1*). Yeasts must be able to leaven bread efficiently by producing considerable quantities of carbon dioxide, mostly via ethanolic fermentation of various sugars present in the doughs (*2*). Bread dough is a complex rheological matrix, and a freezing process affects both the yeast cell and the gluten matrix (*5*). The lack of resistance to freezing of the baker's yeast gives rise to a decrease in the percentage of living cells able to ferment the maltose of the dough and produce CO_2. Besides, the autolyzed cells liberate various reducing agents, which weaken the S−S linkage in the gluten of the dough, preventing CO_2 retention and compacting the dough mass (*6*).

Saccharomyces cerevisiae strains that showed better freeze−thaw resistance than commercial baker's strains were selected, but these strains did not exhibit both freeze−thaw resistance and high leavening ability simultaneously. There have been attempts to exploit the potentiality of non-*Saccharomyces* yeasts, isolating hybrids with baker's yeast that could leaven the dough efficiently (*7*). However, there is no indication that any hybrid yeast strains may have been produced commercially so far.

Yeast in general survive freezing (high percentages) when frozen quickly at −80 °C. However, for food preservation and in commercial practice, relatively higher temperatures (−20 °C) are applied, so that the survival ratio is lower and damage is

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Table 1. *S. cerevisiae* Strains Used in This Work

strain	*a*	source
A1, A2, A3, A4, AZ1, AZ2, AA1, CL1, and CL2	B	Burns Philp Food, Villarrubia, Córdoba, Spain; strains used to ferment sweet doughs
ACA21	W	A. Casas, Universidad de Sevilla, Spain
DOG21	B	this study (2-deoxy-D-glucose spontaneous resistant mutant of V1)
DS81	G	Yeast Genetic Stock Center, Berkeley, CA
H1, H2, and H3	B	Burns Philp Food, Villarrubia, Córdoba, Spain; strains with high fermentative capacity
IFI256	W	V. Arroyo, Instituto de Fermentaciones Industriales, Madrid, Spain
MMY1	G	Yeast Genetic Stock Center, Berkeley, CA
S1 and S2	B	Burns Philp Food, Villarrubia, Córdoba, Spain; strains used to ferment lean doughs
V1	B	Compañía General de Levaduras, Valladolid, Spain
V1R	B	this study ([rho°] mutant of V1)
V1A	B	this study (heteroplasmon with V1R nucleus and ACA21 mitochondria)
V1F	B	this study (heteroplasmon with V1R nucleus and IFI256 mitochondria)
V1V	B	this study (heteroplasmon with V1R nucleus and V1 mitochondria)
V2	B	Compañía General de Levaduras, Valladolid, Spain
V2R	B	this study ([rho°] mutant of V2)
V2A	B	this study (heteroplasmon with V2R nucleus and ACA21 mitochondria)
V2F	B	this study (heteroplasmon with V2R nucleus and IFI256 mitochondria)
V2V	B	this study (heteroplasmon with V2R nucleus and V2 mitochondria)
YNN295	G	ALKO Ltd., Helsinki, Finland

[a] G, genetic laboratory line; B, baker's yeasts; W, wine yeasts.

higher. This is attributed to the formation of ice crystals leading to intracellular dehydration and membrane damage. Viability of yeast cells in the frozen dough system lies within the cell membrane, so that the integrity of the membrane is critical to the success or failure of the yeast (*5*). Membrane integrity during freezing that occurs in the presence of high alcohol levels and under high osmotic pressure requires a high phospholipid content to prevent it from breaking (*2*). Yeasts that possess a particular membrane structure are either more sensitive or more tolerant to the lethal effect of freezing than other yeasts (*1*). Experiments conducted over six months using ethanol-tolerant wine yeasts improved fermentation performance in both lean and sweet doughs (*5*). Furthermore, there are reports of an increase in tolerance to ethanol and temperature of heteroplasmons with mitochondria from wine yeast acting as donors (*8*). Transference of mitochondria to baker's yeast from strains highly tolerant to ethanol (*8*) might give rise to heteroplasmons more tolerant during storage at different temperatures and to freezing (*3*).

The relevance of trehalose, not simply as a reserve carbohydrate, may serve as a cell protectant during extreme stress such as desiccation and freezing (*5*). Freeze-tolerant strains showed higher trehalose-accumulating abilities than other strains, but once the cells started to ferment, maintenance of a high trehalose level did not suffice to prevent loss of stress resistance (*2*, *4*, *9*). For this reason, prefermentation of dough destined for the freezer has a very negative impact on the quality of the baked goods and diminishes the shelf life of the frozen dough (*5*). This situation is worse in frozen sweet dough, which contains different added sucrose levels, because yeasts are exposed to high osmotic pressures that limit their growth (*4*). Osmotolerant yeasts of the genus *Torulopsis* or *Zygosaccharomyces* contain high levels of trehalose, but in *S. cerevisiae* baker's yeast several production parameters have to be changed to induce higher levels of this compound (*5*).

In this study we developed new strains of baker's yeast, which improved viability during storage and fermentation of frozen doughs. The procedures used included mitochondria transfer from highly ethanol-tolerant wine yeast (*8*) to baker's yeast recipient strains, hybrid formation between isogenic and nonisogenic baker's yeast (*10*, *11*), and characterization of baker's yeast mutants (*12*) deregulated for sugar metabolism and with improved capacity to ferment sweet doughs.

Table 2. Genetic Characteristics of *S. cerevisiae* Baker's Yeasts

strain	DNA content	% ascus formation	% spore viability	% auxotrophic meiotic products[a]	pattern of electrophoretic karyotype[b]
A1	1.6n	6	1.2	ND	A
A2	1.5n	25	3.7	16	B
A3	1.8n	20	2.5	ND	C
A4	1.4n	32	1.2	50	D
AZ1	1.5n	ND	ND	ND	A
AZ2	1.5n	ND	ND	ND	B
AA1	2.3n	ND	ND	ND	E
CL1	3n	ND	ND	ND	E
CL2	3.2n	ND	ND	ND	E
S1	2.4n	89	28	17	E
S2	2.4n	75	40	7	F
H1	2.3n	61	46	16	F
H2	2.4n	0			G
H3	2.4n	35	52	19	E
V1	2.7n	75	45	21	E
V2	2.7n	60	51	8	F
SD81[c]	2n	90	80	0	

[a] All baker's strains able to sporulate have meiotic products able to mate. [b] Arbitrary classification. Strains with the same letter possess identical chromosomal pattern (number and possition of chromosomal bands). [c] Laboratory diploid control. Results are the average of three experiments with standard deviation of <5%.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this work are listed in **Table 1**. **Table 2** represents the genetic characteristics of *S. cerevisiae* baker's yeast. For heteroplasmon formation we selected as recipient baker's yeast strains V1 and V2 as shown in **Table 1**, due to their high fermentative capacity (*13*−*15*), frequency of sporulation, tetrad formation, and spore viability (*10*). We also selected the highly ethanol-tolerant wine yeasts ACA21 and IFI256 as mitochondria donors (*8*). V1 and V2 are grande strains (with functional mitochondria); V1R and V2R are rho° mutants derived from V1 and V2, which lack mitochondrial DNA (mtDNA) (*10*); heteroplasmons were formed with V1R or V2R nucleus and either ACA21 (V1A, V2A), IFI256 (V1F, V2F), V1 (V1V), or V2 (V2V) mitochondria (*10*). For experiments of hybrid selection we used the following commercial baker's yeasts (**Table 1**): A1, A2, A3, A4, AZ1, AZ2, AA1, CL1, CL2, S1, S2, H1, H2, and H3. From V1 baker's yeast, 2-deoxy-D-glucose (DOG)-resistant mutants were isolated, and mutant DOG21 was obtained (*12*) and characterized with regard to freezing resistance in this study. We used the laboratory yeast YNN295 as the control for karyotype electrophoresis and the

laboratory strains MMY1 and SD81 (**Table 1**) (*10*) as controls of resistance to cycloheximide and sporulation efficiency, respectively.

Media. Media used were complete YP medium [1% Difco (Detroit, MI) yeast extract; 2% bacto peptone], supplemented with 2% dextrose (YPD), 2% maltose (YPM), 3% glycerol (YPG), or 3% glycerol and 0.1% dextrose (YPDG). For the isolation of cycloheximide-resistant mutants (CYH^R mutants), YPD was supplemented with 0.1−10 mg L^{-1} cycloheximide (YPDcyh). Minimal medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate; 0.5% ammonium sulfate) was supplemented with 2% dextrose (SD), 3% glycerol (SG), or 2% dextrose and 0.1% proline as the nitrogen source instead of ammonium salt (SDP). For the isolation of parafluorphenylalanine-resistant mutants (PPA^R mutants), SDP was supplemented with 0.01−2.0 mg L^{-1} parafluorphenylalanine (SDPppa). For hybrid isolation SDP was supplemented with CYH and PPA (SDPcyhppa). Presporulation (PRE1), sporulation (SPO1), and protoplast regeneration (SOS) media employed were described elsewhere (*10*). Beet molasses (72% sucrose) obtained from Unión Alcoholera Española, S.A. (Granada, Spain) diluted 20 times (3.6% sucrose) was also used (*11*). Media were solidified by the addition of 2% agar.

Enzymes and Chemicals. Proteinase K and sucrose were obtained from Merck, A.G. (Darmstadt, Germany), and Zymolyase 2000 from Seikagako (Seikagako kogyo Co Ltd., Tokyo, Japan). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Culture Conditions. Cells were cultivated as described (*14*). Growth rate, μ (h^{-1}), was determined by measuring the increase in A_{660} (*10*).

Total Cell Number and Viability. Cell number (cells mL^{-1}) was estimated by diluting the samples in water, measuring A_{660}, and counting cells under the microscope, using a hemacytometer. Viability (cfu mL^{-1}) was determined by spreading samples on YPD and counting colonies after 3−4 days of incubation at 30 °C.

Frequency of Petite Mutants. The percentage of petite cells was determined by spreading a midexponential phase culture (5×10^7 cells mL^{-1}) growing in YPD, on YPDG, counting the number of colonies after 3 days of incubation at 30 °C, and measuring colony size (*10*).

CYH^R and PPA^R Mutant Isolation and Stabilization. Once the minimal inhibitory concentrations for the wild types (1.5 mg L^{-1} CYH and 0.5 mg mL^{-1} PPA) were established, baker's yeast strains were pregrown overnight in YPD, harvested during the exponential phase of growth (1×10^7 cells mL^{-1}), washed with sterile distilled water, and plated on YPDcyh (CYH concentrations of 1.5−10 mg L^{-1}) or SDPppa (PPA concentrations of 0.5−2.0 mg mL^{-1}) selective media, at a concentration of 10^8 cells per plate. Colonies appearing after 6−12 days of incubation were subcultured on the same medium for several cycles; each time samples were taken, washed, and plated on YPD, SDP, and YPDcyh and SDPppa solid media, and the percentage of colonies resistant to the toxics was determined to assess the stability of the mutants. Only mutants with >90% CYH^R or PPA^R cells were selected.

Sporulation and Tetrad Analysis. Standard protocols for baker's yeast were used to induce sporulation and to carry out tetrad analysis (*14*).

Protoplast Formation, Protoplast Fusion, and Heteroplasmon or Hybrid Selection. The isolation and fusion of protoplasts from baker's yeasts have been previously described (*10*). After incubation to allow for cell wall regeneration (*10*), the fusion products were replica-plated to appropriate selective media and incubated at 30 °C for 4−12 days until colonies were visible. The colonies were transferred to selective media, and the heteroplasmons or hybrids were characterized.

Transfer of Mitochondria and Recovery of Original Functional Mitochondria from the Heteroplasmons. Active mitochondria were transferred from ACA21 or IFI256 grande wine yeasts to spontaneous petite (rho°) V1 or V2 baker's receptor strains following procedures described in ref *10*. The original functional mitochondria of baker's yeast were returned to spontaneous petite (rho°) mutants of heteroplasmons with the nucleus of the V1 or V2 strain and mitochondria of ACA21 or IFI256 wine yeasts, following procedures previously described (*10*).

mtDNA Restriction Analysis. The mtDNA of the strains was purified, precipitated, washed, and dried as described by Martínez et al. (*16*). Yeast DNA was digested with the appropriate restriction enzymes after incubation with 10 μL of the DNA solution at 37 °C for 12 h. The mtDNA restriction patterns were observed using 0.8% agarose electrophoresis (*16*).

Electrophoretic Karyotype and DNA Content. The basic procedure for chromosomal DNA preparation was that used by Naumov et al. (*17*). The gel was prepared with 0.5% TBE buffer (*12*) and 1% agarose. The system used was a CHEF-DRII gel electrophoresis apparatus from Bio-Rad. Electrophoresis was carried out as previously described (*15*). DNA content per cell was determined as described (*14*).

Analytical Procedures. *Enzyme Assay of Maltase and Invertase and Measurement of Trehalose Content.* Cells were broken by the addition of 1.0 g of glass beads (0.5 mm diameter) and shaken in a vortex for 1 min to obtain crude extracts (*13*). Invertase and maltase were assayed by monitoring liberated glucose in a YSI27 glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). The standard assay mixture contained 20 μL of crude extract obtained from 20 mL of either YPD, molasses, or YPM medium and 20 μL of either sucrose or maltose at 5% (w/v). Maltase was also assayed with *p*-nitrophenyl β-D-glucopyranoside (pNPG) as previously described (*12*). One unit of activity was defined as the amount of enzyme required to liberate 1 nmol of glucose per microgram of protein per minute at 30 °C. Trehalose content was determined after 30 min of incubation of 30 μL of crude extract obtained from 20 mL of molasses cultures with 50 μL of solution containing 1 unit mL^{-1} trehalase. Free glucose was then measured by the glucose oxidase procedure (*12*).

Protein Determination. Total protein was determined either following the procedure of Lowry et al. (*18*) or according to the method of Bradford (*19*) using the Bio-Rad protein assay dye reagent and bovine serum albumin as the protein standard.

Viability after Storage at 4 and 22 or 30 °C. Cells were cultivated in YPD or molasses to stationary phase (*12*). The cells were then centrifuged and washed, and viability, cell number, and wet weight were determined. Aliquots of the cultures were filtered. The filters were weighed and kept at 4, 22, or 30 °C. To determine dry weight, samples were filtered and the filters incubated at 50 °C to constant weight. Periodically, aliquots from 4, 22, or 30 °C were obtained and cell viabilities determined.

Resistance to Freezing and Determination of the Capacity To Leaven Dough. To determine freezing resistance and leavening capacity under laboratory conditions, 20 mL tubes were prepared containing 3.5 mL of distilled water and 2 g of wheat flour (El Calamar, La Algaba, Sevilla, Spain) of W160−180 for plain doughs and W230−240 for sweet doughs (*20*). The tubes were inoculated to final percentages of yeast (previously grown in molasses until stationary phase, 5×10^8 cells mL^{-1}) of 2.5% (plain doughs) or 5% (sweet doughs)(wet weight) with regard to the flour's weight, as per standard industrial protocols (*12*, *21*). The tubes, supplemented or not with 26% sucrose and inoculated with the yeast, were frozen at −20 °C. Sample tubes were taken periodically, thawed, and incubated without shaking at 30 °C. The increase in volume was monitored every 10 min for 2−3 h for plain doughs or for 3−4 h for sweet doughs (*11*−*15*). Viability and trehalose contents were also measured following procedures previously described (*12*, *22*).

Baking. Yeasts were grown in molasses to late stationary phase (~5×10^8 cells mL^{-1}, 30 g L^{-1} wet weight). Elaboration of lean bakery products was carried out by mixing wheat flour (W160−180) with 20 g of salt and 30 or 50 g of yeast (wet weight), either the parental V1 or the mutant DOG21, per kilogram of flour. Elaboration of sweet bakery products was carried out by mixing wheat flour (W230−240) with 200 g of sucrose, 20 g of salt, 50 g of lard, 2 eggs, and 30 or 45 g of yeast (wet weight), either the parental V1 or the mutant DOG21, per kilogram of flour (*12*). The mixtures were preincubated at 40 °C and 60% humidity for 30 min and then frozen at −20 °C (frozen doughs); alternatively, they were incubated for 40 min to 3 h and then baked at 210 °C for 10−30 min (fresh doughs). Frozen doughs were stored at −20 °C for 48 h−7 days. Periodically, frozen doughs were thawed and then incubated at 40 °C and 60% humidity for 40 min−3 h and finally baked at 210 °C for 10−30 min. The products were assessed with regard to their texture and organoleptic properties by 30−40 nonexpert people following standard procedures for quality evaluation (*21*).

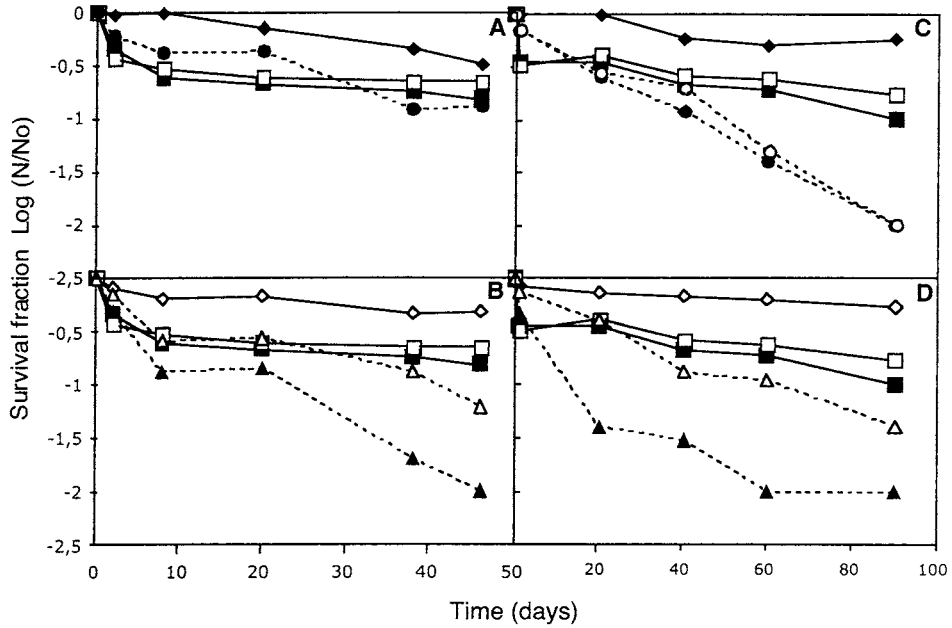

Figure 1. Survival fraction [Log N/N_0, where N is the number of cells surviving the treatment and N_0 is the number of cells before treatment (zero time)] during cell storage at −20 °C of wine strains ACA21 (◆) and IFI256 (◇), baker's yeasts V1 (■) and V2 (□), and heteroplasmons possessing the nucleus of V1 or V2 and the mitochondria of ACA21 [V1A (●) or V2A (○)] or IFI256 [V1F (▲) or V2F (△)]. After heteroplasmon formation, cells were aliquoted and kept at −20 °C. After freezing, one series of samples was taken periodically for ∼2 months and viability was determined (A and B). Another series of samples was maintained at −20 °C for 3 years and then spread on YPG solid medium. Colonies were selected, and the cells were washed, aliquoted, and kept at −20 °C. After freezing, samples were taken periodically for ∼3 months and viability was determined (C and D). Results are the average of five to six experiments in triplicate, with standard deviations of <10%.

All data shown are the average of three to six experiments, with standard deviations of <10%.

RESULTS AND DISCUSSION

Resistance to Freezing and Storage Stability at 4 and 30 °C of V1 and V2 Baker's Yeast Heteroplasmons. Viabilities were determined for baker's strains V1 and V2 and wine yeasts ACA21 and IFI256, in frozen cells kept at −20 °C (**Figure 1**), refrigerated at 4 °C (**Figure 2**), or stored at 30 °C (**Figure 3**). Viability was higher in strain IFI256 in all cases (**Figures 1**−**3**) and in ACA21 wine yeasts after freezing and refrigerating (**Figures 1** and **2**). The capacity of yeasts to appropriately ferment in frozen doughs or after storage of the cells at refrigerated (4 °C) or room temperature (20−30 °C) depends on the cell membrane (*2*), which must remain pliable and intact (*5*). Lipid composition affects membrane functions (*23*, *26*) regulating membrane fluidity and allowing high viability. Yeast membranes contain a mixture of phospholipids and sterols (*23*) that are synthesized under aerobic conditions and when yeasts possess functional mitochondria (grande) (*8*, *24*, *25*). The high viability of wine yeasts of **Figures 1**−**3** suggested that wine yeast mitochondria were partly responsible for tolerance to temperature and freezing, as well as to ethanol and other membrane solvents (*8*). Wine yeasts probably maintain respiratory metabolism, biosynthesis of phospholipids, ergosterol, and unsaturated fatty acids, and therefore membrane integrity and high viability, under conditions where other yeasts do not survive (*8*, *27*) (**Figures 1** and **2**) and where the frequency of cells with nonfunctional mitochondria (petite) dramatically increases. The decrease in the viability of strain ACA21 at 30 °C indicated that there were genes in strain ACA21 involved in tolerance/sensitivity to storage at 30 °C (**Figure 3**) different from those involved in tolerance at −20 and 4 °C (**Figures 1** and **2**).

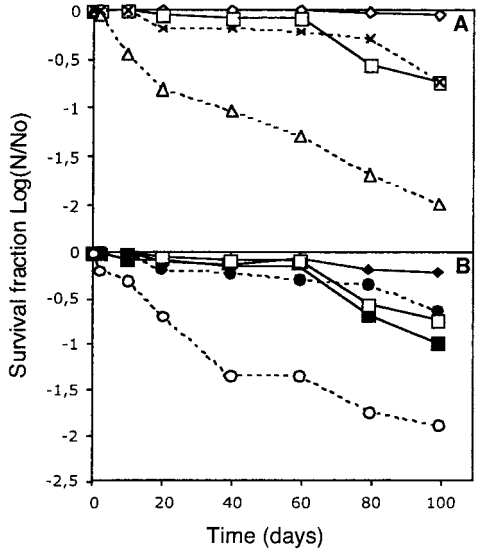

Figure 2. Survival fraction [Log N/N_0, where N is the number of cells surviving the treatment and N_0 is the number of cells before treatment (zero time)] during cell storage at 4 °C of baker's strains V1 (■) and V2 (□), wine yeasts ACA21 (◆) and IFI256 (◇), and heteroplasmons possessing the nucleus of V1 and the mitochondria of ACA21 [V1A (●)] or the nucleus of V2 and the mitochondria of ACA21 [V2A (○)] or IFI256 [V2F (△)] or after recovering its own mitochondria (V2V) (×). After heteroplasmon formation, cells were aliquoted, filtered, and kept at −20 °C for 3 years. Then samples were spread on YPG solid medium. Colonies were selected, and the cells were washed, aliquoted, filtered, and kept at 4 °C. Samples were taken periodically for ∼3 months, and viability was determined. Results are the average of five to six experiments in triplicate, with standard deviations of <10%.

It has been reported that, under extreme conditions such as high concentration of ethanol or high temperature, under which

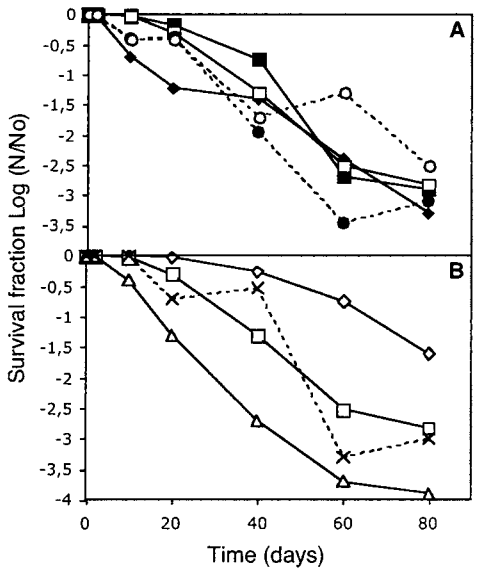

Figure 3. Survival fraction [Log N/N_0, where N is the number of cells surviving the treatment and N_0 is the number of cells before treatment (zero time)] during cell storage at 30 °C of baker's strains V1 (■) and V2 (□), wine yeasts ACA21 (◆) and IFI256 (◇), and heteroplasmons possessing the nucleus of V1 and the mitochondria of ACA21 [V1A (●)] or the nucleus of V2 and the mitochondria of ACA21 [V2A (○)] or IFI256 [V2F (△)] or after V2 had recovered its own mitochondria [V2V (×)]. After heteroplasmon formation, cells were aliquoted, filtered, and kept at −20 °C for 3 years. Then samples were spread on YPG solid medium. Colonies were selected, and the cells were washed, aliquoted, filtered, and kept at 30 °C. Samples were taken periodically for ∼3 months and viability was determined. Results are the average of five to six experiments in triplicate, with standard deviations of <10%.

cells survive but do not divide, transfer of wine yeast mitochondria to a more sensitive yeast strain leads to a lower death rate, lower petite frequency, and an increase in ethanol tolerance and thermotolerance in the recipient strain (*8*). In this study mitochondria were transferred from wine (IFI256 and ACA21 strains) to baker's yeasts (V1 and V2), after isolation of spontaneous mutants that lack mtDNA from the baker's strains, following protocols previously described (*10*). Viability of heteroplasmons was measured; this viability was higher than that of the baker's strains (i.e., V1A, **Figure 1A**) immediately after the cells had been frozen and after one or several days (**Figures 1A**,**B**) of maintenance at −20 °C but lower when the storage time at −20 °C was prolonged (**Figure 1**). Similar results were obtained when the strains were stored at 4 and 30 °C (data not shown). There were also differences in viability between heteroplasmons with the same nucleus but with mitochondria from either ACA21 or IFI256 (**Figure 1**). The fact that the combination of the same nucleus with mitochondria from different strains gave different frequencies in viability (i.e., V1A and V1F, **Figure 1**) indicates the importance that the interactions of nucleus and mitochondria have on cell viability and mitochondria stability (*8*, *10*). The interaction of nucleus and mitochondria resulted in an increase in the frequency of petite mutants, from 0.1−0.5% in V1 and V2 strains, 0.2% in ACA21, and 0.5% in IFI256 to >5% in heteroplasmons V1A and V2A and >15% in heteroplasmons V1F and V2F. mtDNA restriction analysis of heteroplasmons V1A, V2A, or V1F or V2F indicated the same mtDNA pattern as that of the donors ACA21 or IFI256 (*10*). Therefore, the drop in viability of heteroplasmons was exclusively due to incompatibility between mitochondrial and nuclear genomes and not the result of mtDNA reorganizations. In addition, when mitochondria from V1 or V2 were transferred to heteroplasmons after isolation of the rho° mutants, the new strains (V1V and V2V) displayed viabilities similar to that of V1 or V2 original strains (**Figures 2** and **3**). The decrease in viability was not the result of nuclear chromosome reorganization after heteroplasmon formation, because the electrophoretic karyotype of heteroplasmons V1A, V2A, or V1F or V2F (data not shown) had the same chromosomal pattern as the strain V1 or V2 (*13*). To check the possibility that lack of viability was due to instability of the newly formed heteroplasmons, these were kept for three years at −20 °C, and after these three years, samples were plated on YPG, where only grande cells can grow. Grande colonies from each heteroplasmon were isolated, and the experiments of viability at −20 °C (**Figure 1C**,**D**), 4 °C (**Figure 2**), and 30 °C (**Figure 3**) carried out again with results comparable to those described above (**Figure 1A**,**B**).

Wine strains may be genetically quite distinct from baker's yeast, so that although they belong to the same species, *S. cerevisiae*, the lack of isogenicity accounts for the apparent incompatibility found between nuclei and mitochondria from different sources. The lack of improvement in leavening ability and freeze−thaw resistance in dough by hybrids between freeze-tolerant strains and a baker's yeast strain has also been attributed to the contribution of mitochondrial genome derived from the baker's yeast to the baking performance (*28*). When in this study mitochondria from wine yeasts were transferred to baker's strains and frequencies of sporulation and tetrad formation were analyzed, these frequencies also decreased either to no sporulation at all or to <50% in both parameters, with a substantial increase in the frequency of petite mutants (*10*). The improvement found when wine mitochondria were transferred to laboratory strains (*29*) might indicate a greater proximity between wine and laboratory strains than between these and the baker's strains. On the other hand, heteroplasmon formation using compatible yeasts may result in the isolation of stable strains more resistant to freezing and storage at different temperatures.

Freezing Resistance of Hybrids between Baker's Yeasts. Freezing tolerance is polygenic (*2*), and so there is probably more than a single gene responsible for the character. It is therefore expected that in nonisogenic baker's strains, genes involved in freezing tolerance have complementary functions (*29*). The baker's strains described in **Table 1** were characterized with regard to their genetic features such as DNA content, electrophoretic karyotype, frequency of ascus formation, and spore viability (**Table 2**). This characterization made it possible to assemble the strains in seven groups based on their specific karyotype (chromosomal band number and size): group A, formed by A1 and AZ1; group B, A2 and AZ2; group C, A3; group D, A4; group E, V1, H3, S1, AA1, CL1, and CL2; group F, V2, H1, and S2; and group G, H2. Yeasts with the same specific karyotype behave similarly with regard to their growth rate, μ (h^{-1}), in YPD (0.39−0.50 h^{-1}) or in molasses (0.28−0.36 h^{-1}), their yield in these media (23−30 g L^{-1}), and their fermentative capacity (to increase the volume of plain doughs) (7.8−9.8 mL), but their DNA contents were not the same. In group E, DNA content varied from 2.3n to 3.2n (**Table 2**), which indicated different aneuploidies in the strains that do not seem to affect their physiological characteristics. Spontaneous CYH^R (0.75−1 mg L^{-1}) or PPA^R (0.5−1 mg mL^{-1}) mutants were obtained from each of the different baker's strains to select hybrids between pairs or mutants. Due to the DNA content of the strains, mutations were expected to be dominant. However, no stable hybrids (colonies with vigorous

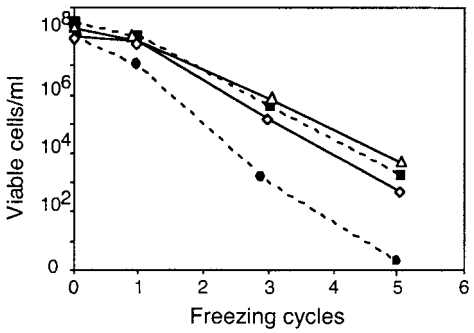

Figure 4. Viability of the baker's yeast S1 (◇) and A2 (△) and of hybrids between S1 and A2 meiotic products (A/S) (■) or between S1 meiotic products (S/S) (●). Once the hybrids had formed, cells were aliquoted and kept at −20 °C. After 1 week, aliquots were thawed, samples were taken for cell viability, and the rest were frozen again. The process was repeated each week for five cycles. The number of cells that remained viable was determined each time. Results are the average of three to four experiments in triplicate, with standard deviations of <10%.

growth on selective media) could be obtained after protoplast fusion, which was attributed to the high ploidy of the parentals (**Table 2**).

All strains tested except H2 were able to sporulate and produce viable spores (**Table 2**). In some industrial strains (i.e., flor yeasts) for which a polygenic character such as ethanol tolerance had been analyzed, some meiotic products showed this feature, indicating that the characteristic was homozygosis or that it was governed by several redundant genes (*29*). If that were the case in baker's strains, the meiotic products of some strains might behave as the parentals, and the hybrids formed could improve their characteristics by complementation (*29*). Tetrads from the different baker's strains were micromanipulated, and those meiotic products resistant to the toxics, with capacity to mate, or auxotrophs were selected. Meiotic products from A and S baker's strains (A, either A1, A2, A3, A4, AZ1, or AA1; S, either S1 or S2; **Figure 4**) or from the same strain S (either S1 or S2; **Figure 4**) were mixed and incubated in YPD for 24 h to allow conjugation. Samples were then spread on selective media. Colonies with vigorous growth were obtained only from crosses between meiotic products of A2 and S1 (A/S hybrids; **Figure 4**) and between meiotic products of S1 alone (S/S hybrids; **Figure 4**). About 500 independent A/S colonies and 300 S/S colonies were isolated, pooled, and subjected to five freeze−thaw cycles, because resistance to repeated freeze−thaw procedures is the main characteristic for a superior frozen dough yeast (*5*). A/S cells displayed a viability that was intermediate between those of their parentals (**Figure 4**). In reports on freeze−thaw resistance of hybrids between a commercial yeast strain—available for bread-making by the frozen-dough method—and a baker's yeast, leavening activity on doughs of various white breads and sweet goods also was intermediate between those of the parentals, but not better, for either fresh or frozen doughs (*28*). However, the S/S cells were in all cases more sensitive than S1 (**Figure 4**). In this study, the traits of the successful parental strains were polygenic and probably unlinked (*5, 29*), with a remote chance of producing an offspring possessing all of the desired characteristics of both parents. Results indicate that freezing resistance is polygenic but that either the parentals were heterozygous or that there are probably dominant alleles involved in sensitivity.

Characterization of the Baker's Yeast Mutant DOG21 with Regard to Its Baking Abilities on Fresh and Frozen Doughs. From the three mutants of baker's yeast V1 that were resistant to 2-deoxy-D-glucose and with altered metabolism of maltase and invertase (*12*), only one—DOG21—grew in molasses with the same growth rate and yield as the wild type and possessed increased trehalose content (**Table 3**). Trehalose protects cell membranes during extreme stress such as desiccation and freezing (*5*), and strains selected for bread-making by the frozen-dough method possessed the common ability to accumulate higher trehalose content than other nonresistant strains (*28*). However, when fermentation conditions were modified to increase the cell's trehalose levels, yeast with higher trehalose concentrations were not cryoresistant (*5*). In addition, naturally isolated strains other than *S. cerevisiae*, which have freeze−thaw resistance in dough, lacked maltose fermentation (*7*), but osmotolerant yeast and mutants with a constitutive *MAL* locus—which possess high maltase activity in glucose-supplemented media—contain high levels of trehalose.

Table 3. Physiological Characterization of the *S. cerevisiae* Baker's Strain V1 and the Mutant DOG21

characteristic	strain V1	mutant DOG21
enzymatic activities and trehalose content when grown in molasses		
maltase specific activity[a]	0.008 (1)	0.02 (2.5)
invertase specific activity[b]	10.0 (1)	24.0 (2.4)
trehalose content[c]	20.4 (1)	33.8 (1.7)
viability after 12 weeks at −20 °C		
in plain doughs (%)	3.0 (1)	2.3 (0.8)
in sweet doughs (%)	2.4 (1)	3.0 (1.2)
fermentative capacity before freezing and after 12 weeks at −20 °C[d]		
in plain doughs		
before freezing	11.0 (1)	10.5 (0.95)
at −20 °C	0.8 (1)	1.9 (2.40)
in sweet doughs		
before freezing	10.0 (1)	12.0 (1.20)
at −20 °C	1.1 (1)	3.0 (2.80)

[a] Nanomoles of nitrophenol liberated/min × μg of protein. [b] Nanomoles of glucose liberated/min × μg of protein. [c] Milligrams per gram of cell dry weight. [d] Leavening capacity measured as increase in the dough volume (mL) under laboratory conditions. Data in parentheses are given in relative values, where those of strain V1 are considered to be 1 under any condition. Absolute values are also shown. Results are the average of two to six experiments, carried out in triplicate, with standard deviations of <5%.

In this study we tried to establish whether there was a positive correlation between increased maltase activity in the presence of glucose and trehalose accumulation and between this parameter and resistance to freezing and storage. The V1 baker's strain and its mutant DOG21 (*12*), which possesses elevated levels of maltase and invertase under repression condition, were cultivated in molasses to early stationary phase [24 h of culture, $\sim(1-2) \times 10^8$ cells mL^{-1}] or late stationary phase (32 h of culture, $\sim 5 \times 10^8$ cells mL^{-1}], following standard industrial protocols (*11*). In some experiments, baker's strains A2 and S1, which possess a different (A2) and the same (S1) pattern of electrophoretic karyotype as V1 (**Table 2**), were also included. Results obtained with strains A2 and S1 were almost identical to those of strain V1 (**Table 3**) in all cases. DOG21 strain grown in molasses possessed 2.5× higher maltase and invertase activities compared to V1 (**Table 3**), whereas in YPM medium both invertase and maltase were ∼0.5× compared to both of V1 (*12*). Trehalose content in molasses was also higher in DOG21 than in V1 and the other baker's yeasts (**Table 3**). When cells were kept frozen at −20 °C (**Figure 5**), refrigerated at 4 °C (**Figure 6A**), or stored at 22 °C (**Figure 6B**) for different periods of time, and viability measured, DOG21 showed viability slightly higher than its parental (**Figures 5** and **6**). This increase in viability, together with the higher specific activities

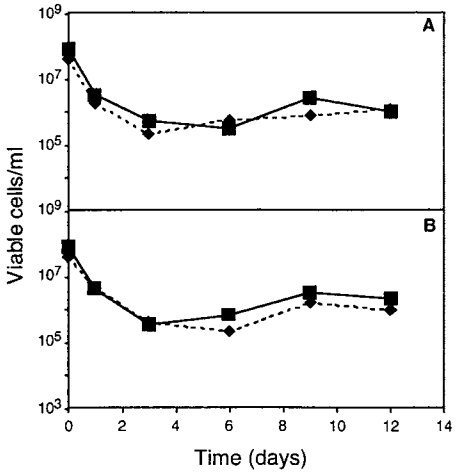

Figure 5. Viability of baker's strain V1 (◆) and the mutant DOG21 (■) after prefermentation of aliquoted sample tubes of plain (A) and sweet doughs (B) and storage at −20 °C. At different times after freezing, tubes with prefermented doughs were thawed and samples taken to determine cell viability. Results are the average of three experiments in triplicate, with standard deviations of <10%.

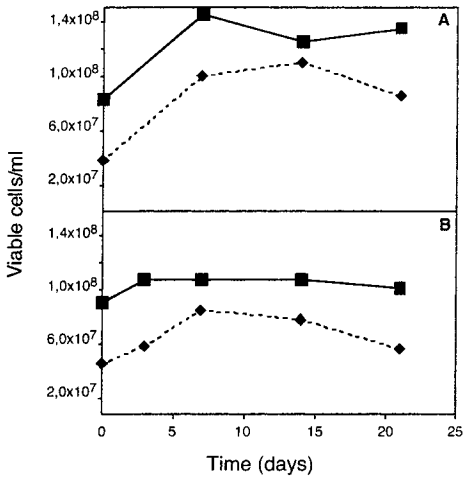

Figure 6. Viability of baker's strain V1 (◆) and mutant DOG21 (■). Cells were aliquoted and kept at 4 °C (A) or 22 °C (B). At different times aliquots were taken to measure cell viability. Results are the average of three experiments in triplicate, with standard deviations of <10%.

for maltase and invertase of DOG21 mutant, was also observed during measurements of fermentative capacity. DOG21 mutant always produced maximal volumes higher than V1 both in lean and in sweet doughs (**Figure 7**). The differences in final volumes of doughs fermented by V1 or DOG21 strains increased as the storage at −20° C was continued (**Figure 7**).

Baking experiments were also carried out with V1 and DOG21 strains. Cells were mixed with plain (**Figures 8** and **9**) or sweet (**Figure 10**) doughs. Pieces of sweet dough weighed 50−55 g (**Figure 10**). Pieces of plain doughs weighed 100−110 g, although in some experiments we prepared pieces of 50−55, 60−65, and 80−85 g (**Figure 9**). After prefermentation for 30 min, half of the pieces of doughs were frozen at −20 °C and thawed and fermented after different periods of storage (2−7 days). The other half were fermented for different times to obtain optimal volume and dough texture: 40 min for plain doughs fermented using strain DOG21; 2 h for plain doughs fermented using strain V1; 1 h and 30 min for sweet doughs fermented using strain DOG21; and 3 h for sweet doughs fermented using strain V1. An increase in the fermentative capacity of strain

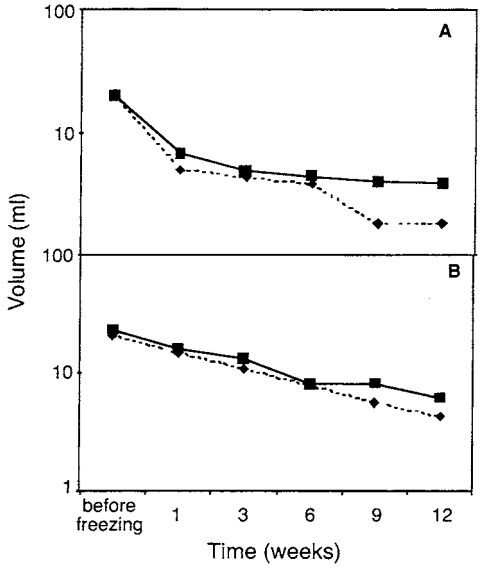

Figure 7. Leavening capacity measured as increase in dough volume (mL) of baker's strain V1 (◆) and mutant DOG21 (■), after prefermentation and storage at −20 °C of aliquoted sample tubes of plain (A) and sweet (B) doughs. At different times after freezing, tubes with prefermented doughs were thawed and incubated at 30 °C. Leavening capacity was determined. Results are the average of three experiments in triplicate, with standard errors of <5%.

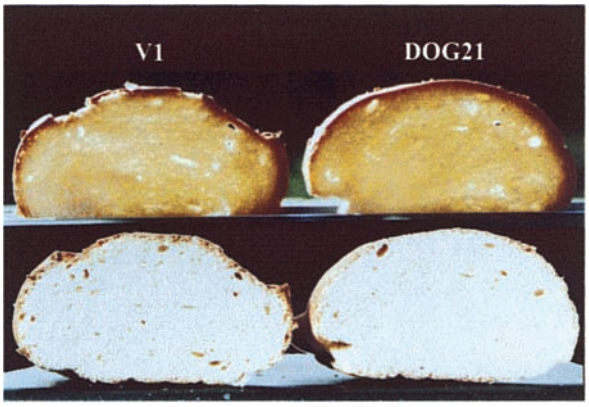

Figure 8. Cross sections of plain fresh doughs fermented with strain V1 or mutant DOG21. Pieces fermented with mutant DOG21 showed the best volume, elasticity, regularity, and size of the alveoli and suitability for slicing.

DOG21 resulted in a shortening the time required to ferment fresh (**Figures 8** and **10**) and frozen doughs (**Figures 9** and **10**) and a considerable increase in the volume of both plain doughs (**Figures 8** and **9**) and, above all, sweet doughs (**Figure 10**) fermented using mutant DOG21. Similar results were obtained when different percentages of yeasts (3, 4.5, or 5%) or flours with different W values (160, 170, or 180 for plain doughs; 230−240 for sweet doughs) were used.

Baked pieces of sweet and lean doughs were examined by 30−40 nonexpert tasters for volume, flavor, and texture following standard procedures (*21*). Those fermented using mutant DOG21 were the best evaluated with regard to each of the three parameters—volume, texture, and flavor. Pieces fermented using mutant DOG21 were also the best with respect to external and internal aspects of the pieces—browning, volume and density, elasticity, color, consistency, suitability for slicing, and regularity and size of the alveoli (pore structure) of both plain and sweet bakery products (**Figures 8−10**).

Figure 9. Whole pieces of plain fresh doughs preferemented, stored at −20 °C for 7 days, and further fermented with strain V1 or mutant DOG21. Pieces fermented with mutant DOG21 showed the best volume, elasticity, regularity, and size of the alveoli and suitability for slicing.

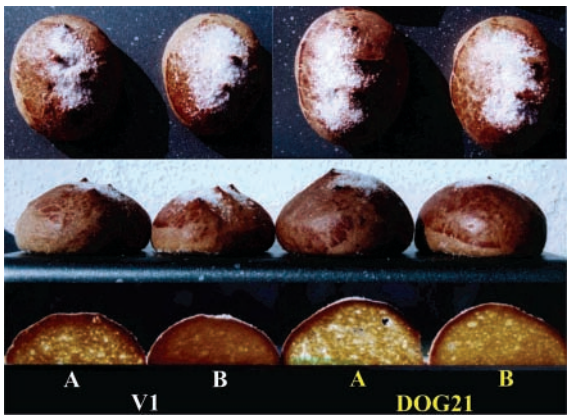

Figure 10. Cross sections and whole pieces of sweet fresh doughs fermented (A) or prefermented, stored at −20 °C for 2 days, and further fermented (B) with strain V1 or mutant DOG21. Pieces fermented with mutant DOG21 showed the best volume, elasticity, regularity, and size of the alveoli and suitability for slicing.

For rapid dough fermentation, the yeast property traditionally required is invertase activity to hydrolyze the higher glucofructans as quickly as possible, at a high maltose fermentation rate. There has been significant interest in strains with deregulated maltase and maltase permease activities. There has also been interest in the fermentation of sweet doughs, in which sucrose is added to the mix, and the construction of novel strains with deregulated, enhanced invertase activity (*12*).

In mutant DOG21 a positive correlation exists between maltase activity under repression conditions (molasses) (**Table 3**), trehalose content (**Table 3**), and resistance to storage at different temperatures (**Figures 5**−**7**), both as filtered cells or in prefermented doughs (**Figures 8**−**10**). The most desirable property of these new industrial strains is genetic stability and physiological reproducibility. This mutant was reported to be genetically very stable, its growth rate in molasses and fermentation capacities in fresh and frozen plain and sweet doughs were improved with regard to the wild type (*12*), and it can be immediately used for industrial processes.

Conclusions. Transient improvement in viability of baker's yeast heteroplasmons using mitochondria from wine yeasts after the cells had been frozen was due to incompatibility between mitochondrial and nuclear genomes of the strains used. Heteroplasmon formation using more compatible yeasts might result in the isolation of stable strains more resistant to freezing and storage at various temperatures.

After five freeze−thaw cycles, hybrids from nonisogenic baker's yeast displayed viability intermediate between those of their parentals; hybrids from the same baker's yeast strain were in all cases more sensitive than their parental strain.

Mutant DOG21, derived from V1 baker's strain, possesses elevated levels of maltase and invertase under repression conditions, higher trehalose content in molasses, and higher viability than its parental strain when kept frozen at −20 °C, refrigerated at 4 °C, or stored at 22 °C for different periods of time. This increase in viability, together with higher specific activity for maltase and invertase, resulted in an increase in fermentative capacity of strain DOG21, shortening the time required to ferment fresh and frozen doughs and with a considerable increase in the volume of both plain and, above all, sweet doughs. Baked samples were evaluated for taste, flavor, and texture, and those fermented with mutant DOG21 were regarded best with reference to the three parameters.

ABBREVIATIONS USED

YP, complete (yeast/peptone) medium with 2% dextrose (YPD), 2% maltose (YPM), 2% glycerol (YPG), or 0.1% dextrose and 3% glycerol (YPDG); S, minimal (sulfate/ammonia) medium with 2% dextrose (SD) or 3% glycerol (SG); SDP, minimal (sulfate/dextrose/proline) medium; M, molasses; CYH, cycloheximide; PPA, parafluorphenylalanine; DOG, 2-deoxy-D-glucose; pNPG, *p*-nitrophenyl β-D-glucopyranoside; TBE, TRIS borate EDTA buffer; W, alveograph value ($\times 10^3$ ergs); μ, growth rate (h^{-1}); CYH^R mutant, cycloheximide-resistant mutant; PPA^R mutant, parafluorphenylalanine-resistant mutant; rho° mutant, petite mutant lacking mitochondrial DNA; mutant DOG21, 2-deoxy-D-glucose-resistant mutant.

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