Induction of Petite Yeast Mutants by Membrane-Active Agents

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Ethanol proved to be a strong mutagenic agent of *Saccharomyces* mitochondrial DNA. Other active membrane solvents, such as *tert*-butanol, isopropanol, and sodium dodecyl sulfate, also turned out to be powerful petite mutation [*rho⁻*] inducers. Mutants defective in ergosterol synthesis (*erg* mutants) showed an extremely high frequency of spontaneous petite cells, suggesting that mitochondrial membrane alterations that were caused either by changes in its composition, as in the *erg* mutants, or by the effects of organic solvents resulted in an increase in the proportion of petite mutants. Wine yeast strains were generally more tolerant to the mutagenic effects of alcohols on mitochondrial DNA and more sensitive to the effect of sodium dodecyl sulfate than laboratory strains. However, resistance to petite mutation formation in laboratory strains was increased by mitochondrial transfer from alcohol-tolerant wine yeasts. Hence, the stability of the [*rho⁺*] mitochondrial DNA in either the presence or absence of solvents depends in part on the nature of the mitochondrial DNA itself. The low frequency of petite mutants found in wine yeast-laboratory yeast hybrids and the fact that the high frequency of petite mutants of a particular wine spore segregated meiotically indicated that many nuclear genes also play an important role in the mitochondrial genome in both the presence and absence of membrane solvents.

Respiration-deficient petite mutants [*rho⁻*] can be induced by a variety of chemical agents which affect hydrophobic interactions and that directly transform cells from [*rho⁺*] to [*rho⁻*] under nonproliferating conditions (1). Of these agents, 24% ethanol effectively induces respiration-deficient mutants with no specific nuclear effects (4), thus suggesting that the mutagenic action of ethanol involves damage to the mitochondrial membranes (4, 15). This suggestion is supported by reports on the isolation of petite mutants induced by a variety of chemical agents, some of which are membrane solvents (1). In fact, alcohols affect numerous biological processes, many of which are associated with the cell membrane (10, 25).

In *Saccharomyces* cells, sterols account for 6% (dry weight) of the protoplasmic membrane, are bound to the mitochondrial particles, and can liquefy and condense the membrane (9, 16). In organelles, such as mitochondria, in which membranes control the function of the organelle, it is essential to know how the sterol is distributed; Bottema and Parks (6) have found that the sterol-to-phospholipid molar ratio was very similar between the inner and outer membranes of yeast mitochondria. Additionally, the types of sterols present affect the physical properties of the mitochondrial membrane so that qualitative changes in the sterol components produce a wide range of fluidities (2, 3, 17, 22).

To see whether differences in the mitochondrial DNA influence the resistance to the mutagenic effects of membrane solvents, the frequencies of spontaneous and induced petite mutants were determined (i) in several wine yeasts resistant to the mutagenic effect of ethanol on mitochondrial DNA (15), (ii) in laboratory yeasts, (iii) in heteroplasmons possessing the nuclei of laboratory yeasts but the mitochondria of wine yeasts, and (iv) in wine yeast-laboratory yeast hybrids. In addition, to study the mechanism by which ethanol mutagenesis occurs in mitochondria, the frequency of petite mutants was determined in both wild-type and *erg*

mutants affected in the synthesis of ergosterol, a membrane compound directly involved in mitochondria functionality (6, 17, 18). Ethanol and other membrane solvents, such as *tert*-butanol, isopropanol, and sodium dodecyl sulfate (SDS), were used as petite mutation inducers.

MATERIALS AND METHODS

Organisms. Strains SM36 and *erg5* are laboratory yeasts (strains of *S. cerevisiae*) unable to synthesize ergosterol. Strain SM36 (*erg3*) is nystatin resistant and accumulates zymosterol and cholesta-7,24-dienol. Strain *erg5* accumulates ergosta-5,7-dienol and zymosterol (20, 21). Both strains were generously provided by A. M. Pierce. ACA, IFI, and FJF wine strains (flor yeasts) have been described previously (5, 12). FSP414/6 is a meiotic product of strain FJF414 (13). FDH1, A4DH, and I82MH are wine yeast-laboratory yeast hybrids obtained after crossing spores of strains FJF414 and ACA4 with laboratory strain D517-4B (FDH1 and A4DH hybrids) and spores of the strain IFI82 with laboratory strain MMY1 (I82MH hybrid) (14). For heteroplasmon formation (15), petite [*rho⁻*] mutants from the laboratory yeasts ABQ21 and K5-5A, which carry a *kar* mutation (8), were obtained after ethidium bromide mutagenesis (23). DS8 is a diploid laboratory control. The strains, genotypes, and sources are shown in Table 1.

Media. Growth media used were YP medium (0.5% Difco yeast extract, 1% Bacto-Peptone) supplemented with either 2% glucose (YPD), 3% glycerol (YPG), or 2% glucose and either ethanol, isopropanol, *tert*-butanol, or SDS at the concentrations indicated. Minimal medium (0.17% Difco yeast nitrogen base without amino acids and with 0.15% ammonium sulfate) prepared with 2% glucose (SD) and supplemented with nystatin for the isolation of nystatin-resistant mutants (8) was also used. Media were solidifed by the addition of 2% agar. To supplement auxotrophic requirements, the appropriate amino acids or bases were added to the minimal medium (23).

Culture procedures. Growth was determined as follows: a single-cell colony was isolated from YPG solid medium and

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TABLE 1. *Saccharomyces* strains used in this work

Strain	*Saccharomyces* species	Genotypic and phenotypic characteristics	Source[a], origin, or reference
K5-5A	S. cerevisiae	MATα his4-Δ15 ade2-1 can1 kar1-1	J. Conde
MMY1	S. cerevisiae	MATα ura3-Δ52 Cyh^R	R. Bailey
D517-4B	S. cerevisiae	MATa ade2 lys9	J. Conde
ABQ21	S. cerevisiae	MATα his4-Δ15 ade2-1 Can^r Nys^r kar1-2	J. Polaina
SM36	S. cerevisiae	MATa erg3	A. M. Pierce
erg5	S. cerevisiae	MATα erg5	A. M. Pierce
ACA4	S. fermentati	MATα/MATa HO/HO	A. Casas
ACA21	Saccharomyces spp.	MATα/MATa HO/HO suc/suc	A. Casas
ACA174	S. cerevisiae	MATα/MATa HO/HO	A. Casas
IFI82	S. cerevisiae	MATα/MATa HO/HO suc/suc	V. Arroyo
IFI256	S. cerevisiae	MATα/MATa HO/HO	V. Arroyo
FJF206	S. rosei	MATα/MATa ho/ho MET/met	J. Conde
FJF414	S. pretoriensis	MATα/MATa ho/ho ME1/met	J. Conde
FSP414/6	S. pretoriensis	MATα met	Spore of FJF414 (14)
FDH1	S. cerevisiae-S. pretoriensis hybrid	MATα/MATa ADE2/ade2 LYS9/lys9 MET/met	FSP414/6 and D517-4B hybrid (14)
A4DH	S. cerevisiae-S. fermentati hybrid	MATα/MATa HO/ho ADE2/ade2 LYS9/lys9	ACA4 spore and D517-4B hybrid (14)
I82MH	S. cerevisiae-S. cerevisiae hybrid	MATα/MATa HO/ho SUC/suc URA3/ura3 Cyh^r/Cyh^s	IFI82 spore and MMY1 hybrid (14)
ABQ21-H1	S. cerevisiae-Saccharomyces sp. heteroplasmon	MATα his4-Δ15 ade2-1 Can^r Nys^r kar1-2	Heteroplasmon with ABQ21 [rho] nucleus and ACA21 mitochondria
ABQ21-N	S. cerevisiae-S. cerevisiae heteroplasmon	MATα his4-Δ15 ade2-1 Can^r Nys^r kar1-2	Heteroplasmon with ABQ21-H1 [rho] nucleus and ABQ21 mitochondria (15)
K5-5A-R	S. cerevisiae	MATα his4-Δ15 ade2-1 can1 kar1-1 Nys^r	Nystatin-resistant mutant of K5-5A (this study)
K5-5A-H	S. cerevisiae-Saccharomyces sp. heteroplasmon	MATα his4-Δ15 ade2-1 can1 kar1-1	Heteroplasmon with K5-5A [rho] nucleus and wine yeast mitochondria (15)
K5-5A-N	S. cerevisiae-S. cerevisiae heteroplasmon	MATα his4-Δ15 ade2-1 can1 kar1-1	Heteroplasmon with K5-5A-H [rho] nucleus and K5-5A mitochondria (15)
DS8	S. cerevisiae	MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1	J. Polaina

[a] Sources are as follows: J. Conde, La Cruz del Campo, Seville, Spain; R. Bailey, Solar Energy Research Institute, Golden, Colo.; J. Polaina, Departamento de Genética, Facultad de Biología, Universidad de Seville, Spain; A. M. Pierce, Department of Chemistry, Simon Fraser University, Burnaby, Canada; A. Casas, Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Seville, Spain; and V. Arroyo, Instituto de Fermentaciones Industriales, Madrid, Spain.

inoculated in YPD liquid medium, and the culture was incubated at 30°C until early stationary phase (1 × 10^8 to 5 × 10^8 cells per ml); 0.5 ml of this culture was inoculated into 100-ml flasks containing 30 ml of either YPD or YPD supplemented with organic solvents at the concentrations indicated. Similar results were obtained when 0.05 ml was inoculated into 10-ml tubes containing 3 ml of media. The cultures were incubated at 30°C and A_{660} was measured periodically in a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.) spectrophotometer. At the same time, cells were counted under the microscope to determine the total cell number and spread on solid YPD to determine the viable cell number. After 3 days of incubation, the final viable cell number was determined by spreading samples on YPD and counting colonies after 3 to 4 days of incubation at 30°C.

Frequency of petite mutants. Among viable cells, the percentage of petite mutants under proliferating conditions was determined after replica plating on YPG the colonies grown on YPD. Petite mutants were known to possess the [rho⁻] genotype (mitochondrial DNA deletion) because they were unable to revert to the [rho⁺] genotype but they recovered the ability to grow on YPG when functional mitochondria was transferred by cytoduction (see below). In some experiments, 0.05-ml samples of an early-stationary-

phase culture were inoculated into 10-ml tubes containing either 3 ml of distilled water (instead of YPD) or 3 ml of distilled water supplemented with different organic solvents at the concentrations indicated. After 9 and 24 h of incubation, samples were taken and the viable cell number and petite mutant formation under nonproliferating conditions were determined as before.

Viability and petite mutant formation under extreme conditions. Flasks containing 200 ml of YPD supplemented with 6% ethanol (vol/vol) were heated with magnetic stirring to 38.5°C to enhance cell death (15). The flasks were then inoculated with 5 × 10^5 cells of an exponential culture (about 10^7 cells per ml) of a particular grande strain or one of its petite mutants, which had been grown in YPD. Viability was determined by taking samples at very short intervals for a period of time and spreading them on YPD. Colonies were counted after 3 to 4 days at 30°C to calculate the frequency of viable cells.

Genetic procedures. Sporulation, micromanipulation, and wine yeast-laboratory yeast hybrid formation were carried out following procedures described previously (13–15). For heteroplasmon formation, functional mitochondria were transferred from grande to petite strains, following methods described previously (8, 15).

TABLE 2. Solvent concentrations which stop growth in
laboratory strain K5-5A and wine yeast ACA4

Strain	Concn of solvent that stops growth			
	SDS (% [wt/vol])	Isopropanol (% [vol/vol])	tert-Butanol (% [vol/vol])	Ethanol (% [vol/vol])
K5-5A	0.3	7.0	4.0	10.0
ACA4	<0.1	>8.0	>5.0	>12.0

Chemicals. Ethanol (ethyl alcohol; CAS (64-17-5), tert-butanol (2-methyl-2-propanol; CAS 75-65-0), SDS (dodecyl sulfate, sodium salt; CAS 151-21-3), and isopropanol (2-propanol; CAS 22739-76-0) were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS

Solvent concentration which prevents growth. The highest solvent concentration at which cell growth occurred or viability was detected was first established in strain K5-5A (representative of laboratory yeasts) and in strain ACA4 (representative of wine yeasts). On the basis of these results (Table 2) the solvent concentrations used in further experiments were 0.1% (wt/vol) SDS, 7% (vol/vol) isopropanol, 4% (vol/vol) tert-butanol, and 10% (vol/vol) ethanol. Viable cells of both strains were found after 3 days of incubation under these extreme conditions.

The behavior of several laboratory and wine yeasts tested was similar to that already shown by laboratory strain K5-5A and wine yeast ACA4, respectively (Table 3).

Although wine yeasts sensitive to SDS were tolerant to alcohols and laboratory yeasts behaved conversely, this empirical association could be changed because tolerance to SDS and tolerance to alcohols are genetically compatible; when wine yeast-laboratory yeast hybrids were constructed, they were tolerant to both SDS and alcohols, growing faster than any parent in all tested solvents (Table 3).

The association was also disrupted in laboratory yeast mutants with altered membrane lipid composition, such as SM36 and erg5, which are unable to synthesize ergosterol (21). Both mutants failed to grow in YPD plus 0.1% SDS as well as in YPD plus any of the alcohols.

Mutagenic effect of membrane solvents on mitochondria. The frequency of petite mutants was determined for viable

TABLE 3. Abilities of laboratory yeasts, wine strains, and wine yeast-laboratory yeast hybrids to grow in YPD with different solvents

Strain	Ability to grow in medium[a]				
	YPD	YPD + 0.1% SDS	YPD + 7% isopropanol	YPD + 4% tert-butanol	YPD + 10% ethanol
Laboratory yeasts	+	+	+/−	− or +/−	−
Wine yeasts	+	− or +/−	+	+ or +/−	+
Wine yeast-laboratory yeast hybrids	+	+	+	+	+
SM36	+	−	−	−	−
erg5	+	+/−	+/−	+/−	−

[a] As measured by the final cell number. +, Final cell number of >10^7 cells; +/−, final cell number of between 10^5 and 10^7 cells; −, final cell number of <10^5 cells. Results are the average of two to three experiments.
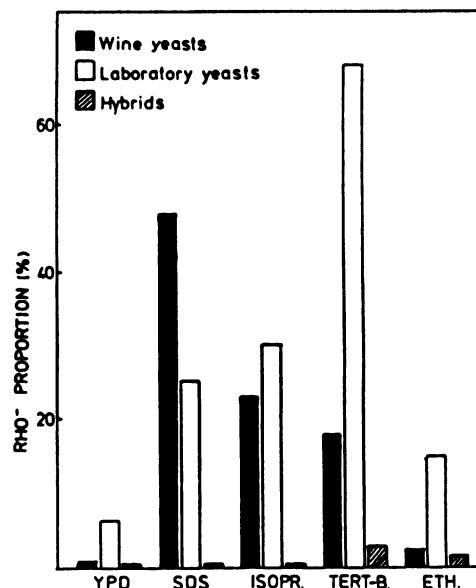

FIG. 1. Average frequencies of petite mutants, either spontaneous (YPD) or induced by alcohol or SDS, of laboratory yeasts, wine strains, and wine yeast-laboratory yeast hybrids. Abbreviations: ISOPR., isopropanol; TERT-B., tert-butanol; ETH, ethanol.

cells in laboratory strains, wine yeasts, and several wine yeast-laboratory yeast hybrids. To be sure that it was induction, rather than selection, of petite mutants that was detected, growth was previously compared in grande and petite strains. In all cases, spontaneous petite mutants obtained from each of the strains under study grew only as well or worse than their grande strain in either YPD or YPD supplemented with the organic solvents (data not shown). The frequency of spontaneous and alcohol-induced petite mutants was lower in the wine yeasts than in laboratory strains (Table 4).

The induction frequency of petite mutants in YPD plus SDS was generally higher in the wine yeasts than in the laboratory strains, although with this solvent the results varied greatly. For some strains, the frequency of induced [rho−] mutants was similar to the spontaneous frequency (i.e., wine strain ACA4), whereas for some other strains this frequency was almost 100% of the population (i.e., wine strains FJF and ACA174).

FJF strains were totally unable to grow in YPD supplemented with SDS, and the viable cell number at the end of the trials was much lower than the initial one. It therefore seems that the high induction frequency of [rho−] mutants could be associated with a high degree of mortality.

The percentage of petite mutants was higher in laboratory strains than in wine yeasts (Fig. 1). tert-Butanol was the most effective mitochondrial mutagen for laboratory strains, whereas SDS was the most effective mutagen for wine yeasts. The wine yeast-laboratory yeast hybrids showed a low rate of induction under any conditions (Table 4C and Fig. 1).

Instability of [rho+] genotype caused by altered membrane composition. The yeast mutants SM36 and erg5 showed a high frequency of spontaneous petite cells (Table 4). These two strains were extremely sensitive to the different solvents; erg5 mutants grew very poorly, and SM36 was completely unable to grow in the presence of solvents. The lack of growth (increase in cell number) was shown to be due

TABLE 4. Percentages of petite mutants [rho⁻] of strains after 3 days of incubation at 30°C in YPD alone and with different solvents

Strain	% Petite mutants[a] of strains grown in:				
	YPD	YPD + 0.1% SDS	YPD + 7% isopropanol	YPD + 4% tert-butanol	YPD + 10% ethanol
Laboratory strains					
K5-5A	6.6 ± 0.5	46.0 ± 30	49 ± 5.2	80 ± 7.0	23 ± 3.0
MMY1	4.8 ± 0.3	13.6 ± 2.4	20 ± 1.3	56 ± 6.2	8 ± 1.0
D517-4B	8.8 ± 0.6	15.9 ± 5.0	33 ± 4.0	68 ± 5.8	15 ± 1.0
DS8	7.0 ± 1.0	17.0 ± 3.0	26 ± 5.1	70 ± 7.2	18 ± 3.2
Wine strains					
ACA4	0.5 ± 0.1	0.6 ± 0.3	2.2 ± 0.2	6.5 ± 0.6	3.3 ± 0.4
ACA21	0.5 ± 0.1	29.0 ± 8.0	15.0 ± 1.8	8.0 ± 1.4	4.0 ± 0.3
ACA174	0.4 ± 0.1	90.0 ± 10	14.0 ± 1.6	13.0 ± 3.0	0.2 ± 0.05
FJF206	3.0 ± 0.1	100.0 ± 0	39.0 ± 8.0	16.0 ± 3.0	1.5 ± 1.0
FJF414	3.2 ± 0.2	70.0 ± 30	31.0 ± 6.0	22.0 ± 3.5	2.5 ± 0.25
IFI82	0.7 ± 0.2	45.0 ± 35	0.8 ± 0.1	2.5 ± 0.3	1.0 ± 0.08
IFI256	0.5 ± 0.1	5.3 ± 1.8	8.5 ± 0.7	13.0 ± 1.5	4.9 ± 0.5
Hybrids[b]					
A4DH	0.2 ± 0.03	1.0 ± 1.0	0.6 ± 0.05	3.2 ± 0.03	3.7 ± 0.4
FDH1	0.6 ± 0.05	0.0 ± 0.0	0.7 ± 0.1	3.8 ± 0.5	1.2 ± 0.2
I82MH	0.8 ± 0.04	0.2 ± 0.2	0.3 ± 0.02	1.3 ± 0.2	0.6 ± 0.1
Mutants and meiotic product[c]					
SM36	18.0 ± 2.0	NV	NV	NV	NV
erg5	26.4 ± 2.5	70.0 ± 10	21.0 ± 3.0	67.0 ± 7.5	9.0 ± 1.0
FSP414/6	13.6 ± 1.5	NV	21.5 ± 4.0	19.6 ± 2.5	26.5 ± 4.0

[a] Results are the average and standard deviation of three to four trials. About 1 × 10³ to 5 × 10³ colonies per strain, per solvent, and per trial were counted, except for strain FJF206 in SDS, when only between 30 to 50 colonies (all of them [rho⁻]) were recovered from each experiment. NV, No viable cells detected.

[b] Hybrids of wine yeasts and laboratory yeast. Approximately three to five individual diploid cells were analyzed from each cross, with similar results.

[c] erg mutants (SM36 and erg5) and a meiotic product of FJF414 wine strain (FSP414/6).

not only to an inhibition of the growth rate but also to a high mortality; viability was measured under extreme conditions of temperature and ethanol (38.5°C and 6% [vol/vol] ethanol), conditions which enhance cell death (15). Mortality in strain SM36, a nystatin-resistant mutant, was higher than mortality observed in the laboratory strain K5-5A (Fig. 2A). This effect was due not to a lack of isogenicity between both strains but to the erg3 mutation carried by the strain SM36; in a spontaneous nystatin-resistant mutant of laboratory strain K5-5A (K5-5A-R), mortality was higher than that observed in the K5-5A wild-type strain (data not shown). Nystatin resistance is frequently associated with alterations of the membrane sterol composition (9).

The decrease in viability in strain K5-5A was accompanied by an increase in the frequency of petite mutants (Fig. 2)
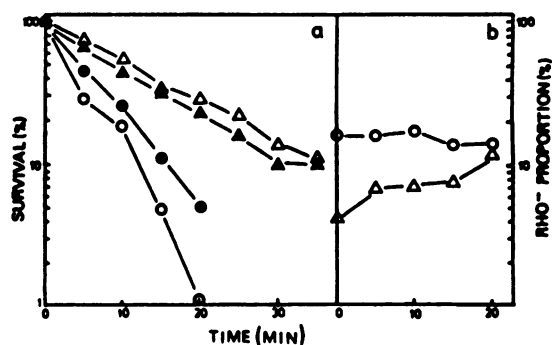

FIG. 2. (a) Percent survival of grande (▲,●) and petite (△,○) cells of laboratory strain K5-5A (▲,△) and the erg mutant strain SM36 (●,○). (b) Petite proportion among survivors of strains K5-5A (△) and SM36 (○). Experimental conditions were YPD + 6% ethanol at 38.5°C.

among the survivors. However, the increase in mortality in strain SM36 was not followed by a similar increase in the frequency of petite cells (Fig. 2). This was a result of a higher sensitivity to ethanol and to temperature by SM36 petite mutants with respect to SM36 grande cells, so that mortality was higher in the former and was balanced by the petite induction rate. Data from Table 4 could be similarly interpreted because the frequency of ethanol-induced petite mutants of the strain erg5 was lower than the spontaneous one. In laboratory strain K5-5A, grande and petite cells displayed the same sensitivity under the conditions used (Fig. 2). The same viability for the grande and petite cells was also observed when similar experiments were carried out with some of the wine yeasts (data not shown).

Under nonproliferating conditions, by using water instead of YPD, a similar increase in the frequency of petite mutants induced by the different solvents was also detected, indicating that growth was not essential for the mutagenic effect to take place (data not shown).

It was observed that the frequency of petite mutants in strain FSP414/6 (a meiotic product of the flor yeast FJF414), either spontaneous or ethanol induced, was substantially higher than that of the parental FJF414 (Table 4 and Fig. 3). The high frequency of petite mutants observed was due to recessive nuclear alleles. This is based on the fact that hybrids between this meiotic product and the laboratory strain D517-4B (FDH1) displayed a much lower frequency of both spontaneous and ethanol-induced petite mutants (Table 4 and Fig. 3), and the meiotic analysis of this hybrid with regard to the spontaneous frequency of petite mutants showed a complex segregation, with several nuclear genes involved (Fig. 3).

Role of mitochondrial DNA in stability of [rho⁺] phenotype. When heteroplasmons (K5-5A-H) possessing the nuclei of
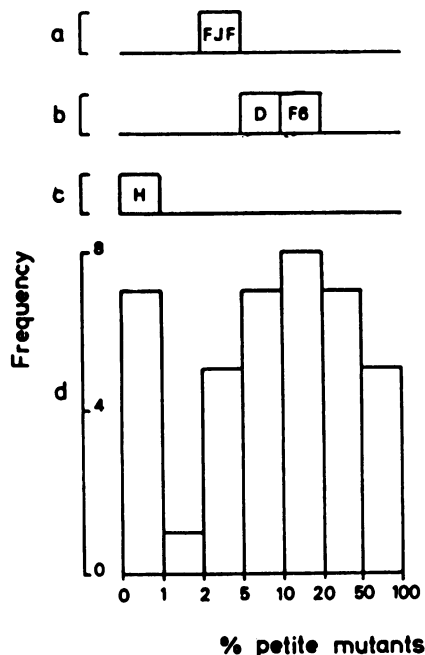
FIG. 3. Range of variation in frequency of spontaneous (YPD) petite mutant formation by yeasts. (a) Flor yeast FJF414 (FJF); (b) meiotic product of FJF414 (FSP414/6 [F6]) and laboratory strain D517-4B (D); (c) wine yeast-laboratory yeast hybrid FDH1 (H), formed between laboratory strain D517-4B and wine meiotic product FSP414/6; (d) meiotic products of the wine yeast-laboratory yeast hybrid FDH1. About 1×10^3 to 3×10^3 colonies per strain were counted.

laboratory strain K5-5A but mitochondria from different wine yeasts were constructed (Table 5), a significant decrease in the frequency of both spontaneous and ethanol-induced petite mutants was observed in the majority of the heteroplasmons formed. When mitochondria of laboratory strain K5-5A were returned to a petite mutant of a K5-5A-H strain (strain K5-5A-N), the behavior of this latter strain was identical to that shown by the original laboratory strain K5-5A (Table 5).

To see whether results obtained with spontaneous and ethanol-induced petite heteroplasmon mutants could be extrapolated to other strains and other alcohols, mitochondria were transferred from wine strain ACA21 to laboratory strain ABQ21 and the frequency of petite mutants was

determined in YPD and in YPD supplemented with 10% (vol/vol) ethanol, 3% (vol/vol) tert-butanol, and 3% (vol/vol) isopropanol. Laboratory strain K5-5A and wine yeast IFI256 were used as controls (Fig. 4). The spontaneous and the alcohol-induced frequency of petite mutants in heteroplasmon ABQ21-H1 was lower than that of laboratory strain ABQ21.

Although stability of the [rho⁺] genotype could be partially transferred via mitochondria, the greatest resistance to petite induction was obtained when non-isogenic hybrids between spores of wine strains and laboratory yeasts were constructed (Table 4). In all the hybrids tested, the frequency of petite mutants, spontaneous or induced by alcohols or SDS, was always either equal to or lower than that of the parents.

DISCUSSION

It is well known that some mutagenic agents, such as ethidium bromide, directly interact with the mitochondrial DNA so that it is the DNA, rather than the mitochondrial membrane, that is involved in the [rho⁻] formation process (26). Other mutagenic agents, such as nitrosoguanidine, also induce [rho⁻] formation by base shifts in DNA (7). However, ethanol seems to be a strong mutagenic agent acting on Saccharomyces mitochondria. Its mutagenic effect may result from damage to the mitochondrial membranes, rather than a direct interaction with DNA (4, 15). Enhancement of thermal death in Saccharomyces cerevisiae by different alkanols correlates with their lipid-buffer partition coefficients (25), suggesting that the thermal death sites of this yeast are associated with a membrane system. In addition, ethanol enhances the lethal effects of high temperatures (thermal death) and decreases the maximal temperature permitting growth (Tmax) (15). Exposure of S. cerevisiae yeasts to high temperature also results in an increased rate of petite mutations, as the mutagenic effect of ethanol is enhanced at high temperatures (15). These observations on the effects of ethanol on Tmax, on thermal death, and on petite mutation suggests that the Tmax sites, the thermal death sites, and the primary target sites of ethanol-enhanced petite mutation are located on a mitochondrial membrane. The results obtained in this study support this view, since other membrane solvents, such as tert-butanol, isopropanol, and SDS are petite mutation inducers and all of them are more powerful mutagenic agents than ethanol (Table 4). It has been observed that constitutive alterations in the membrane composition (SM36 and erg5 strains [20]) also lead to an increase in the frequency of petite mutants (Table 4). This

TABLE 5. Percentages of spontaneous and ethanol-induced petite mutants

Strain[a]	Origin of nucleus	Origin of mitochondria	% [rho⁻] mutants[b]	
			Spontaneous	Ethanol-induced
K5-5A	K5-5A	K5-5A (laboratory strain)	5.6 ± 0.4	23.8 ± 3.5
K5-5A-H	K5-5A	IFI82 (wine strain)	3.0 ± 0.2	7.5 ± 0.6
K5-5A-H	K5-5A	ACA4 (wine strain)	2.3 ± 0.1	15.5 ± 1.2
K5-5A-H	K5-5A	FJF206 (wine strain)	10.3 ± 0.8	12.6 ± 1.1
K5-5A-H	K5-5A	FJF414 (wine strain)	10.6 ± 0.9	6.0 ± 0.5
K5-5A-H	K5-5A	IFI256 (wine strain)	4.4 ± 0.3	ND
K5-5A-H	K5-5A	ACA174 (wine strain)	3.6 ± 0.2	10.8 ± 0.9
K5-5A-N	K5-5A-H	K5-5A (laboratory strain)	5.3 ± 0.4	20.9 ± 1.8

[a] Strains used were laboratory strain K5-5A, heteroplasmons with the nuclei of strain K5-5A but with wine yeast mitochondria (K5-5A-H), and the heteroplasmon control (K5-5A-N), which had the nuclei of strain K5-5A-H [rho⁻] and mitochondria transferred from a K5-5A strain [rho⁺].

[b] Results are the average and standard deviation of five trials. About 500 to 1,000 colonies per strain, per solvent, and per trial were counted. Spontaneous mutants were grown in YPD alone; ethanol-induced mutants were grown in YPD plus 10% (vol/vol) ethanol. ND, Not determined.
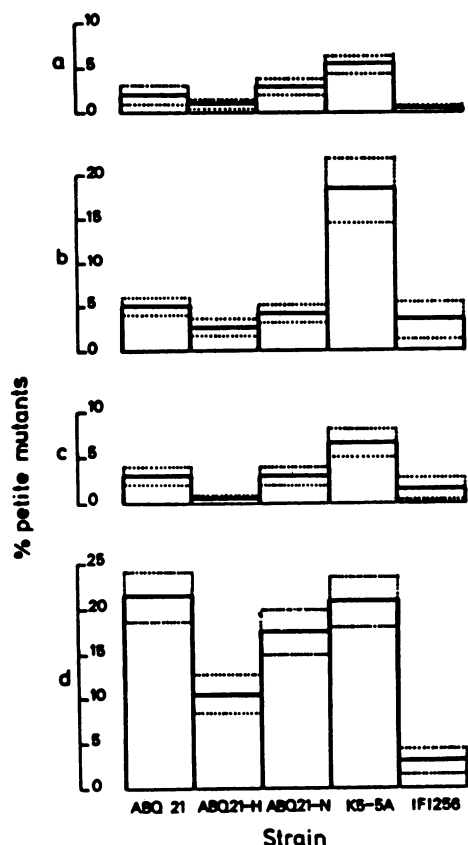
FIG. 4. Percentage of spontaneous (YPD) and alcohol-induced petite mutants of laboratory strain ABQ21, wine yeast-laboratory yeast heteroplasmon ABQ21-H1, laboratory yeast-laboratory yeast heteroplasmon control ABQ21-N, laboratory strain control K5-5A, and wine yeast control IFI256. (a) YPD; (b) YPD + 3% *tert*-butanol; (c) YPD + 3% isopropanol; (d) YPD + 10% ethanol. Data represent the average (———) and standard deviation (······) of four trials. About 500 to 1,000 colonies per strain, per solvent, and per trial were counted.

supports the idea that mitochondrial membrane alterations are involved in the mechanism of petite mutant formation.

Wine yeasts were generally more resistant to spontaneous or organic solvent-induced petite mutant formation (Table 4) than laboratory strains (Table 4 and Fig. 1). When mitochondria were transferred from wine strains to laboratory yeasts (Table 5 and Fig. 4), the heteroplasmons obtained displayed an increase in resistance to spontaneous and induced petite mutation, indicating that the mitochondrial DNA was also directly involved in the degree of resistance to petite formation shown by a particular strain. A high petite frequency was observed in heteroplasmons possessing flor yeast mitochondria, indicating that some sort of interaction may occur between nuclear and mitochondria-coded functions (19). In addition, it has been reported that differences in growth and viability between a laboratory strain and heteroplasmons, possessing the nuclei of the laboratory strain but wine yeast mitochondria, diminished as the ethanol concentration increased (15). This suggests that differences between both strains disappeared at ethanol concentrations at which mitochondria became nonfunctional and that only nuclear functions limited growth (15). Similarly, in addition to the genes that give rise to altered sterol composition, many nuclear genes affect the stability of the grande phenotype, as shown

by the meiotic segregation of the wine yeast-laboratory yeast hybrid FDH1 with regards to petite mutant induction (Fig. 3), growth of ethanol tolerance (14), and above all by the high stability of the [*rho*⁺] genotype (Table 4 and Fig. 1) and the increase in growth of ethanol tolerance (14) of the wine yeast-laboratory yeast hybrids, indicating that nuclear genes from nonisogenic strains are able to complement their functions (14).

A correlation has been observed between the mutagenic effect of a chemical agent and its effect on cell growth (Tables 3 and 4). Although petite mutants could appear by mitochondrial drift as a consequence of cell growth and division (24), these phenomena are not closely associated, since petite mutant induction was also observed when the strains were maintained in distilled water supplemented with different solvents. In accordance with these results, ethanol (4) and other compounds, such as SDS (1), have been used as petite mutation inducers under nonproliferating conditions. The mutagenic effect of the organic solvent can be better correlated with cell death, since both the mutation rate and death rate seem to be closely associated parameters and both are transferable *via* mitochondria (15). In accordance with this suggestion, strain SM36 displayed a higher mortality in the presence of organic solvents together with a higher frequency of spontaneous petite mutants (Tables 3 and 4 and Fig. 2).

These results indicated that the stability of the [*rho*⁺] genotype is influenced by the mitochondrial membrane and the mitochondrial genome, suggesting the existence of a mitochondrial membrane-DNA complex (4, 15). Such a complex may be affected by membrane composition alterations and by membrane solvents (11), giving rise to a high proportion of petite mutations. In addition to the *erg5* and *erg3* mutations which affect the lipid composition of mitochondrial membranes, there are apparently many other nuclear genes involved in the stability of the grande phenotype in the absence and presence of membrane solvents.

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LITERATURE CITED

1. **Bacila, M., and J. Horii.** 1979. Improving the efficiency of alcohol production with respiration-deficient yeast mutants. Trends Biol. Sci. **1:**59–61.
2. **Bailey, R. B., and L. W. Parks.** 1975. Potassium translocation in yeast mitochondria and its relationship to ergosterol biosynthesis. J. Bacteriol. **122:**606–609.
3. **Bailey, R. B., and L. W. Parks.** 1975. Yeast sterol esters and their relationship to the growth of yeasts. J. Bacteriol. **124:**606–612.
4. **Bandas, E. L., and I. A. Zakharov.** 1980. Induction of *rho*⁻ mutations in yeast *Saccharomyces cerevisiae* by ethanol. Mutat. Res. **71:**193–199.
5. **Benítez, T., L. del Castillo, A. Aguilera, J. Conde, and E. Cerdá-Olmedo.** 1983. Selection of wine yeasts for growth and fermentation in the presence of ethanol and sucrose. Appl. Environ. Microbiol. **45:**1429–1436.
6. **Bottema, C. K. and L. W. Parks.** 1980. Sterol analysis of the inner and outer mitochondrial membranes in yeast. Lipids **15:**987–992.
7. **Calderón, I. L., and E. Cerdá-Olmedo.** 1983. Induction by

N-methyl-N'-nitro-N-nitrosoguanidine of nuclear and cytoplasmic mutations in *Saccharomyces cerevisiae*. Mutat. Res. **108**: 133–146.

8. Conde, J., and G. R. Fink. 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA **73**:3651–3655.

9. Gent, M.P.N., and J. H. Prestegard. 1976. Interaction of the polyene antibiotics with lipid bilayer vesicles containing cholesterol. Biochim. Biophys. Acta **426**:17–30.

10. Grisham, C. M., and R. E. Barnett. 1973. The effects of long-chain alcohols on membrane lipids and the (Na^+ + K^+)-ATPase. Biochim. Biophys. Acta **311**:417–422.

11. Ingram, L. O., and N. S. Vreeland. 1980. Differential effects of ethanol and hexanol on the *Escherichia coli* cell envelope. J. Bacteriol. **144**:481–488.

12. Jiménez, J., and T. Benítez. 1986. Characterization of wine yeasts for ethanol production. Appl. Microbiol. Biotechnol. **25**: 150–154.

13. Jiménez, J., and T. Benítez. 1987. Adaptation of yeast cell membranes to ethanol. Appl. Environ. Microbiol. **53**:1196–1198.

14. Jiménez, J., and T. Benítez. 1987. Genetic analysis of highly ethanol-tolerant wine yeasts. Curr. Genet. **12**:421–428.

15. Jiménez, J., and T. Benítez. 1988. Yeast cell viability in high temperature and ethanol depends on the mitochondrial genome. Curr. Genet. **13**:461–469.

16. Larue, R., S. Lafon-Lafourcade, and P. Ribereau-Gayon. 1980. Relationship between the sterol content of yeast cells and their fermentation activity in grape must. Appl. Environ. Microbiol. **39**:808–811.

17. McLean-Bowen, C. A., and L. W. Parks. 1981. Corresponding changes in kynurenine hydroxylase activity, membrane fluidity and sterol composition in *Saccharomyces cerevisiae* mitochondria. J. Bacteriol. **145**:1325–1333.

18. McLean-Bowen, C. A., and L. W. Parks. 1981. The effect of sterol on the energy producing capacity of yeast mitochondria. Chem. Phys. Lipids **29**:137–145.

19. Nagley, P., and A. W. Linnane. 1978. Expression of mitochondrial DNA in *Saccharomyces cerevisiae*: the construction of sets of isonuclear haploid strains containing different specified mitochondrial genomes. Biochem. Biophys. Res. Commun. **85**: 585–592.

20. Pierce, A. M., H. D. Pierce, A. M. Unrau, and A. C. Oehlschlager. 1979. Identification of ergosta-8,24(28)-dien-3β,6α-diol in $\Delta^8 \rightarrow \Delta^7$ sterol isomerase-blocked yeast mutant. Lipids **14**:876–879.

21. Pierce, A. M., A. M. Unrau, A. C. Dehlschlager, and R. A. Woods. 1979. Azasterol inhibitors in yeast. Inhibition of the ^{24}-sterol methyltransferase and the 24-methylene sterol $^{24(28)}$-reductase in sterol mutants of *Saccharomyces cerevisiae*. Can. J. Biochem. **57**:201–208.

22. Proundlock, J. W., L. W. Wheeldon, D. J. Jollow, and A. W. Linnane. 1968. Role of sterols in *Saccharomyces cerevisiae*. Biochem. Biophys. Acta **152**:434–437.

23. Sherman, F., G. R. Fink, and C. W. Lawrence. 1979. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

24. Takahata, N., and T. Muruyama. 1981. A mathematical model of extranuclear genes and the genetic variability maintained in a finite population. Genet. Res. **37**:291–302.

25. van Uden, N. 1984. Temperature profiles of yeasts. Adv. Microb. Physiol. **25**:195–248.

26. Zamaroczy, M., G. Faugeron-Fonty, and G. Bernardi. 1983. Excision sequences in the mitochondrial genome of yeast. Gene **21**:193–202.