

Factors Which Affect the Frequency of Sporulation and Tetrad Formation in *Saccharomyces cerevisiae* Baker's Yeasts

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To clarify the role that respiration, the mitochondrial genome, and interactions of mitochondria and nucleus play on sporulation and to improve the sporogenic ability of several baker's yeasts, an investigation of the effects of different media and culture conditions on baker's yeast sporulation was undertaken. When standard protocols were followed, the sporulation frequency varied between 20 and 60% and the frequency of four-spore asci varied between 1 and 6%. Different presporulation and sporulation media, the use of solid versus liquid media, and incubation at 22 versus 30°C were checked, and the cells were collected from presporulation media in either exponential or stationary phase. Best results, yielding sporulation and four-spore ascus formation frequencies up to 97 and 60%, respectively, were obtained by collection of the cells in exponential phase from liquid presporulation medium with 10% glucose and transfer of them to sporulation medium with 0.5% potassium acetate at 22°C. Under these conditions, the most important factor was the growth phase (exponential versus stationary) at which cells from presporulation medium were collected. Changes in sporulation frequencies were also measured after transfer of mitochondria from different sources to baker's yeasts. When mitochondria from laboratory, baker's, and wine yeasts were transferred to baker's and laboratory petite strains, sporulation and four-spore ascus formation frequencies dropped dramatically either to no sporulation at all or to less than 50% in both parameters. This transfer also resulted in an increase in the frequency of petite mutant formation but yielded similar growth and respiration rates in glycerol. The strains tested recovered their ability to yield maximal sporulation and tetrad formation after recovering their own mitochondria.

Ascospore formation in yeasts is one of two developmental programs that can be undertaken by the cell. When maintained in a complete medium (at the transition from G₁ to M, a proliferative cell cycle), a cell undergoes mitosis, but, under starvation conditions, meiosis occurs, usually followed by production of a four-spore ascus (12, 15). Thus, yeast cells initiate the sporulation program in the G₁ phase of the vegetative cell cycle.

Environmental and hereditary factors influence spore numbers per ascus as well as the frequency of ascus formation (15). Industrial yeast strains, which usually sporulate poorly, rarely form four-spore asci. Moreover, spore viabilities are very low. This has often impeded crossbreeding and genetic characterization (1). Many treatments, including sporulation in a buffer and the use of different carbon sources or nitrogenous compounds, have been reported to affect the frequency of sporulation and the number of spores per ascus (15). Most procedures for inducing sporulation involve a period of vegetative growth (presporulation medium) and then transfer to sporulation medium. It could be expected that cells grown in very rich media are more likely to sporulate when transferred to sporulation medium than cells that are almost nutritionally deprived (2).

Sporulation competency also depends on the development of oxidative ability (15). A large number of genes are differentially expressed in response to oxygen. Among them, many of the genes encoding respiratory functions and functions involved in controlling oxidative damage are induced in aerobic growth (20, 21). In fact, it has been reported that cells grown in acetate or glycerol, neither of which requires a period of re-

spiratory adaptation for the cells on transfer to sporulation medium (3, 6, 7), sporulate maximally if collected during the logarithmic phase of growth (15, 19), whereas cells grown in glucose sporulate best if taken from early-stationary-phase cultures (19). It has also been reported that to optimize sporulation, cells should be grown under conditions in which all glyoxylate and gluconeogenic enzymes are derepressed, i.e., in acetate-containing media (6).

In general, sporulation has a lower maximum temperature and a higher minimum temperature than growth (1, 2, 11, 15). This lower temperature optimum for sporulation has been related to differences in temperature requirements for optimal mitochondrial protein synthesis. Supporting these results, sporulation in yeast has been reported to be controlled by nuclear and mitochondrial genes (13, 14). Ascosporeogenesis occurs only in the presence of mitochondrial respiration, so yeasts cannot sporulate in the presence of antibiotics which inhibit mitochondrial protein synthesis (13). Also, it has been shown that respiration is necessary for the expression of *IME1*, a key gene in the regulation of the meiotic process and sporulation (19). In addition, sporulation is accompanied by a novel pattern of protein synthesis (14). Furthermore, inhibition of mitochondrial protein synthesis causes the disappearance of several of these new polypeptides which are also sensitive to cycloheximide and are localized in the cytosol (14), indicating that expression of some nuclear genes specifically activated during sporulation is impeded by the inhibition of mitochondrial translation (13, 14).

Complementation studies of temperature-sensitive, sporulation-deficient mutants together with the use of reporter genes consisting of sporulation-specific promoters fused to *lacZ* to monitor the sporulation pathway (9) showed the existence of at least 50 loci coding for sporulation-specific functions. Among them was the nuclear *cdc28* mutation, which decreases spontaneous and induced mitochondrial [*rho*⁻] mutability (5). Fur-

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TABLE 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Source
DADI	Unknown	La Cruz del Campo, Seville, Spain
VS	Unknown	La Cruz del Campo, Seville, Spain
CT	Unknown	La Cruz del Campo, Seville, Spain
SB2	Unknown	Horno San Buenaventura, Seville, Spain
IFI256	<i>MATα/MATa HO/HO</i>	V. Arroyo, Instituto de Fermentaciones Industriales, Madrid, Spain
ACA21	<i>MATα/MATa HO/HO suc/suc</i>	A. Casas, Departamento de Microbiología, Universidad de Sevilla, Seville, Spain
DS81	<i>MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1</i>	This study
DS81-R1	<i>MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1 [rho⁰]</i>	This study ([rho ⁰] mutant of DS81)
DS81-D	<i>MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1</i>	This study (heteroplasmon with DS81-R1 nucleus and DS81 mitochondria)
DS81-V	<i>MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1</i>	This study (heteroplasmon with DS81-R1 nucleus and V1 mitochondria)
K5-5A	<i>MATα his4-Δ15 ade2-1 can1 kar1-1</i>	J. Conde, La Cruz del Campo, Seville, Spain
K5-5C	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R</i>	This study (<i>cyh^R</i> mutant of K5-5A)
K5-5C-R1	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R [rho⁰]</i>	This study ([rho ⁰] mutant of K5-5C)
K5-5C-D	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R</i>	This study (heteroplasmon with K5-5C-R1 nucleus and DS81 mitochondria)
K5-5C-V	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R</i>	This study (heteroplasmon with K5-5C-R1 nucleus and V1 mitochondria)
K5-5C-F	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R</i>	This study (heteroplasmon with K5-5C-R1 nucleus and IFI256 mitochondria)
K5-5C-A	<i>MATα his4-Δ15 ade2-1 can1 kar1-1 cyh^R</i>	This study (heteroplasmon with K5-5C-R1 nucleus and ACA21 mitochondria)
V1	Unknown	Compañía General de Levadura, Valladolid, Spain
V1-R1	Unknown	This study ([rho ⁰] mutant of V1)
V1-D	Unknown	This study (heteroplasmon with V1-R1 nucleus and DS81 mitochondria)
V1-V	Unknown	This study (heteroplasmon with V1-R1 nucleus and V1 mitochondria)
V1-F	Unknown	This study (heteroplasmon with V1-R1 nucleus and IFI256 mitochondria)
V1-A	Unknown	This study (heteroplasmon with V1-R1 nucleus and ACA21 mitochondria)
V2	Unknown	Compañía General de Levadura, Valladolid, Spain
V2-R1	Unknown	This study ([rho ⁰] mutant of V2)
V2-F	Unknown	This study (heteroplasmon with V2-R1 nucleus and IFI256 mitochondria)
X-2180-1A	<i>MATa mal gal2 SUC2 CUP1</i>	Yeast Genetic Center, Berkeley, Calif.

thermore, some *cdc28* temperature sensitivity mutations can be suppressed in [*rho*⁻] mutants, pointing to a possible involvement of the mitochondrial genome in the *CDC28*-dependent regulation of cell division in yeast (5).

In this study, the effects of different media on sporulation and four-spore ascus formation were studied to determine whether changes from glucose-rich to acetate-poor medium (implying changes from a mitotic cell cycle to a meiotic one) stimulated sporulation more than did preadaptation to sporulation conditions (caused by growing the cells under oxidative conditions). In preadaptation, the culture is transferred from a presporulation medium with acetate to a sporulation medium which contains only acetate, omitting the respiratory adaptation. The role that respiration plays on sporulation was also studied by transfer of mitochondria from different sources to baker's yeasts.

MATERIALS AND METHODS

Strains. The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. DADI, VS, CT, SB2, V1, and V2 are baker's yeasts; IFI256 and ACA21 are wine yeasts; X2180-1A, DS81, and K5-5A are laboratory strains (DS81 is a diploid strain formed between X2180-1A and its isogenic α strain S288C). The mutation *kar1-1* of strain K5-5A impedes nuclear fusion (4); K5-5C is a spontaneous, cycloheximide-resistant mutant of strain K5-5A; DS81-R1 and K5-5C-R1

are spontaneous petite mutants of strains DS81 and K5-5C, respectively; petite mutants V1-R1 and V2-R1 from the baker's strains V1 and V2, respectively, were obtained after ethidium bromide mutagenesis by methods described elsewhere (18); DS81-D and DS81-V are heteroplasmons possessing the nucleus of strain DS81-R1 but mitochondria from either DS81 or V1; K5-5C-D, K5-5C-V, K5-5C-F, and K5-5C-A are heteroplasmons possessing the nucleus of strain K5-5C-R1 but mitochondria from either DS81, V1, IFI256, or ACA21, respectively; finally, V1-D, V1-V, V1-F, V1-A, and V2-F are heteroplasmons possessing the nucleus of either strain V1-R1 or strain V2-R1 and the mitochondria from either DS81, V1, IFI256, or ACA21.

Enzymes and chemicals. Zymolyase 20000 was purchased from Seikagaku (Seikagaku Kogyo Co., Ltd., Tokyo, Japan); glucose, sorbitol, glycerol, and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Buffer solutions. TE buffer was made with 10 mM Tris and 1 mM EDTA (pH 8); QBT was made with 750 mM sodium chloride, 50 mM MOPS (morpholinepropanesulfonic acid), 15% ethanol, and 0.15% Triton X-100, and the final pH was adjusted to 7; QC buffer contained 1 M sodium chloride, 50 mM MOPS, and 15% ethanol, and the final pH was adjusted to 7; QF was made with 1.25 M sodium chloride, 50 mM Tris, and 15% ethanol, at a final pH of 8.5.

Other solutions. Solution I contained 1 M sorbitol and 100 mM EDTA (pH 7.5), and solution II contained 50 mM Tris and 20 mM EDTA (pH 7.5).

Media. Yeast cells were grown in complete YP medium (0.5% Difco yeast extract, 1% Bacto Peptone) supplemented with 1% glucose (YPD1), with 2% glucose (YPD), with 3% glycerol (YPG), or with 3% glycerol plus 0.1% glucose (YPDG) or in minimal medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate) supplemented with 2% glucose (SD). The presporulation and sporulation media employed were the following: presporulation medium 1 (PRE1), 1% Difco yeast extract-1% Bacto Peptone-1% glucose; PRE2, 1% Difco yeast extract-1% Bacto Peptone-1% potassium acetate; PRE3, 0.3% Difco yeast extract-0.35% Bacto Peptone-1%

glucose–0.1% $MgSO_4$ –0.1% $(NH_4)_2SO_4$ –0.2% KH_2PO_4 ; PRE4, 0.3% Difco yeast extract–0.35% Bacto Peptone–1% potassium acetate–0.1% $MgSO_4$ –0.1% $(NH_4)_2SO_4$ –0.2% KH_2PO_4 ; PRE5, 0.8% Difco yeast extract–0.3% Bacto Peptone–10% glucose; PRE6, 0.8% Difco yeast extract–0.3% Bacto Peptone–5% potassium acetate; sporulation medium 1 (SPO1), 1% potassium acetate–0.1% Difco yeast extract–0.05% glucose; and SPO2, 0.5% potassium acetate. Solid media were supplemented with 2% agar.

For protoplast regeneration, SOS medium containing 1% Difco yeast extract, 2% Bacto Peptone, 2% glucose, 10 mM Cl_2Ca , and 1.2 M sorbitol was used.

Culture conditions. Yeasts were inoculated into 20-ml tubes containing 5 ml of liquid YPD and incubated with rotatory shaking in a New Brunswick incubator at 30°C until the stationary phase was reached (about 10^8 cells per ml). Flasks of 250 ml to 1 liter with 100 to 500 ml of medium were prepared. The flasks were inoculated with the stationary-phase culture until reaching an initial A_{660} of 0.05. After inoculation, the flasks were incubated at 30°C with shaking.

Growth. Growth was determined by measurement of the increase in turbidity in any laboratory medium (A_{660}) with a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.) spectrophotometer. An exponential increase in A_{660} between 0.1 and 0.5 was used to determine the growth rate, μ (units per hour). Previously, a linear relationship between cell number and A_{660} ranging from 0.1 to 0.5 was observed.

Respiration. Respiration was determined as consumed oxygen in a Warburg respirometer at 30°C in YPG with the addition of 0.3 ml of KOH (3 M) to the central well of the Warburg container to absorb CO_2 .

Total cell number. Cell numbers were estimated by dilution of the samples in water, measurement of the A_{660} , and counts of cells under the microscope.

Frequency of petite mutants. Samples were taken periodically from YPD liquid medium and spread on YPDG in order to distinguish between grand (with functional mitochondria) and petite (unable to respire) colonies (18) and to determine the percentage of petite mutants present.

Protoplast formation. Cells were grown in YPD medium until middle exponential phase (about 1×10^7 to 5×10^7 cells per ml, at an A_{660} of 0.5 to 0.7). From this culture, 50 ml was taken, centrifuged, and washed twice with sterile distilled water. The cells were then resuspended in 10 ml of 1.2 M sorbitol; 500 μ l of 2-mercaptoethanol was added, and the mixture was incubated for 15 min at 30°C with gentle shaking. The cells were centrifuged, washed with 10 ml of 1.2 M sorbitol, and newly resuspended in 10 ml of 1.2 M sorbitol to which 100 μ l of the same solution containing 0.5 mg of Zymolyase 20000 had been added. The mixture was incubated at 30°C with gentle shaking for 30 to 45 min, according to the strain, to digest the cell wall. After this time, the protoplasts were collected after centrifugation at $700 \times g$ and after the pellet was washed twice with 1.2 M sorbitol.

Protoplast fusion. Protoplasts from the strains to be fused were mixed and resuspended in a solution containing 40% polyethylene glycol, 10 mM Cl_2Ca , and 10 mM Tris buffer (pH 7.5). The mixture was incubated with gentle shaking at 30°C for 30 min. After the incubation, the protoplasts were collected by centrifugation at $700 \times g$ and washed with 10 ml of 1.2 M sorbitol. The precipitate was then resuspended in 0.5 ml of SOS medium and further incubated at 30°C with gentle shaking for 60 min. After this time, 100- μ l aliquots were taken and inoculated into tubes containing 5 ml of YPD1 medium–2% agar previously stabilized osmotically with 1.2 M sorbitol and maintained liquid at 42°C. After inoculation, the 5-ml cultures were spread onto petri dishes and incubated at 30°C overnight to allow cell wall regeneration. The 5-ml disks were then layered onto petri dishes containing 20 ml of the appropriate selective medium and incubated at 30°C for 4 to 7 days, until colonies were seen. The colonies were newly transferred to selective medium, and the heteroplasmons were characterized.

Genetical procedures. (i) **Sporulation.** Standard protocols were used to induce sporulation (18): cultivation of the cells at 22°C on solid YPD for 24 h, transfer of the grown colonies to SPO1 solid medium, and incubation of the petri dishes for 4 to 7 days. Alternatively, single-colony cells were transferred to solid PRE5 medium, and the petri dishes were incubated for 24 h at 22°C. Then, the cells were newly transferred to solid SPO2 medium and incubated at 22°C for at least 4 days. When indicated, both presporulation and sporulation procedures were carried out at 30°C. When liquid presporulation and sporulation media were used, presporulation medium flasks were inoculated with a stationary-phase culture grown in YPD to reach an initial A_{660} of 0.05. After inoculation, the flasks were incubated at 22°C with shaking until either middle exponential phase (about 1×10^7 to 5×10^7 cells per ml) or stationary phase (about 5×10^8 cells per ml). The cells were then centrifuged, washed twice with distilled water, inoculated into the appropriate sporulation medium, and incubated at 22°C with shaking for at least 4 days.

The percent of asci formed, as well as the number of ascospores per ascus, was determined by counts of cells under the microscope after staining of the spores with malachite green and safranin O, by methods described elsewhere (18). Results are the averages of at least three experiments, and the standard deviations were less than 10%.

(ii) **Transfer of mitochondria.** Active mitochondria were transferred from grande laboratory, baker's, or wine yeasts to the corresponding petite receptor strains. Heterokaryons were formed between two laboratory strains, K5-5C-R1, which lacks functional mitochondria and carries the *kar1-1* mutation (Fig. 1), and X2180-1A as the donor of mitochondria. Heteroplasmons containing both the

nucleus of the K5-5C-R1 laboratory strain and functional mitochondria from the grande strain X2180-1A were selected on YPG medium supplemented with 2 mg of cycloheximide per liter. One of the heteroplasmons, K5-5C-D, was used in this study (Fig. 1) for further transfer of mitochondria.

When active mitochondria were transferred from grande wine (ACA21 and IFI256) or baker's (V1) strain to the K5-5C-R1 strain, different procedures were followed. Wine yeast IFI256 was sporulated, and the asci were digested with Zymolyase (18). A micromanipulated IFI256 meiotic product grown on YPD solid medium was mixed with the K5-5C-R1 strain, and the conjugation mixture was spread on YPD at 30°C and incubated overnight. The cells were then replica plated to YPG supplemented with 2 mg of cycloheximide per liter to select heteroplasmons possessing the nucleus of strain K5-5C-R1 but the IFI256 meiotic product mitochondria. For the ACA21 and V1 strains, protoplasts were first obtained from these yeasts and then further fused to K5-5C-R1 protoplasts, by methods described above. Heterokaryons were then formed between the laboratory K5-5C-R1 petite strain and the wine or the baker's strain. Heteroplasmons containing both the nucleus of petite K5-5C-R1 yeast and functional mitochondria from the grande wine (ACA21) or baker's strain (V1) were selected as before. Heteroplasmons K5-5C-V, K5-5C-F, and K5-5C-A were kept for further transfer of mitochondria.

K5-5C-D, K5-5C-V, K5-5C-F, and K5-5C-A were used as mitochondrial donors for baker's strain V1. Protoplasts from the ethidium bromide petite mutant V1-R1 were fused to protoplasts of strains K5-5C-D, K5-5C-V, K5-5C-F, or K5-5C-A, and the heteroplasmons V1-D, V1-V, V1-F, and V1-A were selected on synthetic galactose (SG) medium as described above. Strain K5-5C-F was also used as a mitochondrial donor for the petite baker's yeast V2-R1. After protoplast fusion and further selection on SG, strain V2-F, containing the nucleus of the baker's yeast V2-R1 but the functional mitochondria of IFI256, was isolated.

(iii) **Recovery of the original functional mitochondria from the heteroplasmons DS81-V, V1-D, V1-F, and V1-A.** The original functional mitochondria of the laboratory strain DS81 (its isogenic haploid X2180-1A) and the baker's yeast V1 were returned to heteroplasmons DS81-V and V1-D, respectively. Mitochondria from DS81 and V1 had previously been transferred to the laboratory petite strain K5-5C-R1 (K5-5C-D and K5-5C-V heteroplasmons, respectively). These heteroplasmons were used as mitochondrial donors for either strain DS81 or strain V1. Previously, spontaneous (DS81-R1) or ethidium bromide-induced petite mutants (V1-R1) had been newly isolated from heteroplasmons DS81-V and V1-D. The new heteroplasmons DS81-D and V1-V were selected on SG after protoplast fusion, as described above.

(iv) **mtDNA restriction analysis.** The mitochondrial DNA (mtDNA) of the strains under study was purified as described by Querol and Barrio (16) with small modifications: firstly, the cells were incubated with Zymolyase for 1 h at 30°C; secondly, after addition of 10% sodium dodecyl sulfate the cells were incubated at 68°C for 45 min; and finally, after being centrifuged at maximum speed, the supernatant containing the DNA was applied on a QIAGEN tip20 column, previously buffered with 1.5 ml of QBT buffer solution. When the DNA was adsorbed, the column was washed twice with 1.5 ml of QC buffer and the DNA was finally eluted with 1 ml of QF buffer. The DNA was precipitated, washed, and dried as described by Querol et al. (17).

Yeast DNA was digested with the appropriate restriction enzymes by incubation of them with 10 μ l of the DNA solution at 37°C for 12 h. The mtDNA restriction patterns were observed by agarose (0.8%) electrophoresis.

RESULTS

Effects on sporulation of presporulation and sporulation carbon concentrations and sources. Sporogenic and four-spore ascus formation abilities of two of the baker's yeasts, strains V1 and V2, were tested under different conditions. These conditions were the use of different concentrations of the carbon sources employed (glucose versus acetate) as presporulation media (PRE1 to PRE6) and the use of two different sporulation media (SPO1 and SPO2). In addition, the cells in either exponential or stationary phase were transferred from presporulation to sporulation medium. Both presporulation and sporulation procedures were carried out at 22°C and in liquid media with rotatory shaking. Analysis of the effects on ascosporeogenesis of presporulation and sporulation carbon sources and concentrations led to the identification of culture conditions which improved both ascus production and four-spore-ascus formation (Table 2). Replacement of acetate with glucose as the presporulation carbon source increased total sporulation. Furthermore, concentrations of 10% glucose, compared with lower concentrations, gave a further increase in total sporulation, and a very high induction of tetrads was

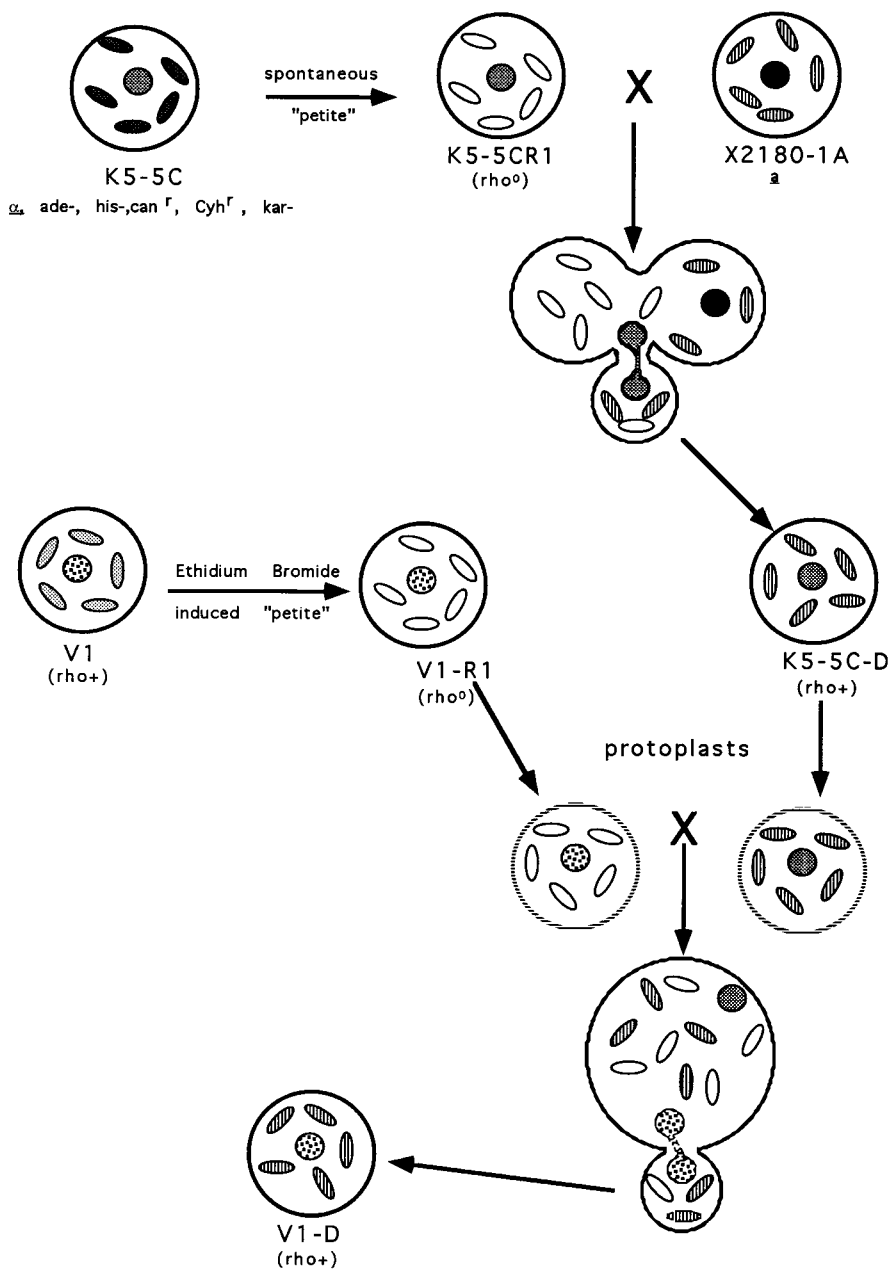


FIG. 1. Formation of the heteroplasm V1-D, which possesses the nucleus of the V1-R1 petite baker's strain and the mitochondria of the DS81 (its isogenic haploid X2180-1A) laboratory strain.

noted. Collecting the cells from presporulation media in the exponential phase rather than the stationary phase led to significant increases in ascus formation.

In all cases, the lowest values for ascus formation were obtained when the cells were collected in the stationary phase, to the point that in some situations the cells of strain V1 did not sporulate at all. The best results, with values of 85 and 77% ascus formation, respectively, were obtained when using PRE5 and SPO2. Therefore, in further experiments, PRE5 and SPO2 were chosen as the best media to induce ascus formation.

Results shown in Table 2 were confirmed by cultivation of the baker's strains V1 and V2 in either presporulation medium PRE5 or PRE6 and transfer of the cells to SPO2 medium after collection of the cells in either the exponential or the stationary

phase of growth. As a control, the laboratory diploid strain DS81 was also used. Figure 2 shows similar results from cultivation of the strains in PRE5 or PRE6 and collection of the cells in exponential phase. When the cells were collected in stationary phase, a dramatic decrease was observed both in the frequency of sporulation and in the frequency of four-spore ascus formation.

Effects of temperature and of solid versus liquid media on sporulation. The experiments to induce sporulation were primarily carried out with the baker's yeasts DADI, VS, CT, SB2, V1, and V2 according to the standard protocols described elsewhere (18). This gave very low frequencies of both ascus (20 to 60%) and especially of four-spore ascus formation (1 to 4%) (Fig. 3A).

TABLE 2. Percentages of ascus formation in baker's yeasts V1 and V2^a

Presporulation medium	Growth phase at time of transfer	Sporulation medium	% Ascus formation in strain:	
			V1	V2
PRE1	Stationary	SPO1	0	40
		SPO2	0	19
	Exponential	SPO1	55	72
		SPO2	47	64
PRE2	Stationary	SPO1	5	57
		SPO2	9	62
	Exponential	SPO1	22	63
		SPO2	85	66
PRE3	Stationary	SPO1	47	38
		SPO2	5	10
	Exponential	SPO1	97	59
		SPO2	52	66
PRE4	Stationary	SPO1	22	40
		SPO2	0	40
	Exponential	SPO1	67	70
		SPO2	61	51
PRE5	Stationary	SPO1	31	36
		SPO2	42	17
	Exponential	SPO1	42	68
		SPO2	85	77

^a Strains were cultivated in different presporulation (PRE1 to PRE5) and sporulation (SPO1 and SPO2) media, and the cells were transferred from presporulation to sporulation media in the exponential or the stationary phase of growth.

To test the effect of incubation in solid versus liquid media, all the baker's yeasts were subjected to sporulation procedures on the selected media described in Table 2 (PRE5 and SPO2). The cells were cultivated at 22°C and for the same length of

time in liquid (Fig. 3B) or solid medium (Fig. 3C), and the results were compared. The percentage of sporulation was substantially higher in all strains in liquid media. In addition, four-spore ascus frequency also increased in almost all strains in liquid media.

When the same baker's strains were subjected to sporulation conditions in liquid PRE5 and SPO2 media but the cells were incubated at 30°C instead of 22°C, the sporulation frequency as well as the percentage of four-spore asci dramatically decreased. This frequency dropped to 1 to 2% in strains like DADI or CT (Fig. 3D).

Transference of mitochondria to the baker's and laboratory yeast strains. To establish the role that mitochondria play in sporulation and to investigate the interactions of nucleus and mitochondria in heteroplasmons, mitochondria were transferred (i) from the baker's strain V1 to the laboratory petite strain DS81-R1, leading to the isolation of the heteroplasmon DS81-V; (ii) from the laboratory strain DS81 and the wine yeasts IFI256 and ACA21 to the petite baker's yeast V1-R1, leading to the heteroplasmons V1-D, V1-F, and V1-A; and (iii) from the wine yeast IFI256 to the petite baker's strain V2-R1, leading to the heteroplasmon V2-F.

The cells were then subjected to the optimal sporulation conditions described above. Heteroplasmons DS81-V, V1-D, and V1-A did not sporulate at all, suggesting incompatibility between the nucleus of the laboratory strain and the mitochondria of the baker's yeast (DS81-V) and vice versa (V1-D) (Fig. 4) and incompatibility of the nucleus of the baker's yeast with the mitochondria of the ACA21 wine yeast (V1-A). In addition, sporulation and, above all, tetrad formation in baker's strains with the mitochondria of the wine yeast IFI256 (V1-F and V2-F heteroplasmons) substantially diminished.

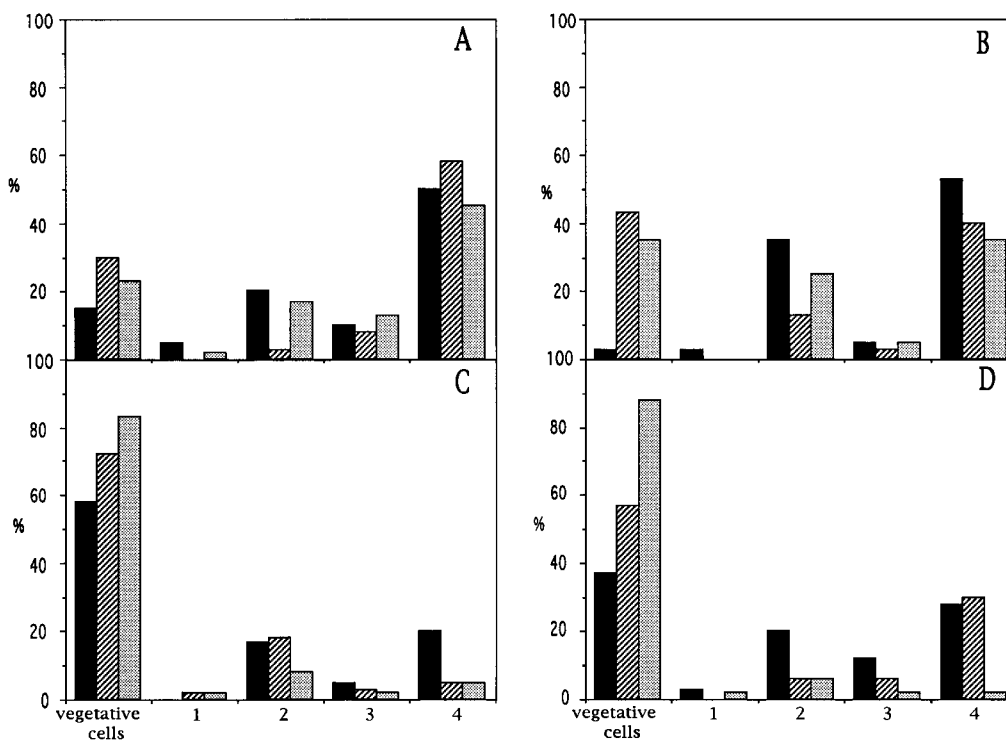


FIG. 2. Percentages of vegetative cells and of one-, two-, three-, and four-spore asci in baker's strains V1 (■) and V2 (▨) and in the laboratory yeast DS81 (▩), after cultivation of the cells in either PRE5 (A and C) or PRE6 medium (B and D) and transfer of the cells from presporulation to sporulation medium in the exponential (A and B) or the stationary (C and D) phase of growth.

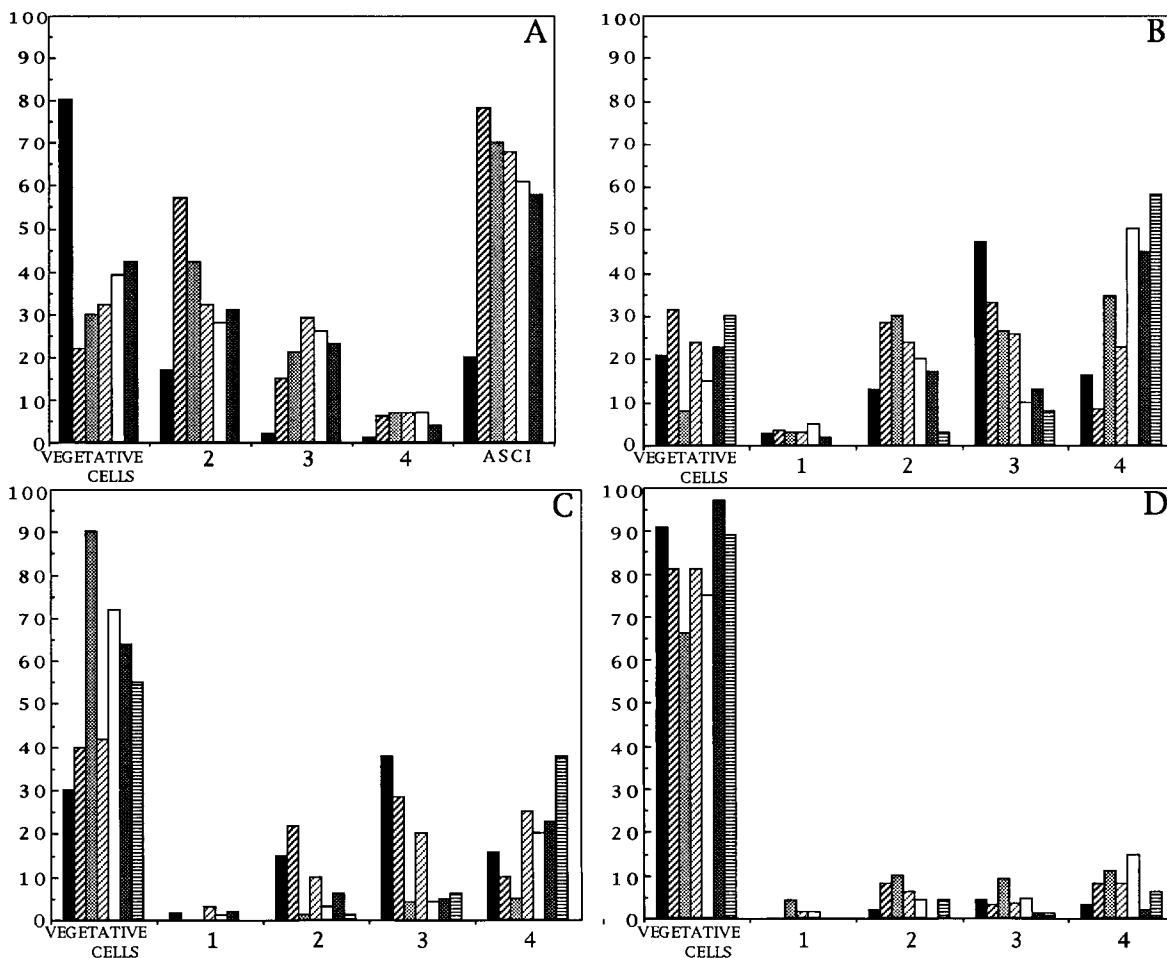


FIG. 3. Percentages of vegetative cells and of one-, two-, three-, and four-spore asci in baker's strains DADI (■), VS (▨), CT (▩), SB2 (▧), V1 (□), and V2 (▤) and the laboratory yeast DS81 (▦) after performance of the following procedures. (A) Presporulation medium, solid YPD; sporulation medium, solid SPO1; temperature, 22°C (standard procedures [18]). (B) Presporulation medium, liquid PRE5; sporulation medium, liquid SPO2; temperature, 22°C. (C) As for panel B, but with solid media. (D) As for panel B, but with incubation at 30°C.

These alterations in the frequencies of sporulation and tetrad formation were exclusively due to the mitochondrial genome and/or the incompatibility between the mitochondria of the donor strain and the nuclei of the receptor yeast cells since, when mitochondria of strain DS81 or V1 were newly transferred to strain DS81 or V1, respectively, the heteroplasms DS81-D and V1-V recovered their ability to sporulate and form four-spore asci with frequencies similar to those of the original strains (Fig. 4).

The observed variations in the sporulation frequencies could be related to the ability of the receptor strains to maintain functional mitochondria. Therefore, the capacity of the heteroplasms to grow and respire on glycerol as a nonfermentable carbon source, as well as the frequency of spontaneous petite formation, was also checked. As Table 3 shows, the spontaneous frequency of petite mutants increases in heteroplasms DS81-V (20% petite compared with 3% in the original DS81 strain), V1-D and V1-F (7% and 4.3%, respectively, compared with <0.5%), and V2-F (2% compared with <0.8%). These frequencies dropped again to their original values when mitochondria were newly transferred to their own strains (DS81-D and V1-V, respectively). However, whereas sporulation frequency in V1-A dropped to zero, the observed

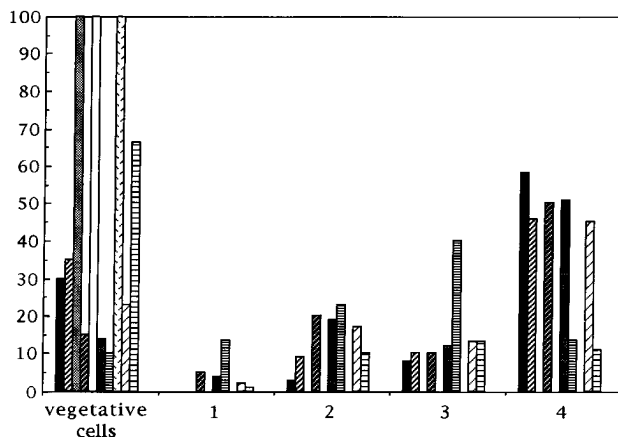


FIG. 4. Percentages of vegetative cells and of one-, two-, three-, and four-spore asci in baker's strains V1 (▨) and V2 (□), the laboratory yeast DS81 (■), and the heteroplasms DS81-D (▨), DS81-V (▩), V1-V (▤), V1-D (□), V1-F (▨), V1-A (▩), and V2-F (▩). Sporulation was induced with PRE5 and SPO2 liquid media and a temperature of 22°C.

TABLE 3. Percentages of petite mutants, growth rates, and respiration rates in YPG medium of the strains described

Strain	Frequency of petite mutants (%)	μ (h^{-1}) ^a	ρ ^b
DS81	3	0.197	263
DS81-D	3.5	0.207	300
DS81-V	20	0.153	347
V1	<0.5	0.290	400
V1-D	7	0.227	371
V1-V	<0.5	0.240	451
V1-I	4.3	0.243	344
V1-A	0.5	0.240	396
V2	<0.8	0.267	330
V2-I	2	0.287	307

^a Growth rate.^b Respiration rate (in microliters of O₂ per 10⁶ cells per hour).

frequencies of petite were the same in the heteroplasmons V1-A and in the original strain V1 (<0.5%).

With regard to growth and respiration in the nonfermentable carbon source (YPG), both the original strains and the heteroplasmons grew with similar growth and respiration rates regardless of the presence of their own mitochondria or of mitochondria from other sources. The fact that, for instance, DS81-V, V1-D, and V1-A did not sporulate at all and nevertheless grew in glycerol with growth rates of 0.153, 0.227, and 0.240 h⁻¹ compared with those of strains DS81 (0.197 h⁻¹) and V1 (0.290 h⁻¹) indicated that incompatibility between the receptor nucleus and the donor mitochondria is specifically related to sporulation functions whereas respiration is carried out perfectly by the donor mitochondria.

In addition, these interactions seem to take place between nucleus and mitochondria and not between mitochondria of the donor and those of the receptor strain (although these were nonfunctional, i.e., with deletions in the mitochondrial genome). This assumption is based on the fact that, as indicated, when restriction fragment length polymorphism (RFLP) of the mtDNA was carried out with the enzymes *AluI*, *HinfI*, and *RsaI* to check the true nature of the heteroplasmons (Fig.

5), no DNA was detected in the receptor strains DS81-R1, V1-R1, and V2-R1 before the donor mitochondria were transferred to them. Besides, RFLP indicated that strains DS81-V, V1-D, V1-V, V1-F, V1-A, and V2-F were true heteroplasmons possessing mitochondria exclusively from the donor strains (Fig. 5).

Finally, the nuclear genomes were tested by electrophoretic karyotyping throughout the mitochondrion transfer experiments. The chromosomal karyotype was the only phenotypic marker that could be used to identify the strains, since the baker's yeasts of this study are all prototrophs. Each strain had a characteristic karyotype, as shown when their chromosomes were individually separated in an electrophoretic contour-clamped homogeneous electric field system. These variations were detected both in the numbers and lengths of the bands, thus allowing each strain to be perfectly distinguishable from the others (data not shown). For this reason, the possibility of chromosome exchange between nuclei (8) was discarded and the true nature of the heteroplasmons was confirmed.

DISCUSSION

In this work, the sporogenic abilities of several baker's yeasts were substantially improved by change of culture conditions (Table 2 and Fig. 3). Preliminary results established that these strains exhibited spore viabilities of up to 65 to 70%. Moreover, some of these strains proved to be heterozygous for some markers, as revealed by the presence of these markers among the meiotic products. Thus, strains should be amenable both to a more detailed genetic analysis and to genetic improvement by hybridization. Improvement of sporulation and four-spore ascus formation frequencies was a prerequisite for carrying out such further analysis. Previous investigations have demonstrated that nutritional and other environmental factors can have a marked influence on ascospore formation in laboratory diploid strains of *S. cerevisiae* (2, 15, 19). Respiratory sufficiency and derepression have been described as being prerequisites for yeast sporulation, so that ascus production in *S. cerevisiae* has been optimized by use of cells grown in glucose and harvested from stationary phase (already shifted from

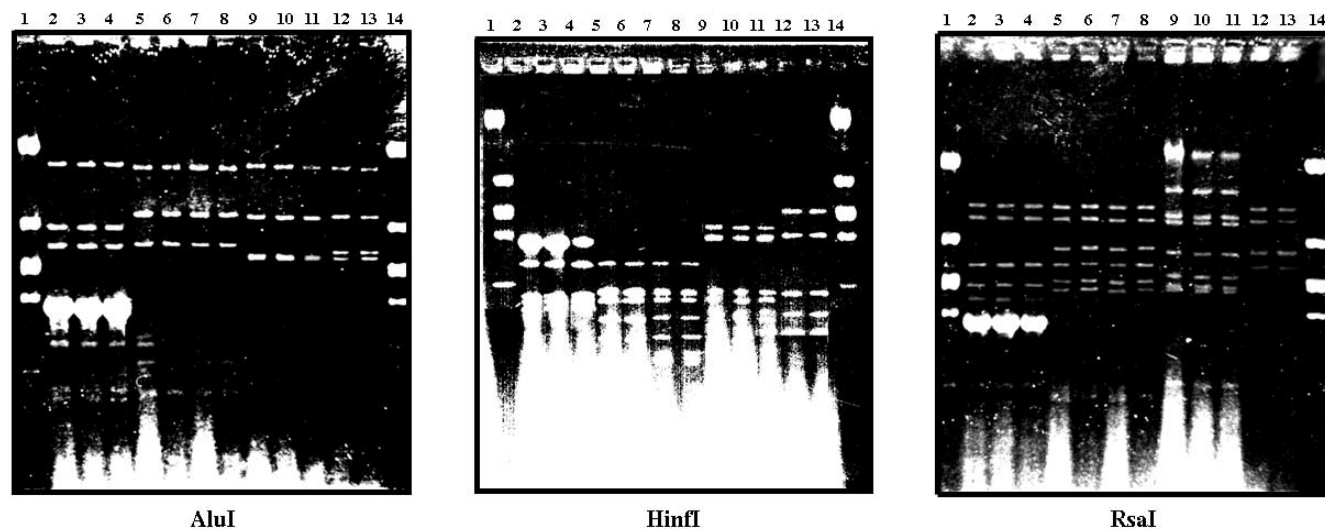


FIG. 5. mtDNA restriction analysis with several restriction endonucleases. Lanes 1 and 14, size markers, which correspond to a mixture of lambda phage DNA fragments obtained with *EcoRI*. Lanes 2 to 13, yeasts DS81 (2), DS81-D (3), V1-D (4), V1 (5), V1-V (6), DS81-V (7), V2 (8), IFI256 (9), V1-F (10), V2-F (11), ACA21 (12), and V1-A (13), respectively. The photographs were scanned in a Howtek Scanmaster 3+, and the images were produced with Adobe Photoshop 2.5 in an Apple Macintosh LC745 8/160.

fermentation to respiratory metabolism) (19) or respiratory-competent cells grown in acetate harvested during exponential phase (15). Furthermore, it was concluded that for maximum sporulation, cells should be grown under conditions in which all glyoxylate bypass and gluconeogenic enzymes are derepressed (2, 3, 6, 15). Similarly, Treinin and Simchen (19) found that when two parallel subcultures, one harvested at logarithmic phase and the other at stationary phase, were shifted from YPD to sporulation medium, sporulation was observed only in the culture obtained from the stationary-phase cells, whereas glucose-grown logarithmic-phase cells did not sporulate. Furthermore, the presence of mitochondrial inhibitors completely inhibited sporulation (19).

In contrast, we found for both laboratory and baker's yeasts that sporulation frequencies were greater for exponential-phase cultures than for stationary-phase cultures. The sporulation frequency was greatest for exponential-phase cultures in 10% glucose, followed by that for exponential-phase cultures in 5% acetate, and followed finally by that for stationary-phase cultures (Fig. 2).

The reason why maximal sporulation was obtained with exponential-phase cultures could rest in the control of the cell cycle: yeast cells embark on the sporulation program from the G_1 phase of the proliferative cell cycle (5, 12). However, progress through a mitotic cell cycle depends upon the presence of nutrients so that, when a nutrient becomes limiting, the cells undergo a shift from G_1 to G_0 (nonproliferative cycle) (12, 15). It is possible that if a fraction of the population is already in G_0 when entering the stationary phase, this state could become irreversible so that the cells cannot enter the meiotic cycle from G_0 .

It has been proposed that the requirement of cells to be grown under derepression conditions for maximum sporulation should not be a strain-dependent response (2). However, in this study, dextrose-grown cells harvested at the exponential phase were able to produce the highest ascus yields, probably because in baker's strains and the laboratory strain DS81 completion of respiratory adaptation occurs during the first few hours in sporulation medium, so that this is sufficient to permit induction of high frequencies of sporulation. The low frequency of petite mutants found in the baker's strains (<0.5 and <0.8% for V1 and V2 strains, respectively; Table 3) indicated that baker's yeast mitochondria are able to adapt rapidly and efficiently to respiratory conditions (7).

The optimum temperature for sporulation has been indicated to be approximately 6°C lower than the optimal temperature for growth, both of which are lower in industrial yeasts than in standard laboratory diploid strains (2), and it coincides with the optimum temperature for mitochondrial protein synthesis (13–15). Brewing and wine strains, with an optimum growth temperature of approximately 27 to 30°C, gave higher sporulation percentages at 21 than at 27°C (2). In line with these results, in this study sporulation dramatically decreased when, under optimal medium conditions, the baker's strains were subjected to sporulation at 30°C compared with 22°C (Fig. 3C and 3D).

Agar culture techniques have been described as used to assess the sporogenic abilities of *S. cerevisiae* laboratory strains (18). In this study, it was evident that standard methods were inadequate for induction of high ascus yields and tetrad formation. A substantial improvement in sporogenic ability was observed with cultivation of baker's strains in liquid rather than in the routinely used agar culture media (Fig. 3A to C). Similar results have been obtained for brewing yeasts (2), and the homogeneity of the populations growing in liquid media (i.e., at the exponential phase of growth) compared with the hetero-

ogeneity existing in single-colony solid media could be the explanation.

The mitochondrial genome has been shown to influence parameters such as tolerance to ethanol, thermotolerance, and resistance to the mutagenic effects of ethanol (10). When mitochondria from different sources were transferred to either baker's or laboratory petite yeast mutants (Fig. 1), sporulation was reduced or absent (Fig. 4). The fact that the combination of the same nucleus (i.e., strain V1) with mitochondria from laboratory or wine strains (V1-D, V1-F, and V1-A) gave such amazingly different frequencies indicates the influence that the mitochondrial genome and especially the interactions of nucleus and mitochondria have on sporulation.

Furthermore, this alteration of the sporulation frequencies was not directly related to the lack of functionality of the mitochondria, as has been described for other systems (10). Although the frequencies of petite mutants increased in most heteroplasmons (Table 3), these increases do not account for the variations observed in sporulation frequencies (Fig. 4). The differences observed (i) were exclusively due to the mitochondrial genome since, when the heteroplasmons recovered their own mitochondria, results for sporulation and tetrad formation were similar to those with the original strains (Fig. 4); (ii) were due to interactions of nucleus and mitochondria and not to recombinogenic events occurring between the mitochondrial genome of the donor and the nonfunctional, deleted mitochondrial genome present in the receptor strain (this conclusion follows from the observation that no mtDNA was present before transfer of mitochondria to the recipient strain, when RFLP was carried out to verify the true nature of the heteroplasmons); and (iii) were specifically related to sporulation and tetrad formation since, in addition to the small variations observed in the frequencies of petite mutants (Table 3), growth and respiration rates of both original strains and heteroplasmons were very similar when the cells were cultivated in glycerol.

Wine and laboratory 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 substantial improvement found when wine mitochondria were transferred to laboratory yeasts (10) might indicate a greater proximity between wine and laboratory strains than between these and the baker's yeasts.

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