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Genetic analysis of highly ethanol-tolerant wine yeasts

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Summary. The genetic basis of ethanol tolerance was investigated in homothallic and heterothallic ethanol-tolerant wine yeasts. All strains were diploid or nearly diploid and able to sporulate. Some possessed recessive lethal alleles. Their meiotic segregation with regard to ethanol tolerance indicates that recessive alleles able to decrease ethanol tolerance were present in the heterozygosis state in the parental wine strains. The number of genes able to spontaneously mutate to alleles of ethanol sensitivity were greater than these found in auxotrophic phenotypes. In homothallic strains segregation in the second generation has to be explained by the simultaneous presence of aneuploidy and ethanol-sensitive alleles. In non-isogenic strains, genes involved in ethanol tolerance had complementary functions. Although a fairly high number of genes were involved at the various tolerance levels of the wine and laboratory strains, different genes limited growth at different ethanol concentrations indicating that ethanol inhibition is the result of the inhibition of different cellular functions with increasing ethanol concentrations.

Key words: Wine yeasts – Homothallic/heterothallic strains – Ethanol-tolerant genes – Ethanol-sensitive genes.

Introduction

Ethanol tolerance in *Saccharomyces* yeast has received increasing attention during the last few years. There is general agreement that in *Saccharomyces* ethanol inhibits growth in a non-competitive way – yeast cell membranes being the primary target of ethanol toxicity (for review see Ingram and Buttke 1984; van Uden 1985; Casey and Ingledew 1986). Ethanol tolerance is strongly influenced by environmental and nutritional conditions (Ingram and Buttke 1984; van Uden 1985; Casey and Ingledew 1986). However, under fixed conditions, different strains differ in their ability to tolerate ethanol and within each strain, ethanol tolerance is a reproducible characteristic, implying that it is genetically determined. Surprisingly, very little is known about the inheritance of ethanol tolerance in yeasts.

It is extremely difficult to isolate ethanol-tolerant mutants by conventional screening and selection techniques. Although mutants of *Sacch. uvarum,* whose fermentation rate in ethanol is higher than that of the wild type, have been obtained in continuous culture (Brown and Oliver 1983), no data is available explaining the genetic basis of this increase in tolerance. In contrast, ethanol-sensitive mutants have been isolated with such a high frequency, and with so many defined complementation groups, that it has been suggested that almost every gene of *Saccharomyces* is able to reduce the ethanol tolerance of a strain (Aguilera and Benítez 1986).

Ismail and Ali (1971) carried out crosses between haploid products showing different levels of tolerance and studied their meiotic segregation at ethanol concentrations very close to that able to completely suppress growth. These workers provide the first direct evidence of a polygenic character of ethanol tolerance in the strains studied.

In this study genetic analyses were carried out to determine those genes which limit growth of *Sacch. cerevisiae* at high ethanol concentrations and those which limit growth at lower ethanol concentrations. The relationship among genes which limit growth at different concentrations of ethanol provides a useful

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Table 1. *Saccharomyces* strains used in this work

Strain	Species	Genotype	Source
ACA4	*Sacch. fermentati*	*MATα/MATa HO/HO*	A. Casas
ACA21	*Sacch. sp*	*MATα/MATa HO/HO suc/suc*	A. Casas
ACA174	*Sacch. cerevisiae*	*MATα/MATa HO/HO*	A. Casas
IFI82	*Sacch. cerevisiae*	*MATα/MATa HO/HO suc/suc*	V. Arroyo
IFI256	*Sacch. cerevisiae*	*MATα/MATa HO/HO*	V. Arroyo
FJF206	*Sacch. rosei*	*MATα/MATa ho/ho MET/met*	J. Conde
FJF414	*Sacch. pretoriensis*	*MATα/MATa ho/ho MET/met*	J. Conde
FSP414/6	*Sacch. pretoriensis*	*MATα met*	This study
DS8	*Sacch. cerevisiae*	*MATα/MATa mal/mal gal2/gal2 SUC2/SUC2 CUP1/CUP1*	A. Aguilera
D517-4B	*Sacch. cerevisiae*	*MATa ade2 lys9*	J. Conde
D517-4BC	*Sacch. cerevisiae*	*MATa ade2 lys9 cyh^R*	This study
D273-11A	*Sacch. cerevisiae*	*MATα ade1 his1*	J. Conde
MMY1	*Sacch. cerevisiae*	*MATα ura3-Δ52 cyh^R*	R. Bailey
FDH1	*Sacch. cerevisiae/ Sacch. pretoriensis* hybrid	*MATα/MATa ADE2/ade2 MET/met LYS9/lys9*	This study

Sources are as follows: A. Casas, Departamento de Microbiologia, Facultad de Biologia, Universidad de Sevilla, Spain; V. Arroyo, Instituto de Fermentaciones Industriales, Madrid, Spain; J. Conde, La Cruz del Campo, S.A. Sevilla, Spain; A. Aguilera, Departamento de Genetica, Facultad de Biologia, Universidad de Sevilla, Spain; R. Bailey, Solar Energy Research Institute, Golden, CO, USA

genetic approach to the study of the overall kinetics of growth inhibition which has previously been studied only from a physiological point of view. Due to the enormous difficulty in isolating highly ethanol-tolerant yeast mutants, this study was carried out in ethanol-tolerant wine yeasts already selected (Benítez et al. 1983; Jiménez and Benítez 1986). Nevertheless, industrial yeast strains are often polyploid or even aneuploid and, as a consequence, do not possess a mating type, have a low degree of sporulation and poor spore viability (Gjermansen and Sigsgaard 1981), rendering genetic analysis of such strains extremely difficult. To study the genetic basis of ethanol tolerance of the wine strains used it was necessary to genetically analyze their ploidy level and their sexual behaviour.

Materials and methods

Organisms. Of the microorganisms used in this study, the ethanol-tolerant wine yeasts ACA4, ACA21, ACA174, IFI82, IFI256, FJF206 and FJF414 have already been described (Benítez et al. 1983; Jiménez and Benítez 1986). FSP414/1 through FSP414/7 are meiotic products of the strain FJF414. Strain DS8 was used as a laboratory diploid control, and the haploid laboratory strains D517-4B, D517-4BC, D273-11A and MMY1 were used to obtain hybrids between wine yeast spores and laboratory yeasts. One of these, FDH1, was obtained by crossing the strain FSP414/6 with D517-4B. Their genotype and source are expressed in Table 1.

Media. Growth media used were YP medium (1% Difco yeast extract; 2% peptone) supplemented with either 2% glucose (YPD) or 2% glucose and different concentrations of ethanol (% v/v) (YPDE). Minimal medium (0.17% Difco yeast nitrogen base without amino acids and with 0.15% ammonium sulphate) was also prepared with either 2% glucose (SD) or 2% glucose and 10% (v/v) ethanol (SDE). Sporulation medium used was SPO (0.1% Difco yeast extract; 1% potassium acetate; 0.05% glucose). Media were solidified by the addition of 2% agar. To supplement auxotrophic requirements, the appropriate amino acids or bases were added to the minimal medium (Sherman et al. 1979). Cycloheximide was used at 10 mg/liter.

Genetical procedure

Sporulation, asci micromanipulation and mating-type determination. To induce sporulation, colonies of the appropriate strain were transferred to sporulation medium SPO and incubated at 22 °C for 6–8 days. Cells were then resuspended in 0.3 ml water and a sample observed under the microscope to determine the percentage of sporulation (asci number/total). For asci dissection, 0.03 ml of helicase (Suc d'*Helix pomatia*, IBF, Clichy, France) was added to the suspension and the mixture incubated to allow digestion of the asci wall. Digested asci were then dissected with a micromanipulator (Lawrence Precision Machine, Lawrence Instruments, OH, USA) on YPD solid medium and the plates incubated afterward at 30 °C for 4–5 days. The proportion of colonies relative to the total number of isolated spores gave the viability percentage of the meiotic products. Mating was attempted on YPD by separately mixing descendants of several tetrads of each strain with the two tester strains, D517-4B (*MATa*) and D273-11A (*MATα*), listed in Table 1. The conjugation mixture was first incubated at 30 °C for 5–6 h and then observed under the microscope to determine whether or not zygotes had been formed.

Hybrid formation. For the heterothallic strains (FJF206 and FJF414), hybrids between wine yeasts and laboratory yeasts

Table 2. Sporulation (%), viability (%) and sexual behaviour of the spores derived from the wine strains

Strain	Sporulation	Spore number/ asci	Time of asci digestion (min)	Spore viability	Sexual behaviour of the spores
ACA4	85	2, 3, 4[a]	15	100 (17)[b]	All sporulate
ACA21	100	2, 3, 4	15	61 (15)	All sporulate
ACA174	23	2, 4	25	12 (13)	All sporulate
IFI82	90	2, 3, 4	10	77 (11)	All sporulate
IFI256	100	2	15	87 (16)	All sporulate
FJF206	78	2, 3, 4	60	50 (10)	All conjugate
FJF414	82	2, 3, 4	45	17 (10)	All conjugate
DS8	92	4	15	100 (10)	All conjugate

[a] The most frequent situation has been underlined
[b] The number of micromanipulated tetrads are shown in parentheses

were selected by micromanipulating the zygotes formed between meiotic products of these strains, of either mating type, and the laboratory strains D517-4B or D273-11A.

For homothallic strains, asci were digested, the spores were centrifuged, washed with distilled water several times, and finally mixed in the proportion 1:3 in 0.05 ml YPD with either the laboratory strain MMY1 (α mating type and Cyh^R) or D517-4BC (*a* mating type). The latter is a spontaneous cycloheximide-resistant mutant of the strain D517-4B (Table 1) whose resistance was verified to be due to a dominant mutation as in strain MMY1. Drops of the mixed strains were placed on YPD medium and the plates incubated at 30 °C for 6 h. Subsequently, the cells were appropriately diluted, plated on minimal medium SD supplemented with cycloheximide and incubated for 6 days at 30 °C.

Some characteristics of the wine strains and their meiotic products. Auxotrophies. Auxotrophs were determined after asci digestion by replica-plating colinies grown on YPD to SD minimal medium and complete medium YPD. The plates were incubated at 30 °C for 4–5 days and those colonies unable to grow on SD were further characterized by testing them on SD supplemented with different amino acids as described by Sherman et al. (1979).

"Flor" characteristic. The capacity to grow as a thin layer on the liquid surface ("flor") was tested for by inoculating 10 ml tubes containing 3 ml of YPDE (8% ethanol) with 0.05 ml of an stationary phase culture of the appropriate strain and incubating the cultures without shaking at 25 °C for 15–20 days.

Determination of growth as a measure of ethanol tolerance. The generation time (τ) of a particular yeast culture at a desired ethanol concentration expressed its ethanol tolerance. The generation time was determined as follows. Aliquots of 0.05 ml of an early stationary-phase culture were inoculated into 10 ml tubes containing 3 ml of either SDE or YPDE with the desired ethanol concentration. The tubes were incubated at 30 °C and absorbance read at 660 nm (A660), was periodically measured by direct insertion of the culture tubes into a Spectronic 20 (Bausch and Lomb, Belgium) spectrophotometer. At the same time, cells were counted under the microscope and a linear relationship was observed between cell number and the absorbance at 660 nm within the range 0.1 to 0.5. The generation time was estimated from the exponential increase in absorbance within the range 0.1 to 0.5 versus time.

Results and discussion

Genetic features of the wine strains

When highly ethanol-tolerant wine strains were genetically analyzed, most of them had a fairly high percentage of sporulation (except ACA174). All strains gave rise to 2, 3 and, above all, 4 spores/asci except for strain IFI256 which only produced two spores/asci (Table 2). The genetic control SD8 had a sporulation percentage of 92%, producing 4 spores/asci. Compared to this control, tetrads of some strains, i.e., FJF, were especially difficult to micromanipulate because they required as long as a 1 hour's digestion with helicase to allow ascus dissection.

Spore viability in "flor" yeasts (FJF206 and FJF414) was low when compared to the laboratory strain. The prolonged incubation with helicase could be the cause, however, the systematic survival of only 2 spores from each tetrad of strain FJF206 suggests a genetic reason. FJF206 might be either aneuploid ($2n-1$) or heterozygous for a recessive lethal mutation. Similarly, the systematic survival of no more than 2 spores per asci in FJF414 might indicate either a heterozygosis state for two recessive lethal mutations or the presence of complex aneuploidies. IFI82 produced tetrads with either 2 or 4 viable spores per asci with about equal frequency, also indicating the presence of aneuploidy and/or the heterozygosis state for a recessive lethal mutation located in one extra chromosome. Tetrads did not systematically segregate for spore viability in the remainder of the strains so that mortality in these strains could be due to complex chromosome alterations or aneuploidy.

Five out of 7 viable spores of strain FJF414 and 13 out of 20 viable spores of strain FJF206 were unable to grow on minimal medium SD. Using the tests of Sherman et al. (1979) they were all shown to be auxotrophic for methionine. This auxotrophy segregated

independently from that of mortality. All other spores in these and the remainder of the strains were prototrophic. Whereas the heterozygosis condition for auxotrophy in "flor" yeasts could have been acquired either by earlier hybridizations or by spontaneous mutation, chromosome loss or the existence of recessive lethal alleles can only be acquired by mutation. Our observation suggests that sporulation rate is lower than mutation rate in the natural environment. These results are in agreement with published findings by Sancho et al. (1986) who suggested the existence of sexual isolation in yeast populations during wine production.

"Flor" yeasts (FJF206 and FJF414) are those able to form "velum" (Santa Maria and Vidal 1973). "Flor" formation was also analyzed in FJF strains and their meiotic products since this feature is normally associated with high ethanol tolerance (Santa Maria and Vidal 1973). All the meiotic products showed this feature indicating that either the strains are homozygous for, or that this characteristic is governed by, several redundant genes. Strains ACA21 and IFI82 are unable to ferment sucrose (Jiménez and Benítez 1986) and as this feature did not segregate in the spores of these strains either, it is likely that they carry no active invertase (*SUC*) genes (Mortimer and Hawthorne 1969).

With regard to further hybridization experiments the main feature to be considered when examining the meiotic products of the wine strains was their ability to mate. All meiotic products of strains FJF206 and FJF414 were able to mate with laboratory strains of either the α or *a* mating type, indicating that the first two strains are heterothallic. Even though spore viability was low, about half of their meiotic products conjugated with α mating strains and half with *a* mating strains. The "flor" strains described previously segregated both homothallic and heterothallic spores in a 2:2 fashion (Santa Maria and Vidal 1973). In the remainder of the strains the meiotic products did not mate. They all sporulated either when subjected to the appropriate sporulation conditions (SPO medium) or, surprisingly, after prolonged incubation on YPD (more than 3 weeks at room temperature), indicating that these strains are homothallic.

Hybrid formation

Meiotic products of the heterothallic strains FJF206 and FJF414 were crossed with the laboratory strains D517-4B (*a* mating type) or D273-11A (α mating type). Zygotes from one of the crosses formed between the strain FSP414/6, a meiotic product of strain FJF414 (α mating type and Met^-, see Table 1), and D517-4B, were micromanipulated on YPD medium. The resulting colonies were prototrophic and mitotically stable. The hybrid cells were able to form "velum" ("flor" characteristic), indicating that this is a dominant feature. When subjected to sporulation conditions, the hybrids produced 4 spores/asci. These asci were easily digested with helicase (10 min incubation) and dissected afterwards on YPD. Spore viability was nearly 100%. The genetic markers *MATa/MATα*, *ade2*, *lys9* and *met* gave a 2:2 segregation. Both the 2:2 segregation of the analyzed markers and spore viability indicated that the hybrid was diploid or nearly diploid and that in consequence the wine yeast spore FSP414/6 was haploid or nearly so. The high fertility and regularity of meiotic segregation of these hybrids cast doubt on whether the parent strains can realistically be ascribed to different species.

Analysis of the genetic feature "flor" rendered a digenic segregation for this characteristic – tetrads segregated $4^-:0^+$ (recombinant ditype) (1 tetrad), $3^-:1^+$ (tetratype) (8 tetrads) and $2^-:2^+$ (parental ditype) (1 tetrad) (+, presence of the feature; –, absence). This indicates that each of the dominant alleles of the two genes involved in this characteristic has to be present for the phenotype to be expressed. Santa Maria and Vidal (1973) found only one such gene segregating in crosses between six different strains of wine yeasts. Presumably the recessive allele of a second gene is present in the laboratory strains used in this study but not in any of the six wine yeasts studied by these authors. However, more complex situations with more than two genes involved cannot be discarded.

Hybrids between spores of the homothallic strains IFI256, IFI82, ACA4 and ACA21 and the cycloheximide-resistant laboratory yeasts MMY1 (*MATα*) and D517-4BC (*MATa*) were obtained by the selection method described in Materials and methods. They were recovered at a low frequency (about 1×10^{-4}). Tetrads from one, obtained from IFI82 x MMY1, showed a nearly 100% spore viability and segregated sucrose segregation versus non-fermentation, uracil requirement versus uracil independence and homotallism versus heterotallism 2:2 in every case. Similarly, tetrads of a D517-4BC x ACA4 hybrid showed 82% spore viability and a 2:2 segregation for *ade2, lys9* and homothallism versus heterothallism in three complete tetrads analysed. These results indicate that the strains analysed were indeed hybrids between the laboratory and wine yeasts and that the wine yeasts yielded nearly haploid spores after meiosis.

Ethanol tolerance of homothallic wine strains and their meiotic products

The wine yeast ACA21 was one of the most ethanol-tolerant – its average generation time in SDE (10% ethanol) being 7.3 h (Fig. 1). This ethanol concentra-

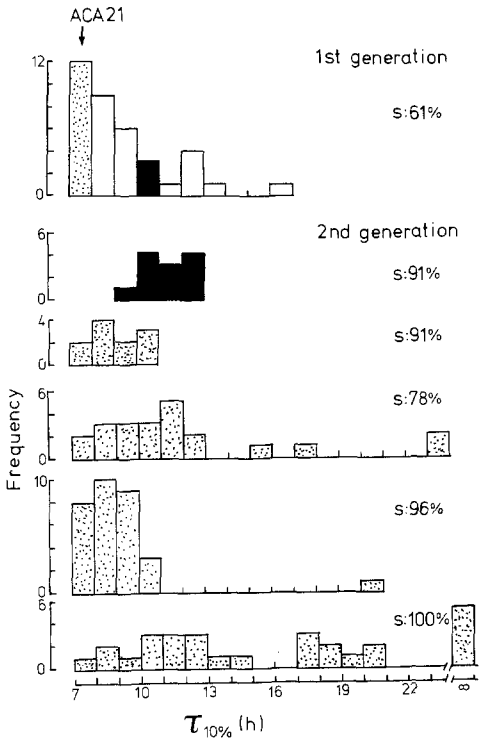

Fig. 1. Range of variation in generation time (h) (τ) of the meiotic products of the strain ACA21 (first generation) and of some of the meiotic products of the first generation (second generation) in SDE (10% ethanol) ($\tau_{10\%}$). The *arrow* shows the generation time of parental strain ACA21. The four dotted histograms of the 2nd generation represent the segregation obtained after analyzing four meiotic products of ACA21 as tolerant as the parental (*dotted bar* of the 1st generation). The shady histogram of the 2nd generation represents the segregation obtained after analyzing a less tolerant meiotic product of ACA21 (*shaded bar* of the 1st generation). *s*: survival

tion prevents growth in all the laboratory strains tested. Thirty-seven single-spore isolates of ACA21 were grown in order to analyze alcohol-tolerance. Their growth was either equal to or slower than that of the parental ACA21 but it was never faster than the latter. This variability prevented the use of classical genetic methods to study ethanol tolerance of these strains since appropriate selection of homothallic-heterothallic hybrids could not be assured and thus the actual ethanol tolerance of the hybridized wine spores could not be observed. Two consecutive generations of homothallic strains were analyzed as an alternative procedure.

The simplest interpretation of the observed meiotic segregation is the existence of recessive alleles which in the homozygosis state reduce ethanol tolerance. These alleles probably appear by spontaneous mutation as almost any gene can mutate to the recessive condition which confers ethanol sensitivity (Aguilera and Benítez 1986).

Since ACA21 is homothallic, once a certain meiotic product has inherited one set of a certain gene combination, and has duplicated this genetic material (due to homothallism), that meiotic product will be homozygous for all its genes and no further segregation should be observed. However, the single-spore isolates obtained from a certain number of meiotic products (second generation) once again displayed an enormous variability for ethanol tolerance even more variability than the first generation (Fig. 1). They were either less or equally tolerant than the parental spores (when the parental spore used was highly tolerant, dotted bars in Fig. 1) or even slightly more tolerant (when the parental spore was less tolerant, shady bars in Fig. 1), but never more tolerant than the parental ACA21.

Segregation in the second generation cannot just be explained by a heterozygosis condition also but by the simultaneous presence of aneuploidy and heterozygosis. Those heterozygous genes involved in ethanol tolerance and located in the basic genome segregated in the first generation whereas those heterozygous alleles located in the extra chromosome(s) segregated in further generations. When τ was determined in SD the values were similar in both the parental strains and all the meiotic products suggesting that all meiotic products were prototrophic. Segregation for growth in ethanol compared to the lack of segregation for auxotrophic features suggests that ethanol tolerance is under polygenic control, as reported by Ismail and Ali (1971), and that, in homothallic strains, there is a large number of genes which can spontaneously mutate to alleles of ethanol sensitivity. This number is greater than that involved in auxotrophic phenotypes.

The consequence of the homothallic nature of ACA21 is that after meiosis the subsequent generations have an even number of extra chromosomes and a tendency to euploidy which, in turn, gives rise to more viable products. In fact, this viability was 61% in the first generation, increasing up to 78–100% in the second generation.

Whereas no meiotic product was more tolerant than the parental ACA21, such increased tolerance was obtained after crossing them with laboratory strains, indicating that in non-isogenic strains genes involved in the upper limit of ethanol tolerance were able to complement their functions.

Similar results were obtained when ACA174 and ACA4 were analyzed (data not shown).

Ethanol tolerance of heterothallic wine strains and their meiotic products

Because some of the meiotic products of the FJF strains are auxotrophic, ethanol tolerance was studied in strain

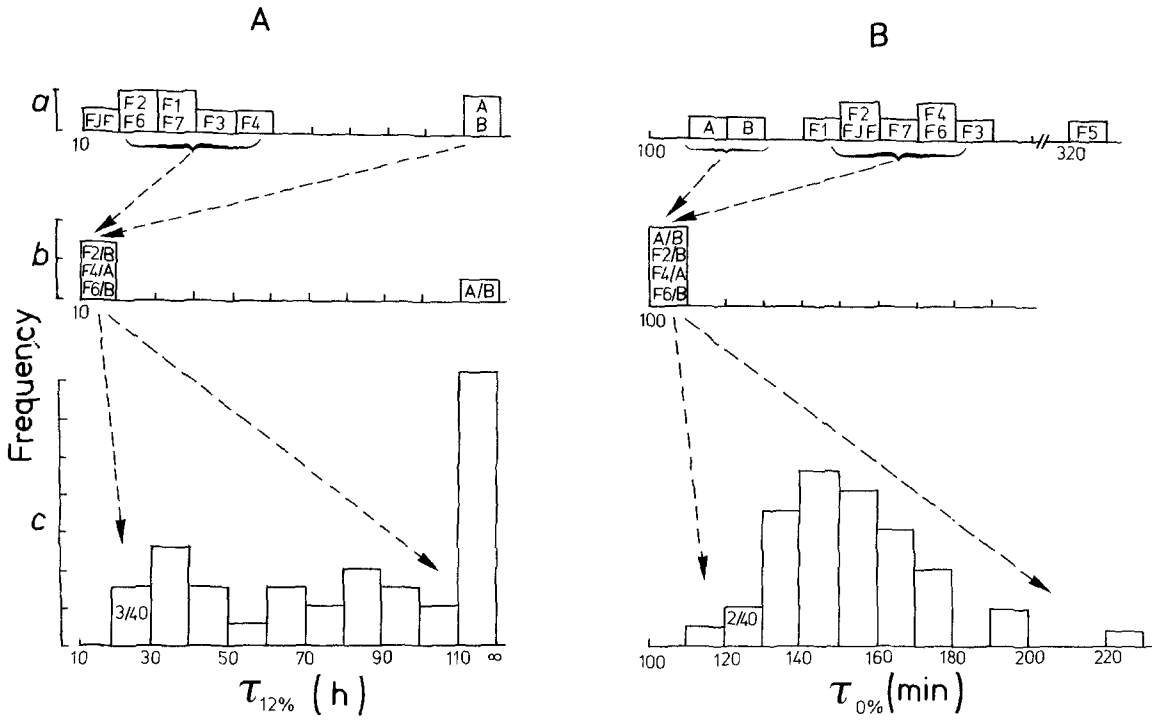

Fig. 2A, B. Range of variation in the generation time of *a* the genetic strains D273-11A (*A*) and D517-4B (*B*), the strain FJF414 (*FJF*) and the wine spores FSP414/1 to FSP414/7 of this strain (*F1* to *F7*); *b* some of the hybrids (*F2/B, F4/A, F6/B*) between spores of the FJF414 strain (FSP414/1 to FSP414/7) and the genetic strains D273-11A and D517-4B and the diploid control formed between both laboratory strains (*A/B*); *c* the meiotic products of the hybrid FDH1, formed by the wine spore FSP414/6 and the genetic strain D517-4B. **A** YPDE (12% ethanol) ($\tau_{12\%}$), **B** YPD (τ_0)

FJF414 (more ethanol-tolerant than FJF206) and its meiotic products, in complete medium supplemented with 12% ethanol (YPDE (12%)). The average generation time of the parental strain in this medium was 18.8 h. The ethanol tolerance of the meiotic products displayed a great variability and spores were less tolerant than the parental strain, probably due to recessive mutations which decreased their ethanol tolerance. Laboratory strains D273-11A and D517-4B were unable to grow under these conditions (Fig. 2). Since only one gene is involved in auxotrophic features (*met*), it can be concluded that in heterothallic strains also the number of genes able to mutate spontaneously to ethanol-sensitive alleles is higher than the number involved in the auxotrophies. Therefore, the affected genes probably code for functions involved in the general metabolism of the cells and are neither specifically nor exclusively involved in the ethanol-tolerance mechanism. Hybrids formed between laboratory strains and spores of the strain FJF414 were more ethanol-tolerant than the parental spore (τ = 15 h), indicating that genes from the laboratory strain are able to complement the recessive alleles present in the wine spores causing such spores to grow slower than the parental strains. Since such hybrids grow in ethanol even better than wine strain FJF414, the genes involved must be different in the different strains.

Ten complete tetrads (40 spores), meiotic products of hybrid FDH1 (FSP414/6 x D517-4B), were analyzed for ethanol tolerance. In the simplest case, when the genes involved are not linked and if they all have similar additive effects on that trait, the number of genes (n) involved in the different tolerances between the wine spore FSP414/6 and the laboratory parental strain D517-4B can be estimated from the proportion of spores (p) as tolerant as the most ethanol-tolerant parental strain ($p = (1/2)^n$). As Fig. 2 shows, 3 out of 40 spores had the same degree of tolerance as the wine spore parental strain, indicating that such difference in the upper limit of ethanol tolerance were determined by at least 3 or 4 diallelic genes. This number is probably higher since different mutations may affect gene expression with different intensities and/or some gene products may be more important than others with regard to ethanol tolerance. No meiotic product was as tolerant as wine strain FJF414 or hybrid FDH1. Probably more genes determine ethanol tolerance in wine strain FJF414 and hybrid FDH1 than in the laboratory yeast strains. Most of the reported physiological results implicate cell membranes as being important determinants of alcohol

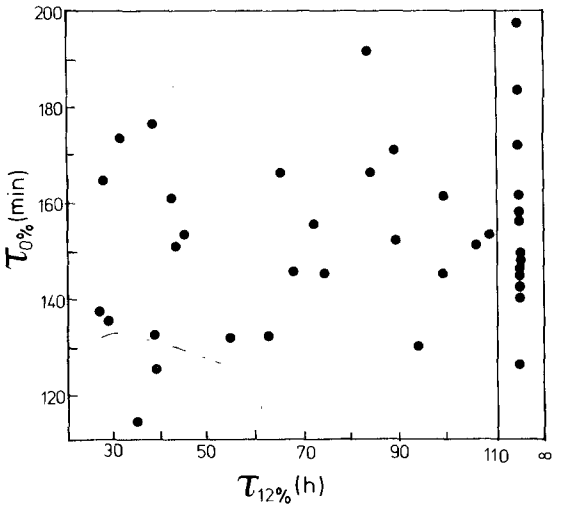

Fig. 3. Relationship between generation time (min) (τ) of the meiotic products of the wine-genetic hybrid FDH1 in YPD (τ_0) versus YPDE (12% ethanol) ($\tau_{12\%}$)

resistance (see Ingram and Buttke 1984), so it could be that the aforementioned genes code for cell membrane functions. In fact, it has recently been found that the ethanol tolerance of the cell plasma membrane of FSP414/6 is very different from that of D517-4B (Jiménez and Benítez 1987) and that these differences correlated highly with their growing ethanol tolerance (Jiménez and van Uden 1985; Jiménez and Benítez 1987). Consequently, whereas any gene can reduce ethanol tolerance (Aguilera and Benítez 1986), genes coding for membrane functions may be responsible for an increase in ethanol tolerance in *Sacch. cerevisiae.*

Kinetics of ethanol-inhibited growth

As is shown in Fig. 2, FJF414 grew faster than the laboratory strains in YPDE 12% ethanol; however, the former strain grew very slowly in comparison to laboratory ones grown in the absence of ethanol. As happened in YPDE 12% ethanol, the meiotic products of FJF414 segregated for growth in YPD (Fig. 2): some grew either equal to, slightly faster, or slower than the parental yeast. This suggests that there are several genes able to limit the growth of these strains in the absence of ethanol as well. The slow growth of FJF414 and its meiotic products in YPD (see Fig. 2) was probably due to recessive alleles since wine-genetic hybrids grew as quickly in YPD as did the genetic diploids formed by D517-4B and D273-11A. In addition, the segregation for growth observed in YPD amongst the meiotic products of FDH1, one of the wine yeast-laboratory yeast hybrids, indicates that there were several genes involved in this feature. Their recessive alleles limit growth of "flor" yeasts in the absence of ethanol. In consequence, growth can be limited by several genes in the presence of 12% ethanol or in the absence of ethanol. However, no relationship could be found between both set of genes (Fig. 3) since the meiotic products which grew faster in YPD did not necessarily do so in the presence of 12% ethanol and those which grew slower in the absence of ethanol were not necessarily slower in 12% ethanol.

The segregation for growth shown by these meiotic products at 5% and 8% ethanol (Fig. 4) indicated that there were several genes able to limit growth under these conditions. As before, there was no correlation between genes which limited growth at 0%, 5% and 8% ethanol (as shown in Fig. 4, spores with similar generation time in YPD behave differently in YPDE 5% ethanol and YPDE 8% ethanol). Thus, ethanol inhibition of growth must be the result of an inhibition of different cellular functions with increasing ethanol concentrations.

In analyzing the "flor" strains it was observed that these strains hardly ever conjugate in their natural environment and that therefore, since many genes are involved in high ethanol tolerance, this feature has probably been acquired by mutation and consequent selection in an accumulative way. However, as a practical approach, the reported results suggest that non-isogenic hybridization might provide the most useful method to improve ethanol tolerance. This has been shown empiri-

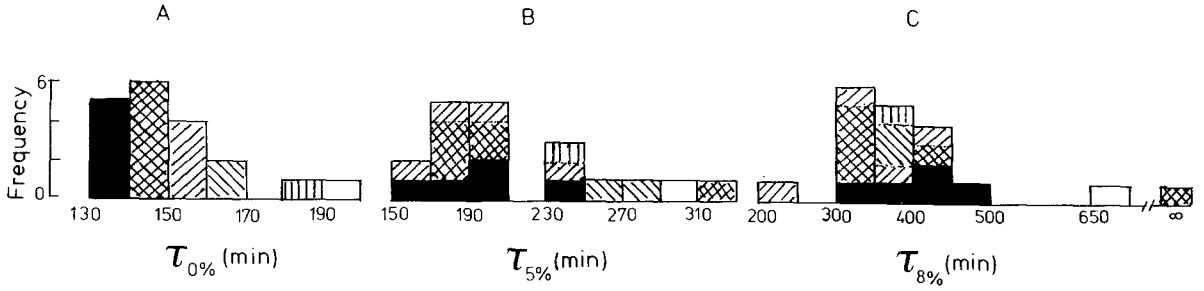

Fig. 4A–C. Range of variation in the generation time (min) (τ) of meiotic products (five complete tetrads) of the wine-genetic hybrid FDH1 in **A** YPD (τ_0), **B** YPDE (5% ethanol) ($\tau_{5\%}$) and **C** YPDE (8% ethanol) ($\tau_{8\%}$). Those meiotic products with similar generation time in YPD are distinctly labeled (**A**) to identify their generation time in YPDE (5% ethanol) (**B**) and YPDE (8% ethanol) (**C**)

cally by other authors (Panchal et al. 1982; Seki et al. 1983). In addition, the selection of the most ethanol-tolerant hybrids has to be carried out in a wide range of ethanol concentrations.

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References

Aguilera A, Benítez T (1986) Arch Microbiol 143:337–344
Benítez T, Del Castillo L, Aguilera A, Conde J, Cerda-Olmedo E (1983) Appl Environ Microbiol 45:1429–1436
Brown SW, Oliver SG (1983) Eur J Appl Microbiol Biotechnol 16:119–122
Casey GP, Ingledew WM (1986) Crit Rev Microbiol 13:219–280
Gjermansen C, Sigsgaard P (1981) Carlsberg Res Commun 46: 1–11
Ingram LO, Buttke TM (1984) Adv Microb Physiol 25:253–300
Ismail AA, Ali AMM (1971) Folia Microbiologica 16:350–354
Jiménez J, van Uden N (1985) Biotechnol Bioeng 27:1596–1598
Jiménez J, Benítez T (1986) Appl Microbiol Biotechnol 25: 150–154
Jiménez J, Benítez T (1987) Appl Environ Microbiol 54:1196–1198
Mortimer RK, Hawthorne DC (1969) Yeast genetics. In: Rose AH, Harrison JS (eds) The yeasts. Academic Press, New York, pp 386–460
Panchal CJ, Peacock L, Stewart GG (1982) Biotechnol Lett 4:639–642
Sancho ED, Hernandez E, Rodriguez-Navarro A (1986) Appl Environ Microbiol 51:395–397
Santa Maria J, Vidal D (1973) J Bacteriol 113:1078–1080
Seki T, Myoga S, Limtong S, Uedono S, Kumnuanta J, Taguchi H (1983) Biotechnol Lett 5:351–356
Sherman F, Fink GR, Lawrence CW (1979) Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor New York
van Uden N (1985) Annu Rep Ferment Proc 8:11–58

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