Ethanol-sensitive mutants of *Saccharomyces cerevisiae*

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**Abstract.** *Saccharomyces cerevisiae* mutants unable to grow at ethanol concentrations at which the wild type strain S288C does grow, have been isolated. Some of them show additional phenotypic alterations in colony size, temperature sensitivity and viability in ethanol, which cosegregate with the growth sensitivity in ethanol. 21 selected monogenic ethanol-sensitive mutants define 20 complementation groups, denominated ETA1 to ETA20, which indicates that there is a high number of genes involved in the ethanol tolerance/sensitivity mechanism.

Out of 21 selected monogenic mutants, 20 are not altered in the glycolytic pathway since, when maintained in glucose-supplemented medium, they can produce as much ethanol as the wild type and at about the same velocity. Nor do any of the mutants seem to be altered in the lipid biosynthetic pathway since, whether grown in the absence or in the presence of ethanol, their concentration of fatty acids and ergosterol is similar to that of the wild type under the same conditions. Therefore growth sensitivity to ethanol does not seem necessarily to be related to carbohydrate or lipid metabolism.

**Key words:** Ethanol-sensitive mutants — Lipid composition — Fermentative capacity — Viability in ethanol

Little is known about either the physiological or the genetic bases by which some microorganism species such as *Saccharomyces cerevisiae* are more tolerant to ethanol than others. Even within the same species, there is enormous variability with respect to the ethanol concentration at which different strains can grow and ferment (Benitez et al. 1983).

It has been observed in natural strains of *Saccharomyces* that this tolerance is regulated by a polygenic system (Ismail and Ali 1971; Day et al. 1975). However, the literature on the genetic basis of ethanol tolerance is not very extensive and there are hardly any reports on laboratory mutants with altered ethanol tolerance/sensitivity. Some mutants have been described in *Escherichia coli* which are more resistant to cell lysis in the presence of ethanol (Fried and Novick 1973; Ingram and Vreeland 1980) and one mutant of *S. uvarum* whose fermentation rate in ethanol is higher than that of the wild type has been obtained in continuous culture (Brown and Oliver 1983).

With regard to the physiological basis of ethanol tolerance, it is mainly the relationship between cell membrane composition and ethanol tolerance/sensitivity (measured as fermentation capacity and viability in ethanol) that has been studied, principally in bacteria (Buttke and Ingram 1978, 1980) and yeasts (Beaven et al. 1982). It has been reported that different yeast strains produce higher concentrations of ethanol (that is, they possess higher fermentation capacity) or are more viable in the presence of ethanol when cultivated in medium supplemented with either unsaturated fatty acids or ergosterol, which are incorporated in their cell membranes (Hayashida et al. 1976; Thomas et al. 1978; Rose and Beaven 1982; Janssens et al. 1983). There is also considerable information about the relationship between the increase in unsaturated fatty acids in the cell membrane and the increase in ethanol tolerance in *E. coli* (Ingram 1976; Ingram et al. 1980; Ingram and Vreeland 1980). Similarly, in yeasts a decrease in ergosterol content of the cell membrane has been directly related to a decrease in cell viability in the presence of ethanol (Less et al. 1980; Larue et al. 1980).

To determine the genetic basis of ethanol tolerance in *S. cerevisiae*, ethanol-sensitive mutants have been isolated and characterized in the present study, mainly with reference to their lipid composition, fermentation capacity and cell viability.

**Materials and methods**

**Strains**

*Saccharomyces cerevisiae* strains S288C (genotype MATa mal gal2 SUC2 CUP1) and X2180-1A (isogenic to S288C but MATa) were obtained from the Yeast Genetic Stock Center, Berkeley, CA, USA. From the latter, a spontaneous mutant auxotroph for histidine, AH28X, was isolated and used in this study. The ethanol-sensitive mutants MSE1 to MSE20 and PSA1 will be described below.

**Culture procedures**

Strains were grown at 30°C in YP medium (Difco yeast extract, 1%; peptone, 2%) supplemented with either 2% d-glucose (YPD), 3% glycerol (YPG) or 2% d-glucose and...
different molar concentrations of ethanol (YPDE[M]), or on SD agar (0.17% Difco yeast nitrogen base w/o amino acids and (NH₄)₂SO₄, 0.5% ammonium sulphate, 2% d-glucose and 2% agar). Nystatin (Sigma Chemical Co., St. Louis, MO, USA) was used at final concentrations varying from 0.5 to 2 mg/l. Diploids were sporulated on SPO agar (Difco yeast extract, 0.1%; potassium acetate, 1%; d-glucose, 0.05%; agar, 2%) after incubation for 15 d at 22°C, following methods already described (Sherman et al. 1979).

Growth, expressed as growth rate (μ), was determined by measuring the increase in turbidity at 660 nm and in parallel by counting cell number under the microscope. For absorbance at 660 nm (A₆₆₀) ranging from 0.10 to 0.5, a linear relationship between A₆₆₀ and cell number values was observed. Absorbance (A₆₆₀) was quantified directly by insertion of the culture tubes into a Spectronic 20 (Bausch and Lomb, Belgium) spectrophotometer.

Fermentation rate, v, and fermentation capacity (maximal ethanol production) were determined by measuring periodically and for 48 h at a time glucose consumption and ethanol production from a YP culture medium supplemented with either 20 or 27% (w/v) glucose, incubated at 30°C. This culture had been inoculated with cells previously grown in YPD medium. Higher concentrations of glucose were not tested, since it has previously been observed that product yield and the final amount of ethanol production diminished when the initial substrate concentration was over 27% (w/v) glucose (data not shown).

Viability in ethanol was determined by maintaining cells previously grown in YPD medium, in phosphate buffer 67 mM pH 4.5 supplemented with 2.5 M ethanol for 48 h at 30°C, plating them on YPD agar and subsequently counting the number of colonies formed. Viability of strain S288C, which turned out to be 50%, was considered as the unit (100%).

Viability in distilled water (osmosensitivity) was checked by plating on YPD agar and counting the number of colonies formed after maintaining cells in distilled water for 24 h at 30°C.

All the experiments were carried out under aerobic conditions.

Mutagenesis and mutant selection

Early exponential phase cells of the strain S288C grown in YPD medium at 30°C with aeration were treated with N-methyl-N'-nitro-N-nitosourea (nitrosourea) (Sigma Chemical Co., St. Louis, MO, USA) following the procedure described by Calderón and Cerdá-Olmedo (1983), and maintained in YPD medium for 8 h. The percentage of cells which remained viable (survivors) after the mutagenic treatment (defining the viable cell number as the number of cells able to form colonies on YPD agar) was about 30%.

Mutagenized cells were spread onto Petri dishes containing YPD agar. The colonies grown after 2 d at 30°C were transferred by the criss-cross technique (Sherman et al. 1979) to Petri dishes containing YPDE (1.2 M) agar and incubated for 3 d at 30°C. It had previously been observed that after 3 d at 30°C under aerobic conditions the evaporated ethanol from the Petri dishes was less than 10% of the initial ethanol concentration. Those colonies unable to grow were checked again under the same conditions, and subsequently cultivated in 15 ml tubes containing 2.5 ml YPD medium at 30°C for 24 h. From these cultures, 20 μl were inoculated into 15 ml tubes containing either 3.5 ml YPD or 3.5 ml YPDE (1 M) medium and incubated at 30°C for 3–4 d. Throughout this experiment, growth was determined periodically. Those mutants with growth rates, μ, in YPD medium similar to that of the wild type (about 0.37 h⁻¹) but either unable to grow in YPDE (1 M) medium after 3–4 d or with a growth rate in this medium of 0.02 h⁻¹ or less were selected as ethanol-sensitive mutants [the wild type strain S288C grows in YPDE (1 M) medium with a growth rate of 0.15 h⁻¹]. Finally these mutants were spread onto YPG and SD agar and incubated for 2 d at 30°C to establish whether any of them were auxotroph or possessed a "petite" phenotype.

Genetic analysis

The selected mutants were crossed with the strain AH28X and the formed zygotes (and afterward the tetrads which appeared after subjecting the diploids to sporulation conditions) were isolated by micromanipulation on Petri dishes with YPD agar, using a micromanipulator (Lawrence Precision Machines, Berkeley, CA, USA) fixed to an inverted Nikon microscope (Model NS) and incubated at 30°C. Tetrads were digested with Suc D'Helix pomatia diluted 1/10 in water (L'Industrie Biologique Francaise, Clichy, France) and incubated for 15 min at room temperature (Sherman et al. 1979).

Once grown on YPD agar, the meiotic products of each sporulated diploid were checked at 30°C on YPDE (1.2 M) and YPDE (1.5 M) agar to test ethanol sensitivity segregation, on SD agar to test histidine marker segregation and on YPD agar at 37°C to test thermosensitivity. Cell size and morphology of the mutants and the wild type strain S288C were observed in a phase contrast microscope Wild M 20 equipped with an automatic photographic system.

To determine the number of complementation groups defined by the ethanol-sensitive mutants, spores from the diploids formed by each mutant and the strain AH28X were checked for ethanol sensitivity and for mating type, and crossed with each of the other mutants. Zygotes were micromanipulated on YPD agar and after 24 h at 30°C diploid cells were inoculated into YPDE (1 M) medium and incubated for 48 h at 30°C.

Analytical determinations

Ethanol. Measurements of ethanol were carried out following the method described by Kaplan and Giotti (1957). In some experiments ethanol was determined by mixing 0.5 ml of each sample with 0.5 ml of a 2% (v/v) isopropanol solution and measuring the ethanol content in a Hewlett-Packard 5880 gas-liquid chromatograph (FID) (Janssens et al. 1983).

Glucose. 20 μl of diluted samples were injected using a microsyringe, in a glucose analyzer YSI 27 (Yellow Springs Instruments, Yellow Springs, OH, USA) equipped with a glucose-oxidase membrane YSI 2365 from Aspergillus niger.

Lipids. Cells were grown overnight in YPD medium at 30°C with aeration and then inoculated either in YPD or in YPDE medium supplemented with different concentrations of ethanol (0.35–1.05 M) according to the strain. The cultures were then incubated at 30°C with aeration until the early