# SACCHAROMYCES CEREVISIAE:

# SORBITOL-DEPENDENT FRAGILE MUTANTS

by

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SUMMARY

Fragile mutants have been isolated among sorbitol-dependent S. cerevisiae. The mutants are also sensitive to high temperature and to rifampicin. They grow exponentially in presence of 10% sorbitol at 30°C, but are very fragile: when resuspended in buffers, they release 50 to 90% of their RNA. These characteristics permit the study of unstable structures and rapid processes in actively growing cells.

## INTRODUCTION

The study of bacterial biochemistry has been aided by the use of spheroplasts and fragile cells, but a similar analysis of yeasts, as model eukaryotes, is difficult. Yeast spheroplasts can be obtained by the use of lytic enzymes. However, the active enzyme preparations are expensive mixtures of variable potency, and the obtained spheroplasts do not grow exponentially, growth being restricted to short time periods only.

An alternative procedure may be the isolation of fragile yeast mutants analogous to the sucrose-dependent fragile strains of E. coli (1). We report here the isolation of several fragile yeast mutants and their partial characterization.

## MATERIALS AND METHODS

Saccharomyces cerevisiae strains A364a, 368, H-2 (from the collection of Dr. L. Hartwell, University of Washington) and S288C (from the collection of Dr. G. Fink, Cornell University) were used in these studies. One of them, H-2, is a mutant already known to have a membrane defect [(2) and Dr. L. Hartwell, personal communication]. A364a has the following markers: ade-1$^-$, ura-1$^-$, gal-1$^-$, try-1$^-$, his-7$^-$, lys-2$^-$, trp-1$^-$, and is the parental strain of H-2 and 368 (2). S288C is of α mating type and has the mal$^-$ and gal-2$^-$ markers.

The basic selection was of sorbitol-dependent yeast colonies. The original yeast strains were mutagenized with ethyl methane sulfonate (4). The mutagenized cells were plated on rich medium (YPD, ref. 2) plus 10% sorbitol and incubated at 30°C. After replica plating on media without sorbitol, about 0.1% of the colonies grew only in the presence of sorbitol. These sorbitol-dependent colonies were tested for fragility, as indicated in the text. Occasionally, as indicated in the Figure legends, standard fractionation techniques were applied to the lysates.

## RESULTS AND DISCUSSION

While many sorbitol-dependent colonies were obtained from wild type strains (A364a or S288C), and a number of them showed initial fragility in liquid cultures, none of the first lots tested was stable - all reverted more or less rapidly. Stable fragile strains were first obtained by remutagenizing H-2 or 368, which already show some indications of membrane lesion and are temperature sensitive (2,3) The mutants obtained - SY 15 (from H-2) and SY17 (from 368) grew exponentially in liquid media containing 10% sorbitol and showed increased sensitivity to rifampicin (a gift of Dr. L. Silvestri, Gruppo Lepetit SPA, Milan). Since the parental strain A364a (like all wild type yeast strains) is not sensitive even to high doses of rifampicin, the sensitivity of SY15 and SY17 to this drug may be related to an increased permeability and fragility of the cell wall or membrane. To test this possibility S288C was mutagenized and rifampicin sensitive colonies selected at 30°C in the presence of 10% sorbitol. Among $10^4$ colonies tested, one was isolated which does not grow even after 5 days on YPD+10% sorbitol plates

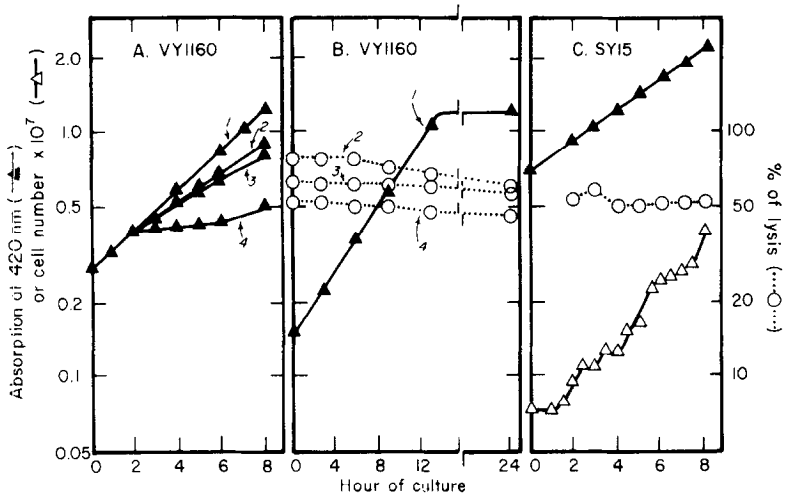


Fig. 1. Growth and lysis of fragile yeast strains.

A. Growth of S. cerevisiae VY1160 in YM-5 media supplemented with different osmotic stabilizers. VY1160 was grown in YM 5+10% sorbitol medium at 30°C. At $A_{420}$ = 0.420 the culture was chilled, and the cells harvested and washed with cold 10% sorbitol. Equal amounts of cells were suspended in YM 5 medium prewarmed to 30°C, with different osmotic stabilizers, and the absorbancy followed at the times indicated. Curve 1, YM 5+10% sorbitol; curve 2, YM 5+10% sucrose; curve 3, YM 5+10% polyethyleneglycol; curve 4, YM 5 without stabilizer.

B. Percentage of lysis of S. cerevisiae VY1160 at different stages of growth. VY1160 was inoculated from an overnight culture in YM 5+10% sorbitol medium containing 0.2 µCi/ml of $^3$H-uracil (specific activity 54 Ci/mmole, from the Radiochemical Centre, Amersham, England). The exponential growth at 30°C was followed by measuring the absorbancy at 420 nm (curve 1; a reading of 0.1 corresponds to 1 x $10^6$ cells/ml). At the times indicated aliquots were taken out from the culture, the cells collected on Millipore membrane filters, and the percentage of cell lysis determined.

Each filter was placed in a scintillation vial containing 2 ml of distilled water or another lysing medium, and the cells suspended by a Vortex mixer. After 30 sec the suspension was transferred from the vial to a tube and centrifuged at 3000 rpm for 10 min. Portions were sampled from the supernatant and the remaining supernatant decanted carefully and discarded. Both supernatant and pellet fractions were precipitated with 5% cold trichloroacetic acid (final conc.), filtered on Whatman GF/C glass-fiber filters, dried, and counted with a toluene-PPO-POPOP phosphor in a Packard scintillation spectrometer. The counts/min in the total supernatant divided by the total counts/min (supernatant plus pellet) is designated as percentage of lysis. Cells were lysed in water (curve 2); 1% sodium dodecyl sulfate (curve 3) or 0.5% sodium deoxycholate (curve 4).

C. Lysis of synchronized S. cerevisiae SY17 growing in YM 5+10% sorbitol medium. Cells were synchronized in a 15 to 50% sucrose graident according to the provedure of Mitchison and Vincent (5). Synchronized cells were then reinoculated into growth medium. (---Δ---), cell count per ml, as followed in a Neubauer hemocytometer. (---▲---), absorbancy at 420 nm; (---o---), percentage of lysis in distilled water.

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containing 350 µg/ml of rifampicin. The rare mutant thus obtained, VY1160, is stable, grows in a fragile form in liquid media supplemented with 10% sorbitol, and is sensitive to increased temperatures of cultivation (38°C).

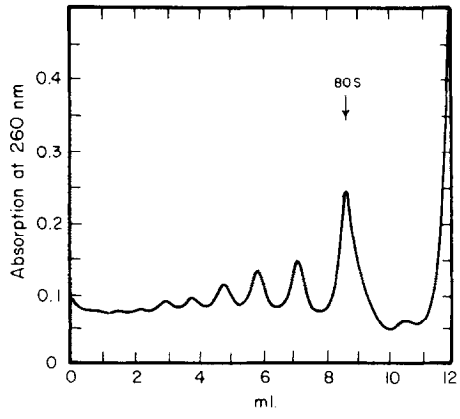


Fig. 2. Zonal sedimentation analysis of a lysate from SY15. To 50 ml of log phase culture of SY15 in YM 5+10% sorbitol medium, 100 µg/ml of cycloheximide (Sigma Chemical Co.) were added to stop protein synthesis. Cells were then immediately collected on ice and harvested by centrifugation. The cells were washed in 50 ml of 10 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml cycloheximide and 10% sorbitol, then suspended in 5 ml of the same buffer without sorbitol and stirred in the cold for 10 min to achieve uniform lysis. The suspension was centrifuged at 25,000 x g for 30 min and the supernatant adjusted with buffer to an optical density at 260 nm of 10.0 per ml. 0.5 ml of this supernatant was layered on a 15-30% sucrose density gradient made up in the same buffer and centrifuged at 34,000 rpm in the SB 206 rotor of an IEC B60 ultracentrifuge. The gradient was pumped from the bottom through a continuous flow cell of a Gilford 2000 recording spectrophotometer to monitor the absorbancy at 260 nm.

Thus, the stable fragile yeast mutants isolated to date are all simultaneously sensitive to rifampicin and to increased temperatures. Consequently, it seems that the change to fragility in yeast cells may not be a one-step mutation, but is of complex nature. This is further suggested by the failure to obtain spontaneous revertants from VY1160. Of about $10^{10}$ cells plated, no revertants have been found capable of growth on: a) YPD plates without sorbitol at 30°C; b) YPD+10% sorbitol plates at 38°C.

Figure 1A shows that the requirement for sorbitol of the fragile yeast mutants is not absolute; other osmotic stabilizers like 10% sucrose or 10% polyethyleneglycol can support growth of fragile mutants. Removal of the osmotic stabilizer from the culture causes an immediate cessation of biosynthesis of macromolecules (RNA and protein); the optical density of the suspension remains unchanged for up to 6 hr, though much of the cell contents are released and measured as follows.

Figure 1B shows the percentage of lysis during different stages of growth.

VY1160 grows exponentially with a doubling time of about 4 hours. About 80% of the cells lyse in water and the percentage of lysis changes little during growth, though it is somewhat lower at later stages of growth. The same is true when cells are lysed in different lysing media, such as 1% sodium dodecyl sulfate or 0.5% sodium deoxycholate. The other fragile mutants, SY15 and SY17, behaved similarly to VY1160, though only 50 to 60% of their cellular RNA was released during lysis in water or other media.

The reason for the incomplete lysis of the cells remains unknown, but synchronized cultures show a fairly constant degree of fragility at different stages of the cell cycle (see Figure 1C for data with strain SY17). Therefore, the lysed cells are a random fraction of the population, and cell fragility is not a temporary pattern appearing only in a certain stage of the cell cycle or the cell growth. Polyribosomes are released, and show a distribution in zonal sedimentation (Fig. 2) very similar to that in lysates from yeast spheroplasts (2). In other experiments (data not shown) we have found that DNA and precursors of ribosomal RNA are released from cells lysed in water, and that the percentage of lysis determined by protein (6) or DNA (7) release is identical to that estimated by the release of RNA. These data indicate that nuclear contents are released from the lysed cells and that lysis represents essentially a complete lysis of most cells, rather than a partial lysis of all cells.

The fragile yeast mutants obtained thus far are all sensitive to increased temperatures (38°C) of cultivation, though in the case of VY1160 no selection to temperature sensitivity was carried out. The increased sensitivity to elevated temperatures may well be connected to cell fragility. Consistent with this notion, when liquid cultures of fragile mutants are shifted from 30°C to 38°C, cells grow for about 3 hours at a reduced rate and then stop growing.

Estimation of cell lysis showed an increased fragility with increase of the temperature of cultivation. At the lowest temperature employed (23°C) the cells grow faster, but the percentage of lysed cells is only 3-8% for SY15 and 35-43% for VY1160. At intermediate temperatures (30°C), the cells grow more slowly, and the

percentage lysis of the two strains is 50-67% and 73-82%. At the highest temperature (38°C), practically the whole cell population is lysed (75 to 80% of SY15 and 86-91% of VY1160), but growth after the temperature shift is restricted.

Thus, cultivation at 30°C gives optimal rates of growth, comparable to those of the parental strains, while the percentage of cell lysis of the fragile mutants is still high enough to allow further biochemical studies. Consequently, the mutants can be used to obtain bulk amounts of many cellular components in proportions characteristic of their level *in vivo*. These mutants can provide a more accurate sampling of yeast cells by much less extreme and faster treatments than those required for the production of spheroplasts or for conventional techniques of ballistic cell disruption. Furthermore, the fragile yeast mutants show also increased sensitivity to amphotericin B (8), and to rifampicin (8,9) and various other inhibitors of macromolecular biosynthesis to which wild type yeast are known to be completely resistant *in vivo*.

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