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ORIGINAL PAPER

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Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*

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Abstract The yeast *Saccharomyces cerevisiae* exhibits high ethanol tolerance compared with other microorganisms. The mechanism of ethanol tolerance in yeast is thought to be regulated by many genes. To identify some of these genes, we screened for ethanol-sensitive *S. cerevisiae* strains among a collection of mutants obtained using transposon mutagenesis. Five ethanol-sensitive (*ets*) mutants were isolated from approximately 7000 mutants created by transforming yeast cells with a transposon (mTn-*lacZ*/*LEU2*)-mutagenized genomic library. Although these mutants grew normally in a rich medium, they could not grow in the same medium containing 6% ethanol. Sequence analysis of the *ets* mutants revealed that the transposon was inserted in the coding regions of *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2*. We constructed deletion mutants for these genes by a PCR-directed disruption method and confirmed that the disruptants, like the *ets* mutants, were ethanol sensitive. Thus, these five genes are indeed required for growth under ethanol stress. These mutants were also more sensitive than normal cells to Calcofluor white, a drug that affects cell wall architecture, and Zymolyase, a yeast lytic enzyme containing mainly β-1,3- glucanase, indicating that the integrity of the cell wall plays an important role in ethanol tolerance in *S. cerevisiae*.

Keywords *Saccharomyces cerevisiae* · Ethanol sensitivity · Transposon mutagenesis

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Introduction

It is well known that *Saccharomyces cerevisiae* is more tolerant to ethanol than other microorganisms. Though many physiological investigations into the ethanol tolerance of yeast have been carried out, little is known about the genetic mechanisms that underlie ethanol tolerance. Aguilera and Benitez (1986) isolated 21 monogenic ethanol-sensitive mutants in *S. cerevisiae* and defined 20 complementation groups. They reported that about one-third of the ethanol-sensitive mutants were also temperature sensitive (Aguilera and Benitez 1986). These results indicate that many genes are involved in mediating ethanol tolerance in *S. cerevisiae*, and suggest that the mechanism responsible partially overlaps with the mechanism required for tolerance to heat. For example, loss of function of the heat shock protein Hsp104 abolishes both heat and ethanol tolerance (Mager and Moradas-Ferreira 1993). However, few genes required for ethanol tolerance have been identified. Although heat and ethanol stresses reduce the level of H+ ATPase, a plasma membrane protein, they stimulate its activity (Alexandre et al. 1993). These findings indicate that some of the biochemical changes induced by ethanol exposure are identical to those induced by heat shock in yeast (Piper 1995). Nevertheless, the effects of ethanol and heat stress on yeast are not always the same. Although pre-exposure to mild heat shock leads to acquisition of ethanol tolerance, pre-exposure to ethanol does not make cells tolerant to high temperature (Van Uden 1984; Costa et al. 1993). In addition, lipid composition (Beaven et al. 1982) and trehalose content (Mansure et al. 1994) have been shown to play important roles in ethanol tolerance in *S. cerevisiae*. Thus, the ethanol tolerance mechanism of yeast is complex, and many genes and factors remain to be analyzed.

The aim of this study was to identify new genes involved in ethanol tolerance and to gain new insights into the genetics of ethanol tolerance of yeast. To do this, we used the method of gene disruption by random

transposon insertion. This method has been used to identify and analyze genes that are involved in cell morphology (Chun and Goebl 1996) and cell wall biosynthesis (Lussier et al. 1997) in *S. cerevisiae*. Since it is easier to identify genes by identifying the location of a transposon insertion than by using the traditional complementation cloning method, the transposon mutagenesis method provides a useful means of screening genes comprehensively, provided that the gene disruptants remain viable.

Materials and methods

Strains and media

Saccharomyces cerevisiae YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) was used as the recipient strain for transposon mutagenesis and gene disruption. YPAD medium (1% yeast extract, 2% Bacto-peptone, 0.01% adenine sulfate, 2% glucose) was used as a rich medium for yeast growth. Synthetic complete medium (SC; 0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, supplemented with appropriate nutrients) was used for transformation of yeast. For all procedures involving *E. coli*, strain JM109 was used. LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) containing 100 μg/ml ampicillin was used for growth of *E. coli*.

Transposon mutagenesis

The method is outlined in Fig. 1. A yeast genomic library that had been mutagenized by random insertion of the transposon mTn-*lacZ*/*LEU2* (Burns et al. 1994) was kindly provided by Dr. Michael Snyder (Yale University, New Haven, Conn.). Yeast DNA fragments with random mutations were excised from the library by digestion with *Not*I, and used for transformation of YPH499. Transformants carrying random insertions of the transposon as a result of homologous recombination-replacement were selected on SC medium containing 1 M sorbitol but lacking leucine. Sorbitol was added to the medium to increase the transformation efficiency. It was not used for the screening of the *ets* mutants, and thus would not have biased the type of mutants recovered.

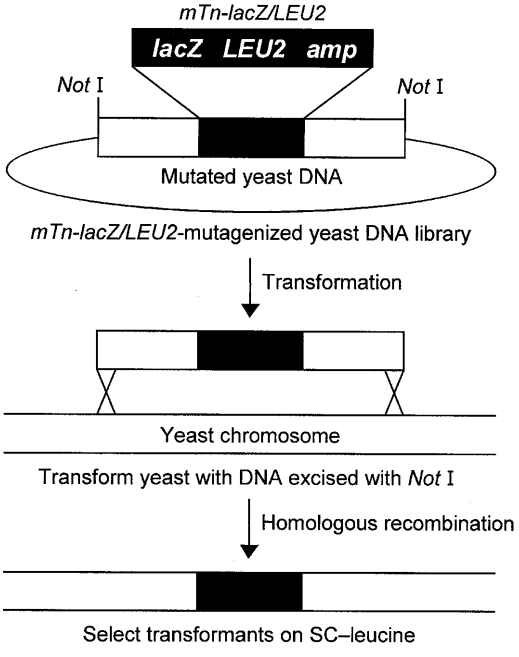


Fig. 1 Diagrammatic representation of the scheme used for transposon mutagenesis with an mTn-*lacZ*/*LEU2*-mutagenized genomic library

Isolation of ethanol-sensitive mutants

The transformants were replica plated onto YPAD plates and YPAD plates containing 6% ethanol, and were incubated at 30°C for 3 days. Mutants that could grow normally on YPAD plates but not, or only slowly, on YPAD plates containing 6% ethanol were selected. To confirm that these mutants were sensitive to ethanol, they were subjected to an ethanol sensitivity test as follows. Cells were grown in YPAD medium with shaking to an OD_{660} value of 1.0, and diluted 10-, 100- and 1000-fold with sterile water. Aliquots (5 μl) of each dilution were spotted onto YPAD agar plates containing no ethanol or 6% ethanol, and incubated at 30°C for 3 days. The amount of ethanol that evaporated from the agar plates during the incubation period was estimated to be less than 10% of the initial concentration (Aguilera and Benitez 1986). Mutants that grew on the latter plates were isolated and are referred to as ethanol-sensitive (*ets*) mutants.

Identification of genes that can cause the ethanol-sensitive phenotype when mutated

The sites of mTn-*lacZ*/*LEU2* insertion in the mutants were determined by rescuing a genomic DNA fragment immediately adjacent to the *lacZ* sequence derived from *E. coli*. The *ets* mutants were transformed with 1 μg of *Bam*HI-digested *pRSQ2-URA3* plasmid (kindly provided by Dr. M. Snyder) that carried the *E. coli* replication origin, β-lactamase as a selectable marker for *E. coli*, *URA3* as a selectable marker for yeast and partial sequences of *lacZ* at both ends of the digested fragment. Transformants were selected on SC-leucine-uracil plates containing 1 M sorbitol after incubation at 30°C for 3 days. Genomic DNA was extracted from each transformant, digested with *Eco*RI and then circularized by self-ligation. This plasmid was propagated in *E. coli* and sequenced to determine the DNA sequence flanking the *lacZ* sequence, using the primer (5′-GTTTTCCCAGTCACGAC-3′). The position of transposon insertion was determined using the BLAST service of the *Saccharomyces* genome database at Stanford University (http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd).

Gene disruptions

The entire ORFs of *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2* were replaced by a *HIS3* marker using the PCR-directed gene disruption method (Baudin et al. 1993; Lorenz et al. 1995). PCR primers used for the gene disruption are shown in Table 1. The PCR product was used for transformation of YPH499 and transformants were selected on SC-histidine plates containing 1 M sorbitol. Disruption of the appropriate gene was confirmed by PCR analysis with the primers shown in Table 2.

Calcofluor white sensitivity test

Yeast cells grown in YPAD medium with shaking to an OD_{660} value of 1.0 at 30°C were harvested, washed with sterile water, and serially diluted three times by factors of 10. Aliquots (5 μl) of each dilution series were spotted onto YPAD agar plates containing 20 μg/ml Calcofluor white and incubated at 30°C for 3 days.

Zymolyase sensitivity test

Yeast cells were grown in YPAD liquid medium with shaking at 30°C for 48 h. Cells were harvested, washed with sterile water, and

Table 1 Oligonucleotide primers used for the production of gene disruption cassettes

Primer	Sequence (5′→3′)
BEM2-1	TGCCTTTTCTGGATAGACACAAAAAAAACAAA TAACGAAGCAGGAGTCTAGCCTCCTCTAGTACACTCTAT
BEM2-2	AATTTTTTCTCTCTCAGCAGTGGATTGTATA CATTTACCACGAAAATTGTCGCCTCGTTCAGAATGACACG
PAT1-1	AAGGAAGCAAAGGTTTTAACCGGAAGTAAGAGCAGCAA GAAGCACTAGCAGCCTCCTCTAGTACACTCTAT
PAT1-2	GGGAGAAAAAAAAATACATGCGTAAGTACATTAAAATTA CAGGAAAAATCCGCCTCGTTCAGAATGACACG
ROM2-1	GCTCTATTACTGCTGACTTAATTGGA CAATTCATCTCTTTTCCTGCGGTTGCCTCCTCTAGTACACTCTAT
ROM2-2	TATGCTTTTTATTCTAAAGAAAATAAGGAAAGTCTATATACGTTGCTATC CGCCTCGTTCAGAATGACACG
VPS34-1	ACAACAATAACATCTCCGTGAAGCATTGAGGGAAGGGTTTAACTCCAA CAGCCTCCTCTAGTACACTCTAT
VPS34-2	TATAATTAGAGTGACGAAATTTAAATTTTGAAGCACCAATTATCAAC CAACGCCTCGTTCAGAATGACACG
ADA2-1	TTCGATAAAATATCAGCGTAGTCTGAAAATATATACATTAAGCAAAAA GAGCCTCCTCTAGTACACTCTAT
ADA2-2	GGCCAATAATAACTAGTGACAATTGTAGTTACTTTTCAATTTTTTTTTT GCGCCTCGTTCAGAATGACACG

Table 2 Oligonucleotides used for confirmation of gene disruptions

Primer	Sequence (5′→3′)
BEM2-3	AGAATGGATGTTGATAAGAGT
BEM2-4	GGGTTCGCTAATCAAGGTGTC
PAT1-3	TCATCGTCTTTCCCCTCATCA
PAT1-4	GGTGCTGTTCCCTGGTTTTGT
ROM2-3	GAGAATTCCGTAGATGAGCAC
ROM2-4	CCAAATAAAAAGACACCAGAT
VPS34-3	TCCAGTGTCCTTTGTAGTCAT
VPS34-4	TCTGTTCGTCCCGTATCTTAT
ADA2-3	TTCCCATAACGCTTTCAATCT
ADA2-4	GAGACCAACCCAGCATTACGC

diluted to an OD_{660} value of 1.0 in 5 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 0.04% NaN_3 and 40 μg/ml Zymolyase 20T (Seikagakukogyo). The reaction mixture was incubated at 30°C with gentle shaking and the optical density at 660 nm was measured periodically.

Results

Screening for ethanol-sensitive mutants

Approximately 7000 transformants were first selected as leucine prototrophs and replica-plated to both YPAD plates and YPAD plates containing 6% ethanol. After incubation at 30°C for 3 days, 260 clones were found that could grow on YPAD but not at all (5 clones) or only slowly (255 clones) on YPAD containing 6% ethanol. The five clones that did not grow at all on medium containing 6% ethanol were selected as candidates for ethanol sensitive (*ets*) mutants. As shown in Fig. 2, these five mutants, designated as *ets1–5*, grew well on YPAD plates but could not grow on YPAD containing 6% ethanol.

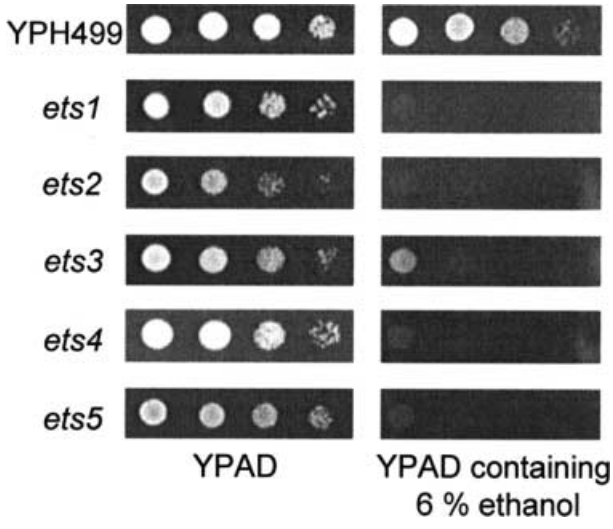


Fig. 2 Growth of *ets* mutants on YPAD plates containing ethanol. YPH499 (wild-type) and *ets* mutants (Nos. 1–5) were grown to an OD_{660} value of 1.0 in YPAD liquid medium. Five-microliter aliquots of serial dilutions (*left to right*) of cell suspension were placed on YPAD plates containing 0% or 6% ethanol, and incubated at 30°C for 48 h

Identification of mTn-lacZ/LEU2 insertion positions in *ets* mutants

From each *ets* mutant, yeast chromosomal DNA adjacent to the mTn-*lacZ*/*LEU2* was rescued in *E. coli* and sequenced. The transposon was found to be integrated into the ORFs of *BEM2*/*YER155C* in *ets1*, *PAT1*/*YCR077C* in *ets2*, *ROM2*/*YLR371W* in *ets3*, *VPS34*/*YLR240W* in *ets4* and *ADA2*/*YDR448W* in *ets5*. The transposon insertion positions and reported functions of all identified genes are summarized in Table 3.

Table 3 Identified genes that can cause the ethanol-sensitive phenotype when mutated

Strain	Disrupted gene	Insertion position[a]	Function[b]	References
ets1	*BEM2/YER155C*	6028 bp	GTPase activating protein for Rho1p, which is involved in cell polarity and cell wall maintenance	Kim et al. (1994), Wang and Bretscher (1995), Chen et al. (1996) Cid et al. (1998)
ets2	*PAT1/YCR077C*	70 bp	Topoisomerase II-associated protein which is required for faithful chromosome transmission	Rodriguez-Cousino et al. (1995), Wang et al. (1996)
ets3	*ROM2/YLR371W*	367 bp	GDP-GTP exchanging factor for Rho1p, which is activated by cell wall defects	Lussier et al. (1997), Schmidt et al. (1997), Ozaki et al. (1996)
ets4	*VPS34/YLR240W*	28 bp	Phosphatidylinositol 3-kinase which is required for vacuolar protein sorting	Herman and Emr (1990), Schu et al. (1993), Stack et al. (1993)
ets5	*ADA2/YDR448W*	117 bp	Component of the nucleosomal histone acetyltransferase complex	Berger et al. (1992), Marcus et al. (1994), Welihinda et al. (1997)

[a]Insertion position is given with respect to the initiator ATG of each coding sequence
[b]From the Yeast Protein Database (YPD)

Growth of gene disruptants under ethanol stress

When the entire ORFs of *BEM2, PAT1, ROM2, VPS34* or *ADA2* in the YPH499 strain were individually replaced by the *HIS3* gene, the five gene disruptants could grow normally on YPAD plates at 30°C, but not on YPAD plates containing 6% ethanol (Fig. 3A). In addition, in liquid medium, the growth rate of each of the disruptants was slightly lower than that of the wild type, and none of the disruptants showed any growth in medium containing 6% ethanol (Fig. 3B). Therefore, the ethanol-sensitive phenotype of each of the *ets* mutants was the result of disruption of a single gene. In other words, all five of the genes identified in this study are required for growth under ethanol stress.

Osmotic stabilization permits growth of the disruptants

Δ*rom2* cells have a temperature-sensitive phenotype, which is partially suppressed in the presence of 1 M sorbitol in the medium (Ozaki et al. 1996). The growth of Δ*rom2* cells under ethanol stress was significantly enhanced by the addition of 1 M sorbitol (Fig. 4). A similar result was observed on addition of 0.5 M NaCl (data not shown), indicating that the growth of Δ*rom2* under these conditions is a consequence of osmotic support. The growth of Δ*pat1* cells under ethanol stress was partially restored by the addition of 1 M sorbitol after incubation for 2 days. On the other hand, growth of the Δ*bem2*, Δ*vps34* and Δ*ada2* disruptants in the ethanol-containing medium was not restored by the addition of an osmotic stabilizer. These results suggest that the ethanol-sensitive phenotype of Δ*rom2* cells is caused by cell lysis, and that the cell wall biosynthesis pathway controlled by the Pkc1-MAP kinase cascade is required for growth under ethanol stress.

Growth of gene disruptants at high temperature

The growth rates of each of the gene disruptants identified in this study were previously found to be temperature sensitive (Herman and Emr 1990; Pina et al. 1993; Peterson et al. 1994; Ozaki et al. 1996; Rieger et al. 1997). We expected this because temperature sensitivity is closely related to ethanol sensitivity. However, temperature sensitivity is strain dependent (Schmidt et al. 1997). Thus we examined the growth of the five disruptants at high temperature (37°C). As shown in Fig. 5, they showed various behaviors at the elevated temperature, despite showing similar ethanol sensitivity. Δ*bem2* and Δ*vps34* cells were not able to grow on YPAD plates at 37°C even when 1 M sorbitol was added to the medium as an osmotic stabilizer. Δ*ada2* cells could grow at 37°C. Δ*rom2* and Δ*pat1* cells could not grow at 37°C but growth was restored by the addition of 1 M sorbitol. These results are discussed below.

Cell wall integrity of *ets* mutants

All disruptants were more sensitive to Calcofluor white and Zymolyase than the wild-type cells (Fig. 6), indicating that all have defects in the cell wall.

Discussion

In this study, in a screen of 7000 transposon-mutagenized mutants, we found five mutant strains that could not grow on ethanol-containing medium. Each of the mutants had a single disrupted gene: *BEM2*, *PAT1*, *ROM2*, *VPS34* or *ADA2*. None of these five genes has previously been associated with an ethanol-sensitive phenotype. However, whether these genes have specific roles under ethanol stress is not known. Further studies will be needed to answer this question. The mutants were obtained by screening only 7000 transposon-mutagenized mutants.

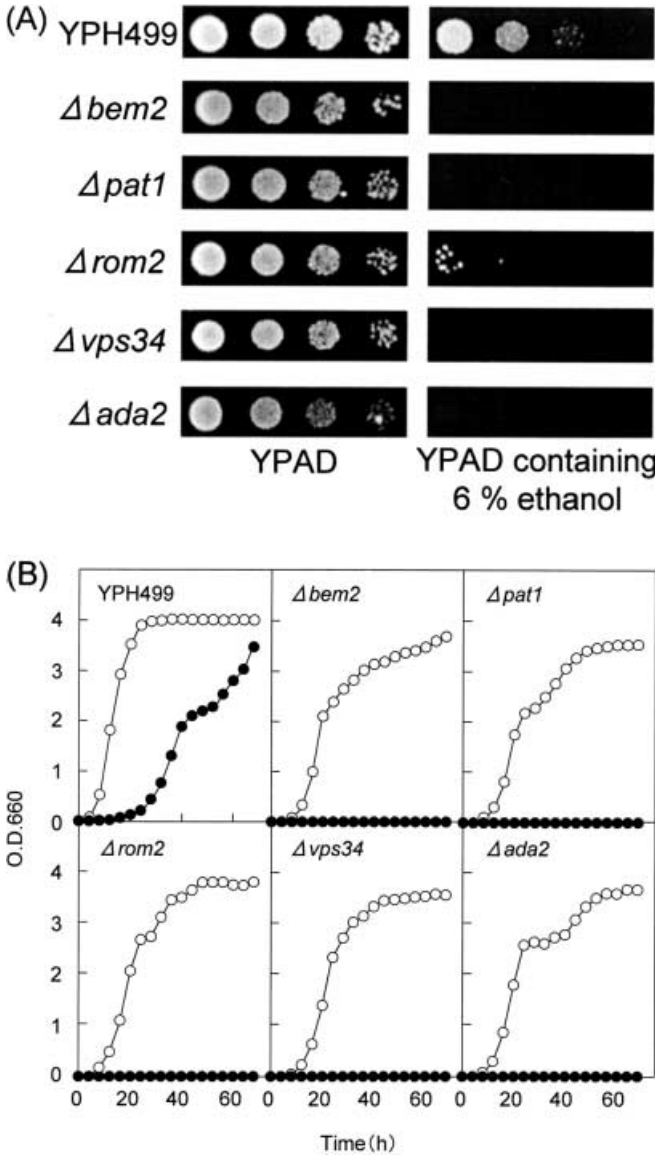


Fig. 3A, B Growth of the gene disruptants on YPAD medium containing ethanol. **A** YPH499 (wild-type) cells and gene disruptants were grown to an OD_{660} value of 1.0 in YPAD liquid medium. Five-microliter aliquots of serial dilutions (*left to right*) of cell suspension were spotted on YPAD agar plates containing 0% or 6% ethanol, and incubated for 48 h at 30°C. **B** Wild-type cells and gene disruptants grown in 5 ml of YPAD liquid medium with shaking for 24 h at 30°C were washed and diluted to an OD_{660} value of 1.0 with sterile water. Aliquots (0.1 ml) of these cell suspensions were used to inoculate YPAD liquid medium containing 0% or 6% ethanol. Growth was examined over a time course of 70 h using a Bio-photo recorder (ADVANTEC). *Open circles*, 0% ethanol; *filled circles*, 6% ethanol

This is far less than the number of mutants (35,000) that would need to be screened to ensure comprehensive coverage of the whole yeast genome (Chun and Goebl 1996). Many genes may be required for ethanol tolerance as reported previously (Aguilera and Benitez 1986). Thus, identification of additional ethanol-sensitive mutants will be necessary to understand the mechanism responsible for ethanol tolerance in *S. cerevisiae*.

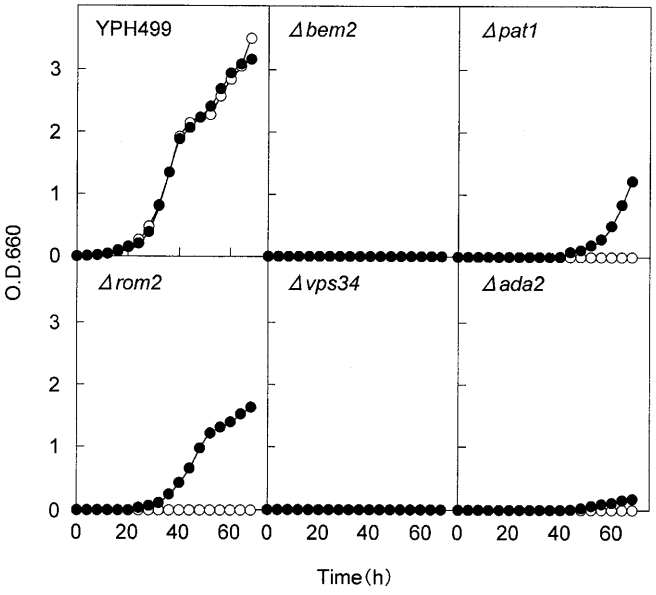


Fig. 4 Effect of an osmotic stabilizer on the ethanol sensitivity of gene disruptants. Wild-type cells and gene disruptants grown in 5 ml of YPAD liquid medium with shaking for 24 hours at 30°C were washed and diluted to an O.D.660 value of 1.0 with sterilized water. 0.1 ml of these cell suspensions was added to YPAD liquid medium containing 6% ethanol and 1 M sorbitol as an osmotic stabilizer. Growth was examined over a time course of 70 hours by using a Bio-photo recorder (ADVANTEC). *Open circles* , YPAD containing 6% ethanol; filled circles, YPAD containing 6% ethanol and 1 M sorbitol

Ethanol sensitivity and high-temperature sensitivity are in part related

Piper (1995) has pointed out that the heat-shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. Four of the disruptants examined here, Δ*bem2*, Δ*pat1*, Δ*rom2* and Δ*vps34*, showed temperature sensitivity as well as ethanol sensitivity. This finding is consistent with the hypothesis that ethanol and high-temperature stress share the same signal transduction pathway and result in similar cellular responses. However, in our experiments, Δ*ada2* cells grew well at 37°C; this contrasts with the results of previously reported experiments, in which Δ*ada2* cells with a different genetic background were found to be temperature sensitive (Pina et al. 1993). This finding indicates that a gene required for growth under ethanol stress is not always necessary for growth at high temperature. One possible explanation is that some cellular processes are especially sensitive to the presence of ethanol but not to high temperature.

Cell wall integrity is needed for ethanol tolerance in yeast

The suppression of the ethanol and temperature sensitivity of Δ*rom2* and Δ*pat1* by an osmotic stabilizer led us to the hypothesis that weakening of the cell wall can contribute to ethanol sensitivity; the growth of Δ*pat1*

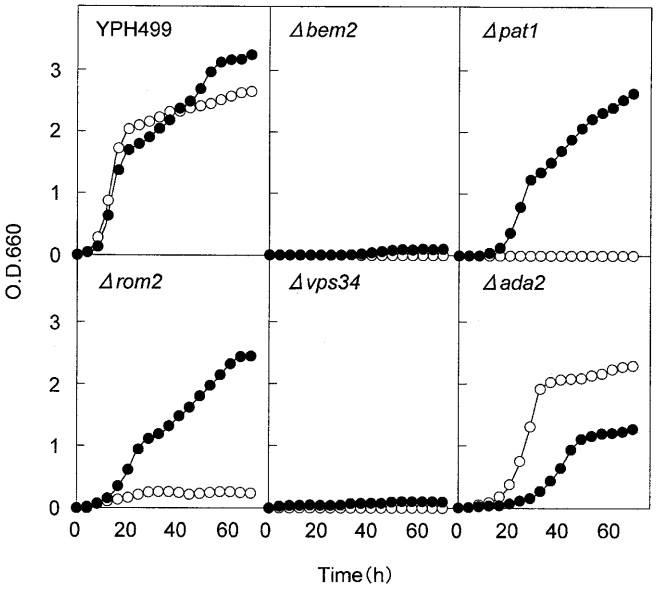
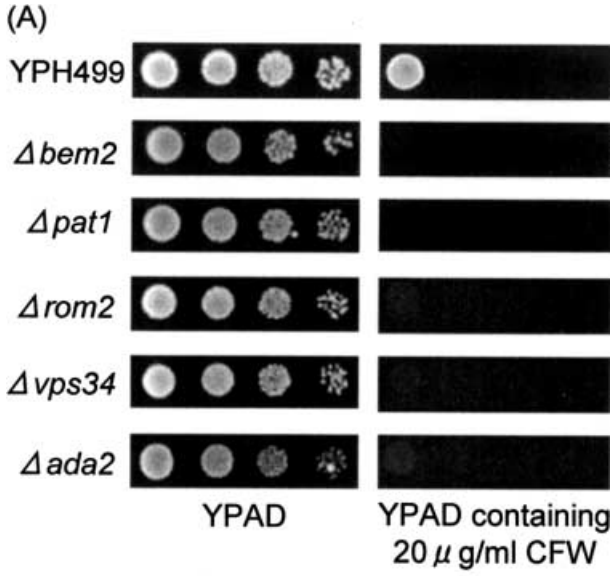


Fig. 5 Growth curves for gene disruptants at high temperature. Wild-type cells and gene disruptants grown in 5 ml of YPAD liquid medium with shaking for 24 h at 30°C were washed and diluted to an OD_{660} value of 1.0 with sterile water. Aliquots (0.1 ml) of these cell suspensions were then added to YPAD liquid medium. Growth at 37°C was examined over a period of 70 h using a Bio-photo recorder (ADVANTEC). *Open circles*, YPAD; *filled circles*, YPAD containing 1 M sorbitol



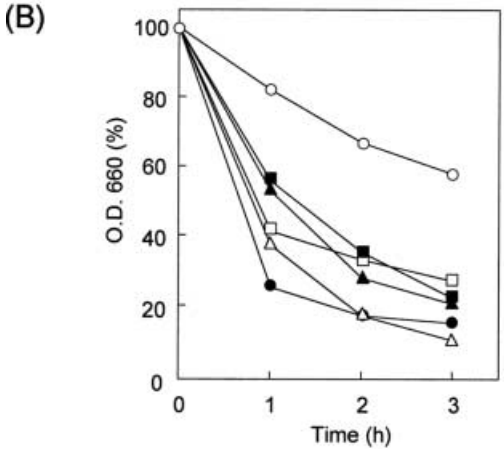


Fig. 6A, B Zymolyase and Calcofluor white sensitivity of the ethanol-sensitive gene disruptants. **A** Growth of the gene disruptants on YPAD medium containing Calcofluor white. YPH499 (wild-type) cells and gene disruptants were grown to an OD_{660} value of 1.0 in YPAD liquid medium. Five-microliter aliquots of serial dilutions (*left to right*) of cell suspension were spotted on YPAD agar plates containing 20 μg/ml Calcofluor white (CFW) and incubated at 30°C for 48 h. **B** Zymolyase sensitivity of the gene disruptants. Cultures of YPH499 (*open circles*), Δ*bem2* (*filled circles*), Δ*pat1* (*open squares*), Δ*rom2* (*filled squares*), Δ*vps34* (*open triangles*) and Δ*ada2* (*filled triangles*) cells grown in YPAD with shaking for 48 h at 30°C were harvested, washed with water and diluted to an OD_{660} value of 1.0 with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.04% NaN_3. After the addition of 0.1 ml of 2 mg/ml Zymolyase 20T, the OD_{660} value was measured periodically

cells under ethanol stress was only partially restored by 1 M sorbitol. The reason for the long lag period before resumption of growth by Δ*pat1* cells under ethanol stress is unclear. One possibility is that there are other unidentified factors that complement the function of *PAT1*. Previously, *ROM2* was identified as a gene involved in cell wall biosynthesis using transposon mutagenesis, and the *rom2* mutant exhibited increased sensitivity to Calcofluor white, a cell surface-perturbing agent that interacts specifically with cell wall chitin and inhibits cell wall synthesis (Lussier et al. 1997). Many mutants with weakened cell walls are known to be sensitive to this agent. Zymolyase is also useful for monitoring cell wall integrity (Shimoi et al. 1998). This enzyme preparation is mainly comprised of β-1, 3-glucanase and protease, which synergistically digest yeast cell walls (Klis 1994). All five mutants showed sensitivity to Calcofluor white and Zymolyase, indicating that they have damaged cell walls. These results suggest that cell wall integrity is needed for ethanol tolerance in yeast. They also raise the possibility that all mutants with cell wall defects show reduced tolerance to ethanol.

Potential roles of the five identified genes in ethanol stress

BEM2 encodes a GTPase-activating protein and *ROM2* encodes a GDP/GTP exchange factor for the small GTP binding protein Rho1 (Kim et al. 1994; Ozaki et al. 1996; Lussier et al. 1997). Rho1 modulates cell wall biosynthesis by controlling Pkc1 (Nonaka et al. 1995) and β-1, 3-glucan synthase (Drgonova et al. 1996; Qadota et al. 1996). Pkc1 regulates the transcription of many genes involved in cell wall biosynthesis through the MAP kinase pathway which mediates cell integrity (Cid et al. 1995). High temperature activates this pathway

(Kamada et al. 1995) and deletion of *ROM2* or genes for proteins that participate in the MAP kinase pathway leads to a temperature-sensitive cell lysis phenotype that is suppressible by an osmotic stabilizer (Cid et al. 1995). Suppression of the ethanol sensitivity of the *ROM2* disruptant by osmotic support suggests that the ethanol stress is also transmitted to the downstream cell wall biosynthesis pathway through this pathway.

On the other hand, disruption of *BEM2* causes constitutive activation of Rho1, which is toxic to yeast cells (Madaule et al. 1987; Schmidt et al. 1997). The normal function of the *BEM2* gene is also essential for maintaining cell wall integrity (Cid et al. 1998). Considering that *ROM2* and *BEM2* have opposing effects on Rho1, proper recycling of Rho1 between the GDP- and GTP-binding forms might be required for growth under ethanol stress.

VPS34, which encodes phosphatidylinositol (PI)-3 kinase, is required for protein sorting from the endoplasmic reticulum to the vacuole (Herman and Emr 1990; Schu et al. 1993; Stack et al. 1993). *S. cerevisiae* Δ*vps34* cells are sensitive to high osmotic pressure (Herman and Emr 1990). A *vps34* disruptant of *Schizosaccharomyces pombe* was found to be sensitive to stresses such as high temperature, high osmotic pressure and high concentrations of monovalent and divalent cations (Kimura et al. 1995). We found *VPS34* is required for ethanol tolerance and cell wall integrity. These findings suggest that *VPS34* plays a significant role in many stress responses, including the stress response to ethanol exposure, in yeast.

PAT1, which encodes a protein associated with topoisomerase, is required for accurate chromosome transmission during cell division (Wang et al. 1996). Yeast cells lacking a functional Pat1 exhibit a number of phenotypic abnormalities, including reduced growth rate, reduced viability and increased chromosome missegregation during both mitosis and meiosis (Rodriguez-Cousino et al. 1995; Wang et al. 1996). Ethanol may exacerbate these abnormalities. When *E. coli* cells are exposed to heat shock, topoisomerase (DNA gyrase) catalyzes an immediate but transient relaxation of DNA (Kataoka et al. 1996), which affects the expression of various genes during heat shock (Mizushima et al. 1993). These results suggest that Pat1 might be associated with stress responses in a similar manner, although topoisomerase is not known to be involved in stress responses in yeast. On the other hand, the growth defect of Δ*pat1* cells at high temperature and under ethanol stress are suppressed by the addition of an osmotic stabilizer, suggesting some relationship between Pat1 and the Pkc1-MAP kinase pathway via one or more unknown intermediates.

ADA2 encodes a component of the nucleosomal histone acetyltransferase complex (Gcn5/Ada), which is required for the maximal activation of a subset of acidic activators (Berger et al. 1992; Marcus et al. 1994). This complex is reported to be required for the induction of molecular chaperones of the endoplasmic reticulum, such Kar2 and Pdi1, in response to unfolded protein stress. Ada5 is another component of this complex and the deletion of this gene results in reduced induction of the chaperones by unfolded proteins but not by heat shock (Welihinda et al. 1997). If Ada2 is also involved in the stress response induced by unfolded proteins in the presence of ethanol, it is reasonable that Δ*ada2* cells would be ethanol-sensitive but not temperature-sensitive.

In conclusion, we have shown that *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2* are required for yeast growth in ethanol-containing medium. Not all of these genes are required for growth at high temperature. Moreover, we demonstrated that cell wall integrity is important in ethanol tolerance. More comprehensive screening of ethanol-sensitive mutants should lead to new insights into the mechanism that enables yeast cells to tolerate ethanol and enable the breeding of yeast strains with higher ethanol tolerances. Such strains would be of great value in industrial ethanol fermentations.

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