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Research Article

Disruption of *URA7* and *GAL6* improves the ethanol tolerance and fermentation capacity of *Saccharomyces cerevisiae*

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Abstract

Screening of the homozygous diploid yeast deletion pool of 4741 non-essential genes identified two null mutants (Δ*ura7* and Δ*gal6*) that grew faster than the wild-type strain in medium containing 8% v/v ethanol. The survival rate of the *gal6* disruptant in 10% ethanol was higher than that of the wild-type strain. On the other hand, the glucose consumption rate of the *ura7* disruptant was better than that of the wild-type strain in buffer containing ethanol. Both disruptants were more resistant to zymolyase, a yeast lytic enzyme containing mainly β-1,3-glucanase, indicating that the integrity of the cell wall became more resistance to ethanol stress. The *gal6* disruptant was also more resistant to Calcofluor white, but the *ura7* disruptant was more sensitive to Calcofluor white than the wild-type strain. Furthermore, the mutant strains had a higher content of oleic acid (C18 : 1) in the presence of ethanol compared to the wild-type strain, suggesting that the disruptants cope with ethanol stress not only by modifying the cell wall integrity but also the membrane fluidity. When the cells were grown in medium containing 5% ethanol at 15 °C, the *gal6* and *ura7* disruptants showed 40% and 14% increases in the glucose consumption rate, respectively.

Keywords: ***Saccharomyces cerevisiae*; ethanol resistance; *URA7*; *GAL6*; fermentation**

Introduction

Since *Saccharomyces cerevisiae* is well known to produce a high concentration of ethanol, it is commonly used for brewing and fuel ethanol production. Since ethanol is toxic to cells, the ethanol tolerance of *S. cerevisiae*, which is closely related to ethanol productivity (Jones, 1989), is an important factor in industrial ethanol production. There have been numerous studies on this issue (for reviews, see Attfield, 1997; Casey and Ingledew, 1986) and the structural and functional alterations in yeast cells in the presence of ethanol have been described (Alexandre *et al*., 1994; Lloyd *et al*., 1993).

Although many physiological investigations into the tolerance of *S. cerevisiae* to ethanol have been carried out, little is known about the genetic mechanisms. To determine the genetic basis of ethanol tolerance in *S. cerevisiae*, many researchers have isolated and characterized mutants. However, most of the approaches were to isolate ethanol-sensitive mutants in order to identify genes that are necessary for growth under ethanol stress. For example, Aguilera and Benitez (1986) isolated 21 ethanol-sensitive mutants and classified them into 20 complementation groups, but they could not identify the genes responsible for ethanol sensitivity. Takahashi *et al*. (2001) screened ethanol-sensitive mutants based on Tn-mediated mutagenesis and

identified five genes responsible for this phenotype. Kubota *et al*. (2004) searched for ethanol-sensitive mutants among 4847 strains carrying non-essential gene deletions and concluded that at least 256 genes are important for cell growth in the presence of ethanol. Similarly, van Voorst *et al*. (2006) searched for ethanol-sensitive mutants among 4868 non-essential gene deletion strains and identified 14 strains that did not grow and 32 strains that showed a clear slow-growth phenotype in the presence of 6% ethanol.

However, the overexpression of such genes did not necessarily confer ethanol tolerance on the cells. Inoue *et al*. (2000) isolated ethanol-sensitive mutants from sake yeast and identified one with a mutation in *erg6*; however, overexpression of *ERG6* did not enhance the ethanol tolerance of *S. cerevisiae* with respect to growth in the presence of 5% or 7% ethanol or viability in 10% ethanol.

Despite the studies described above, the mechanism of ethanol tolerance still remains unclear because of its complexity. Since tolerance to high concentrations of ethanol is one of the most desirable characteristics in industry, isolation of ethanol tolerant mutants would have direct industrial applications. However, it is quite rare to isolate ethanol-tolerant mutants directly, presumably due to the technical difficulties of isolating such mutants and partly because of the polygenic systems of ethanol tolerance. For example, an ethanol-tolerant mutant was bred from industrial sake yeasts and genome-wide gene expression profiling identified the high level expression of several stress-responsive genes, but specific gene mutations were not identified (Ogawa *et al*., 2000).

The aim of this study was to identify new genes involved in ethanol tolerance (not sensitivity) in order to understand its genetic basis in *S. cerevisiae*. We directly screened a pool of yeast deletion strains in order to identify ethanol-tolerant mutants, with the criterion of faster growth in the presence of 8% ethanol. We identified Δ*ura7* and Δ*gal6* null mutants, and characterized their lipid composition, fermentation capacity and cell viability.

Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in Table 1. Pools of 4741 homozygous diploid yeast deletion strains were obtained from Invitrogen (CA, USA). Since sake yeast produces more than 20% v/v ethanol in sake brewing, sake yeast strain K7 was used as an ethanol-tolerant yeast control. Yeast cells were grown in YPAD rich medium (Uemura and Wickner, 1988) and synthetic complete medium (SC; Sherman *et al*., 1986). As a carbon source, 2% w/v glucose (Glc) was added unless otherwise specified.

Growth in liquid media was examined by monitoring the turbidity of the cells at 630 nm with an automatic detector (Bio-Plotter, Toyo-Sokki, Japan).

Table 1. Strains used

Strain	Genotype	Relevant characteristics
BY4743	*MAT***a**/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2*	BY4741 × BY4742 (Brachmann *et al*., 1998)
BY4743-*Δura7*	*MAT***a**/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2Δura7::KanMX4/Δura7::KanMX4*	Homozygous *Δura7* disruptant of BY4743
BY4743-*Δgal6*	*MAT***a**/*MAT α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2Δgal6::KanMX4/Δgal6::KanMX4*	Homozygous *Δgal6* disruptant of BY4743
BY4743-*Δrim21*	*MAT***a**/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2Δ rim21::KanMX4/Δ rim21::KanMX4*	Homozygous *Δ rim21* disruptant of BY4743
BY4743-*Δalg9*	*MAT***a**/*MAT α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2Δ alg9::KanMX4/Δ alg9::KanMX4*	Homozygous *Δ alg9* disruptant of BY4743
K7	*MAT***a**/*MATα*	Sake yeast Kyokai No. 7, Brewing Society of Japan

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Genomic DNA preparation, PCR and DNA microarray hybridization

Genomic DNA was extracted with the Genomic Tip 100/G kit (Qiagen) PCR and DNA microarray hybridizations were performed basically as described by Winzeler *et al*. (1999). Briefly, UPTAG DNA sequences were amplified from genomic DNA by means of PCR with B-U1- and Cy3-labelled B-U2-comp primers. The amplified sequences were hybridized to oligonucleotide arrays, spotting 20mer oligonucleotides corresponding to the UPTAG bar-code sequences of all disruptants onto glass microscope slides. The arrays were then scanned with GenePix 4000B (Axon Instruments) and image analysis was performed with GenePix Pro (Axon Instruments).

Use of glucose in the buffer

Log-phase cells grown in YPAD at 30 °C to an $OD_{600} = 1.0$ were washed once with B61 buffer (0.1 M KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 2 μM $Fe_2(SO_4)_3$, adjusted to pH 6.1 with KOH), and resuspended in 2× B61 buffer to $OD_{600} = 20$. At time zero, 9 ml 40% w/v glucose or 9 ml 40% glucose with 10% v/v ethanol was added to 9 ml cell suspension in 25 ml L-shaped test tubes, which were incubated at 15 °C with gentle shaking in a Monod-type rocking shaker (Monod-mini, Taitec, Japan). The cultures were periodically sampled and glucose use was measured in the culture supernatants.

Viability of cells in ethanol

Log-phase cells ($OD_{600} = 1.0$) grown in SC medium with 2% Glc at 30 °C were washed once with water and resuspended in 10 ml SC (2% Glc) containing 10% v/v ethanol to $OD_{600} = 0.2$ and incubated at 30 °C for 24 h with gentle shaking. Viable cell counts were determined by spreading dilutions of cells on YPAD agar plates.

Zymolyase sensitivity test

Log phase cells ($OD_{600} = 1.0$) grown in YPAD at 30 °C were washed once with water, and resuspended in 0.1 M Na-phosphate buffer, pH 7.5, to $OD_{600} = 1.5$. At time zero, zymolyase 100T solution was added to a final concentration of 10 μg/ml and the decrease in turbidity was measured at 30 °C with an automatic detector (Bio-Plotter, Toyo-Sokki, Japan).

Calcofluor white sensitivity test

Log phase cells ($OD_{600} = 1.0$) grown in YPAD at 30 °C were harvested, washed once with water, and serially diluted five times by factors of 10; 5 μl of each dilution series were spotted onto YPAD plates with or without Calcofluor white (CFW) and incubated at 30 °C for 2 days.

Fatty acid analysis

Total fatty acid contents were determined by gas chromatographic analysis as described (Kainou *et al*., 2006). Fatty acid composition was calculated based on the area of each peak, and the amount was determined by comparison with the methylheptadecanoate standard.

Glucose and ethanol assays

Glucose and ethanol concentrations were determined spectrophotometrically by using the Glucose CII test WAKO kit (Wako, Japan) and ethanol assay F-kit (Roche Diagnostics) according to the manufacturers' protocols.

Results and discussion

Isolation of ethanol-tolerant mutants

There are several definitions of 'ethanol tolerance', including the effects of ethanol on yeast growth, fermentation rate and viability. Since one of the most widely used methods to define ethanol tolerance is to determine the growth rate, we isolated mutants that can grow faster than the wild-type strain in the presence of ethanol. To determine the optimal ethanol concentration, a wild-type diploid strain BY4743, grown overnight at 30 °C in rich medium supplemented with 2% glucose (YPAD), was inoculated into fresh YPAD supplemented with various concentrations of ethanol, and growth rates were compared. As the concentration of ethanol increased, the growth rate of BY4743 decreased (Figure 1). Since the growth was very poor in the presence of 10% v/v ethanol and the effect of

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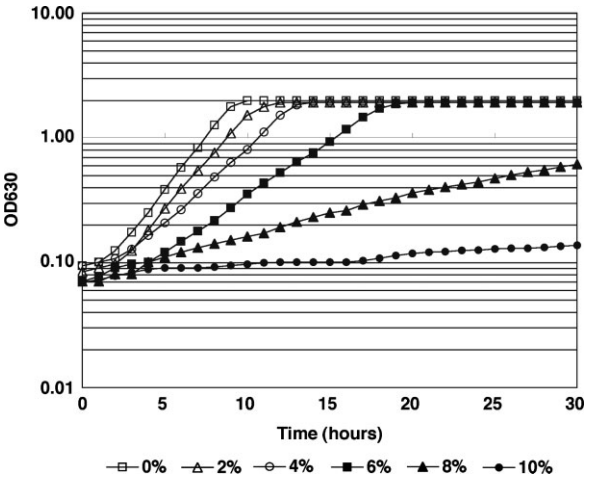

Figure 1. Growth of the wild-type strain in the presence of ethanol. The wild-type strain BY4743 was grown at 30 °C in YPAD containing various concentrations of ethanol after pre-culturing in YPAD overnight in the absence of ethanol. Turbidity was monitored using an automatic detector (Bio-Plotter, Toyo-Sokki, Japan). Open squares, open triangles, open circles, closed squares, closed triangles and closed circles indicate the growth of BY4743 in the presence of 0%, 2%, 4%, 6%, 8% and 10% ethanol, respectively

ethanol toxicity was small in 6% ethanol, we chose 8% ethanol for the mutant selection.

The frozen stock of the pools of 4741 homozygous diploid yeast deletion strains (Invitrogen, CA, USA) was diluted 40-fold into YPAD and subcultured at 30 °C until $OD_{600} = 0.5$. Then the culture was diluted 10-fold into YPAD containing 8% ethanol and the cells were subsequently grown at 30 °C until $OD_{600} = 1.0$. Similar dilution–cultivation processes were repeated three times more after 100-fold (once) and 1000-fold (twice) dilutions. Cells were harvested from the final culture and the genomic DNA was extracted. Bar-code DNA fragments corresponding to the UPTAG were amplified by PCR and the population of disruptants was examined by using DNA microarrays spotted with 20mer oligonucleotides corresponding to the UPTAG bar-code sequences on glass microscope slides. Experiments were repeated twice by using independently grown cells.

Initially, 44 candidate strains were obtained based on the duplicate experiments. The corresponding individual homozygous diploid deletion mutants were examined for their growth in YPAD containing 8% ethanol to confirm their tolerance to ethanol. Disruptants of *ura7* and *gal6* showed clear reproducible results. The remaining deletion strains displayed no or only a weak growth phenotype in liquid medium containing 8% ethanol. As a control ethanol tolerant strain, industrial sake yeast K7 was also examined for its growth in the presence of ethanol. In the absence of ethanol, the doubling times of all strains were approximately the same, although strain K7 grew slightly faster (Table 2). In the presence of ethanol, the growth of all of the strains, including K7, was drastically affected. However, the doubling times of the *ura7* and *gal6* disruptants were almost half that of the wild-type strain BY4743. Their doubling times were slower than that of K7, but the difference was small (Table 2). To examine their dose–response characteristics, we measured their growth under 7% and 9% ethanol conditions. Under all conditions, the growth of K7 was the fastest, and although the difference was small, the growth of the *ura7* disruptant was slightly faster than that of the *gal6* disruptant (Table 2).

Glucose uptake by the mutants in the presence of ethanol

Two conceivable possibilities for faster growth in ethanol are either the acquisition of higher

Table 2. Growth of mutants in YPAD in the presence of ethanol

		Doubling time (h) in YPAD			
Strain	**Relevant genotype**	**+0% Ethanol**	**+7% Ethanol**	**+8% Ethanol**	**+9% Ethanol**
BY4743	Wild-type	1.60 ± 0.01	5.84 ± 0.57	11.78 ± 1.35	16.85 ± 0.38
BY4743-*Δura7*	*Δura7::KanMX4*	1.78 ± 0.03	5.00 ± 0.06	6.63 ± 0.45	14.23 ± 0.08
BY4743-*Δgal6*	*Δgal6::KanMX4*	1.56 ± 0.07	5.66 ± 0.51	7.03 ± 0.32	16.06 ± 1.13
K7	Sake yeast	1.35 ± 0.07	3.58 ± 0.26	5.00 ± 0.72	10.71 ± 0.25

The wild-type strain BY4743, its *Δura7* and *Δgal6* derivatives and sake yeast K7 were grown in YPAD with 0%, 7%, 8% and 9% v/v ethanol at 30 °C after pre-culturing in YPAD overnight at 30 °C in the absence of ethanol.

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fermentation capacity or improved viability in the presence of ethanol. To determine whether the disruptants had a higher fermentation capacity, the glucose consumption rate was examined. Log-phase cells grown in YPAD were harvested and suspended in 0.1 M potassium phosphate buffer, pH 6.1, containing 20% w/v glucose or 20% glucose and 5% v/v ethanol, and incubated at 15 °C for 7 days. In the actual fermentation, the concentration of ethanol increases gradually, but we added an initial 5% concentration of ethanol in the buffer in order to reach a high (stressful) concentration of ethanol faster. The sudden addition of a high concentration of ethanol to log-phase cells typically causes a significant decrease in viability, but since the effect of 6% ethanol on cell growth was small (Figure 1), we expected that a 5% initial concentration of ethanol would not cause such an effect. As shown in Figure 2, the glucose consumption profile of the *gal6* mutant was the same as that of the wild-type strain in buffer containing 5% ethanol at 15 °C. In contrast, the glucose consumption profile of the *ura7* disruptant was better than that of the wild-type strain (Figure 2B). The rate was 13–18% higher throughout the incubation period (Figure 2C). It was also high in the absence of initial ethanol (6–12%) (Figure 2A), indicating a higher intrinsic fermentation capacity. Similar results were obtained in a short-term incubation. The same log-phase cells were suspended in 0.1 M potassium phosphate buffer, pH 6.1, containing 2% glucose with 0% or 10% ethanol, and the cells were incubated at 30 °C for either 2 h (with 0% ethanol) or 4 h (with 10% ethanol) to determine the linear range of glucose uptake. Consistent with the previous result, glucose consumption rates (mM/min/l) of the *ura7* disruptant were 26% and 24% higher than that of the wild-type strain with or without ethanol, respectively, whereas the values of the *gal6* disruptant were almost the same as in the wild-type strain (Table 3).

Viability in the presence of ethanol

We then determined whether the selected mutants were more viable in ethanol. The survival ratio of the *gal6* disruptant ($26.3 \pm 0.3\%$) was 3.35-fold higher than that of the wild-type strain ($7.8 \pm 0.5\%$) following 24 h exposure to 10% ethanol, whereas the survival ratio of the *ura7* disruptant ($7.3 \pm 0.7\%$) was almost the same as that of the

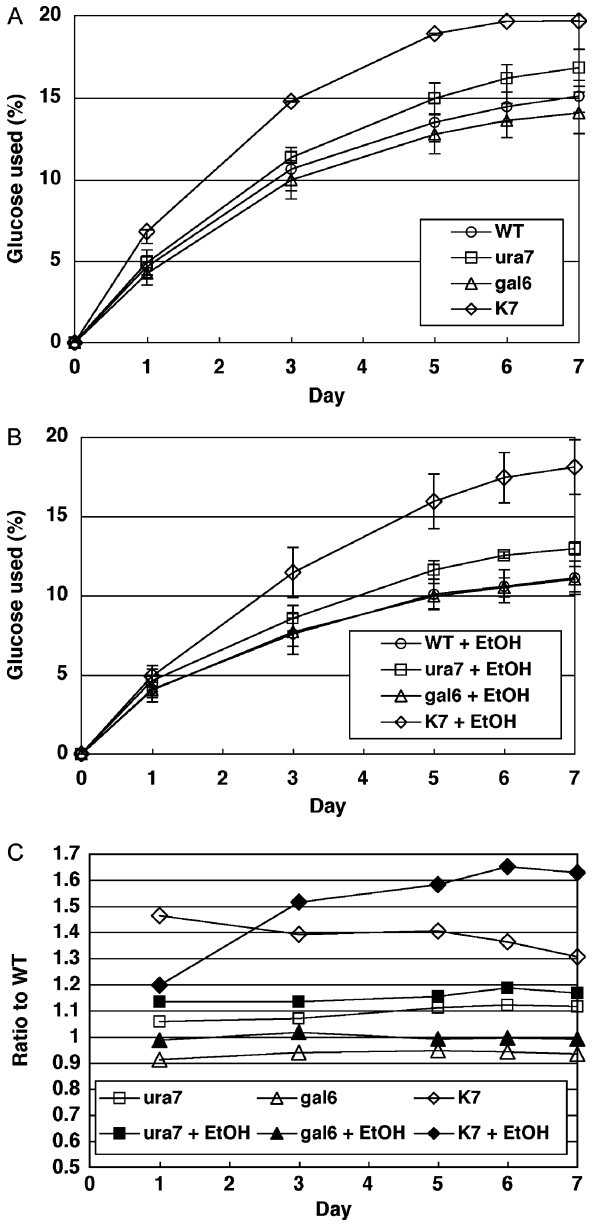

Figure 2. Use of glucose in the buffer at 15 °C. Log phase cells ($OD_{600} = 1.0$) grown in YPAD at 30 °C were washed once with B61 buffer and resuspended in 2× B61 buffer at $OD_{600} = 20$. At time zero, 9 ml 40% glucose (A) or 9 ml 40% glucose with 10% EtOH (B) was added to 9 ml cell suspension to almost fill the L-shaped test tubes, and the cells were incubated at 15 °C with gentle shaking in a Monod-type incubator (Monod-mini, Taitec, Japan). Circles, squares, triangles and diamonds indicate the wild-type, *Δura7, Δgal6* and K7 strains, respectively. (C) Relative glucose use compared to the wild-type strain. Symbols are the same as in (A, B). Open and closed symbols indicate growth in the absence (A) and presence (B) of ethanol

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Table 3. Glucose flux in the buffer

		Rate of glucose uptake (m mole/min/l)			
		Without ethanol		With 10% v/v ethanol	
Strain	**Relevant genotype**	**(m mole/min/l)**	**Ratio**	**(m mole/min/l)**	**Ratio**
BY4743	Wild-type	0.677	(1)	0.253	(1)
BY4743-*Δura7*	*Δura7::KanMX4*	0.836	1.24	0.319	1.26
BY4743-*Δgal6*	*Δgal6::KanMX4*	0.710	1.05	0.264	1.04
K7	Sake yeast	0.979	1.45	0.308	1.22

Log phase cells (OD_{600} = 1.0) grown in YPAD at 30 °C were washed once with B61 buffer, and resuspended in the same buffer at OD_{600} = 20. After 30 min pre-incubation at 30 °C with shaking, glucose was added to 2% in the presence or absence of a final concentration of 10% v/v ethanol, and the cells were incubated at 30 °C with gentle shaking. The cultures were periodically sampled and glucose use was measured by using the supernatants.

wild-type strain. This effect was not due to growth even though cells were incubated in SC (2% Glc) at 30 °C, because the mutant cells as well as the wild-type cells barely grow in the presence of 10% ethanol (see Figure 1).

Cell wall integrity of the mutants

To address the ethanol tolerance mechanism, we examined the cell wall integrity of the wild-type and mutants by measuring their sensitivity to zymolyase and Calcofluor white (CFW). Since zymolyase is mainly composed of β-1,3-glucanase and its primary target is β-1,3-glucan of the cell wall, it is useful for monitoring cell wall integrity (Shimoi *et al*., 1998). Calcofluor white (CFW), a negatively charged fluorescent dye, interacts specifically with cell wall chitin and inhibits cell wall synthesis (Lussier *et al*., 1997). Takahashi *et al*. (2001) showed that all of their ethanol sensitive mutants were more sensitive to zymolyase and CFW compared to the parental strain. In contrast, the *ura7* and *gal6* disruptants were more resistant to zymolyase (Figure 3), suggesting that stronger cell wall integrity is one of the mechanisms for their resistance to ethanol stress. The *gal6* disruptant was also more resistant to CFW but, interestingly, the *ura7* disruptant and the industrial sake yeast K7 were more sensitive to CFW (Figure 4). The CFW-sensitive phenotype of the *ura7* disruptant agreed well with the previous result of Lussier *et al*. (1997). They identified genes involved in cell assembly by screening mutants, based on their altered sensitivity to CFW. The CFW sensitivity did not always coincide with the zymolyase sensitivity. For instance, *IMP2′* (*YIL154C*) is hypersentitive to CFW but resistant to zymolyase, and contrarily *YIL146C* is resistant to CFW and hypersensitive to zymolyase (Lussier *et al*., 1997). Since the targets of zymolyase and CFW are different, these results indicated that the alterations of cell wall integrity in the *ura7* and *gal6* disruptants were different.

Fatty acid composition of the mutants

It is known that the fatty acid composition of the membrane plays an important role in ethanol tolerance. Thus, we examined the fatty acid composition of total cellular lipids in cells that were or

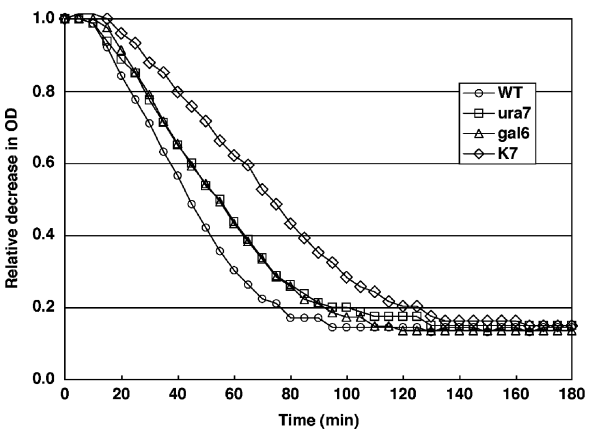

Figure 3. Zymolyase sensitivity. Log phase cells (OD_{600} = 1.0) grown in YPAD were washed once with water and exposed to zymolyase 100T (10 μg/ml) at 30 °C in 0.1 M Na-phosphate buffer, pH 7.5, at OD_{600} = 1.5. The decrease in the turbidity was monitored using an automatic detector (Bio-Plotter, Toyo-Sokki, Japan). Symbols are the same as in Figure 2

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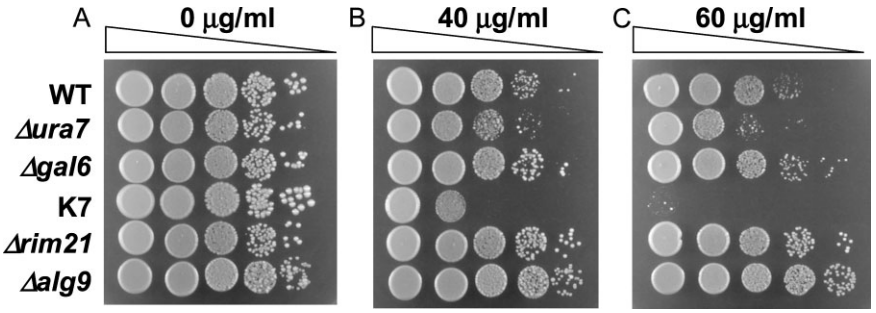

Figure 4. Calcofluor white sensitivity of the ethanol-resistant disruptants. BY4743 (wild-type) and its homozygous diploid deletion mutants were grown to $OD_{600} = 1.0$ in YPAD. Aliquots (5 μl) of serial dilutions of cell suspensions were spotted on YPAD plates containing 0, 20 and 40 μg/ml Calcofluor white (CFW). The plates were incubated at 30 °C for 2 days and then photographed. K7 is an industrial sake yeast. The *rim21* (Castrejon *et al*., 2006) and *alg9* (Lussier *et al*., 1997) disruptants were used as control CFW-resistant mutants

were not exposed to ethanol stress conditions. The unsaturated fatty acid composition of *S. cerevisiae* is relatively simple, consisting almost exclusively of the mono-unsaturated fatty acids (UFAs) palmitoleic acid (C16 : 1) and oleic acid (C18 : 1), with the former predominant. In the absence of ethanol stress, a significant difference was not observed among the strains, including the sake yeast strain K7. Exposure to ethanol resulted in an increase in the amount of C18 : 1 to about 45% in the mutants, which is between the levels of K7 (50%) and the wild-type strain BY4743 (41%). The C16 : 1 content decreased to 30% in laboratory strains, whereas it was retained at approximately 40% in K7 (Figure 5). Although the correlation between ethanol tolerance and the increased degree of unsaturated fatty acyl residues in the membrane phospholipids, which increases the membrane fluidity, is well documented (Thomas *et al*., 1978; Beavan *et al*., 1982; Mishra and Prasad, 1988; Mishra and Prasad, 1989; Jones, 1989; Guerzoni *et al*., 1997; for reviews, see Casey and Ingledew, 1986; Suutari and Laakso, 1994), a causal relationship has not yet been established. You *et al*. (2003) examined the effects of different unsaturated fatty acid compositions on the growth-inhibiting effects of ethanol by systematically altering the UFA composition in *S. cerevisiae*. These results demonstrated that oleic acid is the most efficacious UFA in overcoming the toxic effects of ethanol in growing yeast cells. Our results agreed well with their observation and indicated that the mutant acquired ethanol tolerance from the structural change in the plasma membrane as well as the structural change in the cell wall.

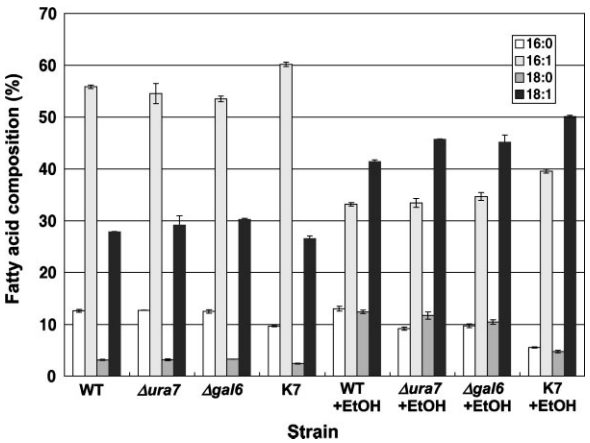

Figure 5. Fatty acid composition in yeast grown in the presence of ethanol. Cells were grown at 30 °C for 3 days in YPAD or for 6 days in YPAD containing 8% ethanol and the amount of fatty acids was examined. White bars, palmitic acid (C16 : 0); light grey bars, palmitoleic acid (C16 : 1); dark grey bars, stearic acid (C18 : 0); black bars, oleic acid (C18 : 1)

Fermentation in ethanol

Our ultimate goal is to gain new insights into the mechanism of resistance to ethanol, and to enable the construction of yeast strains with higher ethanol tolerance, because such strains would be of great value in industrial ethanol fermentation. Thus, we examined whether the *ura7* and *gal6* mutants acquired an improved fermentation capability. Cells were grown at 15 °C in 18 ml 50 mM citrate, pH 6.2, buffered SC medium containing 20% w/v glucose, with or without 5% v/v

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ethanol, starting from $OD_{600} = 0.05$ after pre-culturing overnight in SC with 2% glucose at 30 °C. In one set of samples, 5% ethanol was added in advance, as explained in the section 'Glucose uptake of the mutants in the presence of ethanol'. In the absence of added ethanol, no difference was observed in the glucose consumption between the mutants and the parental wild-type strain (Figure 6A). No difference was observed in the OD profile or the viability profile either (data not shown). Since the glucose consumption after a 20 day incubation was around 10%, the final concentration of ethanol was not thought to be high enough to cause stress. However, in the presence of a 5% initial concentration of ethanol, the fermentation rate of the *gal6* and *ura7* disruptants was higher than that of the wild-type strain (Figure 6B). Glucose consumption in the *gal6* disruptant started to increase on day 8 and the final glucose consumption on day 26 was 6.53%, which was 40% higher than that of the wild-type strain. The effect was smaller in the *ura7* mutant, where the final glucose consumption was about 14% higher than that of the wild-type strain. In accordance with the increased glucose consumption, the ethanol production also increased 1.82-fold and 1.65-fold in the *gal6* and *ura7* mutants, respectively.

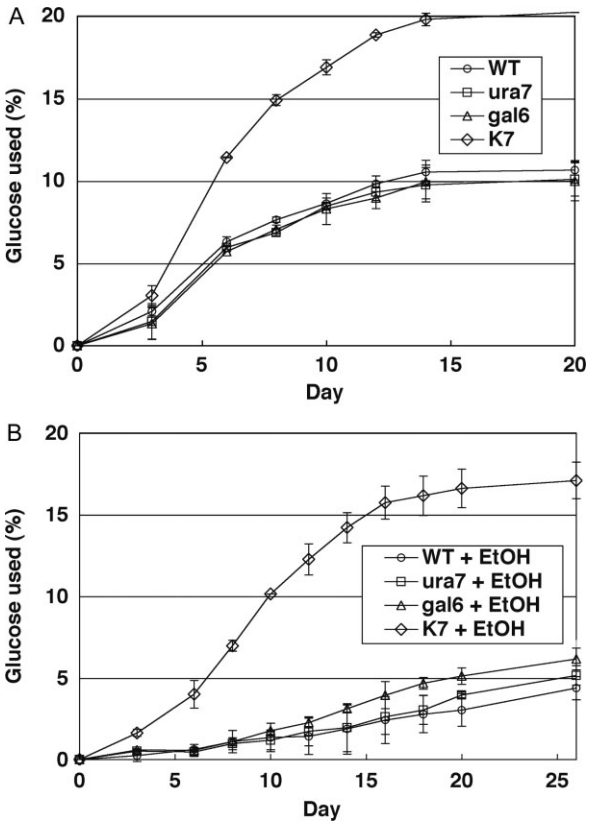

Figure 6. Glucose consumption, growth and viability of yeast in the media. Cells were grown in 18 ml SC medium containing 20% glucose and 50 mM citrate buffer, pH 6.2, in the presence or absence of 5% ethanol at 15 °C, starting from $OD_{600} = 0.05$ after pre-culturing in SC (2% Glc) overnight at 30 °C. The cells were incubated as described in the legend to Figure 2. (A, B) Glucose consumption of the cells grown in the absence (A) and presence (B) of 5% ethanol. Symbols are the same as in Figure 2

Potential role of the identified genes in ethanol stress

We isolated both mutants as strains that showed faster growth in the presence of ethanol. From the analysis of cell wall integrity and fatty acid composition, both strains had characteristics more similar to the ethanol tolerant sake yeast strain K7 than to the parental wild-type strain BY4743. However, the glucose consumption rate showed that only the *ura7* disruptant possessed a higher fermentation capacity (Figure 2). In contrast, only the *gal6* disruptant was more viable in the presence of ethanol. The overall fermentation was improved in both strains when the cells were grown at 15 °C, which is the common temperature for sake making (Figure 6).

URA7 encodes CTP synthetase, the enzyme that catalyses the ATP-dependent conversion of UTP to CTP (Ozier-Kalogeropoulos *et al*., 1994). This final step in the pathway of CTP biosynthesis is important not only for balancing nucleotide pools but also for synthesizing membrane phospholipids (Ostrander *et al*., 1998). Membrane lipid composition is very important for the tolerance to various kinds of stress including ethanol. Since repression of *URA7* has been observed during wine fermentation (Rossignol *et al*., 2003) and in short-term ethanol stress (Alexandre *et al*., 2001), the repression of *URA7*, and presumably the subsequent alteration of the phospholipid composition in the membrane, might be the mechanism for ethanol tolerance in this strain.

GAL6 encodes an aminopeptidase in the cysteine protease family, and is involved in the biological process of proteolysis and drug metabolism. Interestingly, it is also known to have a negative regulatory effect on *GAL* gene expression by

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binding to the upstream activation sequences of galactose-inducible gene promoters (Xu and Johnston, 1994; Zheng *et al*., 1997). A null mutation of *GAL6* caused an increase in respiro-fermentative metabolism, and the consumption of galactose increased 24% when the cells were grown in galactose (Ostergaard *et al*., 2000). Examination of the array data of a *gal6* null mutant grown in raffinose (Ideker *et al*., 2001) indicated that *HSP12* (43-fold) and *HSP26* (33-fold) are the two most upregulated genes in the *gal6* disruptant.

HSP12 encodes a plasma membrane-localized protein that protects membranes from desiccation and ethanol-induced stress (Sales *et al*., 2000). *HSP26* encodes a small heat shock protein with chaperone activity (Haslbeck *et al*., 1999). Since *HSP12* and *HSP26* are known to be involved in ethanol resistance of the strain and their expression is upregulated by ethanol stress (Aranda *et al*., 2002; Alexandre *et al*., 2001), it is quite likely that the ethanol-tolerant phenotype of the strain can be attributed to the upregulation of *HSP12* and *HSP26* in the *gal6* null mutant.

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