RESEARCH ARTICLE

Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in *Saccharomyces cerevisiae*

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Abstract

We quantified the growth behavior of all available single gene deletion strains of budding yeast under ethanol stress. Genome-wide analyses enabled the extraction of the genes and determination of the functional categories required for growth under this condition. Statistical analyses revealed that the growth of 446 deletion strains under stress induced by 8% ethanol was defective. We classified these deleted genes into known functional categories, and found that many were important for growth under ethanol stress including several categories that have not been characterized, such as peroxisome. We also performed genome-wide screening under osmotic stress and identified 329 osmotic-sensitive strains. We excluded these strains from the 446 ethanol-sensitive strains to extract the genes whose deletion caused sensitivity to ethanol-specific (359 genes), osmotic-specific (242 genes), and both stresses (87 genes). We also extracted the functional categories that are specifically important for growth under ethanol stress. The genes and functional categories identified in the analysis might provide clues to improving ethanol stress tolerance among yeast cells.

Introduction

The yeast *Saccharomyces cerevisiae* is used in the production of alcoholic beverages such as beer and wine, and its importance in the production of biofuels from biomass resources has recently increased. During bio-production processes, yeast cells are generally exposed to several types of environmental stresses that decrease their productivity, such as high ethanol concentrations, osmotic pressure, and oxidative stresses (Attfield, 1997; Gibson *et al.*, 2007). Ethanol is a major stress factor that interferes with the growth and production of ethanol by yeast cells in the production process of alcoholic beverages and biofuels. Hence, the responses of yeast cells to ethanol have been investigated in detail in order to improve their tolerance to ethanol stress. For example, yeast cells respond to ethanol stress by altering the unsaturated fatty acid composition of the cellular membrane (You *et al.*, 2003), accumulating trehalose (Lucero *et al.*, 2000), selectively exporting mRNA from the nucleus (Takemura *et al.*, 2004), and forming P-bodies (Izawa *et al.*, 2007).

Studies of the genome-wide response to ethanol stress using microarrays and comprehensive expression data have resulted in the construction of some ethanol-tolerant yeast strains (Hirasawa *et al.*, 2007). However, the construction of deletant or overexpression strains from those genes that are detected in transcriptional studies to alter their expression patterns in response to ethanol stress does not always lead to a change in the ethanol stress response. Thus, the effect of gene manipulation – such as deletion and overexpression – on the acquisition of ethanol tolerance in yeast cells remains obscure. Therefore, analysis of phenotypic changes such as sensitivity and tolerance to ethanol stress is important for improving the ethanol tolerance of yeast cells by genetic modifications.

Here, we report a high-resolution quantitative analysis of growth behavior under ethanol stress in a collection of yeast strains with a single gene deletion (Winzeler *et al.*, 1999) to elucidate the functional categories of genes involved in survival under ethanol stress. This collection of strains is a powerful tool with which to determine the effects of gene deletion under specific conditions (Scherens & Goffeau, 2004). We cultured deletion strains individually with and without ethanol, and determined their growth curves to evaluate the effect of gene deletion. Other studies have

screened genes that are important for growth under ethanol stress from collections of deletion strains (Kubota *et al*., 2004; Fujita *et al*., 2006; van Voorst *et al*., 2006); however, ethanol-sensitive strains were screened in these studies by colony formation on ethanol-supplemented agar, and their growth behavior was not quantified. In contrast, we quantified the specific growth rates of each deletion strain in a liquid medium with and without ethanol and statistically analyzed the effect of the gene deletion. In order to focus on genes that are involved in growth under stress, we excluded data for deletion strains with growth defects under nonstress conditions from further analysis. We also measured the growth of each strain under osmotic stress induced by adding NaCl to the medium. By comparing growth both under ethanol and under osmotic stresses, we identified which genes are generally important for the stress response and which are specifically important for either ethanol or osmotic stress. We screened novel genes and gene categories involved in growth under ethanol stress using high-throughput cultivation and statistical analysis.

Materials and methods

Medium and yeast strains

We evaluated the growth of single gene deletion strains derived from *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) or BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) purchased from Open Biosystems. All strains were cultured in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose).

We prepared culture stocks by culturing deletion strains on plates containing YPD agar (YPD medium containing 2% agar) containing 150 μg mL^{-1} of geneticin. Colonies generated from each strain were suspended in 15% glycerol in 96-well microtiter plates and stored at −80 °C. Some deletion strains that proliferated slowly on the YPD plates with geneticin were removed; 4729 single gene deletion strains were eventually analyzed in this study.

Cultivation system of deletion strains in a microtiter plate

We prepared precultures by shaking deletion strains at 1050 strokes min^{-1} on a microplate shaker, TITRAMAX1000 (Heidolph Instruments, Germany), in 100 μL of YPD medium in 96-well microtiter plates (Corning Inc.) for 24 h at 30 °C. Thereafter, 2 μL of preculture was inoculated in 100 μL of warm YPD medium, and cells were cultured at 30 °C without shaking. To investigate the growth behavior of deletion strains under stress, 25 μL of 40% (v/v) ethanol (final concentration, 8%) or 25 μL of 5 M NaCl (final concentration, 1 M NaCl) was added to the culture medium 4 h later, at the time corresponding to the mid-log phase. Sterilized water (25 μL) was added to the culture medium to provide the nonstress condition. Growth of the deletion strains was monitored at 0, 2, 4, 5.5, 7, and 9 h under the nonstress condition and at 0, 2, 4, 7, 9, and 11 h under the stress condition by measuring the $OD_{600\,nm}$ of each well using the microplate reader 1420 ARVO (PerkinElmer Inc.). Before measuring the OD, the plates were agitated for 1 min at 1050 strokes min^{-1} on a shaker. We used the *HIS3* deletion strain of BY4742 (BY4742Δ*his3*) as a standard because the *HIS3* deletion did not alter the phenotype from the wild-type strain BY4742. The standard strain was cultured together with the deletion strains (4 wells per plate). The standard strain was located at identical positions in all plates. A 96-well microtiter plate comprises eight rows and 12 columns. The standard strain was located at (3, 4), (3, 9), (6, 4), and (6, 9), where the values in parentheses represent the position (row, column) in a 96-well plate. We did not use the outermost wells of the 96-well plate, which are the first and eighth rows and the first and 12th columns in order to avoid the effect of evaporation of the medium. All strains were separately cultured twice under nonstress and stress conditions.

Calculation of specific growth rate

Raw OD data obtained by the microplate reader were processed as follows: the OD of blank wells containing only medium was subtracted from the raw OD data. Then, to compensate for the nonlinearity of the OD value in case of high cell density, ODs of several samples were measured both by the microplate reader and by the spectrophotometer, UVmini-1240 (Shimadzu Corporation, Japan), at 660 nm. Subsequently, we introduced the following relationships for the compensation of raw OD data of the microplate reader. We used different relationships before and after changing the environment because the culture volume was changed by the addition of ethanol, NaCl, or water. For data before the addition of ethanol, NaCl, or water, we used:

$$OD' = 18.706 \times OD^3 - 13.941 \times OD^2 + 21.289 \times OD,$$

while for data after the addition of ethanol, NaCl, or water, we used:

$$OD' = 15.140 \times OD^3 - 10.744 \times OD^2 + 19.586 \times OD$$

where OD is the value obtained using the microplate reader and OD′ is the calibrated OD. The specific growth rate under the nonstress condition was calculated by linear regression using the natural logarithm of calibrated OD values at 5.5, 7, and 9 h, and that under the stress conditions was calculated using the natural logarithm of calibrated OD values at 7, 9, and 11 h. The robustness of the calculation of the specific growth rate using only three time points was confirmed by a good correlation between the specific growth

rates obtained by three time points (7, 9, and 11 h under 8% ethanol) and a greater number of time points (7, 8, 9, 10, and 11 h under 8% ethanol) (data not shown). This indicated that the calculation of the growth rate using three time points was sufficiently accurate; therefore, we used three time points for calculating the specific growth rate.

To compare the specific growth rates of deletion strains cultured in different microtiter plates, specific growth rates were normalized with those of the standard strain cultured in each plate using the formula

$$\mu'_{i,j,k} = \mu_{i,j,k}\left(\frac{\mu_{std,k}}{\bar{\mu}_{std,j,k}}\right)$$

where $\mu_{i,j,k}$ is the specific growth rate of strain i in plate j under condition k, $\bar{\mu}_{std,j,k}$is the average of the specific growth rate of the standard strain from four wells in plate j under condition k, $\mu_{std,k}$ is the median of all specific growth rates of the standard strain under condition k, and $\mu'_{i,j,k}$ is the normalized specific growth rate. The normalized specific growth rates of all deletion strains under each condition are shown in Supporting Information, Table S1.

Functional analysis of gene products

Functions of gene products were classified using the Functional Catalogue (FunCat) described in the Munich Information Center for Protein Sequences (MIPS) database (http://mips.gsf.de/genre/proj/yeast/, Ruepp *et al.*, 2004) and the *Saccharomyces* genome database (SGD, http://www.yeastgenome.org/). We applied a hypergeometric test to evaluate whether or not a functional category was overrepresented by genes deleted from strains exhibiting stress sensitivity by the following formula:

$$P = \sum_{i=k}^{n} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}},$$

where N is the total number of genes, M is the number of genes related to a functional category from the total genes, n is the number of genes in the selected genes, and k is the number of genes related to a functional category from among the selected genes.

Randomization test

Ten thousand sets of randomized data were obtained by random sampling of the same number of the genes of ethanol-sensitive strains from the genes of all the deletion strains. Then the distributions of the proportion of each category in the randomized data sets were calculated and *P*-values of the observed data were computed by the two-sided hypothesis test with a normal distribution assumption.

Construction of single gene overexpression strains

For the construction of single gene overexpression strains *LDB19*, *MEH1*, *PRO2*, and *YNL335W*, the DNA fragment corresponding to each gene was amplified by a PCR from the BY4742Δ*his3* genomic DNA using KOD plus DNA polymerase (Toyobo Co. Ltd, Japan) and the following sets of primers: 5′-TCACGGTACCATACGTTTTACCATG-3 and 5-AAATCTCGAGTAAATACCTTTAACG-3 for *LDB19*, 5-TAGTGGGTACCACGACAGATTTAAG-3 and 5-AGCTTCTAGACCAATGATGTTATAC-3 for *MEH1*, 5-GAGTAGGTACCAAAAGGAGCACAGG-3 and 5-ACGTCCTCTAGACATGGAACTTAGC-3 for *PRO2*, and 5-CTCCGGTACCAAAGAATCAATCATG-3 and 5-TGTTCATTCTAGACTCGCCTCATTG-3 for *YNL335W* (the underlined sequence represents the artificially introduced restriction enzyme sites for cloning into the expression vector). Each amplified PCR product was cloned into the appropriate restriction sites in the YIp-type vector pAURΔCENARS (Hirasawa *et al.*, 2007). The sequence of each cloned gene was confirmed using the automated DNA sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems). The plasmids carrying each gene were introduced into the BY4742Δ*his3* by the lithium acetate method (Gietz & Woods, 2002). In the constructed strains, the cloned genes were constitutively expressed from the *ADH1* promoter on pAURΔCENARS.

Results and discussion

Identification of strains sensitive or tolerant to ethanol stress

To analyze the effects of single gene deletion on growth under ethanol and osmotic stress, we obtained growth data for all available single gene deletion strains of *S. cerevisiae* under nonstress and stress conditions. Stress conditions were created by the addition of ethanol (final concentration, 8%) or NaCl (final concentration, 1 M) to culture broth at the mid-log phase. We calculated the specific growth rates of strains under all culture conditions. Figure 1 shows that the measurement reproducibility of the specific growth rates of deletion strains was high.

We simultaneously obtained 720 sets of information regarding the specific growth rates of the standard strain from the cultivation of all the deletion strains in 90 microtiter plates twice under each condition. In each plate, the standard strain was contained in four wells. Table 1 shows the average and SD of the specific growth rates of 720 growth data for the standard strain under each condition. The coefficient of variance of the growth rate of the standard strain under the nonstress condition was 1%, indicating that

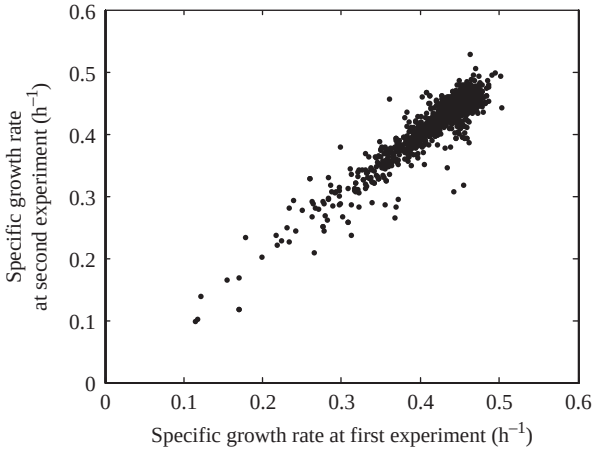

Fig. 1. Reproducibility of culture experiments of all deletion strains under nonstress conditions. Correlation between specific growth rates of all deletion strains at first and second culture experiments. Horizontal and vertical axes represent specific growth rates under the nonstress condition in the first and second experiments, respectively. Each dot corresponds to a strain with a single gene deletion.

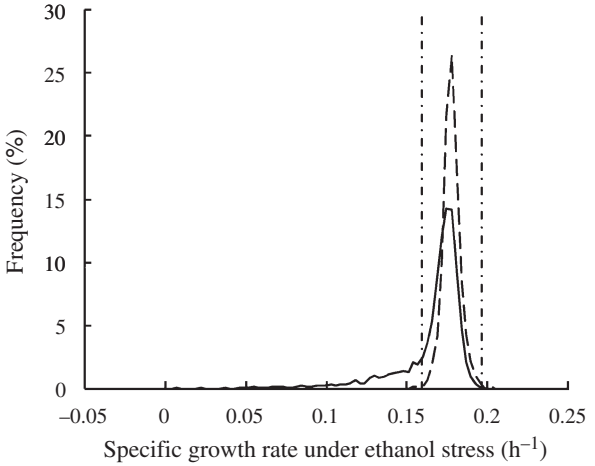

Fig. 2. Identification of strains sensitive or tolerant to ethanol stress. The dashed line represents the frequency of specific growth rates of standard strain under ethanol stress; the solid line represents the frequency of all average specific growth rates of deletion strains under ethanol stress. Dashed–dotted lines represent thresholds of specific growth rates for judging ethanol-sensitive and -tolerant strains.

Table 1. Comparison of average values of specific growth rates of the standard strain and thresholds of specific growth rates to discern sensitive and tolerant deletion strains

Condition	Average specific growth rate (h^{-1})	SD (h^{-1})	Threshold for sensitive strains (h^{-1})*	Threshold for tolerant strains (h^{-1})*
Nonstress	0.452	4.4×10^{-3}	0.437	0.466
8% Ethanol	0.178	5.6×10^{-3}	0.160	0.196
1 M NaCl	0.203	7.6×10^{-3}	0.178	0.229

*Thresholds were defined as average specific growth rate ± 3.3 SDs.

Table 2. Numbers of deletion strains categorized by specific growth rates

	Sensitive	No change	Tolerant
Nonstress	591 (0)	4095 (4095)	43 (43)
8% Ethanol	864 (446)	3862 (3690)	3 (2)
1 M NaCl	637 (329)	4080 (3799)	12 (10)

Numbers in parentheses represent the number of deletion strains in each category not including those exhibiting growth defects under nonstress conditions.

the specific growth rates of deletion strains could be evaluated in our culture system at a high resolution.

From all the deletion strains available in the library, in order to identify those deletion strains that were tolerant or sensitive to stress, we statistically compared the specific growth rates of the deletion and standard strains. Because the distribution of the growth rates of the standard strain described above was bell shaped (Fig. 2), we applied the empirical rule that 99.9% of the data lay within 3.3 SD of the mean. Thus, deletion strains with growth rates outside the range of 3.3 SD of the mean could be regarded as stress tolerant or sensitive, with significantly different growth rates from the standard strain ($P < 0.001$) (Fig. 2). We defined stress-sensitive deletion strains as those whose growth rates in both independent cultures were lower than the threshold value. Although the average growth rate of two cultures is generally used for such analyses, we used a conservative definition because the average growth rate is often unreliable when the data include outliers. Table 1 shows the threshold values of specific growth rates used to identify sensitive and tolerant strains under each condition.

Table 2 shows the numbers of stress-sensitive and -tolerant deletion strains under each condition. Some strains identified as stress sensitive also exhibited a significantly lower growth rate under the nonstress condition than that of the standard strain, as also reported by Warringer *et al.* (2003).

Comparison of data with those from other studies

We compared our data with the findings of others who have similarly examined the effect of a single gene deletion on growth under ethanol stress (Kubota *et al.*, 2004; Fujita *et al.*, 2006; van Voorst *et al.*, 2006). Table 3 shows that our data overlapped with *c.* 70% of that from the ethanol-sensitive strains identified by others. The ethanol-sensitive strains

Table 3. Overlapping of published and present data regarding ethanol-sensitive strains

	No. of ethanol-sensitive strains	Kubota *et al*. (2004)	van Voorst *et al*. (2006)	Fujita *et al*. (2006)	Present study
Kubota *et al*. (2004)	256 (114)		21 (5)	61 (27)	183 (63)
van Voorst *et al*. (2006)	46 (20)			11 (5)	38 (14)
Fujita *et al*. (2006)	137 (56)				102 (33)
Present study	864 (446)				

Numbers in parentheses represent the number of deletion strains not including those with growth defects under the non-stress condition identified in the present study.

identified in these studies included those with defective growth under the nonstress condition identified in the present study, as represented in parentheses in Table 3. Because in the present study we quantified the growth of individual strains, we could exclude deletion strains with defective growth under the nonstress condition and thus define the effect of a single gene deletion on the growth under stress more precisely. Thus, we excluded data from such strains from the following analyses to specifically determine which genes are required for growth under ethanol stress (Table 2). In the study reported by Warringer *et al*. (2003), the authors calculated the phenotype index by dividing the growth rate under the stress condition by that under the nonstress condition in order to take into consideration the growth defects due to the deletion. However, in this method, when a deletion strain exhibits severe growth defects in both the nonstress and the stress conditions, the calculated phenotype index often becomes large enough to be classified as a stress-tolerant strain. To exclude such cases, we removed the strains exhibiting growth defects in the nonstress condition from the analysis and used the specific growth rate itself for analysis.

Among the other three studies, only four deletion strains of *GIM4*, *GIM5*, *SMI1*, and *VPS36* were commonly observed to be sensitive to ethanol; these strains were also identified to be ethanol sensitive in the present study. Such a low overlap of the ethanol-sensitive strains among these studies could be due to the difference in the medium (liquid or solid) and the ethanol concentrations used. The deletion strains of *GIM4* and *GIM5* encoding the subunits of the heterohexameric cochaperone prefolding complex also exhibited growth defects under the nonstress condition in the present study; therefore, we consider that these genes are required not only for ethanol tolerance but also for normal growth. *VPS36* encodes one of the components of the Endosomal Sorting Complex Required for Transport (ESCRT)-II. Notably, all the deletion strains of the genes encoding the components of ESCRT complexes – *MVB12*, *SRN2*, *STP22*, and *VPS28* for ESCRT-I; *SNF8* and *VPS25* for ESCRT-II; and *DID4*, *SNF7*, and *VPS24* for ESCRT-III – also exhibited sensitivity to ethanol. The ESCRT complex is involved in the mechanism of protein sorting to multivesicular bodies (Bowers & Stevens, 2005); thus, protein sorting is an important process for growth under ethanol. Smi1p is responsible for coordinating cell cycle progression with cell wall integrity. The ethanol sensitivity of this gene deletion was consistent with a previous study that reported the importance of the regulator of the cell cycle progression under ethanol (Kubota *et al*., 2004).

Functional categorization of the genes whose deletions resulted in ethanol sensitivity

We classified the genes whose deletions specifically resulted in ethanol sensitivity into functional categories using MIPS (Ruepp *et al*., 2004). Table 4 lists the functional categories of over-represented genes deleted from strains that were sensitive to 8% ethanol ($P < 0.01$). Functional categories containing a large number of genes related to growth under ethanol stress might have important cellular functions for ethanol stress sensitivity and tolerance.

The deletion strains of the genes classified into the category 'metabolism of tryptophan' – *TRP1*, *TRP2*, *TRP3*, *TRP4*, *TRP5*, *ARO1*, *ARO2*, and *ARO7* – were sensitive to 8% ethanol. We have previously described the ethanol sensitivity of *TRP1-5* deletion strains and the ethanol tolerance of strains overexpressing *TRP1-3* and *TRP5* (Hirasawa *et al*., 2007). Here, we found that the strains with deleted *ARO1*, *ARO2*, and *ARO7* genes were ethanol sensitive. Both *ARO1* and *ARO2* are required for the biosynthesis of chorismate, a precursor of aromatic amino acids, and the deletion of either of them causes tryptophan, tyrosine, and phenylalanine auxotrophy (Lucchini *et al*., 1978). Figure 3 summarizes the pathway of aromatic amino acid biosynthesis and the sensitivity of these deletion strains to stress. The ethanol sensitivity of the *ARO1* and *ARO2* deletion strains might be due to the absence of tryptophan biosynthesis. *ARO7* encodes chorismate mutase, which converts chorismate to prephenate – a precursor of tyrosine and phenylalanine. The *ARO7* mutant was a tyrosine and phenylalanine auxotroph (Ball *et al*., 1986), indicating that the simultaneous loss of tyrosine and phenylalanine biosynthesis is related to the ethanol sensitivity of the strain with *ARO7* deletion.

We found that the deletion mutants of the genes overrepresented in the functional categories of 'vesicular

Table 4. Representative functional categories of over-represented genes whose deletion strains were sensitive to 8% ethanol

MIPS functional category number	MIPS functional category	*P*-value	No. of genes among selected genes (446)	No. of genes among all genes (4138)
01.01.09.06	Metabolism of tryptophan	1.01E – 04	8	16
01.01.09.06.01	Biosynthesis of tryptophan	2.49E – 04	5	7
01.05.04	Regulation of C-compound and carbohydrate utilization	2.99E – 03	18	84
01.20.15.03	Biosynthesis of ubiquinone	4.58E – 03	3	4
02.11	Electron transport and membrane-associated energy conservation	2.04E – 03	11	39
02.13.03	Aerobic respiration	1.07E – 11	26	56
11.04.03.01	Splicing	2.52E – 03	9	29
12.01.01	Ribosomal proteins	5.70E – 11	36	105
12.07	Translational control	2.92E – 03	10	35
12.10	Aminoacyl-tRNA-synthetases	2.14E – 03	6	14
14.04	Protein targeting, sorting, and translocation	8.66E – 08	39	151
14.10	Assembly of protein complexes	2.07E – 08	31	100
16.07	Structural protein	3.57E – 03	7	20
20.01.21	RNA transport	6.56E – 03	9	33
20.09.01	Nuclear transport	4.17E – 04	10	28
20.09.07	Vesicular transport (Golgi network, etc.)	3.82E – 03	21	106
20.09.07.03	ER to Golgi transport	3.26E – 03	9	30
20.09.10	Peroxisomal transport	6.64E – 03	6	17
20.09.13	Vacuolar transport	5.71E – 06	28	108
30.01.05.05.01	Small GTPase-mediated signal transduction	2.55E – 03	11	40
42.10.05	Nuclear membrane	6.54E – 03	4	8
42.16	Mitochondrion	7.99E – 10	36	114
43.01.03.05	Budding, cell polarity, and filament formation	8.68E – 03	33	202

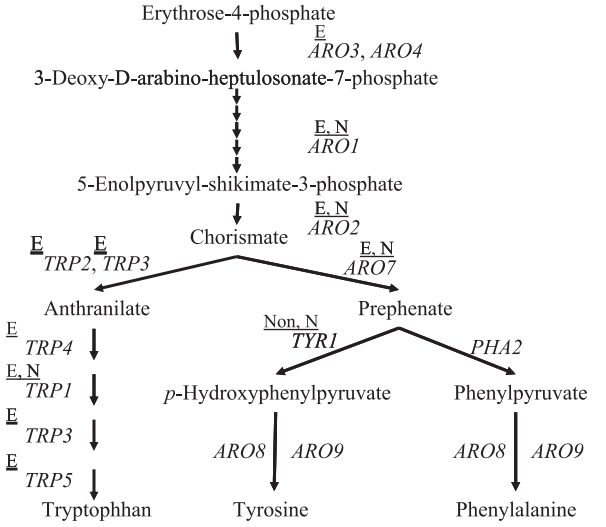

Fig. 3. Aromatic amino acids biosynthesis pathway. This figure is based on 'superpathway of phenylalanine, tyrosine and tryptophan biosynthesis pathway' described in SGD; non, E, and N, on the gene represent the sensitivity of its deletion strain under nonstress, 8% ethanol, and 1 M NaCl stresses, respectively.

transport (Golgi network, etc.)' and 'vacuolar transport' including *VPS* genes (*VPS4*, *VPS24*, *VPS25*, *VPS28*, *VPS30*, *VPS35*, *VPS36*, *VPS38*, *VPS54*, *VPS68*, and *VPS74*) were sensitive to 8% ethanol. The importance of this function with respect to ethanol stress has been described above, and this result was consistent with previous studies (Kubota *et al.*, 2004; van Voorst *et al.*, 2006).

Functional categories of 'aerobic respiration' and 'mitochondrion' showed a very low *P*-value. Genes contained in these categories were involved in mitochondrial functions such as ubiquinone (coenzyme Q) biosynthesis (*COQ5*, *COQ9*, and *COQ10*), cytochrome *c* oxidase (*COX7*, *COX9*, *COX11*, *COX12*, *COX14*, *COX16*, *COX18*, and *COX23*), and mitochondrial ribosomal protein (*MRP1*, *MRP49*, *MRPL6*, *MRPL7*, *MRPL13*, *MRPL20*, *MRPL22*, *MRPL25*, *MRPL27*, *MRPL32*, *MRPL33*, *MRPL37*, *MRPL38*, *MRPL40*, *MRPL49*, *MRPS5*, *MRPS8*, *MRPS17*, and *MRPS28*). Some relationships between mitochondrial function and ethanol tolerance were reported, for example, ethanol sensitivity of the respiratory-deficient strains (Aguilera & Benítez, 1985) and dependence on the mitochondrial genome (Jiménez & Benítez, 1988). Thus, the deletion of these genes caused respiratory deficiency and resulted in the ethanol sensitivity of these deletion strains.

Ethanol sensitivity of strains with a *PEX* gene deletion

We found that the genes in deletion strains exhibiting sensitivity to 8% ethanol were over-represented in the functional category 'peroxisomal transport,' in which strains

with deletions of the *PEX8*, *PEX14*, *PEX15*, *PEX17*, *PEX19*, and *PEX22* genes were scored as ethanol sensitive. Interestingly, the proteins encoded by these genes are involved in peroxisome transport or in the peroxisomal membrane-protein import machinery (Brown & Baker, 2003); further, deletion strains of the genes (*PEX1*, *PEX4*, *PEX10*, and *PEX12*) that are not included in the 'peroxisomal transport' category but whose products are involved in the peroxisome transport machinery were also sensitive to 8% ethanol. The gene names in each category are summarized in Table 5. In contrast, all deletion strains of the *PEX* genes whose products are involved in peroxisome targeting signaling (PTS2) and regulation of peroxisome size and numbers were not sensitive to 8% ethanol (Table 5).

According to the SGD and previous studies (Erdmann *et al.*, 1989; Brocard *et al.*, 1997; Huhse *et al.*, 1998; Koller *et al.*, 1999; Hettema *et al.*, 2000; Rehling *et al.*, 2000; Albertini *et al.*, 2001; Birschmann *et al.*, 2005), the categorized phenotype of all ethanol-sensitive strains with *PEX* gene deletions is a 'lack of morphologically recognizable peroxisomes.' Thus, the disappearance of peroxisomes themselves might cause the ethanol sensitivity of these strains. Furthermore, our results showed that all the strains with deletions of genes encoding peroxisomal matrix enzymes, including those for lysine biosynthesis and the β-oxidation of fatty acids (Breitling *et al.*, 2002), were not sensitive to 8% ethanol (Table 5). The finding that the growth properties of the deletion strains of the enzymes that are functionally associated with peroxisomes and those of *PEX* deletion strains did not correspond also supports the notion that the absence of peroxisomes results in ethanol sensitivity. This suggested that peroxisomes serve an unknown function(s) associated with growth under ethanol stress.

Deletion strains severely sensitive to ethanol

Deletion strains of *LDB19*, *MEH1*, *PRO2*, and *YNL335W* exhibited the severest sensitivity to 8% ethanol as shown in Fig. 4. The *LDB19* deletion strain exhibited a shortened telomere length and reduced affinity for the alcian blue dye (Askree *et al.*, 2004; Corbacho *et al.*, 2005). However, the relationship between these functions and ethanol stress is not clear. With respect to *MEH1*, it was reported that the deletion of this gene results in a defect in vacuolar acidification (Gao *et al.*, 2005). It is known that ethanol stress induces intracellular acidification (Rosa & Sá-Correia, 1996) and that the transportation of intracellular H^+ to the vacuole by H^+ V-ATPase is important for recovery from intracellular acidification (Fujita *et al.*, 2006). Thus, the ethanol sensitivity of the *MEH1* deletion strain might be due to a defect in the H^+ transportation to the vacuole. *PRO2* is involved in proline biosynthesis. It has been reported that strains accumulating proline exhibited ethanol tolerance (Takagi *et al.*, 2005). In our data, the deletion strain of *PRO1*, which encodes for another component of proline biosynthesis, was also ethanol sensitive. These results suggest that proline is important for ethanol tolerance and that the ethanol sensitivity of the *PRO2* deletion strain might be associated with the deficiency in proline biosynthesis. *YNL335W* encodes only a hypothetical protein; however, our results suggested a function for the product of this gene and its relationship with ethanol tolerance. To further investigate the importance of these genes with respect to ethanol stress tolerance, we constructed the overexpression stains in which each of these genes was constitutively expressed. In this construction, the standard strain BY4742-Δ*his3* was used as the host strain for overexpression. However, the growth rates of these overexpression strains in the ethanol stress condition were identical to those of the standard strain. The results indicated that although these genes are important for growth under the ethanol stress condition, the amount of their products is not important for improving ethanol stress tolerance.

Relationship between gene expression and genes of ethanol-sensitive deletion strains

Previously, we have performed genome-wide expression analysis under ethanol stress in yeast (Hirasawa *et al.*, 2007). Here, to investigate the relationship between the change in the expression levels before and after addition of the stress and the effect of gene deletion on the ethanol stress

Table 5. List of genes associated with peroxisomes

Category	Gene
Peroxisome transport machinery	*PEX1*, *PEX2*, *PEX4*, *PEX6**, *PEX8*, *PEX10*, *PEX12*, *PEX13*, *PEX14*, *PEX15*, *PEX17*, *PEX22*
Peroxisomal membrane protein import machinery	*PEX3*, *PEX19*
Peroxisome targeting signaling (PTS2) receptor	*PEX7*, *PEX18*, *PEX21*
Regulation of peroxisome size and numbers	*PEX11*, *PEX25*, *PEX27*, *PEX28*, *PEX29*, *PEX30*, *PEX31*, *PEX32**
β-Oxidation of fatty acids	*CAT2*, *CTA1*, *DCI1*, *ECI1*, *FOX2*, *IDP3*, *MDH3*, *POT1*, *POX1*, *PXA1*, *PXA2*, *SPS19*
Lysine biosynthesis	*LYS1*, *LYS2*, *LYS4*, *LYS5*, *LYS9*, *LYS12*, *LYS14*

Underlined genes represent the genes whose deletions resulted in sensitivity to 8% ethanol; the asterisk indicates genes whose deletions resulted in growth defects under nonstress conditions.

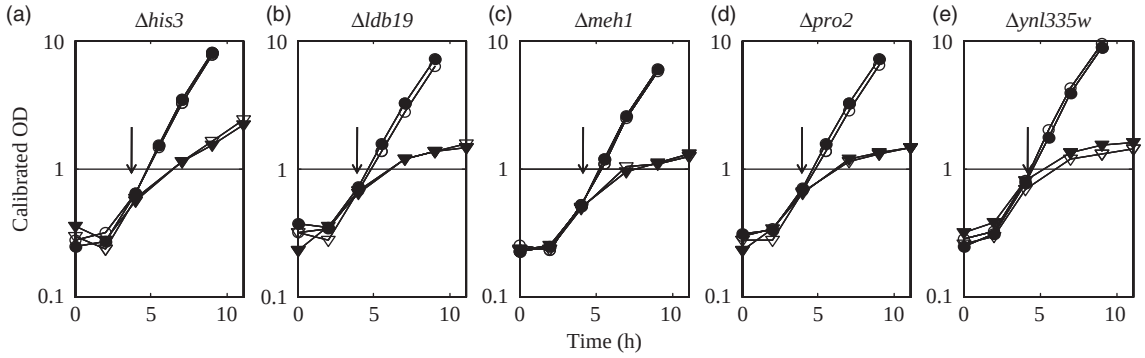

Fig. 4. Growth of ethanol-sensitive strains. BY4742Δ*his3* (a), which was used as the standard strain, and the severest ethanol-sensitive strains, *LDB19* (b), *MEH1* (c), *PRO2* (d), and *YNL335W* (e) deletion strains, were cultivated without (circles) and with (triangles) 8% ethanol addition. Open and filled symbols represent the duplicate data. Arrows indicate the time of ethanol addition.

response, we analyzed how the genes screened from the phenotypic data of the deletion strains change their expression levels after the addition of the stress using the data obtained from the genome-wide expression analysis. As shown in Fig. 5, among the genes whose deletion strains showed ethanol sensitivity, the proportion of genes that were upregulated (ratio > 2) after the addition of ethanol was significantly low ($P < 0.05$, randomization test) in comparison with all the genes examined (4729 in total). Also, the proportion of genes that did not change their expression levels was significantly high ($P < 0.01$, randomization test). In the up-regulated genes whose deletion strains showed ethanol sensitivity, genes for heat shock protein (*HSP26*) and trehalose synthesis (*TPS1*) were included. Genes involved in ergosterol biosynthesis (*ERG2*, *ERG3*, *ERG5*, and *ERG6*) were included in the downregulated genes. The genes whose expression was not altered included those related to peroxisomal and mitochondrial functions whereas these same genes were identified as required for growth in the presence of ethanol in our screening. Genes that are upregulated under stress conditions are often selected as a target gene for manipulation (i.e. deletion or overexpression) to construct ethanol-tolerant strains. However, our result suggested that the genes whose expression levels were not altered under the ethanol-stressed condition contained a higher proportion of genes important for growth under the ethanol-stressed condition than that of the upregulated genes.

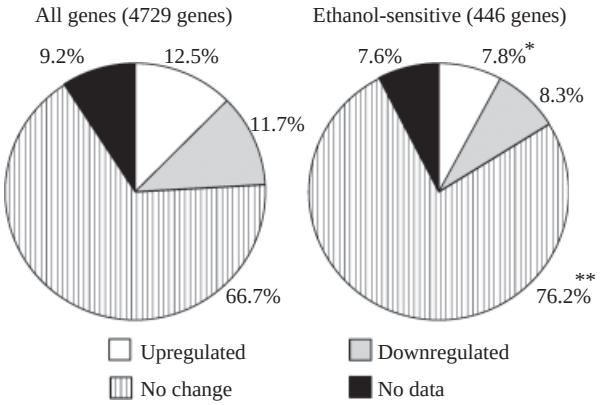

Fig. 5. Relationship between genes of ethanol-sensitive strains and gene expression dynamics. White, upregulated genes; light gray, downregulated genes; stripe, genes whose gene expression levels did not change; dark, no gene expression data under ethanol. Each number beside the circle graph represents the proportion of the genes in each category. The number with (*) indicates that the proportions were significantly low ($P < 0.05$), and the number with (**) indicates that the proportions were significantly high ($P < 0.01$) in 446 ethanol-sensitive strains than that in all the 4729 deletion strains.

Comparison of strains sensitive to ethanol and osmotic stress

We identified the genes that are important for growth under ethanol stress. We then investigated whether these genes are generally important for the stress response by analyzing the growth behavior of deletion strains under osmotic stress, which is usually encountered during bioproduction processes. We aimed to determine which of the genes were specifically important for growth under ethanol stress by subtracting those strains that were sensitive to osmotic stress induced by 1 M NaCl. The relationship between the specific growth rates observed with 8% ethanol and 1 M NaCl is shown in Fig. 6, and the numbers of deletion strains exhibiting sensitivity and tolerance to 8% ethanol and/or 1 M NaCl are described in Table 6. Figure 6 shows that the growth of almost all strains that were cross-sensitive to ethanol and osmotic stress was also defective under the nonstress condition. We excluded the strains showing defective growth under the nonstress condition and found that the stress-sensitive strains were roughly classifiable into groups that were specifically sensitive to either ethanol or

osmotic stress. Notably, fewer deletion strains were sensitive to both 8% ethanol and 1 M NaCl (87 strains) than to either 8% ethanol or 1 M NaCl (359 and 242 strains, respectively). These findings indicate that the mechanisms of the response to ethanol and osmotic stress are considerably different.

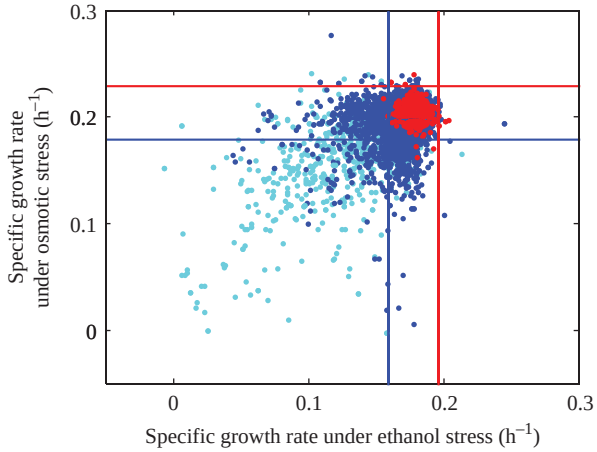

Fig. 6. Relationship between specific growth rates of deletion strains under 8% ethanol and 1 M NaCl stress. Red circles, standard strain; blue circles, deletion strains that did not exhibit growth defects under the nonstress condition; light blue circles, deletion strains with growth defects under the nonstress condition. For deletion strains, the average of the specific growth rates of each deletion strains in the two experiments was used. Red and blue lines represent the thresholds for the identification of the strains tolerant or sensitive to both 8% ethanol and 1 M NaCl, respectively.

After subtracting deletion strains sensitive to osmotic stress from those that were ethanol sensitive, we examined the functional categories to which the resulting group belonged. Table 7 shows that the categories screened were similar to those before subtracting the strains that were sensitive to osmotic stress, except for categories such as 'regulation of C-compound and carbohydrate utilization' and 'small GTPase-mediated signal transduction.' Here, the functional categories of 'peptide binding,' 'temperature perception and response,' and 'peroxisome' were identified to be specifically important for growth under ethanol stress. Identification of the 'temperature perception and response' was consistent with the report by Piper (1995). Notably, the appearance of the functional category 'peroxisome' and persistence of all deletion strains of the genes classified into the 'peroxisomal transport' category indicated that peroxisomes are specifically related to growth under ethanol stress.

The over-represented genes for deletion strains that were sensitive to both 8% ethanol and 1 M NaCl were found in

Table 6. Relationship between strains sensitive/tolerant to ethanol and osmotic stress

	1 M NaCl		
	Sensitive	No change	Tolerant
8% Ethanol			
Tolerant	0	2	0
No change	242	3444	4
Sensitive	87	353	6

Table 7. Representative functional categories of over-represented genes whose deletion strains were sensitive to ethanol but not osmotic stress

MIPS functional category number	MIPS functional category	*P*-value	No. of genes among selected genes (359)	No. of genes among all genes (4138)
01.01.09.06.01	Biosynthesis of tryptophan	1.58E − 03	4	7
01.20.15.03	Biosynthesis of ubiquinone	2.42E − 03	3	4
02.11	Electron transport and membrane-associated energy conservation	3.25E − 04	11	39
02.13.03	Aerobic respiration	6.17E − 14	26	56
11.04.03.01	Splicing	2.50E − 03	8	29
12.01.01	Ribosomal proteins	2.58E − 12	34	105
12.07	Translational control	5.42E − 04	10	35
12.10	Aminoacyl-tRNA-synthetases	6.73E − 04	6	14
14.04	Protein targeting, sorting, and translocation	9.67E − 06	30	151
14.10	Assembly of protein complexes	2.18E − 09	29	100
16.02	Peptide binding	7.51E − 03	2	2
16.07	Structural protein	5.55E − 03	6	20
20.01.21	RNA transport	1.48E − 03	9	33
20.09.01	Nuclear transport	1.96E − 03	8	28
20.09.07.03	ER to Golgi transport	6.94E − 04	9	30
20.09.10	Peroxisomal transport	2.21E − 03	6	17
20.09.13	Vacuolar transport	2.02E − 03	19	108
34.11.09	Temperature perception and response	6.91E − 03	5	15
42.10.05	Nuclear membrane	2.94E − 03	4	8
42.16	Mitochondrion	1.32E − 12	36	114
42.19	Peroxisome	3.94E − 03	8	31

the functional categories 'metabolism of phenylalanine,' 'metabolism of tyrosine,' 'metabolism of tryptophan,' 'isoprenoid biosynthesis,' and 'budding, cell polarity, and filament formation' (Table 8). The 'metabolism of phenylalanine,' 'metabolism of tyrosine,' and 'metabolism of tryptophan' categories include *ARO1, ARO2*, and *ARO7*, whose products are involved in the biosynthesis of the precursors of aromatic amino acids. However, unlike ethanol stress, *TRP2-5* deletion strains were not sensitive to osmotic stress (Fig. 3). This finding was consistent with those of González *et al*. (2007). Thus, the osmotic sensitivity of the *ARO1* and *ARO2* deletion strains was not due to the absence of tryptophan biosynthesis. The Aro7 protein is involved in tyrosine and phenylalanine biosynthesis; thus, the simultaneous loss of tyrosine and phenylalanine biosynthesis might be associated with the osmotic sensitivity of the *ARO1*, *ARO2*, and *ARO7* deletion strains. The products of the genes including *ERG2*, *ERG3*, and *ERG6* categorized into the 'isoprenoid biosynthesis' category are involved in ergosterol biosynthesis; further, strains with a deletion of these genes were sensitive to 8% ethanol and 1 M NaCl. Ergosterol is one of the major components of the cellular membrane and is associated with plasma membrane fluidity. This result indicated that ergosterol is important for stress tolerance, which supports previous findings such as a higher ergosterol content in strains that are tolerant to ethanol stress (del Castillo Agudo, 1992) and that the *ERG3* and *ERG6* deletion strains adapt over time to osmotic stress (Warringer *et al*., 2003; Fernandez-Ricaud *et al*., 2005).

We also found that the functional categories in which osmotic stress-specific sensitive genes were over-represented comprised 'phosphate utilization,' 'G1/S transition of mitotic cell cycle,' and 'stress response' (Table 9). Under osmotic stress, the *HOG1* gene product controls cell cycle progression and is involved in cell cycle arrest at the G1 and G2 phases (Bellí *et al*., 2001; Escoté *et al*., 2004; Clotet *et al*., 2006). We found that deletion strains of the genes classified into the 'G2/M transition of mitotic cell cycle' category were sensitive to both ethanol and osmotic stress, whereas deletion strains of the genes classified into 'G1/S transition of mitotic cell cycle' were specifically sensitive to osmotic stress, suggesting the importance of cell cycle regulation for growth under osmotic stress.

Of the genes classified into the 'phosphate utilization' category, those important for the response to osmotic stress – such as *STE20*, *SSK2*, *PBS2*, and *HOG1*, which are related to the signal transduction pathway for osmotic stress response [the high-osmolarity glycerol (HOG) pathway; Hohmann, 2002] – were included. The growth of all of these mutants was severely defective under osmotic stress.

Table 8. Representative functional categories of over-represented genes whose deletion strains were sensitive to both ethanol and osmotic stresses

MIPS functional category number	MIPS functional category	*P*-value	No. of genes in selected genes (87)	No. of genes in all genes (4138)
01.01.09.04	Metabolism of phenylalanine	1.72E – 03	3	12
01.01.09.05	Metabolism of tyrosine	1.31E – 03	3	11
01.01.09.06	Metabolism of tryptophan	2.74E – 04	4	16
01.06.01.07	Isoprenoid biosynthesis	6.82E – 03	3	19
10.03.01.01.09	G2/M transition of mitotic cell cycle	7.90E – 03	3	20
11.02.03.04.03	Transcriptional repressor	9.08E – 03	3	21
14.04	Protein targeting, sorting, and translocation	4.07E – 03	9	151
20.09.07.05	Intra Golgi transport	4.93E – 03	3	17
20.09.13	Vacuolar transport	3.77E – 04	9	108
32.01.03	Osmotic and salt stress response	9.36E – 03	4	40
43.01.03.05	Budding, cell polarity, and filament formation	9.20E – 04	12	202
43.01.03.09	Development of asco-, basidio-, or zygospore	8.66E – 03	7	112

Table 9. Representative functional categories of over-represented genes whose deletion strains were sensitive to osmotic but not ethanol stress

MIPS functional category number	MIPS functional category	*P*-value	No. of genes in selected genes (242)	No. of genes in all genes (4138)
01.01.06.04.01	Biosynthesis of threonine	9.83E – 03	2	3
01.04.01	Phosphate utilization	7.16E – 03	26	270
10.03.01.01.03	G1/S transition of mitotic cell cycle	4.15E – 04	7	25
10.03.04.01	Centromere/kinetochore complex maturation	9.83E – 03	2	3
32.01	Stress response	3.63E – 03	30	310
32.01.01	Oxydative stress response	5.98E – 03	8	48

Deletion strains specifically tolerant to ethanol or osmotic stress

With respect to the stress-tolerant strains, two deletion strains of *CYB5* and *YOR139C* were specifically ethanol-tolerant, and 10 deletion stains of *ALD6*, *HOC1*, *PRO1*, *SCP160*, *SKY1*, *TIP41*, *UBP6*, *YKL161C*, *YNR004W*, and *YNR036C* were specifically NaCl-tolerant (Table 10). No deletion strains exhibited a resistance to both ethanol and NaCl.

Among the two genes whose deletions specifically showed ethanol tolerance, *YOR139C* is a dubious ORF that is located at the complementary strand of *SFL1*, which is involved in the repression of flocculation-related genes (Robertson & Fink, 1998). *CYB5* is involved in sterol and lipid biosynthesis (Lamb *et al*., 1999); these processes are known to be important for ethanol tolerance (del Castillo Agudo, 1992; You *et al*., 2003). A more detailed analysis of these genes will provide useful information for understanding the mechanisms of ethanol tolerance.

In the 10 genes whose deletions exhibited osmotic tolerance, the *ALD6* and *SKY1* deletion strains are known as osmotic-tolerant strains (Forment *et al*., 2002; Fernandez-Ricaud *et al*., 2005), suggesting that this list was thought to contain valuable information for osmotic tolerance. *PRO1* is involved in proline biosynthesis and its deletion causes proline auxotrophy. The *PRO1* deletion strain was reported to be tolerant to NaCl and FK506 (Butcher & Schreiber, 2004). The osmotic tolerance of the proline overproduction strain (Sekine *et al*., 2007) and the osmotic sensitivity of the *PRO2* deletion strain in the present study, which was also required for proline biosynthesis, suggested the importance of proline in the osmotic stress response. The relationship of seven other genes with osmotic tolerance was not identified and further analyses would be required to elucidate the same. Interestingly, six of the 10 osmotolerant strains, i.e. the *ALD6*, *HOC1*, *PRO1*, *SCP160, SKY1*, and *YNR004W* deletion strains, exhibited sensitivity to ethanol; however, we could not find any common biological features among these genes. This difference and the absence of overlapping in the tolerant strains between ethanol and osmotic stress suggested different response mechanisms to these stresses in yeast.

Here, we developed a high-resolution culture system and quantified the growth of individual strains with a single gene deletion under nonstress, 8% ethanol, and 1 M NaCl stress conditions. This collection of strains allows the analysis of phenotypic changes caused by a single gene deletion under specific conditions. Quantitative data revealed that genes in the functional categories of 'peroxisome' were required for growth under ethanol stress, which was not known previously. The identification of these functional categories in strains cultured on agar plates and measurements of colony sizes produced by deletion strains are difficult because the differences in growth rates between standard and ethanol-sensitive strains are small. However, with our method, we obtained precise growth data under osmotic stress, and screened strains that were specifically sensitive to ethanol stress. Quantitative analysis of the growth of deletion strains will help to generate ethanol-tolerant yeast strains for the bioproduction of useful chemical compounds such as bioethanol. In addition, we identified some deletion strains that tolerated two stress conditions. Further analysis of these strains should reveal the mechanisms of ethanol tolerance.

Table 10. Genes whose deletion strains were tolerant to ethanol or osmotic stress

Name	Description
Ethanol tolerant	
CYB5	Cytochrome b5, involved in the sterol and lipid biosynthesis pathways
YOR139C	Dubious open reading frame; partially overlaps *SFL1*
Osmotic tolerant	
ALD6	Cytosolic aldehyde dehydrogenase
HOC1	Alpha-1,6-mannosyltransferase involved in cell wall mannan biosynthesis
PRO1	γ-Glutamyl kinase that catalyzes the first step in proline biosynthesis
SCP160	Essential RNA-binding G protein effector of mating response pathway
SKY1	SR protein kinase involved in mRNA metabolism and cation homeostasis
TIP41	Negatively regulates the TOR signaling pathway
UBP6	Ubiquitin-specific protease
YKL161C	Protein kinase implicated in the Slt2p mitogen-activated (MAP) kinase signaling pathway
YNR004W	Putative protein of unknown function
YNR036C	Mitochondrial protein

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References

Aguilera A & Benítez T (1985) Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae*. *Arch Microbiol* **142**: 389–392.

Albertini M, Girzalsky W, Veenhuis M & Kunau WH (2001) Pex12p of *Saccharomyces cerevisiae* is a component of a multi-protein complex essential for peroxisomal matrix protein import. *Eur J Cell Biol* **80**: 257–270.

Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, Coker C, Krauskopf A, Kupiec M & McEachern MJ (2004) A genome-wide screen for *Saccharomyces cerevisiae* deletion mutants that affect telomere length. *P Natl Acad Sci USA* **101**: 8658–8663.

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. *Nat Biotechnol* **15**: 1351–1357.

Ball SG, Wickner RB, Cottarel G, Schaus M & Tirtiaux C (1986) Molecular cloning and characterization of *ARO7-OSM2*, a single yeast gene necessary for chorismate mutase activity and growth in hypertonic medium. *Mol Gen Genet* **205**: 326–330.

Bellí G, Garí E, Aldea M & Herrero E (2001) Osmotic stress causes a G1 cell cycle delay and downregulation of Cln3/Cdc28 activity in *Saccharomyces cerevisiae*. *Mol Microbiol* **39**: 1022–1035.

Birschmann I, Rosenkranz K, Erdmann R & Kunau WH (2005) Structural and functional analysis of the interaction of the AAA-peroxins Pex1p and Pex6p. *FEBS J* **272**: 47–58.

Bowers K & Stevens TH (2005) Protein transport from the late Golgi to the vacuole in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1744**: 438–454.

Breitling R, Sharif O, Hartman ML & Krisans SK (2002) Loss of compartmentalization causes misregulation of lysine biosynthesis in peroxisome-deficient yeast cells. *Eukaryot Cell* **1**: 978–986.

Brocard C, Lametschwandtner G, Koudelka R & Hartig A (1997) Pex14p is a member of the protein linkage map of Pex5p. *EMBO J* **16**: 5491–5500.

Brown LA & Baker A (2003) Peroxisome biogenesis and the role of protein import. *J Cell Mol Med* **7**: 388–400.

Butcher RA & Schreiber SL (2004) Identification of Ald6p as the target of a class of small-molecule suppressors of FK506 and their use in network dissection. *Proc Natl Acad Sci USA* **101**: 7868–7873.

Clotet J, Escoté X, Adrover MA, Yaakov G, Garí E, Aldea M, de Nadal E & Posas F (2006) Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity. *EMBO J* **25**: 2338–2346.

Corbacho I, Olivero I & Hernández LM (2005) A genome-wide screen for *Saccharomyces cerevisiae* nonessential genes involved in mannosyl phosphate transfer to mannoprotein-linked oligosaccharides. *Fungal Genet Biol* **42**: 773–790.

del Castillo Agudo L (1992) Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. *Appl Microbiol Biotechnol* **37**: 647–651.

Erdmann R, Veenhuis M, Mertens D & Kunau WH (1989) Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *P Natl Acad Sci USA* **86**: 5419–5423.

Escoté X, Zapater M, Clotet J & Posas F (2004) Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1. *Nat Cell Biol* **6**: 997–1002.

Fernandez-Ricaud L, Warringer J, Ericson E, Pylvänäinen I, Kemp GJ, Nerman O & Blomberg A (2005) PROPHECY – a database for high-resolution phenomics. *Nucleic Acids Res* **33**: D369–D373.

Forment J, Mulet JM, Vicente O & Serrano R (2002) The yeast SR protein kinase Sky1p modulates salt tolerance, membrane potential and the Trk1,2 potassium transporter. *Biochim Biophys Acta* **1565**: 36–40.

Fujita K, Matsuyama A, Kobayashi Y & Iwahashi H (2006) The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols. *FEMS Yeast Res* **6**: 744–750.

Gao XD, Wang J, Keppler-Ross S & Dean N (2005) *ERS1* encodes a functional homologue of the human lysosomal cystine transporter. *FEBS J* **272**: 2497–2511.

Gibson BR, Lawrence SJ, Leclaire JP, Powell CD & Smart KA (2007) Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol Rev* **31**: 535–569.

Gietz RD & Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350**: 87–96.

González A, Larroy C, Biosca JA & Ariño J (2007) Use of the *TRP1* auxotrophic marker for gene disruption and phenotypic analysis in yeast: a note of warning. *FEMS Yeast Res* **8**: 2–5.

Hettema EH, Girzalsky W, van Den Berg M, Erdmann R & Distel B (2000) *Saccharomyces cerevisiae* pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J* **19**: 223–233.

Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H & Shioya S (2007) Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. *J Biotechnol* **131**: 34–44.

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66**: 300–372.

Huhse B, Rehling P, Albertini M, Blank L, Meller K & Kunau WH (1998) Pex17p of *Saccharomyces cerevisiae* is a novel peroxin and component of the peroxisomal protein translocation machinery. *J Cell Biol* **140**: 49–60.

Izawa S, Kita T, Ikeda K, Miki T & Inoue Y (2007) Formation of cytoplasmic P-bodies in sake yeast during Japanese sake brewing and wine making. *Biosci Biotechnol Biochem* **71**: 2800–2807.

Jiménez J & Benítez T (1988) Yeast cell viability under conditions of high temperature and ethanol concentrations depends on the mitochondrial genome. *Curr Genet* **13**: 461–469.

Koller A, Snyder WB, Faber KN, Wenzel TJ, Rangell L, Keller GA & Subramani S (1999) Pex22p of *Pichia pastoris*, essential for peroxisomal matrix protein import, anchors the ubiquitin-conjugating enzyme, Pex4p, on the peroxisomal membrane. *J Cell Biol* **146**: 99–112.

Kubota S, Takeo I, Kume K, Kanai M, Shitamukai A, Mizunuma M, Miyakawa T, Shimoi H, Iefuji H & Hirata D (2004) Effect of ethanol on cell growth of budding yeast: genes that are

important for cell growth in the presence of ethanol. *Biosci Biotechnol Biochem* **68**: 968–972.

Lamb DC, Kelly DE, Manning NJ, Kaderbhai MA & Kelly SL (1999) Biodiversity of the P450 catalytic cycle: yeast cytochrome *b5*/NADH cytochrome *b5* reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction. *FEBS Lett* **462**: 283–288.

Lucchini G, Biraghi A, Carbone ML, de Scrilli A & Magni GE (1978) Effect of mutation in the aromatic amino acid pathway on sporulation of *Saccharomyces cerevisiae*. *J Bacteriol* **136**: 55–62.

Lucero P, Peñalver E, Moreno E & Lagunas R (2000) Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **66**: 4456–4461.

Piper PW (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol Lett* **134**: 121–127.

Rehling P, Skaletz-Rorowski A, Girzalsky W, Voorn-Brouwer T, Franse MM, Distel B, Veenhuis M, Kunau WH & Erdmann R (2000) Pex8p, an intraperoxisomal peroxin of *Saccharomyces cerevisiae* required for protein transport into peroxisomes binds the PTS1 receptor pex5p. *J Biol Chem* **275**: 3593–3602.

Robertson LS & Fink GR (1998) The three yeast A kinases have specific signaling functions in pseudohyphal growth. *P Natl Acad Sci USA* **95**: 13783–13787.

Rosa MF & Sá-Correia I (1996) Intracellular acidification does not account for inhibition of *Saccharomyces cerevisiae* growth in the presence of ethanol. *FEMS Microbiol Lett* **135**: 271–274.

Ruepp A, Zollner A, Maier D *et al.* (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res* **32**: 5539–5545.

Scherens B & Goffeau A (2004) The uses of genome-wide yeast mutant collections. *Genome Biol* **5**: 229.

Sekine T, Kawaguchi A, Hamano Y & Takagi H (2007) Desensitization of feedback inhibition of the *Saccharomyces cerevisiae* gamma-glutamyl kinase enhances proline accumulation and freezing tolerance. *Appl Environ Microbiol* **73**: 4011–4019.

Takagi H, Takaoka M, Kawaguchi A & Kubo Y (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 8656–8662.

Takemura R, Inoue Y & Izawa S (2004) Stress response in yeast mRNA export factor: reversible changes in Rat8p localization are caused by ethanol stress but not heat shock. *J Cell Sci* **117**: 4189–4197.

van Voorst F, Houghton-Larsen J, Jonson L, Kielland-Brandt MC & Brandt A (2006) Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress. *Yeast* **23**: 351–359.

Warringer J, Ericson E, Fernandez L, Nerman O & Blomberg A (2003) High-resolution yeast phenomics resolves different physiological features in the saline response. *Proc Natl Acad Sci USA* **100**: 15724–15729.

Winzeler EA, Shoemaker DD, Astromoff A *et al.* (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**: 901–906.

You KM, Rosenfield CL & Knipple DC (2003) Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Appl Environ Microbiol* **69**: 1499–1503.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Specific growth rates of single gene deletion strains.

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