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Research Article

Elucidation of ethanol tolerance mechanisms in *Saccharomyces cerevisiae* by global metabolite profiling

Sooah Kim[1], Jungyeon Kim[1], Ju Hwan Song[1], Young Hoon Jung[1], Il-Sup Choi[1], Wonja Choi[2], Yong-Cheol Park[3], Jin-Ho Seo[4] and Kyoung Heon Kim[1]

[1] Department of Biotechnology, Graduate School, Korea University, Seoul, South Korea
[2] Department of Life Sciences, Ewha Womans University, Seoul, South Korea
[3] Department of Bio and Fermentation Convergence Technology, Kookmin University, Seoul, South Korea
[4] Department of Agricultural Biotechnology and Center for Food and Bioconvergence, Seoul National University, Seoul, South Korea

Ethanol, the major fermentation product of yeast, is a stress factor in yeast. We previously constructed an ethanol-tolerant mutant yeast iETS3 by using the global transcriptional machinery engineering. However, the ethanol-tolerance mechanism has not been systematically investigated. In this study, global metabolite profiling was carried out, mainly by gas chromatography/time-of-flight mass spectrometry (GC/TOF MS), to investigate the mechanisms of ethanol tolerance in iETS3. A total of 108 intracellular metabolites were identified by GC/TOF MS and high performance liquid chromatography, and these metabolites were mostly intermediates of the central carbon metabolism. The metabolite profiles of iETS3 and BY4741, cultured with or without ethanol, were significantly different based on principal component and hierarchical clustering analyses. Our metabolomic analyses identified the compositional changes in cell membranes and the activation of glutamate metabolism and the trehalose synthetic pathway as the possible mechanisms for the ethanol tolerance. These metabolic traits can be considered possible targets for further improvement of ethanol tolerance in the mutant. For example, the *KGD1* deletion mutant, with up-regulated glutamate metabolism, showed increased tolerance to ethanol. This study has demonstrated that metabolomics can be a useful tool for strain improvement and phenotypic analysis of microorganisms under stress.

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1 Introduction

Saccharomyces cerevisiae has attracted increased attention due to its ability to produce ethanol from lignocellulose [1, 2]. However, the ethanol that accumulates in the culture media during the fermentation process is a major source of stress for *S. cerevisiae*, which ultimately restricts ethanol yields [3–5]. The production of ethanol ceases upon reaching a threshold level in the culture media as ethanol inhibits cell growth and viability, destroys mitochondrial DNA and normal membrane structure, inactivates cellular enzymes, and affects cellular transport systems [6–9]. Therefore, enhancing ethanol tolerance in *S. cerevisiae* is important for increasing ethanol yield. Many studies have been conducted to create ethanol-tolerant strains of *S. cerevisiae* [10–12]. In one such study, *S. cerevisiae* iETS3, with enhanced ethanol tolerance, was developed using global transcriptional machinery engineering (gTME) [10]. However, these studies have usually been limited to screening and obtaining tolerant

Correspondence: Prof. Kyoung Heon Kim, Department of Biotechnology, Graduate School, Korea University, 145 Anam-ro, Seoul 02841, South Korea
E-mail: khekim@korea.ac.kr

Abbreviations: **GC/TOF MS**, gas chromatography/time-of-flight mass spectrometry; **gTME**, global transcriptional machinery engineering; **HCA**, hierarchical clustering analysis; **HPLC**, high performance liquid chromatography; **KGD1**, α-ketoglutarate dehydrogenase; **NIST**, National Institute of Standards and Technology; **PCA**, principal component analysis; **TCA**, tricarboxylic acid

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mutants without an in-depth examination of the mutant phenotypes. It is necessary to understand how ethanol affects the yeast and how the ethanol-tolerant yeast copes with the ethanol stress.

S. cerevisiae is estimated to have fewer than 1000 metabolites [13]. Levels of metabolites can represent the overall response of biological systems to various genetic or environmental changes. As a result, metabolic data can be linked to physiological states and particularly to specific biochemical processes in yeast. Metabolomics, the study of global changes in the entire set of metabolites in a given organism, has been used due to its ability to identify key target metabolites and provide a holistic view of metabolism [14]. Therefore, using this approach, a greater insight into the response and tolerance to ethanol stress can be obtained, because this is a powerful tool for studying the metabolism and physiology of yeast. In the present study, we have performed metabolomics analysis on an ethanol-tolerant mutant of *S. cerevisiae*, iETS3 [10] to investigate the metabolic traits and mechanisms of ethanol tolerance. To accomplish this, we used gas chromatography/time-of-flight mass spectrometry (GC/TOF MS), high performance liquid chromatography (HPLC), and multivariate statistical analysis.

2 Materials and methods

2.1 Yeast strains and cultivation

Wild type *S. cerevisiae* BY4741 and its ethanol-tolerant mutant strain, iETS3 [10], were cultivated in 200 mL YPD broth (1% w/v yeast extract, 2% peptone, and 2% glucose) in a 500 mL baffled flask. For ethanol stress, ethanol was initially added to the culture medium at a final concentration of 5% v/v before fermentation. The culture was incubated at 30°C with agitation at 200 rpm. Cell growth was monitored by measuring the absorbance of the culture broth at 600 nm, which was converted to cell mass concentration.

2.2 Metabolite extraction

Metabolite extraction and analysis were conducted following previously described methods [15]. Briefly, cell culture (1 mL) was collected and immediately vacuum-filtered through a nylon membrane filter (0.45 µm pore size, 30 mm diameter; Whatman, Piscataway, NJ). After the cells were washed with distilled water, the cells were extracted with an acetonitrile/water mixture (1:1, v/v). The extracted metabolite sample was completely dried in the vacuum concentrator and stored at -80°C until derivatization before the analysis by GC/TOF MS. The metabolite extract was derivatized in the following two steps: the first step with 5 µL of 40 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich, St. Louis, MO) at 30°C for 90 min and the second step with 45 µL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Fluka, Buchs, Switzerland) at 37°C for 30 min. A mixture of fatty acid methyl esters was added to the derivatized metabolite sample as the internal retention index marker.

2.3 GC/TOF MS and HPLC analyses

Metabolite samples were analyzed by GC/TOF MS. Metabolite analysis was conducted using an Agilent 7890A GC (Agilent Technologies, Wilmington, DE) coupled with a Pegasus HT TOF MS (LECO, St. Joseph, MI). The derivatized metabolite sample (1 µL) was injected into an RTX-5Sil MS column (30 m × 0.25 mm, 0.25 µm film thickness; Restek, Bellefonte, PA) with an additional 10-m-long integrated guard column in splitless mode. The oven temperature was initially set at 50°C, held for 1 min, and then increased to 330°C at a rate of 20°C/min, where it was finally held for 5 min. The mass spectra of the metabolites were in the mass range of 85 to 500 m/z at an acquisition rate of 10 spectra/s. The ion source and transfer line temperatures were 250°C and 280°C, respectively, and the ionization occurred at an electron impact of 70 eV. The GC/TOF MS data were preprocessed using Leco Chroma TOF software (ver. 3.34; St. Joseph, MI) with automated peak detection and mass spectral deconvolution. The data were further processed using an in-house software, BinBase [16, 17], to remove noise peaks and to identity metabolites by comparing their mass spectra and retention indices with those of the National Institute of Standards and Technology (NIST) library (NIST, Gaithersburg, MD) and of a customized reference mass spectral library. All processed abundance data obtained based on peak intensities from GC/TOF MS were then normalized against factors which were calculated by intensities of the sum of identified metabolites [18]. To determine the concentrations of ethanol and acetate produced during fermentation, HPLC equipped with a refractive index detector (Agilent 1100, Agilent Technologies, Waldbronn, Germany) and an Aminex HPX-87H column (Bio-Rad, Hercules, CA) was used. A mobile phase of 0.01 N H_2SO_4 was eluted in the column at a constant flow rate of 0.5 mL/min at 65°C.

2.4 Spot assay and growth profile of *KGD1* deletion mutant

To compare the ethanol tolerance of *S. cerevisiae* BY4741 and the *KGD1* deletion (*kgd1*Δ) mutant strains, spot assays were performed. The *kgd1*Δ mutant was obtained from the yeast deletion collection (Open Biosystems, GE Dharmacon, Lafayette, CO) [19]. BY4741 and *kgd1*Δ strains, grown to an absorbance of 3.0 at 600 nm, were 10-fold serially diluted, and their aliquots (3 µL) were spotted onto solid yeast synthetic complete media (YSCD; 1.7 g/L of yeast nitrogen base without amino acids, 20 g/L

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of glucose, and 0.79 g/L of complete supplement mixture) with 0% v/v, 10%, or 20% ethanol. The plates were incubated at 30°C for three to four days. To investigate the growth profiles of wild-type *S. cerevisiae* BY4741 and the *kgd1*Δ mutant, yeast strains were cultivated in YPD broth with 5% v/v ethanol or without ethanol at 30°C with agitation at 200 rpm. The cell growth was determined by the absorbance of the culture broth at 600 nm. For measuring the level of metabolites related to glutamate metabolism, BY4741 and *kgd1*Δ strains cultivated in the absence of ethanol were collected at the mid-exponential phase, vacuum-filtered, extracted, and analyzed using GC/TOF MS as described above.

2.5 Statistical analyses

Statistica (ver. 7.1; StatSoft, Tulsa, OK) was used as the statistical software for multivariate and univariate analyses of metabolite data sets from a total of 24 samples consisting of six biological replicates and four different groups [16, 17, 20, 21]. Principal component analysis (PCA) was used to obtain the intrinsic variance in data sets [22]. Hierarchical clustering analysis (HCA), based on the Pearson correlation and complete linkage methods, was performed using a MultiExperiment Viewer (Dana-Farber Cancer Institute, Boston, MA) to cluster both groups of identified metabolites [23].

3 Results

3.1 Effect of ethanol on BY4741 and iETS3 cell growth

Wild-type *S. cerevisiae* BY4741 and its ethanol-tolerant mutant, iETS3, were cultivated in the absence or presence of 5% v/v initial ethanol (Fig. 1). The specific growth rates were 0.41, 0.28, 0.39, and 0.24 h^{-1} for iETS3 without and with ethanol and BY4741 without and with ethanol, respectively. Thus, the specific growth rates of BY4741 and iETS3 decreased by 68 and 62%, respectively, with the addition of 5% ethanol. Specifically, the decrease in specific growth rate in the presence of ethanol was higher for BY4741 than for iETS3. Ethanol is known to inhibit cell growth and affect viability even in ethanologenic yeast [7]. The higher growth rate of iETS3 compared to that of BY4741, regardless of ethanol stress, observed here, was consistent with those of previous studies [10, 24]. Also, the final concentration of ethanol of iETS3 with initial absence of ethanol was 20% higher than that of BY4741, and glucose was consumed faster by iETS3 than by BY4741 (Supporting information, Fig. S1). These results suggested that defense mechanisms against ethanol, found in this study, were more activated in iETS3 than in BY4741.

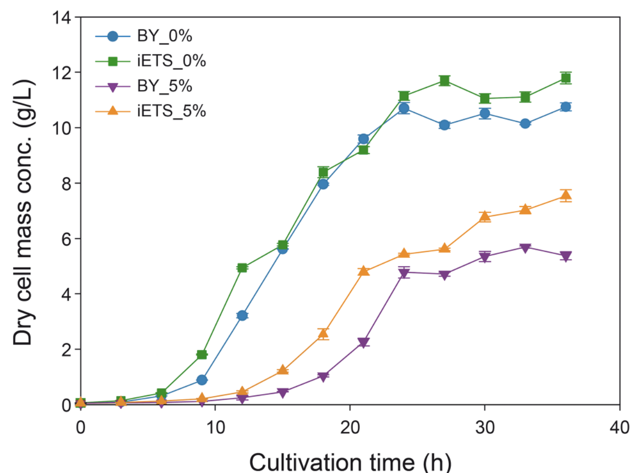

Figure 1. Effect of ethanol on cell growth in *S. cerevisiae* strains iETS3 (ethanol-tolerant mutant) and BY4741 (wild type). BY_0%, BY4741 grown in YPD medium without ethanol; iETS_0%, iETS3 grown in YPD without ethanol; BY_5%, BY4741 grown in YPD with ethanol (5% v/v); iETS_5%, iETS3 grown in YPD with ethanol (5% v/v).

3.2 Intracellular metabolites analyzed by GC/TOF MS and HPLC

GC/TOF MS and HPLC were used to analyze intracellular metabolites in six replicates of iETS3 and BY4741 from the mid-exponential growth phase. Over 600 metabolite peaks with unique m/z values were detected from these GC/TOF MS analyses, and 106 metabolites were identified. In addition, intracellular ethanol and acetate were analyzed by HPLC. All of the metabolites identified in this study were grouped into various chemical classes, including amines, amino acids, fatty acids, organic acids, phosphates, and sugars and sugar alcohols (Table 1). The number of identified metabolites was significantly higher here than in other studies that were examining the tolerance of *S. cerevisiae* to inhibitors (<89 metabolites) [2, 25, 26]. In this study, organic acids were the most frequently detected, accounting for 22.2% of all the identified metabolites. High numbers of amino acids, sugars and sugar alcohols, and fatty acids were also detected, accounting for 21.3, 20.4, and 18.5% of the total number of identified metabolites, respectively. Low numbers of amines (8.3%) and phosphates (5.6%) were detected in this analysis. The metabolites identified in this study are the major intermediates in the central carbon metabolism, including glycolysis, pentose phosphate pathway, tricarboxylic acid (TCA) cycle, and biosynthesis of amino acids, fatty acids, and nucleotides. For example, xylulose, a key metabolite in the pentose phosphate pathway, is converted to ethanol in yeast [27]. Fatty acids, especially mono-unsaturated fatty acids such as palmitoleic and oleic acids, which were detected in this study, are the major components of cell walls and cell membranes of *S. cerevisiae* [28].

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Table 1. Intracellular metabolites identified from *S. cerevisiae* iETS3 and BY4741.

Class	Metabolite	
Organic acids	acetate[b]	mannonate NIST[a]
	adipate	2-methylglycerate NIST[a]
	aminomalonate	nicotinate
	citramalate	octyl phthalate
	citrate	orotate
	fumarate	2-oxogluconate NIST[a]
	gluconate	pantothenate
	glycerate	pyruvate
	glycolate	ribonate
	2-hydroxyglutarate	succinate
	lactate	terephtalate
	malate	threonate
Amino acids	alanine	methionine
	asparagine	*N*-methylalanine
	asparagine dehydrated	ornithine
	aspartate	oxoproline
	carbamoyl-aspartate	phenylalanine
	glutamate	proline
	glutamine	serine
	glycine	threonine
	homoserine	tryptophan
	isoleucine	tyrosine
	leucine	valine
	lysine	
Sugars and sugar alcohols	3,6-anhydro-D-hexose	maltotriose
	arabitol	mannose
	cellobiose	myo-inositol
	erythritol	ribose
	fructose	sorbitol
	galactinol	sucrose
	β-gentiobiose	threitol
	glucose	threose
	glycerol	trehalose
	levoglucosan	xylose
	maltose	xylulose NIST[a]
Fatty acids	arachidic acid	2-monopalmitin
	β-sitosterol	myristic acid
	capric acid	octadecanol
	icosenoic acid	oleic acid
	isopalmitic acid	palmitic acid
	lauric acid	palmitoleic acid
	lignoceric acid	pelargonic acid
	methylhexadecanoic acid	pentadecanoic acid
	1-monoolein	squalene
	1-monopalmitin	stearic acid
Amines	citrulline	UDP-*N*-acetylglucosamine
	hydroxylamine	uracil
	hypoxanthine	urea
	inosine	uridine
	putrescine	
Phosphates	adenosine-5-monophosphate	glucose-6-phosphate
	fructose-6-phosphate	glycerol-3-phosphate
	glucose-1-phosphate	phosphoric acid
Miscellaneous	2,6-D-butylphenol NIST[a]	ethanol[b]
	2,5-dihydroxypyrazine NIST[a]	salicylaldehyde

a) Metabolites annotated by National Institute of Standards and Technology (NIST) mass spectral search but not verified by authentic chemical references
b) Metabolites analyzed by HPLC

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3.3 Discrimination of intracellular metabolite profiles of iETS3 and BY4741

The profiles of 108 intracellular metabolites identified from iETS3 and BY4741 (at mid-exponential phase) cultivated with or without 5% ethanol were statistically compared by PCA. The PCA score plot, composed of two principal components, PC1 and PC2, is shown in Fig. 2. The ellipse represents a 95% confidence level in the Hotelling T2 control chart, and PC1 and PC2 account for 46.6 and 13.2% of the total variance, respectively. The PCA model was excellent in both fit and prediction, showing 0.60 of R^2X and 0.73 of Q^2 cumulative to PC2, respectively. The four groups in the score plot, composed of the two strains with and without ethanol, were separated well by PC1 and PC2 (Fig. 2). The mean values of PC1 and PC2, respectively, were 4.07 and -1.79 for BY4741 without ethanol (BY_0%), 7.01 and -2.53 for BY4741 with 5% ethanol (BY_5%), -11.43 and -1.85 for iETS3 without ethanol (iETS_0%), and 0.35 and 6.16 for iETS3 with 5% ethanol (iETS_5%). Especially, PC1 significantly influenced the separation of strains. For PCA, loading scores indicate how each original variable contributes to the newly generated variables, PC1, PC2, and so on. Among the 108 metabolites of this study, 73 metabolites, including tryptophan, isopalmitic acid, β-gentiobiose, uridine, and hypoxanthine, contributed positively to PC1, indicating the higher levels of these metabolites in BY4741 than in iETS3. However, 35 metabolites, including phosphoric acid, lysine, glutamine, and myristic acid, contributed negatively to PC1, indicating the higher levels of these metabolites in iETS3 than in BY4741. In the loading plot of PC2, 63 metabolites, including pyruvate, carbamoyl-aspartate, pentadecanoic acid, citric acid, and tyrosine, were the major positive contributors, indicating the higher level of these metabolites in iETS3 with 5% ethanol than in other groups. In contrast, the other 45 metabolites, including oxoproline, proline, arachidic acid, 2-oxoglutarate, and orotate, negatively affected PC2, indicating the higher levels of these metabolites in BY4741 with 5% ethanol than in other groups. The loading scores of the top 20 metabolites that contributed highly to PC1 and PC2 are listed in Supporting information, Table S1.

HCA using the Pearson correlation and the complete linkage method was performed on the 108 identified metabolites (Fig. 3). The six replicates in each group were similar in metabolite pattern. However, in accordance with the PCA results in Fig. 2, the metabolite profiles obtained by HCA differed significantly among the four groups. The metabolite profile of each strain was very similar regardless of initial ethanol concentration. Certain individual metabolites showed markedly different abundances depending on their groups. These results indicated that metabolite profiles significantly differed depending on the strains and the presence of ethanol.

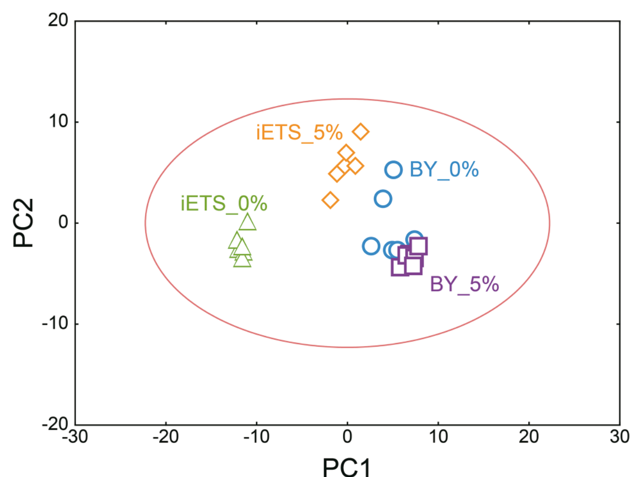

Figure 2. Principal component analysis of the intracellular metabolites of *S. cerevisiae* iETS3 and BY4741 cultivated until mid-exponential growth phase in YPD medium with or without ethanol. BY_0%, BY4741 without ethanol; iETS_0%, iETS3 without ethanol; BY_5%, BY4741 with ethanol (5% v/v); and iETS_5%, iETS3 with ethanol (5% v/v). The ellipse represents the Hotelling T2 at a 95% confidence level.

3.4 Comparison of intracellular metabolite abundances of iETS3 and BY4741

To reveal the ethanol tolerance mechanism of iETS3, we compared the metabolite changes of iETS3 and BY4741 grown with 0% ethanol, using a Student's *t*-test ($p < 0.05$). The levels of 21 metabolites such as glutamine, lysine, citrulline, and glutamate were increased in iETS3 (Supporting information, Table S2). However, 53 metabolites including palmitic acid, stearic acid, oleic acid, orotate, and TCA intermediates (i.e. succinate, citrate, and malate), were decreased in iETS3. Although, fatty acids such as palmitic acid, stearic acid, and oleic acid decreased in iETS3, the relative ratio of mono-unsaturated fatty acids increased in iETS3. Based on the increased metabolites in iETS3, it is considered that some metabolisms including glutamate metabolism, trehalose synthesis, and biosynthesis of cell membrane components could be the mechanisms of ethanol tolerance in iETS3.

The trehalose synthesis and cell membrane composition changes were already reported to be associated with ethanol tolerance by others [28–32]. Among the significantly increased metabolites in iETS3, the abundances of metabolites associated with glutamate metabolism such as glutamine, citrulline, and glutamate were two to 4.5-fold higher in iETS3 than in BY4741. Glutamate and glutamine are produced as a result of assimilation of ammonia. More specifically, α-ketoglutarate is converted to glutamate by glutamate dehydrogenase, and converted to succinyl-CoA in TCA cycle by the α-ketoglutarate dehydrogenase complex, encoded by the *KGD1*, *KGD2*, and *LDP1* genes. To validate the effect of glutamate accumulation on ethanol tolerance, we selected the *kgd1*Δ mutant.

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In our metabolite profile analysis, glutamate accumulation possibly occurred due to the increased metabolic flux from α-ketoglutarate to glutamate, resulting from the decreased flux from α-ketoglutarate to succinyl-CoA. The wild type and *kgd1*Δ strains were spotted and incubated on solid YSCD media with 0, 10, and 20% ethanol for three to four days, after which the *kgd1*Δ mutant had a higher density than wild type on the 10 and 20% ethanol spots (Fig. 4A). In addition, the *kgd1*Δ mutant grew faster than wild type in the liquid media, regardless of initial ethanol

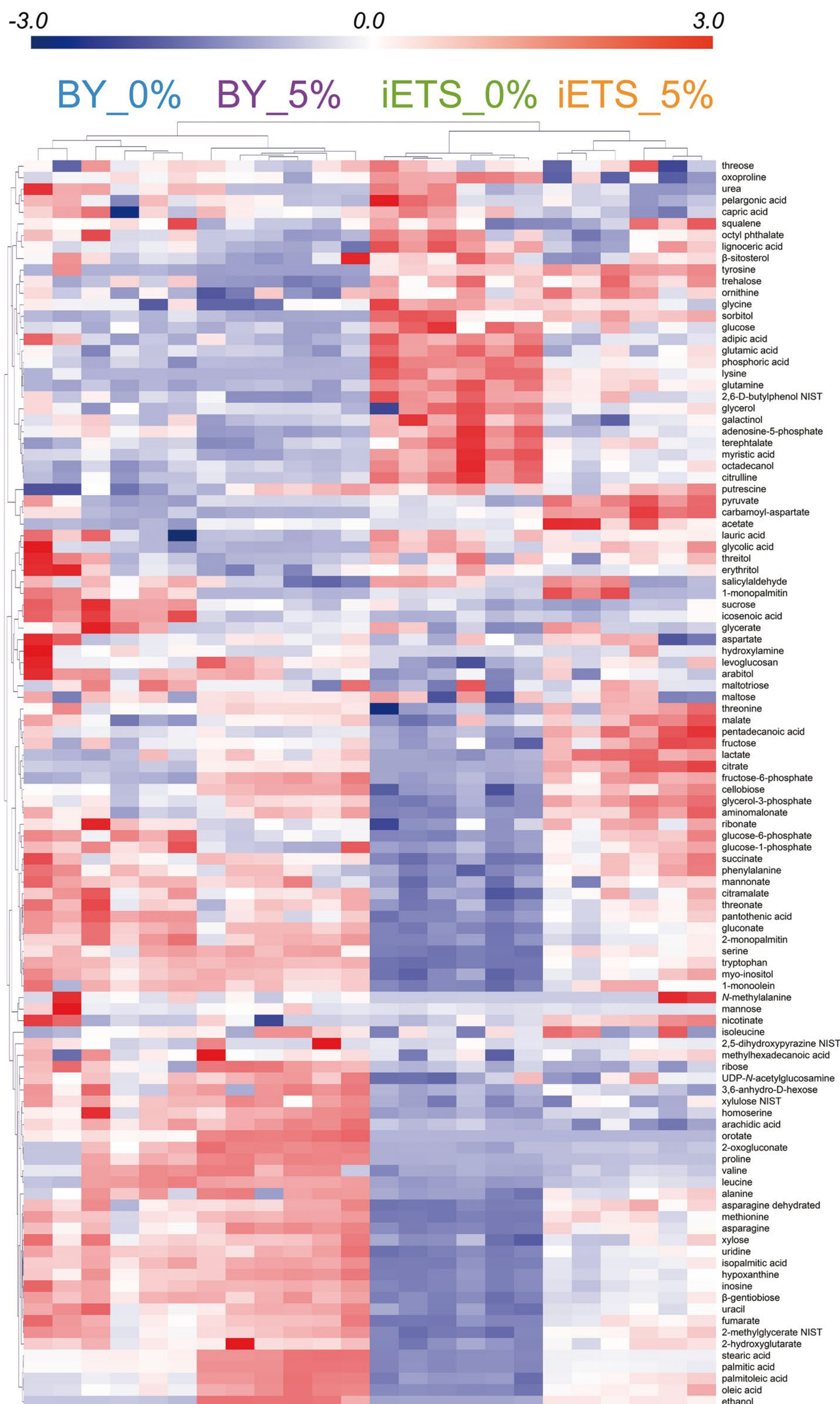

Figure 3. Hierarchical clustering of intracellular metabolite profiles of *S. cerevisiae* iETS3 or BY4741 cultivated in YPD medium with or without ethanol. BY_0%, BY4741 without ethanol; iETS_0%, iETS3 without ethanol; BY_5%, BY4741 with ethanol (5% v/v); and iETS_5%, iETS3 with ethanol (5% v/v).

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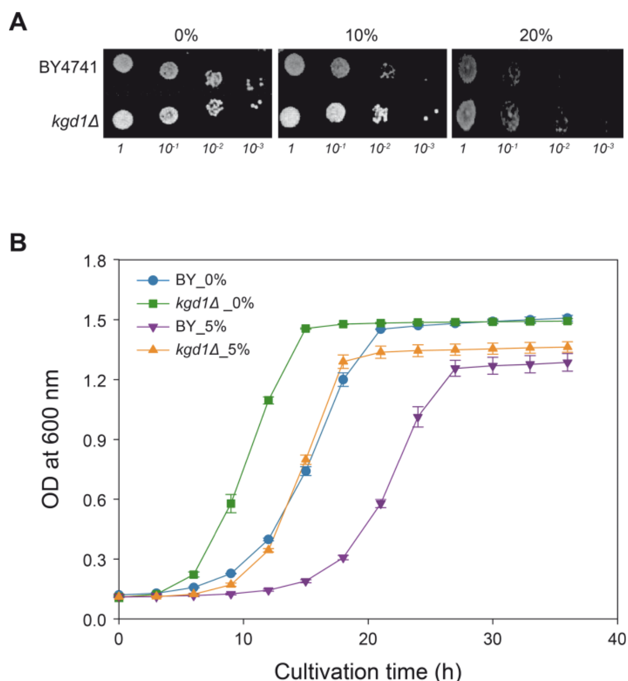

Figure 4. (**A**) Spot assay of *S. cerevisiae* BY4741 and *kgd1*Δ mutant on YSCD media containing 0, 10, and 20% ethanol. 1, cells without dilution; 10^{-1}, 10-fold dilution; 10^{-2}, 100-fold dilution; 10^{-3}, 1000-fold dilution. (**B**) Growth profile of *S. cerevisiae* BY4741 (wild type) and *kgd1*Δ mutant (ethanol-tolerant mutant). BY_0%, BY4741 grown in YPD medium without ethanol; *kgd1*Δ _0%, *kgd1*Δ mutant grown in YPD without ethanol; BY_5%, BY4741 grown in YPD with ethanol (5% v/v); and *kgd1*Δ_5%, *kgd1*Δ mutant grown in YPD with ethanol (5% v/v).

concentration. The *kgd1*Δ mutant also had a shorter lag than the wild type in the presence of 5% ethanol (Fig. 4B). We found that the intensity of metabolites related to glutamate metabolism in the *kgd1*Δ mutant was 1.7 to 3.1-fold higher than that of BY4741 (Supporting information, Fig. S2), suggesting that the change of glutamate metabolism affected ethanol tolerance in this strain. These data clearly demonstrated that glutamate metabolism is a possible metabolic signature responsible for ethanol tolerance in iETS3.

4 Discussion

S. cerevisiae iETS3 is an ethanol-tolerant mutant strain of *S. cerevisiae* BY4741 and has been previously studied [10, 24]. However, the mechanism of phenotypic differences between the two strains has not been revealed. To investigate metabolic changes and identify metabolic traits for ethanol tolerance in yeast, metabolite profiles of *S. cerevisiae* BY4741 and iETS3 were conducted using GC/TOF MS. The metabolite profiles were significantly different between BY4741 and iETS3 by PCA and HCA. Using the metabolomic analysis results, glutamate metabolism, trehalose synthesis, and compositional changes in cell membranes were found to be associated with ethanol tolerance in yeast.

Especially, in this study, the levels of glutamate, glutamine, citrulline, ornithine, and putrescine, which are directly or indirectly associated with glutamate metabolism, significantly increased in iETS3 compared to BY4741, indicating the activation in glutamate metabolism in the ethanol tolerant strain. In previous studies, production of putrescine was increased upon tolerance of osmotic, pH, oxidative, and salt stresses [33–35]. However, there have been few studies on stress tolerance in yeast related to glutamate metabolism. Metabolomics can be used for functional genomics because metabolome data can be often correlated with functional genomics [36, 37]. Based on these metabolic results, we set out to increase the production of metabolites associated with glutamate metabolism; therefore, we obtained the *kgd1*Δ mutant from Open Biosystems and found that this strain was more ethanol tolerant than wild type. These results clearly revealed that the *kgd1*Δ, which activates glutamate metabolism, enhances tolerance to ethanol.

Trehalose is converted from glucose-6-phosphate [38], and it plays key roles in reducing membrane permeability and ensuring proper folding of proteins [26, 39]. The accumulation of trehalose was previously found to confer tolerance to oxidative damage under ethanol and heat stresses [31]. In an ethanol-tolerant mutant of sake yeast, the abundance of trehalose, as well as the expression of *TPS1* and *TPS2* genes involved in the biosynthesis of trehalose, were higher than in the wild type [32]. In addition, in *Escherichia coli*, a high cellular level of trehalose led to significant improvement in cell growth under stress [40]. In this study, trehalose synthesis was found to be up-regulated in the ethanol-tolerant mutant. The intracellular abundances of trehalose and glucose-6-phosphate in iETS3 were 1.4-fold higher and 3.7-fold lower, respectively, than those in BY4741, implying that glucose-6-phostate was more actively converted to trehalose in iETS3 than in BY4741. Trehalose may function as a protectant under ethanol stress in iETS3 and might have improved the cell growth of this strain. These results indicate that active trehalose synthesis in iETS3 confers ethanol tolerance.

Mono-unsaturated fatty acids, including palmitoleic acid and oleic acid, are important components of cell membranes in *S. cerevisiae*. A high concentration of ethanol in the culture medium is known to affect the integrity and fluidity of the cell membrane [41]. A previous study revealed that the abundance ratio of mono-unsaturated fatty acids was higher in ethanol-tolerant yeast strains to prevent interdigitation and to maintain optimal membrane thickness [29, 30]. In accordance with previous studies, herein, the abundance ratio of mono-unsaturated fatty acids, such as palmitoleic acid and oleic acid, in iETS3 was 1.5-fold higher than in BY4741. This sug-

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gests that in the ethanol-tolerant iETS3 strain, the metabolism of mono-unsaturated fatty acids is more active, to maintain membrane integrity. In conclusion, the metabolic results of this study demonstrated that global metabolite profiling could be a powerful tool for phenotypic analysis of microorganisms under external stress. In addition, these metabolic markers could be important targets for further improvement and development of ethanol-tolerant strains at the molecular level.

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The authors declare no conflict of interest for this study.

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Cover illustration

This regular issue of BTJ includes articles on bioprocess engineering and biochemical engineering. Also it contains two special articles to the topic "Eukaryotic Synthetic Biology". The cover shows mouse embryonic stem cells proliferating in a microfluidic culture device capable of monitoring specific oxygen uptake rates in real time. Monitoring occurs without disruption of the cell culture and label-free. Image is provided by Alexandre Super, Nicolas Jaccard, Marco Paulo Cardoso Marques, Rhys Jarred Macown, Lewis D. Griffin, Farlan Singh Veraitch and Nicolas Szita authors of "Real-time monitoring of specific oxygen uptake rates of embryonic stem cells in a microfluidic cell culture device" (http://dx.doi.org/10.1002/biot.201500479).

Biotechnology Journal – list of articles published in the September 2016 issue.

Forum
Tailor-made biocatalysts enzymes for the fine chemical industry in China
Yu Jiang, Rongsheng Tao and Sheng Yang
http://dx.doi.org/10.1002/biot.201500682

Commentary
Omics insights into production-scale bioreactors
Susan T. Sharfstein
http://dx.doi.org/10.1002/biot.201600338

Review
Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production
Fabian Steinebach, Thomas Müller-Späth and Massimo Morbidelli
http://dx.doi.org/10.1002/biot.201500354

Biotech Method
Facile construction of random gene mutagenesis library for directed evolution without the use of restriction enzymes in *Escherichia coli*
Jae-Eung Kim, Rui Huang, Hui Chen, Chun You and Y.-H. Percival Zhang
http://dx.doi.org/10.1002/biot.201600121

Biotech Method
Augmenting Chinese hamster genome assembly by identifying regions of high confidence
Nandita Vishwanathan, Arpan A. Bandyopadhyay, Hsu-Yuan Fu, Mohit Sharma, Kathryn C. Johnson, Joann Mudge, Thiruvarangan Ramaraj, Getiria Onsongo, Kevin A. T. Silverstein, Nitya M. Jacob, Huong Le, George Karypis and Wei-Shou Hu
http://dx.doi.org/10.1002/biot.201500455

Research Article
The MYpop toolbox: Putting yeast stress responses in cellular context on single cell and population scales
Thomas Spiesser, Clemens Kühn, Marcus Krantz and Edda Klipp
http://dx.doi.org/10.1002/biot.201500344

Research Article
Cell-to-cell heterogeneity emerges as consequence of metabolic cooperation in a synthetic yeast community
Kate Campbell, Jakob Vowinckel and Markus Ralser
http://dx.doi.org/10.1002/biot.201500301

Research Article
Real-time monitoring of specific oxygen uptake rates of embryonic stem cells in a microfluidic cell culture device
Alexandre Super, Nicolas Jaccard, Marco Paulo Cardoso Marques, Rhys Jarred Macown, Lewis D. Griffin, Farlan Singh Veraitch and Nicolas Szita
http://dx.doi.org/10.1002/biot.201500479

Research Article
Combined metabolomics and proteomics reveals hypoxia as a cause of lower productivity on scale-up to a 5000-liter CHO bioprocess
Yuanwei Gao, Somak Ray, Shujia Dai, Alexander R. Ivanov, Nicholas R. Abu-Absi, Amanda M. Lewis, Zhuangrong Huang, Zizhuo Xing, Michael C. Borys, Zheng Jian Li and Barry L. Karger
http://dx.doi.org/10.1002/biot.201600030

Research Article
Porting the synthetic D-glucaric acid pathway from *Escherichia coli* to *Saccharomyces cerevisiae*
Amita Gupta, Michael A. Hicks, Shawn P. Manchester and Kristala L. J. Prather
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Research Article

Production of a tumor-targeting antibody with a human-compatible glycosylation profile in *N. benthamiana* hairy root cultures

Chiara Lonoce, Reda Salem, Carla Marusic, Philippe V. Jutras, Andrea Scaloni, Anna Maria Salzano, Sergio Lucretti, Herta Steinkellner, Eugenio Benvenuto and Marcello Donini

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Research Article

Elucidation of ethanol tolerance mechanisms in *Saccharomyces cerevisiae* by global metabolite profiling

Sooah Kim, Jungyeon Kim, Ju Hwan Song, Young Hoon Jung, Il-Sup Choi, Wonja Choi, Yong-Cheol Park, Jin-Ho Seo and Kyoung Heon Kim

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Research Article

Enabling xylose utilization in *Yarrowia lipolytica* for lipid production

Haibo Li and Hal S. Alper

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