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Changes and roles of membrane compositions in the adaptation of *Saccharomyces cerevisiae* to ethanol

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Bioethanol fermentation by *Saccharomyces cerevisiae* is often stressed by the accumulation of ethanol. Cell membrane is the first assaulting target of ethanol. Ethanol-adapted *S. cerevisiae* strains provide opportunity to shed light on membrane functions in the ethanol tolerance. This study aimed at clarifying the roles of cell membrane in the ethanol tolerance of *S. cerevisiae* through comparing membrane components between *S. cerevisiae* parental strain and ethanol-adapted strains. A directed evolutionary engineering was performed to obtain the ethanol-adapted *S. cerevisiae* strains. The parental, ethanol-adapted M5 and M10 strains were selected to be compared the percentage of viable cells after exposing to ethanol stress and cell membrane compositions (i.e., ergosterol, trehalose, and fatty acids). Compared with the parental strain, M5 or M10 strain had higher survival rate in the presence of 10% v/v ethanol. Compared with that in the parental strain, contents of trehalose, ergosterol, and fatty acids increased about 15.7, 12.1, and 29.3%, respectively, in M5 strain, and about 47.5, 107.8, and 61.5%, respectively, in M10 strain. Moreover, expression differences of genes involved in fatty acids metabolisms among the parental, M5 and M10 strains were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), and results demonstrated that M5 or M10 strain had higher expression of *ACC1* and *OLE1* than the parental strain. These results indicated that although being exposed to step-wise increased ethanol, *S. cerevisiae* cells might remodel membrane components or structure to adapt to the ethanol stress.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

With the depletion of traditional petrochemical fuels and the increasing concerns of environmental issues, renewable biofuels including bioethanol would be of great strategic importance [1]. Application of bioethanol would contribute to the alleviating of environmental pollution and can ease off the energy crisis [2, 3]. *Saccharomyces cerevisiae* is the most used fermentative microorganism for bioethanol production [4]. However, bioethanol fermentation by *S. cerevisiae* is often stressed by the accumulation of ethanol which is considered to be the major hindrance to the complete bioethanol fermentation [4–6].

Under ethanol stress, *S. cerevisiae* cell membrane is one of the targets of ethanol [7]. Yeast cell membrane lipid compositions play important functions in counteracting against ethanol stress and keeping cell viability. For example, levels of trehalose [8, 9], ergosterol [5, 10], oleic acid, palmitoleic acid, and phosphatidylinositol (PI) in the plasma membrane [5, 11] can increase in response to ethanol stress and have been reported to promote *S. cerevisiae* cell viability under ethanol stress and confer ethanol tolerance to the cells. Some previous papers have reported that stability of the cell wall or membrane can be a major factor contributing to the increased tolerance to ethanol in *S. cerevisiae* [12].

Although much progress has been made, there are still some problems needed to be further clarified. For example, previous studies about the function of

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membrane lipid compositions in the ethanol tolerance of *S. cerevisiae* are still controversial. Ethanol affects the fluidity of the plasma membrane [5], the integrity of the cell membrane of *S. cerevisiae* was compromised [7] and the membrane structure becomes loose under ethanol stress. One view suggested that the factors such as ergosterol that make the plasma membrane more rigid are important for cells to maintain membrane stability and survival under conditions of ethanol stress. Moreover, our previous results also suggested that *S. cerevisiae* cells changed the levels of hexadecanoic, octadecanoic, and palmitoleic acids, to try to maintain the integrity of their plasma membrane through decreasing membrane fluidity in the presence of ethanol stress [13]. However, another view indicated that maintaining membrane fluidity is essential to maintaining the *S. cerevisiae* cell viability [14].

Moreover, although *S. cerevisiae* has evolved a series of stress responses to ethanol stress to maintain the cell survival [6, 15], *S. cerevisiae* growth is still inhibited by accumulated ethanol in the bioethanol fermentation. Therefore, to increase the final bioethanol yield, ethanologenic strains with higher tolerance to ethanol stress are highly desirable [12, 16, 17]. Some methods have been attempted to develop the ethanol-tolerant or adapted *S. cerevisiae* strain. Among these approaches, exposing *S. cerevisiae* cells to a stepwise increase in the concentration of ethanol is an effective way to obtain high ethanol-adapted *S. cerevisiae* strains [15, 18]. Microorganisms including yeast cells can tolerate and adapt to environmental perturbations through direct evolutionary engineering with a gradual inducing progress [4, 15]. For example, an ethanol-tolerant strain *S. cerevisiae* NRRL Y-50316 was obtained through enforced evolutionary adaption [4]. The ethanol-sensitive strain *S. cerevisiae* W303-1A was subjected to three rounds of turbidostat with increasing contents of external ethanol to select ethanol tolerant mutants [12, 19]. In our previous work, a set of ethanol-adapted *S. cerevisiae* strains (included M5 and M10 strains which are, respectively, adapted to 5 and 10% v/v ethanol) was also generated through a directed evolutionary engineering and showed higher tolerance to ethanol than the parental strain. Ethanol-adapted *S. cerevisiae* strains also provided the opportunity to shed some light on the functions of membrane compositions accounting for the improved ethanol tolerance of *S. cerevisiae*. The understanding of such functions will facilitate the rational design of industrial ethanologenic strains with higher ethanol yield [15, 20].

In this study, the parental strain, ethanol-adapted M5 and M10 strains were selected to be compared the percentage of viable cells after exposing to ethanol stress and cell membrane compositions. Moreover, expression differences of genes involved in fatty acid (FA) metabolisms among the parental strain, ethanol-adapted M5 and M10 cells were also analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), thereby contributing to a better understanding of the functions of membrane compositions in the ethanol tolerance of *S. cerevisiae*.

Materials and methods

Strain, media, and culture conditions

S. cerevisiae CEC B9S-15 used in this study was cultured in YPD broth (2% glucose, 1% yeast extract, 2% peptone) at 30 °C and shaken at 170 rpm in 250 ml cotton-plugged flasks.

To obtain ethanol-adapted *S. cerevisiae* strains, repetitive cultivation was carried out in the YPD broth with an increase of ethanol concentration in 1% v/v increment. *S. cerevisiae* CEC B9S-15 was precultured in YPD broth at 30 °C for 24 h. Then the precultured yeast cells were transferred to the fresh medium containing 1% v/v ethanol and the optical density of the initial cells determined at 600 nm was adjusted to 0.1. After the repetitive cultivations for stable growth under 1% ethanol has been completed, the culture was sequentially transferred to the fresh YPD broth containing a higher ethanol concentration, followed by repetitive cultivations under the same ethanol concentration. Such repetitive cultivations were performed till the ethanol concentration changed to 10% v/v. The obtained ethanol-adapted *S. cerevisiae* strains were named as M1, M2, M3, M4, M5, M6, M7, M8, M9, and M10, respectively. Ethanol-adapted M5 and M10 strains were selected for subsequent experiments.

Determination of growth curve

The growth curve of the *S. cerevisiae* parental strain cells, M5 and M10 cells cultured in the YPD broth either with or without ethanol was determined every 12 h by measuring the optical density of the culture at 600 nm with a 752 visible spectrophotometer (Shanghai Optical Instrument Factory, Shanghai, China). For the ethanol-treated cells, 5 or 10% v/v ethanol (final concentration) was, respectively, added to the YPD broth at the beginning of the primary culture. The experiment was conducted in triplicate.

Determination of cell survival rate

Precultured CEC B9S-15 parental strain cells, M5 and M10 cells were, respectively, exposed to a challenge with 10% v/v ethanol for 0.5 and 2 h, respectively. After being exposed to ethanol stress, the CFU of parental strain

cells, M5, and M10 cells were counted using plate assay. In comparison with the control treatment without ethanol stress, the percentage of viable yeast cells stressed by 10% v/v ethanol was calculated. The experiment was conducted in triplicate.

Determination of ergosterol content

S. cerevisiae CEC B9S-15 parental strain cells, M5, and M10 cells were, respectively, cultivated in YPD broth without or with 10% v/v ethanol. Cells were harvested after 8 h of incubation and dried in vacuum drying oven. After addition of 0.8 g KOH and 3.2 ml of 60% v/v ethanol into 50 ml ground-in conical flask containing 0.1 g dried cells, the samples were saponified at 80 °C for 2 h and cooled to room temperature. One milliliter of deionized water was added into the sample before extraction (vigorous shaking for 15 min) with 2.5 ml petroleum ether. After static layering, the supernatant was transferred to tube and stored (−20 °C) for measurement of ergosterol content by liquid chromatography (LC) analysis. Before LC analysis, the sample was filtered through 0.22 μm microporous membrane. LC analysis was performed on a LC-20A HPLC system (Shimadzu Co., Japan) equipped with two LC-20AT pumps and a SPD-20A UV-Vis detector and a CBM-102 chromatography work station. The chromatographic column was Diamonsil C18 column (5 μm, 4.6 × 250 mm) which was maintained at 30 °C. The mobile phase was methanol/water (97:3, v/v). The flow rate was 1.8 ml min^{-1} and injection volume was 20 ml. The peak was monitored at 280 nm. The experiment was conducted in triplicate.

Determination of trehalose content

S. cerevisiae CEC B9S-15 parental strain cells, M5 and M10 cells were, respectively, cultivated in YPD broth without or with 10% v/v ethanol. Twenty milliliters of the cultures were harvested after 8 h of incubation and disrupted by ultrasonication in SCIENTZ IID Noise Isolating Tamber (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China). Samples were sonicated for 20 min in pulse mode (40 s on and 20 s off) at 20 kHz. After standing for 6 h at room temperature, samples were centrifuged at 5000 rpm for 10 min. The supernatant was filtered through 0.22 μm microporous membrane and used for measurement of trehalose content by LC analysis. LC analysis was performed on a Waters Sugar-Pak1 column (Waters, USA) and a refractive index detector (0.5 ml min^{-1}, 80 °C). The experiment was conducted in triplicate.

Moreover, Pearson correlation analysis was performed using SPSS13.0 software to determine the connection between trehalose content and ergosterol content.

Determination of free fatty acids

S. cerevisiae CEC B9S-15 parental strain cells, M5, and M10 cells were, respectively, cultivated in YPD broth containing 10% v/v ethanol. Cultured cells were harvested by centrifugation (5000 rpm, 10 min) after 8 h of incubation. According to 6 ml HCl (4 mol L^{-1}) per gram harvested cells, HCL was added into and turbulence mixed with the sample. After standing for 30 min at room temperature, the sample was placed at boiling water bath for 3 min and immediately cooled at −20 °C. Double volumes of chloroform-methanol (1:1, v/v) were added into the sample prior to centrifugation (5000 rpm, 5 min). The chloroform layer was collected and equal volume of 0.1% NaCl was added into the chloroform layer before centrifugation (5000 rpm, 5 min). The supernatant was collected and stored at −20 °C until use.

Samples were dried through evaporation. For esterification, 2 ml of sulfuric acid in methanol (1% v/v) was added for every 50 mg dried samples prior to incubation at 70 °C for 60 min. FA methyl ester was extracted with 2 ml n-hexane twice. The supernatants from above two n-hexane extraction steps were pooled together and stored at −20 °C until gas chromatography (GC) analysis of free fatty acids (FFAs) contents. GC analysis was performed on a GC system (Shimadzu Co., Japan) equipped with a DB-FFAP capillary column (30 m × 320 μm i.d., 0.5 μm film thickness; Agilent J&W Scientific, Folsom, CA). Samples (1 μL) were injected into the DB-FFAP capillary column by split injection mode with a split ratio of 30:1 using an AOC-20i autoinjector (Shimadzu Co., Japan). The flow-rate of the carrier gas, helium, was 8.9 ml min^{-1} and that of hydrogen and air supplied to the detector 40 and 400 ml min^{-1}, respectively. The temperature of the injection block was 250 °C and that of the FID 280 °C. The initial oven temperature was heated to 50 °C for 1 min, raised to 150 °C at a rate of 10 °C min^{-1}, maintained at 150 °C for 2 min, raised to 228 °C at a rate of 5 °C min^{-1}, and then maintained at 228 °C for 32 min. The experiment was conducted in triplicate.

Reverse transcription of total RNA and quantitative real-time PCR

Two genes which are responsible for encoding rate-limiting enzymes in FAs related metabolisms were chosen for detection in this study. Specifically, *ACC1* and *OLE1*, which are, respectively, responsible for encoding acetyl-CoA carboxylase and stearoyl-CoA 9-desaturase, were chosen. For measurement of expression of *ACC1* and *OLE1*, samples were harvested from exponential phase cells of *S. cerevisiae* CEC B9S-15 parental, M5 and M10 strains cultured in the YPD broth

with or without 10% v/v ethanol. Pellets were quickly frozen in liquid nitrogen for RNA extraction or store at −80 °C until use. Total RNA in *S. cerevisiae* cells was extracted according to the manual instruction of Trizol (Invitrogen, USA), and used for synthesis of cDNA. RNA integrity and purity were evaluated by gel electrophoresis and OD_{260}/OD_{280} ratio. cDNA was synthesized using the extracted RNA, M-MLV reverse transcriptase, and random primer according to the manual instruction (Promega, USA). cDNA from this step was stored at −20 °C for using as the template for qRT-PCR. Relative quantification of genes was performed on an Eppendorf Mastercycler® RealPlex^2. Primers of selected ethanol metabolism related genes and β-actin (*ACT1*) were designed using Primer Premier 5 (https://www.PremierBiosoft.com) with manual editing, and listed in Table 1. For each amplification reaction, a total of 20 μl was used consisting of 2.5 μl cDNA template, 10 μl real SYBR mixture, 0.3 μl of forward, and reverse primer (10 μM each), and 6.9 μl H_2O. Amplifications were performed after an initial denaturation at 95 °C for 5 min followed by 40 cycles of PCR at 95 °C for 15 s, at 60 °C for 25 s, and 72 °C for 30 s. The melting curve of each PCR product was determined to ensure the specific amplification of the target gene. The mRNA levels of the selected genes were normalized to that of *ACT1* gene in the same sample. The mean fold change in abundance of transcripts was determined according to the method of Livak and Schmittgen [21]. The experiment was conducted in triplicate.

Statistical analysis

Data were analyzed by independent-samples *T*-test with SPSS13.0 for Windows, and standard error of the mean (SEM) was used error bar. Differences showing p values less than 0.05 were considered statistically significant.

Results

Cell growth differences among the *S. cerevisiae* parental, M5, and M10 strains

In the *S. cerevisiae* parental strain cultures, the addition of 5 or 10% v/v ethanol inhibited the cell growth (Supporting Information Fig. S1a). Though M5 or M10 has adapted to 5 or 10% v/v ethanol, respectively, these two strains were also inhibited by 5 or 10% v/v ethanol contained in the medium (Supporting Information Figs. S1b and c). In the case of no ethanol stress, the difference in growth among the parental strain, M5 and M10 was not significant ($p > 0.05$) (Fig. 1a). However, under the same ethanol stress condition (i.e., 5 or 10% v/v ethanol), the growth of M5 or M10, especially M10 strain, was superior to that of the parental strain ($p < 0.05$) (Fig. 1b and c).

Differences of cell adaption to ethanol among the *S. cerevisiae* parental, M5, and M10 strains

The survival rates of parental, M5, and M10 cells in the presence of 10% v/v ethanol stress all decreased with the time (Fig. 2). After having been exposed to 10% v/v ethanol for 0.5 h (Fig. 2a) or 2 h (Fig. 2b), both M5 and M10 cells had a much higher survival rate than that of the parental strain cells ($p < 0.001$ for both 0.5 and 2 h). Moreover, M10 cells also survived much more than M5 cells after having been exposed to the challenge of 10% v/v ethanol for both 0.5 h ($p < 0.001$) and 2 h ($p < 0.001$) (Fig. 2).

Differences of ergosterol content among the *S. cerevisiae* parental, M5, and M10 strains

Compared with that in the parental strain, the ergosterol content of M5 or M10 in the absence or presence of 10% v/v ethanol stress increased obviously ($p < 0.001$) (Fig. 3). Moreover, M10 strain had much higher ergosterol content than M5 strain ($p < 0.001$) (Fig. 3).

Differences of trehalose content among the *S. cerevisiae* parental, M5, and M10 strains

Compared with that in the parental strain and M5 strain, the trehalose content of M10 in the absence ethanol stress increased obviously ($p < 0.05$) (Fig. 4a). However, in the case of no ethanol stress, the difference in trehalsoe between the parental strain and M5 strain was not significant ($p > 0.05$) (Fig. 4a). Compared with that in the parental strain, the trehalose content of M5 or M10 in the presence of 10% v/v ethanol stress increased obviously ($p < 0.05$ for M5, $p < 0.001$ for M10) (Fig. 4b).

Table 1. Oligonucleotides used in this study.

Primers	Sequences (5′–3′)
ACC1-F/R	GTTATTTCTAAGGAACTGGAATGGACC/TTGATGCTTCGCCTACCTGATG
ACT1-F/R	CTCCAATGAACCCTAAATCAAACAG/CGGAAGAGTACAAGGACAAAACG
OLE1-F/R	TCTACGCTATCTTCGGTTGTGCT/CCTTGTATTTTGGATTTGGCTTCA

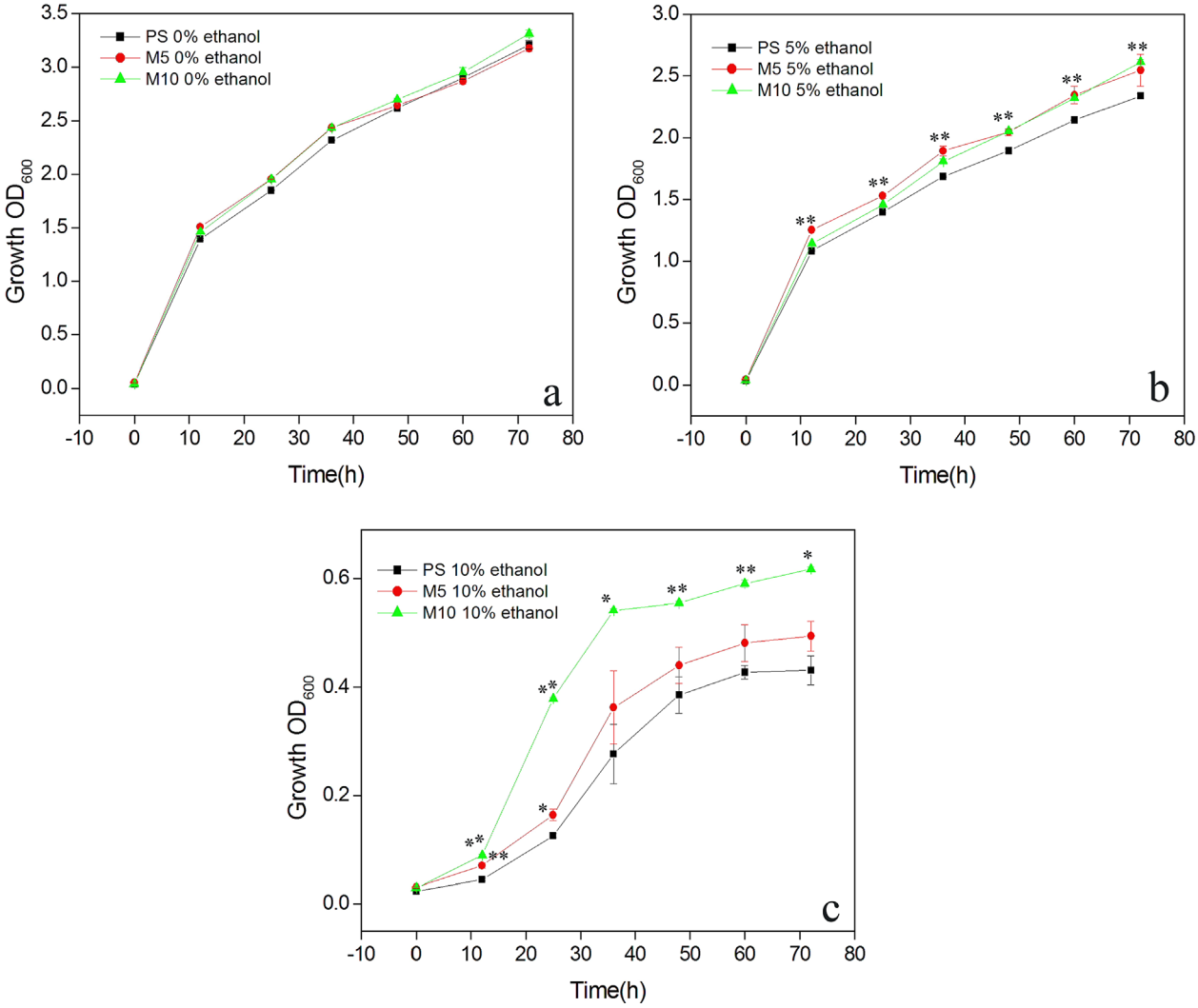

Figure 1. Growth differences of *S. cerevisiae* the parental, M5 and M10 strains in the absence or presence of ethanol. Growth curve in the (a) absence of ethanol, (b) presence of 5% v/v ethanol, and (c) presence of 10% v/v ethanol. $^{*}p < 0.05$ compared to that parental strain; $^{**}p < 0.01$ compared to the parental strain.

Moreover, M10 strain had much higher trehalose content than M5 strain ($p < 0.01$) (Fig. 4b).

Differences of contents of fatty acids among the *S. cerevisiae* parental, M5, and M10 strains

The content differences of FAs among the parental, M5, and M10 strains under the same incubation conditions with exogenous ethanol stress were compared. Under the culture condition with 10% v/v ethanol stress, both M5 and M10 had higher contents of hexadecanoic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), octadecanoic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$), and total FFAs than the parental strain ($p < 0.01$); moreover, compared with that in M5 strain, contents of hexadecanoic acid, octadecanoic acid, oleic acid, and total FFAs all increased in M10 strain ($p < 0.01$) (Fig. 5a; Supporting Information Fig. S2). Furthermore, interestingly, compared with that parental strain, M5, and M10 had higher percent of unsaturated fatty acids (UFAs) ($p < 0.001$) but lower percent of saturated fatty acids (SFAs) ($p < 0.001$) (Fig. 5b).

Quantitative analysis of transcriptional levels of the parental, M5, and M10 strains

In order to gain insights into the function of FFAs in the higher tolerance of M5 or M10 to ethanol than that of the parental strain, differences of transcriptional levels of *ACC1* and *OLE1* which are, respectively, responsible for encoding rate-limiting enzymes in biosynthesis of FFAs

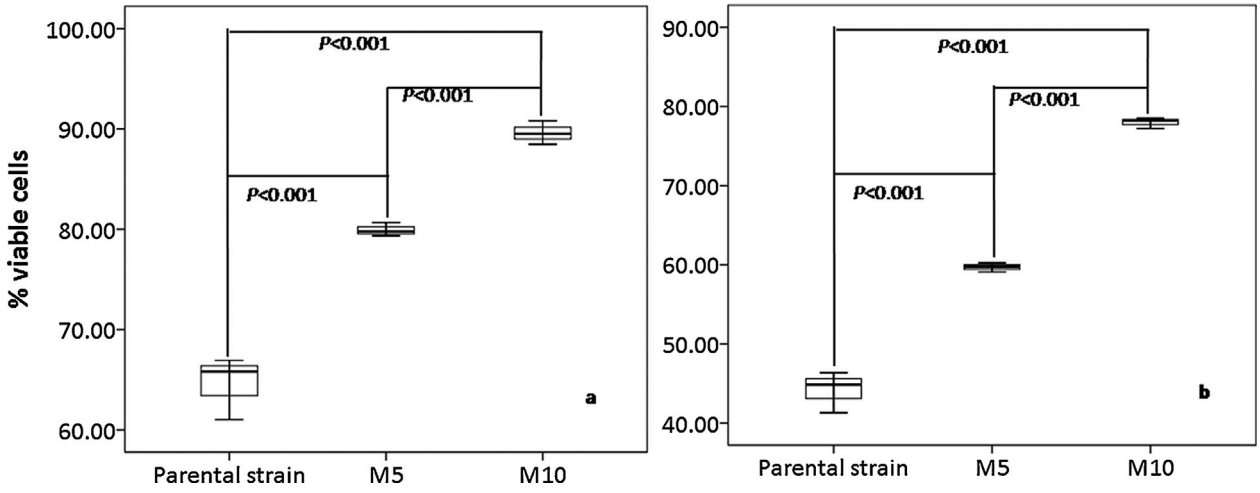

Figure 2. Survival rate of the *S. cerevisiae* parental, M5 and M10 cells after exposing to 10% v/v ethanol for (a) 0.5 h and (b) 2 h.

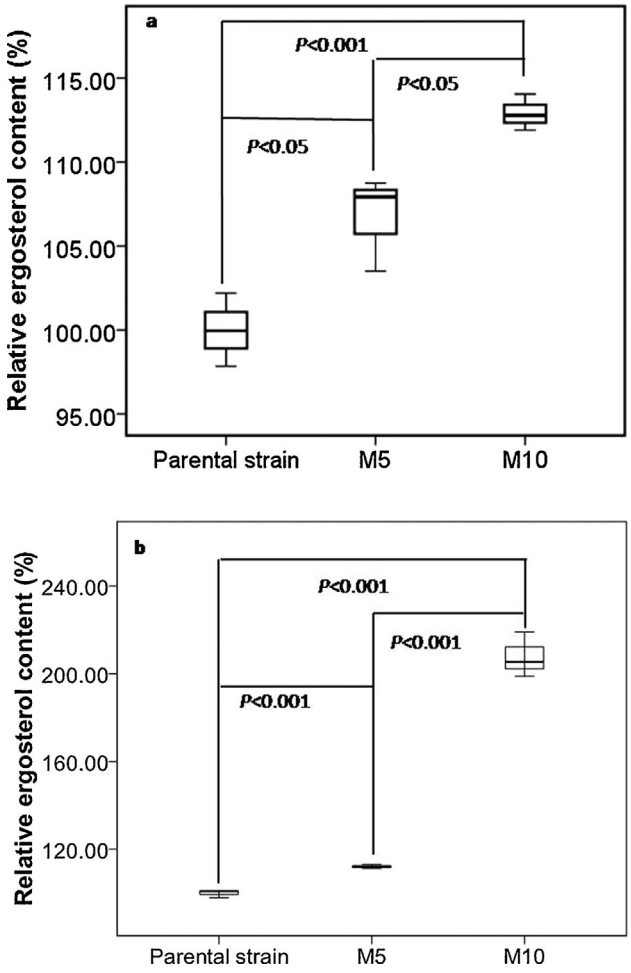

Figure 3. Differences of content of ergosterol in the (a) absence or (b) presence of 10% v/v ethanol stress among the *S. cerevisiae* parental, M5, and M10 strains.

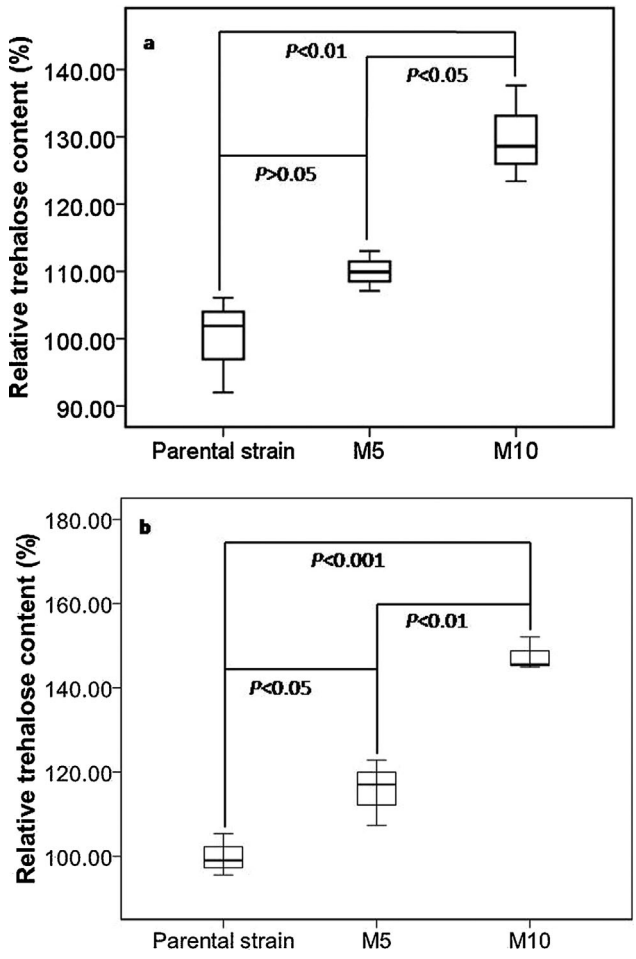

Figure 4. Differences of content of trehalose in the (a) absence or (b) presence of 10% v/v ethanol stress among the *S. cerevisiae* parental, M5, and M10 strains.

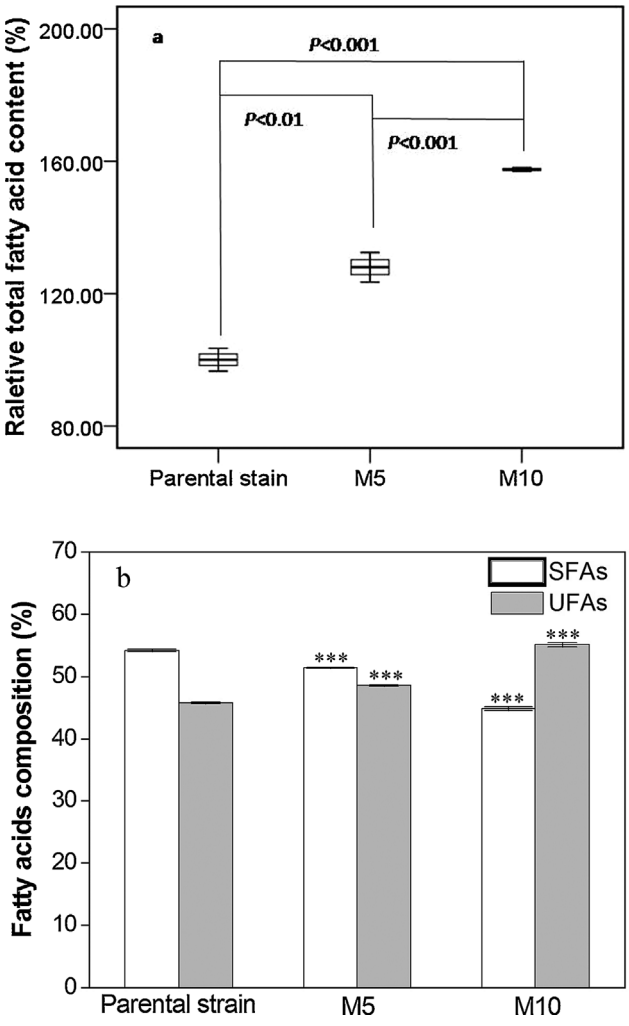

Figure 5. Differences of (a) total fatty acid content and (b) percentages of saturated and unsaturated fatty acids among the *S. cerevisiae* parental, M5, and M10 strains in the presence of 10% v/v ethanol. ***$P<0.01$ compared to the parental strain.

and monounsaturated fatty acid (MUFAs) at the exponential phase between the parental strain and M5 or M10 strain under the same incubation condition with or without 10% v/v exogenous ethanol stress were determined by qRT-PCR. Under the culture with or without 10% v/v ethanol, both M5 and M10 strains had higher expressions of *ACC1* and *OLE1* than the parental strain ($p < 0.05$) (Fig. 6).

Discussion

To increase the final bioethanol production, ethanologenic strains with higher tolerance to ethanol stress are highly desirable [16, 17]. Through a directed evolutionary engineering, a set of ethanol-adapted *S. cerevisiae* strains including M5 and M10 strains used in this study was created from the parental strain. Though being inhibited by 10% v/v ethanol (Supporting Information Figs. S1b and c), the growth of M5 or M10 was still superior to that of the parental strain in the presence of 5 or 10% ethanol ($p < 0.05$) (Fig. 1b and c). Moreover, compared with that of the parental strain, much higher cell survival rate of M5 and M10, especially M10 strain, in the presence of 10% v/v ethanol stress illustrated more tolerance or adaptation of M5 and M10 to ethanol stress than that of the parental strain (Fig. 2).

Ethanol stress has been reported to have negative effects on *S. cerevisiae* plasma membrane associated processes [22], and our previous results also indicated that the yeast cell membrane is the first assaulting target of ethanol [7]. To survive under the ethanol stress, *S. cerevisiae* cells might regulate their membrane compositions to stabilize the plasma membrane structure [5, 23]. The cell disruption results (Fig. 2) might preliminarily suggest better protective function of cell membrane of ethanol-adapted M5 and M10 strains than that of parental strain. Moreover, to some extent, such protection might be the consequence of remodeling of the structure or compositions of M5 or M10 strain during the directed evolution. In other words, during the domestication process, the ethanol-adapted M5 or M10 strains might change the plasma membrane components or other intracellular compositions to acquire higher ethanol tolerance and counteract the ethanol stress. Indeed, the membrane compositions of M5 or M10 strain did change in comparison to the parental strain whether the ethanol stress was present or not. The contents of trehalose and ergosterol of M5 strain increased about 13.0 and 6.7%, respectively, than that in the parent strain; and the contents of trehalose and ergosterol of M10 strain also increased about 29.9 and 12.9%, respectively, than that in the parent strain (Figs. 3a and 4a). The ethanol stress further promoted the increase tendency of the membrane components. The contents of trehalose, ergosterol, and FAs of M5 strain increased about 15.7, 12.1, and 29.3%, respectively, than that in the parent strain; and the contents of trehalose, ergosterol, and FAs of M10 strain also increased about 47.5, 107.8, and 61.5%, respectively, than that in the parent strain (Figs. 3b, 4b, and 5a). These results suggested that the regulation of cellular membrane components might be correlated with the enhanced ethanol tolerance of the ethanol-tolerant strains [24].

Ergosterol is the most abundant sterol in the *S. cerevisiae* cell plasma membrane [5, 24, 25] and plays

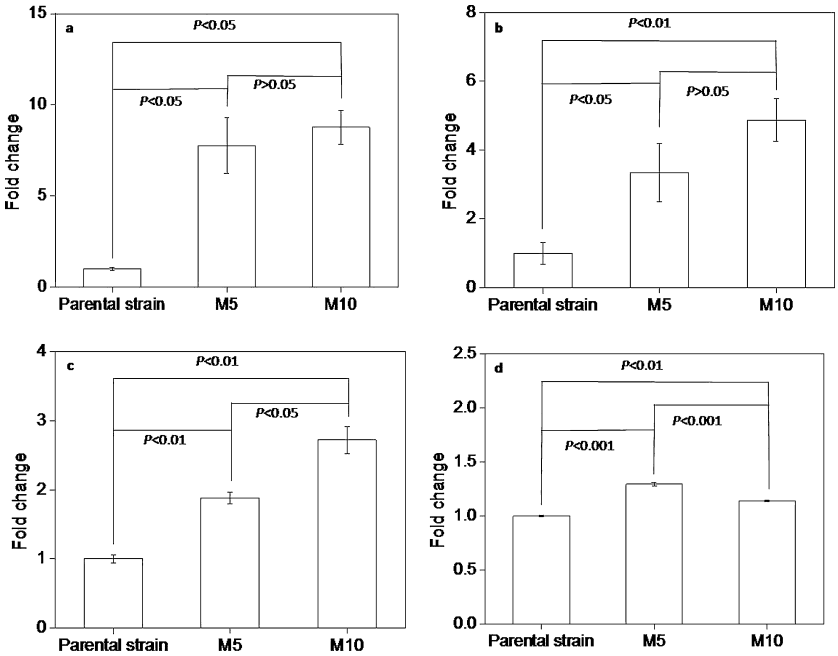

Figure 6. Change of transcriptional levels of genes involved in fatty acid related metabolisms between the *S. cerevisiae* parental strain and ethanol-adapted strains (M5 and M10) either (a and c) in the absence of exogenous ethanol or (b and d) in the presence of 10% v/v ethanol. (a and b) *ACC1*. (c and d) *OLE1*. All results were normalized to the *ACT1* transcriptional level in the same sample.

important role in stabilizing the yeast plasma membrane [6]. Ergosterol content directly correlates with ethanol tolerance of *S. cerevisiae* [24]. The ATPase activity of the plasma membrane and ethanol tolerance were proven to be correlated at the 96.64% level with oleic acid and ergosterol in yeast plasma membrane [5]. Higher ergosterol level promoted the yeast cell viability and contributed to the greater tolerance of cell to ethanol [5, 10] through increasing membrane rigidity [5] and promoting the formation of an effective membrane barrier which blocked the entry of ethanol into cells [10]. In this study, ergosterol might increase in ethanol-adapted strains in response to step-wise increased ethanol during the directed evolution process and the increased ergosterol level in ethanol-adapted M5 or M10 strain might also confer higher ethanol tolerance to *S. cerevisiae* cells and avoid significant changes in cell physiology. Being consistent with what previously reported by Aguilera and his colleagues [5], the strain with the highest ergosterol content (M10 strain in this study) was the most tolerant of ethanol.

Besides ergosterol, accumulation of trehalose could also help cells to survive stresses through stabilizing membrane and preventing the denaturation of proteins, nucleic acids and other macromolecules [24, 26]. Trehalose plays an important role in the stress tolerance of *S. cerevisiae* [9], and its content is widely considered to be an important parameter for indexing stress tolerance of yeast cells [23, 27]. Trehalose can accumulate induced by ethanol stress and function as protectant against ethanol in *S. cerevisiae* [28]. Moreover, any changes in sterols including ergosterol in the cell membrane would result in the trehalose accumulation [24, 29]. To some extent, the positive correlation between ergosterol and trehalose ($p < 0.001$, $r = 0.934$) (Supplementary Fig. S3) also confirmed the promoting effect of ergosterol on the accumulation of trehalose. The higher trehalose content in either M5 or M10 strain should be gradually induced by the step-wise increased ethanol and the accumulated ergosterol in the cell membrane, and would also contribute to the higher ethanol tolerance of M5 or M10 strain.

Moreover, FAs especially unsaturated fatty acids (UFAs) are also necessities of cell membrane and considered as the critical membrane components as ergosterol [23], and play important roles in ethanol resistance of cells [6, 30]. Another previous study even reported that membrane lipid composition played more functions in stress tolerance than ergosterol, trehalose, or HSPs [31]. *S. cerevisiae* cell contains trace or almost no polyunsaturated fatty acids (PUFAs) [14]. Palmitoleic acid and oleic acid, which are the predominant UFAs in *S. cerevisiae* [6, 32], are formed by the oxygen- and NADH-dependent desaturation of hexadecanoic acid and

octadecanoic acid catalyzed by Δ9-fatty acid desaturase (the rate-limiting enzyme of biosynthesis of MUFAs) which is encoded by *OLE1*e. Previous studies showed that oleic acid is the main UFA that can overcome the inhibitory effect of ethanol in yeast cells [6, 32]. In this study, higher transcriptional level of *ACC1*, which is responsible for encoding acetyl-CoA carboxylase (the rate-limiting enzyme of FAs synthesis), in M5 or M10 strain ($p < 0.05$) (Fig. 6) contributed to higher contents of total FAs in both M5 and M10 strains in comparison to that in the parental strain ($p < 0.01$) (Fig. 5a; Supporting Information Fig. S2). Interestingly, compared with the parental strain, higher percentage of UFAs but lower percentage of SFAs caused by higher transcriptional level of *OLE1* ($p < 0.05$) (Fig. 6) was detected in M5 or M10 strain (Fig. 5b). Increased FAs, especially the UFAs (i.e., palmitoleic acid and oleic acid) might contribute to the remodeling of membrane components and enhanced the membrane fluidity in ethanol-adapted M5 or M10 strain.

At first glance, the consequence of increased FAs especially UFAs contrasted with changes of ergosterol and trehalose contents. Higher concentrations of UFAs might lead to more membrane fluidity, and one viewpoint suggested that keeping membrane fluidity is essential to maintain the *S. cerevisiae* cell viability [14]. However, high ergosterol or trehalose content in *S. cerevisiae* was considered to be associated with decreased membrane fluidity [5]. The functions of ergosterol, trehalose, and FAs in ethanol tolerance of yeast cells might be not so straightforward. Based on these results, we proposed our hypothesis about higher ethanol tolerance of ethanol-adapted M5 or M10 strain. Ethanol stress can assault and damage the cell membrane and cause the loose membrane structure. Increased ergosterol or trehalose might maintain the integrity of cell membrane through keeping the cross-linking of different membrane molecules to antagonize the inhibitory effect of ethanol stress. In the other hand, increased UFAs enhanced the membrane fluidity in ethanol-adapted strain. Increase of membrane fluidity might make cell morphology more changeable to better adapt to the ethanol stress. In other words, although the integrity of the membrane of the parental strain was damaged by ethanol stress, the cell membrane of ethanol-adapted strain was still a cross-linked whole with increased fluidity and contributed to the maintaining of plasma membrane functionality.

In summary, compared with the parental strain, ethanol-adapted M5 or M10, especially M10 strain had higher tolerance to ethanol stress through directed evolutionary engineering. Although being exposed to step-wise increased ethanol pressure, *S. cerevisiae* cells might remodel membrane components or structure to adapt to the ethanol stress. These results highlighted our current understanding of the roles of cell membrane functioned in the tolerance of *S. cerevisiae* to ethanol. Understanding of the molecular basis of *S. cerevisiae* tolerance to ethanol will facilitate the rational design of feasible industrial ethanologenic strains with higher ethanol tolerance.

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Conflict of interest

The authors have declared no conflicts of interest.

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