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ORIGINAL RESEARCH PAPER

Deletion of *JJJ1* improves acetic acid tolerance and bioethanol fermentation performance of *Saccharomyces cerevisiae* strains

Xuechang Wu · Lijie Zhang · Xinna Jin · Yahong Fang · Ke Zhang · Lei Qi · Daoqiong Zheng

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Abstract
Objectives To improve tolerance to acetic acid that is present in lignocellulosic hydrolysates and affects bioethanol production by *Saccharomyces cerevisiae*.
Results *Saccharomyces cerevisiae* strains with improved tolerance to acetic acid were obtained through deletion of the *JJJ1* gene. The lag phase of the *JJJ1* deletion mutant BYΔJJJ1 was ~16 h shorter than that of the parent strain, BY4741, when the fermentation medium contained 4.5 g acetic acid/l. Additionally, the specific ethanol production rate of BYΔJJJ1 was increased (0.057 g/g h) compared to that of the parent strain (0.051 g/g h). Comparative transcription and physiological analyses revealed higher long chain fatty acid, trehalose, and catalase contents might be critical factors responsible for the acetic acid resistance of *JJJ1* knockout strains.
Conclusions *JJJ1* deletion improves acetic acid tolerance and ethanol fermentation performance of *S. cerevisiae*.

Keywords Acetic acid · Bioethanol · *JJJ1* · *Saccharomyces cerevisiae* · Tolerance

Introduction

Bioethanol production from agricultural and forestry residues and municipal wastes has been of great concern because these lignocellulosic biomasses are more widely available compared to cornstarch and sucrose biomasses. Pretreatment processes, such as steam explosion and dilute acid pretreatment, are necessary to make the cellulose in lignocellulosic feedstocks more accessible for hydrolysis into monosaccharides before fermentation (Martins et al. 2015). These pretreatment processes inevitably produce certain toxic inhibitors (Qureshi et al. 2015).

Acetic acid, which inhibits cell growth and affects fermentation performance, is a major inhibitor formed by hemicellulose degradation (Palmqvist and Hahn-Hagerdal 2000). Undissociated acetic acid inflow into the cytosol decreases the cytosolic pH, induces reactive oxygen species (ROS) accumulation, and causes apoptosis in yeast (Guaragnella et al. 2010; Mollapour and Piper 2007). To date, much effort has been made to obtain genetic engineered yeast strains

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with improved acetic acid tolerance. Examples of such genetic manipulations include deletion of the Fps1 protein to reduce acetic acid uptake (Zhang et al. 2011), deletion of the major facilitator superfamily member Qdr3 (Ma et al. 2015), overexpression of the acetic acid-activated transcription factor Haa1 (Tanaka et al. 2012), and overexpression of the *ELO1* gene, which is involved in the elongation of fatty acids (Zheng et al. 2013). Each of these genetic manipulations has improved acetic acid tolerance of *S. cerevisiae* but high concentrations of acetic acid are still harmful to these engineered mutants.

J-Proteins, also known as Hsp40 s, are co-chaperones of Hsp70 chaperones (Walsh et al. 2004). Jjj1, a specialized cytosolic J-protein, is associated with the biogenesis of the large ribosomal subunit. More specifically, Jjj1 stimulates the ATPase activity of Hsp70 Ssa1 (Demoinet et al. 2007). Here, we found that the deletion of *JJJ1* greatly enhanced acetic acid tolerance of *S. cerevisiae* strains, thus improving the ethanol fermentation rate in the presence of this weak acid. The mechanisms underlying the improved phenotypes of the ΔJJJ1 mutant were also determined.

Materials and methods

Strains and media

All the strains used in this study are listed in Table 1. Strain BYΔJJJ1 was constructed by deleting the *JJJ1* gene from BY4741. *S. cerevisiae* strain YJSH1 is a haploid strain produced from the industrial bioethanol production strain YJS329 (Zheng et al. 2012). The yeast growth medium used in this work was YPD, which contains 10 g yeast extract/l, 20 g peptone/l, and 20 g glucose/l (pH adjusted to 5.5). The basic fermentation medium (pH 4.0) used in this work contains 5 g yeast extract/l, 10 g peptone/l, and 100 g glucose/l.

Genetic manipulation

A *JJJ1* disruption cassette was obtained by PCR with the plasmid pUG6 as a template (Gueldener et al. 2002). *Saccharomyces cerevisiae* cells were transformed using the LiAc/SS carrier DNA/PEG method. The ΔJJJ1 mutant strains were screened on YPD medium containing 300 μg G418/ml. The G418-resistant *KanMX* gene was excised with Cre recombinase and expressed from the pSH65 plasmid (Gueldener et al. 2002). Heterogeneous expression of *JJJ1* in BYΔJJJ1 was performed by cloning the *JJJ1* ORF into the *EcoR*I and *Kpn*I sites of the pYES2 plasmid (Invitrogen, Carlsbad, CA, USA). *FPS1* deletion and *ELO1* overexpression strains were obtained using the methods described in our previous studies (Zheng et al. 2011b, 2013). The primers used in this study are listed in Supplementary Table 1.

Stress tolerance test and ethanol fermentation

Saccharomyces cerevisiae was cultivated in 100 ml flasks containing 25 ml liquid YPD medium (pH 5.5) for 24 h at 30 °C. Cells were collected for stress tolerance and fermentation tests. Two types of fermentation medium were used in this study: (I) basic fermentation medium (pH 4.0; 5 g yeast extract/l, 10 g peptone/l, and 100 g glucose/l), also defined as normal fermentation medium and (II) acetic acid containing

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strains	Description	Reference
BY4741	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	Gift from Oliver Valerius
BY4741 + v	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; pYES2	This study
BYΔJJJ1	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; jjj1Δ*	This study
BYΔJJJ1 + v	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; jjj1Δ*; pYES2	This study
BYΔJJJ1 + JJJ1	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; jjj1Δ*; pYES2-JJJ1	This study
YJSH1	*MATa*;	Zheng et al. (2012)
YJSHΔJJJ1	*MATa*; *jjj1Δ*	This study
BYFPS1	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *fps1Δ*; pYES2	This study
BYELO1	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; jjj1Δ*; pYES2-ELO1	This study

fermentation medium (pH 4.0; 5 g yeast extract/l, 10 g peptone/l, 100 g glucose/l, and 4.5 g acetic acid/l). Fermentation conditions were maintained for 3–4 days at 30 °C. The concentrations of ethanol and glucose were measured using HPLC with an Aminex HPX-87H column (Bio-Rad) as previously described (Zheng et al. 2011a).

Measurement of intracellular acetic acid

Yeast cells were pre-cultured in 25 ml YPD medium for 18 h at 30 °C. Cells (4 ml in suspension) were collected, centrifuged at 12,000×*g* for 1 min and suspended in 2 ml YPD medium containing 4.5 g acetic acid/l. The cells were treated for 0, 0.5, 1, or 2 h. Acetic acid-challenged cells were washed twice with normal saline and were then suspended in 2 ml distilled water. To extract intracellular metabolites, the cells were boiled at 100 °C for 10 min. Acetic acid was evaluated using HPLC with an Aminex HPX-87H column and eluting with 4 mM H_2SO_4 at 1 ml/min. Four ml cells were collected and dried at 95 °C for 5 h for dry weight analysis. The amount of intracellular acetic acid was represented as mg per g dry wt.

Determination of physiological attributes

Yeast cells were cultivated in 25 ml YPD medium at 30 °C at 200 rpm for 24 h and then collected and transferred into 25 ml YPD medium with 4.5 g acetic acid/l for 0, 1, or 2 h. The cells from 2 ml medium were collected by centrifugation at 5000×*g* for 5 min and washed twice before assaying for physiological attributes. The trehalose content was extracted with 0.5 M trichloroacetic acid and determined using the anthrone method (Zheng et al. 2011a). Fatty acids were extracted and analyzed by GC with a DSQ II MS detector on a DB-5 MS capillary column (Tao et al. 2012). Catalase (CAT) activity was measured using a commercial kit. One unit of CAT activity was defined as the loss of 1 μmol H_2O_2/s mg protein.

qRT-PCR

Total RNA was extracted from yeast cells using the Fungal RNA out kit (Tiandz, Beijing, China) according to the manufacturer's instructions. RNA samples were reverse transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA eraser (TaKaRa, Dalian, China).Samples were tested in triplicate in a 96-well plate with a final volume of 20 μL. The relative expression of genes was quantified using the comparative $2^{-\Delta\Delta CT}$ method with *ACT1* as the reference gene. The primers used to determine the expression of the *TPS1*, *TPS2*, *TPS3*, *TSL1*, *ATH1*, *NTH1*, *FPS1*, *TPO2*, *TPO3*, *ELO1*, *ELO2*, *ELO3*, *CTT1*, and *CTA1* genes are listed in Supplementary Table 2.

Results and discussion

Improved stress tolerance and ethanol fermentation performance in *S. cerevisiae* *JJJ1* deletion strains

JJJ1 encodes a chaperone that facilitates the function of Ssa1p in ribosome biogenesis (Woolford and Baserga 2013). The loss of *JJJ1* results in multiple phenotypic changes for yeast cells, including increased cold sensitivity and acquisition of tolerance to oxidative stress (Brown et al. 2006). To determine the role of *JJJ1* in the response to the inhibitors in lignocellulosic hydrolysate, this gene was knocked out from *S. cerevisiae* strain BY4741. The stress tolerance to inhibitors and ethanol fermentation performance of the wild-type strain and *JJJ1* null mutant were then examined.

To determine the stress tolerance of BY4741 and BYΔJJJ1, a colony-forming test was performed using YPD plates and YPD plates with individual inhibitors (4.5 g acetic acid/l, 1 g furfural/l, 2 g 5-hydroxymethylfurfural (5-HMF)/l, 1.1 g vanillin/l or 120 g ethanol/l) that can often be found in steam- and dilute acid-pretreated lignocellulosic hydrolysate (Fig. 1). The BYΔJJJ1 strain exhibited slightly slower growth on normal YPD plates compared to the BY4741 strain. This phenotypic change might be due to the ribosome biogenesis defect of the *JJJ1* mutant strain (Demoinet et al. 2007; Meyer et al. 2007). BYΔJJJ1 showed strikingly faster growth on YPD plates with 4.5 g acetic acid/l, but no obvious differences in the tolerance to furfural, 5-HMF, vanillin, or ethanol were observed between the two strains (Fig. 1).

Under normal fermentation conditions, no striking differences in the biomass formation, fermentation rate, or ethanol production were observed between BY4741 and BYΔJJJ1 (Fig. 2a, b). To adjust the physiological status to resist acetic acid in the

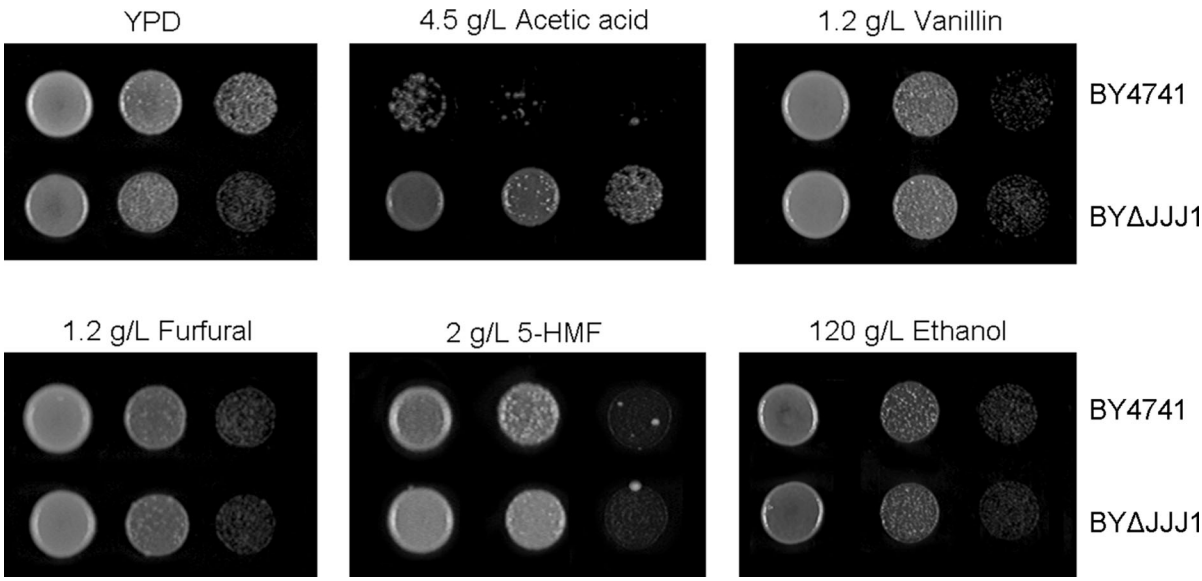

Fig. 1 Stress tolerance to individual inhibitors of BY4741 and BYΔJJJ1. Cells were cultured in 25 ml liquid YPD medium for 24 h at 30 °C. Cells were then collected and 10-fold serially diluted with sterile water at an initial density of 1 (OD_{600}). Cell suspensions (4 µl) were spotted on YPD plates and YPD plates with the indicated inhibitors. The plates were incubated at 30 °C for 1–3 days. Experiments were duplicated three times, and one experimental result is shown

fermentation medium, a longer lag phase was required to start growth and fermentation for BY4741 and BYΔJJJ1 (Fig. 2c, d). However, the lag phase of BYΔJJJ1 was approx. 16 h shorter than that of BY4741. Similarly, BYΔJJJ1 depleted glucose approx. 15 h earlier than did BY4741 (Fig. 2d). The specific ethanol production rate of BYΔJJJ1 was 0.057 g/l h, in contrast to 0.051 g/l h for the control strain BY4741, but the final concentrations of ethanol of these two strains were similar (Fig. 2b). To confirm the phenotypic effect on ethanol fermentation observed in the *JJJ1* deletion strain, a *JJJ1* expression plasmid was transformed into the BYΔJJJ1 strain. The increased fermentation rate of the BYΔJJJ1 strain disappeared in the resulting transformant, BYΔJJJ1 + JJJ1 (Supplementary Fig. 1a). Moreover, deletion of *JJJ1* could also increase the ethanol fermentation rate of the industrial *S. cerevisiae* strain YJSH1 (Supplementary Fig. 1B). These results demonstrate that *JJJ1* deletion confers an improved fermentation performance to both laboratory and industrial *S. cerevisiae* strains in the presence of acetic acid.

We have suggested that *FPS1* deletion and *ELO1* overexpression could improve the acetic acid tolerance of *S. cerevisiae* (Zheng et al. 2011b, 2013). Compared to these genetic manipulations, *JJJ1* deletion resulted in a greater improvement in the acetic acid resistance of the *S. cerevisiae* strain BY4741, as indicated by a faster ethanol production rate of BYΔJJJ1 + v (this strain was obtained by transformation of empty pYES2 into BYΔJJJ1; the aim of this manipulation is just to offset the effect of plasmid when compared to other strains with plasmid) than that of the BYΔFPS1 and BYELO1 strains in fermentation medium with 4.5 g acetic acid/l (Fig. 3; the backgrounds of these strains are provided in Table 1).

Mechanisms underlying the acetic acid tolerance of the ΔJJJ1 mutant

The mechanisms that regulate acetic acid tolerance in *S. cerevisiae* may involve activation or deactivation of membrane transporters of acetic acid (Mollapour and Piper 2007; Tanaka et al. 2012), membrane composition modification (Lindberg et al. 2013), trehalose synthesis (Yoshiyama et al. 2015), and induction of antioxidative factors (Liu et al. 2014). To gain an insight into the mechanism of the acetic acid tolerance induced by *JJJ1* deletion, intracellular acetic acid and certain physiological attributes of BY4741 and BYΔJJJ1 were determined.

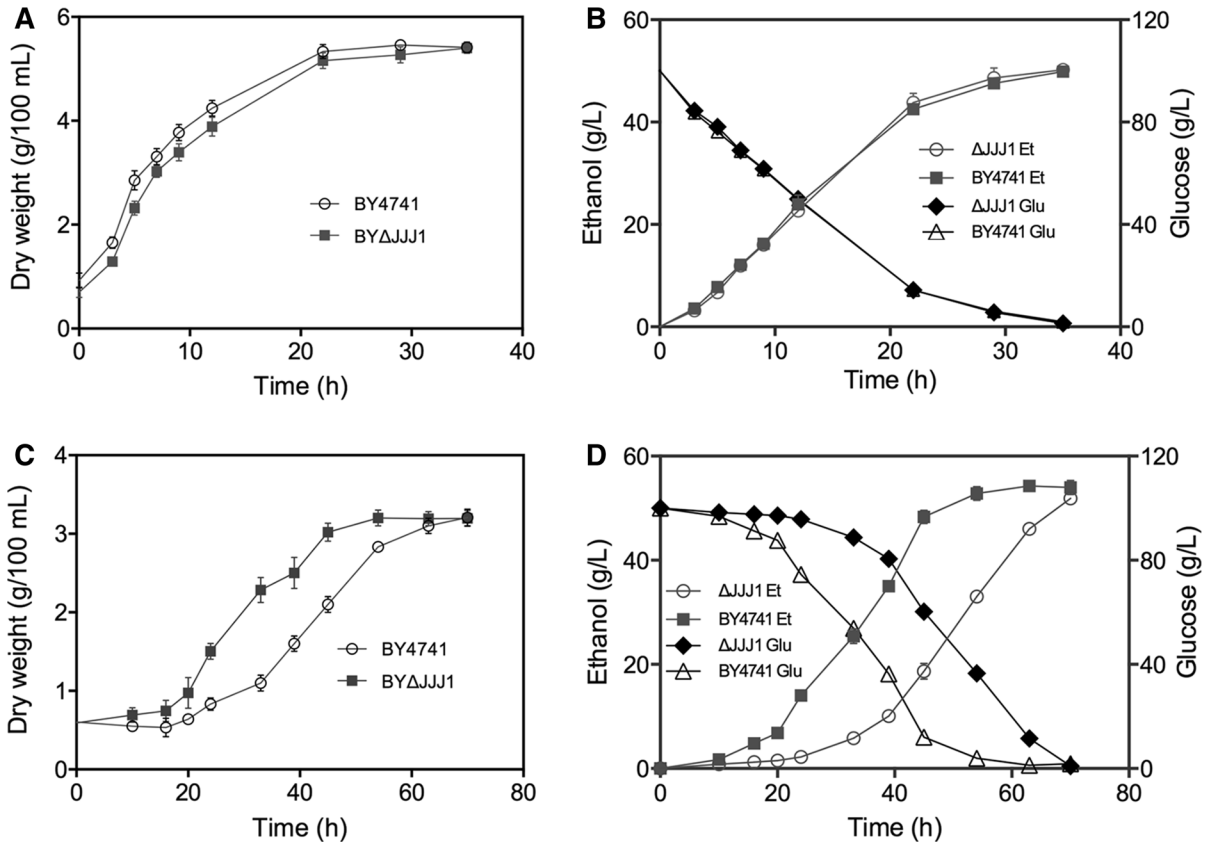

Fig. 2 Fermentation performance comparison of wild-type and *JJJ1* mutant strains. Yeast cells were pre-cultured in 25 ml liquid YPD medium for 24 h at 30 °C. Cells were then collected and incubated in fermentation medium at an initial OD_{600} of 1. Glucose (Glu) and ethanol (Et) were determined as described in the see Materials and methods. **a** Biomass formation of strains BY4741 and BYΔJJJ1 in basic fermentation medium. **b** Ethanol production and glucose consumption of strains BY4741 and BYΔJJJ1 in basic fermentation medium. **c** Biomass formation of strains BY4741 and BYΔJJJ1 in acetic acid (4.5 g/l) containing fermentation medium. **d** Ethanol production and glucose consumption of strains BY4741 and BYΔJJJ1 in basic fermentation medium with 4.5 g acetic acid/l. The data are presented as the mean ± SD of three independent experiments

Intracellular acetic acid and fatty acid composition

As shown in Fig. 4a, the intracellular acetic acid content of wild-type cells increased with treatment time. BYΔJJJ1 had 15, 7.8, and 12 % lower intracellular acetic acid concentrations compared to BY4741 at 0.5 h, 1 h and 2 h, respectively (Fig. 4a; t test, $P < 0.05$). To prevent intracellular accumulation of acetic acid, the plasma membrane channel, Fps1p, which is involved in the uptake of acetic acid, is downregulated and deactivated when yeast cells are exposed to this acid (Mollapour and Piper 2007). The upregulation of the major facilitator superfamily multidrug transporters, Tpo2p and Tpo3p, is required for reducing the intracellular concentration of the acetate anion (Mira et al. 2011). The reduction in the intracellular acetic acid concentration in BYΔJJJ1 cells might be explained by a limitation in the diffusional entry of acetic acid, a higher efficiency of acetic acid export, or a combination of both. After BY4741 cells were transferred to acetic acid-containing YPD liquid medium, the expression of the *FPS1* gene at 2 h was downregulated by 41 %, while the expression of *TPO2* and *TPO3* was upregulated by 3.5-fold and 3.2-fold, respectively, compared to the levels at 0 time (Fig. 4b; t test, $P < 0.01$). However, the expression of these genes showed no significant differences between these two strains, regardless of the presence or absence of acetic acid stress (Fig. 4b). These results suggest that *JJJ1* deletion does not regulate acetic acid diffusion via affecting the expression levels of membrane transporters.

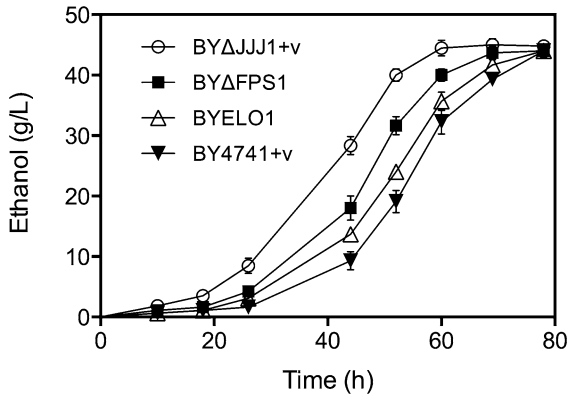

Fig. 3 Comparison of ethanol fermentation between the ΔJJJ1 mutant and other engineered strains with improved acetic acid tolerance. BY4741 + v, BYFPS1, BYELO1, and BYΔJJJ1 + v cells were cultured in 25 ml YPD for 24 h. Cells were collected and incubated in 100 ml fermentation medium at an initial OD_{600} value of 1 with 4.5 g acetic acid/l. Ethanol production was determined during the fermentation process. The data are presented as the mean ± SD of three independent experiments

In addition to the mediation of acetic acid fluxes by membrane transporters, the composition of the plasma membrane can also influence the transmembrane diffusion of acetic acid (Lindahl et al. 2015). In *S. cerevisiae*, fatty acids are mainly divided into three categories based on the length of carbon chain: short chain (lauric acid, 12:0 and myristic acid, 14:0), medium chain (palmitic acid, 16:0 and palmitoleic acid, 16:1), and long chain (oleic acid, 18:1 and stearic acid, 18:0) fatty acids. Our previous studies demonstrated that acetic acid affects the membrane integrity of yeast (Zheng et al. 2011b) and that a higher proportion of long chain fatty acids contributes to the acetic acid tolerance of yeast (Zheng et al. 2013). In the present study, application of acetic acid increased the contents of 18:0 and 18:1 in both strains, resulting in increases of 37 and 29 % in the ratio of long chain fatty acids of BY4741 and BYΔJJJ1, respectively (Table 1; $P < 0.01$, t test). Compared to BY4741, BYΔJJJ1 had 6.3 and 12.3 % higher proportions of long chain fatty acids than those of BY4741 in YPD before and after acetic acid treatment, respectively (Table 2; $P < 0.05$, t test). These alterations might be due to higher expression levels of the genes *ELO1*, *ELO2*, and *ELO3*, which are involved in fatty acid elongation, in BYΔJJJ1 (Fig. 4c; t test, $P < 0.05$).Concurrently, a decrease in the ratio of saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) to unsaturated fatty acids

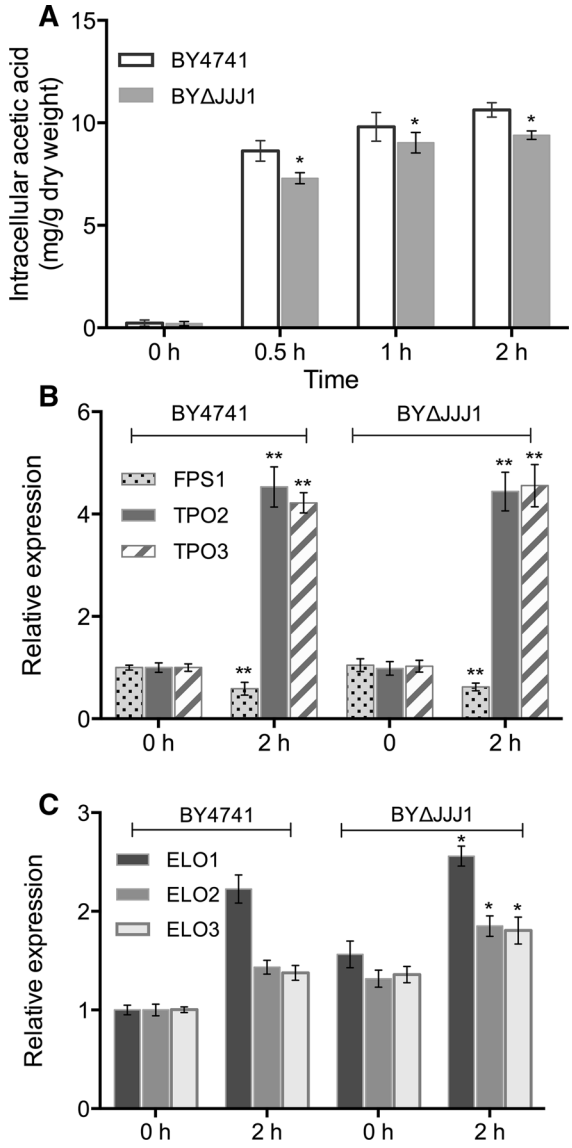

Fig. 4 Intracellular acetic acid and expression of membrane transporters associated with acetic acid diffusion. Cells pre-cultured in 25 ml YPD were collected and transferred into fresh YPD with 4.5 g/l acetic acid for 0 h, 0.5 h, 1 h, or 2 h. **a** The intracellular acetic acid was extracted and determined as described in the see Materials and methods. *Asterisk* indicates significant level of $P < 0.05$ between strains. **b** Determination of the relative mRNA expression levels of the genes *FPS1*, *TPO2*, and *TPO3* in BY4741 and BYΔJJJ1. Total RNA was extracted for qRT-PCR at the time points 0 and 2 h of acetic acid treatment. The expression levels of these three genes in BY4741 at 0 h were normalized as 1. *Double asterisk* indicates significant level of $P < 0.01$ between 0 h and 2 h (C). Relative expression levels of genes *ELO1*, *ELO2*, and *ELO3* in BY4741 and BYΔJJJ1. *Asterisk* indicates significant level of $P < 0.05$ between 0 and 2 h. The bars indicate the standard deviation here and in other figures

(16:1 and 18:1) was observed after the acetic acid treatment, but the ratio of saturated to unsaturated fatty acids was similar between these strains (Table 1).

Decanoic acid (10:0) stress changes the fatty acid composition of the yeast plasma membrane, resulting in a transition from 16:1 to 18:1 (Alexandre et al. 1996). The authors argued that this change might partly explain the increased in vivo activity of membrane H^+-ATPase, a protein that exports H^+ to maintain the intracellular pH. In this work, we also observed that BYΔJJJ1 showed a higher proportion of 18:1 compared to BY4741 in both the presence and absence of acetic acid. A higher proportion of long chain fatty acids in BYΔJJJ1 might also facilitate the synthesis of sphingolipid, which makes membranes less permeable under acetic acid stress (Lindahl et al. 2015).

Trehalose accumulation in BY4741 and BYΔJJJ1

Trehalose accumulation is associated with acetic acid tolerance in yeast (Yoshiyama et al. 2015). Figure 5a shows that strains BY4741 and BYΔJJJ1 had similar trehalose contents in YPD medium. Exposure to 4.5 g acetic acid/l resulted in a decrease in the trehalose content in these two strains (Fig. 5a; t test, $P < 0.01$). The trehalose content of BYΔJJJ1 was 38 and 54 % higher than that of BY4741 under acetic acid treatment for 1 and 2 h, respectively (Fig. 5a; t test, $P < 0.01$). Given the strong positive correlation between trehalose and acetic acid resistance (Yoshiyama et al. 2015), the higher trehalose content under acetic acid stress could be one factor underlying the improved tolerance of the ΔJJJ1 mutant.

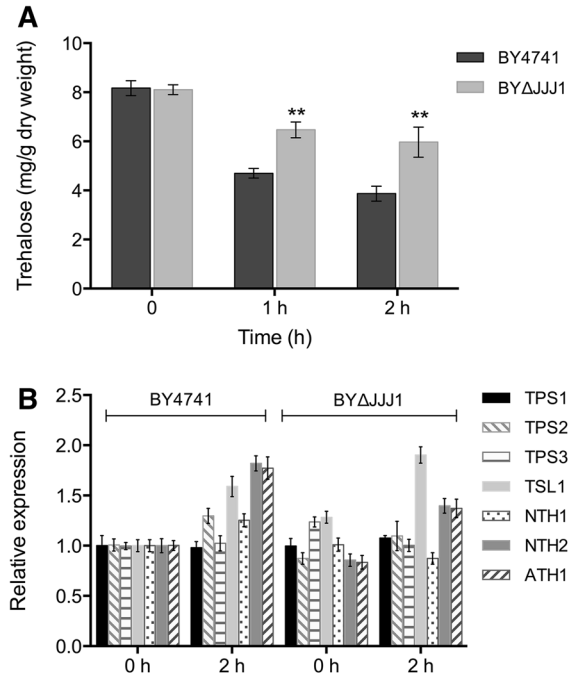

Fig. 5 Intracellular trehalose levels and expression of genes involved in trehalose metabolism. Yeast cells were cultured and treated with acetic acid in the same way described in Fig. 4. **a** Comparison of the trehalose contents of BY4741 and BYΔJJJ1. *Double asterisk* indicates significant level of $P < 0.01$ between BY4741 and BYΔJJJ1. **b** The relative expression of the *TPS1*, *TPS2*, *TPS3*, *TSL1*, *NTH1*, *NTH2*, and *ATH1* genes in BY4741 and BYΔJJJ1. The expression levels of these genes in BY4741 at 0 h in were normalized to 1

The intracellular trehalose content is the result of a well-regulated equilibrium between enzymatic synthesis and degradation (Eleutherio et al. 2015). In the

Table 2 Composition of fatty acids in the plasma membrane of the BY4741 and BYΔJJJ1 strains

Strains[a]	Fatty acid composition (%)[b]						Δ/mol[c]
	Short		Medium		Long		
	12:0	14:0	16:0	16:1	18:0	18:1	
BY4741	0.5 ± 0.1	0.9 ± 0.3	22.7 ± 1.8	37.2 ± 2.3	2.5 ± 0.3	13.7 ± 0.5	0.8
BYΔJJJ1	0.4 ± 0.1	0.8 ± 0.2	24.5 ± 2.1	38.0 ± 2.6	3.5 ± 0.3	16.3 ± 0.8	0.81
BY4741 + AA[d]	0.6 ± 0.2	0.7 ± 0.2	25.7 ± 1.7	32.2 ± 3.1	5.8 ± 0.2	17.8 ± 0.7	0.77
BYΔJJJ1 + AA	0.5 ± 0	0.4 ± 0.1	26.2 ± 1.5	32.5 ± 2.7	6.8 ± 0.4	19.0 ± 0.9	0.77

[a] Yeast cells were pre-cultured in 25 ml YPD for 24 h. Cells were collected and transferred into 25 ml fresh YPD or YPD with 4.5 g/l acetic acid (AA) for 6 h. The fatty acids were extracted and analyzed as described in the see Materials and methods

[b] Fatty acids are presented as the ratio of the number of carbon atoms: number of unsaturated linkages

[c] Δ/mol (unsaturation index) was calculated as: Δ/mol = [1 × (% monoene) + 2 × (% diene)/100

[d] AA indicates that acetic acid was added into the medium as described in the see Materials and methods

present study, acetic acid treatment increased the expression of the three genes *NTH1*, *NTH2,* and *ATH1* encoding trehalases in BY4741 and BYΔJJJ1 (Fig. 5b; t test, $P < 0.01$). The *TPS1*, *TPS2*, *TPS3*, and *TSL1* genes, which are involved in trehalose biosynthesis, showed different expression patterns in response to acetic acid: *TPS2* and *TSL1* were unregulated in both strains (t test, $P < 0.01$), while *TPS1* and *TPS3* were not affected in the two strains (Fig. 5b). The decreased trehalose content observed in BY4741 and BYΔJJJ1 suggests that the mobilization process of trehalose prevailed over the synthesis process under acetic acid stress. The genes *NTH1*, *NTH2*, and *ATH1* showed 30, 23, and 23 %, respectively, lower expression levels in the ΔJJJ1 mutant than in BY4741 under acetic acid stress (Fig. 5b; t test $P < 0.01$), but no significant differences in the expression of the genes *TPS1*, *TPS2*, and *TPS3* were observed between the two strains. It is likely that the different expression levels of genes encoding trehalases are responsible for the different trehalose contents between BY4741 and BYΔJJJ1 in the presence of acetic acid.

Acetic acid causes intracellular acidification, which is partly counteracted by the activity of Pma1p, a plasma membrane H^+-ATPase pump that consumes up to 60 % of cellular ATP during weak acid stress (Holyoak et al. 1996). Additionally, acetic acid inhibits nutrient uptake and the glycolysis process, thereby reducing intracellular ATP generation (Ding et al. 2013). It is not surprising that acetic acid would activate the mobilization of trehalose, an intracellular energy reserve, to compensate for the cellular ATP depletion. However, this mobilization may come at the cost of the cell's trehalose-dependent stress resistance.

Catalase activities of BY4741 and BYΔJJJ1

Acetic acid can induce intracellular H_2O_2 accumulation, resulting in apoptotic cell death of yeast. Upregulation of CAT activity is necessary to resist acetic acid stress by eliminating H_2O_2 in yeast cells (Giannattasio et al. 2005). As showed in Fig. 6a, no obvious differences in CAT activity were observed between BY4741 and BYΔJJJ1 cultured in YPD medium (t test, $P > 0.05$). However, decreased CAT activity was observed in acetic acid-challenged cells of BY4741 and BYΔJJJ1 (Fig. 6a; t test, $P < 0.05$). The qRT-PCR results indicate that the genes (*CTA1* and *CTT1*) encoding catalases in the two strains were all downregulated after the application of acetic acid (Fig. 6b; t test, $P < 0.01$). The expression of *CTT1* and *CTA1* in BYΔJJJ1 was 33.5 and 50 % higher, respectively, than that of BY4741 (Fig. 6b; t test, $P < 0.01$), explaining the higher CAT activity in BYΔJJJ1 under acetic acid stress.

Like trehalose, CAT represents an important anti-stress factor that is induced when yeast cells are exposed to environmental stressors, such as heat, osmotic pressure, and ethanol (Jamieson 1998). However, in the present study, both trehalose content and CAT activity were repressed under acetic acid stress, indicating the difference between the toxic mechanisms of acetic acid and other stressors. One possible explanation for this difference is that acetic acid can cause intracellular energy crisis (ATP depletion),

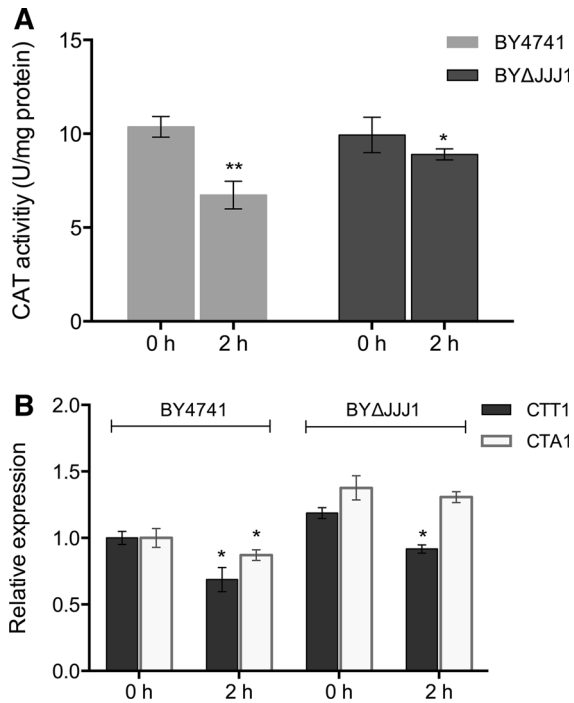

Fig. 6 Catalase (CAT) activity and expression levels of the genes *CTT1* and *CTA1*. StrainsBY4741 and BYΔJJJ1 were cultured in 25 ml YPD for 24 h and then transferred into fresh YPD with 4.5 g/l acetic acid. **a** Determination of the CAT activities of BY4741 and BYΔJJJ1 at the time points 0 and 2 h. CAT activity was defined as the degradation of 1 μmol H_2O_2 per s mg protein. *Asterisk* and *double asterisk* indicates significant level of $P < 0.05$ and $P < 0.01$, respectively, between 0 and 2 h. **b** The relative expression of the *CTT1* and *CTA1* genes in BY4741 and BYΔJJJ1 was determined at acetic acid treatment time points of 0 h and 2 h, respectively. The expression levels of these two genes in BY4741 at 0 h were normalized to 1. *Asterisk* indicates significant level of $P < 0.05$ between 0 and 2 h

which represses trehalose biosynthesis and CAT expression. Noteworthy, the *JJJ1* knockout mutant maintained its trehalose content and catalase activity under acetic acid stress, which then assisted in ameliorating the cytotoxicity caused by acetic acid.

Conclusion

This work demonstrates that *JJJ1* deletion is powerful for improving the acetic acid tolerance and ethanol fermentation performances of *S. cerevisiae*. Yeast strains with *JJJ1* knockout showed a shorter lag phase and an increased ethanol production rate compared with those of the parent strain in the presence of acetic acid. Deletion of *JJJ1* resulted in multiple physiological changes that prepare yeast cells to resist acetic acid. Our observations indicate that higher long chain fatty acid concentrations, intracellular trehalose levels, and CAT activity are important factors contributing to the improved acetic acid tolerance of ΔJJJ1 mutants.

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Supporting information Supplementary Table 1—Primers used for genetic manipulations.

Supplementary Table 2—Primers used in qRT-PCR.

Supplementary Figure 1—(A) Expression of JJJ1 in BYΔJJJ1 restores the phenotypic change (improvement of the fermentation rate) conferred by JJJ1 deletion in *S. cerevisiae*.

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