Research Article

Improvement of D-Lactic acid production in *Saccharomyces cerevisiae* under acidic conditions by evolutionary and rational metabolic engineering†

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Abbreviations: ADH, alcohol dehydrogenase; **GPD,** glycerol-3-phosphate dehydrogenase; **HPLC,** high performance liquid chromatography; **LA,** lactic acid; **LDH,** lactate dehydrogenase; **PDC,** pyruvate decarboxylase

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Abstract

Microbial lactic acid (LA) production under acidic fermentation conditions is favorable to reduce the production cost, but circumventing LA toxicity is a major challenge. We generated D-LA-producing *Saccharomyces cerevisiae* strain JHY5610 by expressing D-lactate dehydrogenase gene (*Lm. ldhA*) from *Leuconostoc mesenteroides*, while deleting genes involved in ethanol production (*ADH1*, *ADH2*, *ADH3*, *ADH4*, and *ADH5*), glycerol production (*GPD1* and *GPD2*), and degradation of D-LA (*DLD1*). Adaptive laboratory evolution of JHY5610 led to a strain JHY5710 having higher LA tolerance and D-LA-production capability. Genome sequencing of JHY5710 revealed that $SUR1^{I245S}$ mutation increases LA tolerance and D-LA-production, whereas a loss-of-function mutation of *ERF2* only contributes to increasing D-LA production. Introduction of both $SUR1^{I245S}$ and *erf2Δ* mutations into JHY5610 largely mimicked the D-LA-production capability of JHY5710, suggesting that these two mutations, which could modulate sphingolipid production and protein palmitoylation, are mainly responsible for the improved D-LA production in JHY5710. JHY5710 was further improved by deleting *PDC1* encoding pyruvate decarboxylase and additional integration of *Lm. ldhA* gene. The resulting strain JHY5730 produced up to 82.6 g/L of D-LA with a yield of 0.83 g/g glucose and a productivity of 1.50 g/(L·h) in fed-batch fermentation at pH 3.5.

1 Introduction

Saccharomyces cerevisiae, having high acid tolerance, is a promising host for lactic acid (LA) production under acidic fermentation conditions [1, 2]. If LA is produced under acidic fermentation conditions, the utilization of neutralizing reagent such as $CaCO_3$ and the following LA recovery process can be avoided, thus reducing the cost of LA production [3, 4]. Metabolic engineering strategies for LA production in *S. cerevisiae* have been focused on the reduction of major fermentation products such as ethanol and glycerol, while introducing heterologous genes encoding stereospecific lactate dehydrogenase (LDH), which can produce either L-LA or D-LA from pyruvate. To reduce the pyruvate flux toward ethanol production, genes encoding pyruvate decarboxylase (*PDC1*, *PDC5*, and *PDC6*) and alcohol dehydrogenase (*ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, and *SFA1*) have been deleted in various combinations [5-8]. In addition, glycerol formation has been reduced by deleting glycerol-3-phosphate dehydrogenase genes (*GPD1* and *GPD2*) [9, 10].

Elimination of natural fermentation products can enhance LA production yield in *S. cerevisiae*. However, LA toxicity is a major limiting factor for high-titer production of LA. Lactic acid, which dissociates into the acid anion and proton in the cytosolic pH, inhibits cell growth by modifying various cellular components such as proteins and lipids [11, 12]. In addition, exportation of the protons and acid anions through H^+-ATPase and efflux pumps, respectively, requires large ATP consumption [3]. Transport of lactate anion into the cells is mediated by proton symporters Jen1 and Ady2 [13]. However, under acidic fermentation conditions, extracellular lactic acid can diffuse into the cytosol through the plasma membrane and dissociates into lactate and proton in the cytosol. Subsequent export of these ions and regeneration of extracellular lactic acid might generate a futile cycle causing energy depletion

[3, 11]. Especially, the genetically engineered cells with impaired ethanol and glycerol production pathways have growth defects and reduced LA tolerance than wild type [10, 14]. Therefore, increasing LA tolerance is a key challenge to produce efficient LA-producing strains.

Several efforts have been made to increase LA tolerance in *S. cerevisiae* either by using rational design based on known LA tolerance mechanisms or by genome-wide screening of gene-deletion or knock-down libraries [15-17]. However, limited knowledge and complexity of LA tolerance mechanisms make it difficult to generate LA-tolerant strains just by modifying a few target genes. Furthermore, a given gene can exert different effects on LA tolerance depending on the physiological conditions of each strain. Therefore, adaptive laboratory evolution would be an efficient strategy to improve LA tolerance of individual engineered strain having different genetic background and metabolic property [14]. Identification of the mutated genes in the evolved strains could be used for reverse engineering as well as for understanding novel tolerance mechanisms [18, 19].

In this study, we generated LA-producing strain JHY5610 by expressing D-lactate dehydrogenase gene (*ldhA*, LEUM_1756) from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293, while deleting 5 ADH genes (*ADH1* to *ADH5*), *GPD1* and *GPD2*, and *DLD1* encoding D-lactate dehydrogenase. Adaptive laboratory evolution and further metabolic engineering of JHY5610 led to a highly efficient strain JHY5730 having improved D-LA production capability under acidic conditions.

2 Materials and methods

2.1 Strains and media and culture conditions

All yeast strains used in this study are described in Table S1. *S. cerevisiae* CEN.PK2-1C strain (*MAT***a** *ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*) was used as a parental strain in this study. All yeast cells were cultivated in YP medium (20 g/L peptone and 10 g/L yeast extract) supplemented with 20, 50, or 100 g/L glucose. A_{600} of 1 of pre-cultured cells were inoculated in 5 mL of YPD medium containing 50 g/L glucose in a 50 mL screw cap conical tube, and then cultured at 30˚C with shaking at 170 rpm. For shake flask fermentation, A_{600} of 1 of pre-cultured cells were cultivated in 10 mL of YPD medium containing 100 g/L glucose in a 100 mL flask.

Fed-batch fermentation was performed in 500 mL YPD medium containing 60 g/L glucose using a 1 L bench-top fermentor FMT-DS (Fermentec, Korea) at 30ºC with agitation speed of 450 rpm and air flow rate of 1.0 vvm. The pH of the culture medium was maintained at 3.5 by using 4 N NaOH. Strain JHY5730 was pre-cultured in YPD medium containing 20 g/L glucose and inoculated into the fermentor with initial A_{600} of 10. The feeding solution (800 g/L glucose) was intermittently added to the culture medium when the glucose concentration was lower than 5 g/L.

2.2 Plasmid construction

All plasmids used in this study are described in Table S1. To construct the integration plasmids containing mutated genes, each open reading frame (ORF) flanked by its own promoter and terminator was amplified by PCR from the genomic DNA of the evolved strain JHY5710, and cloned between *Bam*HI and *Sal*I sites of pUG27MCS plasmid, resulting in plasmids pUG27MCS-*CIT2*^{V45F} INT, pUG27MCS-*NCL1*^{I626T} INT, and pUG27MCS-*SUR1*^{I245S} INT. To construct pUG6MCS-GPD-*Lm.ldhA* INT plasmid, a DNA fragment spanning from *TDH3* promoter to *CYC1* terminator (P_{TDH3}-*Lm.ldhA*-T_{CYC1}) was amplified by PCR from p425GPD-*Lm.ldhA* plasmid and cloned between *Apa*1 and *Nhe*1 sites of pUG6MCS, which had been generated by introducing additional cloning sites (*Pac*I, *Nhe*I, *Bam*HI, *Sma*I, *Eco*RI, *Apa*I, *Kpn*I, and *Asc*I) into pUG6.

2.3 Construction of genetically manipulated strains and adaptive evolution

Various genetically manipulated strains were generated by PCR-mediated homologous recombination [18]. To generate deletion and integration strains, gene-specific cassettes were amplified by PCR from pUG6MCS-, pUG27- or pUG27MCS-based plasmids using target-specific primer pairs, and then introduced into *S. cerevisiae* strains.

To develop LA-tolerant strain, adaptive laboratory evolution of the strain JHY5610 was carried out by growing cells in YPD medium with a gradual increase in LA concentrations from 10 g/L to 40 g/L during 15 subcultures. During the cultivation, growth rate and the titers of metabolites were measured for characterization of the evolved strains. After the final subculture, LA-tolerant colonies were isolated on solid medium containing 40

g/L LA and the most efficient strain JHY5710 was selected based on the D-LA production and glucose consumption levels. Strain JHY5720 was generated by deleting *PDC1* in JHY5710. An additional copy of *Lm.ldhA* gene (P_{TEF1}-*Lm.ldhA*-T_{CYC1}), PCR amplified from genomic DNA of strain JHY5210, was integrated into the genome of JHY5710 by replacing the *PDC1* ORF, resulting in strain JHY5730. All genetic modifications were confirmed by PCR using specific primer pairs.

2.4 Analytical methods

To determine the concentration of acetate, ethanol, glucose, and lactate, all samples collected from culture supernatant were filtered through a 0.22 μm syringe filter and quantified by high performance liquid chromatography (HPLC). HPLC was performed in UltiMate 3000 HPLC system (Thermo Fishers Scientific) equipped with Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm, 5 μm) at 60ºC with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min and refractive index (RI) as a detector keeping at 35ºC. Cell growth was monitored by determining optical density at 600 nm.

2.5 Whole genome sequencing analysis

Whole genome sequencing of the evolved strain JHY5710 and its sequence analysis in comparison with strain JHY5210 were performed as described previously [14]. Indels were detected by using Creator/Indel Realigner of Genome Analysis Toolkit (GATK) and VarScan software (ver. 2.3.7), and Single nucleotide variants (SNVs) were detected using MuTect (ver 1.1.7).

3 Results

3.1 Development of a D-LA-producing *S. cerevisiae* strain with reduced ethanol and glycerol production

In our previous study, we generated a D-LA-producing *S. cerevisiae* strain JHY5210 by expressing D-lactate dehydrogenase gene from *L. mesenteroides* (*Lm.ldhA*) while deleting genes involved in ethanol formation (*ADH1, PDC1*), glycerol formation (*GPD1* and *GPD2*), and D-LA consumption (*JEN1* and *DLD1*) (Fig. 1A and B) [10]. Adaptive laboratory evolution of JHY5210 resulted in an LA-tolerant strain JHY5310 having improved glucose consumption and D-LA production levels (Fig. 1B) [10]. However, ethanol was accumulated as a major byproduct in these strains with a yield of around 0.06 g/g glucose (Fig. 1C).

Therefore, as an effort to reduce ethanol production, we generated another D-LA-producing platform strain using JHY605 (*adh1Δadh2Δadh3Δadh4Δadh5Δgpd1Δgpd2Δ*) [20], as a parental strain. In JHY605, which lacks major 5 ADH genes and *GPD1* and *GPD2*, we deleted *DLD1* by integrating *TDH3* promoter-controlled *Lm.ldhA* into the *DLD1* locus of JHY605. However, *JEN1*, encoding monocarboxylate transporter, was not deleted since *JEN1* deletion showed a marginal effect on D-LA production in combination with *DLD1* deletion in our previous study [10]. The resulting strain JHY5610 produced 24.7 g/L D-LA, consuming 38.8 g/L glucose in YPD medium containing 50 g/L glucose (Fig. 1C). Compared to the previously generated strain JHY5210, JHY5610 showed an 18% increase in glucose uptake level and a 44% increase in D-LA production level (Fig. 1C). In addition, JHY5610 showed reduced ethanol titer during the exponential growth phase and a 15% decrease in final ethanol yield, supporting the effects of additional *ADH2* to *ADH5* deletion on reducing ethanol

production. However, JHY5610 still produced a significant amount of ethanol (2.1 g/L), suggesting that other remaining ADH genes such as *SFA1*, *AHD6*, *ADH7* might be involved in the residual ethanol production in this strain.

3.2 Improvement of D-LA production by adaptive laboratory evolution

Although JHY5610 showed better D-LA production performance than JHY5210, growth limitation by the inhibitory effect of D-LA is still a major bottleneck to high-titer D-LA production. Therefore, to further enhance D-LA production ability of JHY5610, we carried out adaptive laboratory evolution by cultivating the cells with a gradual increase in LA levels from 10 g/L to 40 g/L during serial subcultures for 15 times. Among several LA-tolerant candidate strains, JHY5710, having the highest glucose consumption and D-LA production levels, was selected (Supplementary Fig. S1). Compared to the unevolved strain JHY5610, JHY5710 showed improved growth both on YPD medium and YPD medium containing LA (Fig. 2A). Unlike JHY5610, whose growth stopped before consuming all the glucose in the medium, JHY5710 consumed all the glucose (50 g/L), producing 30% more D-LA and 52% less ethanol than did JHY5610 (Fig. 2B).

3.3 *SUR1*^{I245S} mutation and loss-of-function mutation of *ERF2* in JHY5710 improved LA tolerance and LA production

In order to identify genes contributing to the improvement of LA tolerance and D-LA production in JHY5710, whole genome sequencing analysis of JHY5710 was carried out. In comparison with the genome of the unevolved strain, five genes carrying mutations were

found in the genome of JHY5710 (Table S2). A nonsense mutation was found in *BSD2* (bypass Sod1 defects), which changes the codon for Arg242 to stop codon. *ERF2* (effect on Ras function) gene has a single nucleotide deletion at position 261, resulting in a frameshift mutation after Pro87. In addition, point mutations were found in *CIT2* (citrate synthase), *NCL1* (nuclear protein), and *SUR1* (suppressor of rvs161 and rvs167 mutations). Any gene duplication or insertion was not identified.

To examine the functions of the genetic mutations found in the genome of JHY5710, we individually introduced each mutation to the parental strain JHY5610. A nonsense mutation in *BSD2* and a frameshift mutation in *ERF2* were mimicked by deleting each gene. To reconstruct the mutant carrying missense mutation in *CIT2*, *NCL1*, or *SUR1* gene, the wild-type gene in the chromosome of JHY5610 was replaced by each mutated ORF. The 5 mutant strains were first tested for LA tolerance on YPD solid medium containing 1% LA (Fig. S2A). Among the mutants, only JHY5615 harboring $SUR1^{I245S}$ mutation showed enhanced LA tolerance compared to JHY5610, but to a lesser extent than JHY5710 (Fig. 2A, Fig S2A). Sur1 is a catalytic subunit of mannosylinositol phosphorylceramide (MIPC) synthase involved in sphingolipid biosynthesis, and it was previously reported that deletion of *SUR1* conferred LA sensitivity in *S. cerevisiae* [17, 21, 22]. The other 4 mutants showed reduced LA tolerance (Fig. S2A).

Next, we examined LA production levels in these mutants. In comparison with JHY5610, JHY5612 (*erf2Δ*) as well as JHY5615 showed increased glucose uptake and D-LA production levels (Fig. 2B). Erf2, a subunit of palmitoyltransferase, is required for posttranslational modification of several proteins such as Ras1 and Ras2 by palmitoyl lipid moieties [23, 24]. The other mutants showed similar or slightly reduced D-LA production levels (Fig. S2B). To examine any synergistic effects of *erf2Δ* and $SUR1^{I245S}$ mutations, we

introduced both mutations into JHY5610, generating JHY5617 (*erf2Δ SUR1*^{I245S}). This double mutant strain showed higher LA tolerance than JHY5615 even though *erf2Δ* mutation alone reduced LA tolerance (Fig. 2A). Moreover, the double mutant strain JHY5617 showed high glucose uptake and D-LA production levels comparable to those of JHY5710 (Fig. 2B), suggesting that these two mutations might be mainly responsible for increasing D-LA production in JHY5710. Although *SUR1*^{I245S} mutation led to increased LA tolerance and production, *erf2Δ* improved D-LA production without conferring LA tolerance. Therefore, not a dominant mutation, but multiple mutations with different functions might contribute together to the phenotype of JHY5710 having high LA-tolerance and high D-LA production capability.

3.4 Increase in D-LA production by disrupting *PDC1*

In JHY5710, D-LA production level enhanced significantly with decreased ethanol production, but acetate was accumulated up to 2.9 g/L as a major byproduct (Fig. 3). Therefore, to reduce acetate formation from pyruvate through acetaldehyde (Fig. 1), we deleted *PDC1* gene in JHY5710, resulting in strain JHY5720. Deletion of *PDC1* led to a significant reduction in both acetate and ethanol production levels (Fig. 3), resulting in 11% higher D-LA production level (41.2 g/L) compared to JHY5710 (Fig. 3). We also generated strain JHY5730, by additional integration of *Lm.ldhA* gene under the control of *TEF1* promoter into the *PDC1* locus of JHY5710. JHY5730 showed similar fermentation profile to that of JHY5720 (Fig. 3), suggesting that the extra copy of LDH gene might not contribute to D-LA production at least under our fermentation conditions with 50 g/L glucose. The

fermentation profiles of JHY5610, 5710, 5720, and 5730 shown in Fig. 3 are compared in Table 1.

Next, we further examined D-LA production ability of JHY5730 in YPD medium containing 100 g/L glucose in comparison with our previously developed strain JHY5320 (Fig. 4A). JHY5320, having two copies of genome-integrated *Lm.ldhA* gene, is a derivative of JHY5310, a LA-tolerant strain generated by adaptive evolution of JHY5210 (Fig. 1B). In flask fermentation without pH control, JHY5730 showed significantly improved glucose consumption and LA production levels compared to JHY5320, suggesting that JHY5730 has higher tolerance to the growth inhibitory effects of the produced LA than JHY5320. JHY5320 consumed only 57.8 g/L glucose, producing 45.2 g/L D-LA. On the other hand, strain JHY5730 showed 70% higher glucose consumption level (98.2 g/L), and produced D-LA up to 69.7 g/L with a yield of 0.71 g/g glucose and a productivity of 0.97 g/(L·h) (Fig. 4A, Table 1).

We also evaluated D-LA production capability of JHY5730 in fed-batch fermentation at pH 3.5 using a 1 L bench-top fermentor. In fed-batch fermentation, by consuming 100.0 g/L glucose, up to 82.6 g/L of D-LA was produced with a yield of 0.83 g/g (Fig. 4B, Table 1). Moreover, D-LA productivity was improved to 1.50 g/(L·h). These results demonstrate that our strain JHY5730 is a highly efficient D-LA producer under acidic fermentation conditions.

4 Discussion

Adaptive laboratory evolution has been demonstrated as a powerful tool for strain development for industrial applications. In this study, we performed adaptive laboratory evolution of a D-LA-producing *S. cerevisiae* strain JHY5610, having minimized ethanol and glycerol production and D-LA degradation activity, to develop improved strain JHY5710. JHY5710 was further engineered to generate more efficient strain JHY5730. Previously, we used similar strategy of combining metabolic engineering and adaptive laboratory evolution, generating JHY5310 and JHY5320 (Fig. 1B) [10]. Although JHY5320 and JHY5730 have similar genetic modifications including deletion of *ADH1*, *GPD1*, *GPD2*, *PDC1*, *DLD1*, and integration of two copies of LDH gene, newly developed JHY5730 showed about 1.7-fold increase in glucose consumption and 1.5-fold increase in D-LA production levels under acidic fermentation conditions compared to JHY5320 (Fig. 4A), implying the diversity of genetic mutations that can be introduced during the adaptive laboratory evolution process.

In the case of JHY5310, inactivation of *GSF2*, involved in maturation and secretion of certain type of hexose transporters such as Hxt1 to the plasma membrane, was turned out to be mainly responsible for the increase in LA tolerance and LA production capability [14]. In *GSF2* deletion mutant, reduced functional localization of Hxt1 and subsequent reduction of glucose uptake rate might alleviate glucose repression, resulting in activation of respiratory pathway [14, 25]. More efficient ATP synthesis through respiration might rescue the growth defects of LA-producing strain, where ATP depletion caused by extensive export of protons and lactate anions can be detrimental. On the contrary, *GSF2* deletion in wild type strain exerted a negative effect on LA tolerance, suggesting that even the same genetic

modifications can cause completely different effects depending on cellular metabolic status [14].

In JHY5710, we identified *SUR1*^{I245S} point mutation and a loss of function mutation of *ERF2* as major contributors to the improved D-LA production. Although *SUR1*^{I245S} mutation increased both LA tolerance and production, *ERF2* deletion was effective only in improving D-LA production. *ERF2* deletion alone led to a decrease in LA tolerance, but combination of the two mutations led to synergistic effects on LA tolerance and LA production, suggesting that these mutations and possibility yet unidentified mutations might contribute together to the phenotype of JHY5710. Sur1 is a catalytic subunit of mannosylinositol phosphorylceramide (MIPC) synthase involved in MIPC production from inositolphosphorylceramide (IPC) [22]. IPC, MIPC, and mannosyldiinositol phosphorylceramide [M(IP)2C] are the three sphingolipid classes in *S. cerevisiae* [21]. *Zygosaccharomyces bailii*, a yeast having high tolerance to acetic acid, increases plasma membrane sphingolipids in response to acetic acid [26, 27]. It has been suggested that high sphingolipid content in the plasma membrane might increase acetic acid tolerance by reducing the rate of acetic acid diffusion into the cell [26]. *SUR1*^{I245S} mutation might increase LA tolerance by altering the sphingolipid compositions in the plasma membrane, but further studies are necessary to clarify the effects of sphingolipid on LA permeability and the effect of *SUR1*^{I245S} mutation on Sur1 activity. At least, I245 is not located in the catalytic domain of Sur1, but in the cytosolic domain between two transmembrane domains spanning the membrane of the Golgi apparatus.

ERF2 encodes a DHHC protein family of protein acyltransferases involved in protein palmitoylation [23]. Known Erf2 target proteins include G proteins such as Ras1, Ras2, Rho2, and Rho3, and G protein alpha subunits Gpa1 and Gpa2 [24]. Deletion of *ERF2* might reduce

proper membrane localization of these target proteins, but it is not clear yet how *ERF2* inactivation leads to an increase in LA production in JHY5710. Since Ras proteins and Gpa2 are involved in glucose signaling via PKA pathway [24], alternations in glucose signaling might affect the regulation of cell growth control in response to the toxic effect of LA, but it needs further studies to elucidated detailed underlying mechanisms.

In conclusion, our study demonstrates the great potential of combining metabolic engineering and adaptive laboratory evolution in strain development as well as discovering novel cellular regulatory mechanisms.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

[1] Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 16 years research on lactic acid production with yeast - ready for the market? *Biotechnol. Genet. Eng. Rev.* 2010, *27*, 229-256.

[2] Song, J.-Y., Park, J.-S., Kang, C. D., Cho, H.-Y., *et al.*, Introduction of a bacterial acetyl-CoA synthesis pathway improves lactic acid production in *Saccharomyces cerevisiae*. *Metab. Eng.* 2016, *35*, 38-45.

[3] van Maris, A. J. A., Winkler, A. A., Porro, D., van Dijken, J. P., Pronk, J. T., Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: Possible consequence of energy-dependent lactate export. *Appl. Environ. Microbiol.* 2004, *70*, 2898-2905.

[4] Vaidya, A. N., Pandey, R. A., Mudliar, S., Kumar, M. S., *et al.*, Production and recovery of lactic acid for polylactide-an overview. *Crit. Rev. Environ. Sci. Technol.* 2005, *35*, 429-467.

[5] Lee, J. Y., Kang, C. D., Lee, S. H., Park, Y. K., Cho, K. M., Engineering cellular redox balance in *Saccharomyces cerevisiae* for improved production of L-lactic acid. *Biotechnol. Bioeng.* 2015, *112*, 751-758.

[6] Ishida, N., Suzuki, T., Tokuhiro, K., Nagamori, E., *et al.*, D-Lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 2006, *101*, 172-177.

[7] Ishida, N., Saitoh, S., Onishi, T., Tokuhiro, K., *et al.*, The effect of pyruvate decarboxylase gene knockout in *Saccharomyces cerevisiae* on L-lactic acid production. *Biosci., Biotechnol., Biochem.* 2006, *70*, 1148-1153.

[8] Tokuhiro, K., Ishida, N., Nagamori, E., Saitoh, S., *et al.*, Double mutation of the *PDC1* and *ADH1* genes improves lactate production in the yeast *Saccharomyces cerevisiae* expressing the bovine lactate dehydrogenase gene. *Appl. Microbiol. Biotechnol.* 2009, *82*,

883-890.

[9] Ida, Y., Hirasawa, T., Furusawa, C., Shimizu, H., Utilization of *Saccharomyces cerevisiae* recombinant strain incapable of both ethanol and glycerol biosynthesis for anaerobic bioproduction. *Appl. Microbiol. Biotechnol.* 2013, *97*, 4811-4819.

[10] Baek, S. H., Kwon, E. Y., Kim, Y. H., Hahn, J. S., Metabolic engineering and adaptive evolution for efficient production of D-lactic acid in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 2016, *100*, 2737-2748.

[11] Abbott, D. A., Suir, E., van Maris, A. J., Pronk, J. T., Physiological and transcriptional responses to high concentrations of lactic acid in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 2008, *74*, 5759-5768.

[12] Valli, M., Sauer, M., Branduardi, P., Borth, N., *et al.*, Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. *Appl. Environ. Microbiol.* 2006, *72*, 5492-5499.

[13] Pacheco, A., Talaia, G., Sá-Pessoa, J., Bessa, D., *et al.*, Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. *FEMS Yeast Res.* 2012, *12*, 375-381.

[14] Baek, S. H., Kwon, E. Y., Kim, S. Y., Hahn, J. S., *GSF2* deletion increases lactic acid production by alleviating glucose repression in *Saccharomyces cerevisiae*. *Sci. Rep.* 2016, *6*.

[15] Dato, L., Berterame, N., Ricci, M., Paganoni, P., *et al.*, Changes in *SAM2* expression affect lactic acid tolerance and lactic acid production in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 2014, *13*, 147.

[16] Suzuki, T., Sakamoto, T., Sugiyama, M., Ishida, N., *et al.*, Disruption of multiple genes whose deletion causes lactic-acid resistance improves lactic-acid resistance and productivity in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 2013, *115*, 467-474.

[17] Kawahata, M., Masaki, K., Fujii, T., Iefuji, H., Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res.* 2006, *6*, 924-936.

[18] Kildegaard, K. R., Hallstrom, B. M., Blicher, T. H., Sonnenschein, N., *et al.*, Evolution reveals a glutathione-dependent mechanism of 3-hydroxypropionic acid tolerance. *Metab. Eng.* 2014, *26*, 57-66.

[19] Caspeta, L., Chen, Y., Ghiaci, P., Feizi, A., *et al.*, Altered sterol composition renders yeast thermotolerant. *Science* 2014, *346*, 75-78.

[20] Kim, S., Hahn, J.-S., Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing. *Metab. Eng.* 2015, *31*, 94-101.

[21] Dickson, R. C., Sumanasekera, C., Lester, R. L., Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. *Prog. Lipid Res.* 2006, *45*, 447-465.

[22] Cowart, L. A., Obeid, L. M., Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. *Biochim. Biophys. Acta* 2007, *1771*, 421-431.

[23] Bartels, D. J., Mitchell, D. A., Dong, X. W., Deschenes, R. J., Erf2, a novel gene product that affects the localization and palmitoylation of Ras2 in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 1999, *19*, 6775-6787.

[24] Roth, A. F., Wan, J., Bailey, A. O., Sun, B., *et al.*, Global analysis of protein palmitoylation in yeast. *Cell* 2006, *125*, 1003-1013.

[25] Sherwood, P. W., Carlson, M., Efficient export of the glucose transporter Hxt1p from the endoplasmic reticulum requires Gsf2p. *Proc. Natl. Acad. Sci. U. S. A.* 1999, *96*, 7415-7420.

[26] Lindahl, L., Genheden, S., Eriksson, L. A., Olsson, L., Bettiga, M., Sphingolipids

contribute to acetic acid resistance in *Zygosaccharomyces bailii*. *Biotechnol. Bioeng.* 2016, *113*, 744-753.

[27] Lindberg, L., Santos, A. X., Riezman, H., Olsson, L., Bettiga, M., Lipidomic profiling of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* reveals critical changes in lipid composition in response to acetic acid stress. *PloS one* 2013, *8*, e73936.

Table 1. Comparison of LA production in engineered *S. cerevisiae* strains

Strain	Fermentation time (h)	Cell density (A_{600})	Consumed glucose (g/L)	D-LA (g/L)	Ethanol (g/L)	Yield of D-LA (g/g glucose)	Productivity of D-LA (g/(L·h))
Batch fermentation (conical tube) (N*=3)							
JHY5610	96	8.2±0.6	38.2±1.3	26.8±0.5	2.1±0.1	0.70	0.28
JHY5710	96	13.4±0.2	49.3±0.6	37.0±0.6	1.1±0.0	0.75	0.39
JHY5720	96	12.5±0.3	50.0±0.1	41.2±0.3	0.7±0.1	0.82	0.43
JHY5730	96	13.1±0.2	50.1±0.0	41.2±0.1	0.7±0.0	0.82	0.43
Batch fermentation (flask) (N=3)							
JHY5320	72	17.8±0.6	57.8±2.5	45.2±0.6	0.8±0.1	0.78	0.63
JHY5730	72	25.8±0.1	98.2±1.7	69.7±0.7	0.0	0.71	0.97
Fed-batch fermentation (fermentor) (N=1)							
JHY5730	55	26.0	100.0	82.6	0.36	0.83	1.50

*Number of experiments

Figure legends

Figure 1. Metabolic engineering strategies to produce D-LA in *S. cerevisiae*. (**A**) Metabolic pathways for the production of D-LA in *S. cerevisiae*. Pyruvate, produced by glycolysis, is converted to D-LA by heterologous D-Ldh or ethanol by the sequential reactions of native PDC and ADH. Extracellular lactate is transported into cells via Jen1 transporter, and D-lactate can be oxidized to pyruvate by Dld1. DHAP, dihydroxyacetone phosphate; GAP, glycealdehyde-3-phosphate; G3P, glycerol-3-phosphate. (**B**) Schematic diagrams representing the development process of D-LA-producing strains from *S. cerevisiae* CEN.PK2-1C by using rational metabolic engineering and adaptive evolution. Strains JHY5210, 5310, and 5320 shown on the left side were generated in our previous study [10], and strains JHY5610, 5710, 5720, and 5730 shown on the right side were generated in this study. Deleted genes are indicated. P_{TEF1} or P_{TDH3} promoter and T_{CYC1} terminator were used to express *Lm.ldhA* as indicated. Detailed genotypes of these strains are described in Table 1. (**C**) D-LA production in the LA-producing strains JHY5210 and JHY5610. Cells were cultivated in YPD medium containing 50 g/L of glucose with initial A_{600} of 1. Cell growth, consumption of glucose, and production of D-LA, ethanol, and acetate were monitored for 96 h. Error bars indicate standard deviations of three independent experiments.

Figure 2 Effect of the mutated genes in JHY5710 on LA tolerance and LA production. (**A**) Cells were grown in YPD medium and then A_{600} of 1 cells were serially diluted and spotted onto YPD solid medium with or without 1% LA. JHY5612, JHY5615, and JHY5617, having the indicated genetic modifications, are derivatives of JHY5610. (**B**) Improvement of D-LA

production by adaptive evolution, *ERF2* deletion, and *SUR1*^{I245S} mutation. Cells were cultured in YPD medium containing 50 g/L glucose. Cell growth, residual glucose, D-LA, and ethanol levels were determined for 96 h. Error bars indicate standard deviations of three independent experiments.

Figure 3. Improvement of D-LA production by *PDC1* deletion and additional expression of *Lm.ldh*A. The indicated strains were grown in YPD medium containing 50 g/L glucose. JHY5720 and JHY5730, having the indicated genetic modifications, are derivatives of JHY5710. Cell growth, residual glucose, D-LA, ethanol, and acetate levels were detected during growth. Error bars indicate standard deviations of three independent experiments.

Figure 4. D-LA production in JHY5730. (**A**) Comparison of D-LA production profiles between two evolved LA-tolerant strains in shake flask culture. Evolved strains JHY5320 generated previously [10] and JHY5730 generated in this study were cultured in YPD medium containing 100 g/L glucose, and D-LA and glucose levels were determined for 72 h. Error bars indicate standard deviations of three independent experiments. (**B**) Fed-batch fermentation of JHY5730 under acidic conditions. Strain JHY5730 was cultivated in YPD medium containing 60 g/L glucose with initial A_{600} of 10 in a 1 L bioreactor. The pH was controlled to 3.5. Additional glucose was added into culture medium using the feeding solution (800 g/L glucose) before glucose was completely consumed.

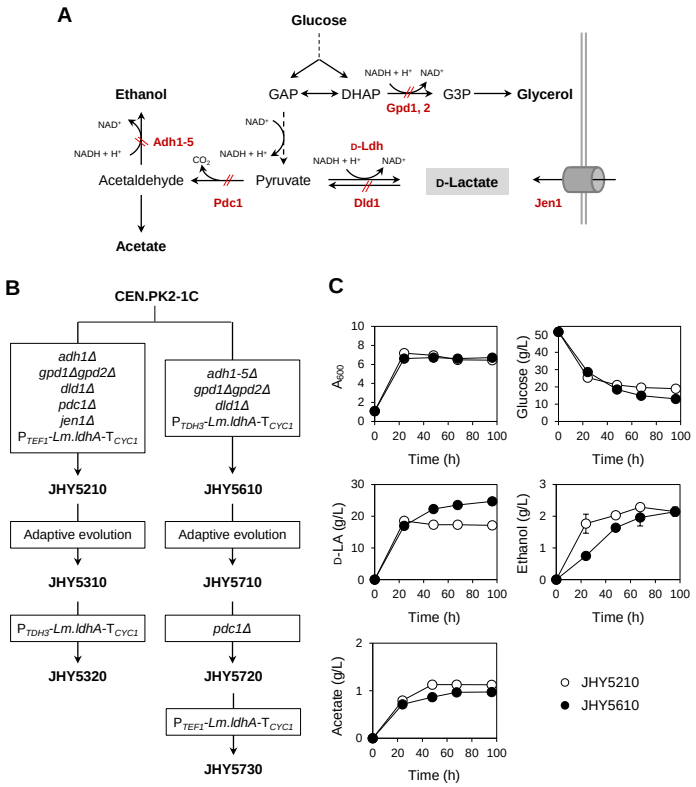

Figure 1

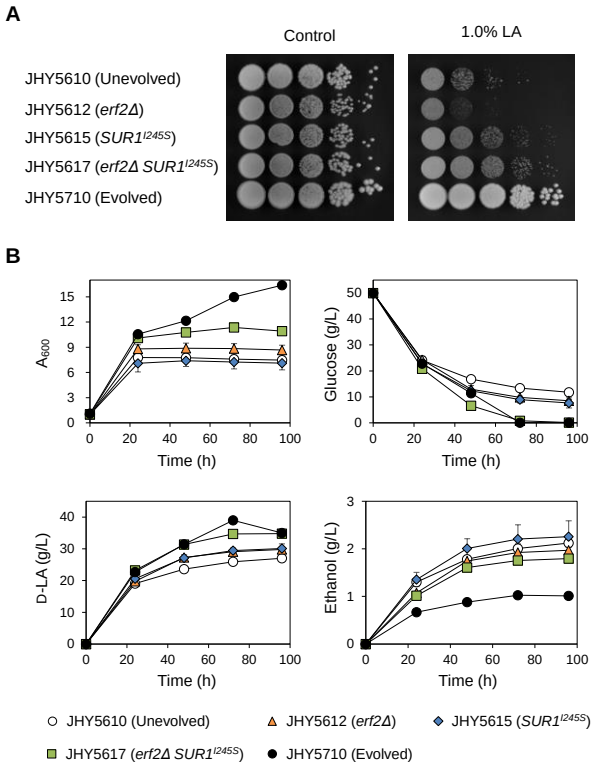

Figure 2

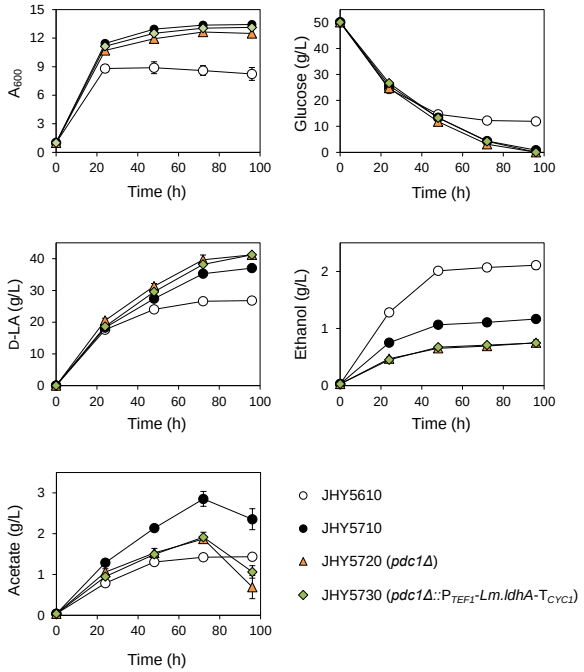

Figure 3

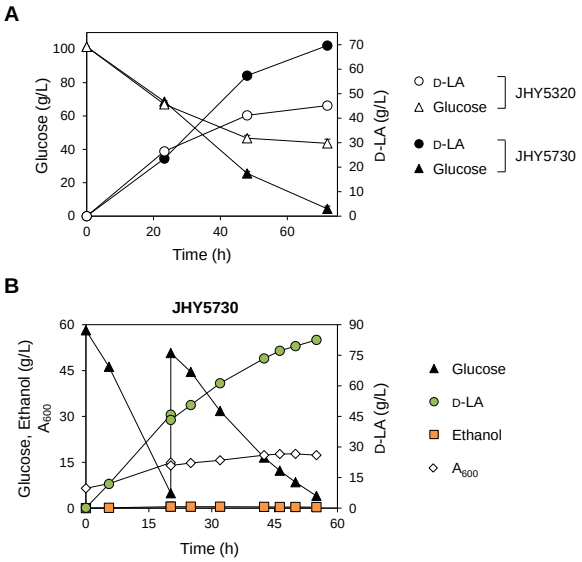

Figure 4