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Introduction of a bacterial acetyl-CoA synthesis pathway improves lactic acid production in *Saccharomyces cerevisiae*

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ABSTRACT

Acid-tolerant *Saccharomyces cerevisiae* was engineered to produce lactic acid by expressing heterologous lactate dehydrogenase (*LDH*) genes, while attenuating several key pathway genes, including glycerol-3-phosphate dehydrogenase1 (*GPD1*) and cytochrome-c oxidoreductase2 (*CYB2*). In order to increase the yield of lactic acid further, the ethanol production pathway was attenuated by disrupting the pyruvate decarboxylase1 (*PDC1*) and alcohol dehydrogenase1 (*ADH1*) genes. Despite an increase in lactic acid yield, severe reduction of the growth rate and glucose consumption rate owing to the absence of ADH1 caused a considerable decrease in the overall productivity. In Δ*adh1* cells, the levels of acetyl-CoA, a key precursor for biologically applicable components, could be insufficient for normal cell growth. To increase the cellular supply of acetyl-CoA, we introduced bacterial acetylating acetaldehyde dehydrogenase (A-ALD) enzyme (EC 1.2.1.10) genes into the lactic acid-producing *S. cerevisiae*. *Escherichia coli*-derived A-ALD genes, *mhpF* and *eutE*, were expressed and effectively complemented the attenuated acetaldehyde dehydrogenase (ALD)/acetyl-CoA synthetase (ACS) pathway in the yeast. The engineered strain, possessing a heterologous acetyl-CoA synthetic pathway, showed an increased glucose consumption rate and higher productivity of lactic acid fermentation. The production of lactic acid was reached at 142 g/L with production yield of 0.89 g/g and productivity of 3.55 g L^{-1} h^{-1} under fed-batch fermentation in bioreactor. This study demonstrates a novel approach that improves productivity of lactic acid by metabolic engineering of the acetyl-CoA biosynthetic pathway in yeast.

1. Introduction

Lactic acid has broad industrial applications. One of its attractive uses is as a monomer for polylactic acid (PLA), a raw material for biodegradable plastics (Choi et al., 2015). Lactic acid has usually been produced using lactic acid bacteria, the natural hosts for lactic acid fermentation. There have been various trials to develop the bacterial strains overproducing lactic acid and attempts to improve the economics (Johnson and Beckham, 2015; Kim et al., 2013, 2015). However, yeasts such as *Saccharomyces cerevisiae* have recently received increasing attention as microorganisms for industrial lactic acid production because of their acid tolerance (Porro et al., 1995).

Saccharomyces cerevisiae does not make lactic acid naturally, yet expression of heterologous L-lactate dehydrogenase (*L-LDH*) genes enables the production of L-lactic acid by a recombinant *S. cerevisiae* strain (Dequin and Barre, 1994). Since yeast has a strong tendency for ethanol production, reduction or inactivation of the ethanol synthetic pathway is essentially required for high-yield production of lactic acid. Disruption of pyruvate decarboxylases (PDCs), which catalyze the first step of ethanol fermentation from pyruvate, is an effective metabolic approach to inhibit ethanol production in *S. cerevisiae*. However, a PDC-negative strain showed severe growth defects in glucose-containing medium and required C2 carbon sources (ethanol or acetate) for growth, even under glucose-limiting conditions (Flikweert et al., 1996). In spite of various attempts to restore growth for industrial purposes, including evolutionary engineering (van Maris et al., 2004), rational approaches to improving a PDC-negative strain are still limited. The second enzyme of ethanol fermentation is alcohol dehydrogenase (ADH), which converts acetaldehyde to ethanol. *S. cerevisiae* possesses multiple ADH genes, and the null mutant of *ADH1*, encoding a major cytosolic alcohol dehydrogenase, diminishes ethanol production. A strain lacking *adh1* is able to grow on glucose medium, yet it shows severe growth retardation under anaerobic conditions (Paquin and Williamson, 1986). The poor growth of ethanol pathway mutants has been interpreted as the

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result of a disturbed redox balance on fermentative growth. Since such phenotypes result in reduced productivity despite high yield, improvement of ethanol pathway mutants is one of the most critical issues in engineering of industrial yeast strains.

Acetyl-CoA is the starting intermediate of the tricarboxylic acid (TCA) cycle and a key precursor for various biosynthetic pathways, including amino acids, fatty acids, and polyketides. It also acts as a signaling molecule, activating cell growth and proliferation by promoting the acetylation of histones at growth genes, and its levels indicate nutrients and growth conditions (Cai et al., 2011; Shi and Tu, 2013). Yeast has multiple routes of acetyl-CoA synthesis, which are dependent on various compartments and growth conditions. Direct conversion of pyruvate to acetyl-CoA, catalyzed by the pyruvate dehydrogenase (PDH) complex, occurs in mitochondria under respiratory growth (Pronk et al., 1994). The PDH complex is the target of glucose repression, and mitochondrial acetyl-CoA is also supplied for other compartments by the glyoxylate cycle or the carnitine/acetyl-carnitine shuttle (van Roermund et al., 1999). During fermentative growth of *S. cerevisiae*, cytosolic acetyl-CoA is produced by a 'PDH-bypass' from acetaldehyde via acetaldehyde dehydrogenases (ALD2-6) and acetyl-CoA synthetases (ACS1/2). The ALDs convert acetaldehyde to acetate using NAD or NADP as cofactors. Even though the multiple ALDs play overlapping roles in the cells, a null mutation in *ALD6*, encoding a major cytosolic aldehyde dehydrogenase, was found to reduce cell growth (Meaden et al., 1997). The next step, charged to ACS, is the synthesis of acetyl-CoA from acetate, involving the hydrolysis of ATP to AMP. Because of its high energy consumption, the activity of ACS is tightly regulated by transcriptional and posttranslational mechanisms and is not increased by simple over-expression of ACS genes (de Jong-Gubbels et al., 1997; Kratzer and Schuller, 1997; Starai et al., 2005). Since acetyl-CoA is also a key precursor for fuels and chemicals, there have been various attempts to increase acetyl-CoA synthesis in yeast. In previous reports, over-expression of endogenous ALD genes and a heterologous-ACS gene (*Salmonella enterica* ACS^{L641P}) increased the acetyl-CoA-driven products (Chen et al., 2013; Shiba et al., 2007).

In this work, we tried to increase the acetyl-CoA supply in lactic acid-producing strains, not for the direct usage of acetyl-CoA as building blocks for biotechnology products, but rather for the improvement of cell growth or carbon catabolic rates in *adh1* null mutants. We achieved a significant increase in lactic acid productivity in Δ*adh1* strains by introduction of a bacterial acetylating acetaldehyde dehydrogenase pathway, which converts acetaldehyde to acetyl-CoA directly, instead of enhancing the ALD–ACS pathway in yeast.

2. Materials and methods

2.1. Yeast genetics and cultivation

Based on *S. cerevisiae* CEN.PK2-1C, lactic acid-producing yeast strains were constructed and are listed in Table 1. For gene deletion, the open reading frame of each gene was replaced with an epitope-tagged URA3 cassette or P_{CCW12}-LDH/URA3 cassettes (Schneider et al., 1995) (US14336945). An ALD6-overproducing strain was generated by replacement of the promoter region of the *ALD6* gene (−272 to −1) with a *GPD* promoter. The *S. cerevisiae* codon-optimized *S. enterica* ACS^{L641P} ($SeACS^{L641P}$) gene under the control of a *TEF1* promoter (Chen et al., 2013) was integrated into the chromosome using the nonfunctional *leu2* locus. The *Escherichia coli* acetylating acetaldehyde dehydrogenase (A-ALD) *MhpF* gene was codon-optimized and synthesized using DNA 2.0 (Menlo Park, CA, USA) and cloned under the control of a *GPD* promoter for expression in yeast. Another A-ALD gene, *EutE,* was polymerase chain reaction (PCR)-amplified from the *E. coli* genome and cloned under the control of a *GPD* promoter. The heterologous genes were introduced into the yeast genome together with appropriate auxotrophic marker genes, and synthetic yeast drop-out medium (Sigma Aldrich; St. Louis, MO, USA) was used for marker selection. Yeast strains were cultivated in YP medium (1% (w/v) yeast extract, 2% peptone). Carbon sources were 2% glucose for aerobic standard growth and 8% glucose for micro-aerobic fermentation. The aerobic cultures and seed cultures were grown at 30 °C with shaking at 230 rpm. For lactic acid fermentation in flasks, cells were inoculated from an overnight seed culture at an optical density at 600 nm (OD_{600}) of 3.0 into fermentation medium in flat-capped flasks and grown at 30 °C with shaking at 90 rpm.

Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Source
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3_112 his3Δ1 MAL2−8^C SUC2*	EUROSCARF
SP2005	CEN.PK2-1C *pdc1Δ cyb2Δ gpd1Δ* $P_{ccw12}-LDH$	This work
SP2006	SP2005 *adh1Δ*	This work
SP1050	SP2006 $PGPD-ALD6\ PTEF1-SeACS^{L641P}$	This work
SP1061	SP2006 $P_{GPD}-mhpF$	This work
SP1081	SP2006 $P_{GPD}-mhpF$ *ald6Δ*	This work
SP1130	SP2006 $P_{GPD}-mhpF$ *ald6Δ* $P_{GPD}-eutE$	This work
SP1175	SP1130 *adh2Δ*	This work
SP2065	SP1130 *adh6Δ*	This work
SP2075	SP1130 *adh2Δ adh6Δ*	This work

2.2. Lactic acid production by fed-batch fermentation

Seed cultures were prepared by growing L-lactic acid-producing *S. cerevisiae* in 100 mL YPD medium in 500-mL Erlenmeyer flasks at 34 °C with shaking at 280 rpm for 10 h. Fed-batch fermentations were performed in a 2-L jar fermentor (New Brunswick Fermentation Systems, USA) containing 1 L medium, at 36 °C with shaking at 475 rpm at an air flow rate of 0.1 L/min. The medium was composed of 60 g/L glucose, 5 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_47H_2O$, 500 mg/L KH_2PO_4, 100 mg/L inositol, 100 mg/L leucine, 50 mg/L nicotinamide, 50 mg/L calcium pantothenate, 50 mg/L valine, 100 mg/L tryptophan, 50 mg/L histidine, 100 mg/L threonine, 100 mg/L uracil, 10 mg/L $FeSO_47H_2O$, 50 μg/L $MnSO_45H_2O$, 200 μg/L $CaCl_2\cdot 2H_2O$, 100 μg/L $CuSO_45H_2O$, 200 μg/L $ZnSO_47H_2O$, 10 μg/L $(NH_4)_6Mo_7O_{24}$, and 20 μg/L $Na_2B_4O_710H_2O$. The feed medium was composed of 700 g/L glucose, 5 g/L yeast extract, 10 g/L $MgSO_47H_2O$, 150 mg/L nicotinamide, and 150 mg/L calcium pantothenate, and was fed continuously to maintain a residual glucose concentration of approximately 10 g/L during the fermentation. The pH was controlled at 4.7 using a 5 N $Ca(OH)_2$ slurry during the initial 20 h of fermentation, after which the pH was not further controlled.

2.3. mRNA levels and enzyme activities

Total RNA was prepared from exponentially growing cells using the RNeasy Mini Kit (Qiagen; Venlo, Netherlands). cDNA conversion was carried out using a SuperScript® VILO cDNA Synthesis Kit (Invitrogen; Carlsbad, CA, USA). Quantitative real-time PCR was performed using the FastStart SYBR Green Master (Roche Diagnostics; Mannheim, Germany) according to the manufacturer's protocol. The CFX96TM Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA) was used for gene expression analysis. Standard curve, expression normalization, and standard error values were obtained with CFX Manager™ software version 1.5 (Bio-Rad), which employs the ΔΔCt method. Normalization was performed with the *TAF10* gene. Reverse transcription-quantitative PCR (RT-qPCR) products were verified using melting curve and peak analysis. For

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determination of acetylating acetaldehyde dehydrogenase activities, yeast cells were cultivated to the exponential phase, and whole-cell crude extracts were prepared by bead beating as previously described (Song et al., 2012). Activity measurement of A-ALD was conducted spectrophotometrically by monitoring the reduction of NAD to $NADH_2$ at 340 nm as reported elsewhere (Clark and Cronan, 1980).

2.4. Analytical methods

Supernatants from flask and bioreactor cultures were analyzed using high-performance liquid chromatography (HPLC). After filtering culture supernatants using 0.45-μm syringe filters, L-lactic acid, glucose, acetate, glycerol, and ethanol were quantified using the Waters e2695 Separation Module instrument equipped with a Waters 2414 Differential Refractometer and a Waters 2998 Photodiode Array Detector (Waters; Milford, MA, USA). An Aminex HPX-87H Organic Acid Analysis Column (300 × 7.8 mm; Bio-Rad) was equilibrated with 2.5 mM H_2SO_4 in water at 60 °C at a 0.5 mL/min flow rate. Culture samples for intracellular metabolite analysis were taken from bioreactors and treated as previously described, with minor modifications (Ohashi et al., 2008). Briefly, 1×10^9 cells were filtered using 0.45-μm pore-size filters and washed with Milli-Q-water. Cells were quenched by 2 mL methanol and lysed by sonication. After chloroform extraction to remove phospholipids, aqueous layers were transferred to the Ultrafree-MC ultrafilter cup and centrifuged at 9100 × *g* and 4 C for 4 h to remove the proteins. Samples were dried and stored at −80 C. D-camphor-10-sulfonic-acid sodium salt was used as the internal standard. Intracellular metabolites were quantified using the Agilent CE capillary electrophoresis system connected to an Agilent 6530 time-of flight mass spectrometer (TOF-MS). Electrospray ionization-TOF MS was operated in the negative ion mode of 3.5 kV, and a flow rate of heated dry nitrogen gas was maintained at 10 psig.

3. Results

3.1. Effect of Δadh1 in lactic acid producing yeast.

In order to generate L-lactic acid producing yeast, the acid-tolerant *S. cerevisiae* CEN.PK2 strain was genetically engineered as previously described (US14336945). Triple deletion of the pyruvate decarboxylase1 (*PDC1*), cytochrome-c oxidoreductase2 (*CYB2*), and glycerol-3-phosphate dehydrogenase1 (*GPD1*) genes and replacing them with the *Pelodiscus sinensis japonicas* LDH and *Bos taurus* LDH genes generated the SP2005 strain, which produced 40 g/L of lactic acid under micro-aerobic flask culture condition in YP medium, containing 80 g/L of glucose. However, this

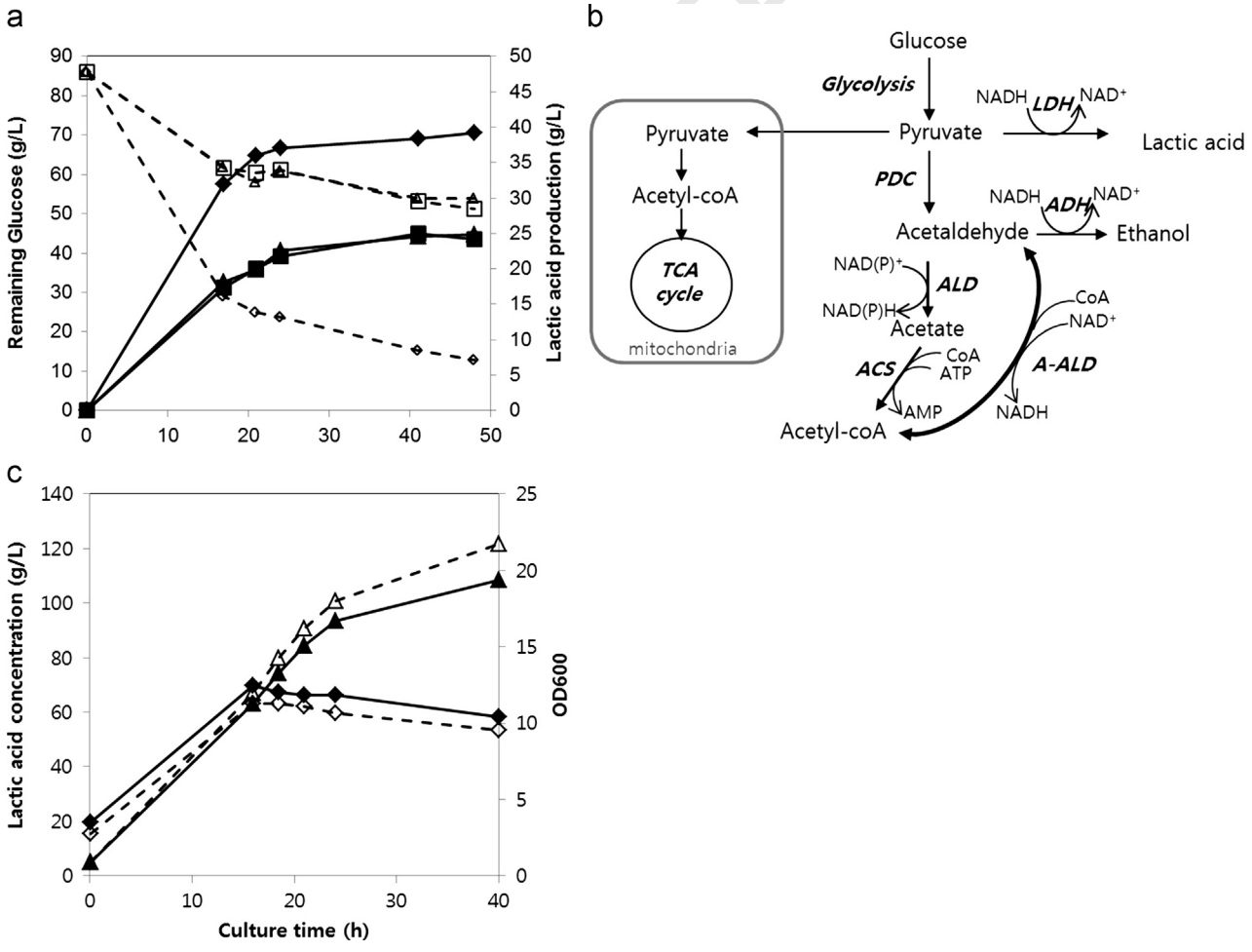

Fig. 1. Effects of modification of ethanol and acetyl-CoA synthetic pathway in lactic acid producing *S.cerevisiae*. (a) Lactic acid fermentation in flask culture. SP2005 (diamonds), SP2006 (squares), and SP1050 (triangles) strains were inoculated from exponentially growing seed culture at an OD_{600} of 3.0 in YP medium with 8% glucose and grown at 30 °C with shaking at 90 rpm. The concentration of glucose (open symbols and dashed lines) and lactic acid (closed symbols and solid lines) in the culture medium were monitored by HPLC. (b) Metabolic pathways which were engineered in this study. (c) Lactic acid fermentation in bioreactor. SP2006 (open symbols and dashed lines) and SP1050 (closed symbols and solid lines) strains were cultured in a 2-L bioreactor as described in the Materials and methods. Cell growth was monitored by measuring the OD_{600} (diamonds) and concentration of lactic acid (triangles) in culture medium were monitored by HPLC.

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strain also produced 15 g/L of ethanol, causing a relatively low lactic acid yield of 0.5 (g/g) (Fig. 1a).

For maximizing the yield of lactic acid production in the *Saccharomyces* strains, it is necessary to minimize the ethanol production flux. In *S. cerevisiae*, the generation of ethanol in Δ*pdc1* mutants is not significantly reduced compared with the PDC1 strain because of the compensational expression of minor PDC genes (Hohmann and Cederberg, 1990). On the other hand, the deletion of the *ADH1* gene, encoding major cytosolic alcohol dehydrogenase, diminishes most of the catalytic activity reducing acetaldehyde to ethanol in the cells (Tokuhiro et al., 2009). For the repression of the ethanol synthetic pathway, we deleted the *ADH1* gene in SP2005. The *adh1* null mutant (SP2006) showed growth retardation, resulting in a 26% lower specific growth rate than SP2005 (0.28 ± 0.001 and 0.38 ± 0.002, respectively) and a maximum OD_{600} of approximately 10 (Table S1). Under micro-aerobic condition, SP2006 produced only 1.4 g/L of ethanol, less than one tenth that of SP2005. At the same time, SP2006 produced 25 g/L of lactic acid, resulting in a lactic acid yield of 0.78 g/g (Fig. 1a). When compared with SP2005, the lactic acid production yield in the Δ*adh1* cells (SP2006) increased by 1.5-fold, yet the lactic acid titer was decreased dramatically because of reduced growth and glucose consumption rates.

3.2. Over-expression of the ALD–ACS pathway in lactic acid-producing yeast

In *S. cerevisiae*, the ADH reaction is the major step for reoxidation of NADH under fermentative conditions. Therefore, it has been presumed that inactivation of ADH1 causes an imbalance of the NADH/NAD ratio, resulting in reduced carbon flux in glycolysis. However, the introduction of more copies of LDH, which also oxidizes NADH, only partially improved glucose consumption and growth (our unpublished data), and it turns out that reduced growth and productivity in the Δ*adh1* strain cannot simply be explained as consequence of redox imbalance.

To enhance cell growth and productivity, we assessed the requirements for increasing the acetyl-CoA pool in *adh1* null mutants. In the case of the PDC-negative strain, in which the first step of the PDH bypass is blocked, depletion of cytosolic acetyl-CoA causes a growth defect of the cells (Flikweert et al., 1999). In Δ*pdc1*Δ*adh1* double mutants, the conversion of pyruvate to acetaldehyde is functional. However, more than 15-fold-accumulated intracellular acetaldehyde, caused by the *ADH1* deletion (Table S1), could affect the activities of ALDs by substrate inhibition (Eggert et al., 2012). We suspected that the resulting supply of acetyl-CoA was not sufficient for normal cell growth in SP2006.

For an efficient conversion of acetaldehyde to acetyl-CoA, the endogenous cytosolic ALD–ACS pathway was overexpressed (Fig. 1b). The promoter of the *ALD6* gene, encoding the major cytosolic aldehyde dehydrogenase, was replaced with the strong *GPD* promoter, and *S. enterica* ACS^{L641P} ($SeACS^{L641P}$) was introduced under the control of the *TEF1* promoter, in order to avoid the usual posttranslational regulation of ACS in yeast. The resulting strain SP1050 showed a slightly increased growth rate ($0.30 \pm 0.006\ h^{-1}$) under aerobic conditions, but lactic acid production and growth characteristics under micro-aerobic conditions were not different from the reference (Fig. 1a). Interestingly, lactic acid production was reduced in SP1050, and the titer was 10–20% lower than that of SP2006 in the fed-batch bioreactor (Figs. 1c and S1). These results indicate that strengthening of the ALD–ACS pathway has no beneficial effect on lactic acid production in yeast.

3.3. Introduction of bacterial acetylating acetaldehyde dehydrogenase into Δadh1

Induced ACS activities originating from $SeACS^{L641P}$ were not controlled by posttranslational regulation in *S. cerevisiae*. Because of its high-energy cost, it may cause overconsumption of ATP in the cells, affecting cell viability, particularly under low-pH fermentative conditions. To overcome the limitations of the endogenous pathway, we tried to introduce the foreign pathway to replace or support for the role of ALD–ACS. The bacteria-originated acetylating acetaldehyde dehydrogenase (A-ALD, EC 1.2.1.10) converts acetaldehyde to acetyl-CoA directly without energy consumption (Fig. 1b). There are several types of A-ALD genes in various prokaryotes that catalyze reversible conversion of acetaldehyde to acetyl-CoA, unlike the ALD–ACS pathway that hydrolyzes ATP. Most of the reported A-ALDs have bifunctional activities or are part of bifunctional enzyme complexes (Manjasetty et al., 2003). *E. coli* MhpF also functions as a bifunctional aldolase (MhpE)/A-ALD(MhpF) complex in the 3-(3-hydroxyphenyl)propionate catabolic pathway (Lee et al., 2006). Naturally, *mhpEF* genes are coexpressed, but MhpF is active as a monomer without oligomerization (Fischer et al., 2013). In previous studies, *mhpF* was functionally expressed in *S. cerevisiae* (Guadalupe Medina et al., 2010). Therefore, we tested the effects of MhpF in SP2006. The *S. cerevisiae* codon-optimized *mhpF* was introduced into SP2006 under the control of the strong *GPD* promoter. The expression of the *mhpF* gene and the catalytic activity of its product were confirmed in the resulting SP1061 cells (Fig. 2a). In spite of MhpF activities, the growth rate and lactic acid production of SP1061 was not drastically enhanced over that of SP2006 (Table S1, Fig. 2b).

Acetate generated from acetaldehyde by ALD is another byproduct in lactic acid fermentation. Accumulation of acetate causes aggravated acid stress and limited lactic acid yield. Even the SP1061 strain that still possesses native ALD genes also produced more than 1.8 g/L of acetate during lactic acid fermentation (Fig. S1). Since the bacterial A-ALD generates acetyl-CoA without producing acetate, we expected that the heterologous pathway could effectively replace the role of the ALD–ACS pathway in the yeast, resulting in positive effects on the productivity and yield of lactic acid. To diminish the natural flux from acetaldehyde to acetate, the *ALD6* gene was deleted in the SP1061 strain. In the resulting SP1081 strain, extracellular acetate levels were reduced to the non-detectable range under micro-aerobic conditions, and lactic acid production was drastically improved. The glucose consumption was enhanced by approximately 27% compared to SP2006, and lactic acid production was also increased by about 30%, with yields reaching more than 0.8 g/g in flask fermentation (Fig. 2b). These results indicate that the function of endogenous ALD/ACS was successfully complemented by overexpressing bacterial A-ALD and knocking out native *ALD6*.

3.4. Improvement of lactic acid production by alternative acetyl-CoA synthesis pathway

To further improve lactic acid production, we evaluated the effects of enhanced expression of A-ALD. Even though protein expression and catalytic activity of MhpF was detected in yeast cells possessing the *mhpF* gene, they showed limited effects on the enhancement of lactic acid production. We tried to increase the A-ALD activities in those cells by introducing additional copies of *mhpF* genes. SP1061 was constructed, containing the multi-copy plasmid pRS426 carrying the *mhpF* genes, and mRNA levels of *mhpF* and catalytic activities of A-ALD were determined in these cells. In spite of a more than 10-fold increase of mRNA levels, A-ALD activity in cell crude extracts was only about 2.8-fold higher

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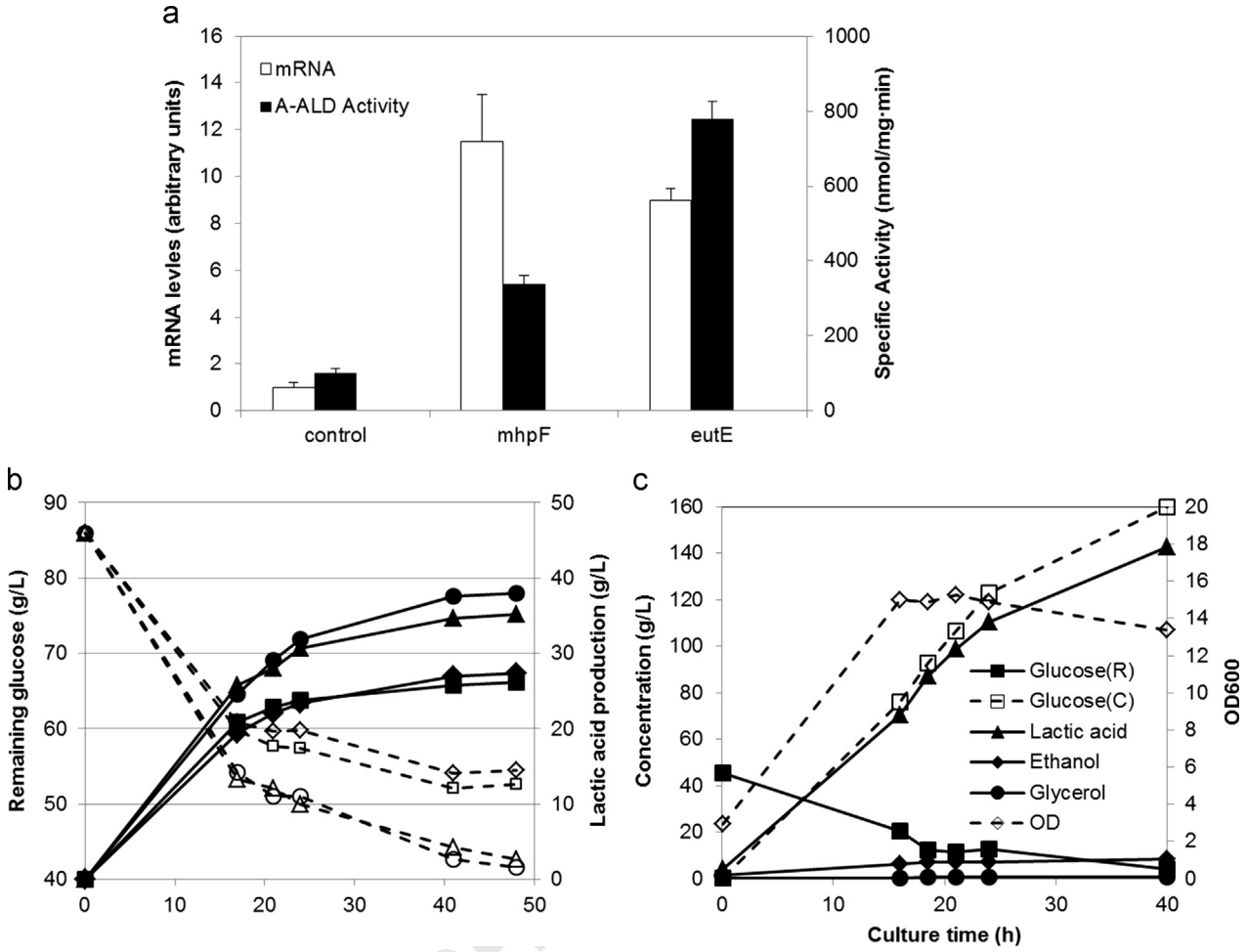

Fig. 2. Introduction of acetylating-acetaldehyde dehydrogenase in lactic acid producing yeast. (a) mRNA levels of the *mhpF* and *eutE* genes were determined by quantitative PCR (open bars), and catalytic activities of A-ALD were measured (solid bar) from SP1061 cells possessing control vector (pRS426,control), pRS426-mhpF (mhpF), and pRS426-eutE (eutE). Error bars represent SDs from three independent experiments. (b) Lactic acid fermentation in flask culture. SP2006 (diamonds), SP1061 (squares), SP1081 (triangles), and SP1130 (circles) strains were inoculated from exponentially growing seed culture at an OD_{600} of 3.0 in YP medium with 8% glucose and grown at 30 °C with shaking at 90 rpm. The concentration of glucose (open symbols and dashed lines) and lactic acid (closed symbols and solid lines) in the culture medium were monitored. (c) Fermentation profile of the SP1130 strain in the fed-batch bioreactor. Cells were cultured in a 2-L jar bioreactor as described in the Materials and methods. Glucose(R) and glucose(C) represent residual glucose in the medium and total amount of glucose consumed, respectively.

than that in the control (Fig. 2a). The inconsistency between the expression of the *mphF* gene and its catalytic activity may be caused by limited stability of heterologous proteins. To solve this problem of heterologous gene expression, other A-ALDs are required that present higher activities in yeast. According to Kozak's recent report on expression of heterologous A-ALD genes in yeast, EutE of *E. coli* showed the best performance in *S. cerevisiae* (Kozak et al., 2014). When the plasmid carrying the e*utE* gene under the control of the *GPD* promoter was introduced into SP1061, the specific activity of A-ALD increased 6.5-fold, which was two times higher compared with *mhpF* under the same conditions (Fig. 2a). Based on these results, *E. coli* EutE, was selected for further study.

The e*utE* gene was introduced and expressed under the control of the *GPD* promoter in SP1081, resulting in SP1130. Under lactic acid-producing conditions in flasks, glucose consumption and lactic acid production in SP1130 increased up to 44 g/L and 37 g/L, respectively (Fig. 2b). The lactic acid yield of the flask fermentation also increased up to 0.85 g/g. In particular, the titer of lactic acid production in SP1130 was comparable to the titer in the ADH1 wild type strain (39 g/L, Fig. 1a). Under aerobic conditions, growth rates of SP1081 and SP1130 (0.27 ± 0.001 and 0.29 ± 0.004, respectively) were not significantly different from those of SP2006, but their maximum OD_{600} values were increased to approximately 13 and 16, respectively (Table S1).

Under the fed-batch fermentation in a 2-L jar fermentor, SP1130 cells produced 142 g/L of lactic acid in 40 h (Fig. 2c). During the fermentation, production of ethanol and glycerol was limited about 8 g/L and 0.7 g/L, respectively, and acetate production was less than 0.1 g/L. Compared to SP2006, the lactic acid productivity in SP1130 was improved by about 20%, up to 3.55 g L^{-1} h^{-1}, and lactic acid yield was up to 0.89 g/g (Table S4).

To better understand the influence of introduction of a heterologous acetyl-CoA synthesis pathway into lactic acid-producing cells, we carried out an intracellular metabolite analysis on the strains SP2006 and SP1130 (Table 2, the full list of analyzed components and levels is provided in Table S2). Cells were collected from 20-h fed-batch bioreactor cultures, in which cell growth reached a maximum OD_{600} and lactic acid productivities were highest. To determine the levels of central metabolites we used Ohashi's metabolite extraction and CE/TOF-MS analytical methods which are especially effective for the detection of phosphate-rich metabolites and cofactors including coenzyme A (Ohashi et al., 2008). Intracellular metabolites levels including glycolytic intermediates showed good reproducibility in our duplicate experiments. For the most part, compounds of the TCA cycle or pentose phosphate pathway were detected at similar or

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increased levels in SP1130 compared to SP2006. In contrast, the earlier intermediates in glycolysis were reduced in SP1130. At the same time, ATP levels in SP1130 were almost two-fold higher than those in the reference strain. These tendencies in intracellular metabolites could be interpreted as an improved carbon flux from glucose uptake to pyruvate, the substrate of lactic acid, and increased supply of biosynthetic intermediates for energy metabolism. In the SP1130 strain, taking an alternative acetyl-CoA synthetic pathway, the determined levels of intracellular acetyl-CoA were 1.8-fold higher than those in SP2006. Since acetyl-CoA could be converted to other metabolites in the cells, we also measured the levels of amino acids which were synthetized from various metabolic intermediates, thereby reflecting their flux. Most of the amino acids generated from glycolytic intermediates were not significantly different in the two strains. However, among the five amino acids whose levels in SP1130 were more than 30% higher than in SP2006, four amino acids (aspartate, asparagine, glutamate, and arginine) are generated from oxaloacetate or α-ketoglutarate and require acetyl-CoA for their biosynthesis. These results suggest that the supply of acetyl-CoA in SP1130 is improved compared to SP2006.

Table 2
Intracellular metabolite concentrations in SP2006 and SP1130 (nmol/10^7 cells). Cells were harvested from 20-h fermentor cultures. Average values with standard deviations from two replicates are given.

Metabolites	SP2006	SP1130
Glucose-6-phosphate	95.92 ± 0.65	15.91 ± 1.19*
Fructose-6-phosphate	11.31 ± 0.18	4.81 ± 1.24*
Fructose-1,6-bisphosphate	168.08 ± 8.14	65.81 ± 0.51*
Dihydroxyacetone phosphate	250.29 ± 15.63	108.78 ± 2.18*
3-phosphoglycerate	47.66 ± 4.89	30.94 ± 2.34
2-phosphoglycerate	9.20 ± 0.48	9.47 ± 0.14
Phosphoenolpyruvate	11.12 ± 1.39	8.75 ± 1.60
Glycerol-3-phosphate	9.87 ± 0.35	9.14 ± 0.3
Acetyl-CoA	1.61 ± 0.14	2.91 ± 0.03*
Isocitrate	1037.75 ± 18.10	1544.67 ± 4.62*
Cis-Aconitate	9.49 ± 0.77	12.38 ± 1.82
a-Ketoglutarate	42.20 ± 0.30	30.65 ± 0.07*
Succinate	85.95 ± 1.13	94.28 ± 1.81
Fumatare	14.60 ± 0.58	18.47 ± 0.16*
Malate	105.76 ± 0.34	159.86 ± 0.02*
6-phosphogluconate	0.88 ± 0.23	2.12 ± 0.19*
Ribulose-5-phosphate	4.76 ± 0.15	8.97 ± 0.23*
Ribose-5-phosphate	3.67 ± 0.12	6.92 ± 0.17*
Sedoheptulose-7-phosphate	8.08 ± 0.72	13.90 ± 0.35*
Erythrose-4-phosphate	13.81 ± 0.46	16.75 ± 0.02*
ATP	84.00 ± 8.45	157.90 ± 7.65*
ADP	7.38 ± 0.72	10.20 ± 0.13
AMP	13.79 ± 0.42	13.98 ± 0.08
Arginine	313.47 ± 1.92	524.74 ± 7.62*
Asparagine	385.40 ± 2.96	509.01 ± 3.01*
Glutamate	3011.42 ± 54.40	4351.52 ± 80.82*
Aspartate	301.16 ± 0.13	432.16 ± 4.35*
Tyrosine	39.30 ± 0.41	61.04 ± 1.22*

* Significant difference between two strains, obtained by *t*-test ($P < 0.05$).

3.5. Development of high-yield lactate producing yeast by emission of alcohol dehydrogenases

Despite lacking ADH1, SP1130 still generated ethanol with a yield of 0.05 g/g. In order to achieve a higher yield of lactic acid production via reduction of ethanol fermentation, we carried out additional engineering of the ethanol pathway. Using reference strain S288C in the genome database (www.yeastgenome.org), we annotated five genes as *ADH2* through *ADH6*, encoding ADH isozymes in CEN.PK2. To examine the effects of the other ADHs in a lactic acid-producing strain, we disrupted one of those ADH genes in SP1130. Under micro-aerobic flask conditions, the production of ethanol in the double mutants was compared. The deletion of *adh2* or *adh6* led to a reduction in ethanol production by 60% or 40%, respectively (Table 3), whereas the disruption of other ADH genes did not show a significant effect on ethanol production in SP1130 (Table S3). In the *adh2* null mutant, SP1175, the lactic acid titer was similar to that of the reference strain. At the same time, the lactic acid yield increased up to 0.9 g/g in flask cultures (Table 3). These results are consistent with the idea that a decrease in ethanol flux increases the lactic acid yield. To minimize ethanol production, we also eliminated the *adh6* gene in SP1175. The resulting strain SP2075, a triple ADH deletion mutant, generated only 0.5 g/L of ethanol in flask cultures. However, glucose consumption was also reduced in these cells, and the lactic acid titer was decreased by 75% compared to SP1175 (Table 3). Hence, the simple deletion of more ADH genes is not beneficial to the development of lactic acid-producing strains. It reveals that the remaining ethanol pathway is still important for the maintenance of the productivity in yeast. Further studies are required for maximizing productivities and yield of lactic acid production.

4. Discussion

S. cerevisiae has a naturally optimized metabolic system for ethanol fermentation and is able to maintain growth and fermentative productivity under a broad range of oxygen levels. The lactic acid-producing pathway, catalyzed by LDH, competes with the ethanol pathway for pyruvate and NADH pools of the cells. Therefore, the ethanol pathway should be inhibited to increase the yield of lactic acid production. Since *S. cerevisiae* has a regulatory system controlling respiratory functions under glucose-replete conditions (Schuller, 2003), inhibition of the ethanol pathway by depletion of ADH resulted in growth retardation, even under aerobic conditions. Glucose consumption rate was reduced to 40% by the *adh1* null mutation, and lactic acid production was also reduced despite high yield.

In this work, by introducing an alternative pathway for the production of acetyl-CoA, we improved glucose consumption rate and lactic acid productivity in *adh1* null mutants. Even though the expression of bacterial A-ALD in *S. cerevisiae* was reported previously, major targets were the reductive catalytic activities from acetyl-CoA to acetaldehyde for ethanol production (Guadalupe

Table 3
Lactic acid fermentation of engineered strains with deletions of multiple ADH genes. Samples of micro-aerobic cultures at 48h were analyzed by HPLC. Average values with standard deviations from triplicates are given.

Strain	ADH deletion	Glucose consumption (g/L)	Titer (g/L)		Yield (g/g)	
			Lactic acid	Ethanol	Lactic acid	Ethanol
SP1130	*adh1*	43 ± 0.7	34 ± 0.7	2.3 ± 0.4	0.80 ± 0.01	0.05 ± 0.01
SP1175	*adh1 adh2*	40 ± 1.2	36 ± 2.0	1.4 ± 0.25	0.90 ± 0.03	0.03 ± 0.005
SP2065	*adh1 adh6*	39 ± 2.3	33 ± 2.5	0.9 ± 0.1	0.83 ± 0.04	0.02 ± 0.001
SP2075	*adh1 adh2 adh6*	29 ± 1.5	27 ± 1.5	0.5 ± 0.12	0.93 ± 0.004	0.02 ± 0.003

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Medina et al., 2010). In an attempt to use A-ALD for acetyl-CoA supply for the production of isoprenoids, the production of mevalonate, the precursor of isoprenoids, was decreased by introduction of A-ALD because of its reversible activities (US patent, US8415136). In our *adh1* null mutants, replacing the reaction from acetaldehyde to acetyl-CoA with A-ALD complemented natural pathway without increasing ethanol production and resulted in enhanced lactic acid production. Since intracellular acetaldehyde is accumulated in Δ*adh1* cells about 15-fold compared with ADH1 cells, the kinetic equilibrium of the enzyme may have shifted direction from acetaldehyde to acetyl-CoA in our strain.

Acetyl-CoA metabolism in *S. cerevisiae* is compartmentalized and differently regulated (Krivoruchko et al., 2015).Mitochondria, in which TCA cycle takes place, is important for the supply of the metabolic intermediates generated from acetyl-CoA. However, under microaerobic condition, respiratory metabolic activities are low and the TCA cycle in mitochondria contributes minor roles in maintaining redox balance and supporting metabolic intermediates. In this condition, the level of acetyl-CoA in mitochondria could be less important for cell growth. While, the limited cytosolic acetyl-CoA pool in the cells attenuated ethanol pathway could be critical for their growth, because cytosolic acetyl-CoA is key precursor for the various building blocks such as amino acids, fatty acids, and sterols. Therefore, enhancement of cytosolic acetyl-CoA could be one of the most effective strategies for the cell engineering (Nielsen, 2014). In this study, we successfully increased intracellular levels of acetyl-CoA by introducing bacterial A-ALD while deleting *ALD6*. Although the intracellular acetyl-CoA concentrations we detected reflect both cytosolic and mitochondrial acetyl-CoA pools, A-ALD, expressed without any targeting sequences, might initially contribute to increasing cytosolic acetyl-CoA levels. In addition, considering the small mitochondrial volume fraction (1–2% of total cell volume) (Rafelski et al., 2012; Uchida et al., 2011), and minor roles of mitochondria under our microaerobic culture conditions, the increased acetyl-CoA levels in SP1130 might be mainly derived from cytosol. The increased acetyl-CoA levels might serve as a driving force to increase the synthesis of acetyl-CoA-originated amino acids in SP1130. Unlike the situation with other biochemicals, the lactic acid production pathway does not require the acetyl-CoA pool as a substrate or an intermediate. Nevertheless, higher supply of acetyl-CoA in lactic acid-producing yeast causes more lactic acid production with higher yield. In the SP1130 strain, glucose consumption rate and lactic acid productivity were increased. The metabolic flux from glucose to terminal metabolites was also improved in the cells. Acetyl-CoA is not only a key intermediate for the metabolic pathway but also participates in various regulatory mechanisms. Cells use the molecule as an indicator for environmental conditions and cellular metabolic activities. According to this signal, various biological reactions, such as cell proliferation, nutrient uptake, metabolism, and stress response, are regulated in transcriptional or posttranslational manners. In SP2006, in which the major metabolic flux to ethanol is diminished and stress caused by lactic acid production is accumulated, turning on the 'activating signal' induced by acetyl-CoA could accelerate metabolic activities and result in enhanced productivity of lactic acid fermentation.

Previously, overproduction of acetyl-CoA by engineering of the ALD–ACS pathway in yeast was applied to the production of other biochemicals (Shiba et al., 2007). The main concept of the strategy was uncontrolled hyper-activation of ACS activity. However, when we first applied this scheme to lactic acid production, it caused reduced lactic acid production in low-pH fermentation. As a weak acid, dissociated form of lactic acid can be accumulated in the cells under low pH condition. When the cells produce lactic acid in high concentration, not only protons but also anion of the organic acid cause the toxic effects on the cells including intracellular acidification, turgor pressure, perturbation of metal homeostasis and amino acid homeostasis. (Bayrock and Ingledew, 2004; Suzuki et al., 2012). The primary defense mechanisms to acid stress arising from lactic acid fermentation are proton out-pumping or lactic acid export of the cells (Martinez-Munoz and Kane, 2008; Pacheco et al., 2012). As these are energy-consuming reactions, the overconsumption of ATP by hyper-active ACS could be hazardous to lactic acid-producing strains. Considering this, the introduction of A-ALD, saving ATP, and reducing acetate is a more proper approach to lactic acid production. Supporting this hypothesis, attenuation of the activity of the ALD–ACS pathway resulted in an increase of lactic acid production, while ATP levels were also increased in the cells.

Interestingly, despite enhanced glucose consumption and lactic acid productivity, cell growth under fermentative conditions did not drastically change in all engineered Δ*adh1* strains tested in this study (Fig. S1). This could be related to the hazardous effects of acetaldehyde, which was highly accumulated by the *adh1* null mutation and was not significantly reduced by the engineering (Table S1). More studies are needed to elucidate the exact mechanism underlying the growth retardation by ADH depletion.

In summary, by introduction of a heterologous acetyl-CoA-producing pathway, we successfully increased the productivity of lactic acid in *adh1* null strains. It turns out that the replacement of ALD–ACS pathways with bacterial A-ALD is a novel and critical strategy for developing hyper-lactic acid-producing *S. cerevisiae* strains. Our results also suggest that enhancement of the acetyl-CoA supply via an energy-saving pathway can be applied to the development of yeast strains with a diminished ethanol pathway for various industrial purposes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.09.006.

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