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Production of itaconic acid using metabolically engineered *Escherichia coli*

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An *Escherichia coli* system was engineered for the heterologous production of itaconic acid via the expression of *cis*-aconitate decarboxylase gene (*cad*), and then maximal itaconic acid levels produced by engineered *E. coli* were evaluated. Expression of *cad* in *E. coli* grown in Luria-Bertani (LB) medium without glucose in a test tube resulted in 0.07 g/L itaconic acid production after 78 h at 20°C. To increase itaconic acid production, *E. coli* recombinants were constructed by inactivating the isocitrate dehydrogenase gene (*icd*) and/or the isocitrate lyase gene (*aceA*). Expression of *cad* and inactivation of *icd* resulted in 0.35 g/L itaconic acid production after 78 h, whereas *aceA* inactivation had no effect on itaconic acid production. The intracellular itaconate concentration in the Δicd strain was higher than that in the *cad*-expressing strain without *icd* inactivation, which suggests that the extracellular secretion of itaconate in *E. coli* is the rate-determining step during itaconic acid production. pH-stat cultivation using the *cad*-expressing Δicd strain in LB medium with 3% glucose in a jar fermenter resulted in 1.71 g/L itaconic acid production after 97 h at 28°C. To further increase itaconic acid production, the aconitase B gene (*acnB*) was overexpressed in the *cad*-expressing Δicd strain. Simultaneous overexpression of *acnB* with the expression of *cad* in the Δicd strain led to 4.34 g/L itaconic acid production after 105 h. Our findings indicate that *icd* inactivation and *acnB* overexpression considerably enhance itaconic acid production in *cad*-expressing *E. coli*.

Key words: aconitase; *cis*-aconitate decarboxylase; isocitrate dehydrogenase; isocitrate lyase; itaconic acid; metabolic engineering

Introduction

Over the past several decades, there has been substantial interest in itaconic acid as a dicarboxyvinyl monomer produced by microbial fermentation of biomass (Kobayashi and Nakamura, 1964; Okabe et al., 2009; Willke and Vorlop, 2001). Owing to its useful properties, itaconic acid is used in the manufacturing of synthetic polymers, such as plastics and resins (Milson and Meers, 1985; Tate, 1981). Graft polymers with an itaconic acid-based main chain and oligolactate side chain have been synthesized and characterized in our laboratory (Ishimoto et al., 2012; Okada et al., 2012). The polymers consisting of itaconic acid had high biomass content and are therefore thought to contribute to carbon emission reduction.

Itaconic acid is industrially produced from sugars, such as glucose, by the fungus *Aspergillus terreus* via submerged fermentation (Bonnarme et al., 1995). To date, many researchers have reported increased productivity of a native itaconic acid producer, *A. terreus*, by using techniques associated with mutation breeding (Yahiro et al., 1995) and fermentation conditions (Okabe et al., 1993; Park et al., 1994; Riscaldati et al., 2000; Träger et al., 1989; Yahiro et al., 1997). Specifically, the spontaneous mutant *A. terreus* TN-484 is used as a target microorganism for optimizing the fermentation process. In a previous study, a typical yield of 82.4 g of itaconic acid per 160 g of supplied glucose was obtained after 6 days of fermentation using *A. terreus* TN-484 (Yahiro et al., 1995).

Remarkable progress has recently been made using genetic engineering tools for metabolic engineering. Additionally, genetic engineering of *A. terreus* has been reported to improve itaconic acid production. For example, Lin et al. (2004) reported that *Vitreoscilla* hemoglobin expression enhanced itaconic acid production in *A. terreus*. Tevz et al. (2010) also reported that itaconic acid production can be

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enhanced in *A. terreus* by inserting modified *A. niger* 6-phosphofructo-1-kinase genes.

Recently, the *cis*-aconitate decarboxylase gene (*cad*) was identified, which encodes the key enzyme catalyzing the decarboxylation of *cis*-aconitate in the tricarboxylic acid (TCA) cycle (Kanamasa et al., 2008). This clone enables the introduction of *cad* into heterologous hosts using the TCA cycle to produce itaconic acid, and the hosts expressing *cad* can be modified to improve itaconic acid production. For example, Li et al. (2012) demonstrated *cad* expression in *A. niger*, which resulted in itaconic acid production of 1.9 g/L from glucose after 168 h of cultivation. Blumhoff et al. (2013) also demonstrated that overexpression of aconitase genes together with the expression of *cad* in both mitochondria and cytosol in *A. niger* resulted in the itaconic acid production of 1.1 g/L after 240–312 h of cultivation. This suggests that the overexpression of the aconitase gene along with the expression of *cad* for itaconic acid production could be an adaptation for citric acid producers. More recently, van der Straat et al. (2014) demonstrated that overexpression of a mitochondrial transporter gene and a plasma membrane transporter gene together with the expression of *cad* in an oxaloacetate hydrolase and glucose oxidase-deficient *A. niger* resulted in the itaconic acid production of 7.1 g/L at 78 h cultivation after induction of gene expression. In addition to these reports, introduction of *cad* into *Escherichia coli* has been reported by Li et al. (2011). They demonstrated *cad* expression under an inducible T7 promoter in *E. coli* cultivated in Luria-Bertani (LB) medium at 20°C, which resulted in itaconic acid production of 0.08 g/L after overnight cultivation.

In general, it takes a relatively long time to cultivate fungi for itaconic acid production. Compared to fungi, *E. coli* seems to be a more suitable producer of itaconic acid because of its high growth rate; furthermore, gene manipulation of *E. coli* is easier than that of fungi (Yu et al., 2011). Additionally, Jantama et al. (2008) demonstrated that pH-stat cultivation of *E. coli* mutants with glucose resulted in a high production of succinic acid (86.5 g/L; 120 h cultivation) and malic acid (69.2 g/L; 144 h cultivation). These results suggest that *E. coli* has the potential to produce dicarboxylic acids, such as succinic acid, malic acid, and itaconic acid. However, itaconic acid production in *cad*-expressing *E. coli* continues to remain low. Therefore, there is a need for developing other techniques to increase itaconic acid production in *E. coli*.

In contrast to only *cad* expression in *E. coli*, interruption of metabolic flow downstream of the itaconate precursor *cis*-aconitate in the TCA cycle in *cad*-expressing *E. coli* seems to improve the flow from *cis*-aconitate to itaconate; thus, *E. coli* is beneficial for itaconic acid production. This interruption can be achieved by inactivating the isocitrate dehydrogenase gene (*icd*) and isocitrate lyase gene (*aceA*), which encode for enzymes metabolizing isocitrate derived from *cis*-aconitate in the TCA cycle. The aims of this study were to enhance the itaconate-generation productivity of *E. coli* by *icd* and/or *aceA* inactivation, thus improving the metabolic flow from *cis*-aconitate to itaconate, and to demonstrate itaconic acid production by pH-stat cultivation with a medium containing glucose as the carbon source to further improve itaconic acid production. In this study, we

evaluated maximal itaconic acid levels produced by engineered *E. coli*.

Here we observed that *icd* inactivation led to an increase in citric acid production in the culture during pH-stat cultivation. Thus, the overexpression of a major aconitase gene (*acnB*) in the *cad*-expressing Δicd strain was also demonstrated to improve the metabolic flow to itaconate via citrate from glucose during pH-stat cultivation. The present study concluded that engineering by *icd* inactivation and *acnB* overexpression is beneficial for itaconic acid production in *cad*-expressing *E. coli*, and 4.34 g/L of itaconic acid can be produced from glucose by engineered *E. coli* cultivated for 105 h.

Materials and Methods

Strains and media. Bacterial strains and plasmids used and developed in this study are listed in Table 1. All *E. coli* strains were grown in LB medium supplemented with 0, 1, and 3% glucose. When required, antibiotics were used at the following concentrations: kanamycin, 50 mg/L; chloramphenicol, 50 mg/L; and carbenicillin, 50 mg/L. Restriction and ligation enzymes were purchased from Roche Applied Science (Penzburg, Germany), Thermo Fisher Scientific (Waltham, MA), New England Biolabs (Beverly, MA), and TOYOBO Co., Ltd. (Osaka, Japan). DNA isolation and manipulation were performed according to standard protocols (Sambrook and Russell, 2001). The overnight cultures of recombinant *E. coli* cultivated in LB medium at 37°C for 18 h were inoculated in fresh LB medium to an optical density of 0.1 at 600 nm (OD₆₀₀). The cultures were subsequently incubated at 20 or 28°C for an appropriate amount of time. After the cultures reached an OD₆₀₀ of 0.4–0.6, isopropylthio- β -D-galactoside (IPTG) was added at a final concentration of 0.1 mM for *cad* and/or *acnB* expression.

Construction of recombinant plasmids. Plasmid construction for *cad* expression was performed in the following manner: according to the deposited *CAD1* sequence from itaconic acid producer *A. terreus* (GenBank database accession no. AB326105), *cad* was synthesized by Biomatik Co., Ltd. (Ontario, Canada). The synthesized *cad* gene was amplified by polymerase chain reaction (PCR) with KOD plus DNA polymerase (TOYOBO), *CAD1* as a template, and a primer set of *cad*-F1 (5'-gcgccatggccaacagagtgcgtcacaacgc-3') and *cad*-R1 (5'-gcgctgcagttacccaagggggactaacggggc-3') under the following conditions: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and polymerization at 68°C for 1 min. After *cad* was subcloned at the *Nco*I and *Pst*I sites of a modified plasmid from pVUB3 (Cote-Sierra et al., 1998), which contains unique *Eco*RI site downstream of *rrnB* terminator, the *Nco*I-*Eco*RI fragment containing *cad* and *rrnB* terminator was cloned into the pETHis expression vector (Chen and Hai, 1994) at the *Nco*I and *Eco*RI sites to create pETHis-*cad*.

Plasmid construction for *acnB* expression was performed in the following manner: according to the deposited sequence of *acnB* from *E. coli* (GenBank database accession no. U41560), a primer set for PCR to amplify *acnB* was constructed. *acnB* was amplified by performing PCR with KOD plus DNA polymerase, with BW25113 genome DNA as a

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Description	Source
<i>Escherichia coli</i> strains		
BW25113	$\Delta(\text{araD-araB})567$, $\Delta \text{lacZ4787}(\text{::rrnB-3})$, λDE3 , $rph-1$, $\Delta(\text{rhaD-rhaB})568$, $hsdR514$	NBRP
JW1122	BW25113 (<i>icd</i> ::Kan ^r)	NBRP
JW3975	BW25113 (<i>aceA</i> ::Kan ^r)	NBRP
SO01	BW25113 integrated with λDE3 prophage	This study
SO02	SO01 with disruption of <i>icd</i>	This study
SO03	SO01 with disruption of <i>aceA</i>	This study
SO04	SO01 with disruption of <i>icd</i> and <i>aceA</i>	This study
SO05	SO01 carrying pLysS and pETHis plasmids	This study
SO06	SO01 carrying pLysS and pETHis-cad plasmids	This study
SO07	SO02 carrying pLysS and pETHis-cad plasmids	This study
SO08	SO03 carrying pLysS and pETHis-cad plasmids	This study
SO09	SO04 carrying pLysS and pETHis-cad plasmids	This study
SO10	SO07 carrying pRSF-1b plasmid	This study
SO11	SO07 carrying pRSF-acnB plasmid	This study
Plasmids		
pETHis	T7 promoter, ColE1 ori, Amp ^r	NBRP
pLysS	Cm ^r	Novagen
pRSF-1b	T7 promoter, RSF1030 ori, Kan ^r	Novagen
pETHis-cad	pETHis carrying <i>cad</i>	This study
pRSF-acnB	pRSF-1b carrying <i>acnB</i>	This study
pCP20	FLP-containing plasmid, Amp ^r	CGSC
pKD46	Red recombinase expression plasmid, Kan ^r	CGSC

template, and a primer set of *acnB*-F1 (5'-gagggatcccggtc tagaagaataacc-3') and *acnB*-R1 (5'-gctctcgagttaaaccgcagctc gg-3') under the following conditions: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and polymerization at 68°C for 1 min. The amplified *acnB* was cloned at the *Bam*HI and *Xho*I sites of the pRSF-1b plasmid (Novagen) to create pRSF-acnB.

Metabolic pathway engineering. The related genes were deleted based on λ Red-mediated bacterial chromosome modification (Datsenko and Wanner, 2000). Initially, *E. coli* JW1122 and JW3975 were transformed with pCP20, and the transformants were obtained by cultivation at 30°C. The transformants were incubated at 43°C for 18 h to express FLP recombinase for elimination of the kanamycin resistance marker from their genomes; therefore, this procedure resulted in the SO02 and SO03 mutants.

The fragment containing the *icd*::Kan^r region was amplified by PCR with KOD plus DNA polymerase and a primer set of *icd*-F2 (5'-gagagtgtgatacatcagccctggtgctcacc-3') and *icd*-R2 (5'-gtctgtcagcgaagctaggtcagcctgaacagatac-3') under the following conditions: denaturation at 94°C for 4 min, followed by 30 cycles consisting of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and polymerization at 68°C for 3 min. The amplified fragment containing the *icd*::Kan^r region was introduced into *E. coli* JW3975 transformed with pRK46, and the cells were subsequently incubated at 30°C for 18 h using a medium supplemented with 1 mM arabinose to induce *icd*::Kan^r region recombination into the *icd* region of the genome. The recombinant *E. coli* JW3975 *icd*::Kan^r was transformed with pCP20 and then subjected to specific conditions to eliminate the kanamycin resistance marker from its genome, resulting in the SO04 mutant.

To confirm the desired mutants, agarose gel electrophoresis was performed using PCR amplicons of respective deleted regions using mutant genomic DNA as the template with the following primer sets: *icd*-F1 (5'-aacgtggtggcagac

gagcaaacacagtag-3') and *icd*-R1 (5'-cccgtaataaatttaacaaactac ggc-3'), and *aceA*-F1 (5'-taccgcctgttagcgtaaaccaccac-3') and *aceA*-R1 (5'-ggcctacagtcagcaacgggtgtgttcg-3'). The λDE3 Lysogenization Kit (Merck KGaA, Darmstadt, Germany) was used for integration of the λDE3 prophage into the mutant chromosomes to construct DE3 lysogenized strains. *E. coli* strains integrated with the λDE3 prophage were transformed with pETHis-cad to express *cad*. The helper plasmid pLysS was also introduced into engineered *E. coli* strains to suppress basal *cad* expression. Another recombinant plasmid, pRSF-acnB, was introduced into the resulting *E. coli* strain to overexpress *acnB*. The metabolic pathway engineered for itaconic acid production in this study is illustrated in Fig. 1.

pH-stat cultivation. Before being transferred into a jar fermenter, the *E. coli* recombinant cells were first grown in 100 ml preculture LB medium in a 500 ml shaking flask for 18 h at 37°C with shaking. All the batch cultivations were performed in a 2 L jar fermenter, M-1000B (Tokyo Rikaki-

Fig. 1. Biosynthetic pathway of itaconate production using glucose, and metabolic engineering of *Escherichia coli* BW25113 (DE3) by *cad* expression, *acnB* overexpression, and *icd* and/or *aceA* inactivation.

kai Co., Ltd., Tokyo, Japan), with a working volume of 1.5 L. The agitation speed was maintained at 300 rpm and the temperature at 28°C. The aeration rate was maintained at 1.5 L/min. pH was controlled at 6.8 with automatic addition of 0.1 M NaOH/1 M NH₄OH or 1 M phosphoric acid. After the cultures reached OD₆₀₀ of 0.4–0.6, IPTG was added at a final concentration of 0.1 mM for *cad* and/or *acnB* expression. When foam on the medium was frequently observed (at an OD₆₀₀ of 2.4 in all cultivations), 100 µl of the anti-foaming agent PE-L (Wako Pure Chemical Industries, Osaka, Japan) was added.

Analytical methods. The itaconic acid and acetic acid produced in the culture supernatant and intracellular itaconate concentration in disrupted cells were quantified using a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a SCR-102H column (Shimadzu). Itaconic acid and acetic acid were eluted using 0.1% perchloric acid solution. The flow rate was 0.9 ml/min, and the eluate was monitored by absorbance at 210 nm. A commercial itaconic acid reagent (Wako Pure Chemical Industries) was used as a control for itaconic acid quantification. Glucose and citric acid concentrations in the culture supernatant were measured using commercial kits: Glucose CII-Test Wako (Wako Pure Chemical Industries) and Enzytec Citric Acid (R-Biopharm AG, Darmstadt, Germany), respectively. Protein concentration was measured using protein assay CBB solution (Nacalai Tesque, Inc., Kyoto, Japan) based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

Cad specific activity in *E. coli* transformants was assayed according to previously described methods (Kanamasa et al., 2008). Aconitase specific activity in *E. coli* transformants was assayed in the following manner: briefly, 10 µl of the crude cell extract was incubated with 30 µl of 60 mM *cis*-aconitic acid solution and 250 µl of 0.2 M sodium phosphate buffer (pH 6.2) for 10 min at 37°C. The enzyme reaction was terminated by the addition of 10 µl of concentrated HCl. The released citric acid was measured by using Enzytec Citric Acid. One unit (U) corresponds to 1 µmol citric acid formed per min.

Specific growth rate (μ) was calculated as the slope of the regression line, from a plot between $\ln(X/X_0)$ and time (t) during the exponential growth period, where X (g-cell/L) and X_0 (g-cell/L) are the cell concentration at t (h) and at the beginning of the exponential phase, respectively.

Results and Discussion

Itaconic acid production by *cad* expression

Expression of *cad* cloned into the pETHis vector containing the inducible promoter P_{T7} was demonstrated in the SO06 strain at 20°C for 18 h in LB medium in a test tube after induction with IPTG. In this study, *cad* expression was performed by the T7 promoter expression system at 20°C according to a previous study by Li et al. (2011). Introduction of pLysS considerably improved Cad protein expression levels (data not shown). HPLC analysis revealed that cultivation for 18 h at 20°C produced itaconic acid (0.03 g/L), suggesting that *cad* expression in a heterologous host elicits itaconic acid production.

Effect of *icd* and/or *aceA* inactivation on itaconic acid production

To enhance itaconic acid production in *E. coli*, metabolic engineering was used to improve the metabolic flow from the itaconate precursor *cis*-aconitate to itaconate. Using the λ Red/ET recombination method, *icd* and/or *aceA* were inactivated, which resulted in the following engineered strains: SO02, SO03, and SO04. Agarose gel electrophoresis showed that the respective gene regions in these mutants were deleted (data not shown). Using the constructed mutants, *cad* was expressed by introducing pETHis-*cad* and pLysS, and then profiles of itaconic acid production in *E. coli* were investigated at 20°C in test tubes. Itaconic acid production was observed in the engineered strains, and maximal levels were produced when itaconic acid production ceased at 78 h.

The SO06 strain produced maximal itaconic acid levels at 0.07 g/L (Table 2). Interestingly, *cad* expression along with *icd* inactivation in *E. coli* produced 0.35 g/L of itaconic acid (Table 2). These results indicate that *icd* inactivation considerably enhances itaconic acid production in *cad*-expressing *E. coli*. On the other hand, the growth rate of the SO07 strain was somewhat slower compared with only *cad* expression, because the abolition of Icd activity in *E. coli* affects the respiratory system and electron transport chain (Kabir and Shimizu, 2004). It was also suggested that protein synthesis levels in the SO07 strain were suppressed by depletion of a series of amino acids (glutamine, glutamate, proline, and arginine) biosynthesized from α -ketoglutarate, presumably because *icd* encodes the key enzyme that metabolizes

Table 2. Cell yield (X), itaconic acid production (P), specific product yield ($Y_{P/X}$), and intracellular itaconate concentration (intIA) from engineered *E. coli* strains cultivated for 18 h and 78 h in LB medium.

Strains	Cultivation time ^a (h)	X (g-cell/L)	P (mg-IA/L)	$Y_{P/X}$ (mg-IA/g-cell)	intIA (mg-IA/g-cell)
SO06	18	1.1 ± 0.0	26.7 ± 5.4	24.5 ± 5.0	1.5 ± 0.4
	78	1.4 ± 0.0	71.6 ± 3.2	53.2 ± 3.0	1.7 ± 0.5
SO07	18	0.4 ± 0.0	169.0 ± 2.0	406.8 ± 7.0	6.8 ± 0.3
	78	1.1 ± 0.0	346.3 ± 3.1	316.5 ± 6.6	8.6 ± 3.0
SO08	18	0.6 ± 0.0	16.1 ± 1.0	29.3 ± 2.4	2.6 ± 0.3
	78	1.1 ± 0.0	60.1 ± 3.6	57.4 ± 2.9	1.9 ± 0.1
SO09	18	0.4 ± 0.0	21.5 ± 1.0	49.8 ± 3.0	3.5 ± 0.2
	78	0.8 ± 0.0	67.4 ± 8.6	89.3 ± 10.9	2.3 ± 0.2

^a18 h, early-middle log phase; 78 h, prolonged stationary phase.

Each strain was cultivated at 20°C in 2 ml of LB medium. After the cultures reached an OD₆₀₀ of 0.4–0.6, IPTG was added at a final concentration of 0.1 mmol/L for *cad* gene expression. Each experiment was repeated 3 times independently.

isocitrate to α -ketoglutarate. In fact, the SO07 strain growth rate substantially improved when cultivated in a minimum medium supplemented with glutamate compared with cultivation without glutamate (data not shown). In this study, we used LB medium in which a series of amino acids is contained. Therefore, the SO07 strain could grow in LB medium without any serious problem. On the other hand, *aceA* inactivation led to a similar tendency of maximal itaconic acid levels (0.06 g/L) but a decrease in cell growth compared with only *cad* expression (Table 2). In the culture supernatants after 54 h cultivation, the SO08 and SO06 strains produced 0.15 and 0.04 g/L of acetic acid, respectively (data not shown). In general, *E. coli* strains release acetic acid into the culture during aerobic growth and then reuptake it as a carbon source (Wagner et al., 1972). Thus, it was suggested that *aceA* inactivation led to inactivation of the glyoxylate shunt; consequently, acetic acid released during cultivation was not reassimilated. Therefore, cell growth of the SO08 strain was lower than that of the SO06 strain. Inactivation of both *icd* and *aceA* as well as inactivation of only *aceA* showed similar cell growth and itaconic acid levels (0.07 g/L) (Table 2). Therefore, it was concluded that inactivation of only *icd* is more effective for itaconic acid production than inactivation of only *aceA* or both of *icd* and *aceA*.

Analysis of intracellular itaconate concentrations in engineered *E. coli* during the cultivation revealed that intracellular itaconate concentration in the SO07 strain increased (6.8 mg/g-cell, 18 h cultivation; 8.6 mg/g-cell, 78 h cultivation) compared with other strains (<3.5 mg/g-cell) (Table 2). This suggests that extracellular secretion of itaconate in *E. coli* is the rate-determining step during itaconic acid production.

To investigate the effect of gene inactivation on *cad* expression level and thus protein product function, intracellular Cad activity was assayed. As a result, Cad activities were estimated to be 0.0 ± 0.0 (the parent strain; SO01), 5.0 ± 1.0 (SO06), 6.0 ± 2.7 (SO07), 5.7 ± 0.4 (SO08), and 2.7 ± 0.0 (SO09) U/mg. The enzymatic assay for Cad activities suggested that *icd* inactivation improves itaconic acid production rather than a change in Cad activity level.

pH-stat cultivations using media with glucose

In general, glucose is recognized as a readily available carbon source for industrial fermentation. Itaconic acid fermentation is also industrially performed by *A. terreus* from sugars such as glucose as a carbon source (Bonnarme et al., 1995). Additionally, a study by Li et al. (2011) on itaconic acid production in *E. coli* was performed using LB medium without additional glucose, and results suggested that it is possible to enhance itaconic acid production in *E. coli* by adding glucose to the medium. To increase itaconic acid production and investigate the effect of additional glucose on itaconic acid production, the SO07 strain was cultivated in LB medium containing 0, 1, or 3% glucose at optimal temperature (28°C) for itaconic acid production. The cultures were maintained at pH 6.8 during all cultivations, because the results of cultivations with LB medium supplemented with and without glucose yielded negligible pH change in cultures without pH maintenance.

Cultivation using LB medium without glucose resulted in

μ of $0.04 \pm 0.00 \text{ h}^{-1}$, maximal cell growth of 1.50 g/L after 54 h, and maximal itaconic acid production of 0.19 g/L after 44 h (Fig. 2A). On the other hand, 1% glucose supplementation led to an increase of each fermentative property (μ , $0.43 \pm 0.03 \text{ h}^{-1}$; maximal cell growth, 2.26 g/L after 49 h; maximal itaconic acid production, 1.09 g/L after 59 h) (Fig. 2B). In this case, itaconic acid production ceased when glucose depletion occurred for 59 h.

To further improve itaconic acid production, cultivation was performed by increasing the glucose concentration to 3%. Compared with 1% glucose, 3% glucose concentration yielded maximal cell growth (5.16 g/L at 107 h) and itaconic acid production (1.71 g/L at 97 h) that were nearly 2.3 and 1.6-times greater, respectively, while the specific growth rate ($\mu = 0.35 \pm 0.02 \text{ h}^{-1}$) was similar (Fig. 2C). In this case, itaconic acid production ceased after 97 h, when glucose was mostly depleted. Consequently, the specific itaconic acid productivity during 107 h cultivation with 3% glucose was estimated to be 0.33 g/g-cell, which is lower than that observed on using 1% glucose (0.48 g/g-cell), suggesting that glucose was utilized for cell growth rather than itaconic acid production, even if the glucose concentration was increased to more than 1%. Unexpectedly, diauxic growth was observed on using 3% glucose, being a reproducible phenomenon without contamination (Fig. 2C). The concentration of acetic acid in the culture decreased after second growth occurred. This is presumably explainable as "acetate switch," which is a phenomenon of transition from acetate production (dissimilation) to acetate utilization (assimilation) (Wolfe, 2005). In addition, it was suggested that *icd* inactivation led to activation of the glyoxylate shunt. Consequently, cell growth was presumably stimulated by assimilation of acetic acid in the glyoxylate shunt activated. The exact reason is unclear, but that may be one reason why

Fig. 2. pH-stat cultivations of engineered *E. coli* SO07.

The recombinant expressing *cad* was cultivated in LB medium supplemented with 0 (A), 1 (B), and 3% glucose (C). Solid circles, cell growth; open circles, itaconic acid (IA) concentration; solid squares, citric acid (CA) concentration; open squares, acetic acid (AA) concentration; open triangle, residual glucose. This cultivation was performed in duplicate, and the average is represented with error bars indicating standard deviations.

diauxic growth occurred. To clarify the mechanism underlying this result, further study is needed.

Interestingly, maximal concentrations of citric acid in the cultures increased in a glucose concentration-dependent manner (0% glucose, 0.20 g/L after 44 h; 1% glucose, 0.82 g/L after 59 h; 3% glucose, 1.52 g/L after 97 h) (Fig. 2). On the other hand, no isocitric acid or *cis*-aconitic acid release was detected in any culture during cultivation (data not shown). These results indicate that glucose supplementation improved itaconic acid production. However, aconitase activity does not seem to be enough to produce itaconate via citrate from glucose. Therefore, glucose supplementation was preferentially utilized for cell growth, and citric acid production was elevated in a glucose concentration-dependent manner.

Effect of *acnB* overexpression on itaconic acid production during pH-stat cultivation

Blumhöff et al. (2013) demonstrated that co-overexpression of aconitase genes with *cad* expression in a citric acid producer, *A. niger*, results in improved itaconic acid production. To improve metabolic flow to itaconate from citrate in engineered *E. coli*, we conducted a similar analysis using *E. coli*. To date, 3 types of aconitase (*AcnA*, *AcnB*, and *PrpD*) have been found in *E. coli*, and it is thought that *AcnB* is the major aconitase for logarithmic growth in aerobic *E. coli* cultures (Walden, 2002). Therefore, we performed *acnB* overexpression in engineered *E. coli*. *acnB* overexpression using a compatible expression vector pRSF-1b containing a T7 promoter in the SO07 strain resulted in an increase of specific aconitase activity in the SO11 strain (1.84 U/mg) compared to the SO10 strain (0.44 U/mg), while specific *Cad* activity was similar (2.1 and 2.5 U/mg, respectively) (Fig. 3).

Cultivation using the SO11 strain in LB medium with 3% glucose showed maximal itaconic acid production at 4.34 g/L after 105 h (Fig. 4). Compared to *acnB* non-overexpression, both maximal cell growth (2.36 g/L after 18 h) and maximal concentration of citric acid in the culture (0.99 g/L after 105 h) were decreased as expected, despite an increase of specific growth rate ($\mu = 0.48 \pm 0.08 \text{ h}^{-1}$). Additionally, glucose consumption and itaconic acid production rates during cultivation, especially in the early phase, considerably improved compared with *acnB* non-overexpression. This result showed that overexpression of *acnB* improved the metabolic flow to itaconate via citrate in engineered *E. coli*.

However, citric acid was observed to a certain degree in the culture, despite the overexpression of *acnB*. Aconitase catalyzes the dehydration of citrate or isocitrate to *cis*-aconitate as well as the hydration of *cis*-aconitate to citrate or isocitrate. Generally, the concentrations in a mixture are citrate $\gg$ isocitrate $>$ *cis*-aconitate (Blumhöff et al., 2013; Schomburg et al., 2013). Therefore, it seems possible that itaconic acid production will be further improved by aconitase activity improvement to convert more citrate to *cis*-aconitate. Surprisingly, maximal concentration of acetic acid in the culture (9.04 g/L after 105 h) increased considerably compared to *acnB* non-overexpression (1.52 g/L after 87 h). Overexpression of *acnB* likely suppressed glyoxylate shunt activity, resulting in the interruption of acetyl-CoA metabolism and suppression of acetate

Fig. 3. Specific *Cad* and aconitase activities in engineered *E. coli* strains.

Specific *Cad* and aconitase activities in SO10 and SO11 strains were measured after cultivation in 2 ml of LB medium at 28°C for 18 h. After the cultures reached an OD₆₀₀ of 0.4–0.6, IPTG was added at a final concentration of 0.1 mM for *cad* and *acnB* expression. This assay was performed at least in duplicate, and the average is represented with error bars indicating standard deviations.

Fig. 4. pH-stat cultivations of engineered *E. coli* SO11.

The recombinant expressing *cad* and overexpressing *acnB* was cultivated in LB medium supplemented with 3% glucose. Solid circles, cell growth; open squares, itaconic acid (IA) concentration; solid squares, citric acid (CA) concentration; open squares, acetic acid (AA) concentration; open triangle, residual glucose. This cultivation was performed in duplicate, and the average is represented with error bars indicating standard deviations.

switch. Consequently, it was suggested that the concentration of acetic acid derived from acetyl-CoA in the culture increased.

The itaconic acid levels achieved in this study are comparable to those reported in a previous study using *A. niger* (van der Straat et al., 2014), but are still much low compared to that reported for the industrial itaconic acid producer *A. terreus*. However, itaconic acid production in *E. coli* can be further enhanced by a metabolic approach, such as inactivation of undesired metabolic pathways involved in acetate production. The technique developed in this study is a promising new approach to enhance itaconic acid production in heterologous itaconic acid producers.

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