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^{13}C-metabolic flux analysis for mevalonate-producing strain of *Escherichia coli*

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Mevalonate (MVA) is used to produce various useful products such as drugs, cosmetics and food additives. An MVA-producing strain of *Escherichia coli* (engineered) was constructed by introducing *mvaES* genes from *Enterococcus faecalis*. The engineered strain produced 1.84 mmol/gDCW/h yielding 22% (C-mol/C-mol) of MVA from glucose in the aerobic exponential growth phase. The mass balance analysis revealed that the MVA yield of the engineered strain was close to the upper limit at the biomass yield. Since MVA is synthesized from acetyl-CoA using NADPH as a cofactor, the production of MVA affects central metabolism in terms of carbon utilization and NADPH requirements. The reason for this highly efficient MVA production was investigated based on ^{13}C-metabolic flux analysis. The estimated flux distributions revealed that the fluxes of acetate formation and the TCA cycle in the engineered strain were lower than those in the control strain. Although the oxidative pentose phosphate pathway is considered as the NADPH generating pathway in *E. coli*, no difference of the flux was observed between the control and engineered strains. The production/consumption balance of NADPH suggested that additional requirement of NADPH for MVA synthesis was obtained from the transhydrogenase reaction in the engineered strain. Comparison between the measured flux distribution and the ideal values for MVA production proposes a strategy for further engineering to improve the MVA production in *E. coli*.

[**Key words:** ^{13}C-metabolic flux analysis; Mevalonate; *Escherichia coli*; [1-^{13}C] glucose; Flux distribution; Redox balance; Flux balance analysis]

Mevalonate (MVA) is a valuable precursor for the synthesis of drugs, cosmetics, and food additives (1–4), and can be produced by fermentation using microorganisms. MVA is synthesized from acetyl-CoA (AcCoA) in central carbon metabolism, and is subsequently used for the synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as essential building blocks of isoprenoids (5). High MVA-producing organisms have been obtained by screening techniques (6,7). It has been reported that *Saccharomycopsis fibuligera* ADK8107 produces 19 g L^{-1} of MVA in 12 days (7). Since this yeast strain consumes MVA for isoprene synthesis, loss of the MVA product is natural for their survival and maintenance (8,9).

Bacteria used for MVA production, such as *Escherichia coli*, do not possess the biosynthetic pathway via MVA, which have been investigated to avoid MVA consumption (9). *E. coli* produces IPP and DMAPP using an alternative pathway from pyruvate (Pyr) and glyceraldehyde 3-phosphate (GAP) (10). The heterologous enzymes of the mevalonate pathway from *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* have been studied for the efficient production of MVA and its derivatives in *E. coli* (1,4,9,11). Tabata and Hashimoto (9) achieved 47 g L^{-1} of MVA production for 2 days under fed-batch culture by introducing *mvaES* genes from *E. faecalis* as these genes function most effectively in *E. coli*. Since MVA is synthesized from AcCoA using NADPH as a cofactor (12), MVA production does affect central metabolism in terms of NADPH requirements and carbon utilization. It is important to optimize the flux distribution in central carbon metabolism for further improvement of MVA production because NADPH is generated by some reactions in these metabolic pathways. However, currently, little is known about the flux distribution in the central carbon metabolism of the MVA-producing strains.

In the present study, an MVA producing *E. coli* (engineered) strain was also constructed by introduction of *mvaES* genes from *E. faecalis*. The effect of MVA production on the central carbon metabolism was investigated by ^{13}C-metabolic flux analysis (^{13}C-MFA). ^{13}C-MFA is a method to experimentally estimate the flux distribution in pathways based on mass balance constraints and isotope labeling measurements (13,14). The flux distribution suggested that additional requirements of NADPH for MVA synthesis are obtained from the transhydrogenase reaction. The flux distributions provide a strategy for rational engineering to improve the production of MVA.

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MATERIALS AND METHODS

Bacterial strains and culture condition All *E. coli* strains were grown as pre-pre-cultures overnight at 37°C in Lennox medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 1 g L^{-1} glucose). Pre-pre-cultures were transferred to

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50 ml of M9 medium (17 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 3.1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, 0.5 g L^{-1} NaCl, 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$, 15 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 8.1 mg L^{-1} $FeCl_3$, 1 mg L^{-1} $MnSO_4 \cdot 4H_2O$, 1.7 mg L^{-1} $ZnCl_2$, 0.43 mg L^{-1} $CuCl_2 \cdot 2H_2O$, 0.6 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.6 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$, and 4 g L^{-1} glucose) to form pre-cultures with an initial optical density of 0.05 at 600 nm (OD_{600}). Pre-culture was performed for reducing the risk of interfusion of rich culture medium. After 24 h, pre-cultures were inoculated in 50 ml of M9 medium containing [1-^{13}C] glucose as the main culture with an initial OD_{600} of 0.05. [1-^{13}C] glucose was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Kanamycin (0.03 g L^{-1}) and isopropylthiogalactoside (IPTG, 24 mg L^{-1}) was added whenever necessary. Dry cell mass was determined by filtration, washing of cells, and drying at 70°C until a constant biomass of an equivalent of 25 mL culture volume at an OD_{600} of 1.0 was obtained.

Construction of the plasmid and strain The *E. coli* strains used in this study are derived from MG1655(DE3) (15). The plasmid pCOLADuet-1 (Merck KGaA, Darmstadt, Germany) was used as the vector. Acetoacetyl-CoA synthase/3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase gene (*mvaE*) and HMG-CoA synthase *mvaS* genes derived from *E. faecalis* were synthesized by GeneArt (Thermo Fisher Scientific, MA, USA) according to *E. coli* codon usage. These genes were trimmed with *Nde*I and *Xho*I, and then subcloned into pCOLADuet-1 to generate pCOLADuet-1/*mvaES* (*mvaE* and *mvaS* expression plasmid, T7 promoter, Km^R). *E. coli* MG1655(DE3) was transformed with pCOLADuet-1 (control) and pCOLADuet-1/*mvaES* (engineered), respectively.

Analyses of dry cell weight and extracellular metabolites Cell growth was monitored by measuring OD_{600} using the UVmini-1240 (Shimadzu, Kyoto, Japan). Dry cell weight was calculated using a conversion coefficient of 0.3 gDCW L^{-1} OD_{600}^{-1}. The concentrations of glucose and organic acids (lactate, formate, acetate, succinate and MVA) in the supernatant of the culture broth were determined by an HPLC system (Shimadzu) equipped with an Aminex HPX-87X column (Bio-Rad, Hercules, CA, USA), UV/vis detector (SPD-20A), and a refractive index detector (RID-10A). The column temperature was set to 65°C, and 1.5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.5 ml min^{-1}. The supernatant of the culture broth was obtained by centrifugation at 18,000 ×*g* for 5 min at 4°C, and then filtered through a Millex HV 0.45-μm filter (Merck KGaA).

Flux prediction by simulation analysis Constraint-based Flux Balance Analysis (FBA) was performed by using the core metabolic model of *E. coli* K-12, named as ecoli_core_model obtained from BiGG Models (16). All calculations were performed using a solver for linear programming, the GNU Linear Programming Kit in Matlab and the COBRA Toolbox (17). The MVA synthesis pathway was added to this model. The glucose uptake rate was set to the experimental value of 7.74 and 8.21 mmol/gDCW/h for control and engineered strains, respectively. In both strains, the non-growth-associated ATP maintenance requirement rate was set to the default value (8.39 mmol/gDCW/h), which was estimated using experimental data from continuous cultures with glucose as a carbon source (18).

To illustrate the relationship between the biomass yield and the MVA yield, the maximum growth rate was calculated using an objective function as a maximization of biomass production. The maximum and minimum fluxes of MVA production were calculated using an objective function as the maximization or minimization of MVA production with a constraint of each fixed growth rate from zero to the maximum value. The MVA production rate and the growth rate were converted to carbon yields using coefficient 6 C-mol/mol-MVA and 63.1 C-mol/gDCW, respectively.

The flux distribution with theoretical MVA production yield was calculated by FBA using an objective function as a maximization of MVA production. To minimize the Euclidian norm of internal fluxes, the optional parameter minNorm of optimizeCbModel function was set to 'one'. The glucose uptake rate was set to the experimentally measured value (8.21 mmol/gDCW/h) of the engineered strain.

Analysis of ^{13}C-enrichment of proteinogenic amino acids ^{13}C-enrichment of proteinogenic amino acids was measured by GC–MS. Broth culture (7.5 ml) was taken from the flask, and centrifuged at 7000 ×*g*, for 5 min at 4°C. The cell pellet was then washed twice with 0.9% NaCl, and hydrolyzed in 6 mol/L HCl at 105°C for 18 h. The resulting proteinogenic acids were derivatized with *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide containing *tert*-butyldimethylchlorosilane in acetonitrile at 105°C for 1 h, and then analyzed by a GC–MS [Agilent 7890A GC and 5975C Mass Selective Detector (Agilent Technologies, Santa Clara, USA)] equipped with a DB-5MS+DG column (Agilent Technologies). The analytical conditions used are described elsewhere (19). The data obtained from GC–MS were corrected by reduction of the natural abundance ratio of C, H, O, N, and Si isotopes. Cumulative ^{13}C-enrichment from 5 to 6 h after cultivation was calculated using the following equation:

$$MDV_{5-6h} = \frac{MDV_{6h} \times OD_{6h} - MDV_{5h} \times OD_{5h}}{OD_{6h} - OD_{5h}} \quad (1)$$

where MDV_{xh} is ^{13}C-enrichment at x h, and OD_{xh} is the cell concentration at x h.

^{13}C-metabolic flux analysis ^{13}C-MFA was performed using the OpenMebius software (20), which is based on the elementary metabolite units framework (21) in Matlab (MathWorks Inc., Natick, MA, USA). The building block and cofactor requirements for *E. coli* biomass formation was referred to in a previous study (22). The metabolic pathway model of *E. coli* that was used in this study has been described previously (19). The following MVA synthesis pathway was added. The amino acid fragments were chosen according to the previous study (23). Metabolic fluxes were estimated by minimizing the residual sum of squares between the experimentally measured and model predicted ^{13}C-enrichment using the fmincon optimization solver in the Matlab toolbox. The standard deviation (SD) of ^{13}C-enrichment was set to 0.01. The 95% confidence intervals were calculated using a grid search method as described in previous studies (24).

Malic enzyme activity measurement The crude cell extract was prepared from M9 batch cultures that were harvested at 2 h after the addition of IPTG. Cells were washed twice with 0.9% NaCl, and disrupted by sonication. The activity of two types of malic enzymes (SfcA and MaeB) was measured as described in a previous study (25). Enzyme activities were measured spectrophotometrically at 30°C by monitoring NADPH production at 340 nm with the UVmini-1240 system (Shimadzu). The reaction was started by addition of 10 μL of crude cell extract. The protein concentration in the supernatant was determined by using the Bradford method.

RESULTS AND DISCUSSION

Growth characteristics of the MVA-producing strain The control and MVA-producing (engineered) strains were constructed by transforming *E. coli* MG1655(DE3) with pCOLADuet-1 and pCOLADuet-1/*mvaES* harboring acetoacetyl-CoA synthase/HMG-CoA reductase gene (*mvaE*) and HMG-CoA synthase gene (*mvaS*) from *E. faecalis*, respectively. The codon usage of *mvaES* genes and its expression vector were modified from the previous report (9). The control and engineered strains were grown in batch cultures on glucose under aerobic conditions. IPTG was added at 4 h after inoculation for inducing the expression of *mvaES*. Fig. 1 shows the cell growth and metabolite production of the two strains. Growth parameters are summarized in Table 1. These specific rates were calculated at the exponential phase between 4 and 6.5 h. Both

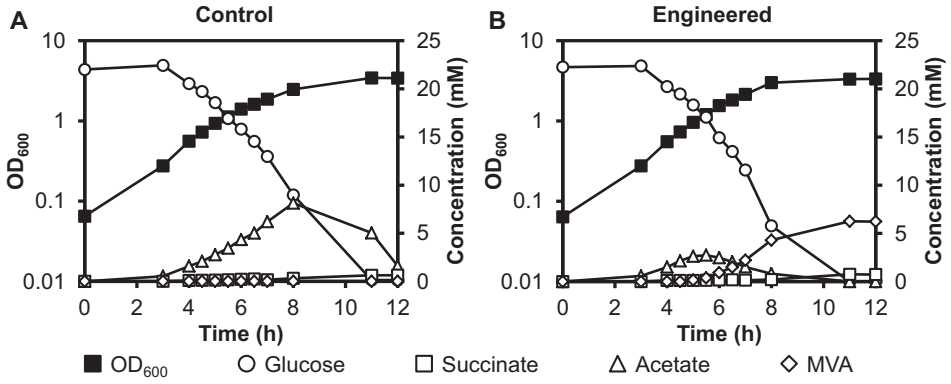

FIG. 1. Growth characteristics of control (A) and engineered (B) strains in batch cultures grown on glucose under aerobic conditions. Closed squares, biomass; circles, glucose; triangles, acetate; open squares, succinate; diamonds, MVA. Data shown are mean ± SD (n = 3).