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Metabolic impact of nutrient starvation in mevalonate-producing *Escherichia coli*

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HIGHLIGHTS

- Sulfur starvation achieved highest mevalonate yield of 0.61 C-mol/C-mol in *E. coli*.
- Degradation of MvaE enzyme caused a large TCA cycle flux under nitrogen starvation.
- TCA cycle flux was suppressed under magnesium or sulfur starvation.
- Mevalonate synthesis was reduced by NADPH supply shortage under magnesium starvation.
- Low TCA cycle flux and enough NADPH production led high yield in sulfur starvation.

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ABSTRACT

The aim of this work was to enhance mevalonate yield from glucose in *Escherichia coli* by essential nutrient starvations and to reveal these effects on the central carbon metabolism. Stationary phase culture without essential nutrients such as nitrogen, sulfur, and magnesium was evaluated using an engineered *E. coli* introducing *mvaE* and *mvaS* genes from *Enterococcus faecalis*. Sulfur starvation resulted in the highest mevalonate yield of 0.61 C-mol C-mol^{-1} from glucose. The metabolic impacts of nutrient starvation were investigated by ^{13}C-metabolic flux analysis. Under nitrogen starvation, the flux of the TCA cycle was large, causing high CO_2 production. This was caused by degradation of mevalonate synthesis pathway enzymes. Under magnesium starvation, NADPH production was decreased, which limited mevalonate synthesis and promoted an overflow of acetate. Sulfur starvation not only suppressed the TCA cycle flux, but also supplied NADPH for mevalonate synthesis.

1. Introduction

Mevalonate is a valuable precursor of terpenoids for drugs, cosmetics, fragrance, and coloring agents, and is an intermediate of a biosynthesis pathway for isoprenoids (Kuzuyama et al., 2004; Tabata and Hashimoto, 2004). This metabolite is synthesized from acetyl-CoA, the intermediate that links glycolysis to the TCA cycle, using NADPH as a cofactor. Mevalonate production has been studied in *Saccharomycopsis fibuligera*, which possesses a native mevalonate synthesis pathway, obtained by screening techniques (Tamura et al., 1968). *S. fibuligera* ADK8107, developed by Asahi Denka Co., Ltd. produced 19 g L^{-1} of mevalonate in 12 days (Kuzuyama et al., 2010). To further improve the productivity by metabolic engineering procedures, *Escherichia coli* has been used as a host because genetic manipulation is simple in this species (Tabata and Hashimoto, 2004). Because *E. coli* does not contain the mevalonate synthesis pathway, mevalonate synthesis genes derived from various species were introduced and mevalonate production was examined (Xiong et al., 2014). Heterologous expression of the mevalonate synthesis genes, *mvaE* and *mvaS*, derived from *Enterococcus faecalis* were used to achieve 47 g L^{-1} mevalonate production in 2 days (Tabata and Hashimoto, 2004). It has been reported that an *E. coli* strain expressing codon-optimized *mvaE* and *mvaS* under the T7 promoter produced mevalonate with a yield of 0.22 C-mol Cmol^{-1} during the exponential growth phase (Wada et al., 2017). Mass balance analysis suggested that there is no room for further mevalonate production at the biomass yield (Wada et al., 2017). Previous studies have suggested that mevalonate production can be enhanced by growth inhibition under sulfur starvation conditions (Li et al., 2016).

In bio-production, nutrient restriction can inhibit cell growth and lead to a stationary phase. Many nutrients are essential for *E. coli* growth, such as nitrogen, sulfur, phosphorus, and trace metal elements. It has been reported that metabolic state varies depends on the restricted nutrients in the parent strain (Chubukov and

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Sauer, 2014). For example, intracellular alpha-ketoglutarate (αKG) accumulates under nitrogen starvation conditions (Chubukov and Sauer, 2014). Because the phosphotransferase system is inhibited by αKG, glucose consumption rate is decreased (Doucette et al., 2011). Sulfur starvation suppresses the synthesis of amino acids containing thiol groups, and promotes decomposition of these amino acids (Shimizu, 2013). Furthermore, iron-sulfur (Fe-S) clusters are not produced under sulfur starvation conditions (Johnson et al., 2005). Because Fe-S clusters are required for various metabolic enzymes, the corresponding reactions are suppressed. Similarly, because magnesium is a cofactor of many metabolic enzymes, magnesium starvation would suppress various metabolic reactions (Chubukov and Sauer, 2014). However, the effects of such nutrient starvations on the metabolism of a mevalonate-producing strain remain unclear.

In the present study, mevalonate production was evaluated in a synthetic medium containing glucose as the sole carbon source without essential nutrients such as nitrogen, sulfur or magnesium using a mevalonate producing *E. coli* strain containing *mvaE* and *mvaS* from *E. faecalis*. The cause of the differences in mevalonate yield following restricted nutrition culture was investigated by ^{13}C-metabolic flux analysis (^{13}C-MFA).

2. Methods

2.1. Strains and medium

Escherichia coli K-12 MG1655(DE3) was used as the host strain. Codon-optimized *mvaE* and *mvaS* from *E. faecalis* were inserted into the multi-cloning site of the pCOLADuet-1 vector (Novagen). *E. coli* cells were transformed with this plasmid for mevalonate production by electroporation (Wada et al., 2017). *pntAB*, *sthA* and *zwf* were deleted by P1 phage transduction (Datsenko and Wanner, 2000). Gene deletions were confirmed by colony PCR using primers targeting sites upstream and downstream of the deletion (Table S1).

M9 medium (44.4 mM glucose, 47.2 mM Na_2HPO_4, 22.7 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.05 mM $FeCl_3$, and 0.1 mM $CaCl_2$) supplemented with 1/1000 vol of trace metal solution (1 mg/L $MnCl_2$ $4H_2O$, 1.7 mg/L $ZnCl_2$, 0.43 mg/L $CuCo_2$ $2H_2O$, 0.60 mg/L $CoCl_2$ $6H_2O$, and 0.60 mg/L Na_2MoO_4 $2H_2O$) was used for cultivation. For nitrogen starvation, 18.7 mM NH_4Cl was removed. For magnesium starvation, 1 mM $MgSO_4$ was replaced with 1 mM Na_2SO_4. For sulfur starvation, 1 mM $MgSO_4$ was replaced with 1 mM $MgCl_2$ (Chubukov and Sauer, 2014).

2.2. Culture conditions

Cell concentration was measured as the absorbance at 600 nm (OD_{600}) using the UVmini-1240 (Shimadzu, Kyoto, Japan). Dry cell weight (DCW) was calculated using a conversion coefficient of 0.3 gDCW L^{-1} OD_{600}^{-1}. In preculture, a single colony was inoculated into 5 mL L medium in a test tube containing 30 mg/L kanamycin and cultured at 37 °C at 150 rpm for 17 h. The preculture was inoculated into 75 mL M9 medium in a 200-mL baffled flask at an OD_{600} of 0.05 and cultured at 37 °C at 150 rpm. At an OD_{600} of 0.5, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 24 h, the cells were collected by centrifugation at 4000*g* for 5 min at room temperature and washed with M9 medium without glucose. The obtained cells were inoculated in 50 mL each nutrient-free medium in 200-mL baffled flask at OD_{600} of 9, and cultured at 37 °C at 150 rpm.

2.3. Measurement of extracellular metabolites

Concentrations of extracellular metabolites such as glucose, mevalonate, D-lactate, formate, acetate, succinate, and ethanol were measured by HPLC (Shimadzu) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Measurement conditions have been described previously (Wada et al., 2017).

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An appropriate amount of cells ([OD_{600}] x culture volume [mL] = 30) was harvested by centrifugation at 15,000*g* for 10 min at 4 °C and the cell pellet was resuspended in 1 M Tris-HCl (pH 7.0). The cells were disrupted by sonication using a UD-100 sonicator (Tomy Seiko, Osaka, Japan) at an output of 50 with 5 s on/off cycles on ice. The samples were centrifuged at 20,000*g* for 5 min to remove debris. The total protein amount was quantified by Bradford assay (Bio-Rad). After denaturation at 95 °C for 3 min, the samples (containing 0.8 mg protein) were analyzed on a 10% SDS-PAGE gel. The gel was stained with Coomassie brilliant blue.

2.5. Measurement of intracellular cofactor levels

Cofactor levels were measured using EnzyChrom $NADP^+$/NADPH and NAD^+/NADH assay kits (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instruction. An appropriate amount of cells ([OD_{600}] × culture volume [mL] = 8) was harvested by centrifugation and the cell pellet was resuspended in 1 mL cold phosphate-buffered saline.

2.6. ^{13}C-labeling experiment

For flux estimation, the carbon source of the stationary phase medium was replaced with [1-^{13}C] glucose. The 3 mL culture was obtained at 2.5 and 3.5 h during the stationary phase, and was immediately quenched by mixing with 4 volumes of cold 60% (v/v) methanol containing 10 mM ammonium acetate. The quenched cells were collected by filtration using a PTFE membrane filter with 0.5 μm pore size (ADVANTECH, Taipei, Taiwan). After adding 640 μL of water and 1.6 mL of chloroform, the sample was mixed for 1 min and ultra-sonicated for 1 min, and then centrifuged 3700*g* for 20 min at 4 °C. The supernatant was evaporated at room temperature, and derivatized with *tert*-butyl dimethylsilylation (TBDMS) for pyruvate, αKG, phosphoenolpyruvate (PEP), citrate (Cit), and 3-phosphoglycerate (3PG) or trimethylsilylation (TMS) for fumarate (Fum), fructose 1,6-bisphosphate (FBP), and mevalonate.

For TBDMS derivatization, 50 μL of methoxyamine hydrochloride in pyridine (40 mg mL^{-1}) was added to the dried sample and incubated for 90 min at 37 °C. Then, 50 μL of *N*-Methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide + 1% *tert*-butyldimethylchlorosilane was added and incubated for 30 min at 60 °C. Pyruvate (m/z = 174), αKG (m/z = 346), PEP (m/z = 453), Cit (m/z = 591), 3PG (m/z = 585), and their mass isotopomers were measured by gas chromatography mass spectrometry (GC/MS) (Agilent 7890A GC and 5975C MSD, Agilent Technologies, Santa Clara, CA, USA) in the selected ion monitoring mode under following conditions: column, DB-5MS + DG (30 m, 0.25 mm, 0.25 μm, Agilent Technologies); carrier gas, helium; inlet temperature, 250 °C; injection mode, split (1:10). Oven temperature was set as follows: 150 °C for 2 min, increased by 3 °C/min to 270 °C, and then increased by 10 °C/min to 300 °C maintained at 300 °C for 5 min. For TMS derivatization, 20 μL of methoxyamine hydrochloride in pyridine (40 mg mL^{-1}) was added to the dried sample and incubated for 90 min at 30 °C. Then, 80 μL of *N*-Methyl-*N*-trimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane was added and incubated for 30 min at 37 °C. Fum (m/z = 346), FBP (m/z = 453),

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