ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Farnesol Production From *Escherichia coli* by Harnessing the Exogenous Mevalonate Pathway

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ABSTRACT: Farnesol (FOH) production has been carried out in metabolically engineered *Escherichia coli*. FOH is formed through the depyrophosphorylation of farnesyl pyrophosphate (FPP), which is synthesized from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) by FPP synthase. In order to increase FPP synthesis, *E. coli* was metabolically engineered to overexpress *ispA* and to utilize the foreign mevalonate (MVA) pathway for the efficient synthesis of IPP and DMAPP. Two-phase culture using a decane overlay of the culture broth was applied to reduce volatile loss of FOH produced during culture and to extract FOH from the culture broth. A FOH production of 135.5 mg/L was obtained from the recombinant *E. coli* harboring the pTispA and pSNA plasmids for *ispA* overexpression and MVA pathway utilization, respectively. It is interesting to observe that a large amount of FOH could be produced from *E. coli* without FOH synthase by the augmentation of FPP synthesis. Introduction of the exogenous MVA pathway enabled the dramatic production of FOH by *E. coli* while no detectable FOH production was observed in the endogenous MEP pathway-only control.

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KEYWORDS: farnesol; mevalonate pathway; FPP synthase; phosphatase; *E. coli*

Introduction

(*E*,*E*)-Farnesol (1-hydroxy-3, 7, 11-trimethyl-2, 6, 10-dodecatriene; $C_{15}H_{26}O$, abbreviated FOH) is an acyclic sesquiterpene widely distributed in nature as an odoriferous component (Lapczynski et al., 2008; Lee et al., 2007). It can be used to enhance the odors of sweet floral perfumes by its distinctive aroma. It is also known to have various biological functions such as signal transduction, quorum-sensing, cell proliferation, apoptosis, and suppression of fungal filamentation (Nickerson et al., 2006; Shea and Del Poeta, 2006; Shirtliff et al., 2009). It has also been developed as the starting material for anticancer pharmaceuticals (Goldberg et al., 2009). Additionally, the properties of low hygroscopicity and high energy content also make FOH an ideal candidate for biofuel molecules (Rude and Schirmer, 2009).

FOH production was observed in a culture of *Saccharomyces cerevisiae* when squalene (SQ) synthase was deficient due to loss-of-function mutation or SQ synthase inhibitors (Asadollahi et al., 2008; Muramatsu et al., 2008a). The resulting FPP could be directed to the formation of FOH in the yeast. Optimization of culture condition such as alkaline pH (Muramatsu et al., 2009) and supplementation with oils and detergents (Muramatsu et al., 2008b) could increase FOH production in yeast. It has been reported that FOH was produced at a oncerntration of approximately 80 mg/L in a mevalonate (MVA) pathway engineered yeast strain after 8 days culture (Takahashi et al., 2007). However, few studies have been carried out using *Escherichia coli* as a host for FOH production. *E. coli* offers great advantages in terms of a higher growth rate and easier genetic manipulation than other industrial organisms including *S. cerevisiae*. FOH production from *E. coli* by in vitro phosphatase treatment has been reported give a concentration of 389 μg/L (Ohto et al., 2009b). Such low

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production and complicated operation make its implementation in an industrial process a distant goal. Thus, there is strong interest in metabolic engineering to improve FOH production. FOH can be generated from the depyrophosphorylation of (*E,E*)-farnesyl pyrophosphate (FPP) by FOH synthase in plants (Cheng et al., 2007) or by promiscuous phosphatases in microorganisms (Fig. 1). Some lipid phosphatases such as LPP1 and DPP1, have been found to hydrolyze FPP to FOH in *S. cerevisiae* (Faulkner et al., 1999). The truncated alkaline phosphatase encoded by *pho8* of *S. cerevisiae* has also demonstrated the ability to convert FPP into FOH (Song, 2006). Acid or alkaline phosphatases have been used to hydrolyze FPP in vitro (Mackie and Overton, 1977; Song, 2003). FPP synthase encoded by *ispA* can catalyze FPP formation by the head-to-tail condensation of one dimethylallyl pyrophosphate (DMAPP) and two isopentenyl pyrophosphates (IPP) (Fujisaki et al., 1990). These two basic building blocks have been found to be produced either by the methylerythritol phosphate (MEP) pathway in plant plastids and most prokaryotes including *E. coli*, or by MVA pathway in eukaryotes and some bacteria. Interconversion of IPP and DMAPP can be catalyzed by the IPP isomerase encoded by *idi* (Anderson et al., 1989). Overexpression of the *idi* and *ispA* genes increased FOH production in *E. coli*, where FOH production was measured by treatment with potato acid phosphatase for the in vitro depyrophosphorylation of FPP (Ohto et al., 2009b). To the best of our knowledge, there has been no report of direct FOH production in *E. coli*. In this study, *E. coli* was metabolically engineered for direct FOH formation by increasing FPP synthesis through use of the MVA pathway and *ispA* overexpression. We also applied two-phase culture for FOH production using a decane overlay of the culture broth to reduce the volatile loss of FOH.

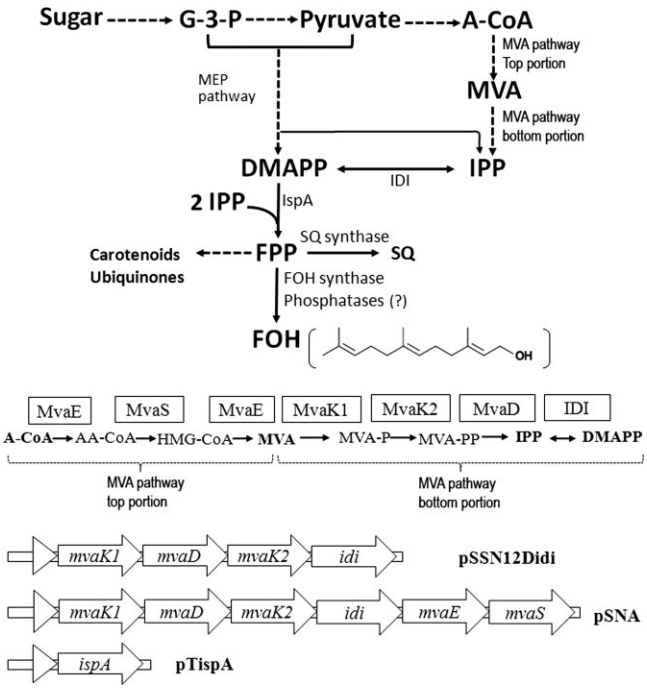

Figure 1. Pathway of FOH formation via IPP, DMAPP, and FPP and depiction of the synthetic operons used in this study. IPP and DMAPP are synthesized by the MEP or MVA pathway. The MEP pathway is initiated by the condensation of glyceraldehyde 3-phosphate and pyruvate. The MVA pathway can be divided into the top portion (acetyl-CoA to MVA) and bottom portion (MVA to IPP and DMAPP). FPP is synthesized by IspA through the condensation of 1 DMAPP and 2 IPPs. The solid and dashed arrows represent single and multiple-enzyme reactions, respectively. Plasmid pSSN12Didi and pSNA are composed of bottom portion of the MVA pathway (Yoon et al., 2007) and the entire MVA pathway (Yoon et al., 2009), respectively. The open arrows and triangles represent genes and promoters, respectively. Abbreviations of the pathway intermediates are as follows: G-3-P, glyceraldehyde 3-phosphate; MEP, methyl-erythritol phosphate; A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVA-P, mevalonate 5-phosphate; MVA-PP, mevalonate pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; FPP, (*E,E*)-farnesyl pyrophosphate; FOH, farnesol; SQ, squalene. The abbreviations of enzymes are as follows: MvaE, bifunctional acetoacetyl-CoA thiolase and HMG-CoA reductase. MvaS, HMG-CoA synthase; MvaK1, mevalonate kinase; MvaK2, phosphomevalonate kinase; MvaD, mevalonate pyrophosphate decarboxylase; IDI, IPP isomerase; IspA, FPP synthase; phosphatases (?), endogenous promiscuous phosphatases of *E. coli*.

Materials and Methods

Chemical Regents

All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The plasmid preparation and gel extraction kits were purchased from Qiagen (Valencia, CA). Farnesol, *n*-decane, and *n*-dodecane are Sigma (St. Louis, MO) products. For the PCR reaction, *Pfu* DNA polymerase and oligonucleotides were ordered from SolGent (Daejeon, Korea).

Bacterial Strains

The strains used in this study are listed in Table I. *E. coli* DH5α was used for all DNA manipulations and FOH production. *E. coli* SF7N (Fujisaki et al., 2005) was an *ispA* knockout stain of *E. coli* W3110, and was used to determine the effect of *ispA* on FOH production. *E. coli* BW25113 and Keio collection mutants (Baba et al., 2006) of *E. coli* BW25113 Δ*phoA* (JW0374), Δ*aphA* (JW4015), Δ*bacA* (JW3029), Δ*pgpA* (JW0408), and Δ*pgpB* (JW1270) were used to investigate the putative phosphatases responsible for FOH formation. *E. coli* BW25113 Δ*pgpAB* containing a double deletion of *pgpA* and *pgpB* was created by P1 transduction using JW0408 and JW1270. Before transduction, the kanamycin antibiotic marker gene was removed from the JW1270 mutant using plasmid pCP20 (Datsenko and Wanner 2000) to make it a recipient cell. The JW0408 mutant was used as a donor cell. Genomic DNA from the transductants was extracted and subjected to PCR. The primer pairs of pgpA-F and pgpA-R, and pgpB-F and pgpB-R (Table I) were designed to flank *pgpA* and *pgpB*, respectively, and were used to verify successful P1 transduction.

Table I. Strains, plasmids, and primers used in this work.

Name	Description	Refs.
Strains		
DH5α	F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1 endA1, hsdR17(rK^- mK^+), phoA, supE44, λ^-, thi	ATCC 53868
W3110	F-, lambda-, IN(rrnD-rrnE)1, rph-1	ATCC 27325
SF7N	W3110 *ΔispA*	Fujisaki et al. (2005)
BW25113	Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), $lambda^-$, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*	
JW0374	BW25113 *ΔphoA*	Baba et al. (2006)
JW4015	BW25113 *ΔaphA*	Baba et al. (2006)
JW3029	BW25113 *ΔbacA*	Baba et al. (2006)
JW0408	BW25113 *ΔpgpA*	Baba et al. (2006)
JW1270	BW25113 *ΔpgpB*	Baba et al. (2006)
JW1270408	BW25113 *ΔpgpA*, Δ*pgpB*	This study
Plasmids		
pSTV28	P_{lac} expression vector, pACYC184 origin, *lacZ*, Cm^r	Takara Co., Ltd
pSSN12Didi	pSTV28 containing *mvaK1*, *mvaK2*, and *mvaD* of *S. pneumoniae*, and *idi* of *E. coli*	Yoon et al. (2007)
pSNA	pSTV28 containing *mvaE* and *mvaS* of *E. faecalis*, *mvaK1*, *mvaK2*, and *mvaD* of *S. pneumoniae*, and *idi* of *E. coli*	Yoon et al. (2009)
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	Amersham Bioscience
pTispA	pTrc99A vector containing *ispA* of *E. coli*	This study
pBBR1MCS2	Broad-host-range (bhr) cloning vector, K^r_m	Kovach et al. (1995)
pBBRispA	pBBR1MCS2 vector containing *ispA* of *E. coli*	This study
pCP20	Temperature-sensitive replication, thermal induction of FLP synthesis, Amp^r	Datsenko and Wanner (2000)
Primers		
ispA-F1	GCGAATTCATG GAC TTT CCG CAG CAA C	This study
ispA-F2	GCGTCGAC AGGAGATATACCATGGACTTTCCGCAGCAAC	This study
ispA-R	GCTGCAGTTATTTATTACGCTGGATGTAG	This study
pgpB-F	TTCCTGGAACTGGCGTTTTCAG	This study
pgpB-R	GATGATTTATGGGCTAAATTCG	This study
pgpAref-F	CGAACCTGTTACATTAGACTGG	This study
pgpAref-R	ACAAGCGTCACATCAGGCATCG	This study

Plasmids Construction

Basic molecular biological techniques, including genomic DNA preparation, restriction enzyme digestion, and transformation were carried out as described in the literature (Sambrook and Russell, 2001). Briefly, the *ispA* fragment was amplified from the *E. coli* genome using *Pfu* DNA polymerase (SolGent). For the construction of pTispA, ispA-F1, and ispA-R primers were used to perform the PCR reaction. This resulting PCR fragment was inserted between *Eco*RI and *Pst*I of pTrc99A after the corresponding restriction enzyme digestion. In a similar manner, the *ispA* fragment amplified by ispA-F2 and ispA-R was cloned into pBBR1MCS2 to obtain pBBRispA after digestion with *Sal*I and *Pst*I. Each primer sequence was listed in Table I.

Media and Culture Conditions

E. coli was grown in 2YT medium (16 g tryptone, 5 g sodium chloride, and 10 g yeast extracts per 1 L) at 37°C for gene manipulation and at 29°C for FOH production. Antibiotics (ampicillin at 100 μg/mL, chloramphenicol at 50 μg/mL, and kanamycin at 50 μg/mL) were added as required. Glycerol served as a carbon source and was supplied at a concentration of 1% (v/v) unless otherwise specified. MVA was prepared from mevalonolactone (Sigma) as previously described (Kim et al., 1992), and fed at a concentration of 5 mM for IPP synthesis when bottom portion of the MVA pathway was employed. To avoid the volatile loss of FOH, 1 mL of decane was initially overlaid on 5 mL of culture broth for solubilization of FOH and in situ extraction of FOH from the culture broth. Cell growth was monitored by optical density at 600 nm (OD600).

FOH Production Analysis and Quantification

FOH was extracted with ethyl acetate from culture broth without the decane overlay. In two-phase culture, the decane phase was collected and centrifuged for 10 min at 12,000 rpm to remove cell debris and subsequently analyzed for FOH using thin-layer chromatography (TLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). For the TLC assay, silica plates were developed with hexane/acetone (3:1) and visualized with potassium permanganate. GC-MS analysis was run on a Double-Focusing Mass Spectrometer GC Mate II (JEOL, Tokyo, Japan). FOH production was quantified using an Agilent Technologies 7890A Gas Chromatograph equipped with a flame ionization detector (FID). Samples were injected at a split ratio of 1:10. Each analyte from the 1 μL samples was separated on a 19091J HP-5 column (30 m, length; 0.32 mm, internal diameter; 250 μm, film thickness). The oven temperature was initially held at 80°C for 1 min

and increased at a rate of 10°C/min to 250°C where it was held for 1 min. Nitrogen was used as the carrier gas with an inlet pressure of 39 psi. The detector was maintained at 260°C. Dodecane was used as an internal standard.

Quantification of Residual Glycerol

Culture broth was centrifuged and the supernatant was injected for residual glycerol quantification. Glycerol concentration was determined by high performance liquid chromatography (Shimadzu, Kyoto, Japan) with a refractive index detector using a Kromasil KR100-5NH_2 column (250 mm × 4.6 mm, Eka Chemicals, Bohus, Sweden). The mobile phase was 75% acetonitrile in water at 35°C and a rate of 1.5 mL/min.

Results

Application of the Bottom Portion of MVA Pathway for FOH Production

In yeast, FOH is probably generated through FPP hydrolysis by endogenous phosphatases, which are induced by an increased intracellular FPP level (Asadollahi et al., 2008; Muramatsu et al., 2008a). Analogously, we hypothesized that *E. coli* could produce FOH under cellular conditions of an increased intracellular FPP level through metabolic engineering.

A foreign MVA pathway has been shown to synthesize IPP and DMAPP efficiently in *E. coli* (Martin et al., 2003). In our previous work, we have demonstrated that the introduction of the MVA pathway into *E. coli* enhanced the production of carotenoids which are also composed of IPP and DMAPP (Yoon et al., 2007, 2009). Thus, we introduced the bottom portion of MVA pathway into *E. coli* for FOH production by the transformation of pSSN12Didi, which performed well in the synthesis of IPP and DMAPP from MVA (Yoon et al., 2007). However, FOH production was not observed in recombinant *E. coli* culture with the supplementation of 5 mM MVA (data not shown). This result was thought to be caused by the volatile loss of FOH during culture. Two-phase cultures have been successfully employed to collect toxic, water-immiscible or volatile compounds (Leon et al., 2003; Newman et al., 2006; Verhoef et al., 2009). Because of its high hydrophobicity (log $P_{O/W}$, 5.6) and low volatility, decane was chosen to extract and solubilize FOH from culture broth in this study. Decane toxicity in the cell and FOH retainability in decane were first tested with a 1 mL of decane overlay on 5 mL of culture broth. As expected, the decane overlay in the two-phase culture did not affect growth, and FOH could be solubilized in the decane phase with negligible volatile loss (Fig. 2). Thereafter, the two-phase culture of, 1 mL of decane overlaid to 5 mL of culture broth was applied to further studies.

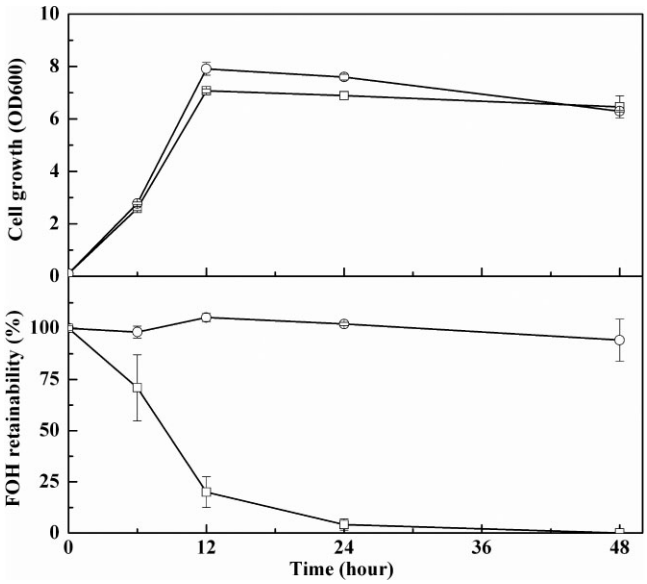

Figure 2. Effects of the decane overlay on cell growth and FOH retainability during culture. *E. coli* DH5α strain harboring empty pTrc99A vector was cultivated with (○) and without (□) 1 mL of decane overlay on 5 mL of culture broth, and cell growth was measured at various time intervals (upper). FOH at a concentration of 200 mg/L was added to culture with (○) and without (□) the decane overlay and the residual amount of FOH in the culture was measured at various time intervals (lower). FOH retainability is represented as a percentage of the residual FOH amount in comparison with the 200 mg/L of FOH initially added.

Two-phase culture of *E. coli* DH5α (pSSN12Didi) was carried out in 2YT medium containing 5 mM MVA and 1% glycerol at 29°C for 48 h (Fig. 3A–C). The decane phase of the two-phase culture was collected to analyze the FOH content by TLC, GC, and GC-MS. In the TLC analysis (Fig. 3A), a spot with an R_f value 0.43 was observed in the collected decane phase sample (lane 1), which was the same as the FOH standard dissolved in decane (lane 2). In the GC analysis (Fig. 3B), there was a main peak at 10.4 min in the collected decane phase sample, which corresponded to the reference solution of the standard FOH compound dissolved in decane. Mass spectrometry confirmed that the peak at 10.4 min was FOH (Fig. 3C). However, the peak was not observed in two-phase culture without the addition of 5 mM MVA. The inability of pSSN12Didi to produce IPP and DMAPP without the addition of MVA suggested that the IPP and DMAPP generated by native MEP pathway are not enough to be coupled to form FOH. The formation of FOH from FPP was further confirmed by blocking FPP synthesis. The *ispA* deletion mutant *E. coli* SF7N (Fujisaki et al., 2005) and the wild-type strain *E. coli* W3110 were transformed with pSSN12Didi and cultivated by the two-phase culture with the addition of 5 mM MVA (Fig. 3D). In the GC chromatogram, the FOH peak was observed in *E. coli* W3110 culture, whereas no peak was observed with *E. coli* SF7N. It was found that the *ispA* gene encoding the FPP synthase was critical for FOH production in *E. coli*. Because

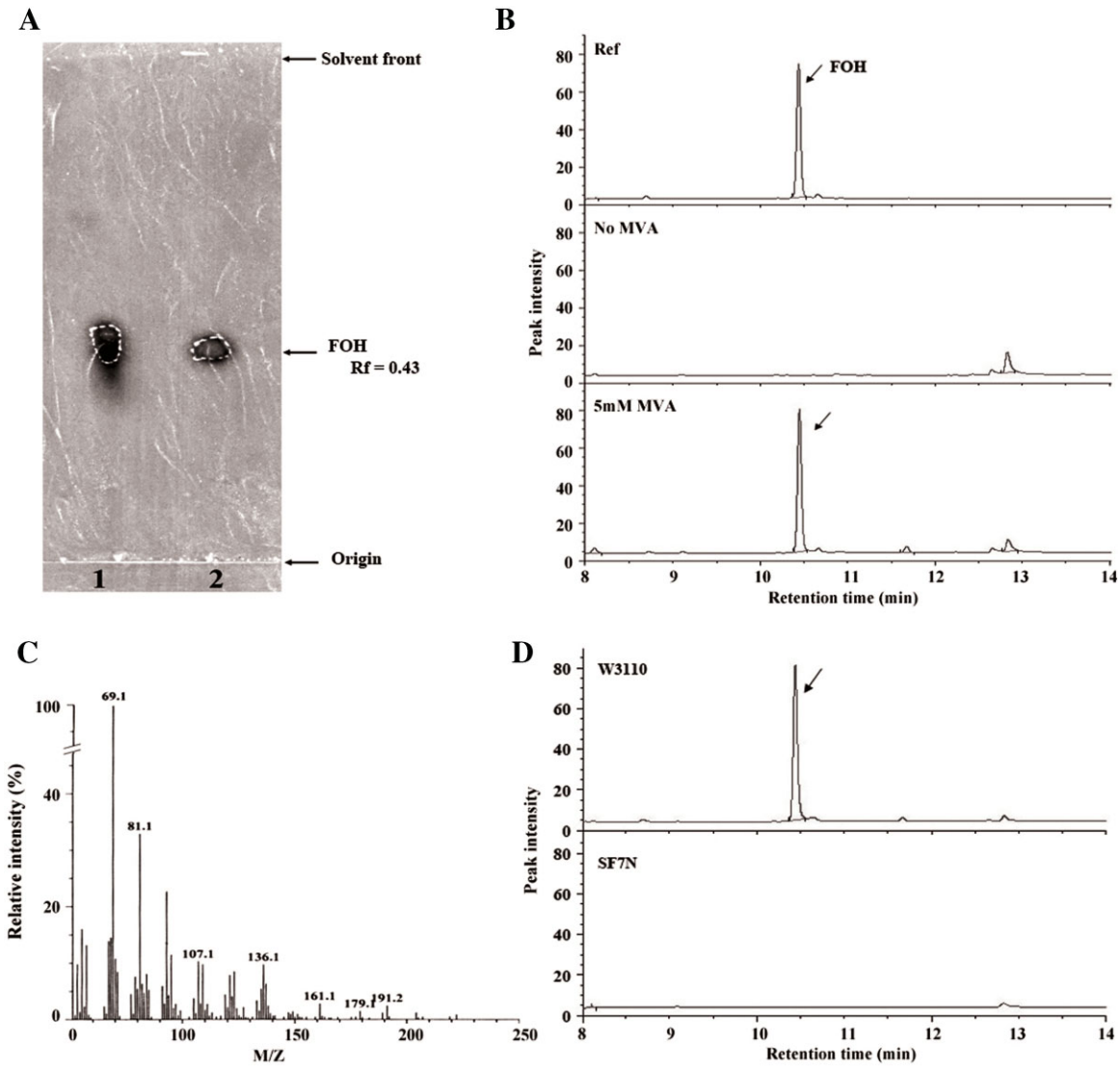

Figure 3. Identification of FOH produced from *E. coli* DH5α (pSSN12Didi) supplemented with exogenous MVA by TLC (**A**), GC (**B**) and GC-MS (**C**), and FOH production by *E. coli* W3110 (pSSN12Didi) and *E. coli* SF7N (Δ*ispA*, pSSN12Didi) (**D**). In the TLC chromatogram, **lanes 1 and 2** are a sample of the decane phase collected from two-phase culture and the FOH standard solution dissolved in decane, respectively (A). In the GC chromatogram, "no MVA" and "5 mM MVA" represent the GC analysis of culture supplemented with 0 mM and 5 mM mevalonate, respectively. The FOH standard solution (Ref) was used as a control. The peak corresponding to The FOH standard at 10.4 min is indicated by an arrow (B). The peak at 10.4 min was applied to GC-MS (C). FPP synthase deletion mutant, *E. coli* SF7N (Δ*ispA*, pSSN12Didi), and its wild-type strain, *E. coli* W3110 (pSSN12Didi), were compared with respect to FOH formation using GC analysis (D).

there is no endogenous FOH synthase in *E. coli*, FOH production has been studied by in vitro phosphatase treatment of cell lysate (Ohto et al., 2009b). This study indicates that FOH is produced by endogenous phosphatases of *E. coli*. It represents the first report of FOH production from enhanced IPP and DMAPP in an engineered *E. coli* strain.

Effect of the Overexpression of FPP Synthase on FOH Production

To increase FPP synthesis and FOH production, *ispA* overexpression was carried out using pTrc99A, a high-copy vector with a strong *trc* promoter and pBBR1MCS2, a low-copy vector with a *lac* promoter. Plasmids, pTispA and pBBRispA, were constructed by cloning *ispA* into pTrc99A and pBBR1MCS2, respectively. The plasmids were transformed into *E. coli* DH5α (pSSN12Didi) to yield *E. coli* (pSSN12Didi, pTispA) and *E. coli* (pSSN12Didi, pBBRispA). The double transformants were cultivated in 2YT medium containing 5 mM MVA at 29°C for 48 h using two-phase culture and the FOH production is shown in Figure 4. Overexpression of *ispA* using pTrc99A improved FOH production compared with pBBR1MCS2. After 48 h, a concentration of 106.2 mg/L of FOH was obtained in a culture of *E. coli* (pSSN12Didi, pTispA), which was

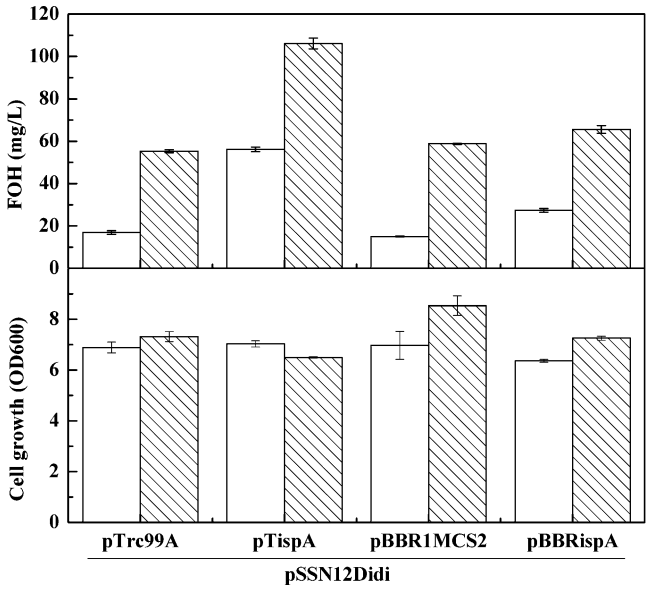

Figure 4. Effect of *ispA* overexpression on FOH production and cell growth in *E. coli* DH5α strain harboring plasmid pSSN12Didi expressing bottom portion of the MVA pathway with exogenous 5 mM MVA supplementation. Open and striped bars represent the results obtained at 24 and 48 h of culture, respectively.

significantly higher than the 65.5 mg/L of FOH produced by *E. coli* (pSSN12Didi, pBBRispA). FOH production by *E. coli* (pSSN12Didi, pTispA) at 24 and 48 h was threefold (56.2 vs. 17.0 mg/L) and twofold (106 vs. 55.3 mg/L) higher than that of *E. coli* (pSSN12Didi, pTrc99A), respectively. After 24 h, a concentration of 56.2 mg/L of FOH was obtained from *E. coli* (pSSN12Didi, pTispA) and was similar to the concentration of 55.3 mg/L from *E. coli* (pSSN12Didi, pTrc99A) at 48 h; even *ispA* overexpression using the low-copy pBBR1MCS2 vector almost doubled FOH production (27.4 vs. 15.0 mg/L) at 24 h. This result suggested that FOH production could be accelerated by *ispA* overexpression. There was no significant difference in cell growth amongst cultures containing different pTrc99A and pBBR1MCS2 vectors. Overexpression of *ispA* did not affect cell growth. It was concluded that FPP availability was improved as a consequence of ispA overexpression from pTispA, which led to increased FOH productivity.

FOH Production From the Entire Synthetic MVA Pathway

In the abovementioned work, FOH production was achieved in *E. coli* through the bottom portion of MVA pathway with the addition of MVA. However MVA is not an economic or abundant substrate. To achieve FOH production from cost-effective starting materials such as glycerol, an abundant byproduct of the biodiesel industry, pSSN12Didi was replaced with pSNA, which contains the entire synthetic MVA pathway and produces IPP and DMAPP without addition of MVA. As shown in Figure 5, FOH was produced in *E. coli* DH5α strain harboring pSNA and pTispA (*E. coli* (pSNA, pTispA)) with addition of 1% (v/v) glycerol. FOH production was improved from 76.6 mg/L without induction to 121 mg/L with 0.1 mM IPTG induction. FOH production was only slightly increased by prolonging the culture time to 72 h (data not shown). Thereafter, FOH production was carried out using *E. coli* (pSNA, pTispA) for 48 h with 0.1 mM IPTG induction.

To determine whether glycerol availability limits FOH production, various concentrations of glycerol ranging 0–4% (v/v) were added (Fig. 6). FOH production was dependent on the amount of supplied glycerol. Without the addition of glycerol, FOH production was 14.1 mg/L, approximately 10-fold lower than the 135.5 mg/L of FOH obtained with the addition of 1% (v/v) glycerol. However, glycerol concentrations above 1% (v/v) did not result in the further improvement of FOH production; the addition of 4% (v/v) glycerol reduced production to 42.4 mg/L and impaired cell growth to a level comparable to that of 0.5% (v/v) glycerol.

The residual glycerol concentration in the culture was analyzed. Glycerol depletion was observed in the culture containing 0.5% (v/v) glycerol, whereas glycerol consumption was around 0.7% (v/v) in the other culture with more added glycerol. IPP and DMAPP synthesis by plasmid pSNA is initiated from acetyl-CoA (Fig. 1). The powerful MVA pathway efficiently branches acetyl-CoA to build IPP and DMAPP. If insufficient glycerol is added, assimilation of the

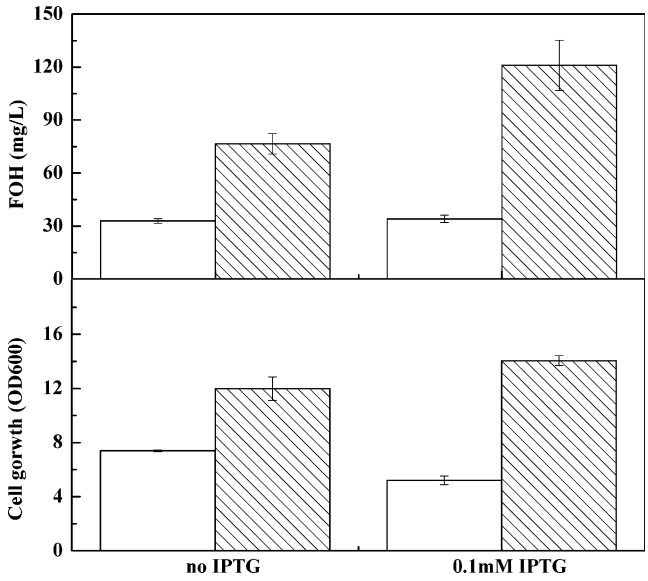

Figure 5. FOH production and cell growth in *E. coli* DH5α strain harboring plasmid pSNA expressing the entire synthetic MVA pathway and pTispA. Open and striped bars represent the results obtained at 24 and 48 h of culture, respectively.

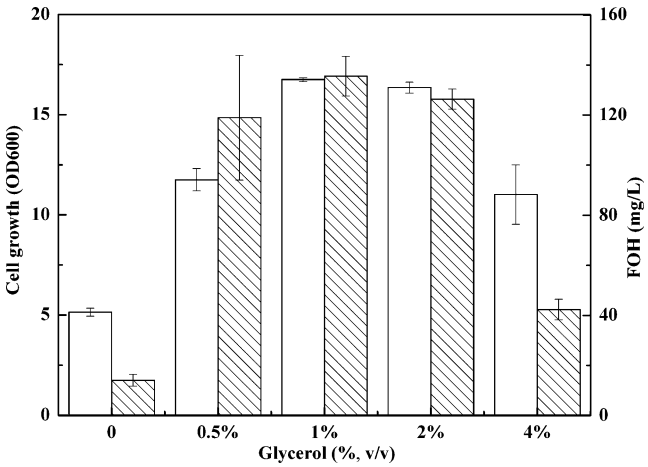

Figure 6. Effect of glycerol concentration on FOH production and cell growth in *E. coli* DH5α strain harboring the plasmids pSNA and pTispA. Culture was carried out with 0.1 mM IPTG for 48 h. Open and striped bars represent cell growth and FOH production, respectively.

carbon source into acetyl-coA would be limited, and FOH synthesis would have to compete with central metabolism. This competition results in cell growth inhibition and low FOH production. A supply of glycerol can alleviate the shortage of acetyl-CoA, however, extraordinary high glycerol concentrations (e.g., 4% (v/v)), might change the medium properties such as osmosis, which has been reported to affect microorganism viability (Karlgren et al., 2005; Mille et al., 2005). Under such unfavorable condition, the capacity of *E. coli* to produce FOH might be weakened and led to low FOH production after the addition of 4% (v/v) glycerol. Thus, intermittent feedings with the optimal glycerol concentration using a fermentor would be appropriate for higher level of FOH production.

Investigation of Putative Phosphatases Catalyzing FOH Formation

Several phosphatases were investigated in *E. coli*, and included PhoA, AphA, BacA, PgpA, and PgpB, the putative enzymes that functionally catalyze FOH formation. PhoA and AphA are an alkaline phosphatase and an acid phosphatase, respectively, and catalyze the hydrolysis of a wide variety of phosphoesters (Holtz and Kantrowitz, 1999; Passariello et al., 2006). BacA and PgpB exhibit undecaprenyl pyrophosphate (UPP) phosphatase activity and are known as a UPP phosphatase (El Ghachi et al., 2004; Touze et al., 2008). UPP phosphatase may recognize FPP as a substrate because UPP and FPP are the same kind of isoprenyl pyrophosphates, although UPP has a longer isoprenyl chain than FPP. PgpB was recently reported to display phosphatase activity toward FPP in an in vitro assay (Touze et al., 2008,). PgpB is known as a phosphatidylglycerol (PGP) phosphatase (Icho, 1988), although PGP phosphatase activity is not the principal role of PgpB. PgpA, the other PGP phosphatase, was also considered to be one of the possible FPP phosphatases because PgpA may have a different principal activity like PgpB (Funk et al., 1992). FOH production was decreased in the deletion mutants when compared with the control wild-type strain, *E. coli* BW25113 (Fig. 7). A significant decrease in FOH production was observed in deletions of *aphA*, *pgpA*, and *pgpB*. There was no significant difference in cell growth between the control strain and the phosphatase deletion mutants. In the double deletion mutant (Δ*pgpAB*) of *pgpA* and *pgpB*, there was no further significant decrease in observed FOH production when compared with that of the *pgpA* mutant (Δ*pgpA*). It was suspected that the contribution from phosphatases other than PgpA and PgpB was increased to reduce the toxicity of FPP accumulation in the double deletion mutant (Δ*pgpAB*). PhoA, AphA, BacA, PgpA, and PgpB were overexpressed in *E. coli*, but FOH production was not increased by their overexpression (data not shown). This result suggests that expression levels of the phosphatases are not the limiting factors for increased FOH production in *E. coli*.

Discussion

Engineering of the heterologous MVA pathway in *E. coli* has been reported to enhance production of isoprenoids such as

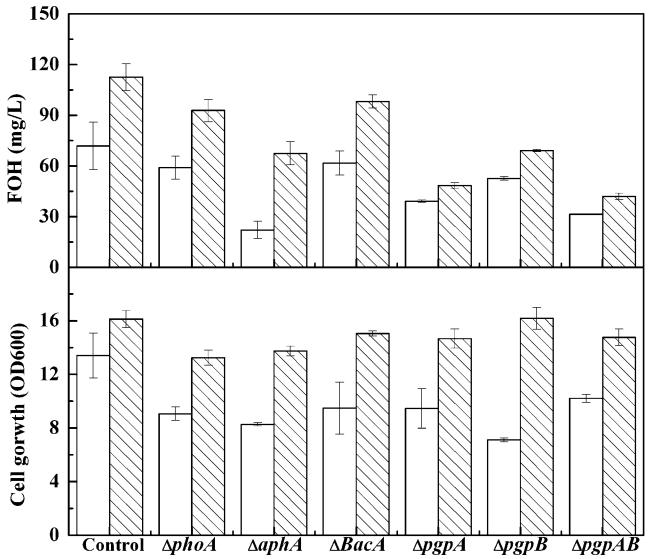

Figure 7. Effect of the deletion of promiscuous phosphatases on FOH production and cell growth. The control *E. coli* BW25113 strian and its mutants containing deletions of the *phoA*, *AphA*, *bacA*, *pgpA*, or *pgpB* genes were compared with regard to FOH production. The double deletion of *pgpA* and *pgpB* is represented by Δ*pgpAB*. Plasmids pSSN12Didi and pTispA were cotransformed into each *E. coli* strain for FOH production. Open and striped bars represent the results obtained at 24 and 48 h of culture, respectively.

amorphadiene (Martin et al., 2003), carotenoids (Yoon et al., 2007, 2009) and Coenzyme Q_{10} (Zahiri et al., 2006) by increasing the supply of IPP and DMAPP. Likewise, in this study we report FOH production from *E. coli* harboring the MVA pathway. FOH production was only observed upon introduction of the MVA pathway into *E. coli* (Fig. 3B). It confirms the great potential for the use of heterologous MVA pathway for biosynthesis of IPP and DMAPP in metabolic engineering of *E. coli*. FOH is derived from depyrophosphorylation of its direct precursor FPP. FPP synthesis from IPP and DMAPP by the FPP synthase encoded by *ispA* is regarded as a critical factor for FOH production. FOH production was not observed in *E. coli* SF7N, an *ispA* deletion mutant (Fig. 3D), whereas FOH production in *E. coli* DH5α was increased twofold by overexpression of *ispA* using the pTrc99A vector (Fig. 4). This result suggests that the sufficient supply of isoprenyl pyrophosphates, IPP, DMAPP, and FPP generated through the MVA pathway and *ispA* overexpression lead to FOH formation in *E. coli*.

Since the intracellular accumulation of FPP is known to be toxic to cells (Martin et al., 2003; Withers et al., 2007), the high intracellular FPP level in the engineered *E. coli* was hypothesized to force endogenous phosphatases to hydrolyze FPP to FOH. The investigation of putative phosphatases indicated that the depyrophosphorylation of FPP to FOH is not mediated by a single specific phosphatase, but by several promiscuous phosphatases (Fig. 7). FOH production was not affected by expression level of the putative phosphatases. Thus, it is thought that the phosphatases are not specific for FPP and might have high K_m for FPP. The high K_m of the promiscuous phosphatases could be a bottleneck for FOH production, which is also the most probable reason that FOH formation was only observed in the *E. coli* strain engineered to produce large amount of FPP. We have attempted to express the FOH synthase of *Oryza sativa* (Cheng et al., 2007) in *E. coli*, but its poor protein expression failed to improve FOH production. A similar result has been observed in the expression of another plant sesquiterpene synthase in *E. coli* (Martin et al., 2001). These results suggest that the limiting factor for sesquiterpene synthesis in *E. coli* mostly is poor expression of the plant enzyme, not supply of the FPP precursor. Therefore, enzyme engineering to either make the promiscuous phosphatases specific for FPP or improve plant FOH synthase expression will help develop *E. coli* into much more robust FOH producer.

FOH is a volatile compound and its evaporation from culture broth causes significant loss of the FOH produced during culture. Therefore, a two-phase culture containing a decane overlay on the culture broth was used for FOH production in this study. FOH is highly soluble in decane, but insoluble in water. The FOH produced in culture broth could be efficiently extracted from the broth to overlaid decane phase. Solubilized FOH in decane experienced very little evaporation (Fig. 2). The in situ recovery of FOH from the decane phase during culture can reduce production costs by simplifying both the harvest and purification process of FOH. This in situ FOH separation from the culture broth can also reduce product inhibition and increase FOH production. These characteristics will be of interest in an industrial process.

A previous study reported the production of 389 μg/L of FOH from *E. coli* by overexpressing *idi* and *ispA* (Ohto et al., 2009b). For FOH production, they carried out potato acid phosphatase treatment for the hydrolysis of FPP in cell lysates after cell harvest and lysis. In this study, we report the production of 135.5 mg/L of FOH from *E. coli* with the engineered MVA pathway and *ispA* overexpression, which is a 350-fold increase from the previous report (Ohto et al., 2009b). Moreover, there is no need of an additional in vitro phosphatase treatment for FOH production after culture. There are a few reports on FOH production from yeast. A SQ synthase deficient mutant of *S. cerevisiae* produced 28 mg/L of FOH in culture with the addition of oils and detergents (Muramatsu et al., 2008b). Blockage of SQ synthesis would increase intracellular FPP level, resulting in FOH formation. The *S. cerevisiae* AURGG101 mutation screened as a good yeast strain for FOH production produced 8.6 mg/L of FOH in test tube culture, with overexpression of the HMG-CoA reductase known to be the rate-limiting enzyme of the MVA pathway (Ohto et al., 2009a). The highest FOH production of 145 mg/L from a yeast strain was obtained in a fed batch culture of 7 days using a 5-L jar fermentor. Our engineered *E. coli* strain (pSNA, pTispA) produced 135.5 mg/L of FOH for 48 h. If fed-batch culture using this *E. coli* strain is carried out under optimized culture condition, mass production of FOH can be expected. Moreover, if the FOH synthase effectively working in *E. coli* is created by enzyme engineering of promiscuous phosphatases or plant FOH synthases, *E. coli* can be an ideal optional cell factory for FOH production.

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