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Efficient synthesis of functional isoprenoids from acetoacetate through metabolic pathway-engineered *Escherichia coli*

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Abstract We show here an efficient synthesis system of isoprenoids from acetoacetate as the main substrate. We expressed in *Escherichia coli* a *Streptomyces* mevalonate pathway gene cluster starting from HMG-CoA synthase and including isopentenyl diphosphate isomerase (*idi*) type 2 gene and the yeast *idi* type 1 and rat acetoacetate-CoA ligase (*Aacl*) genes. When the α-humulene synthase (*ZSS1*) gene of shampoo ginger was expressed in this transformant, the resultant *E. coli* produced 958 μg/mL culture of α-humulene with a lithium acetoacetate (LAA) supplement, which was a 13.6-fold increase compared with a control *E. coli* strain expressing only *ZSS1*. Next, we investigated if this *E. coli* strain engineered to utilize acetoacetate can synthesize carotenoids effectively. When the *crtE*, *crtB*, and *crtI* genes required for lycopene synthesis were expressed in the transformant, lycopene amounts reached 12.5 mg/g dry cell weight with addition of LAA, an 11.8-fold increase compared with a control expressing only the three *crt* genes. As for astaxanthin production with the *E. coli* transformant, in which the *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtW* genes were expressed, the total amount of carotenoids produced (astaxanthin, lycopene, and phytoene) was significantly increased to 7.5 times that of a control expressing only the six *crt* genes.

Keywords α-Humulene · Lycopene · Astaxanthin · Sesquiterpene · Carotenoids

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Introduction

Isoprenoids are the most diverse class of natural products (>20,000) including more than 7,000 of sesquiterpenes and 750 of carotenoids (Connolly and Hill 1991; Britton et al. 2001). These isoprenoid compounds can be used as pharmaceuticals, pesticides, functional foods, or raw materials. For example, a sesquiterpene lactone artemisinin, which is included in a shrub *Artemisia annua*, has been used as an antimalarial drug (Enserink 2005), and a monocyclic sesquiterpene α-humulene (α-caryophyllene) is contained in the essential oil of hop (*Humulus lupulus*) that has been utilized as an inevitable ingredient for beer brewery (Connolly and Hill 1991). Lycopene, a red carotenoid pigment contained in tomato and watermelon, was shown to prevent prostate cancer (Giovannucci et al. 1995), and another red carotenoid astaxanthin, which is found in red sea animals such as crab, shrimp, and red fish (red sea bream and salmon), has been reported to inhibit the oxidation of low-density lipoprotein, which is one cause of cardiovascular disease (Iwamoto et al. 2000). However, many of these useful isoprenoids are present in a small quantity in nature or low yielding from their natural

sources. Chemical synthesis of these isoprenoids is often difficult and costly because of their structural complexity. Production of such isoprenoids with metabolically engineered microorganisms offer an alternative approach. Many papers exist reporting biotechnological approaches to the efficient production of the useful isoprenoids using recombinant strains of bacteria such as *Escherichia coli* and *Methylomonas* sp. and yeasts such as *Saccharomyces cerevisiae* and *Candida utilis* (Chang and Keasling 2006; Shimada et al. 1998; Tang et al. 2007; Ye et al. 2007). Among microbes, *E. coli* is the microorganism of choice for biotechnological applications. This is because extensive molecular genetic resources exist, and it is fully amenable to genetic manipulation. As a result, it is widely used as a host for heterologous production of sesquiterpenes, diterpenes, and carotenoids (tetraterpenes; Albrecht et al. 1999; Alper et al. 2005b; Cragg 1998; Farmer and Liao 2000; Kakinuma et al. 2001; Martin et al. 2003b; Vadali et al. 2005; Yokoyama et al. 1998; Yoon et al. 2006; Yuan et al. 2006).

In *E. coli*, isoprenoids are biosynthesized not through the "classic" mevalonate (MVA) pathway but through non-mevalonate [2-*C*-methyl-D-erythritol-4-phosphate (MEP)] pathway (Fig. 1; Rohmer et al. 1993). Isopentenyl diphosphate (IPP) is an initial precursor for isoprenoid biosynthesis (Fig. 1). IPP is isomerized to dimethylallyl diphosphate (DMAPP) by IPP isomerase (Idi), and then this C_5 isomer sequentially combine with IPP to yield geranyl diphosphate (GPP; C_{10}) and farnesyl diphosphate (FPP; C_{15}; Fig. 1). Sesquiterpene, ubiquinone, and dolichol are synthesized from FPP. Addition of further IPP to FPP results in the formation of geranylgeranyl diphosphate (GGPP; C_{20}), which is the precursor of diterpenes and carotenoids (Fig. 1). *E. coli* produces ubiquinone and dolichol and does not synthesize

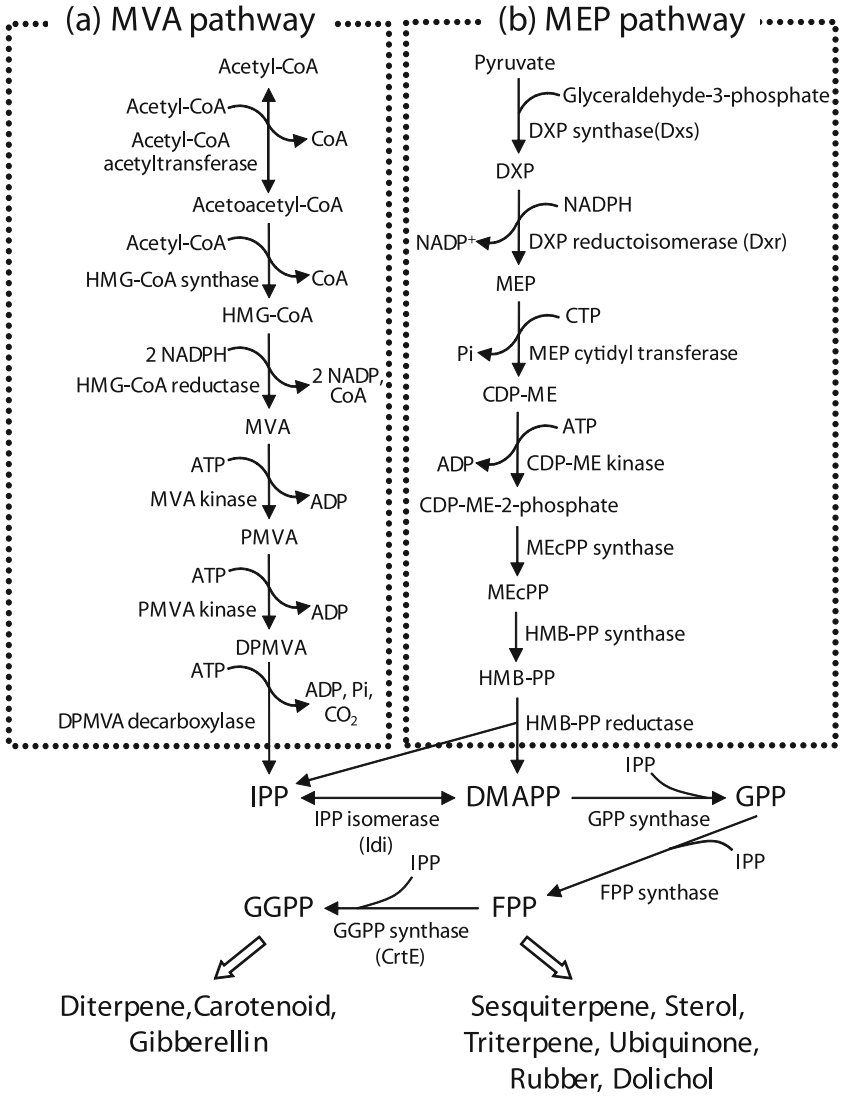

Fig. 1 Isoprenoid biosynthetic pathway. Isoprenoids are synthesized via two independent early biosynthetic pathways: mevalonate (*MVA*) pathway (**a**) and 2-*C*-methyl-D-erythritol-4-phosphate (*MEP*) pathway (**b**). MVA pathway is also illustrated in Fig. 3 in detail

isoprenoids such as monoterpenes, diterpenes, sesquiterpenes, triterpenes, sterols, and carotenoids. Thus, in order to produce these isoprenoids in *E. coli*, it is necessary to introduce a series of biosynthesis genes from FPP to the desired isoprenoids. For example, for lycopene production, a gene cluster encoding GGPP synthase (CrtE), phytoene synthase (CrtB), and phytoene desaturase (CrtI) was introduced into *E. coli* and expressed to synthesize lycopene from intracellular FPP (Misawa et al. 1990). Further expression in *E. coli* of the lycopene cyclase gene (*crtY*), *crtY* and the β-carotene hydroxylase gene (*crtZ*) together, and *crtY*, *crtZ*, and the β-carotene ketolase gene (*crtW*) simultaneously resulted in the accumulation of β-carotene, zeaxanthin, and astaxanthin as the dominant carotenoids, respectively (Misawa et al. 1995; Fraser et al. 1997). However, *E. coli* synthesizes small amounts of the endogenous isoprenoids from low levels of FPP, and amounts of the isoprenoids produced with the recombinant *E. coli* cells were far from the practical use, which was difficult to exceed 1 mg/g dry cell weight (DCW), when the biosynthesis genes starting from FPP were introduced in *E. coli*. In order to overcome this problem, pathway engineering studies have been performed for increasing intracellular concentration of FPP. To date, we know two types of Idi enzymes: type 1 (eukaryote type) and type 2 (prokaryote type; Kaneda et al. 2001). When the *idi* (type 1) gene derived from green alga *Haematococcus pluvialis* or yeast *S. cerevisiae* was introduced in *E. coli* and expressed there, the FPP content was estimated to increase several times, e.g., *E. coli* expressing the *crtE*, *crtB*, *crtI*, and *crtY* genes derived from soil bacterium *Pantoea ananatis* (formerly called *Erwinia uredovora* 20D3) in addition to the *H. pluvialis idi* gene produced 1.3 mg/g DCW of β-carotene (Kajiwara et al. 1997). Similarly, when the intrinsic 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*) or 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*) gene in MEP pathway (Fig. 1) was overexpressed in *E. coli*, the resultant transformant was shown to increase FPP amounts (1.6 mg/g DCW of carotenoids at the highest levels; Albrecht et al. 1999). Yuan et al. (2006) showed that the simultaneous overexpression of *idi*, *dxs*, and other genes resulted in producing higher titers (6 mg/g DCW) of β-carotene. On the other hand, the introduction of heterologous mevalonate pathway genes into *E. coli* has been shown to improve the productivity of sesquiterpenes or carotenoids (Kakinuma et al. 2001; Martin et al. 2003b; Newman et al. 2006; Vadali et al. 2005; Yoon et al. 2006, 2007). These studies also showed that production levels of the desired isoprenoids were significantly increased with addition of exogenous mevalonate in the culture medium. Recently, we isolated two sesquiterpene synthase genes from shampoo ginger *Zingiber zerumbet* Smith and functionally identified them as the genes for α-humulene synthase and β-eudesmol synthase by using *E. coli* expressing mevalonate pathway genes and a D-mevalonate supplement (Yu et al. 2008a, b). We show here an efficient synthesis system of desired isoprenoids, sesquiterpenes such as α-humulene, and carotenoids such as lycopene and astaxanthin, from acetoacetate supplemented to the culture, which is a cheaper compound with simple chemical structure compared with D-mevalonate, by using *E. coli* cells that express the types 1 and 2 *idi* and acetoacetate-CoA ligase (*Aacl*) genes as well as mevalonate pathway genes.

Material and methods

Bacterial strains and genetic manipulations

E. coli DH5α *deo*R, *end*A1, *gyr*A96, *hsd*R17 (r_k^-, m_k^+), *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ (*lac*ZYA-*arg*FV169), φ80*lac*ZΔM15, F^- was used for DNA manipulations. *E. coli* BL21 (DE3) [*E. coli* B, F^-, *dcm*, *omp*T, *hsd*S (rB^-mB^-), *gal*, λ (DE3)] and JM109 [*end*A1, *gyr*A96, *hsd*R17 (r_k^-, m_k^+), *mcr*B^+, *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ (*lac-pro*AB), F′ (*tra*D36, *pro*AB, *lac*I^qZΔM15)] were used as hosts for the production of isoprenoids. The polymerase chain reaction (PCR) amplifications were performed using KOD plus DNA polymerase version 2 (Toyobo, Osaka, Japan) and a thermal cycler (Applied Biosystems, Foster City, CA, USA). Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, CA, USA) or Takara Bio (Ohtsu, Japan). Plasmid DNA was prepared with QIAprep Miniprep Kit (Qiagen, Hilden, Germany). The nucleotide sequences were confirmed by Bigdye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems) and model 3730 DNA analyzer (Applied Biosystems). All recombinant DNA techniques were conducted as described by Sambrook and Russell (2001).

Construction of plasmids

The *tac* promoter (Ptac) and the *rrn*B terminator (TrrnB) were amplified by PCR with vector pTTQ18 (Stark 1987) as the template using two primer pairs: PtacF and PtacR for Ptac and TrrnBF and TrrnBR for TrrnB, respectively (Table 1). These amplified fragments were digested with *Eag*I and *Sal*I and *Hin*dIII and *Cla*I and ligated into the corresponding sites of *E. coli* vector pACYC184 (accession no. X06403). A 6.5-kb fragment included in plasmid pUMV19 (accession no. AB037666; Takagi et al. 2000), which was originated from *Streptomyces* sp. strain CL190, contains a cluster composed of five genes of mevalonate pathway encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase, HMG-CoA reductase, MVA kinase, phospho-

Table 1 List of PCR primers used in this study

Primer Name	Sequence (5′→ 3′)
PtacF	AAACGGCCGAGCTGTTGACAATTAATCATC
PtacR	AAGTCGACCATATGTGTTTCCTGTGTGAAATTGTTA
TrrnBF	GAGAAGCTTCTGTTTTGGCGGATGAGAG
TrrnBR	CCGATCGATCTGCTTTCCTGATGCAAAAAC
MevF	GTCCATATGCAGAAAAGACAAAGGGAGCTG
MevR	GCAGATATCCTAGCGCGCCTCGTAGATG
AhmF	TACGAATTCGATGGAGAGGCAGTCGATGG
AhmR	CGAGGATCCTCAAATAAGAAAGGATTCAACAAATATG
ScidiF	GACGATATCAGGAGGCAGCTATGACTGCCGACAACAATAGTA
ScidiR	CGTAAGCTTACGACTAGTTTATAGCATTCTATGAATTTGCCTGTC
AclF	GACACTAGTAGGAGGCAGCTATGTCCAAGCTGGCACG
AclR	GACAAGCTTTCAGAAGTCCTGCAGCTCAG

Underlines indicate a recognition site of restriction enzyme. Double underlines indicate Shine–Dalgarno sequence

mevalonate (PMVA) kinase and diphosphomevalonate (DPMVA) decarboxylase, and the IPP isomerase (*idi*) type 2 gene (Figs. 2a and 3). This fragment was amplified by PCR using MevF and MevR, with attached *Nde*I and *Eco*RV sites at the 5′-end of respective primers (Table 1). The PCR products were digested with *Nde*I and *Eco*RV and inserted into the corresponding sites between Ptac and TrrnB. This plasmid was designated pAC-Mev as shown in Fig. 2a. To generate pAC-Mev/Scidi (Fig. 2b), the *idi* type 1 gene from *S. cerevisiae* (*Scidi*) was amplified using ScidiF and ScidiR (Table 1) and inserted into the *Eco*RV and *Hin*dIII sites of pAC-Mev. This insertion of the *Scidi* fragment resulted in the generation of a new *Spe*I site at the upstream of the *Hin*dIII site. The acetoacetate-CoA ligase gene (*Aacl*) from *Rattus norvegicus* was PCR-amplified using Mammalian Gene Collection cDNA clone of *Aacl* (clone ID: 5598532, Invitrogen, Carlsbad, CA, USA) as the template with primers AclF and AclR (Table 1), digested, and inserted into the *Spe*I and *Hin*dIII sites of pAC-Mev/Scidi to generate pAC-Mev/Scidi/Aacl (Figs. 2c and 3).

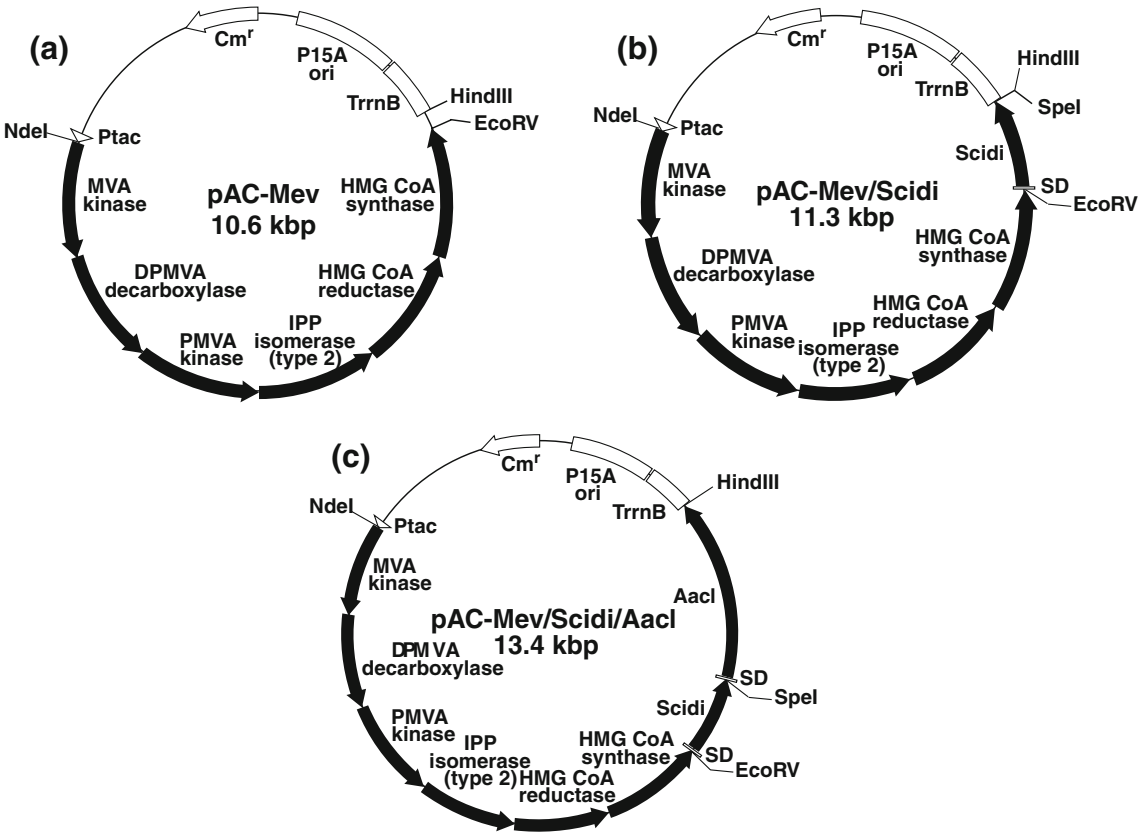

Fig. 2 Structures of plasmids used for isoprenoid pathway engineering

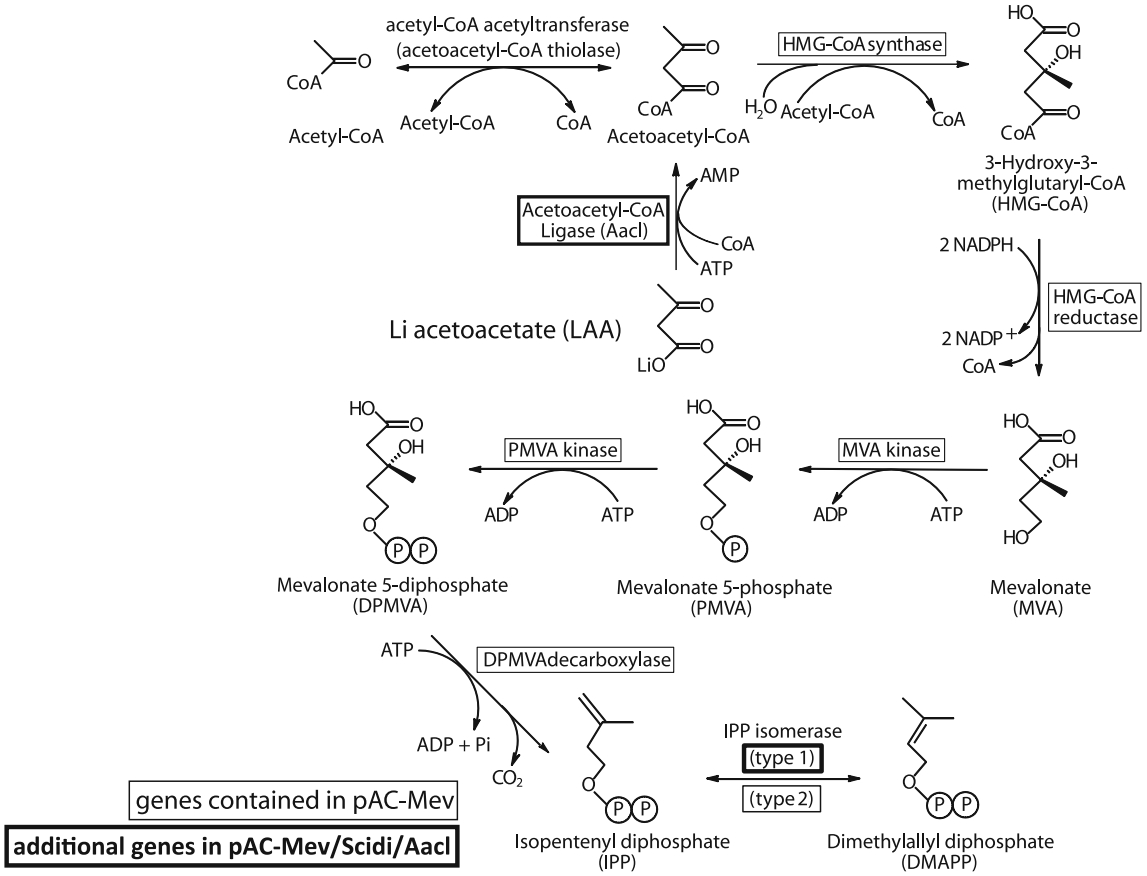

Fig. 3 Mevalonate pathway map containing chemical structures of the substrates and reaction products. Enzyme genes contained in plasmids pAC-Mev and pAC-Mev/Scidi/AacI are also shown in this map

Plasmid pET-ZSS1 was described, which carries the α-humulene synthase gene (cDNA; *ZSS1*) from shampoo ginger *Z. zerumbet* Smith under the control of the T7 promoter of vector pET101/D-TOPO (Invitrogen Japan, Tokyo; Yu et al. 2008a). Plasmid pUC-ZSS1 for higher production of α-humulene was constructed as follows: A coding sequence of the *ZSS1* gene was amplified with pET-ZSS1 (Yu et al. 2008a) as the template using primers AhmF and AhmR (Table 1); the amplified product was digested with *Eco*RI and *Bam*HI and ligated into the corresponding sites of pUC18 (accession no. L08752).

A lycopene-synthesizing plasmid pCRT-EIB, in which the *crtE*, *crtI*, and *crtB* genes from *P. ananatis* (accession no. D90087) are inserted in pUC19 to have transcriptional read through from the *lac* promoter, was described as pCAR-ADE in Misawa et al. (1990). A plasmid for astaxanthin production (pUC-Asta) was created as follows: A 6.9-kb *Xba*I and *Hin*dIII fragment including the six carotenoid biosynthesis genes, *crtE*, *crtX*, *crtY*, *crtI*, *crtB* and *crtZ*, from *P. ananatis* (accession no. D90087) was isolated from pCAR1 (Misawa et al. 1990) and ligated into the corresponding sites (downstream sites of *crtW*) of pUCBre-W containing the *crtW* gene derived from marine bacterium *Brevundimonas* sp. strain SD212 (accession no. AB181388; Choi et al. 2005; Nishida et al. 2005). The resulting plasmid was cleaved with *Bst*EII, filled in with Klenow fragment, and self-ligated to eliminate CrtX activity, thereby generating pUC-Asta.

Growth conditions of isoprenoids-producing *E. coli*

Production of α-humulene in *E. coli* was conducted as described by Newman et al. (2006) with some modifications: *E. coli* carrying plasmids pET-ZSS1 and pAC-Mev, or plasmids pUC-ZSS1 and pAC-Mev, pAC-Mev/Scidi, or pAC-Mev/Scidi/AacI was precultured in a Luria–Bertani (LB) medium containing ampicillin (Ap; 100 μg/mL) and chloramphenicol (Cm; 30 μg/mL) at 30°C for overnight with shaking at 100 rpm. One percent (*v*/*v*) of this culture was inoculated into 50 ml of terrific broth (Sambrook and Russell 2001) containing Ap and Cm and cultivated at 30°C for 3–5 h with shaking at 100 rpm. When the absorbance at 600 nm (A_{600}) of the culture reached 0.5, 1 mM (final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG), 20% (*v*/*v*) dodecane, and 0.5 mg/mL of D-mevalonolactone (MVL; Tokyo Kasei, Tokyo, Japan) or

1 mg/mL of lithium acetoacetate (LAA; Tokyo Kasei) were added and further cultivated at 20°C for 2 days with shaking at 100 rpm. The dodecane phase was extracted and subjected to gas chromatography-mass spectrometry (GC–MS) analysis.

As for carotenoid production by *E. coli*, the lycopene-synthesizing plasmid pCRT-EIB or the astaxanthin-synthesizing plasmid pUC-Asta was cotransformed into *E. coli* along with pAC-Mev, pAC-Mev/Scidi, or pAC-Mev/Scidi/Aacl. Each transformant was precultured in a LB medium containing Ap (100 μg/mL) and Cm (30 μg/mL) at 30°C for overnight with shaking. One percent (*v*/*v*) of this culture was inoculated into 50 ml of 2× yeast extract–tryptone (YT) medium containing Ap and Cm and cultivated at 30°C for 3–5 h with shaking at 100 rpm. When the A_{600} of the culture reached approximately 0.5, 1 mM of IPTG and 1 mg/mL of LAA were added and further cultivated at 20°C for 2 days with shaking at 100 rpm. The *E. coli* cells were harvested by centrifugation and stored at −70°C before analysis.

GC–MS analysis of α-humulene

GC–MS analysis was performed on a Shimadzu GCMS-QP5050A GC–MS system (Shimadzu, Kyoto, Japan) equipped with a DB-WAX capillary column (0.25 mm internal diameter×0.25 μm×30 m, J&W Scientific, Folsom, CA) according to a method by Martin et al. (2003a) with some modifications. Split injections (1 μL) were made at a ratio of 22:1 with an injector temperature of 250°C. The instrument program used was 40°C (held for 3 min), 3°C min^{-1} increment up to 80°C, 5°C min^{-1} increment up to 180°C, and 10°C min^{-1} increment up to 240°C (held for 5 min). Mass spectra were monitored in the mass range of m/z 40–400 with an electron voltage at 70 eV and an interface temperature at 250°C. For quantification by GC–MS, α-humulene purchased from Wako Pure Chemical Industries was used as a standard.

HPLC-PDA analysis of carotenoid

High-performance liquid chromatography with photodiode-array detection (HPLC-PDA) analysis was carried out as described previously (Choi et al. 2005). HPLC-PDA–atmospheric pressure chemical ionization (APCI)-MS analysis was performed by a modification of a previously described method (Choi et al. 2005), using a reverse-phase HPLC column Deverosil C30-UG5 (1.0×250 mm, Nomura chemical) with a Deverosil C30-UG-S precolumn. Column temperature was maintained 40°C. Mobile phases consisted of (a) water–methanol, 5:95, *v*/*v*, and (b) methanol–*tert*-butylmethylether, 30:70, *v*/*v*, and the elute was monitored continuously at 210–600 nm. The crude extracts from *E. coli* transformants were eluted at a rate of 0.1 ml/min with solvent (a) for 15 min, followed by a linear gradient from solvent (a) to (b) for 50 min and hold for 20 min. Mass spectra were monitored continuously m/z 200–1200. The capillary temperature was set to 150°C, the APCI vaporizer temperature was held at 400°C, the capillary voltage was optimized to 23 V, and the sheath nitrogen gas flow was set to 28 (arbitrary units). Authentic samples of carotenoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) or purified from the appropriate *E. coli* transformants expressing various *crt* genes (Nishida et al. 2005).

Results

Comparison of plasmids and host strains on sesquiterpene productivity

We isolated a terpene cyclase gene (*ZSS1*) from shampoo ginger *Z. zerumbet* Smith and identified *ZSS1* as the α-humulene synthase gene that mediates the conversion from FPP to α-humulene (α-caryophyllene; Yu et al. 2008a). Plasmid pET-ZSS1, in which *ZSS1* has transcriptional read through from the T7 promoter, was used in the previous study. In order to compare the productivity of α-humulene, plasmid pUC-ZSS1 was constructed, in which the original translational start ATG of the *ZSS1* gene was designed to be fused to the sequence encoding the additional several amino acid terminus of β-galactosidase (LacZ) in pUC18. We also compared two *E. coli* strains, BL21 (DE3) and JM109 as the productive hosts. Accumulation of α-humulene was not observed in the *E. coli* BL21 (DE3) strain carrying one plasmid pET-ZSS1 or pUC-ZSS1 (Fig. 4a). In contrast, *E. coli* JM109 carrying one plasmid pUC-ZSS1 accumulated 94 μg/mL of α-humulene (Fig. 4a). When plasmid pAC-Mev was cotransformed into *E. coli* BL21 (DE3) with pET-ZSS1 or pUC-ZSS1, the resultant *E. coli* accumulated 416 or 363 μg/mL of α-humulene with addition of 0.5 mg/mL of D-mevalonate lactone to the culture (Fig. 4a). Surprisingly, *E. coli* JM109 carrying pUC-ZSS1 and pAC-Mev produced 1,039 μg/mL of α-humulene with the MVL supplement, which was approximately 2.5-fold increase compared with the BL21 (DE3) strain carrying the same two plasmids (Fig. 4a). This result shows that *E. coli* JM109 strain is superior to BL21 (DE3) strain as the host for carrying the two plasmids pUC-ZSS1 and pAC-Mev to produce the isoprenoid.

Sesquiterpene production with mevalonate pathway-engineered *E. coli* from acetoacetate

We next investigated the efficacy of respective recombinant *E. coli* JM109 strains possessing plasmid pUC-ZSS1 on α-

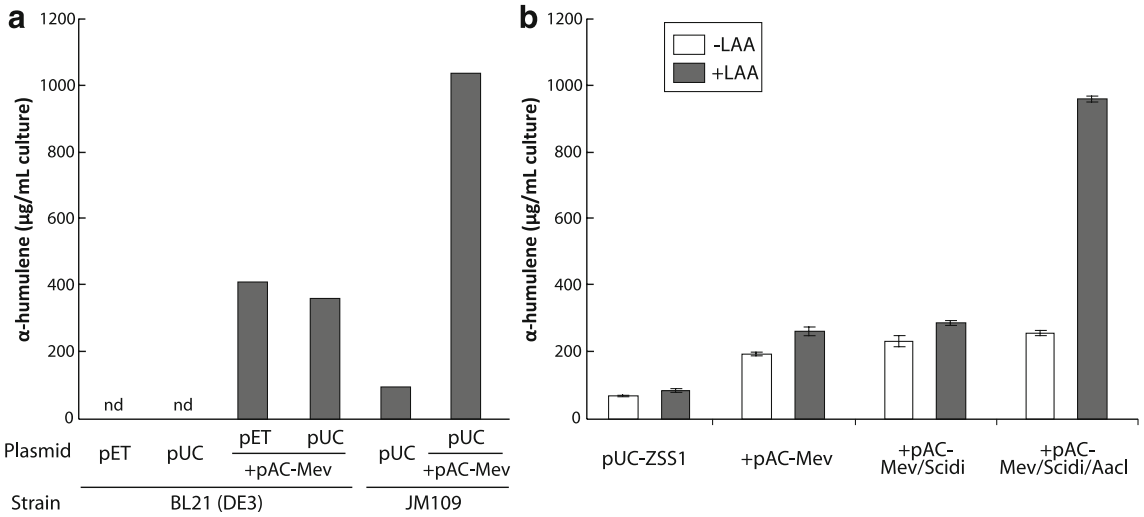

Fig. 4 **a** α-Humulene production by *E. coli* BL21 (DE3) or JM109 harboring respective plasmids; 0.5 mg/mL of D-mevalonolactone (*MVL*) was supplemented in all the cultures. *pET* pET-ZSS1, *pUC* pUC-ZSS1, *nd* not detected. **b** α-Humulene production by *E. coli* JM109 harboring respective plasmids with or without addition of 1 mg/mL of lithium acetoacetate (*LAA*) to the culture medium. Each value is shown as mean ± SE (n=3)

humulene production from acetoacetate as the main substrate. An *E. coli* control strain carrying only one plasmid pUC-ZSS1 produced 70 μg/mL of α-humulene regardless of addition of exogenous acetoacetate salt (lithium acetoacetate; Fig. 4b). *E coli* carrying two plasmids pUC-ZSS1 and pAC-Mev synthesized 194 μg/mL of α-humulene without a LAA supplement, which is a 2.7-fold increase compared with a control strain possessing only pUC-ZSS1 (Fig. 4b). This result indicated that the mevalonate pathway gene cluster in pAC-Mev was functionally active in *E. coli* and effective in enhancing FPP content. It is probably due to the presence of the type 2 IPP isomerase (*idi*) gene (Kaneda et al. 2001) as described in the "Discussion" section. When 1 mg/mL of LAA was supplemented in the culture as the substrate, *E. coli* cells carrying pUC-ZSS1 and pAC-Mev produced 263 μg/mL of α-humulene, which represents a 3.7-fold increase compared with the control strain possessing only pUC-ZSS1 (Fig. 4b), indicating that a supplementation of LAA presumably contributes to further increase of intracellular FPP.

In order to further improve α-humulene productivity, the type 1 *idi* gene from yeast *S. cerevisiae* (*Scidi*) was introduced downstream into the mevalonate pathway gene cluster to construct pAC-Mev/Scidi (Fig. 2b). *E. coli* harboring pUC-ZSS1 and pAC-Mev/Scidi produced 233 μg/mL of α-humulene without LAA, which is slightly higher than the content (194 μg/mL) observed in the case of pAC-Mev (Fig. 4b). When grown in the presence of LAA, the amount of α-humulene produced by *E. coli* harboring the two plasmids was 287 μg/mL, 4.1 times higher than the control strain (Fig. 4b). These results suggest that an additional type 1 IPP isomerase activity can enhance α-humulene production further.

Sramek and Frerman (1975a, b) showed that conversion from acetoacetate to acetoacetyl-CoA was achieved through CoA transfer from acetyl-CoA to acetoacetate by an intrinsic butyrate–acetoacetate CoA-transferase (AtoB and AtoD, EC 2.8.3.9). On the other hand, mammalian and some bacteria posses an enzyme referred to as acetoacetate-CoA ligase (acetoacetyl-CoA synthetase, Aacl; EC 6.2.1.16), which catalyzes the conversion reaction from acetoacetate to acetoacetyl-CoA through direct CoA ligation (Ito et al. 1984, Bergstrom and Edmond 1985). In an attempt to further utilize exogenous acetoacetate, the *Aacl* gene that had been isolated from rat *R. norvegicus* was introduced downstream into *Scidi* to construct pAC-Mev/Scidi/Aacl (Fig. 2c). The amount of α-humulene reached 968 μg/mL in the presence of LAA, which is a 13.6-fold increase compared with the control (Fig. 4b). This productivity of α-humulene was almost equivalent to the use of MVL as the substrate (1,039 μg/mL; Fig. 4a). These data indicate that exogenous LAA is efficiently converted into acetoacetyl-CoA by mammalian acetoacetate-CoA ligase (Aacl) and used as a dominant precursor for isoprenoid biosynthesis in the *E. coli* cells.

Carotenoid production with mevalonate pathway-engineered *E. coli* from acetoacetate

We examined the efficacy of respective recombinant *E. coli* JM109 strains possessing plasmid pCRT-EIB on lycopene production from acetoacetate as the main substrate. Plasmid pCRT-EIB contains GGPP synthase (*crtE*), phytoene

synthase (*crtB*), and phytoene desaturase (*crtI*) genes derived from soil bacterium *P. ananatis*, which mediates the conversion from FPP to lycopene (Misawa et al. 1995). An *E. coli* control strain carrying pCRT-EIB alone produced approximately 1 mg/g dry cell weight (DCW) of lycopene regardless of LAA supplementation (Fig. 5). *E coli* carrying two plasmids pCRT-EIB and pAC-Mev synthesized 4.1 mg/g DCW of lycopene without LAA, which is a 3.9-fold increase compared with the control strain carrying only pCRT-EIB (Fig. 5). Supplemented with 1 mg/mL of LAA, *E coli* (pCRT-EIB and pAC-Mev) produced 9.8 mg/g DCW of lycopene, which represents a 9.3-fold increase compared with the control (Fig. 5). *E. coli* harboring pCRT-EIB and pAC-Mev/Scidi produced 4.2 mg/g DCW of lycopene without LAA, which is equal to the content observed with pAC-Mev. When grown in the presence of LAA, the amount of lycopene produced by *E. coli* carrying the two plasmids reached 12.0 mg/g DCW, which is an 11.4-fold increase compared with the control strain. *E. coli* carrying pCRT-EIB and pAC-Mev/Scidi/Aacl produced 3.5 mg/g DCW of lycopene without LAA, which was similar level to the case of pAC-Mev and pAC-Mev/Scidi. However, with addition of LAA to the culture medium, *E. coli* (pCRT-EIB and pAC-Mev/Scidi/Aacl) produced 12.5 mg/g DCW of lycopene, which was the highest level achieved (11.8 times that of the control; Fig. 5).

Next, we examined whether respective recombinant *E. coli* JM109 strains possessing plasmid pUC-Asta is effective in increasing astaxanthin production. Plasmid pUC-Asta contains the β-carotene ketolase (carotenoid 4,4′-oxygenase; *crtW*) gene that was isolated from marine bacterium *Brevundimonas* sp. strain SD212 (Nishida et al. 2005), and *crtE*, *crtB*, *crtI*, *crtY* (lycopene cyclase), and *crtZ* (β-carotene hydroxylase; carotenoid 3,3′-hydroxylase) genes derived from *P. ananatis* (Misawa et al. 1990, 1995), which was constructed as described in the "Materials and methods" section. An *E. coli* control strain harboring pUC-Asta alone produced about 0.7–0.8 mg/g DCW of astaxanthin (including its *cis*-isomer) regardless of LAA addition, and almost all the carotenoids produced in the cells were found to be astaxanthin (Table 2). As expected from the results of the lycopene-producing recombinants, *E. coli* JM109 carrying pUC-Asta and pAC-Mev, pAC-Mev/Scidi, or pAC-Mev/Scidi/Aacl synthesized approximately 1.2 to 1.5 mg/g DCW of astaxanthin without exogenous LAA supplementation, which is 1.6- to 1.9-fold larger than that of the control strain carrying only pUC-Asta (Table 2). When LAA was added to the cultures, total amount of generated carotenoids was significantly increased to 3.5–7.5 times compared with the control, whereas there was little or no effect on enhancement in astaxanthin content (Table 2). In these cultures, lycopene was the predominant carotenoid, which corresponded to approximately half to 60% of total carotenoids, and precursor carotenoids including phytoene and 3-hydoroxyechinenone were also observed specifically in *E. coli* (pUC-Asta and pAC-Mev/Scidi/Aacl; Table 2).

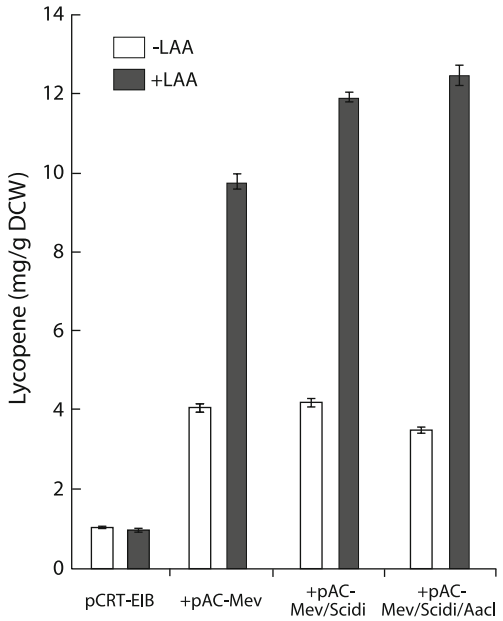

Fig. 5 Lycopene production by *E. coli* JM109 harboring respective plasmids with or without addition of 1 mg/mL of LAA to the culture medium. Each value is shown as mean ± SE (n=3)

Discussion

As mentioned in the "Introduction" section, many biotechnological studies have been focused on efficient production of desirable isoprenoids using *E. coli* as a host. In particular, introduction in *E. coli* of heterologous MVA pathway genes has been very effective in increasing productivity. However, a problem in this instance is that a substrate must be added to the cultures, i.e., D-mevalonate or D-mevalonate lactone. These compounds are considerably expensive due to its complicated chemical structure including one chiral carbon (see Fig. 3). We therefore decided to establish an efficient isoprenoid production system from a lower-cost substrate, acetoacetate which has a simple chemical structure, through engineering upstream isoprenoid biosynthetic pathway in *E. coli*.

We first compared plasmids, pET-ZSS1 and pUC-ZSS1, and *E. coli* host strains, BL21 (DE3) and JM109, on the production of α-humulene. In comparison of the *E. coli* strains carrying a single plasmid pET-ZSS1 or pUC-ZSS1, only the *E. coli* JM109 strain carrying pUC-ZSS1 synthesized detectable amounts of α-humulene (Fig. 4a; data not shown for JM109 carrying pET-ZSS1) and *E. coli* JM109

Table 2 Carotenoid production by *E. coli* JM109 harboring respective plasmids with or without addition of 1 mg/mL of LAA to the culture medium

Plasmids	Carotenoid content μg g^{-1} DCW					
	Astaxanthin	*cis*-Astaxanthin	3-OH echinenone	Lycopene	Phytoene	Total
pUC-Asta (-LAA)	542±11	247±7	3±1	5±1	nd	797±18
pUC-Asta (+LAA)	491±6	210±6	5±<1	5±<1	nd	712±12
+pAC-Mev (-LAA)	882±16	332±3	58±1	7±<1	nd	1,279±19
+pAC-Mev (+LAA)	886±19	322±10	72±3	1,454±35	13±4	2,748±67
+pAC-Mev/Scidi (-LAA)	980±29	396±21	63±4	9±1	nd	1,447±54
+pAC-Mev/Scidi (+LAA)	1,007±13	357±3	83±8	1,665±25	12±2	3,126±51
+pAC-Mev/Scidi/Aacl (-LAA)	1,045±27	427±12	101±4	10±3	4±2	1,589±48
+pAC-Mev/Scidi/Aacl (+LAA)	833±18	260±13	39±4	3,682±103	1,170±42	5,986±144

Each value is shown as mean ± standard error (SE; $n=3$)
nd not detected, *3-OH echinenone* 3-hydroxyechinenone

harboring pAC-Mev in addition to pUC-ZSS1 produced the largest amount of α-humulene. *E. coli* BL21 (DE3) strain is commonly used as a host for the synthesis of a foreign protein using pET vector and has an advantage of deficiency in both *lon* and *ompT* protease genes. But, this strain may be problematic for the stability of introduced plasmids, since there are the intact *recA* and *endA* genes. In addition, we designed plasmid pUC-ZSS1, in which the original start codon of the *ZSS1* was fused to several amino acid residues derived from LacZ. It has been reported that such a hybrid gene is expected to be efficiently expressed using the original ribosomal binding site and start codon of the *lacZ* gene (Choi et al. 2005; Nishida et al. 2005). Thus, we adopted a combination of pUC-ZSS1 and *E. coli* JM109 in subsequent experiments. It was also demonstrated that α-humulene production with *E. coli* JM109 (pUC-ZSS1 and pAC-Mev/Scidi/Aacl) was significantly increased with the presence of LAA (Fig. 4b). This result indicates that the mammalian Aacl fully functions in this metabolically engineered *E. coli* to convert exogenous LAA to acetoacetyl-CoA that is a substrate of MVA pathway. In *R. norvegicus*, the Aacl activity has been reported to play an important role in the rate of ketone body utilization during the developmental process (Nakamoto et al. 1999; Sato et al. 2002). Several bacteria species were reported to possess a homologous gene of *Aacl*, while *E. coli* does not possess it. But, there were few studies on physiological role of this enzyme. α-*Proteobacteria Sinorhizobium meliloti* possessed a Aacl homolog, which was reported to play an essential role in synthesis of acetoacetyl-CoA from acetoacetate in the poly-3-hydroxybutyrate degradation pathway (Cai et al. 2000). It would particularly be interested to compare these bacterial Aacl homologs with the rat Aacl concerning isoprenoid productivity.

We showed in this study that isoprenoid production was increased even in the absence of MVA or LAA, when the 6.5 kb mevalonate gene cluster of *Streptomyces* sp. strain CL190 that was contained in pAC-Mev (Fig. 2a) was expressed in recombinant *E. coli* (Figs. 4b and 5; Table 2). Vadali et al. (2005) also reported that recombinant *E. coli* harboring the 6.5-kb gene cluster produced approximately 4.3 mg/L of lycopene from glucose as the substrate, which corresponds with our result (Fig. 5). Since these *E. coli* transformants that possess only the nonmevalonate pathway are postulated not to utilize the initial substrate acetoacetyl-CoA of the mevalonate enzymes starting from HMG-CoA synthase (Fig. 3) for isoprenoid production, the expression of the type 2 *idi* gene included in the 6.5-kb cluster (Fig. 2a) is very likely to be responsible for enhancing isoprenoid levels.

Results obtained with *E. coli* JM109 (pUC-Asta and pAC-Mev/Scidi/Aacl) showed that the total amount of generated carotenoids was significantly increased up to 7.5 times that of the control strain carrying only pUC-Asta, with a LAA supplement (Table 2). However, there was a little or no effect on enhancement of astaxanthin levels (Table 2). Specifically, lycopene tended to accumulate as the predominant carotenoid (Table 2), suggesting that CrtY activity is relatively weak in the metabolic pathway-engineered *E. coli*, which coincides with a result of our other experiments (Choi et al. 2005). Since lycopene and astaxanthin are two pigments among the most promising carotenoids for improving human health, the carotenoid composition achieved with *E. coli* (pUC-Asta and pAC-Mev/Scidi/Aacl) seems to be interesting for producing "mixed carotenoids" or "multi carotenoids" as a human supplement.

Recently, other approaches to increase productivity of desirable isoprenoids have been reported. Alper et al. (2005a, b) have developed a multiple gene knockout method that increased lycopene biosynthesis in *E. coli*. The triple gene-knockout strain (*ΔgdhA*, *ΔaceE*, *ΔfdhF*)

that was obtained by this method produced 6.6 mg/g DCW of lycopene, which is an approximately 40% increase compared with a parental strain (Alper et al. 2005a). In order to further improve the productivity, this gene knockout method was combined to an overexpression method of a key gene (Jin and Stephanopoulos 2007). One of the best genetically engineered strains accumulated 16 mg/g DCW of lycopene with 5 g/L of glucose in M9 minimal medium (Jin and Stephanopoulos 2007). Combination of various approaches seems to be effective to improve productivity of desirable isoprenoids up to industrially sufficient levels.

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