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Engineering the lycopene synthetic pathway in *E. coli* by comparison of the carotenoid genes of *Pantoea agglomerans* and *Pantoea ananatis*

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Abstract The lycopene synthetic pathway was engineered in *Escherichia coli* using the carotenoid genes (*crtE*, *crtB*, and *crtI*) of *Pantoea agglomerans* and *Pantoea ananatis*. *E. coli* harboring the *P. agglomerans crt* genes produced 27 mg/l of lycopene in 2YT medium without isopropyl-beta-D-thio-galactopyranoside (IPTG) induction, which was twofold higher than that produced by *E. coli* harboring the *P. ananatis crt* genes (12 mg/l lycopene) with 0.1 mM IPTG induction. The *crt* genes of *P. agglomerans* proved better for lycopene production in *E. coli* than those of *P. ananatis*. The *crt* genes of the two bacteria were also compared in *E. coli* harboring the mevalonate bottom pathway, which was capable of providing sufficient carotenoid building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), with exogenous mevalonate supplementation. Lycopene production significantly increased using the mevalonate bottom pathway and 60 mg/l of lycopene was obtained with the *P. agglomerans crt* genes, which was higher than that obtained with the *P. ananatis crt* genes (35 mg/l lycopene). When *crtE* among the *P. ananatis crt* genes was replaced with *P. agglomerans crtE* or *Archaeoglobus fulgidus gps*, both lycopene production and cell growth were similar to that obtained with *P. agglomerans crt* genes. The *crtE* gene was responsible for the observed difference in lycopene production and cell growth between *E. coli* harboring the *crt* genes of *P. agglomerans* and *P. ananatis*. As there was no significant difference in lycopene production between *E. coli* harboring *P. agglomerans crtE* and *A. fulgidus gps*, farnesyl diphosphate (FPP) synthesis was not rate-limiting in *E. coli.*

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Introduction

Carotenoids are a diverse class of C_{40} isoprenoids with multiple physiological and nutritional roles in plants, algae, bacteria, and fungi. Carotenoids have received considerable attention as a result of their interesting properties as pigments, and more importantly, their potential and bene-

ficial effects on human health (Armstrong 1997; Sandmann 2001). Lycopene, in particular, is an effective antioxidant (Sies and Stahl 1998) and has been proposed as a possible treatment for some cancers and other degenerative human conditions (Giovannucci 1999, 2002). Lycopene, the biosynthetic precursor of most cyclic carotenoids, is the linear carotenoid composed of seven isopentenyl diphosphate (IPP) and 1 dimethylallyl diphosphate (DMAPP). High-yield production of carotenoids in engineered microbial hosts requires optimization of the available isoprenoid precursor pool of IPP and DMAPP and balancing the expression of carotenogenic genes for efficient transformation of the precursors to the desired carotenoid compounds (Fig. 1). The optimization of the available isoprenoid precursor pool of IPP and DMAPP has been studied to increase the production of lycopene. Two pathways for the synthesis of IPP and DMAPP exist: the well-known mevalonate pathway and the newly discovered 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Rohmer et al. 1993). The MEP pathway for isoprenoid biosynthesis is essential in most eubacteria, such as *Escherichia coli* (Eisenreich et al. 1998; Lichtenthaler 1999; Lichtenthaler et al. 1997; Rohmer 1999). The MEP pathway has been engineered to increase the supply of isoprenoid precursors in *E. coli* (Farmer and Liao 2001; Kajiwara et al. 1997; Kim and Keasling 2001). As an alternative to using the MEP pathway, a foreign mevalonate pathway was recently introduced into *E. coli* (Martin et al. 2003; Vadali et al. 2005; Yoon et al. 2006). Martin et al. reported that amorphadiene isoprenoid production, obtained by engineering the expression of the *Saccharomyces cerevisiae* mevalonate pathway in *E. coli*, demonstrated an 11-fold increase when compared to the endogenous MEP pathway. Several studies have been performed which investigated balancing the expression of carotenogenic genes for the desired carotenoids compounds. Misawa et al. described the carotenoid pathway in *Pantoea ananatis* (formerly known as *Erwinia uredovora*) (Misawa et al. 1990). Subsequently, the pathway for *Pantoea*

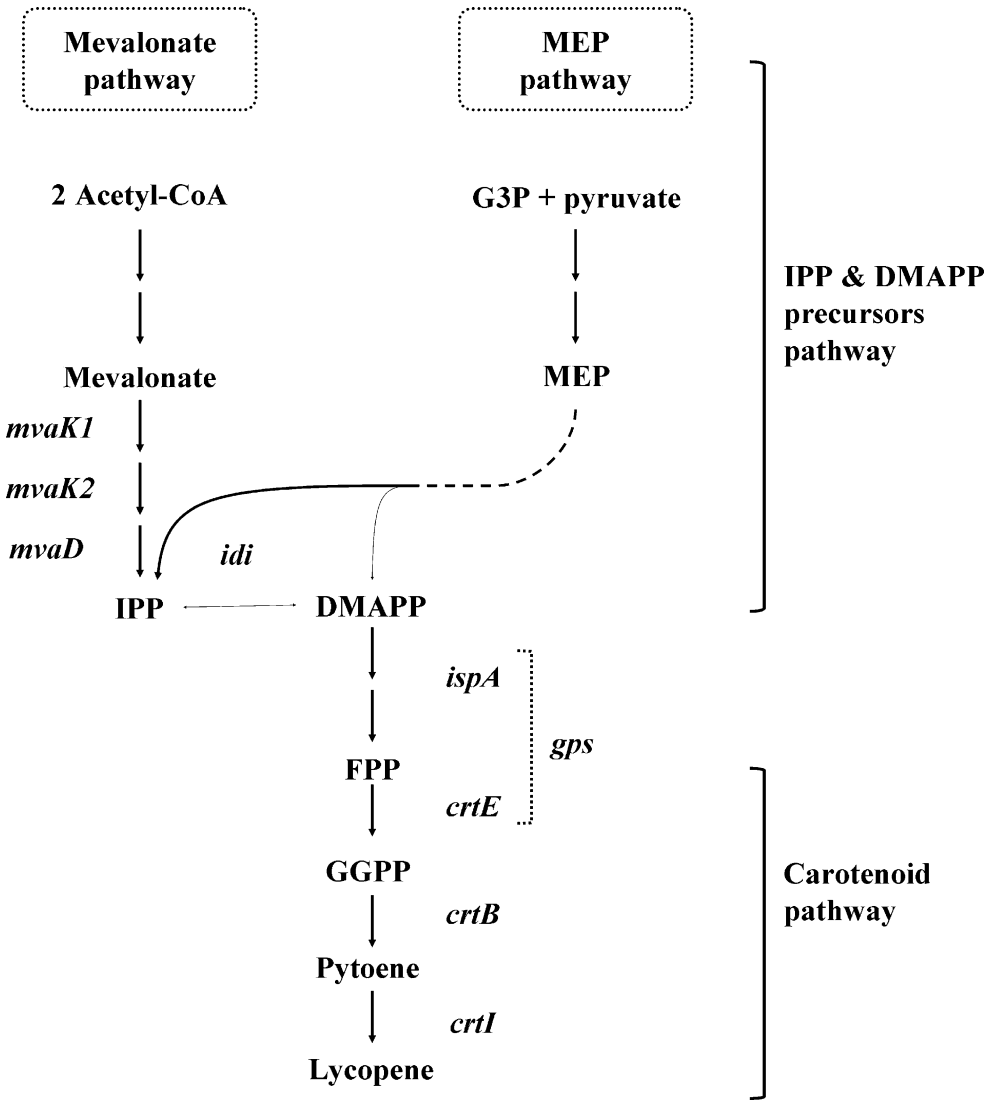

Fig. 1 Pathway for synthesis of lycopene in *E. coli* from a foreign mevalonate pathway and a native MEP pathway for formation of IPP and DMAPP precursors. The mevalonate bottom pathway, composed of *mvaK1*, *mvaK2*, *mvaD*, and *idi*, was used in the present study. Gene symbols and the enzymes they encode: *ispA*, FPP synthase; *crtE*, GGPP synthase; *crtB*, phytoene synthase; *crtI*, phytoene desaturase; *gps*, multifunctional GGPP synthase; *mvaK1*, mevalonate kinase; *mvaK2*, phosphomevalonate kinase; *mvaD*, mevalonate 5-diphosphate decarboxylase; *idi*, IPP isomerase. Pathway intermediates: *FPP* Farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate, *G3P*D -glyceraldehyde-3-phosphate, *MEP* 2-*C*-methyl-D-erythritol-4-phosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate

agglomerans (formerly known as *Erwinia herbicola*) was documented (Hundle et al. 1994; Schnurr et al. 1991). Thus, most of the metabolic engineering on carotenoid production from *E. coli* has been performed using the carotenogenic genes of *P. ananatis* and *P. agglomerans* (Alper et al. 2005; Cunningham et al. 1993; Hundle et al. 1994; Kang et al. 2005; Misawa et al. 1990; Ruther et al. 1997; Wang et al. 1999). However, these studies were restricted to the manipulation of only one pathway. To date, there has been no study performed comparing the two carotenoid synthetic pathways. Therefore, we investigated which of the two carotenoid pathways is better for lycopene production in *E. coli.* As the co-expression of three exogenous genes in *E. coli* cells—geranylgeranyl diphosphate (GGPP) synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*)—is sufficient for the conversion of IPP and farnesyl diphosphate (FPP) to lycopene (Fig. 1), it was found which one of the carotenogenic genes was responsible for the difference in lycopene productivity.

Materials and methods

Bacterial strains and culture conditions

E. coli DH5α (F^- ϕ80d*lac*ZΔM15 Δ (*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44λ^- *thi*-1 *gyr*A96 *rel*A1) was used for gene cloning and lycopene production. *E. coli* was grown in 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) at 37°C for gene cloning and at 29°C for lycopene production on a rotary shaker at 250 rpm. *P. ananatis* KCCM 40419 purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea) was grown in Nutrient Broth (BD Science, Franklin Lakes, NJ) at 26°C for genomic DNA preparation. Ampicillin (100 μg/ml) and chloramphenicol (5 μg/ml) (Sigma Chemical, St. Louis, MO, USA) were added as required. For lycopene production, 0.5% (w/v) glycerol was added as a carbon source. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) (0.1 mM) was used as an inducer for the *trc* promoter. Mevalonate (3.32 mM), prepared as previously described (Kim et al. 1992) from mevalonolactone (Sigma), was supplied for IPP synthesis via the mevalonate bottom pathway. The lycopene production culture was maintained for 48 h and bacterial growth was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}).

Construction of plasmids for lycopene biosynthesis in *E. coli*

Common procedures including genomic DNA preparation, restriction digestions, transformations, and other standard molecular biological techniques were carried out as previously described in Sambrook et al. (Sambrook and Russell 2001). Polymerase chain reaction (PCR) was performed using *pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA), based on a standard protocol. PCR products were cloned into pBluescript for the analysis of DNA sequences. The plasmids, pBluescript, pTrc99A, and pSTV28, were used as vectors for gene cloning and gene expression studies (Table 1).

Plasmid, pT-LYCm4 was constructed on pTrc99A by introducing the *crtE*, *crtB*, and *crtI* genes of *P. agglomerans* for lycopene biosynthesis and the *ipiHP1* gene encoding IPP isomerase of *Haematococcuspluvialis* (Yoon et al. 2006) (Fig. 2a). For the construction of pT-ULYC4, the *idi* gene encoding IPP isomerase was amplified from genomic DNA of *E. coli* using the PCR primers, idi-5 and idi-3 (Table 1). The isolated *idi* gene was excised using *Sma*I and *Sal*I, followed by insertion into the corresponding sites of pTrc99A to create pTidi. The *crtE*, *crtB*, and *crtI* genes of *P. ananatis* were isolated from its genomic DNA by PCR using primers from UcrtE-F, UcrtE-R, UcrtIB-F, and UcrtIB-R (Table 1). The PCR product of *crtE* was digested using *Bsp*HI and *Eco*RI for insertion into the *Nco*I and *Eco*RI sites of pTidi. The PCR product for *crtI* and *crtB* was then introduced behind the *crtE* gene after restriction with *Eco*RI and *Sac*I (Fig. 2b).

The plasmid pT-LUBI was composed of *P. agglomerans crtE*, *P. ananatis crtB* and *crtI*, and *E. coli idi.* For the construction of pT-LUBI, the *crtI*, *crtB*, and *ipiHP1* genes of pT-LYCm4 were replaced with the *crtI*, *crtB*, and *idi* genes of pT-ULYC4. The plasmid pT-LYCm4 was digested using *Xho*I, treated with a Klenow fragment to create a blunt-end, and subsequently excised using *Sal*I to remove *crtI*, *crtB*, and *ipiHP1*. The fragment including *crtI*, *crtB*, and *idi* from pT-ULYC4 was restricted using *Eco*RI, treated with a Klenow fragment, and then excised with *Sal*I. The excised fragment was inserted between the blunt-ended *Xho*I and *Sal*I sites of pT-LYCm4 with the deletion of *crtI*, *crtB*, and *ipiHP1*, resulting in pT-LUBI (Fig. 2c). For the construction of pT-GLYC4, the *gps* gene encoding multifunctional GGPP synthase was isolated from genomic DNA of *Archaeoglobus fulgidus* by PCR using primers GPS-F and GPS-R (Table 1). The PCR product was restricted using *Pci*I and *Eco*RI, and inserted between the *Nco*I and *Eco*RI sites of pTidi. The *crtI* and *crtB* of *P. ananatis*, which were used for the construction of pT-ULYC4, were introduced into *Eco*RI and *Sac*I sites behind *gps* (Fig. 2d). The *E. coli ispA* gene, excised by *Not*I from pAC-LYCm5 (Kang et al. 2005), was inserted into the *Not*I site of pT-LYCm4 to create pT-LYCm5 (Fig 2e). The plasmid pSSN12Didi was constructed on pSTV28 by introducing the *mvaK1*, *mvaK2*, and *mvaD* of *Streptococcus pneumoniae* and the *idi* of *E. coli* (Yoon et al. 2006) (Fig. 2f).

Table 1 Plasmids and primers used in the present study

Plasmids and primers	Description	References
Plasmids		
Pbluescript	P*lac* cloning vector, pUC origin, LacZ, Amp^r	(Stratagen Corp.)
pTrc99A	P*trc* expression vector, pBR322 origin, lacI^q, Amp^r	(Amersham Biosci.)
pSTV28	P*lac* cloning vector, pACYC184 origin, LacZ, Cm^r	(Takara Shuzo Co.)
pT-LYCm4	pTrc99A with *crtE, crtB,* and *crtI* from *P. agglomerans*, and *ipiHP1* from *H. pluvialis*	(Yoon et al. 2006)
pT-ULYC4	pTrc99A with *crtE, crtB,* and *crtI* from *P. ananatis*, and *idi* from *E. coli*	(Present study)
pT-LUBI	pTrc99A with *crtE* from *P. agglomerans*, *crtB* and *crtI* from *P. ananatis*, and *idi* from *E. coli*	(Present study)
pT-GLYC4	pTrc99A with *gps* from *A. fulgidus, crtB* and *crtI* from *P. agglomerans*, and *idi* from *E. coli*	(Present study)
pT-LYCm5	pTrc99A with *crtE, crtB,* and *crtI* from *P. agglomerans*, *ipiHP1* from *H. pluvialis,* and *ispA* from *E. coli.*	(Present study)
pSSN12Didi	pSTV28 with *mvaK1, mvaK2,* and *mvaD* from *S. pneumoniae*, and *idi* from *E. coli*	(Yoon et al. 2006)
Primers[a]		
Idi-5	5′-CCCCGGGAGGA**GAGAAATTATGCAAACGGAAC**-3′	(Yoon et al. 2006)
Idi-3	5′-TGCATGCTTA**TTTAAGCTGGGTAAATG**-3′	(Yoon et al. 2006)
UcrtE-F	5′-CTC**ATGA**C**GGTCTGCGCAAAAAAACACGTTC**-3′	(Present study)
UcrtE-R	5′-GGAATTC**TTAA**C**TGACGGCAGCGAGTTTTTTG**-3′	(Present study)
UcrtIB-F	5′-CGAATTCAG**GAGCGACTACATGAAACCAACTACGG**-3′	(Present study)
UcrtIB-R	5′-GGAGCTCT**TA**G**AGCGGGCGCTGCCAGAGATG**-3′	(Present study)
GPS-F	5′-GA**CATGT**T**GAAGGAGGAAATAGCG**-3′	(Present study)
GPS-R	5′-CAAGCTTA**CTTTTTTCTTGTAACCAAGAAG**-3′	(Present study)

[a] Coding regions are indicated by bold letters, start and stop codons are underlined, and restriction sites are double underlined.

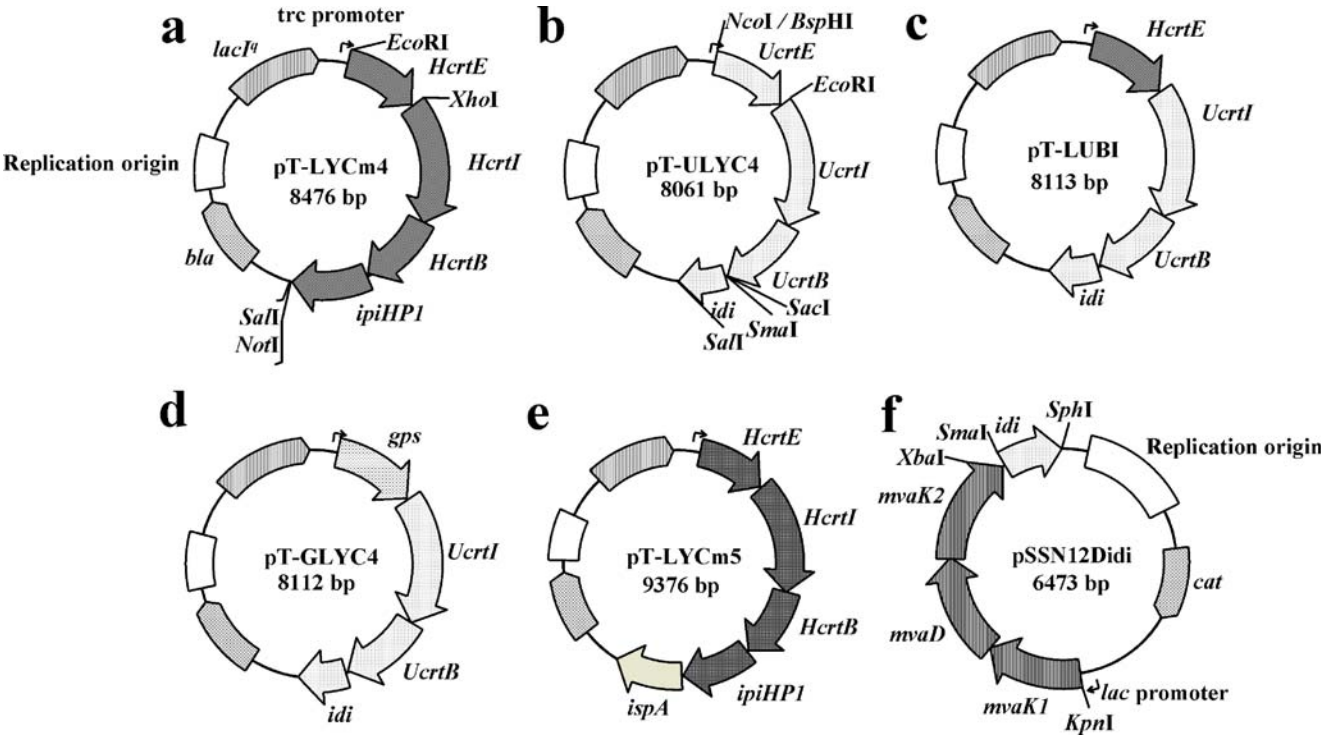

Fig. 2 Schematic representation of the main plasmids used for the metabolic engineering of *E. coli* for lycopene production. For a detailed description see Table 1

Quantification of lycopene production

To determine the lycopene content of the cells, an aliquot of *E. coli* cells was harvested by centrifugation at 14,000 rpm for 1 min and washed once with water. The cells were suspended in acetone (1 ml) and incubated at 55°C for 15 min in the dark. The samples were then centrifuged at 14,000 rpm for 10 min, and the acetone supernatant containing the lycopene was transferred to a new tube. The lycopene content of the extracts was quantified by measuring the absorbance at 475 nm using a Shimadzu UV1601 spectrophotometer (Shimadzu, Kyoto, Japan) and by comparing it with a lycopene standard (Sigma) (Harker and Bramley 1999). The results represent the means±SD from three independent experiments.

Results

Lycopene production from *E. coli* using crt genes of *P. agglomerans* and *P. ananatis*

Lycopene is synthesized in *E. coli* by the co-expression of three exogenous genes of GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*), and further produced by incorporation of the IPP isomerase (*idi* or *ipi*), one of rate-limiting enzymes in carotenoids biosynthesis. The *crtE*, *crtB*, and *crtI* genes of *P. agglomerans* and *P. ananatis* were used for the construction of pT-LYCm4 and pT-ULYC4, respectively, and the IPP isomerase coding gene (*idi* or *ipi*) was also introduced into both plasmids (Fig. 2). Plasmids of pT-LYCm4 and pT-ULYC4 were compared for lycopene production in *E. coli*.

Culture of recombinant *E. coli* harboring pT-LYCm4 and pT-ULYC4 was performed at 30°C for 48 h in 2YT complex medium containing 0.5% glycerol and 100 μg/ml ampicillin (Fig. 3). Cell growth and lycopene production in cells harboring pT-LYCm4 were severely inhibited by the addition of 0.1 mM IPTG, whereas, cell growth and lycopene production were favored with the leaky expression of the carotenogenic genes in 2YT complex medium without IPTG addition. In the presence of 0.1 mM IPTG, the cell growth of *E. coli* harboring pT-LYCm4 abruptly increased after 24 h without concomitant production of lycopene. The high expression of the carotenogenic genes of pT-LYCm4 by 0.1 mM IPTG induction appeared toxic to cells, which may have provoked some physiological or genetic changes after 24 h. FPP is a precursor of respiratory quinones, ubiquinone and menaquinone, and dolichol, involving bacterial cell wall synthesis, which are essential for cell growth and survival.

As carotenoid biosynthesis in *E. coli* must compete with the endogenous pathway for biosynthesis of quinones and dolichol, overall metabolism of the carotenoid producing cell might be perturbed by over-expression of exogenous carotenogenic genes. When the culture broth (cultivated for 24 h) was plated on LB agar, large populations of white mutants, which cannot produce lycopene, were observed after the addition of 0.1 mM IPTG in the culture harboring pT-LYCm4. The *E. coli* cells harboring pT-ULYC4 did not show significant difference in cell growth under different IPTG induction conditions, whereas, lycopene production increased after the addition of 0.1 mM IPTG. The highest lycopene production obtained from *E. coli* harboring pT-ULYC4 was 12 mg/l at 0.1 mM IPTG, which was much lower than 27 mg/l obtained from the *E. coli* harboring pT-LYCm4 without IPTG induction. Therefore, the *crt* genes of *P. agglomerans* appeared to perform better than those of *P. ananatis* for lycopene production in *E. coli*.

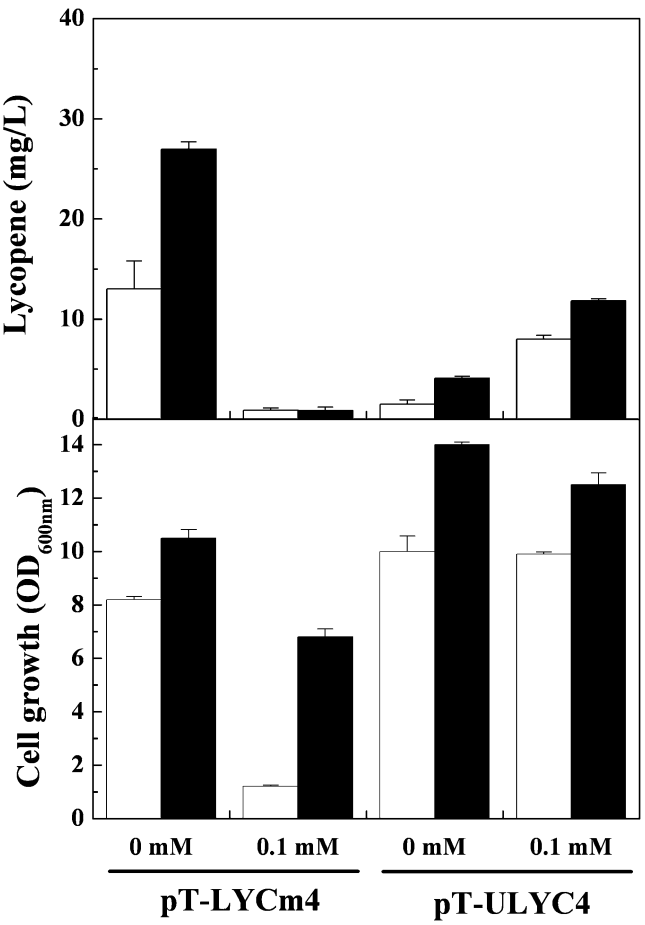

Fig. 3 Comparison of lycopene production and cell growth in 2YT complex medium containing 0.5% (w/v) glycerol between *E. coli* harboring pT-LYCm4 (*P. agglomerans crt* genes) and pT-ULYC4 (*P. ananatis crt* genes). Culture was performed with or without the addition of 0.1 mM IPTG at 30°C for 48 h. *Open bars* and *solid bars* represent the results obtained at 24 and 48 h, respectively

Lycopene production in the presence of the mevalonate bottom pathway

To compare the efficiency of the *crt* genes for lycopene production without limitation of its building blocks, IPP and DMAPP, pSSN12Didi carrying bottom portion (*mvaK1*, *mvaK2*, *mvaD*, and *idi*) of the mevalonate pathway was introduced into *E. coli* harboring pT-LYCm4 and pT-ULYC4 (Fig. 2). The mevalonate pathway has been known to synthesize IPP and DMAPP efficiently in *E. coli* (Martin et al. 2003). The recombinant *E. coli* harboring pSSN12Didi could produce sufficient IPP and DMAPP for lycopene production with an exogenous supply of mevalonate (Yoon et al. 2006). Culture was performed using the same cultivation condition (Fig. 3), with the addition of 3.32 mM mevalonate and 50 μg/ml chloramphenicol (Fig. 4). Co-transformation of pSSN12Didi with pT-LYCm4 and pT-ULYC4 significantly increased lycopene production. In addition, the *E. coli* cells harboring pSSN12Didi and pT-LYCm4 produced 60 mg/l of lycopene without the addition of IPTG, which was approximately twofold higher than that obtained with pT-LYCm4 alone. In the case of pT-ULYC4, lycopene production increased to 35 mg/l by introducing pSSN12Didi after 0.1 mM IPTG induction, which was threefold higher than that obtained with pT-ULYC4 alone. Cell growth generally decreased by co-transformation of pSSN12Didi with pT-LYCm4 or pT-ULYC4, which might be due to the metabolic burden for maintenance of the two plasmids. The severe inhibition of cell growth and lycopene production in *E. coli* harboring pT-LYCm4 and pSSN12Didi were also observed after 0.1 mM IPTG induction. In *E. coli* harboring pT-ULYC4 and pSSN12Didi, the addition of 0.1 mM IPTG increased lycopene production and did not inhibit cell growth. The results concerning IPTG induction were similar to those observed in Fig. 3. As lycopene production from *E. coli* harboring pT-ULYC4 and pSSN12Didi increased after 0.1 mM IPTG addition, higher concentrations of IPTG (up to 1.0 mM) were added to the culture. However, no difference in both lycopene production and cell growth at IPTG concentrations above 0.1 mM were observed (data not shown). It appears that IPTG induction was saturated at a concentration of 0.1 mM. Moreover, after the co-transformation of pSSN12Didi to *E. coli* harboring pT-LYCm4 or pT-ULYC4, the *crt* genes of *P. agglomerans* still appeared more efficient for lycopene production than those of *P. ananatis*.

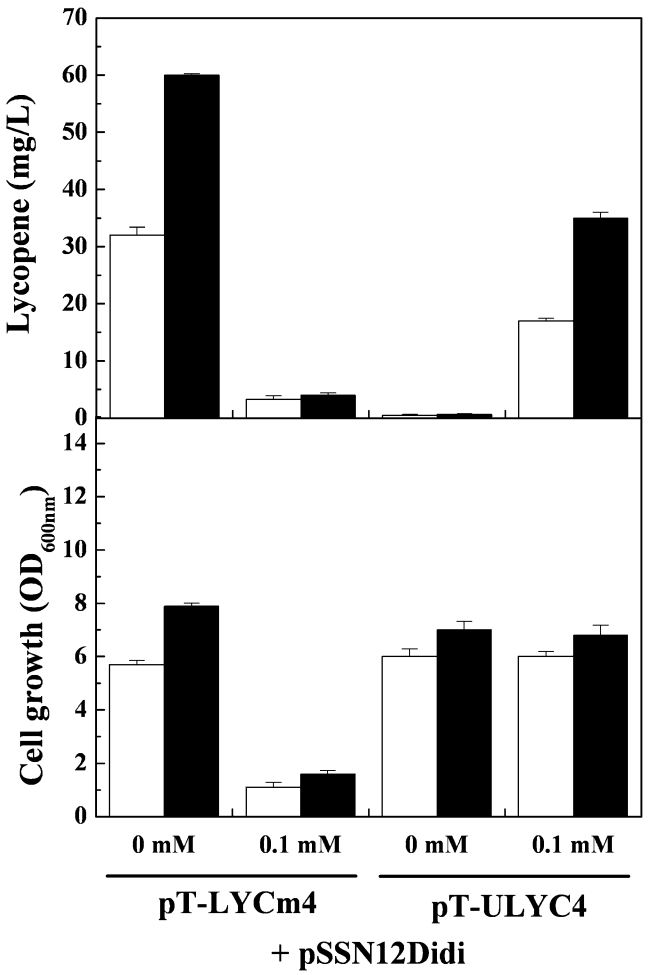

Fig. 4 Effect of the mevalonate bottom pathway, introduced by co-transformation of pSSN12Didi, on lycopene production and cell growth in *E. coli* harboring pT-LYCm4 or pT-ULYC4. Culture was performed in 2YT medium containing 3.32 mM mevalonate and 0.5% (w/v) glycerol with or without the addition of 0.1 mM IPTG at 30°C for 48 h. *Open bars* and *solid bars* represent the results obtained at 24 and 48 h, respectively

Comparison of *crtE* genes of *P. agglomerans* and *P. ananatis* on lycopene production

Wang et al. (1999) have systematically studied the rate-limiting steps in GGPP synthesis by over-expression of different gene combinations using *crtE* of *P. ananatis*, and *idi* and *ispA* of *E. coli*. The conversion from FPP to GGPP, which is mediated by *P. ananatis crtE* encoding GGPP synthase, was reported to be the first functional limiting step for the production of carotenoids in *E. coli*. It was, therefore, suspected that the higher lycopene production from *E. coli* harboring the *P. agglomerans crt* genes (pT-LYCm4) rather than the *P. ananatis crt* genes (pT-ULYC4) might be due to the difference in activity of *crtE* among the *crt* genes.

To identify the reasons involved, pT-LUBI was constructed by replacing all carotenogenic genes, except *crtE*, of pT-LYCm4 with the corresponding genes of pT-ULYC4 (Fig. 2). Recombinant *E. coli* harboring pT-LUBI and pSSN12Didi was cultivated for lycopene production under the same culture conditions used in Fig. 4 (Fig. 5). Although the *crtE* gene was the only difference between pT-LUBI and pT-ULYC4, the result obtained with pT-LUBI and pSSN12Didi was quite different from that of pT-

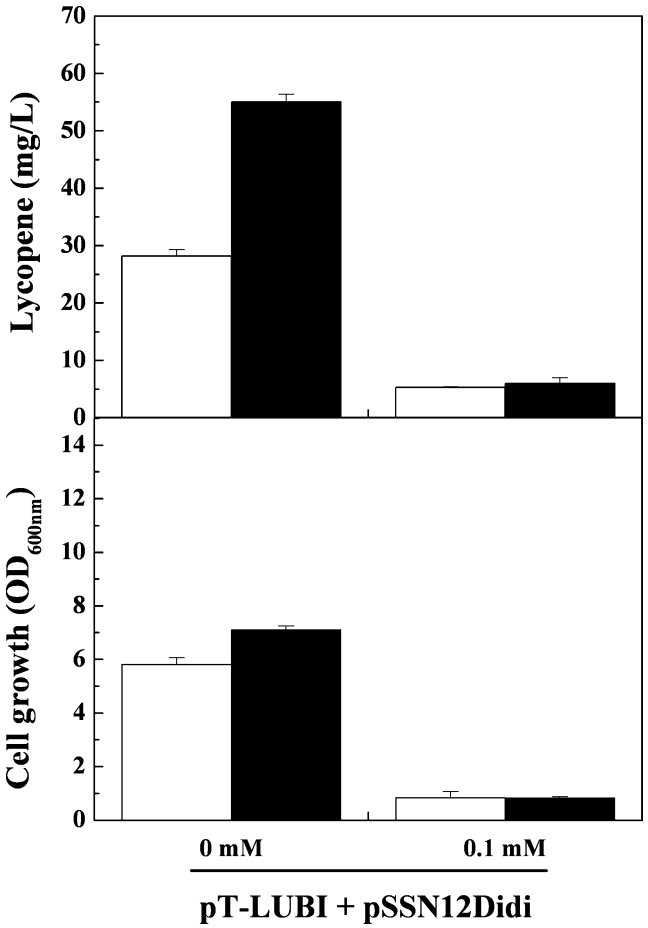

Fig. 5 Replacement of *crtE* among the *P. ananatis crt* genes with *P. agglomerans crtE* enhancing lycopene production. *E. coli* harboring pT-LUBI and pSSN12Didi was cultivated in 2YT medium containing 3.32 mM mevalonate and 0.5% (w/v) glycerol, with or without the addition of 0.1 mM IPTG, at 30°C for 48 h. *Open bars* and *solid bars* represent the results obtained at 24 and 48 h, respectively

ULYC4 and pSSN12Didi (Fig. 4). Both cell growth and lycopene production of *E. coli* harboring pT-LUBI and pSSN12Didi were severely inhibited by the addition of 0.1 mM IPTG, whereas, lycopene production with no addition of IPTG significantly increased to 55 mg/l in normal cell growth. This result was similar to that obtained with pT-LYCm4 and pSSN12Didi (Fig. 4). This result suggests that the high carotenogenic activity of *P. agglomerans crt* genes is achieved by its *crtE* gene and that no difference in carotenogenic activities of *crtB* and *crtI* between *P. agglomerans* and *P. ananatis* exists.

Lycopene production using gps encoding multifunctional GGPP synthase

It has been previously reported that astaxanthin production was significantly increased by over-expression of *gps*, cloned from *A. fulgidus*, in *E. coli* (Wang et al. 1999). The *gps* coding multifunctional GGPP synthase catalyzes the formation of GGPP directly from DMAPP and IPP (Fig. 1). To confirm that *crtE* among the *P. ananatis crt* genes is rate-limiting in lycopene production, pT-GLYC4 was constructed by replacing *crtE* in pT-ULYC4 with *gps* (Fig. 2). Recombinant *E. coli* harboring pT-GLYC4 and pSSN12Didi was cultivated at 30 and 37°C under the same culture condition as in Figs. 4 and 6). When cultivated at 30°C without IPTG induction, lycopene production and cell growth reached 59 mg/l and an OD_{600nm} of 7.5 for 48 h, respectively. Both lycopene production and cell growth were severely inhibited with 0.1 mM IPTG induction. These results are similar to those obtained with pT-LUBI and pSSN12Didi.

It was confirmed that the lower lycopene production of pT-ULYC4 than pT-LYCm4 was due to the low efficiency of the GGPP synthase encoded by *P. ananatiscrtE*. As *gps* has been cloned from a hyperthermophilic *A. fulgidus*, an elevated cultivation temperature of 37°C was applied.

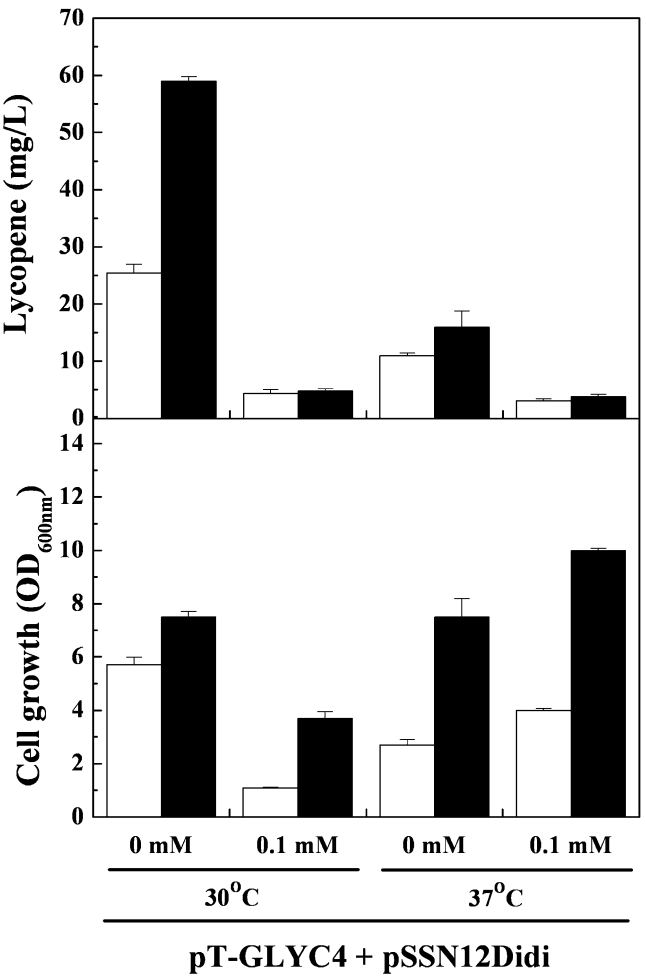

Fig. 6 Replacement of *crtE* among the *P. ananatis crt* genes with the *A. fulgidus gps* enhancing lycopene production. The *E. coli* harboring pT-GLYC4 and pSSN12Didi was cultivated in 2YT medium containing 3.32 mM mevalonate and 0.5% (w/v) glycerol, with or without the addition of 0.1 mM IPTG, at 30 or 37°C for 48 h. *Open bars* and *solid bars* represent the results obtained at 24 and 48 h, respectively

However, 16 mg/l of lycopene was obtained without IPTG induction for 48 h, which was 3.7-fold lower than that obtained at 30°C. Lycopene production at 37°C was also inhibited by 0.1 mM IPTG induction. The decrease in lycopene production at 37°C was also observed using pT-LYCm4, pT-ULYC4, and pT-LUBI (data not shown). It has been previously reported that carotenoid production in *E. coli* is greater at lower temperatures such as 28°C (Lee et al. 2004; Sandmann et al. 1999). The explanation given for this observation was that slower expression of carotenogenic genes yields more active enzymes and carotenoid precursors of prenyl diphosphates can be better provided to carotenoid synthesis when the entire metabolism is slower. As lycopene production obtained with *gps*, possessing function of both *ispA* and *crtE*, was similar to that achieved with *P. agglomerans crtE*, FPP synthesis might not be a rate-limiting step in carotenoid synthesis in the presence of high GGPP synthase activity.

Discussion

Metabolic engineering of the carotenoid synthetic pathway in *E. coli* has been performed mainly using carotenoid genes of *P. ananatis* and *P. agglomerans*. Considerable sequence diversity exists between *crt* genes of *P. ananatis* and *P. agglomerans*, although both belong to the *Pantoea* genus. Based on NCBI BLAST analysis, homologies of *crtE*, *crtB*, and *crtI* between the two bacteria are 55, 64, and 77%, respectively. Sedkova et al. (2005) recently reported these types of sequence diversity in carotenoid synthesis gene clusters from *Entero bacteriaceae* strains. However, there was no report on the direct comparison of carotenoids productivity between the *crt* genes of the two bacteria. Herein, we found that lycopene production from *E. coli* harboring *P. agglomerans crt* genes was significantly higher than that using *P. ananatis crt* genes.

The different lycopene productivity observed between the *crt* genes of *P. agglomerans* and *P. ananatis* was attributed to *crtE*, which has the lowest homology among the *crt* genes. Systematic studies determining rate-limiting steps in carotenoid biosynthesis were reviewed well (Barkovich and Liao 2001; Lee and Schmidt-Dannert 2002). According to the reviews, the systematic studies had been carried out only with *crt* genes of *P. ananatis*. It has been known that FPP synthesis in *E. coli* is rate-limiting in carotenoid production which is increased by overexpression of *E. coli ispA*, or by the replacement of *P. ananatis crtE* with *A. fulgidus gps*. However, in this study comparing the *crt* genes of *P. agglomerans* and *P. ananatis*, we suggest that the rate-limitation of FPP synthesis on cartotenoids production depends on the activity of *crtE* in *E. coli*.

To confirm the suggestion, pT-LYCm5 was constructed by cloning the *E. coli ispA* gene into pT-LYCm4 (Fig. 2). Recombinant *E. coli* harboring pT-LYCm5 and pSSN12Didi was cultivated at 30°C for 48 h (Data not shown here). A lycopene production of 58 mg/l and a cell growth (OD_{600nm}) of 7.2 were observed without any addition of IPTG for 48 h. Both lycopene production and cell growth were severely inhibited after the addition of 0.1 mM IPTG. Conclusively, no difference was observed using pT-LYCm5 and pT-LYCm4. Therefore, we believe that when using *P. agglomerans crtE* with a high activity of GGPP synthesis from FPP, *ispA* is not a rate-limiting step in GGPP synthesis and its overexpression does not increase lycopene production. We also used *H. pluvialis ipi* gene and *E. coli idi* gene encoding IPP isomerase with *crt* genes of the two bacteria for lycopene production.

Lycopene production was not likely to be affected by the difference of IPP isomerase genes. It was confirmed by using pT-LYCm4-1 which was recently constructed by replacing *H. pluvialis ipi* gene of pT-LYCm4 with *E. coli idi* gene (data not shown here). Therefore, to increase lycopene production in *E. coli*, it might be important to choose efficient *crt* genes, especially *crtE* gene because they have considerable sequence diversity according to Sedkova et al. (2005), and the sequence diversity might be a cause of a large difference in carotenogenic activity like that of the *crtE* genes of *P. agglomerans* and *P. ananatis*.

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