

Enhancement of Lycopene Production in *Escherichia coli* by Optimization of the Lycopene Synthetic Pathway

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Abstract Using carotenoid genes of *Erwinia herbicola*, metabolic engineering was carried out for lycopene production with the pAC-LYCO4 plasmid, which was composed of a chromosomal DNA fragment of *E. herbicola* containing the *crtE*, *crtB*, and *crtI* genes under the control of the tetracycline promoter and the *ipi* gene of *Haematococcus pluvialis* with the *trc* promoter. Plasmid pAC-LYCM4 was constructed for efficient expression of the four exogenous genes using a strong RBS sequence and the same tetracycline promoter. The optimized expression construct of pAC-LYCM4 increased lycopene production three times as compared with pAC-LYCO4. pAC-LYCM5 containing *ispA* behind the four exogenous genes was constructed. There was no significant difference in lycopene production and cell growth between pAC-LYCM4 and pAC-LYCM5. FPP synthase encoded by *ispA* was not rate-limiting for lycopene production. Each gene of *crtE*, *crtB*, *crtI*, and *ipi* was overexpressed, using pBAD-*crtE*, pBAD-*crtIB*, and pBAD-*ipiHP1*, in addition to their expression from pAC-LYCM4. However, there was no increase of lycopene production with the additional overexpression of each exogenous gene. The four exogenous genes appeared to be not rate-limiting in cells harboring pAC-LYCM4. When pDdxs, pBAD24 containing *dxs*, was introduced into cells harboring lycopene synthetic plasmids, lycopene production of pAC-LYCO4, pAC-LYCM4, and pAC-LYCM5 was increased by 4.7-, 2.2-, and 2.2-fold, respectively. Lycopene production of pBAD-DXM4 containing *crtE*, *crtB*, *crtI*, *ipi*, and *dxs* was 5.2 mg/g dry cell weight with 0.2% arabinose, which was 8.7-fold higher than that of the initial strain with pAC-LYCO4. Therefore, the present study showed that proper regulation of a metabolically engineered pathway is important for lycopene production.

Key words: Lycopene, carotenoids, metabolic engineering, recombinant *E. coli*, *Erwinia herbicola*

Carotenoids form an important medical and biotechnological class of natural pigments produced by many microorganisms and plants [1, 2, 15–17, 25], and have received considerable attention due to their interesting properties as pigments and, more importantly, their potential beneficial effects on human health. Although more than 600 different carotenoids in nature have been identified, only a few are used industrially: lycopene, β -carotene, canthaxanthin, and astaxanthin are used as food colorants, animal feed additives, and in pharmaceuticals and cosmetics [17]. Lycopene, in particular, is an effective antioxidant [28] and has been proposed as a possible treatment for some cancers and other degenerative human conditions [7, 8].

Lycopene is a linear carotenoid composed of 7 IPP (isopentenyl diphosphate) and 1 DMAPP (dimethylallyl diphosphate), which is the biosynthetic precursor of most cyclic carotenoids, including the useful carotenoids mentioned above. During the last few decades, fast progress has been made within the field of molecular biology of carotenoid biosynthesis in bacteria, fungi, and plants [2, 19, 26]. High-yield production of carotenoids in engineered microbial hosts requires balanced expression of carotenogenic genes for efficient transformation of precursors to the carotenoid compounds desired. Considerable progress has been made in expressing all of the genes necessary to synthesize structurally different carotenoids such as lycopene, β -carotene, and zeaxanthin in *Escherichia coli* [4, 21, 23]. In *E. coli*, the IPP synthetic pathway branches from the glycolytic intermediates pyruvate and glyceraldehyde 3-phosphate (G3P) (Fig. 1). The pathway is called the DXPP

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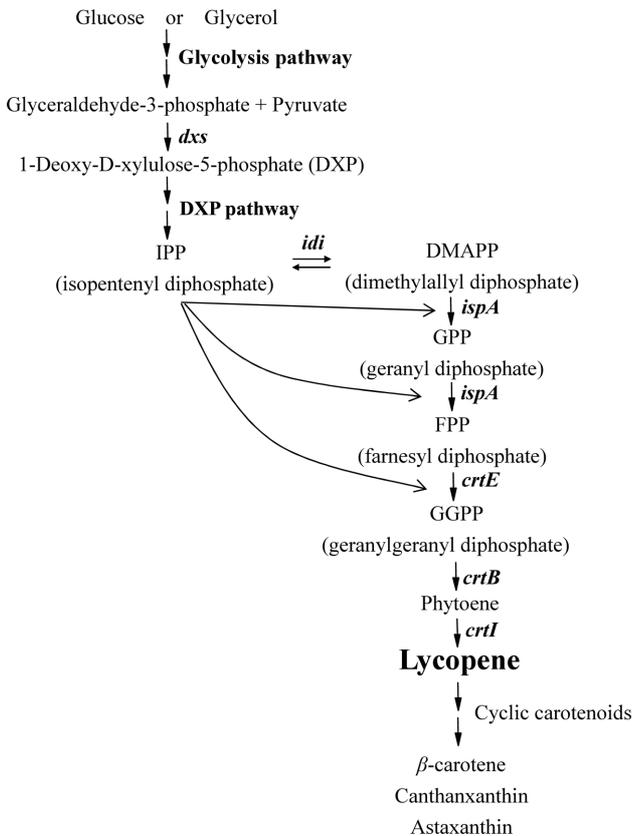


Fig. 1. Biosynthesis of lycopene in *E. coli*.

Lycopene is synthesized via the glycolysis pathway for glyceraldehyde-3-phosphate and pyruvate, the DXP pathway for IPP and DMAPP, isoprenyl diphosphate pathway for FPP, and the foreign lycopene synthesis pathway composed of *crtE*, *crtB*, and *crtI* genes.

pathway [20], to emphasize the function of its intermediates, DXP. Farnesyl diphosphate (FPP) is composed of two IPP molecules and one DMAPP molecule derived from isomerization of IPP. FPP is used for the synthesis of essential components including two electron carriers of the electron transport chain, ubiquinone and menaquinone, and the sugar carrier of cell wall synthesis, dolichol [6, 13]. Lycopene production in *E. coli* requires the introduction of foreign genes *crtE*, *crtB*, and *crtI*, which encode GGPP synthase, phytoene synthase, and phytoene desaturase, respectively (Fig. 1). Misawa *et al.* [21] have described the carotenoid pathway in *Erwinia uredovora*. Shortly afterward, the pathway for *Erwinia herbicola* was documented [10, 27]. Most of the metabolic engineering on carotenoids production from *E. coli* have been carried out using the carotenoid pathway of *Erwinia uredovora* [4, 21–23, 30]. However, there are not enough studies on an optimized expression of the carotenoid synthetic pathway of *Erwinia herbicola*. In this study, we optimized the expression of four exogenous genes of GGPP synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*) from *Erwinia herbicola*, and IPP isomerase (*ipi*) from *Haematococcus pluvialis* to engineer

higher lycopene production levels in *E. coli*. Expression of two endogenous genes of DXP synthase (*dxs*) and FPP synthase (*ispA*) were also optimized for lycopene synthesis in combination of optimal expression of the four exogenous genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

E. coli strain DH5 α ($F^- \phi 80dlacZ\Delta M15 \Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44\lambda^- thi-1 gyrA96 relA1$) was used for gene cloning and expression studies. *E. coli* was grown in 2YT medium at 37°C on a rotary shaker at 200 rpm. Ampicillin (100 $\mu\text{g}/\text{ml}$) and chloramphenicol (50 $\mu\text{g}/\text{ml}$) were added as required. Plasmids pACYC184 [24] and pBAD24 were used as vectors for genes expression studies of lycopene production. Induction with arabinose was carried out as indicated in the results. Plasmid pAC-LYCO4, which expresses the *Erwinia herbicola crtE*, *crtB*, and *crtI* genes necessary for lycopene biosynthesis in *E. coli*, were gifts from F. X. Cunningham of University of Maryland [5, 29]. This plasmid, derived from pACYC184, also contained the *Haematococcus pluvialis ipi* gene that encodes IPP isomerase. Plasmid pDdxs, which was derived from pBAD24 [14] and contained the *E. coli dxs* gene, was also used. Bacterial growth was determined by measuring optical density at 600 nm ($OD_{600\text{ nm}}$). A seed culture was made by inoculating cells into 2YT medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin or 50 $\mu\text{g}/\text{ml}$ chloramphenicol and growing the cells overnight at 37°C. An aliquot of the seed culture was inoculated into 5 ml of 2YT medium containing 0.5% (v/v) glycerol as a carbon source and 100 $\mu\text{g}/\text{ml}$ ampicillin or 50 $\mu\text{g}/\text{ml}$ chloramphenicol as antibiotics, and incubated at 29°C for 48 h to produce lycopene.

PCR Amplification and Plasmid Construction

The genes encoding the three enzymes of the lycopene synthetic pathway, GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*), were isolated by PCR from the pAC-LYCO4 plasmid. The individual genes were spliced together using overlapping extensions from primers crtE-f, crtE-r, crtIB-f, and crtIB-r. The sequences of primers were as follows: crtE-f, 5'-dGAGGAATTCTACGTACTAGTAGGAGGTAATAAATATGCTGAGTGGCAGTAAAG-3'; crtE-r, 5'-dTTCATCCTTTTATACCTCCTCGAGTTATCAGGCGATTTTCATGAC-3'; crtIB-f, 5'-dAAAATCGCCTGATAACTCGAGGAGGTATAAAGGATGAAAAAAAC-3'; crtIB-r, 5'-dGAAGCATATT TTATTACCTCCTCTAGATTACTAAACGGGACGCTGCC-3'. In these primer sequences, the coding regions are indicated by bold letters and the start codons are underlined. Restriction sites, introduced to facilitate subcloning, are double underlined. The gene encoding IPP isomerase (*ipi*) was also isolated

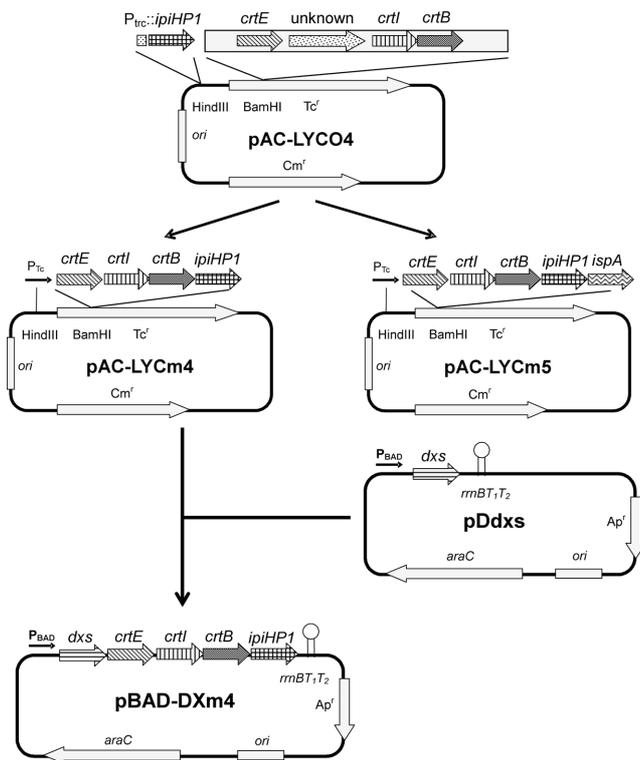


Fig. 2. Schematic diagram of the construction of pBAD-DXm4. The arrow indicates the coding regions. Ap: Ampicillin; Cm: Chloramphenicol; Tc: Tetracycline.

by PCR from plasmid pAC-LYCO4 using primers *ipi-f* and *ipi-r*. The sequences of the primers were as follows: *ipi-f*, 5'-dTCCCGTTTAGTAATCTAGAGGAGGTAATAAAA-TATGCTTCGTTTCGTTGC TC-3'; *ipi-r*, 5'-dGCAGGTCGACGCGGCCGCTTATCACGCTTCGTTGATGTG-3'. The PCR was carried out using *pfx* DNA polymerase (Invitrogen) and a standard PCR protocol. The synthetic operon consisting of *crtE*, *crtB*, and *crtI* was restricted with *Eco*RI and *Xba*I, and the PCR fragment of *ipi* with *Xba*I and *Sal*I. The restricted synthetic operon and *ipi* were moved to the *Eco*RI-*Sal*I sites of pBAD24 to generate pBAD-LYCM4 by three-fragment ligation. The operon of *crtE*, *crtB*, *crtI*, and *ipi* on pBAD-LYCM4 was excised with *Sna*BI and *Ssp*I, and moved to the *Eco*RV site of pACYC184 to generate pAC-LYCM4 (Fig. 2). The *ispA* gene, cloned from *E. coli* using primers *ispA-f* and *ispA-r*, was ligated into the *Not*I site of pAC-LYCM4, resulting in pAC-LYCM5. The sequences of primers were as follows: *ispA-f*, 5'-dGTAAGCGGCCGAGGAGGAATGAGTA-ATGGACTTTCCGCAG-3'; *ispA-r*, 5'-dGTAAGCGGC-CGCGGTACCTCATTATTTATTACGCTGGATGATG-TAG-3'. The *dxs* gene from *E. coli* was ligated into the *Eco*RI-*Sna*BI sites of pBAD-LYCM4 using primers *dxs-f* and *dxs-r*, thereby producing also pBAD-DXm4. The sequences of primers were as follows: *dxs-f*, 5'-dCCGGAATTCATA-ATGAGTTTTGATATTGCC-3'; *dxs-r*, 5'-dCTCCTCG-

AGTACGTATCATTATGCCAGCCAGGCC TTG-3'. The genes of *crtE*, *crtB*, *crtI*, and *ipi* were subcloned into pBAD24 to generate pBAD-*crtE*, pBAD-*crtI*B, and pBAD-*ipiHP1*, respectively. General procedures, including restriction enzyme digestions, transformations, and other standard molecular biology techniques, were carried out as described by Sambrook and Russell [24].

Quantification of Lycopene Production

Dry cell weight was calculated from known volumes of culture harvested by centrifugation at 13,000 ×g for 3 min and washed once with water. The cells were dried overnight at 105°C and weighed. To determine the lycopene content of the cells, an aliquot of *E. coli* cells was harvested by centrifugation at 13,000 ×g for 3 min and washed once with water. The cells were suspended in acetone (200 μl) and incubated at 55°C for 15 min in the dark. The samples were centrifuged at 13,000 ×g for 10 min, and the acetone supernatant containing lycopene was transferred to a clean tube. The lycopene content of the extracts was quantified by measuring absorbance at 470 nm using a Beckman DU Series 640 spectrophotometer and comparing to a lycopene standard (purchased from Sigma). The results are reported as the mean from three independent determinations. The standard deviations were in the range of ±10% of the means.

RESULTS

Reconstruction of Native Lycopene Synthetic Operon from *E. herbicola*

The expression of three exogenous genes, GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*), in *E. coli* cells was sufficient for the conversion of isopentenyl diphosphate (IPP) and farnesyl diphosphate (FPP) to a red-colored carotenoid, lycopene. The carotenoid biosynthetic gene cluster of *E. herbicola* is composed of *crtE*, *crtX*, *crtY*, *crtI*, *crtB*, and *crtZ*. Therefore, plasmid pAC-LYCO4 harboring *crtE*, *crtI*, and *crtB* was constructed by deletion of *crtX*, *crtY*, and *crtZ* from the carotenoid biosynthetic gene cluster of *E. herbicola*, which left an unknown ORF and junk DNA sequence (Fig. 2). The DNA fragment containing *crtE*, *crtI*, and *crtB* was introduced to the *Bam*HI site of tetracycline resistance gene, by the promoter of which the lycopene synthetic genes were constitutively expressed. It has been shown that isomerization of IPP and DMAPP was the rate-limiting step in carotenoid biosynthesis, the production of which was increased by IPP isomerase [12]. The pAC-LYCO4 also has the *Haematococcus pluvialis ipi* gene encoding IPP isomerase under the control of the *trc* promoter. In order to coordinately regulate expression of the above four genes under a promoter and remove the unknown ORF and junk DNA sequence from the DNA

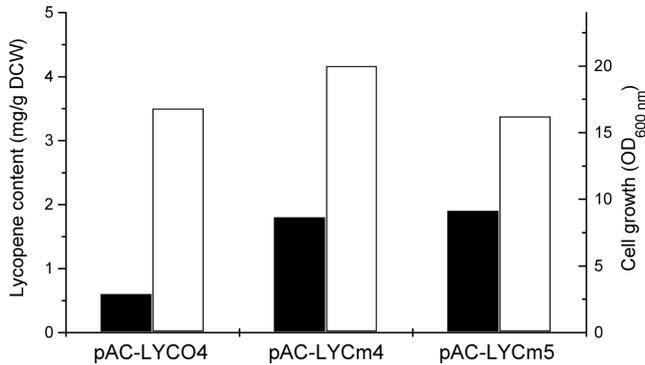


Fig. 3. Effect of optimized expression construct of *crtE*, *crtB*, *crtI*, and *ipi*, and amplification of *ispA* on lycopene production (solid bars) and cell growth (open bars). Plasmids pAC-LYCO4 and pAC-LYCm4 contained *crtE*, *crtB*, *crtI*, and *ipi*, and pAC-LYCM5 was derived by addition of *ispA* to pAC-LYCm4.

fragment containing *crtE*, *crtI*, and *crtB*, plasmid pAC-LYCm4 was constructed from pAC-LYCO4 (Fig. 2). The genes *crtE*, *crtI*, and *crtB* with a modified strong ribosomal binding sequence (RBS) of AGGAGG were isolated by PCR from pAC-LYCO4. The individual genes were spliced together using overlapping extensions of sewing PCR to make an artificial operon with no junk DNA sequences. The *ipi* gene was also isolated by PCR from pAC-LYCO4 and introduced just behind *crtB* of the artificial operon. The four genes were regulated under the same promoter of the tetracycline resistance gene in pAC-LYCm4. The *ispA* encoding FPP synthase was isolated from *E. coli* by PCR and introduced behind *ipi* on pAC-LYCm4 to generate pAC-LYCM5 (Fig. 2). Plasmid pAC-LYCM5 contains all of the genes related to lycopene synthesis from IPP. The *E. coli* harboring each one of pAC-LYCO4, pAC-LYCm4, and pAC-LYCM5 were cultivated in 2YT medium containing 0.5% glycerol and 50 µg/ml chloramphenicol at 29°C for 48 h (Fig. 3). Lycopene contents of the cultures with pAC-LYCO4, pAC-LYCm4, and pAC-LYCM5 were 0.6, 1.8, and 1.8 mg/g dry cell weight, respectively. Consequently, lycopene production was increased three-fold using the plasmids of pAC-LYCm4 and pAC-LYCM5 with the optimized construct of *crtE*, *crtI*, *crtB*, and *ipi*. There was no significant difference in cell growth among the above cultures. The *ispA* gene on pAC-LYCM5 seemed to have no effect on lycopene production and cell growth, as compared with pAC-LYCm4. It means that *ispA* encoding FPP synthase is not rate-limiting, even in the high expression of *crtE*, *crtB*, and *crtI* to increase metabolic flow from FPP to lycopene.

Gene Dosage Effect of *crtE*, *crtI*, *crtB*, and *ipi* on Lycopene Production

The question of which genes among the four exogenous genes were rate-limiting for lycopene production in *E. coli*

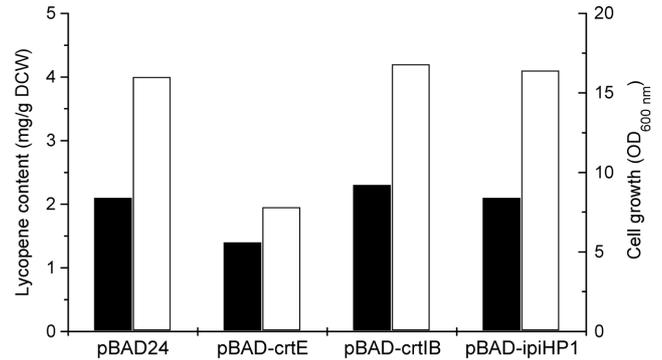


Fig. 4. Effect of further amplification of *crtE*, *crtB*, *crtI*, and *ipi* on lycopene production (solid bars) and cell growth (open bars) in cells harboring pAC-LYCm4.

was studied. Each of *crtE*, *crtB*, *crtI*, and *ipi* were introduced into pBAD24, resulting in pBAD-crtE, pBAD-crtIB, and pBAD-ipiHP1, respectively. Since encoding regions (ORF) of *crtI* and *crtB* are overlapped in native chromosomal DNA structure, they were not separated while cloning into pBAD24: The plasmids were co-transformed into cells harboring pAC-LYCm4. The co-transformants were cultivated in 2YT medium containing 0.5% glycerol, 100 µg/ml ampicillin, and 50 µg/ml chloramphenicol at 29°C for 48 h (Fig. 4). For maximal induction of P_{BAD} promoter, 0.2% arabinose was initially added. Further amplification of *crtI*, *crtB*, and *ipi* genes, using pBAD-crtIB and pBAD-ipiHP1, had no significant effect on cell growth and lycopene production, as compared with pBAD24. However, the amplification of *crtE*, using pBAD-crtE, significantly reduced cell growth to 7.8 OD_{600 nm}, which was a half of other cultures. Lycopene production of pBAD-crtE was slightly decreased. Therefore, there was no rate limitation on lycopene production among the four exogenous genes in cells harboring pAC-LYCm4.

Overexpression of *dxs* Increased Lycopene Production

It has been reported that 1-deoxy-D-xylulose-5-phosphate (DXP) synthesis is the rate-limiting step in IPP synthesis, and overexpression of *dxs* encoding DXP synthase increases lycopene production [9, 14, 18]. Therefore, plasmid pDdxs (pBAD24::*dxs*), which was maximally induced with 0.2% arabinose, was co-transformed into cells harboring pAC-LYCO4, pAC-LYCm4, and pAC-LYCM5. The co-transformants were then cultivated in 2YT medium containing 0.5% glycerol, 0.2% arabinose, 100 µg/ml ampicillin, and 50 µg/ml chloramphenicol at 29°C for 48 h (Fig. 5). From culture harboring pDdxs and pAC-LYCO4, 2.8 mg of lycopene/g dry cell weight was obtained. Lycopene content in pDdxs and pAC-LYCm4 was 3.9 mg/g dry cell weight, and 3.8 mg/g dry cell weight in pDdxs and pAC-LYCM5. Lycopene production of pAC-LYCO4 and pAC-LYCm4 was increased 4.7-fold and 2.2-fold, respectively, by introducing

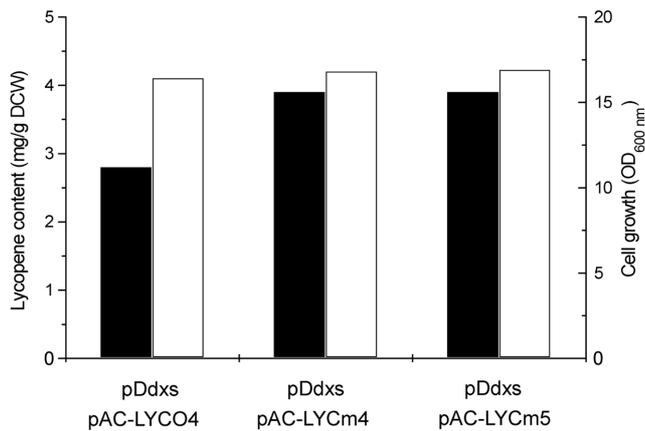


Fig. 5. Effect of overexpression of *dxs* on lycopene production (solid bars) and cell growth (open bars) by introducing pDdxs into cells harboring pAC-LYCO4, pAC-LYCM4, and pAC-LYCM5.

pDdxs. Lycopene production with overexpression of *dxs* was more effective in pAC-LYCO4 than in pAC-LYCM4. The lycopene content of cells harboring pDdxs and pAC-LYCM4 was 1.4-fold higher than that of pAC-LYCO4 and pDdxs. In cells harboring pDdxs and pAC-LYCM5, lycopene production was similar to that of pDdxs and pAC-LYCM4; therefore, the *ispA* gene in pAC-LYCM5 still had no effect on lycopene production even in the presence of pDdxs. There was no significant difference in cell growth among the cultures.

One-Vector System is Better Than Two-Vector System for Lycopene Production

In order to reduce metabolic burden caused by maintaining two plasmids of pDdxs and pAC-LYCM4, the *dxs* of pDdxs and *crtE*, *crtB*, *crtI*, and *ipi* of pAC-LYCM4 were combined into pBAD24 to generate pBAD-Dxm4. The five genes were expressed coordinately under the P_{BAD} promoter,

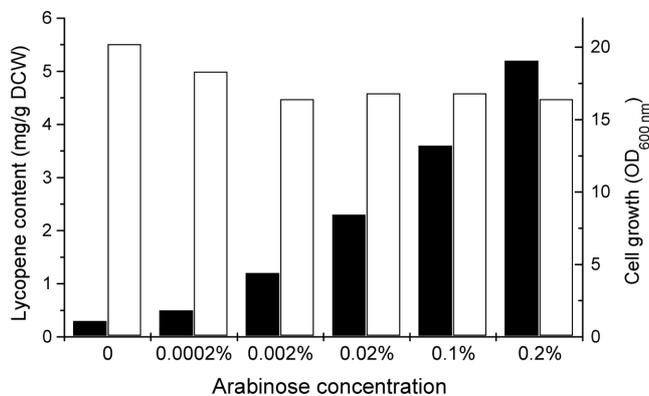


Fig. 6. Lycopene production (solid bars) and cell growth (open bars) of recombinant *E. coli* harboring pBAD-Dxm4, depending on inducer arabinose concentration.

and the gene dose of *crtE*, *crtB*, *crtI*, and *ipi* was increased, because the copy number of pBAD24 was higher than pAC-LYCM4, which was derived from pACYC184. Cells harboring pBAD-Dxm4 were cultivated in 2YT medium containing 0.5% glycerol and 100 µg/ml ampicillin at 29°C for 48 h (Fig. 6). Arabinose was added as indicated to induce the P_{BAD} promoter. The lycopene content of cells harboring pBAD-Dxm4 was significantly increased, depending on the arabinose concentration, and 5.2 mg lycopene/g dry cell weight was obtained at 0.2% arabinose induction. In the absence of arabinose, 0.3 mg of lycopene/g dry cell weight was observed, because genes on pBAD-Dxm4 were tightly regulated under P_{BAD}. There was no significant difference in cell growth, ranging from 16 to 20 of OD_{600nm}, between different arabinose concentrations.

DISCUSSION

Metabolic engineering of the carotenoid synthetic pathway in *E. coli* has been carried out using carotenoid genes of *E. uredoovora* and *E. herbicola*. The expression of three carotenoid genes of *crtE*, *crtB*, and *crtI* is required for lycopene synthesis in *E. coli*. Although they belong to the same genus of *Erwinia*, there is significant difference in the amino acid sequences of *crtE*, *crtB*, and *crtI* between *E. uredoovora* and *E. herbicola*, and based on NCBI BLAST analysis, homologies of *crtE*, *crtB*, and *crtI* between the two photosynthetic bacteria are 55%, 64%, and 77% respectively. Metabolic engineering using carotenoid genes of *E. uredoovora* has mostly been done, whereas there are not enough studies using *E. herbicola* carotenoid genes [22, 30]. Considering the low homology of the *crtE*, *crtB*, and *crtI* genes between the two microorganisms, it was worthwhile to attempt metabolic engineering with carotenoid genes of *E. herbicola*. In order to find the rate-limiting genes among *crtE*, *crtB*, and *crtI-ipi*, each gene was further overexpressed in addition to their expression from pAC-LYCM4 (Fig. 4). There was no significant difference in lycopene production with the additional overexpression of each exogenous gene, suggesting that the four exogenous genes were not rate-limiting in cells harboring pAC-LYCM4. In carotenoids production from *E. coli* using carotenoid genes of *E. uredoovora*, *crtE* encoding GGPP synthase was reported to be the rate-limiting step [3, 23, 30]. It is quite possible that *E. herbicola crtE* is more active in carotenoid production in *E. coli* than *E. uredoovora crtE*. Cells harboring both pBAD-*crtE* and pAC-LYCM4 showed 50% decreased cell growth. Farnesyl pyrophosphate (FPP) is a precursor of respiratory quinines (ubiquinone and menaquinone) and dolichols (sugar carrier lipids), which are essential for cell growth. The additional overexpression of *crtE* encoding GGPP synthase, which converts FPP to GGPP, was thought

to cause deprivation of FPP in the cells and limitation of cell growth. Lycopene synthetic plasmids, pAC-LYCO4, pAC-LYCM4, and pAC-LYCM5, were co-transformed with pDdxs, pBAD24 containing the *dxs* gene. Enhancement of lycopene production with overexpression of *dxs* was found to be more significant in pAC-LYCO4, in comparison with pAC-LYCM4 or pAC-LYCM5 (Fig. 5). Metabolic engineering is to increase metabolic flow to a target product by pulling down or pushing up from the bottom or top of the production pathway. It was suspected that the high expression of carotenoid genes in pAC-LYCM4 and pAC-LYCM5 was strongly pulling down metabolic flow to lycopene synthesis, and pushing up of the metabolic flow with overexpression of *dxs* from pDdxs had a less significant effect on the increase of lycopene production than pAC-LYCO4. To reduce the metabolic burden of using the two-vector system of pAC-LYCM4 and pDdxs and to achieve precise control of carotenoid gene expression, *crtE*, *B*, *I*, and *ipi*, the two plasmids were combined in pBAD-DXM4. At 0.2% arabinose, 5.2 mg/g dry cell weight of lycopene was obtained, which was higher than the 3.9 mg/g dry cell weight of pAC-LYCM4 and pDdxs. The recombinant *E. coli* harboring pBAD-DXM4 produced 8.7-fold higher lycopene than the initial strain with pAC-LYCO4. The present study showed that proper regulation of a metabolically engineered pathway is important. Intracellular accumulation of hydrophobic carotenoids has been known to be toxic to cells and to inhibit cell growth [23]. For mass production of lycopene, it is necessary to separate growth and production phases using a two-stage culture. The two-stage culture can not be accomplished with pAC-LYCM4 because its carotenoid genes are constitutively expressed under the tetracycline promoter. However, pBAD-DXM4 is appropriate for the two-stage culture even when using industrial complex medium because the P_{BAD} promoter has less leaky expression than *lac*-derived promoters in complex medium. The recombinant *E. coli* harboring pBAD-DXM4 might be a good producer of lycopene. Metabolic engineering of the lycopene synthetic pathway for its mass production is expected to give an advantage for production of other industrially important carotenoids derived from lycopene.

Acknowledgments

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