Identification of Genes Affecting Lycopene Accumulation in *Escherichia coli* Using a Shot-Gun Method

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Abstract: Genes enhancing lycopene production in *Escherichia coli* were identified through colorimetric screening of shot-gun library clones constructed with *E. coli* chromosomal DNA. These *E. coli* cells had been engineered to produce lycopene, a red-colored carotenoid, which enabled screening for genes that enhance lycopene production. Six clones with enhanced lycopene production were isolated. Among 13 genes in these clones, *dxs*, *appY*, *crl*, and *rpoS* were found to be involved in enhanced lycopene production. While *dxs* and *rpoS* have been already reported to enhance lycopene production, *appY* and *crl* have not. DXP (1-deoxy-D-xylulose-5-phosphate) synthase is encoded by *dxs* and participates in the rate-limiting step in the synthesis of isopentenyl pyrophosphate (IPP), a building block of lycopene. Sigma S factor, encoded by *rpoS*, regulates transcription of genes induced at the stationary phase. The *appY* and *crl* genes encode transcriptional regulators related to anaerobic energy metabolism and the formation of curli surface fibers, respectively. *E. coli* harboring *appY* plasmids produced 2.8 mg lycopene/g dry cell weight (DCW), the same amount obtained with *dxs* despite the fact that *appY* is not directly involved in the lycopene synthesis pathway. The co-expression of *appY*, *crl*, and *rpoS* with *dxs* synergistically enhanced lycopene production. The co-expression of *appY* with *dxs* produced eight times the amount of lycopene (4.7 mg/g DCW) that was produced without expression of both genes (0.6 mg/g DCW).

Keywords: lycopene; colorimetric screening; *AppY*; *Crl*

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 beneficial effects on human health (Armstrong, 1997; Sandmann, 2001). Lycopene, in particular, is an effective antioxidant and has been proposed as a possible treatment for some cancers and other degenerative human conditions (Giovannucci, 2002; Sies and Stahl, 1998). As a result, the in vivo synthesis of carotenoids has become increasingly necessary, and a number of reports have described their production in recombinant microorganisms (Farmer and Liao, 2000; Jones et al., 2000; Kajiwara et al., 1997; Kim and Keasling, 2001; Lee et al., 2004; Misawa and Shimada, 1997; Sandmann, 2003; Schmidt-Dannert et al., 2000). Carotenoids have been successfully synthesized in non-carotenogenic bacteria and yeast using recombinant DNA techniques (Misawa et al., 1991; Sandmann et al., 1990; Yamano et al., 1994). 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* (Cunningham et al., 1993; Misawa et al., 1990; Ruther et al., 1997). In *E. coli*, the MEP (2-C-methyl-D-erythritol-4-phosphate) pathway is used for IPP and DMAPP synthesis, branching from the glycolytic intermediates, glyceraldehyde-3-phosphate and pyruvate, and for condensing two IPP and one DMAPP to form FPP via the protein encoded by *ispA* (Fig. 1) (Meyer et al., 2003). The co-expression of three exogenous genes in *E. coli* cells—GGPP synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*)—is sufficient for the conversion of IPP and FPP to a red-colored carotenoid, lycopene (Lotan and Hirschberg, 1995). Formation of lycopene in *E. coli* cells can be detected simply by the color of colonies on agar plates. These observations suggest the possibility of establishing a novel system for the identification of genes stimulating lycopene accumulation. This bacterial system could also potentially

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Min Jung Kang and Young Mi Le contributed equally to this work.

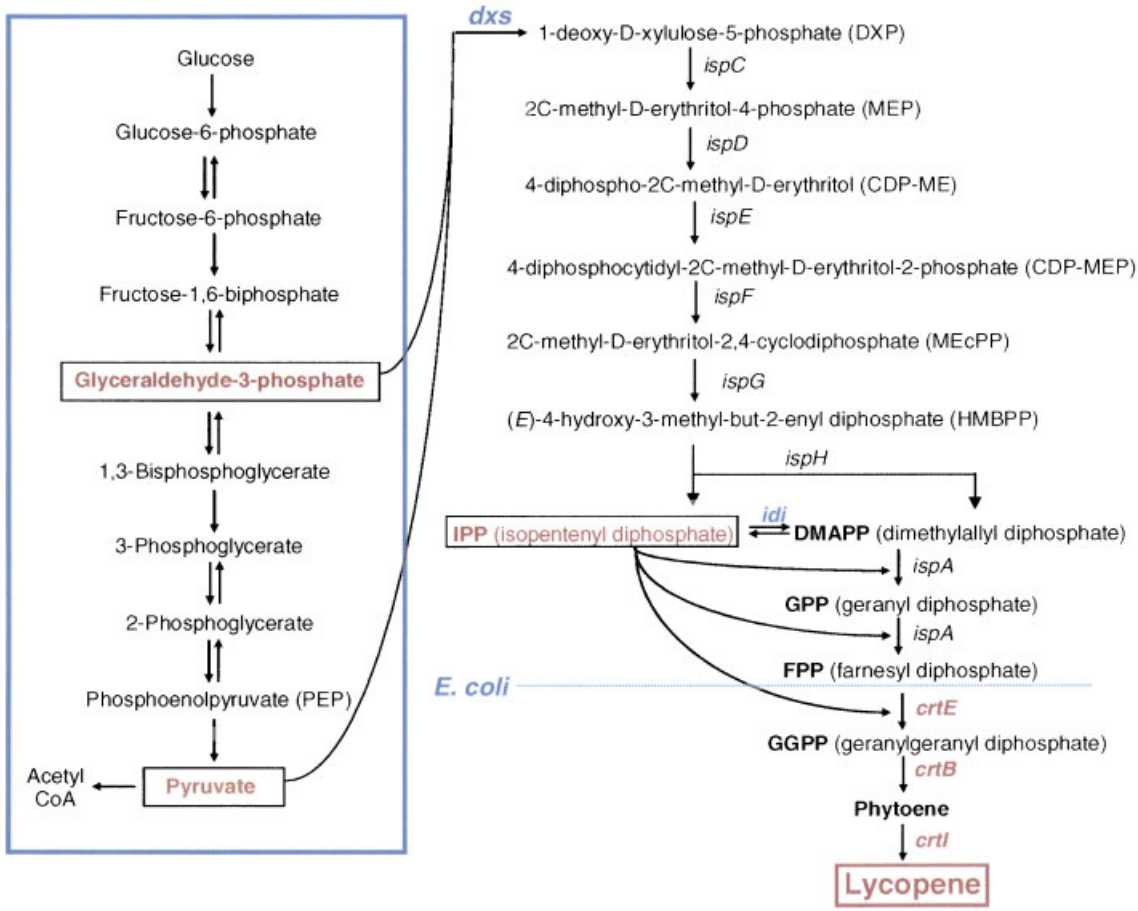

Figure 1. Biosynthesis of lycopene in *E. coli*. Lycopene is synthesized via the glycolysis pathway for glyceraldehyde-3-phosphate and pyruvate, the MEP pathway for IPP and DMAPP, the isoprenyl diphosphate pathway for FPP, and also via the foreign lycopene synthesis pathway composed of *crtE*, *crtB*, and *crtI* genes. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

be used for the isolation of genes that enhance carotenoid accumulation.

In this study, *E. coli* cells were transformed with a plasmid conferring lycopene accumulation, followed by transformation with an *E. coli* genomic DNA library. We found four gene clusters that increased lycopene accumulation in *E. coli*, and we discuss the potential implications for lycopene production in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

E. coli strain 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 expression studies. Unless otherwise stated, *E. coli* was grown in 2YT medium (Sambrook and Russell, 2001) at 37°C on a rotary shaker at 200 rpm. Ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) (both from Sigma) were added as required. The plasmids pBluescript (Stratagene) and pBAD24 (Guzman et al., 1995) were used as vectors for genomic library construction and candidate gene expression studies. Induction with arabinose was carried out as indicated in the Results. Plasmid pAC-LYC04, which expresses the *Erwinia herbicola crtE*, *crtB*, and *crtI* genes necessary for lycopene biosynthesis in *E. coli*, was a gift from F. X. Cunningham of the University of Maryland. This plasmid also contained the *Haematococcus pluvialis ipi* gene that encodes IPP isomerase (Sun et al., 1998).

Growth and Induction Experiments

Bacterial growth was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}). A seed culture was made by inoculating cells into 2YT medium containing 100 μg/mL ampicillin and 50 μg/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 100 μg/mL ampicillin, 50 μg/mL chloramphenicol, and 0.2% arabinose to an OD_{600} of 0.1 and incubated at 29°C for 48 h in order to produce lycopene.

Construction of *E. coli* Genomic Library

E. coli cultures were grown in 1 L of 2YT medium at 37°C and harvested. Their genomic DNA was isolated and purified. Genomic DNA was partially digested with Sau3AI, and the

resulting restriction fragments were fractionated by 0.5% agarose electrophoresis. The region of the gel between 3 and 6 kb was cut out, and the DNA in the gel was isolated using a Qiagen gel extraction kit. The Sau3AI fragments (2.7 pg) were ligated with 1.4 pg of BamHI-digested, dephosphorylated pBluescript and transformed into *E. coli* DH5α. The clones were stored at −70°C. General procedures including restriction enzyme digestions, transformations, and other standard molecular biological techniques were carried out as described in Sambrook et al. (Sambrook and Russell, 2001).

Identification of Genes That Alter Lycopene Accumulation in *E. coli*

The *E. coli* genomic library described above was isolated by the alkaline method. Competent cells containing pAC-LYC04 were transformed with 10 ng of DNA from the genomic library and spread on LB plates containing chloramphenicol (50 μg/mL) and ampicillin (100 μg/mL). The double-transformants were plated at a density of about 100 CFU/plate (on 100-mm Petri dishes) and incubated overnight at 37°C. A further 5 days of incubation at room temperature enabled screening for colonies with relatively increased pigmentation in comparison with control colony harboring pBluescript and pAC-LYCO4.

Quantification of Lycopene Production

Dry cell weight (DCW) 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 the lycopene was transferred to a clean tube. The lycopene content of the extracts was quantified by measuring the absorbance at 470 nm using a Beckman DU Series 640 spectrophotometer and comparison with a lycopene standard (Sigma) (Harker and Bramley, 1999). The reported results represent the mean from three independent determinations. The standard deviations were in the range of ±10% of the means.

DNA Sequencing and Analysis

DNA sequences for both strands were determined by primer walking, using the automated DNA Sequencing Facility at Solgent Co. in Korea. Sequence analysis was performed using the Vector NTI Suite, ver 9.0 (InforMax, North Bethesda, MD) and BLAST 2.1.

RESULTS

Isolation of Genes Conferring Increased Lycopene Accumulation in *E. coli*

To identify genes that enhance lycopene accumulation in *E. coli*, an *E. coli* genomic DNA library was used to transform *E. coli* cells containing pAC-LYC04. Cells transformed by a plasmid containing a gene that stimulated lycopene production became red colonies on LB agar plates. One thousand transformants (of 100,000 colonies) were selected on the basis of reddish colony color and were re-streaked on LB agar plates to observe the difference in color intensity more clearly. Fifty-three of these were cultured in liquid medium to enable accurate comparisons of lycopene production. Six red transformants were finally selected: L4, H36, H39, H70, H72, and H90 (Fig. 2A). As can be seen in Figure 2B, the six red positive clones were much redder than controls harboring

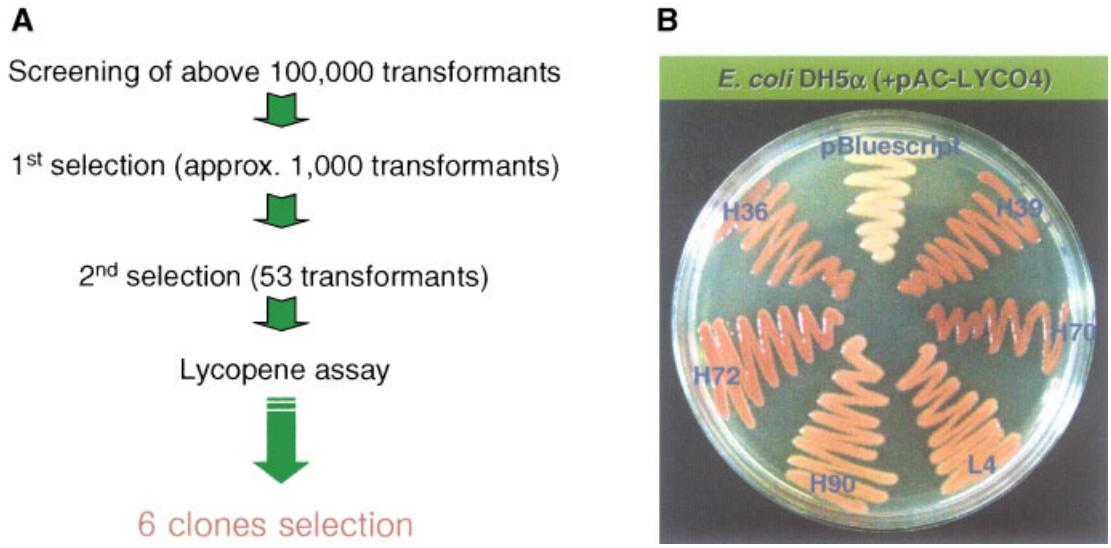

Figure 2. Screening an *E. coli* genomic DNA library to find genes that increase lycopene accumulation in engineered *E. coli*. **A**: Screening schemes for clones positive for lycopene production. **B**: Photos of six positive clones which were streaked on LB plate containing 100 μg/mL ampicillin and 50 μg/mL chloramphenicol, and incubated at 29°C for 2 days. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table I. Identification of the genes in the clones positive for lycopene production by using BLAST searches (following determination of the DNA sequences by primer walking).

Positive clone	Gene & encoded protein	Reference
L4	*crl*: transcriptional regulator of cryptic *csgBA* gene for curli surface fibers	
	phoE: outer membrane pore protein E	
H36	*appY*: regulatory protein affecting *appA* and other genes	
H39	*rpoS*: RNA polymerase, sigma S (sigma38) factor	Sandmann et al. (1990)
H70	*dxs*: DXP synthase in MEP pathway	Harker and Bramley (1999)
H72	*ycgZ*: hypothetical protein	
	ymgA: hypothetical protein	
	ymgB: hypothetical protein	
H90	*dgoK*: 2-oxo-3-deoxygalacto kinase	
	dgoR: transcriptional repressor for galactonate utilization	
	yidX: putative replicase	
	yidA: hypothetical protein	
	yidB: hypothetical protein	

the empty vector (pBluescript) that was used for genomic DNA library construction.

Identification of Genes in the Positive Clones

The pBluescript plasmids that contained inserts of *E. coli* DNA fragments were recovered from the positive clones and analyzed. Insert DNA fragments were sequenced and identified through BLAST searches (Table I). Clone L4 contained *crl* and *phoE*, encoding gene products related to outer membrane structure. The *crl* gene product is a transcriptional regulator of *csgBA* for curli surface fibers, and *phoE* encodes an outer membrane protein induced under limiting phosphate conditions. Clones of H36, H39, and H70 contained just one complete gene, *appY*, *rpoS*, and *dxs*, respectively. The *appY* gene encodes a transcription activator for genes related to anaerobic energy metabolism. The sigma S factor encoded by *rpoS* regulates the transcription of genes expressed during the stationary phase. The *dxs* gene encodes DXP synthase, which mediates the condensation of glyceraldehy-3-phosphate and pyruvate to 1-deoxyxylulose-5-phosphate, the first step of the MEP pathway for the synthesis of IPP, a building block for lycopene. Clone H72 contained three genes encoding hypothetical proteins whose functions are unknown. Clone H90 contained a large DNA fragment composed of five genes. Of these five, *dgoK* and *dgoR* are related to galactonate metabolism, but the functions of *yidX*, *yidA*, and *yidB* are unknown.

Effect of Selected Genes on Lycopene Production

In order to confirm the apparent increases in lycopene production in the positive clones, their pBluescript insert DNA fragments were re-cloned into a pBAD24 expression vector in frame with the P_{BAD} promoter, which enables precise control of expression in a complex medium such as 2YT (used for lycopene production). The reconstructed plasmids based on pBAD24 were named pBAD-L4, pBAD-H36, pBAD-H72, and pBAD-H90 (Fig. 3). In pBAD-L4R, the cloning direction of the insert DNA fragment was opposite to that of pBAD-L4. As *rpoS* and *dxs* have already been reported to increase lycopene production (Table I), these two genes were not pursued in this confirmation study. Lycopene production culture was carried out with recombinant *E. coli* harboring pAC-LYC04 and the reconstructed pBAD24 plasmids in 2YT medium containing 0.2% arabinose, for maximal induction of the P_{BAD} promoter (Table II). The highest level of lycopene production (1.7 mg lycopene/g DCW) was obtained with pBAD-H36, containing the *appY* gene. The other plasmids also enhanced lycopene production by two or three times relative to empty vector controls. There was no significant difference in lycopene production between pBAD-L4 and pBAD-L4R, despite the difference in direction of the insert containing *crl* and *phoE*. The expression of *crl* and/or *phoE* from their own native promoters was evidently sufficient to enhance lycopene production without expression from P_{BAD}, the vector promoter.

In order to investigate individual genes more closely, every gene found in the positive clones was re-cloned individually into pBAD24 using PCR. Only the ORF regions and ribosomal binding sequences of each gene were cloned, without junk sequences or neighboring genes. The resulting plasmids were named pBAD-dxs, pBAD-rpoS, pBAD-crl, pBAD-phoE, and pBAD-appY. Lycopene production culture was performed after transformation of the plasmids into *E. coli* harboring pAC-LYC04 (Table III). Lycopene production increased three to five times in cells with plasmids carrying the *crl*, *rpoS*, *appY*, or *dxs* gene. We found that the *phoE* gene had no effect on lycopene production and concluded that the observed increased lycopene production in clone L4 was owing to the effects of *crl* alone. The highest

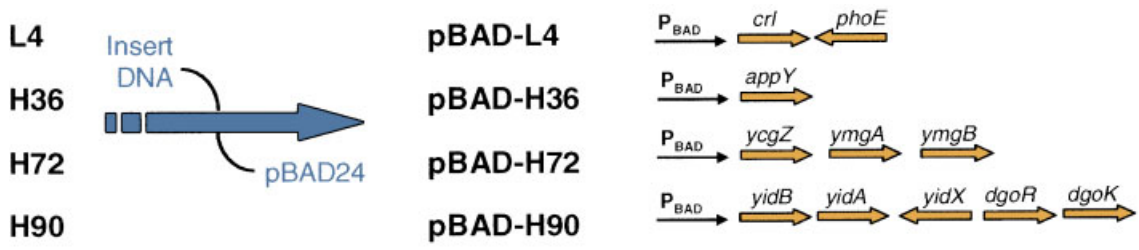

Figure 3. Schematic representation of the reconstructed pBAD24 plasmids containing the positive clones. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table II. Lycopene production with reconstructed pBAD24 plasmids in recombinant *E. coli* harboring pAC-LYC04.

Plasmid	Lycopene content (mg/g dry cell weight)
pBAD24	0.5 ± 0.03
pBAD-L4	1.5 ± 0.05
pBAD-L4R	1.4 ± 0.06
pBAD-H36	1.7 ± 0.05
pBAD-H72	1.1 ± 0.08
pBAD-H90	1.0 ± 0.09

(a) Arabinose at a concentration of 0.2% (w/v) was added initially for maximal induction of P_{BAD} promoter. (b) Culture was carried out at 29°C for 48 h. (c) The values are means ± standard errors for triplicate experiments.

Table IV. Effect of cooperation between *dxs* and *crl*, *rpoS*, and *appY* on lycopene production in recombinant *E. coli* harboring pAC-LYC04.

Plasmid	Lycopene content (mg/g dry cell weight)
pBAD-dxs	2.7 ± 0.07
pBADdxs-crl	3.1 ± 0.11
pBADdxs-rpoS	3.5 ± 0.10
pBADdxs-appY	4.7 ± 0.12

(a) Arabinose at a concentration of 0.2% (w/v) was added initially for maximal induction of P_{BAD} promoter. (b) Culture was carried out at 29°C for 48 h. (c) The values are means ± standard errors for triplicate experiments.

level of lycopene production (2.8 mg lycopene/g DCW) was observed in pBAD-dxs and pBAD-appY. The *dxs* gene occurs in the lycopene synthesis pathway, but the other genes, *crl*, *rpoS*, and *appY*, are not directly related to lycopene synthesis. To investigate the effects of the cooperation between *dxs* and *crl*, *rpoS*, and *appY* on lycopene production, *dxs* was combined with *crl*, *rpoS*, or *appY* in the plasmid constructs pBADdxs-crl, pBADdxs-rpoS, or pBADdxs-appY, respectively. Cooperation between *dxs* and *crl*, *rpoS*, or *appY* further increased lycopene production relative to that observed in cells containing plasmids of each gene alone (Table IV). The combination of *dxs* and *appY* produced the highest amount of lycopene (4.7 mg lycopene/g DCW).

DISCUSSION

The recombinant *E. coli* system involving pAC-LYC04 was used for cloning unknown genes related to lycopene production in *E. coli*. The screening of *E. coli* genomic DNA library did generate six clones containing thirteen genes. The H70 clone containing *dxs* was obtained, indicating that *dxs* is the rate-limiting gene in IPP synthesis for lycopene production. It was suspected that the other genes of MEP pathway (*ispC*, *ispD*, *ispE*, *ispF*, *ispG*, and *ispH)* might not be rate-limiting because no clone containing those gene was detected from the screening system. It has been reported that the amplification of *dxs* enhances carotenoids production in *E. coli*, which coincides with the results of this study (Harker and Bramley, 1999; Kim and Keasling, 2001; Matthews and Wurtzel, 2000). Besides *dxs*, IPP isomerase coding *idi* has also been reported as rate-limiting for carotenoids production

Table III. Effect of each gene, cloned into pBAD24, on lycopene production in recombinant *E. coli* harboring pAC-LYC04.

Plasmid	Lycopene content (mg/g dry cell weight)
pBAD24	0.6 ± 0.03
pBAD-crl	1.5 ± 0.05
pBAD-phoE	0.7 ± 0.04
pBAD-rpoS	1.7 ± 0.08
pBAD-appY	2.8 ± 0.09
pBAD-dxs	2.8 ± 0.07

(a) Arabinose at a concentration of 0.2% (w/v) was added initially for maximal induction of P_{BAD} promoter. (b) Culture was carried out at 29°C for 48 h. (c) The values are means ± standard errors for triplicate experiments.

(Barkovich and Liao, 2001). However, no clone containing *idi* was detected by screening the genomic DNA library of *E. coli* because the pAC-LYC04 plasmid used in our screening system contains the IPP isomerase gene of *Haematococcus pluvialis*, which is sufficiently expressed to allow lycopene production (Sun et al., 1998).

The *rpoS* gene of H39 encodes sigma S factor which stimulates the transcription of genes expressed in the stationary phase or related to stress responses. Sandmann et al. reported that the inactivation of *rpoS* in *E. coli* resulted in an 85% reduction in carotenoid formation (Sandmann et al., 1990). The enhancement of lycopene production by *rpoS* might be the result of higher expression levels of lycopene synthesis enzymes or increased stress resistance to intracellular accumulation of lycopene, which is a very hydrophobic molecule.

The *appY* gene of H36 encodes a transcriptional activator of two energy metabolism operons, *hya* and *cbdAB*-appA which are induced by anaerobiosis. The *hya* operon encodes hydrogenase 1, and the *cbdAB*-appA operon encodes cytochrome bd-II oxidase and acid phosphatase (Atlung et al., 1997). The role of hydrogenase 1 is to recycle H_2 produced during fermentation (Sawers et al., 1986). Hydrogenase 1 would serve for protective or energetic purposes and would be advantageous to bacteria under energy limitation (Laurinavichene and Tsygankov, 2001). It is suggested that lycopene-producing cells experience energy limitation observed under anaerobic conditions because lycopene production was increased with amplification of *appY*, transcriptional activator of the two energy metabolism operons induced by anaerobiosis. Ubiquinone is a component of the electron transport chain, producing energy from aerobic respiration. It is composed of a quinine ring and an isoprenyl side chain containing seven molecules of IPP and one DMAPP molecule (Kainou et al., 2001; Okada et al., 1997). Since cells use same building block molecules of IPP and DMAPP to make ubiquinone and lycopene, they might not be able to synthesis normal quantities of ubiquinone in the presence of active lycopene synthesis. When lycopene production in *E. coli* reached 3.0 mg lycopene/g DCW with a fortified lycopene synthesis pathway, the ubiquinone content decreased from 240 μg/g DCW (without lycopene synthesis) to 45 μg/g DCW (data not shown). Therefore, the role of *appY* for enhanced lycopene production is suspected

to be production of energy via anaerobic metabolic pathway of *hya* and *cbdAB*-appA, to complement the energy insufficiency occurred at low intracellular level of ubiquinone. There is a report which shows blockage of ubiquinone synthesis increases the expression of *cbdAB*-appA operon (Georgellis et al., 2001). It is significant that the highest lycopene production obtained with *dxs* was also achieved with *appY*, which has no direct involvement in lycopene synthesis.

The *crl* and *phoE* genes in L4 respectively encode a transcriptional regulator of *csgBA* for curli surface fiber formation and outer membrane pore protein E for passive diffusion of small molecules (~600 Da) (Arnqvist et al., 1992; Phoenix, 1996). Cells with pBAD-crl increased lycopene production to levels similar to those seen with pBAD-L4, whereas no increase in lycopene production was observed in cells with pBAD-phoE. This indicates that *crl* alone increases lycopene production. Curli is thin aggregate fibers which enable cells to colonize hydrophobic surfaces and to form aggregation clumps (Vidal et al., 1998). Sandmann et al. suggested that hydrophobic carotenoid molecules might be located in cell membranes and that intracellular carotenoid accumulation could be restricted by limited membrane space (Sandmann et al., 1999). It was therefore suggested that some genetic manipulations leading to the formation of additional membrane or novel carotenoid sequestering system such as soluble carotenoid binding proteins were necessary to maximize carotenoid production (Albrecht et al., 1999). When considering curli surface fiber formation by *crl* and accumulation of hydrophobic carotenoid in cells, there could be a positive relationship between *crl* and lycopene production with respect to some hydrophobic interaction of curli fiber molecules and carotenoid. Recently, other function of *crl* has been reported to be stimulation of RpoS activity for transcription of some genes during stationary phase, including *csgBA* (Pratt and Silhavy, 1998; Bougdour et al., 2004). Since *rpoS* of H39 clone showed increase of lycopene production, further investigation is required to see which one, between the hydrophobic interaction with carotenoid and the stimulation of RpoS activity, is a real role of *crl* for enhanced lycopene production.

The H90 clone contained five genes including two galactonate metabolism genes, *dgoK* and *dgoR*, and three genes of unknown function, *yidA*, *yidB*, and *yidX*. The *dgoK* and *dgoR* genes encode 2-oxo-3-deoxygalactonate kinase and a transcriptional repressor for galactonate utilization, respectively (Cooper, 1978). To determine which of these affected lycopene production, the genes were cloned into pBAD24 vector, forming the plasmids pBAD-yidBA, pBAD-yidX, and pBAD-dgoRK, none of which had any effect on lycopene production (data not shown). Further study of these genes is necessary, especially those involved in galactonate metabolism. Galactonate is metabolized by enzymes of *dgoD*, *dgoK*, and *dgoA* to glyceraldehyde-3-phosphate and pyruvate, which are the starting metabolites of the MEP pathway for IPP synthesis. Farmer and Liao (2001) showed that metabolic imbalances in IPP synthesis via the MEP pathway were caused by discrepancies between intracellular pool sizes of glyceraldehyde-3-phosphate and pyruvate during glycolysis.

The H72 clone contained three genes (*ycgZ*, *ymgA*, and *ymgB)* encoding hypothetical proteins. These three genes were separately cloned into plasmids (pBAD-ycgZ, pBAD-ymgA, and pBAD-ymgB) none of which had any effect on lycopene production (data not shown). The observed increase in lycopene production in H72 was therefore suspected to have occurred through the concerted activities of *ycgZ*, *ymgA*, and *ymgB*. Strangely, the culture broth of cells harboring pBAD-ymgA was highly viscous owing to slime formation.

Four genes (*dxs*, *crl*, *rpoS*, and *appY*) were identified as enhancers of lycopene production in our color screening system harboring pAC-LYCO4. IPP isomerase coding *idi* was not detected as an enhancer for lycopene production because it was contained within pAC-LYCO4. These results imply that rate-limiting genes in carotenoid biosynthetic pathway (such as *dxs* and *idi*) are important for high lycopene production but also, some regulatory genes (such as *crl*, *rpoS*, and *appY*) can be used to further increase the production. The global regulatory genes *crl*, *rpoS*, and *appY* seem to produce metabolic conditions favorable for lycopene production. The combination of *dxs* with *crl*, *rpoS*, and *appY* increased lycopene production beyond that observed with *dxs* alone. Cells containing pBADdxs-appY produced 4.7 mg lycopene/g DCW, which is significantly greater than the 2.7 mg lycopene/g DCW produced by cells harboring pBAD-dxs. The mechanisms by which the *crl*, *rpoS*, and *appY* genes enhance lycopene production are not restricted to the lycopene synthesis pathway; thus the over-expression of these three genes can be expected to increase the production of all carotenoids in *E. coli* that have been metabolically engineered for carotenoid production.

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