Metabolic Engineering of the Nonmevalonate Isopentenyl Diphosphate Synthesis Pathway in *Escherichia coli* Enhances Lycopene Production

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Received 11 February 2000; accepted 20 August 2000

Abstract: Isopentenyl diphosphate (IPP) is the common, five-carbon building block in the biosynthesis of all carotenoids. IPP in *Escherichia coli* is synthesized through the nonmevalonate pathway, which has not been completely elucidated. The first reaction of IPP biosynthesis in *E. coli* is the formation of 1-deoxy-D-xylulose-5-phosphate (DXP), catalyzed by DXP synthase and encoded by *dxs*. The second reaction in the pathway is the reduction of DXP to 2-C-methyl-D-erythritol-4-phosphate, catalyzed by DXP reductoisomerase and encoded by *dxr*. To determine if one or more of the reactions in the nonmevalonate pathway controlled flux to IPP, *dxs* and *dxr* were placed on several expression vectors under the control of three different promoters and transformed into three *E. coli* strains (DH5α, XL1-Blue, and JM101) that had been engineered to produce lycopene. Lycopene production was improved significantly in strains transformed with the *dxs* expression vectors. When the *dxs* gene was expressed from the arabinose-inducible *araBAD* promoter (P_{BAD}) on a medium-copy plasmid, lycopene production was twofold higher than when *dxs* was expressed from the IPTG-inducible *trc* and *lac* promoters (P_{trc} and P_{lac}, respectively) on medium-copy and high-copy plasmids. Given the low final densities of cells expressing *dxs* from IPTG-inducible promoters, the low lycopene production was probably due to the metabolic burden of plasmid maintenance and an excessive drain of central metabolic intermediates. At arabinose concentrations between 0 and 1.33 mM, cells expressing both *dxs* and *dxr* from P_{BAD} on a medium-copy plasmid produced 1.4–2.0 times more lycopene than cells expressing *dxs* only. However, at higher arabinose concentrations lycopene production in cells expressing both *dxs* and *dxr* was lower than in cells expressing *dxs* only.

A comparison of the three *E. coli* strains transformed with the arabinose-inducible *dxs* on a medium-copy plasmid revealed that lycopene production was highest in XL1-Blue. *Biotechnol Bioeng* **72**: 408–415, 2001.

Keywords: lycopene; isopentenyl diphosphate; carotenoids

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Contract grant sponsor: the ERC Program of the National Science Foundation

Contract grant number: EEC-9731725

Contract grant sponsor: National Science Foundation

Contract grant number: BES-9911463

Contract grant sponsor: Office of Naval Research

Contract grant number: N00014-00-1-0750

INTRODUCTION

Carotenoids are naturally occurring pigments synthesized as hydrocarbons (carotenes) or oxygenated derivatives (xanthophylls) by plants and microorganisms. Their major functions are protecting against oxidative damage by quenching photosensitizers, interacting with singlet oxygen (Krinsky, 1994), and scavenging peroxy radicals (Conn et al., 1992), thus preventing the accumulation of harmful oxygen species. Many studies indicate that carotenoids play important roles in the prevention of cancer (Bendich, 1993; Gerster, 1993; Rice-Evans et al., 1997) and enhancing immune responses (Jyonouchi et al., 1991). More than 600 naturally occurring carotenoids have been identified to date, most of which are biosynthetic intermediates that accumulate only in trace amounts, making it very difficult to extract sufficient material for purification and application. Currently, carotenoids are used as nutrient supplements, pharmaceuticals, and food colorants (Johnson and Schroeder, 1995).

Recently, carotenoids have been successfully synthesized in noncarotenogenic 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* (Misawa et al., 1990; Ruther et al., 1997).

One of the key factors for high-yield carotenoid production is a sufficient supply of precursors. All carotenoids are synthesized via a common metabolic precursor, isopentenyl diphosphate (IPP; C5). Until recently, the biosynthesis of IPP was generally assumed to proceed exclusively from acetyl-CoA via the classical mevalonate pathway (Fig. 1) (McCaskill and Croteau, 1998).

Recent studies have demonstrated that mevalonate is not the biosynthetic precursor of IPP in all living organisms (Horbach et al., 1993; Rohmer et al., 1993). The existence of an alternative, mevalonate-independent pathway for IPP formation was characterized initially in several species of eubacteria (Rohmer et al., 1993, 1996) and a green alga (Schwender et al., 1996). The first reaction in the nonmevalonate pathway is the transketolase-type condensation reac-

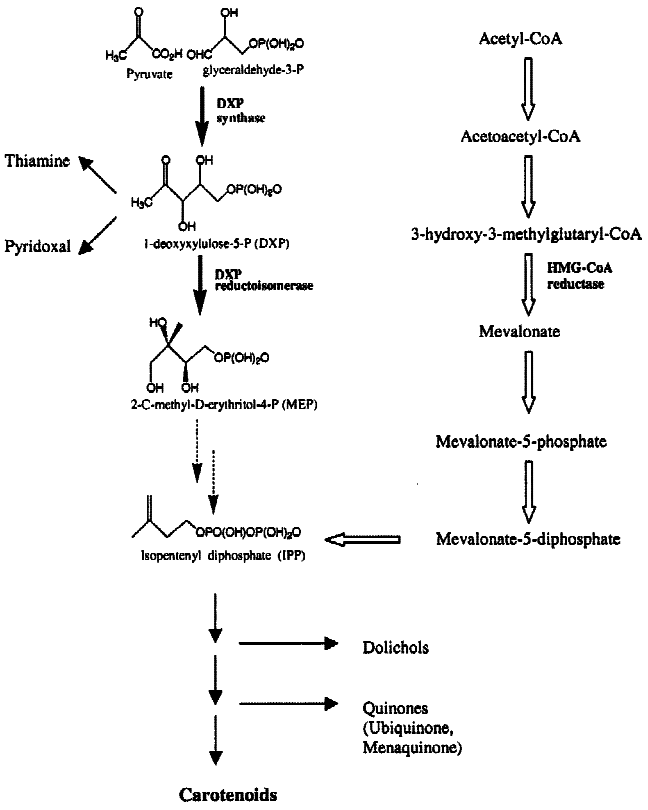

Figure 1. Isopentyl pyrophosphate (IPP) biosynthetic pathways. The mevalonate pathway from acetyl-CoA to IPP via hydroxymethylglutaryl-CoA (HMG-CoA) is shown with hollow arrows. The nonmevalonate pathway from glyceraldehyde-3-phosphate and pyruvate to IPP via 1-deoxy-D-xylulose-5-phosphate (DXP) is shown with solid arrows. Unidentified enzymatic steps in nonmevalonate pathway are indicated by broken arrows.

tion of pyruvate and D-glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP) (Fig. 1). The cloning and characterization of the DXP synthase gene (*dxs*) from *E. coli* has been described (Lois et al., 1998; Sprenger et al., 1997). Recently, a gene (*dxr*) which encodes DXP reductoisomerase responsible for the reduction of DXP to 2-C-methyl-D-erythritol-4-phosphate (MEP), the proposed second step in the nonmevalonate pathway, was cloned from *E. coli* (Takahashi et al., 1998). Although the genes and biochemistry of the remaining steps of this pathway are not known, it may be possible to increase the supply of IPP by enhancing DXP synthase and DXP reductoisomerase enzymatic activities. Indeed, a number of laboratories have reported a twofold increase in lycopene production when the *Bacillus subtilis* or *Synechocystis* sp. 6803 *dxs* genes were expressed in *E. coli* (Harker and Bramley, 1999; Matthews and Wurtzel, 2000). Here, we report the effect of amplification of the *E. coli dxs* and *dxr* genes on production of lycopene, the initial product of the carotenoid pathway from which most carotenoids are derived.

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*E44l$^-$ *thi*-1 *gyr*A96 *rel*A1) was used for gene cloning and expression studies. *E. coli* DH5α, XL1-Blue (*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17(r_k^- m_k^+) *sup*E44 *rel*A1 *lac* [F′ *pro*AB$^+$ *lac*I^{q}Z ΔM15 ::Tn10(Tet^r)]), and JM101 (*sup*E *thi* Δ(*lac-proAB*) [F′ *tra*D36 *pro*AB *lac*I^{q}Z ΔM15]) were used for lycopene production. *E. coli* DYM1 (a *dxr*$^-$ derivative of FS1576 (C600 *thr, leu, thi, lac, thy, recD1009*)), a gift from T. Kuzuyama of the University of Tokyo (Kuzuyama et al., 1999), was used to test for functional expression of *dxr*. *E. coli* was grown in 2×YT medium (Fred et al., 1995) at 37°C on a rotary shaker at 200 rpm (unless otherwise stated). Ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) (all purchased from Sigma, St. Louis, MO) were added as required. Plasmids pTrc99A (Amersham Pharmacia, Cleveland, OH), pBluescript (Stratagene, La Jolla, CA), and pBAD24 (Guzman et al., 1995) were used as vectors for *dxs* and *dxr* expression studies. Induction with isopropyl-β-D-thiogalactoside (IPTG) and arabinose was carried out as indicated in Results. Plasmid pAC-LYC04, which expresses the *Erwinia herbicola crt*E, *crt*B, and *crt*I genes necessary for lycopene biosynthesis in *E. coli*, were gifts from F.X. Cunningham (University of Maryland). This plasmid also contained the *Haematococcus pluvialis ipi* gene that encodes IPP isomerase.

PCR Cloning of *dxs* and *dxr* Genes

The *dxs* gene was cloned by polymerase chain reaction (PCR) from genomic DNA of *E. coli*. The forward primer 5′-CGGAATTCATGAGTTTTGATATTGCCAAA-3′ contained an *Eco*RI restriction site (underlined) and the reverse primer 5′-GGGGTACCTTATGCCAGCCAGGCCTTG-3′ contained a *Kpn*I site after the stop codon of the gene. PCR (25 cycles) using Turbo *Pfu* DNA polymerase (Stratagene) produced a DNA fragment of the expected size (~1.9 kb), which was confirmed to be the *dxs* gene by subsequent sequencing. To clone *dxr* from genomic DNA of *E. coli*, the forward primer containing a *Sac*I restriction site (5′-CCCGAGCTCATGAAGCAACTCACCATTC-3′) and the reverse primer containing an *Xba*I restriction site (5′-GCTCTAGATCAGCTTGCGAGACGCATC-3′) were used. After PCR (25 cycles) a DNA fragment of the expected size (~1.2 kb) was obtained and when sequenced proved to be *dxr* of *E. coli*. The PCR products from both reactions were treated with the restriction enzymes indicated and cloned into the multicloning site of the pTrc99A vector using T4 DNA ligase (Roche, Nutley, NJ). The plasmids containing *dxs* and *dxr* were designated pTdxs and pTdxr, respectively. From pTdxs, the *Nco*I-*Xba*I fragment containing *dxs* was ligated to the corresponding sites of pBAD24 to obtain pDdxs. To combine *dxr* into pDdxs, *dxr* was amplified using the forward primer 5′-GGGGTACCACAC*AGGA*AACAGACCATG-3′, which contains a *Kpn*I restriction site and overlaps the sequence of ribosomal binding site (underlined) of pTrc99A, and the reverse primer used in construction of pTdxr. The PCR product was treated with restriction enzymes *Kpn*I and *Xba*I and ligated into the corresponding sites of pDdxs to obtain

pDdxs/r. The *dxs* PCR product from genomic DNA was cloned into the *Sma*I site of the pBluescript vector to obtain pBdxs.

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 2×YT 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 2×YT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol to an OD_{600} of 0.1 and incubated at 29°C for 24 h. IPTG and arabinose induction studies were initiated at an OD_{600} of 0.8 (unless otherwise stated).

Quantification of Lycopene Production

Dry cell weight was calculated from known volumes of culture harvested by centrifugation at 13,000*g* for 3 min, 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 comparing to a lycopene standard (purchased from Sigma) (Harker and Bramley, 1999). The results are reported as the mean from three independent determinations. The standard deviations were in the range of ±10% of the means.

SDS-PAGE Analysis

E. coli DH5α harboring pBAD24, pDdxs, or pDdxs/r were grown for 15 h under the culture conditions described above. The cultures were induced with arabinose at concentrations between 0–13.3 mM initially. Cells were harvested by centrifugation, resuspended in water, and sonified at 4°C using a Branson Sonifier. After centrifugation (30 min at 38,000*g*) the supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated in 4–12% acrylamide gels (NuPAGE Gel System; Invitrogen, La Jolla, CA) and stained with Coomassie brilliant blue R250.

RESULTS

Confirmation of *dxs* and *dxr* Expression

To confirm that the constructs were producing the desired proteins, extracts of cells harboring pBAD24 (control), pDdxs, and pDdxs/r were analyzed by SDS-PAGE. With increasing inducer concentrations cells harboring pDdxs and pDdxs/r produced increasing amounts of a protein that migrated at 65 kDa, the predicted size of DXP synthase (Fig. 2). Unfortunately, no band corresponding to the size of DXP reductoisomerase (42 kDa) was visible in SDS-PAGE gels of extracts of pDdxs/r-containing cells. To confirm that the *dxr* gene on pDdxs/r was active and producing functional DXP reductoisomerase, pDdxs/r and pBAD24 were transformed into *E. coli* DYM1, a *dxr*$^-$ mutant that will only grow when supplemented with 2-C-methylerythritol (ME), a free alcohol of MEP. The *E. coli* DYM1 harboring pDdxs/r grew on Luria-Bertani (LB) medium in the absence of ME, whereas *E. coli* DYM1 harboring pBAD24 would not. Thus, even though it was not possible to visualize a band corresponding to the predicted size of DXP reductoisomerase on an SDS-PAGE gel, the plasmid-encoded gene produces enough functional enzyme to complement the loss of the chromosomal gene.

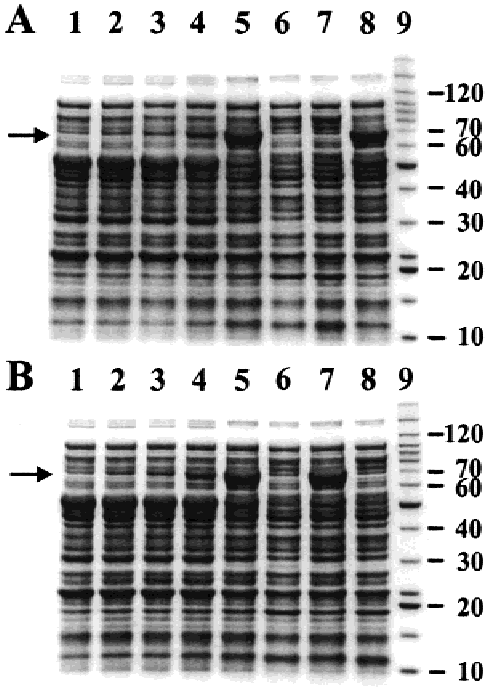

Figure 2. SDS-PAGE analysis of *E. coli* DH5α harboring the various *dxs* and *dxr* constructs. **A**: Lane 1: pDdxs, 0 mM arabinose. Lane 2: pDdxs, 0.013 mM arabinose. Lane 3: pDdxs, 0.133 mM arabinose. Lane 4: pDdxs, 1.33 mM arabinose. Lane 5: pDdxs, 13.3 mM arabinose. Lane 6: pBAD24, 13.3 mM arabinose. Lane 7: pDdxr, 13.3 mM arabinose. Lane 8: pDdxs/r,13.3 mM arabinose. Lane 9: molecular weight marker with molecular weights of selected bands marked on the right. **B**: Lane 1: pDdxs/r, 0 mM arabinose. Lane 2: pDdxs/r, 0.013 mM arabinose. Lane 3: pDdxs/r, 0.133 mM arabinose. Lane 4: pDdxs/r, 1.33 mM arabinose. Lane 5: pDdxs/r, 13.3 mM arabinose. Lane 6: pBAD24, 13.3 mM arabinose. Lane 7: pDdxs, 13.3 mM arabinose. Lane 8: pDdxr,13.3 mM arabinose. Lane 9: molecular weight marker with molecular weights of selected bands marked on the right. In addition to the plasmid listed for each lane of the gel, the cells contained pAC-LYC04. The arrow to the left of each gel indicates the expected size (65 kDa) for Dxs.

Expression of *dxs* and *dxr* Using pTrc99A

E. coli cells transformed with pAC-LYC04 are pigmented pink due to the accumulation of lycopene. *E. coli* DH5α engineered to produce lycopene were transformed with either pTdxs, pTdxr, or pTrc99A (as a control) to determine the effect of *dxs* and *dxr* overexpression on lycopene biosynthesis. In the absence of induction, cells harboring pTdxs exhibited a twofold increase in lycopene production over cells harboring pTrc99A (Fig. 3). When IPTG was added to the culture, cell growth and lycopene production decreased, even in the presence of 0.1 mM IPTG, below levels produced by cells harboring either pTdxr or pTrc99A. In contrast, regardless of the amount of inducer added to the culture, *dxr* expression had no significant effect on lycopene production or cell growth.

Expression of *dxs* Using pBluescript

Because the *trc* promoter of pTrc99A was too strong to control expression of *dxs*, we introduced the *dxs* gene into pBluescript, which carries the weaker *lac* promoter. As with uninduced cells harboring pTdxs, cells harboring pBdxs produced more lycopene than cells harboring pBluescript (Fig. 4). In contrast to the results with the *trc* promoter, there was no difference in lycopene production or cell growth at any IPTG concentration used. As 2×YT medium is very rich and there is no *lacI* gene in pBluescript or in *E. coli* DH5α, the expression of *dxs* on pBdxs was fully induced in the absence of IPTG. Lycopene production by the pBdxs-containing cells was 3.5-fold higher than by the cells harboring pBluescript (control), whereas cell growth was similar. However, growth and lycopene production by the pBdxs-containing culture were lower than by *E. coli* DH5α transformed with pTdxs but not induced (Fig. 3). These differences were probably not due to the expression of *dxs*, as cell growth and lycopene production in transformants harboring pBluescript were lower than that by transformants harboring pTrc99A (Table I). As pBluescript is a high-copy-number vector and pTrc99A is a medium-copy-number vector, the added metabolic burden from the higher copy number of pBluescript relative to pTrc99A may result in lower cell growth and lycopene production. Ruther et al. (1997) reported similar results.

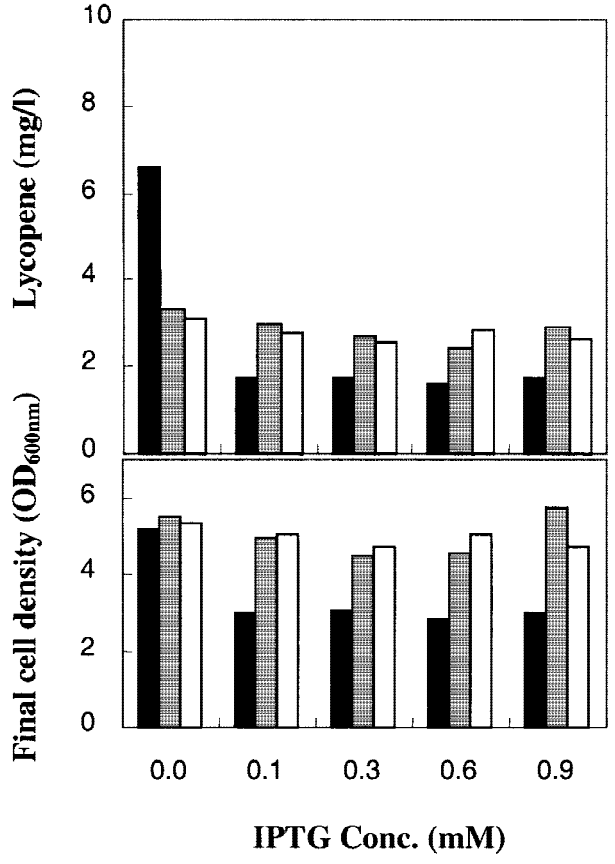

Figure 3. Effect of IPTG concentration on lycopene production and cell growth in *E. coli* DH5α harboring pTdxs (solid bar), pTdxr (gray bar), or pTrc99A (open bar).

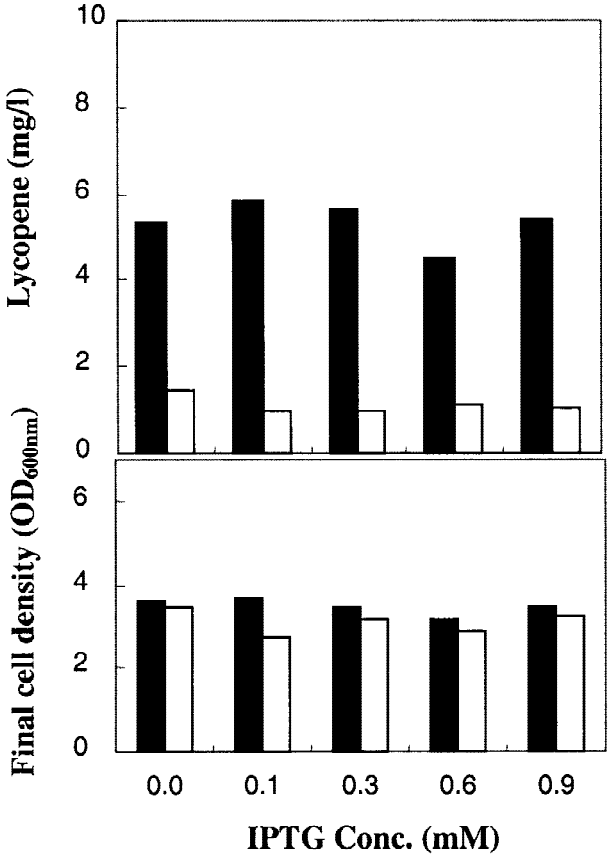

Figure 4. Effect of IPTG concentration on lycopene production and cell growth in *E. coli* DH5α harboring pBdxs (solid bar) or pBluescript (open bar).

Expression of *dxs* Using pBAD24

Because expression of *dxs* from pTrc99A and pBluescript was difficult to control, we introduced the *dxs* gene into

Table I. Various expression vectors and their effect on cell growth and lycopene production[a]

Plasmids	Lycopene (mg/l)	Cell growth (OD_{600})	Lycopene content (mg/g dry cell weight)
pAC-LYC04	3.43	5.55	1.50
pAC-LYC04/pTrc 99A[b]	3.13	5.34	1.42
pAC-LYC04/pTdxs[b]	6.62	5.17	3.12
pAC-LYC04/pBluescript[b]	1.49	3.43	1.06
pAC-LYC04/pBdx[b]	5.32	3.59	3.61
pAC-LYC04/pBAD 24[c]	2.99	5.10	1.43
pAC-LYC04/pDdxs[c]	12.31	5.26	5.69

[a]Measurements were made 24 h of culture.
[b]IPTG was not added.
[c]Arabinose at a concentration of 13.3 mM was added at an OD_{600} of 0.8.

pBAD24, a medium-copy-number plasmid containing the arabinose-inducible *araBAD* promoter (P_{BAD}). This system uses the inexpensive sugar L-arabinose as an inducer. In contrast to the results obtained using the IPTG-inducible systems, the amount of lycopene produced by the cells increased with the arabinose concentration (Fig. 5). Furthermore, cell growth was unaffected by the amount of arabinose added to the culture. When induced with 13.3 mM arabinose, *E. coli* transformed with pDdxs produced 12.3 mg lycopene/L in 24 h. This lycopene production is four times higher than the control and significantly higher than any other genetic construct. Lycopene production in the absence of inducer was slightly higher in strains harboring pDdxs than those harboring pBAD24, most likely due to leaky expression from P_{BAD}.

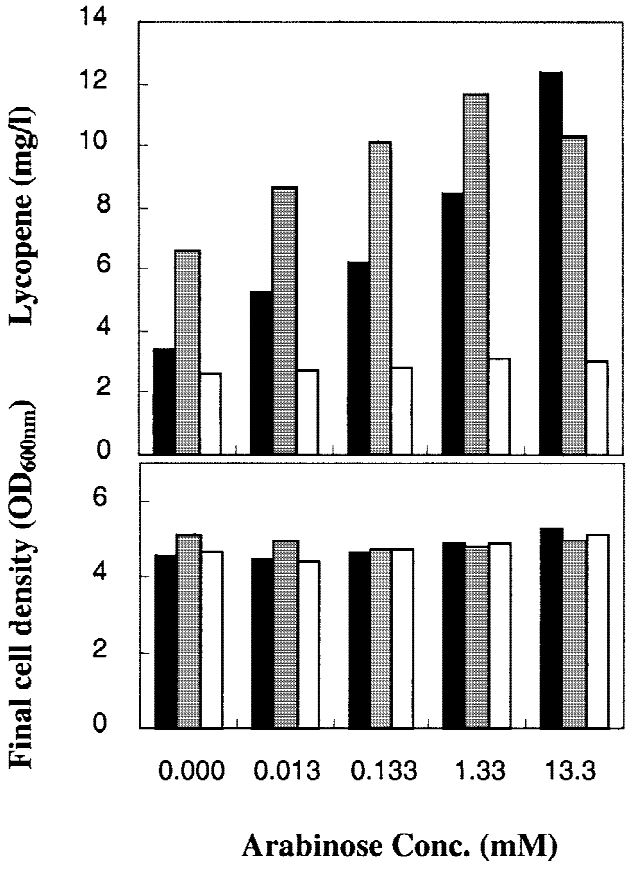

Figure 5. Effect of arabinose concentration on lycopene production and cell growth in *E. coli* DH5α harboring pDdxs (solid bar), pDdxs/r (gray bar), or pBAD24 (open bar).

Lycopene Production in *E. coli* Transformed with pDdxs/r

Since the amplification of *dxs* expression increased production of lycopene, we postulated that further enhancement of lycopene production could be limited by other reactions in the pathway. To determine if amplification of *dxr* expression could improve lycopene production in strains harboring pDdxs, *dxr* was combined with *dxs* of pDdxs to obtain pDdxs/r. At arabinose concentrations between 0–1.33 mM, cells expressing both *dxs* and *dxr* from P_{BAD} produced 1.4–2.0 times more lycopene than cells expressing *dxs* only. However, at higher arabinose concentrations lycopene production in cells expressing both *dxs* and *dxr* was lower than in cells expressing *dxs* only (Fig. 5). There was no significant difference in the growth of cells harboring pDdxs or pDdxs/r at all arabinose concentrations.

Time Course of Lycopene Production in *E. coli* Transformants of pDdxs

It has been reported that carotenoid production ceases as cells enter stationary phase (Kajiwara et al., 1997; Sandmann et al., 1999). In contrast, such secondary metabolites as polyhydroxyalkanoates are known to accumulate well into stationary phase. To determine if it is possible to improve carotenoid production in the stationary phase, cell growth and lycopene production were monitored in *E. coli* DH5α harboring pDdxs and pAC-LYC04. The cells were grown in 50 ml of medium at 29°C. At an OD_{600} of 0.4, arabinose was added to a final concentration of 13.3 mM. During the first 36 h, the *E. coli* cultures expressing *dxs* accumulated lycopene steadily, even though the cells had reached stationary phase after 20 h (Fig. 6). The final lycopene content of the recombinant *dxs* strains was approximately 6-fold that of the control. The highest yield was 22 mg lycopene/L. The results show that increased expression of *dxs* leads to increased lycopene in recombinant *E. coli*, and that DXP synthesis may be the rate-limiting reaction in the mevalonate-independent pathway for IPP biosynthesis.

Comparison of Lycopene Production in Different *E. coli* Strains Transformed with pDdxs or pBAD24

E. coli strains DH5α, XL1-Blue, and JM101 harboring pAC-LYC04 were transformed with either pBAD24 or pDdxs to compare lycopene production in these strains. In cells transformed with pBAD24 (control), DH5α produced more

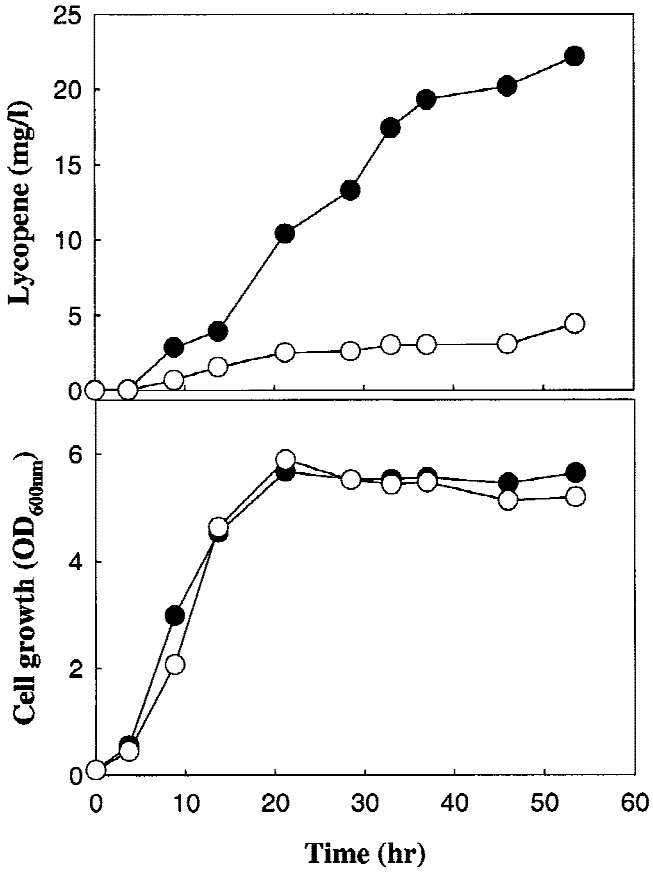

Figure 6. Lycopene accumulation and cell growth in *E. coli* harboring pDdxs (closed circles) or pBAD24 (open circles). Induction was carried out with 13.3 mM arabinose at an OD_{600} of 0.4.

lycopene than XL1-Blue, which produced more lycopene than JM101 (Fig. 7). A similar trend was observed in cells harboring pDdxs; DH5α and XL1-Blue produced significantly more lycopene than JM101. No significant differences in cell growth were observed for all these strains.

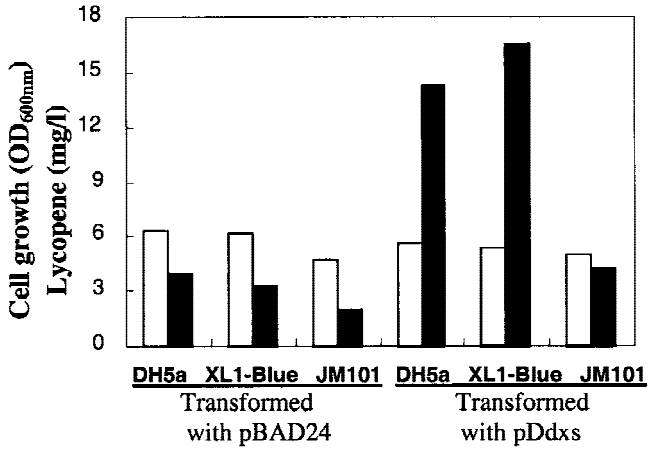

Figure 7. Comparison of lycopene production (solid bars) and growth (open bars) in *E. coli* strains DH5a, XL1-Blue, and JM101 transformed with pBAD24 or pDdxs. Cultivation was carried out for 48 h and 13.3 mM arabinose was added initially for induction.

DISCUSSION

Recently, carotenoids have been successfully synthesized in noncarotenogenic 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 engineering *E. coli* to produce carotenoids with high yield (Farmer and Liao, 2000; Misawa et al., 1990; Ruther et al., 1997). However, the progress has been largely restricted to reactions between IPP and the carotenoids because the enzymes and genes of the IPP synthesis pathway are not completely known. For high-yield carotenoid production, it is also necessary to optimize production of IPP. The first reaction in the mevalonate-independent IPP synthesis pathway is the condensation of (hydroxy)thiamin, derived from the decarboxylation of pyruvate, with glyceraldehyde-3-phosphate to produce 1-deoxy-D-xylulose-5-phosphate (DXP) and is catalyzed by DXP synthase. In this work, we have shown that DXP synthase controls the flux through the mevalonate-independent pathway and that optimized expression of the gene encoding DXP synthase (*dxs*) results in increased lycopene biosynthesis.

We also noted that the type of expression vector and the promoter strength are very important factors in balancing *dxs* expression with overall metabolism (Table I). A control transformant with pAC-LYC04 alone (no second expression vector) was compared to transformants with pAC-LYC04 and the second expression vector (with no *dxs* gene) to investigate the effect of plasmid maintenance on cell growth and lycopene production. A decrease in cell growth and lycopene production was observed when the second expression vector was pBluescript, a high-copy-number plasmid, whereas the medium-copy-number plasmids pTrc99A and pBAD24 did not affect growth. We suspect that the decreased cell growth and lycopene production were due to the burden of maintaining a high-copy-number plasmid.

In addition to the burden from the expression vector, the cells suffered significantly from overexpression of *dxs*. When pBAD24 was used as an expression vector for *dxs*, lycopene production at the maximum arabinose concentration was approximately twofold higher than when pTrc99A was used as the expression vector in the absence of an inducer, IPTG. When IPTG was added to cells harboring pTdxs, cell growth was severely inhibited. Most likely, overexpression of DXP synthase caused a severe drain on glycolytic intermediates (glyceraldehyde-3-phosphate and pyruvate) necessary for balanced growth. The synthetic *trc* promoter of pTrc99A, which consists of the -35 region of the *trp* promoter and -10 region of the *lac* promoter, is quite strong and routinely allows the expression of recombinant proteins to about 15–30% of the total cell protein. Even under conditions of no induction, the specific growth rate of the pTdxs transformant was 0.317 h^{-1} at 29°C, significantly lower than that of pTrc99A (0.410 h^{-1}). In contrast, there was no significant difference in the specific growth rates of transformants harboring pBAD24 (0.402 h^{-1}) or pDdxs (0.419 h^{-1}).

We confirmed expression of *dxs* by SDS-PAGE of extracts of strains containing pDdxs and pDdxs/r. The expression of *dxs* increased with arabinose concentration. Unfortunately, we were unable to observe expression of *dxr* from pDdxs/r due to numerous bands in the region of DXP reductoisomerase. So we confirmed expression of *dxr* by complementing the chromosomal *dxr* mutation in *E. coli* DYM1 with pDdxs/r. Lycopene production from a pDdxs/r-containing culture was significantly higher than that from a pDdxs-containing culture at concentrations of arabinose below 1.33 mM, but the opposite was observed at 13.3 mM arabinose. We suspect that the metabolic burden due to high-level production of DXP synthase and reductoisomerase may be responsible for the reduced lycopene production at high arabinose concentrations.

The time-course lycopene production studies using the pDdxs-containing cells showed that lycopene can be produced in the stationary phase of cell growth, in contrast to previous reports that carotenoid production ceases in the stationary phase (Kajiwara et al., 1997; Sandmann et al., 1999). Harker and Bramley (1999) also reported that amplification of *dxs* of *Bacillus subtilis* and *Synechocystis* sp. 6803 allowed lycopene production in the stationary phase. As the growth of *E. coli* requires DXP for synthesis of thiamine (vitamin B_1), pyridoxal (vitamin B_6), dolichols (sugar carrier lipids), and respiratory quinones (ubiquinone and menaquinone) (Sherman et al., 1989; Sprenger et al., 1997), one might expect the cell to downregulate the flux through the IPP synthesis pathway in the stationary phase. Expression of *dxs* from an inducible promoter may relieve this bottleneck, allowing high-level carotenoid production in the stationary phase. This work shows that metabolic channeling of glycolytic intermediates to IPP synthesis by optimized expression of *dxs* and *dxr* is important for high-level carotenoid production.

Finally, we observed significant strain dependence in lycopene production. Both XL1-Blue and DH5α were significantly better producers of lycopene than JM101, a strain that has been commonly used for carotenoid overproduction. Interestingly, XL1-Blue has also been found to produce the highest levels of polyhydroxyalkanoates (Lee et al., 1994).

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