Microbial production of carotenoids for fortification of foods

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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 non-mevalonate pathway. 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 non-mevalonate 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 (DH5(, 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 (PBAD) on a medium-copy plasmid, lycopene production was 2-fold higher than when *dxs* was expressed from the IPTG-inducible *trc* and *lac* promoters (Ptrc and Plac, 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 PBAD 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.

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 ADDIN ENRfu (Krinsky, 1994), and scavenging peroxy radicals ADDIN ENRfu (Conn *et al.*, 1992), thus preventing the accumulation of harmful oxygen species. Recently, carotenoids have been successfully synthesized in non-carotenogenic bacteria and yeast using recombinant DNA techniques ADDIN ENRfu (Misawa *et al.*, 1991; Sandmann *et al.*, 1990; Yamano *et al.*, 1994).

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 ADDIN ENRfu (McCaskill and Croteau, 1998). Recent studies have demonstrated that mevalonate is not the biosynthetic precursor of IPP in all living organisms ADDIN ENRfu (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 ADDIN ENRfu (Rohmer *et al.*, 1993; Rohmer *et al.*, 1996) and a green alga ADDIN ENRfu (Schwender et al., 1996). The first reaction in the non-mevalonate pathway is the transketolase-type condensation reaction of pyruvate and D-glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP). 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* ADDIN ENRfu (Takahashi *et al.*, 1998). It may be possible to increase the supply of IPP by enhancing DXP synthase and DXP reductoisomerase enzymatic activities.

MATERIALS AND METHODS

Bacterial strains, and plasmids

E. coli DH5(, XL1-Blue and JM101 were used for lycopene production. Plasmids pTrc99A, pBluescript, and pBAD24 were used as vectors for *dxs* and *dxr* expression studies. Plasmid pAC-LYC04 expresses the *Erwinia herbicola crtE, crtB* and *crtI* genes necessary for lycopene biosynthesis in *E. coli,* This plasmid also contained the *Haematococcus pluvialis ipi* gene that encodes IPP isomerase.

PCR cloning of *dxs* and *dxr* genes

The *dxs* and *dxr* genes were cloned by the PCR from genomic DNA of *E. coli*. Plasmid pTrc99A containing *dxs* and *dxr* were designated pTdxs and pTdxr, respectively. Plasmids pDdxs and pDdxs/r were pBAD24 containing *dxs*, and both *dxs* and *dxr*.

Growth and induction experiments

Bacterial growth was determined by measuring the optical density at a wavelength of 600 nm (OD600). A seed culture was made by inoculating cells into 2(YT medium containing 100 (g/ml ampicillin and 50 (g/ml chloramphenicol and growing the cells overnight at 37°C. An aliquot of the seed culture was inoculated into 5ml of 2(YT medium containing 100 (g/ml ampicillin and 50 (g/ml chloramphenicol to an OD600 of 0.1, and incubated at 29°C for 24 h. IPTG and arabinose induction studies were initiated at an OD600 of 0.8 (unless otherwise stated).

RESULTS

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* DH5(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 two-fold increase in lycopene production over cells harboring pTrc99A (Fig. 1). When IPTG was

added to the culture, cell growth and lycopene production decreased, even in 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. 2). 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 nor in *E. coli* DH5(, 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* DH5(transformed with pTdxs but not induced (Fig. 1). 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. 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.* ADDIN ENRfu (1997) reported similar results.

Lycopene production in *E. coli* transformed with pDdxs or pDdxs/r Because expression of *dxs* from pTrc99A and pBluescript was difficult to control, we introduced the *dxs* gene into pBAD24, a medium-copy-number plasmid containing the arabinose-inducible *araBAD* promoter (PBAD). 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. 3). 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 hours. 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 PBAD.

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, the *dxr* gene was combined with the *dxs* of pDdxs to obtain pDdxs/r. At arabinose concentrations between 0 and 1.33 mM, cells expressing both *dxs* and *dxr* from PBAD 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. 3). There was no significant difference in the growth of cells harboring pDdxs or pDdxs/r at all arabinose concentrations.

Comparison of lycopene production in different *E. coli* strains transformed with pDdxs or pBAD24E. coli strains DH5(, XL1-Blue, and JM101 harboring pAC-LYC04 were transformed with either pBAD24 or pDdxs to compare lycopene production in these strains. Lycopene production was highest in XL1-Blue whereas no significant differences in cell growth were observed for all these strains (Fig.4).

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