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ORIGINAL PAPER

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Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase

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Abstract The recently discovered non-mevalonate pathway to isoprenoids, which uses glycolytic intermediates, has been modulated by overexpression of *Escherichia coli* D-1-deoxyxylulose 5-phosphate synthase (DXS) to increase deoxyxylulose 5-phosphate and, consequently, increase the isoprenoid precursor pool in *E. coli*. Carotenoids are a large class of biologically important compounds synthesized from isoprenoid precursors and of interest for metabolic engineering. However, carotenoids are not ordinarily present in *E. coli*. Co-overexpression of *E. coli dxs* with *Erwinia uredovora* gene clusters encoding carotenoid biosynthetic enzymes led to an increased accumulation of the carotenoids lycopene or zeaxanthin over controls not expressing DXS. Thus, rate-controlling enzymes encoded by the carotenogenic gene clusters are responsive to an increase in isoprenoid precursor pools. Levels of accumulated carotenoids were increased up to 10.8 times the levels of controls not overexpressing DXS. Lycopene accumulated to a level as high as 1333 μg/g dw and zeaxanthin accumulated to a level as high as 592 μg/g dw, when pigments were extracted from colonies. Zeaxanthin-producing colonies grew about twice as fast as lycopene-producing colonies throughout a time course of 11 days. Metabolic engineering of carbon flow from simple glucose metabolites to representatives of the largest class of natural products was demonstrated in this model system.

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Introduction

Isoprenoids are a diverse group of natural products found in all organisms. They are derived from the C_5 skeleton of isopentenyl pyrophosphate (IPP). IPP was thought to be strictly derived from the mevalonate pathway, and the enzyme HMGCoA reductase was considered to be a rate-limiting enzyme for this biosynthetic route (Dimster-Denk et al. 1994; Chappell 1995). Recently, a novel non-mevalonate biosynthetic pathway was discovered that operates specifically in plastids of plants (reviewed in Lichtenthaler 1999) as well as in bacteria and in cyanobacteria; in plants, the mevalonate pathway operates in the cytoplasm in parallel with the plastid-localized non-mevalonate IPP pathway (Rohmer et al. 1993; Schwender et al. 1996). In the non-mevalonate route, IPP is derived from deoxyxylulose 5-phosphate (DXP), which in *Escherichia coli* has also been found to be a common precursor in the biosynthesis of vitamins B_1 (thiamin) and B_6 (pyridoxal) (Sprenger et al. 1997). The enzyme D-1-deoxyxylulose 5-phosphate synthase (DXS) is responsible for catalyzing the synthesis of DXP from pyruvate and GAP (glyceraldehyde 3-phosphate) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998). In plants and bacteria, competition for DXP might affect end-product accumulation for different isoprenoid-derived biosynthetic pathways. The enzyme responsible for DXP synthesis might represent a novel rate-controlling enzyme whose expression could be modified to increase substrate availability for pathways under consideration for metabolic engineering.

Carotenoids are one example of isoprenoids for which interest in metabolic engineering relates to their usefulness as coloring agents and as precursors to vitamin A and to retinoids, compounds essential to growth and development (Misawa et al. 1991, 1993; Yamano et al. 1994; Burkhardt et al. 1997; Ruther et al. 1997; Wang et al. 1999). Carotenoids are synthesized in certain bacteria, fungi, and in plastids of plants (as reviewed in Armstrong and Hearst 1996; Cunningham and Gantt

1998). Gene clusters encoding the carotenoid biosynthetic enzymes have been isolated from epiphytic bacteria such as *Erwinia uredovora*, and their introduction into *Escherichia coli* has resulted in carotenoid accumulation (Misawa et al. 1990). In addition to the prospect of bioengineering the accumulation of useful and unusual carotenoids, the expression of carotenogenic genes in *E. coli* represents a unique opportunity for the cloning of new genes by heterologous complementation (Sun et al. 1996), for the functional testing of gene products (Li et al. 1996) and for the examination of flow through the pathway (Kajiwara et al. 1997; Ruther et al. 1997).

The C_{20} isoprenoid geranylgeranyl pyrophosphate (GGPP), produced by GGPP synthase (GGPPS), is the first precursor to the carotenoids, and to a variety of other isoprenoid-derived products, including gibberellins, the phytol chain of chlorophyll, prenylquinones, tocopherols, prenylated proteins, and many secondary metabolites such as taxol and casbene (Chappell 1995). GGPP is formed from four units of IPP, one of which is an isomer (DMAPP). IPP isomerase converts IPP to DMAPP in *E. coli*; DMAPP condensed with consecutive molecules of IPP forms farnesyl pyrophosphate (FPP), geranyl pyrophosphate (GPP) and then GGPP.

In *E. coli*, the presence of GGPP depends on the activity of endogenous IPP isomerase and FPP synthase, and exogenous GGPPS. Recent efforts have focused on increasing the carotenoid accumulation by overexpression of these three enzymes (Wang et al. 1999), as well as by overexpression of enzymes within the carotenoid pathway (Kajiwara et al. 1997; Ruther et al. 1997). Hypothetically, the accumulation of carotenoids also depends on the concentration of IPP as derived from the non-mevalonate pathway. Since the DXS enzyme functions at the junction of three pathways, manipulation of DXS enzyme levels might be as important in enhancing end-product accumulation as in increasing levels of downstream enzymes.

To test the flux relationships between the DXS-mediated isoprenoid pathway and a carotenoid biosynthetic sink in *E. coli*, we examined the effect of overexpressing *E. coli* DXS in combination with carotenoid enzymes encoded by *Erwinia uredovora*. For this purpose, we used an *Escherichia coli dxs* gene construct that had previously been shown to confer a >100-fold increase in DXS activity in *E. coli* cell-free extracts (Sprenger et al. 1997; Lois et al. 1998). The overexpression of *E. coli* DXS, in the presence of the *Erwinia* carotenoid gene cluster, resulted in strikingly rich-colored colonies. We present the time course and extent of carotenoid accumulation in these strains.

Materials and methods

Growth, strains and plasmids

Lycopene (ψ,ψ-carotene) and zeaxanthin (β,β-carotene-3,3′-diol) accumulation in *E. coli* TOP10 F′ (Invitrogen, Carlsbad, Calif.) was accomplished by expression of the *Erwinia uredovora* carotenoid gene cluster present in plasmids pACCRT-*EIB* or pACCAR-25Δ*crtX*, alternatively named pCAR25*delX* (Misawa et al. 1990), as previously described (Wurtzel et al. 1997). Plasmid pACCAR25Δ*crtB* (Misawa et al. 1990), which has a frameshift mutation in the gene encoding phytoene synthase, produces no carotenoid and was used as a negative control. These carotenoid-accumulating strains were transformed using the $CaCl_2$ protocol (Sambrook et al. 1989) with an additional plasmid, pTAC-ORF2 (*dxs*) expressing *E. coli* D-1-deoxyxylulose-5-phosphate synthase (Lois et al. 1998). Bacteria were cultivated in Luria-Bertani (LB) medium in liquid culture or agar plates (Sambrook et al. 1989) supplemented with 50 μg/ml ampicillin to select for pUC-derived plasmids carrying *dxs* and 70 μg/ml chloramphenicol to select for pACY184-derived plasmids carrying the *Erwinia* carotenogenic gene clusters. In some cases DXS expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). Liquid cultures were inoculated from a glycerol stock (Sambrook et al. 1989) prepared from one primary transformant colony. A 10-μl aliquot of the glycerol stock was added to 10 ml LB with antibiotic and grown to stationary phase for 12 h at 37 °C with shaking at 240 rpm. Aliquots (50 μl) of these starter cultures were then inoculated into 50 ml LB with antibiotics in 125-ml Erlenmeyer flasks, incubated at 37 °C with shaking for 30 h, then held at room temperature for an additional 30 h. For growth of colonies on solid medium, cells from a glycerol stock of approx. 500 primary transformant colonies scraped from a plate and mixed together were serially diluted and plated on to LB plates with antibiotics at a density of approx. 150 colonies per 15 × 100-mm Petri dish. These plates were incubated at 37 °C for 8 h and then at room temperature for 2–11 days. All cultures were grown in the dark and shielded from light during all manipulations and subsequent carotenoid extractions.

Carotenoid analysis

Colonies lifted on to dry 5.4-cm^2 nitrocellulose membranes or single colonies lifted onto 7-mm-diameter discs were placed colony-side up in a pool of 50 mM glucose/0.25 mM Tris (pH 8.0) 10 mM EDTA (GTE) on a glass plate or microtiter plate lid and scanned wet on a Hewlett Packard ScanJet 6100C at the following settings: contrast, 200; brightness, 200; scaling, 300%; resolution, 100 dpi; 256 greys. Colony boundaries were selected manually and pixel values determined with ImageQuaNT V4.1b (Molecular Dynamics, Sunnyvale, Calif.). After scanning, cells were released from the filters by vortexing (in the case of single colony lifts) or massaging with GTE (in the case of entire plate lifts) and stored frozen in glycerol until extraction. Alternatively, cells from liquid cultures were collected by centrifugation and weighed wet.

Carotenoids were exhaustively extracted essentially according to the protocol of De Leehnheer and Nelis (1992), except that starting cells were not freeze-dried, but rather suspended in a small volume of GTE. All samplings and extractions were performed at least in duplicate and all samples were extracted simultaneously in parallel. Carotenoids were quantified spectrophotometrically in hexanes at 479 nm and the concentrations of extracts were determined by interpolation of a curve ($r^2 = 0.991$) of lycopene standards (Sigma, St. Louis, Mo.). Intermediates in the carotenoid biosynthetic pathway do not accumulate to significant levels; hence, the "quantified" carotenoid represents the end-product of the pathway engineered on the basis of the carotenoid gene cluster present (Ruther et al. 1997, and data not shown).

Since extracted carotenoids were highly correlated ($r = 0.998$) with the area of colonies pooled for extraction, relative comparisons were based on the concentration of the extract normalized to colony area. Wet and dry colony mass and area relationships were determined by lifting colonies to preweighed membranes, scanning, weighing, then reweighing air-dried colonies until no further weight change occurred. The ratio of colony fresh weight to colony dry weight was 0.24 ± 0.04 ($n = 9$ filters, ±SD) and the ratio of colony area in pixels to dry weight (g) was $4{,}130{,}000 \pm 430{,}000$ ($n = 9$ filters, ±SD).

Results

We tested the hypothesis that the IPP pool available for carotenoid accumulation in *E. coli* is limited by expression of *dxs*, the gene encoding DXS. The *dxs* gene was recently cloned from *E. coli* and expression of its gene product characterized (Sprenger et al. 1997). Lois et al. (1998) have demonstrated high levels of DXS activity in *E. coli* cell-free extracts transformed with the plasmid pTAC-ORF2, which contains *dxs* under the control of a strong promoter. We introduced this plasmid into *E. coli* harboring *Erwinia uredovora* genes needed to confer carotenoid biosynthesis. The *E. coli* strain TOP10 F′ was chosen for transformation on the basis of an earlier study that suggested that carotenoid accumulation was clonally stable and fairly strong in this strain compared to others (Wurtzel et al. 1997). As shown in Fig. 1, colonies accumulating the red lycopene showed a dramatic increase in pigmentation when the overexpressed DXS was present. This increase in accumulation, conferred by the overexpressed DXS, was apparent for both lycopene- and zeaxanthin-accumulating strains and was further quantified as shown in Table 1.

On the basis of values from exhaustively extracted carotenoids from Top10 F′ strains, we have demonstrated a 4.4–10.8-fold increase in lycopene accumulation in colonies grown on agar plates containing pACCRT-*EIB* and *E. coli dxs* (*EIB*+*dxs*) relative to the same strain containing just pACCRT-*EIB* (*EIB*). Similarly, zeaxanthin-accumulating strains exhibited a 2.1–3.9-fold increase when cells expressed both the carotenoid gene cluster of pACCAR25Δ*crtX* and *dxs* (Δ*crtX* + *dxs*) relative to the same strain containing just pACCAR25Δ*crtX* (Δ *crtX*).

Carotenoid accumulation is the product of cell growth and pigment biosynthesis, each of which might vary independently over time. Therefore, we carried out a time-course experiment for lycopene accumulation over 11 days colony culture on plates, as shown in Fig. 2. Pigmentation reached its maximum after 11 days incubation at room temperature in the dark. Lycopene accumulation per gram dry cell weight was consistently two times higher than that of zeaxanthin accumulation during the entire growth period, regardless of increases due to overexpression of DXS (zeaxanthin data are not shown). Over the incubation time of 11 days, lycopene levels in *EIB* + *dxs* increased up to levels 15-fold higher than those for 1 day of growth. In strains expressing *dxs*, increases in pigmentation continued only up to 4 days for zeaxanthin but for up to 11 days for lycopene. In all cases, zeaxanthin-producing colonies grew faster and were nearly twice as large as lycopene-producing colonies; therefore, levels of zeaxanthin production were similar to lycopene production on a per-colony or per-plate basis (data not shown).

Fig. 1 Overexpression of D-1-deoxyxylulose 5-phosphate synthase (DXS) causes an increase in lycopene accumulation in *Escherichia coli* colonies. *E. coli* transformed with the *Erwinia uredovora* gene cluster conferring lycopene biosynthesis without (*left*) or with (*right*) overexpression of *E. coli* DXS. The middle sample shows unpigmented cells

Table 1 Average carotenoid accumulation among strains carrying plasmids with or without the *Escherichia coli dxs* gene. Enzymes encoded by the plasmids are as indicated. Carotenoid content data were taken from 4 days growth on solid medium for zeaxanthin and 11 days growth for lycopene. *GGPPS* Geranylgeranyl pyrophosphate synthase, *PSY* Phytoene synthase, *CRTI* Phytoene Desaturase, *DXS* D-1-deoxyxylulose 5-phosphate synthase, *LCY* Lycopene β-cyclase, *HYD* β-carotene hydroxylase, *g fw* grams fresh weight, *g dw* grams dry weight, for which carotenoid content was normalized to colony surface area

Plasmid	Enzyme(s)	Pigment	Carotenoid (μg/g fw)	Carotenoid (μg/g dw)
pACCRT-*EIB*	GGPPS, PSY, CRTI	Lycopene	38 ± 8	160 ± 32
pACCRT-*EIB* pTAC-ORF2	GGPPS, PSY, CRTI DXS	Lycopene	266 ± 64	1106 ± 270
pACCAR25Δ*crtX*	GGPPS, PSY, CRTI, LCY, HYD	Zeaxanthin	44 ± 8	186 ± 36
pACCAR25Δ*crtX* pTAC-ORF2	GGPPS, PSY, CRTI, LCY, HYD DXS	Zeaxanthin	126 ± 16	526 ± 66
pACCAR25Δ*crtB*	GGPPS, CRTI, LCY, HYD	None	None	None

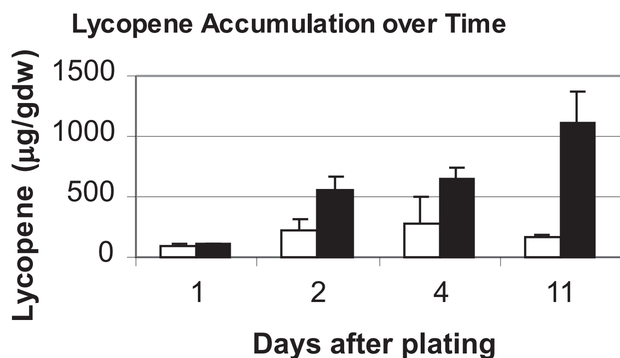

Fig. 2 Time course of carotenoid accumulation in strains containing pACCRT-*EIB* (□) or pACCRT-*EIB* + pTAC-ORF2(DXS) (■). Values are averages of 2–4 determinations and error bars are ±SD

The differences observed when strains were grown on solid medium were further explored by means of growth in liquid culture. Extracts from liquid cultures showed a range of pigment expression similar to that measured in colonies, but with much greater variation (data not shown). We saw a large variation in carefully prepared replicate cultures, as well as large variation among cultures prepared from different primary transformants. IPTG induction of DXS expression at various time points had a negative effect on carotenoid accumulation in liquid cultures as well as in colonies on plates (data not shown).

Discussion

Accumulation of both lycopene and zeaxanthin in *E. coli* was greatly enhanced by overexpression of DXS, suggesting that IPP pools may be limited by DXS expression and activity. Other studies have shown effects on the rate of carotenoid synthesis by causes downstream of the IPP pool. For example, Wang et al. (1999) have shown that co-overexpression of heterologous GGPPS (CrtE) and homologous IPP isomerase, enzymes upstream of the carotenoid biosynthetic pathway but downstream from DXS, increased astaxanthin accumulation in *E. coli* up to a level of 234 μg/g dw. Co-overexpression of genes encoding a novel bifunctional GGPPS (*gps*) and IPP isomerase (*idi*) led to a drastic increase in astaxanthin levels up to 1419 μg/g dw, demonstrating that GGPPS and IPP isomerase are rate-controlling. By modulating IPP precursor levels in the presence of native levels of IPP isomerase and FPP synthase, we obtained a maximum value for accumulation of lycopene that was similar (1333 μg/g dw). Together, these studies clearly indicate that flux into the heterologous carotenoid biosynthetic pathway is influenced by rate-controlling enzyme levels which are also responsive to precursor concentrations.

Overexpression of enzymes within the carotenoid biosynthetic pathway has not led to large increases of carotenoid accumulation. For example, co-transformation of carotenogenic strains harboring pACYC-derived plasmids with an additional pUC-derived plasmid overexpressing one of enzymes of the biosynthetic gene cluster (such as phytoene synthase, phytoene desaturase or lycopene cyclase) had a negative effect on carotenoid accumulation. Only carefully controlled (by growth-phase-specific IPTG induction) overexpression of a terminal enzyme, β-carotene hydroxylase, created a metabolic sink and a 1.3-fold increase in zeaxanthin accumulation to 276 μg/g dw (Ruther et al. 1997).

In contrast, we report a 2.2- to 10.8-fold increase, relative to our average values of extracted carotenoid, when DXS is overexpressed. While our average values for accumulation in Δ*crtX* and *EIB* are similar to values for other carotenoid gene cluster-containing *E. coli* strains in the literature (Ruther et al. 1997; Wurtzel et al. 1997; Wang et al. 1999), we measured increases up to 592 μg/g dw for zeaxanthin and up to 1333 μg/g dw for lycopene with overexpression of DXS. pTAC-TAC expression vectors are IPTG-inducible, but lack tight control of the basal expression level from the strong P_{tac} promoter, leading to DXS expression in the absence of induction (Rohmer et al. 1993). Preliminary experiments with induction of DXS expression by inclusion of IPTG in solid medium or addition of IPTG to liquid cultures at inoculation, at mid-log phase and at stationary phases of growth had a negative effect on lycopene- and zeaxanthin accumulation.

Inclusion of glucose in the growth medium increases astaxanthin pigmentation in *E. coli* overexpressing GGPPS and IPP isomerase in combination with an astaxanthin biosynthetic gene cluster (Wang et al. 1999). Co-overexpression of IPP isomerase in our DXS-overexpressing lines in the presence of glucose may further stimulate carotenoid accumulation in our strains.

Success with manipulation of these coupled pathways in bacteria may be applicable to plants, especially as the carotenoid biosynthetic pathway and the non-mevalonate route to IPP via DXS are plastid-localized. Efforts are underway to extend metabolic engineering of the isoprenoid precursor pool in the presence of a carotenoid biosynthetic sink to the higher plants.

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