Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis

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Abstract Isopentenyl diphosphate (IPP) acts as the common, five-carbon building block in the biosynthesis of all isoprenoids. The first reaction of IPP biosynthesis in *Escherichia coli* is the formation of 1-deoxy-D-xylulose-5-phosphate, catalysed by 1-deoxy-D-xylulose-5-phosphate synthase (*DXPS*). *E. coli* engineered to produce lycopene, was transformed with *dxps* genes cloned from *Bacillus subtilis* and *Synechocystis* sp. 6803. Increases in lycopene levels were observed in strains expressing exogenous *DXPS* compared to controls. The recombinant strains also exhibited elevated levels of ubiquinone-8. These increases corresponded with enhanced DXP synthase activity in the recombinant *E. coli* strains.

Key words: Isoprenoid; Isopentenyl diphosphate; Carotenoid; Bioengineering; Ubiquinone; Biosynthesis

1. Introduction

Isoprenoids constitute the largest class of natural products occurring in nature, with over 29 000 individual compounds identified to date [1]. Chemically, they are extremely diverse in their structure and complexity. The fundamental biological functions performed by isoprenoids ensure they are essential for the normal growth and developmental processes in all living organisms. These include functioning as eukaryotic membrane stabilisers (sterols), plant hormones (gibberellins and abscisic acid), providing pigments for photosynthesis (carotenoids and phytol side chain of chlorophyll), and as carriers for electron transport (menaquinone, plastoquinone and ubiquinone).

All isoprenoids are synthesised via a common metabolic precursor, isopentenyl diphosphate (IPP; C_5). Until recently, the biosynthesis of IPP was generally assumed to proceed exclusively from acetyl-CoA via the classical mevalonate pathway (Fig. 1) [2]. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) catalyses the conversion of hydroxymethylglutaryl-CoA to mevalonate, a key reaction of the mevalonate-dependent IPP biosynthetic pathway. Recent studies have demonstrated that mevalonate is not the biosynthetic precursor of IPP in all living organisms [3,4]. The existence of an alternative, mevalonate-independent pathway for IPP formation was characterised initially in several species of eubacteria [4,5] and a green alga [6]. The pathway was subsequently shown to be operational in the plastids of higher plants [7–10]. 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) (Fig. 1). The cloning and characterisation of the DXP synthase (*dxps*) gene has been described for a number of organisms including *Escherichia coli* [11,12] and higher plants [13–15]. The *CLA1* gene product from *Arabidopsis thaliana* associated with chloroplast development [16] has been shown to exhibit *DXPS* activity [11]. Recently, a gene responsible for the reduction of DXP to 2-*C*-methyl-D-erythritol-4-phosphate, the proposed next step in the non-mevalonate pathway has been cloned from *E. coli* [17].

Here we report the effect of enhancing *DXPS* enzymatic activity on the levels of two end products of the isoprenoid pathway, lycopene and ubiquinone-8 (UQ-8) in *E. coli*. The results suggest applications for *DXPS* in the bioengineering of isoprenoids in biotechnology.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

E. coli strain XL1-blue (Stratagene) was used for gene cloning and expression of plasmids. *E. coli* was grown in Luria Broth media [18] at 37°C on a rotary shaker at 250 rpm (unless otherwise stated). Ampicillin (100 μg/ml), chloramphenicol (50 μg/ml) and 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) (all purchased from Sigma) were added as required. Plasmid pBluescript (Stratagene) was used as a vector for both cloning and expression studies. *Synechocystis* sp. PCC 6803 was obtained from the Institute Pasteur (Paris) and grown in BG11 medium [19] supplemented with 0.5% glucose at 30°C and 2000 lux. *Bacillus subtilis* strain PY79 DNA was a kind gift from P. Wakeley (Royal Holloway, University of London). The construction of plasmid pACCRT-EIB, which expresses the *E. uredovora crtE*, *crtB* and *crtI* genes necessary for lycopene biosynthesis in *E. coli* cells into which it has been introduced, has been described previously [20]. The plasmid used for the expression of *HMGR1* cloned into pBluescript (pHMGR1) has also been described elsewhere [21].

2.2. Recombinant DNA techniques

All recombinant DNA techniques were performed by standard methods [22] or according to supplier's instructions. Genomic DNA was extracted from all organisms using the Qiagen Genomic-tip 20/G kit.

2.3. Cloning of dxps genes

Based on the nucleotide sequence of ORF sll1945 from the genome database for *Synechocystis* sp. PCC 6803 [23], primers were designed to clone the putative *dxps* gene by polymerase chain reaction (PCR). The forward primer 5′-GTCCCAATCCACCATGCACATCAG-3′ overlaps the beginning of the coding sequence. The reverse primer 5′-CCCTCGACAAATGCAAAATGTATC-3′ lies outside the stop codon of the gene. A PCR (25 cycles) using *Pfu* DNA polymerase (Stratagene) produced a DNA fragment of the expected size (∼1.9 kb). Subsequent sequencing of the fragment confirmed the product to be the ORF sll1945. The *B. subtilis dxps* gene was also cloned by PCR using primers designed to amplify the gene encoding the product YqiE, identified in the *B. subtilis* genome database [24]. The forward primer 5′-GATCCGCTATGGATCTT TTATC-3′ contains a modified base substitution at the predicted start codon (underlined) for improved expression in *E. coli*. The reverse primer 5′-ATC-

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TAATCGTTCTTTCTTTGAC-3′ lies outside the stop codon of the *dxps* gene. After PCR (25 cycles) a DNA product of the expected size (~1.9 kb) was obtained, and when sequenced proved to be identical to the gene encoding the product YqiE. The PCR products from both reactions were treated with *Taq* DNA polymerase (GibcoBRL) at 72°C for 10 min to synthesise blunt ended fragments. The fragments were then cloned into the *Eco*RV site of the pBluescript vector (Stratagene) using T_4 DNA ligase (Fermentas) (Fig. 2).

2.4. In vitro DXP synthase assay

E. coli XL1-blue cells, transformed with the appropriate plasmid, were grown at 37°C in Luria Broth medium with appropriate antibiotics to an OD_{620nm} of 0.6, and induced by the addition of 1.0 mM IPTG at 28°C for 2 h. Bacteria were harvested by centrifugation ($6000\times g$ for 10 min) and washed in buffer A (100 mM Tris-HCl (pH 7.5), 1 mM dithioreitol, 0.3 M sucrose). Cells were resuspended to their original volume in buffer B (100 mM Tris (pH 8.0), 1 mM dithioreitol, 0.1 mM phenylmethanesulphonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mg/ml lysozyme). The cells were then incubated at 30°C for 15 min with gentle agitation, and then disrupted by brief sonication at 4°C. The supernatant was recovered and the protein concentration determined using the Bradford assay [25].

An aliquot of the supernatant (100 μl) was transferred to an Eppendorf tube along with 100 μl of assay buffer containing 100 mM Tris (pH 8.0), 3 mM ATP, 3 mM Mn^{2+}, 3 mM Mg^{2+}, 1 mM KF, 1 mM thiamine diphosphate, (0.1%) Tween-60, 0.6 mM DL-glyceraldehyde-3-phosphate, 30 μM [2-^{14}C]pyruvate (0.5 μCi). The mixture was incubated for 2 h at 30°C with gentle agitation. The reaction was stopped by heating the mixture at 80°C for 3 min. After centrifugation at $13\,000\times g$ for 5 min, the supernatant was transferred to a clean tube and evaporated to dryness. The residue was resuspended in methanol (50 μl) and loaded onto a TLC plate (silica gel 60). Chromatograms were developed in *n*-propyl alcohol/ethyl acetate/H_2O (6:1:3 v/v/v).

Enzyme assays were performed with extracts of induced cells expressing either *Synechocystis* sp. PCC 6803 or *B. subtilis DXPS*, as opposed to control assays in which cells contained only the pBluescript vector without insert. TLC analysis of assays expressing one of the *dxps* clones exhibited a major band (R_f 0.14) assumed to be DXP which was not observed in the controls. Quantification of ^{14}C-labelled DXP was achieved by isolation of the reaction product on TLC. The DXP band was scraped off the plate, eluted from the silica using methanol and quantified by liquid-scintillation counting. Enzymatic dephosphorylation of the assay products resulted in the formation of 1-deoxy-D-xylulose (DX), when analysed on TLC (R_f 0.50). When non-radioactive pyruvate was used in the assay, the DXP (R_f 0.12 stained purple) and DX (R_f 0.47 stained blue) were identified by staining with *p*-anisaldehyde/sulphuric acid (3:1). The DXP co-chromatographed with authentic, chemically synthesised DXP which stained purple also. The reaction substrates pyruvate (R_f 0.36 stained yellow), DL-glyceraldehyde-3-phosphate (R_f 0.15 stained orange) and D-glyceraldehyde (R_f 0.74 stained orange) were also observable using this TLC system. In reactions where the assay products were dephosphorylated no DXP was observed on TLC only DX.

2.5. Quantification of lycopene and ubiquinone QB-8 in E. coli

Bacterial growth was determined from the OD_{620nm}. Dry cell weight was calculated from known volumes of culture harvested by centrifugation at $13\,000\times g$ for 5 min, washed once with water and recentrifuged. The cells were lyophilised overnight and the weight of the dried cell pellet determined. The lycopene content of the cells was determined by harvesting aliquots of *E. coli* cells by centrifugation at $13\,000\times g$ for 5 min and washing once in water followed by recentrifuging. The cells were resuspended in acetone (200 μl) and incubated at 68°C for 5 min in the dark. The samples were centrifuged again $13\,000\times g$ for 10 min and the acetone supernatant containing the lycopene was placed in a clean tube. The extract was evaporated to dryness under a stream of N_2 and stored at −20°C in the dark. The lycopene content of the extracts was determined by visible light absorption spectra using a Beckman DU Series 7000 diode array spectrometer. Spectra were recorded in acetone using an $A^{1\%}_{1cm}$ of 3450 [26].

UQ-8 was extracted from cells based on the methods of Yoshida et al. [27]. Cells were collected by centrifugation, washed once with water and then lyophilised overnight. The dried pellet was extracted in *n*-propanol (3 ml) and of *n*-hexane (5 ml) containing 15 μg of UQ-10 as an internal standard, by disruption of the cells using a pestle and mortar. The solvent phase and that obtained by the second extraction from the aqueous phase *n*-hexane (3 ml) were combined and evaporated to dryness under N_2. The residue was resuspended in ethanol and analysed by reversed phase HPLC as described previously [28]. Peaks were identified by comparing their elution profiles with standards for UQ-7, UQ-9 and UQ-10. A standard of UQ-8 was not available, and the UQ-8 peak was identified by its elution profile relative to those of the other standards [29].

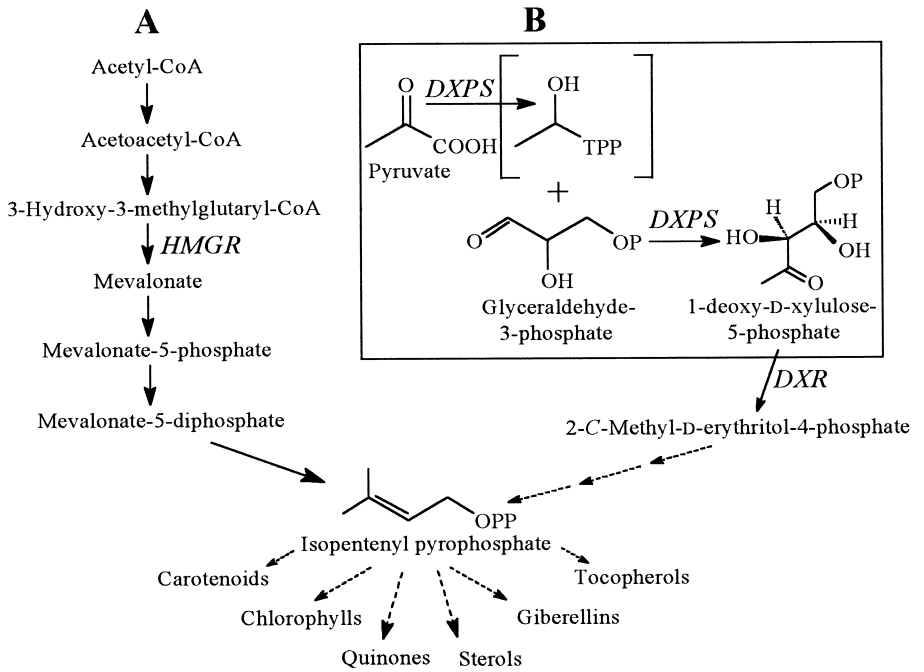

Fig. 1. Mevalonate-dependent (A) and -independent (B) pathways for IPP biosynthesis. Proposed reactions for the biosynthesis of 1-deoxy-D-xylulose-5-phosphate from pyruvate and glyceraldehyde-3-phosphate, catalysed by *DXPS* is shown inside the box.

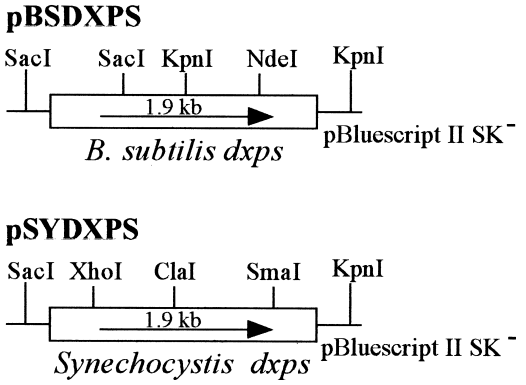

Fig. 2. Structure of plasmids pBSDXPS and pSYDXPS.

3. Results and discussion

3.1. Cloning of the dxps genes

The cloning of *dxps* and the characterisation of the gene product, *DXPS*, from *E. coli* has recently been reported by two research groups [11,12]. The gene product was shown to exhibit DXP synthase activity, which is considered as the first reaction of the mevalonate-independent pathway for IPP biosynthesis (Fig. 1) [5]. Based on the *E. coli dxps* nucleotide sequence homologs of the gene were identified in the eubacterial genomes of *B. subtilis* and *Synechocystis* sp. PCC 6803. The open reading frame sll1945 in the *Synechocystis* sp. 6803 genome was cloned by PCR, ligated into the vector pBluescript, and designated pSYDXPS (Fig. 2). The gene extends over 1920 bp and contains an open reading frame encoding a polypeptide of 640 amino acids, with a predicted molecular mass of 69 kDa. The *dxps* homolog in the *B. subtilis* genome was identified as the ORF encoding the product YqiE. It was cloned by PCR, and introduced into pBluescript to generate plasmid pBSDXPS (Fig. 2). The gene extends over 1899 bp and encodes a polypeptide of 633 amino acids with a predicted molecular mass of 70 kDa.

The amino acid sequence of the *DXPS* proteins of *Synechocystis* sp. 6803 and *B. subtilis* exhibited significant similarity to each other over their entire length (47% identities) and to the *E. coli DXPS* (*B. subtilis* (44% identities) and *Synechocystis* sp. 6803 (46% identities) (Fig. 3). All three polypeptides share two conserved domains; one thought to be involved in thiamin binding [30] and a histidine residue postulated to participate in proton transfer [31], both of which are detailed in

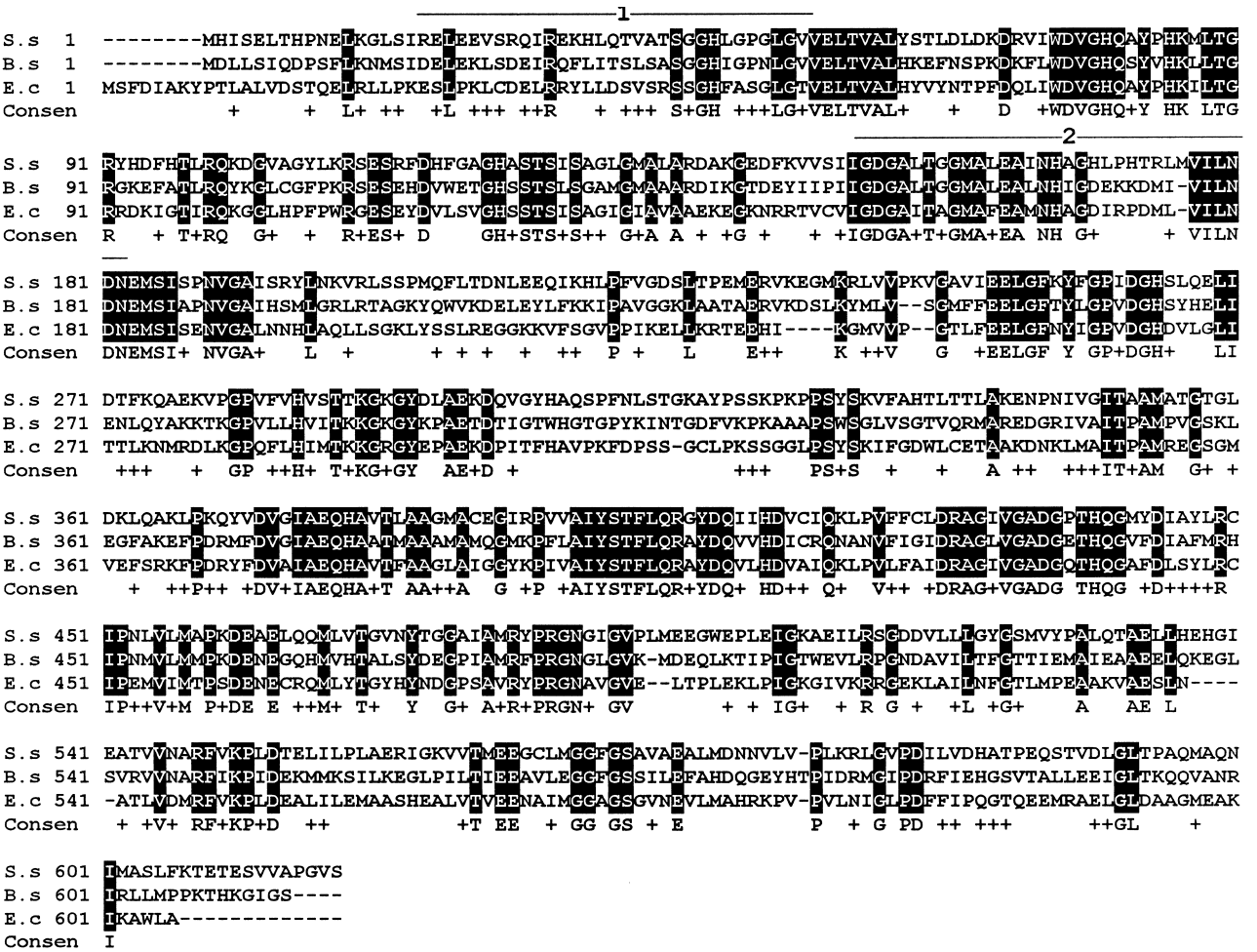

Fig. 3. Amino acid sequence alignment of *DXP* synthases used in the study, *Synechocystis* sp. 6803 (S.s) (GenBank D90903), *B. subtilis* (B.s) (GenBank D84432) and *E. coli* (E.c) (GenBank AF035440). The consensus line (consen) shows residues conserved in all three sequences (upper case letters) or residues which are identical in two sequences and replaced by an equivalent amino acid in the third sequence (+). The conserved histidine domain putatively involved in proton transfer is overlined and numbered 1. The second overlined domain (2) denotes the consensus thiamin pyrophosphate (TPP)-binding motif.

Fig. 3. The existence of a thiamin-binding domain in each of the polypeptides explains the cofactor requirement of thiamin for *DXPS* activity [12]. The high degree of polypeptide sequence identity, particularly the distribution of conserved domains, in all three indicates that they all encode *DXPS* or a closely related gene product.

3.2. Quantification of lycopene and UQ-8 in E. coli transformants

Cells of *E. coli* transformed with pACCRT-EIB [20] are pigmented pink due to the accumulation of lycopene. *E. coli* cells engineered to produce lycopene, were transformed with either pBSDXPS, pSYDXPS, pHMGR, or pBluescript to act as a control, to monitor the effect on lycopene biosynthesis when exogenous *DXPS* was expressed in the cells. The *E. coli* were grown in 50 ml cultures at 30°C with induction by IPTG for 48 h, by which time they had reached the stationary phase of growth. Fig. 4 shows the accumulation of lycopene in the cultures during the 48 h culture period. The graph clearly demonstrates that the *E. coli* cultures expressing exogenous *dxps* accumulated lycopene at a much greater rate than the control culture. The final lycopene content of the recombinant *dxps* strains was approximately double that of the control (Fig. 5). A similar increase was also obtained in *E. coli* cells engineered to produce the colourless carotenoid phytoene (data not shown). Alterations in the endogenous levels of isoprenoids were determined by measuring the ubiquinone content of the cells. In *E. coli*, the major quinones encountered are ubiquinone (UQ-8) and menaquinone (MK-8) [32]. Ubiquinone is a major component of the aerobic respiratory chain. It is estimated that there are approximately 50 molecules of ubiquinone for each of the oxidation complexes in *E. coli* [33]. By measuring an end product which is produced in relatively large quantities, it was conjectured that alterations in the rates of biosynthesis could be readily detected. The UQ-8 content of the recombinant *dxps* strains was 1.5 times greater than the controls (Fig. 5). Lycopene and UQ-8 levels were measured in *E. coli* transformed with *hmgr1* from *A. thaliana*, to monitor if this caused any alterations in the isoprenoid content of the cells. Expression of the *A. thaliana hmgr1* cDNA had no effect of the levels of lycopene nor UQ-8 in the cells (Fig. 5).

The results show that increased expression of *DXPS* leads

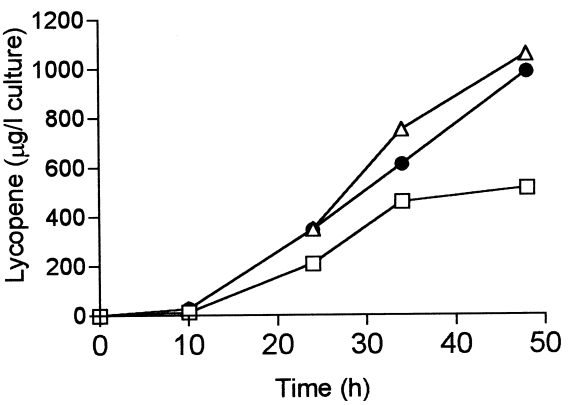

Fig. 4. Lycopene accumulation in recombinant *E. coli* cultures expressing vector only (□), *B. subtilis DXPS* (●) and *Synechocystis* sp. 6803 *DXPS* (△). (Data are means ± S.E.M. from three independent determinations.)

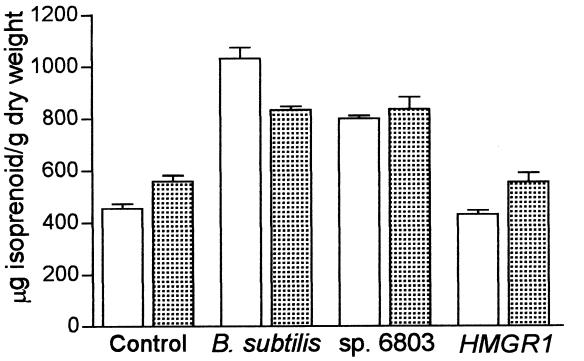

Fig. 5. Lycopene (open columns) and UQ-8 (shaded columns) content of *E. coli* control cultures (vector only) or expressing exogenous *B. subtilis dxps* (*B. subtilis*), *Synechocystis* sp. 6803 *dxps* (sp. 6803) or *A. thaliana hmgr1* (*HMGR1*) genes. (Data are means ± S.E.M. from three independent determinations.)

to increased lycopene and UQ-8 levels in the recombinant *E. coli* cells. This indicates that increasing the rate of DXP synthesis, the initial reaction in the mevalonate-independent pathway for IPP biosynthesis, elevates isoprenoid production. In contrast, expression of *hmgr1* had no effect on isoprenoid biosynthesis, suggesting that mevalonate-dependent IPP biosynthesis has little or no role in IPP synthesis in *E. coli*. Similarity searches of the *E. coli* genome database for proteins of the mevalonate-independent IPP biosynthesis pathway failed to identify any possible homologs in the genome suggesting that this pathway is probably absent in this organism.

3.3. In vitro enzyme activity

The increased levels of carotenoids and UQ-8 in *E. coli* expressing exogenous *DXPS* were hypothesised to be due to increased *DXPS* enzymatic activity in the cells. This was confirmed by preparing cell homogenates from recombinant *E. coli* strains after induction with IPTG. Reaction products were measured over a 2 h period, separated by TLC and quantified by liquid-scintillation counting. The major product obtained from the reaction after incubation with alkaline phosphatase co-chromatographed with chemically synthesised DX. This confirms DXP as the major reaction product in the assay. The putative *DXPS* function of *B. subtilis* ORF encoding the product YqiE and *Synechocystis* sp. 6803 ORF sll1945 has been established by these results. Table 1 shows the specific activity of *DXPS* in the recombinant *E. coli* strains. The results show that *DXPS* activity was increased in *E. coli* expressing endogenous *dxps* genes. This increase was greatest in homogenates containing the *B. subtilis DXPS*, where a 2.0-fold increase was observed compared to the controls. Homogenates containing the *Synechocystis* sp. 6803 *DXPS* exhibited a 1.8-fold increase compared to control reactions. Therefore, increased *DXPS* activity in *E. coli* appears to be responsible for the increased levels of carotenoids and UQ-8 observed in

Table 1
DXP synthase activity in *E. coli* homogenates

	Specific activity (nmol/min/mg protein)	Fold increase in activity
Control	5.8 ± 0.07	1.0
B. subtilis	11.5 ± 0.58	2.0
Syn. sp. 6803	10.4 ± 0.24	1.8

the transgenic strains. The relative increases in carotenoid levels between *E. coli* cultures expressing plasmids pSYNDXSP and pBSDXPS closely resemble the increases observed in the in vitro studies. This suggests that there is a direct relationship between *DXPS* activity and the carotenoid content of the cells. This is not the case for UQ-8 where increases in the levels of UQ-8 are more restricted, which could be due to a rate-limiting reaction later in the UQ-8 biosynthesis pathway [34]. The results support the hypothesis that increased *DXPS* activity in *E. coli* results in increased levels of carotenoids and UQ-8. These data suggest that isoprenoid levels in *E. coli* can be increased by enhancing *DXPS* activity.

Isoprenoids constitute a large group of compounds many of which are of high economic value. The condensation of (hydroxy)thiamin, derived from the decarboxylation of pyruvate, with glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate, is considered to be the first reaction in the mevalonate-independent pathway for IPP and ultimately isoprenoid biosynthesis. The data presented in this paper show that increasing the rate of DXP synthesis in *E. coli* results in increased isoprenoid biosynthesis. This finding could be utilised to optimise the industrial production of isoprenoids from bacteria. The manipulation of enzyme activities important in the biosynthesis of specific isoprenoids in concert with *DXPS* could be employed to bioengineer the production of specific, high value isoprenoids. This technology could also be transferred to plants where increased isoprenoid production could be used for improving crop flavour, fragrance and colour. Alternatively, crops could be engineered to produce increased concentrations of isoprenoids with pharmaceutical and/or nutritional properties.

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