# Engineered Isoprenoid Pathway Enhances Astaxanthin Production in *Escherichia coli*

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**Abstract:** The isoprenoid pathway is a versatile biosynthetic network leading to over 23,000 compounds. Similar to other biosynthetic pathways, the production of isoprenoids in microorganisms is controlled by the supply of precursors, among other factors. To engineer a host that has the capability to supply geranylgeranyl diphosphate (GGPP), a common precursor of isoprenoids, we cloned and overexpressed isopentenyl diphosphate (IPP) isomerase (encoded by *idi*) from *Escherichia coli* and GGPP synthase (encoded by *gps*) from the archaebacterium *Archaeoglobus fulgidus*. The latter was shown to be a multifunctional enzyme converting dimethylallyl diphosphate (DMAPP) to GGPP. These two genes and the gene cluster (*crtBIYZW*) of the marine bacterium *Agrobacterium aurantiacum* were introduced into *E. coli* to produce astaxanthin, an orange pigment and antioxidant. This metabolically engineered strain produces astaxanthin 50 times higher than values reported before. To determine the rate-controlling steps in GGPP production, the IDI-GPS pathway was compared with another construct containing *idi, ispA* (encoding farnesyl diphosphate (FPP) synthase in *E. coli*), and *crtE* (encoding GGPP synthase from *Erwinia uredovora*). Results show that the conversion from FPP to GGPP is the first bottleneck, followed sequentially by IPP isomerization and FPP synthesis. Removal of these bottlenecks results in an *E. coli* strain providing sufficient precursors for in vivo synthesis of isoprenoids.  *Biotechnol Bioeng* **62**: 235–241, 1999.
**Keywords:** isoprenoids; astaxanthin; isopentenyl diphosphate isomerase; geranylgeranyl diphosphate; metabolic engineering

## INTRODUCTION

Isoprenoids are a diverse class of natural products that are derived from successive addition of an active isoprene ($C_5$) unit, isopentenyl diphosphate (IPP), followed by enzymatic reactions such as cyclization, oxidation, reduction, isomerization, and hydroxylation (McGarvey and Croteau, 1995). Carotenoids, gibberellins, sterols, dolichols, ubiquinones, taxol, and prenylated proteins are just a few examples. The synthesis of isoprenoids is described by the visionary ''biogenetic isoprene rule'' (Ruzicka, 1953), which states that various isoprenoid structures are derived from intermediates in the common isoprenoid pathway including IPP, dimethylallyl diphosphate (DMAPP, $C_5$), geranyl diphosphate (GPP, $C_{10}$), farnesyl diphosphate (FPP, $C_{15}$), and geranylgeranyl diphosphate (GGPP, $C_{20}$). Each of these compounds can be the starting substrate for biosynthesis of isoprenoids (Fig. 1). Because of various chemical modifications of these precursors, over 23,000 natural products have been identified (Sacchettini and Poulter, 1997), which include antibiotics, antitumor pharmaceuticals, vitamins, coenzymes, cell membrane constituents, reproductive hormones, defensive agents, mating pheromones, polymers, agrochemicals, feed and food additives, and pigments. Many enzymes in the isoprenoid pathways have been identified, cloned, and structurally elucidated (Lesburg et al., 1997; Starks et al., 1997; Tarshis et al., 1994; Wendt et al., 1997). With the increasing understanding of the structure-function relationship of enzymes in these pathways, novel isoprenoid compounds are expected to be synthesized and identified using native and re-engineered enzymes.

Typical synthesis of isoprenoids relies on in vitro enzymatic reactions in series (Huang et al., 1998). These in vitro methods depend on expensive precursors and are difficult to scale up. Furthermore, they cannot be used for fast screening of isoprenoids produced. Therefore, it is desirable to construct a host organism that is capable of overproducing isoprenoids in vivo from inexpensive carbon sources such as glucose. Such a strain may serve as a host for constructing an in vivo library of isoprenoids in the future. However, one of the most commonly used hosts for metabolic engineering, *Escherichia coli*, has limited supply of the common precursors, IPP, DMAPP, GPP, FPP or GGPP, because the need for such compounds for growth is relatively small. Therefore, the goal of this work is to develop an *E. coli* strain that has increased supply of GGPP. The well-characterized route for IPP synthesis is through the mevalonate pathway, in which IPP is formed from acetyl coenzyme A (acetyl-CoA) via 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and mevalonate. Several bacteria, including *E. coli*, synthesize IPP using a different pathway branching from glycolytic intermediates, glyceraldehyde 3-phosphate (G3P) and pyruvate, although the genes and biochemistry of these steps are not completely known

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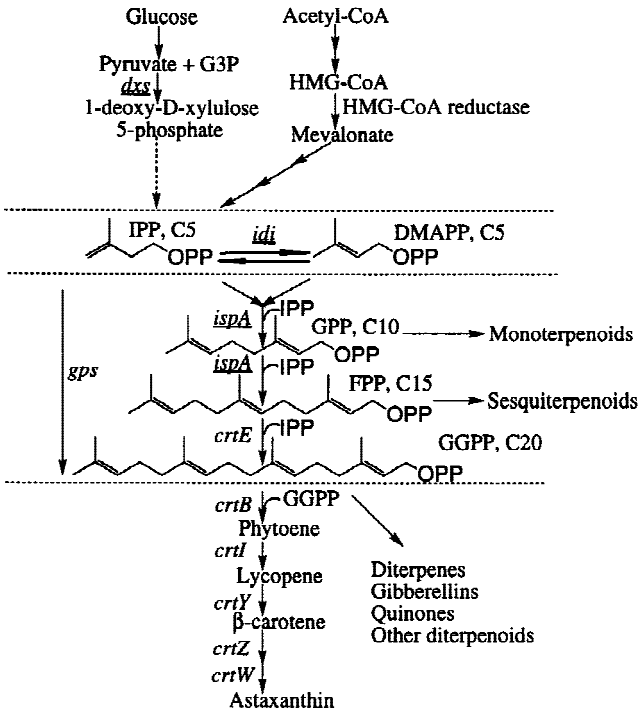


**Figure 1.** The isoprenoid biosynthetic pathway leading to the production of astaxanthin in *E. coli*. The vertical dashed line represents the pyruvate/G3P pathway generating IPP in some bacteria. The genes underlined exist in the wild-type *E. coli*. The mevalonate pathway is also shown.

(Rohmer et al., 1996; White, 1996). Once IPP is formed, it is isomerized to DMAPP through IPP isomerase (coded by *idi*), which, upon successive addition of IPP, leads to FPP through FPP synthase (coded by *ispA*). GGPP is an intermediate product of the multifunctional enzyme octaprenyl diphosphate (OPP) synthase encoded by *ispB* (Asai et al., 1994) and is not known to accumulate in *E. coli*. Therefore, it is desirable to use a GGPP synthase from other organisms that synthesize GGPP as the final enzymatic step, in order to increase the supply of GGPP.

We reconstructed a pathway between IPP and GGPP in *E. coli* using two strategies. One was to convert IPP and DMAPP directly to GGPP through a multifunctional GGPP synthase. The other was to convert IPP and DMAPP to FPP by the *E. coli ispA* gene (Fujisaki et al., 1990) and then convert FPP to GGPP by the *crtE* gene from *Erwinia uredovora* (Misawa et al., 1990). In either case, we needed to increase the level of IPP isomerase, which catalyzes the isomerization between IPP and DMAPP. By homology searching through the genome sequences, we identified the *idi* gene from *E. coli* and the GGPP synthase gene (*gps*) from the archaebacterium *Archaeoglobus fulgidus*. These genes were cloned and simultaneously expressed in *E. coli*. Meanwhile, the *idi*, *ispA*, and *crtE* genes were cloned into separate strains for comparison. To show that the GGPP produced can be effectively directed to an isoprenoid product, we introduced in these strains the genes for astaxanthin biosynthsis, *crtBIYZW* from *Agrobacterium aurantiacum* (Misawa et al., 1995), which converts GGPP to the orange pigment. The conversion to astaxanthin also alleviates potential feedback inhibition by GGPP, and allows easy color screening and quantitation of the flux through the isoprenoid pathway.

## MATERIALS AND METHODS

### Strains, Plasmids, and Culture Conditions

*E. coli* strain VJS676 (as *E. coli* K-12, but $F^-$, $\lambda^-$, and $\Delta$(*argF-lac*)) , a generous gift from Valley Stewart, Cornell University, was used in the expression system, while JM101 ($F'$ *traD36 proA*$^+$ *proB*$^+$ *lacI*$^q$ *lacZ*$\Delta$*M15/supE thi* $\Delta$*(lac-proAB)*) and INV$\alpha$F′ (Invitrogen, Carlsbad, CA) were used in the cloning procedures. Plasmids pAK32 (containing *crtBIYZW* from *A. aurantiacum*) and pACCAR25 ΔcrtX (containing *crtE* and *crtYIBZ* from *E. uredovora*) were gifts from N. Misawa of Kirin Brewery Co., Ltd, Japan. Plasmid pR10 containing *ispA* gene of *E. coli* was from S. Fujisaki of Toho University, Japan. All fermentation studies were carried out in shake-flask cultures with LB medium supplemented with carbon sources indicated.

### PCR Amplification and Plasmid Construction

To clone the *E. coli idi* gene which encodes IPP isomerase, two primers 5′-CGATAAACGCTCACTTGG-3′ and 5′-ACGTTATGCTCACAACCC-3′ were used. The PCR fragment was ligated into pCR2.1 (Invitrogen) to obtain pCW1 using the TA cloning technique. From pCW1, the *Eco*RI fragment containing *idi* was ligated to the *Eco*RI site of pACYC184, which interrupts the chloramphenicol-resistant gene to obtain pCW2. The orientation of the fragment was determined by enzyme digestion map. To clone the putative *gps* gene from *A. fulgidus*, two primers 5′-GGCAATG-TTTTTACGGG-3′ and 5′-TTTAATTCGCCGCTCTTC-3′ were designed to amplify this region. The PCR fragment was first ligated into pCR2.1 (Invitrogen) by TA cloning. The *Kpn*I–*Xba*I fragment of the resulting plasmid was ligated to the corresponding sites of pCL1920 to obtain pCW3. To construct a plasmid containing only *crtE* from *E. uredovora*, the *Hin*dIII fragment of pACCAR25 ΔcrtX containing *crtYIBZ* was removed and remaining fragment was self-ligated to give pAC–crtE. The *Bam*HI–*Hin*dIII fragment of pAC–crtE was then ligated into pCL1921 to give pCW4. To clone the *E. coli ispA* gene, primers were designed to amplify this gene using pR10 as a template. The PCR fragment was first ligated into pCR2.1, from which the *Bam*H1–*Sal*I fragment was ligated into pCL1921 to give pCL–ispA. Furthermore, the *Sal*I–*Hin*dIII fragment from pAC–crtE was ligated into pCL–ispA to give pCW5.

### IPP Isomerase Assay

The growth of *E. coli* and preparation of cell extracts are modified from Fujisaki et al. (1986). Cells with and without

plasmid pCW1 containing *idi* (obtained from the cloning above) were inoculated in 25 mL of L-broth medium for 24 h. The cells were harvested by centrifugation and washed twice with 0.1 *M* potassium phosphate buffer (pH 7.4), then resuspended in the same phosphate buffer containing 1 m*M* 2-mercaptoethanol (buffer A). The cells in the suspension were disrupted by French press, and centrifuged at 14,000*g* for 15 min. The supernatant was used as cell extracts.

Idi activity was identified by an assay developed by A. Ian Scott and co-workers (Huang et al., 1998). Buffer A was diluted twofold and $MgCl_2$ was added to a final concentration of 10 m*M* to prepare the reaction buffer. A 0.2 mL amount of reaction buffer was mixed with 0.3 mL [1-$^{13}$C]IPP and an appropriate amount of cell extracts. After incubation for 3 h at 37°C, $D_2O$ was added. 13C-NMR analysis was performed immediately.

### GGPP Synthase Assay

*E. coli* VJS676 *recA* harboring pCW3 was grown in 25 mL of LB medium containing spectinomycin (50 μg/mL) and induced with 100 μ*M* IPTG at 37°C. The cells were harvested and resuspended in 1 mL of 50 m*M* Tris-HCl buffer, pH 7.0, containing 10 m*M* 2-mercaptoethanol and 1 m*M* EDTA. The cells in the suspension were disrupted by French press. The homogenate was heated at 55°C for 60 min and centrifuged at 14,000*g* for 15 min. The supernatant was used to assay GGPP synthase activity.

GPS activity was identified by an assay developed by Peter A. Edwards and Johan Ericsson of University of California, Los Angeles (personal communication). GGPP synthase activity was assayed in a 20 μL reaction buffer containing 25 m*M* Hepes, pH 7.0, 2 m*M* $MgCl_2$, 5 m*M* KF, 1 m*M* dithiothreitol, 0.5% *n*-octyl-β-glycopyranoside, 10 μ*M* [1-$^{14}$C]IPP, and 10 μ*M* of the indicated allylic isoprenoid diphosphate. The reactions were started by the addition of 10 μL cell-free extracts and were allowed to proceed for 30 min at 55°C. The reactions were stopped by the addition of 400 μL of *n*-butanol saturated with water, followed by extensive vortexing. After the addition of 100 μL of 2 *M* KCl, the samples were vortexed once more and centrifuged to separate the organic and aqueous phases. The butanol phase was transferred to a new tube and an aliquot of 50 μL removed for scintillation counting. To analyze the distribution of products, the remaining butanol phase was evaporated under $N_2$ and the residue was redissolved in *n*-octyl-β-glycopyranoside and dephosphorylated enzymatically as described (Fujii et al., 1982). Following dephosphorylation, the products were extracted with 500 μL of hexane and the extract evaporated with $N_2$. The dephosphorylated and extracted reaction products were applied to the normal phase thin layer chromatography (TLC) using Kieselgel 60 developed with petroleum ether:diethyl ether (60:40). The plates were exposed to film at −80°C. The radioactive products were identified by comparison with unlabeled standards, visualized with iodine vapor.

Human GPS (a gift from Peter Edwards of University of California, Los Angeles) was assayed as the positive control. The assay condition was as described above, and FPP was used as the allylic substrate.

### Quantification of Astaxanthin Accumulated in *E. coli*

*E. coli* VJS676 transformants carrying two or three distinct plasmids were cultured in LB or LB + 0.2% glucose medium containing carbenicillin (100 μg/mL), spectinomycin (50 μg/mL), and tetracycline (10 μg/mL) if necessary at 37°C. Carotenoid pigments were extracted from cells with methanol and concentrated if needed, and subjected to high-performance liquid chromatography (HPLC); the column (4.6 × 250 mm, μBondapak C18; Waters, Milford, MA) was eluted with acetonitrile–methanol–water (49:44:7 v/v) at a flow rate of 1 mL/min. Astaxanthin was identified by co-chromatography with the authentic standard, which was purchased from Sigma (St. Louis, MO).

## RESULTS

### Cloning and Expression of the *E. coli idi* Gene

IPP isomerase has been isolated from yeasts *Saccharomyces cerevisiae* (Anderson et al., 1989), *Schizosaccharomyces pombe* (Hahn and Poulter, 1995), *Phaffia rhodozyma*, green algae *Haematococcus pluvialis* (Kajiwara et al., 1997), and human (Xuan et al., 1994). Since the complete *E. coli* genome has been sequenced (Blattner et al., 1997), it is possible to identify the candidate *idi* gene given the homologous sequences from other organisms. We used the known IPP isomerase protein sequence (Hahn and Poulter, 1995) from *S. pombe* to search for homologous sequence in the *E. coli* genome. Consistent with that reported previously (Hahn et al., 1996), this search gave the open reading frame designated as O182 in the *E. coli* genome, which encodes 182 amino acid and a deduced molecular weight of 20,508. This open reading frame shows 50.82% similarity and 25.14% identity with the *S. pombe* enzyme and also contains the active site residues C80 and E119 identified from *S. cerevisiae* IPP isomerase. Upstream from the start codon, the putative ribosome binding site (RBS) and the promoter region were identified. A pair of primers were designed to amplify this region of chromosome including the RBS and the promoter region by the polymerase chain reaction (PCR). The PCR product was ligated into the cloning vector pCR 2.1 (Invitrogen, Carlsbad, CA) to yield pCW1. To assay the enzyme activity, the *E. coli* strain (INVαF′) containing pCW1 was cultured in L-broth medium for 24 h. The cell extract was assayed for the idi activity using $^{13}$C NMR. Results show that the cell extract containing the putative IPP isomerase generates a chemical shift signal at 24.32 ppm, characteristic of DMAPP (Fig. 2). In contrast, the cell extract from the host strain without the plasmid did not give this signal (data not shown). Therefore, O182 was con-

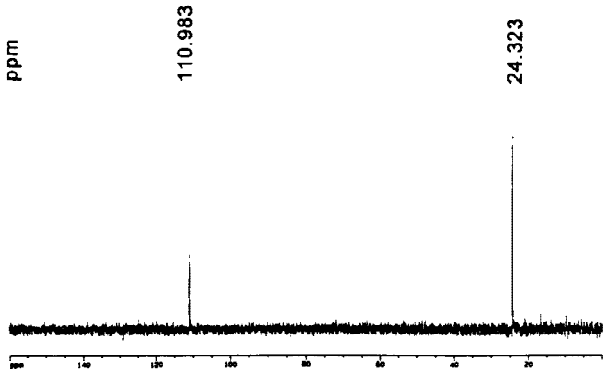


**Figure 2.** $^{13}C$ NMR analysis of the enzymatic isomerization of [1-$^{13}C$]isopentenyl diphosphate (δ 111 ppm) to [1-$^{13}C$]dimethylallyl diphosphate (δ 24.3 ppm). The reaction consisted of a crude cell extract containing the IPP isomerase from *E. coli* and [1-$^{13}C$]IPP as the substrate.

firmed to encode IPP isomerase. The basal level of IPP isomerase expressed from the host chromosome apparently was too low to be detected.

## Cloning and Expression of *gps* from *A. fulgidus*

As mentioned above, *E. coli* synthesizes GGPP only as an intermediate product of a multifunctional enzyme. Therefore, to construct a GGPP producer, it is desirable to use a foreign enzyme that synthesizes GGPP as a final product. Several GGPP synthases have been isolated (Chen et al., 1994), including the chloroplast-related GGPP synthase from *Capsicum annuum* (Kuntz et al., 1992), the archaebacterial bifunctional FPP/GGPP synthases from *Methanobacterium thermoautotrophicum* (Chen and Poulter, 1994) and *Sulfolobus acidocaldarius* (Ohnuma et al., 1994), and the eubacterial GGPP synthases from *E. herbicola* (Armstrong et al., 1990; Math et al., 1992) and *E. uredovora* (Misawa et al., 1990). The archaebacterial bifunctional FPP/GGPP synthase is attractive for our purpose here, because it eliminates the need to amplify another enzyme in the pathway. To isolate such a gene, we searched the genome sequence of *A. fulgidus* (Klenk et al., 1997). The GGPP synthase protein sequence from *S. acidocaldarius* (Ohnuma et al., 1994) was used to find homologous regions in the *A. fulgidus* genome. The best match was found to be an ORF encoding 317 amino acid, which was assigned as AF2286 in the *A. fulgidus* genome. Pairwise alignment indicated a high degree of identity (40.5%) and similarity (60.45%) between the *S. acidocaldarius* GGPP synthase and *A. fulgidus* ORF AF2286. The aspartate-rich domains I and II of prenyltransferases (Ohnuma et al., 1994) are also seen in the amino acid residues 69–95 and 205–224 of ORF AF2286, respectively. On the basis of the high similarity and presence of the conserved domain, the *A. flugidus* ORF AF2286 was predicted to catalyze the multiple steps from DMAPP to GGPP.

This ORF along with the putative RBS was amplified by PCR and inserted into the *Xba*I and *Kpn*I sites of the low-copy number vector pCL1920 to construct pCW3. As an initial screening, we transformed pCW3 into an *E. coli* strain harboring pAK32, which contains the genes needed (*crtBIYZW* from *A. aurantiacum)* to convert GGPP into astaxanthin. The *E. coli* strain with both plasmids produced an orange dye compared to the control strain with pAK32 and pCL1920. This screen gave the initial indication that the cloned ORF encodes *gps.* Although *A. fulgidus* is an extreme thermophile, its GGPP synthase maintains significant activity at 37°C. Because of the satisfactory *gps* expression, the putative RBS (GAGG) from the *A. fulgidus gps* is apparently recognized by *E. coli* ribosomes. The GPS activity was confirmed by an in vitro GGPP synthase enzyme assay. As expected, the result (Fig. 3) shows that this enzyme can utilize substrates DMAPP, GPP, or FPP to produce GGPP. When DMAPP was used as the substrate, GGPP was the only product detected, indicating that this enzyme catalyzes the multiple steps from DMAPP to GGPP.

## Reconstruction of the Isoprenoid Pathway in *E. coli*

In order to construct the complete pathway from DMAPP to GGPP in *E. coli*, we employed two sets of genes. The first set includes *E. coli idi* and *A. fulgidus gps*, whereas the second includes *E. coli idi, ispA,* and *Erwinia crtE.* These genes are expressed from two compatible vectors. Plasmid pCW2 (carrying *idi*) is derived from pACYC184, with *idi* gene inserted into the chloramphenicol resistant gene. Plasmid pCW3 (carrying *gps*) is a pCL1920 (Lerner and Inouye, 1990) derivative, which is spectinomycin-resistant and replicates by the pSC101 origin. Plasmids pCW4 (carrying *crtE*) and pCW5 (carrying *ispA* and *crtE*) are based on pCL1921 (Lerner and Inouye, 1990), which uses the same replicon as pCL1920.

Because GGPP cannot be excreted to the medium, its intracellular accumulation may lead to feedback inhibition.

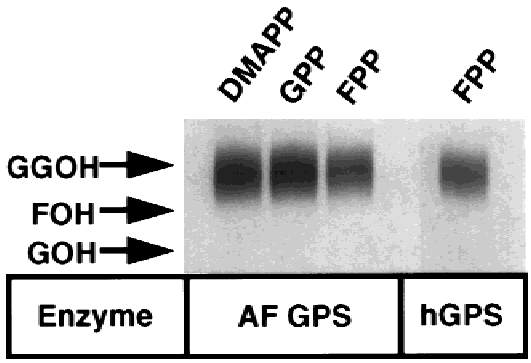


**Figure 3.** Normal phase thin layer radiochromatograms of the products from the GPS assay (prenyltransferase reactions) using various allylic substrates. From left to right lanes, the enzymatic reactions of the *A. fulgidus* GPS using DMAPP, GPP, and FPP as the allylic substrates, respectively, and positive control of human GPS using FPP as the allylic substrate. The band shown was geranylgeraniol compared to the positions of authentic standard alcohols.

To allow for easy color screening and quantitation of pathway flux, GGPP produced is converted to astaxanthin by the gene cluster *crtBIYZW* from *A. aurantiacum.* These genes were expressed from plasmid pAK32, which is a pUC19 derivative, and thus is compatible with the pACYC184- and PCL1920-derived plasmids described above.

### Determining the Rate-Controlling Steps

With these plasmids, a series of strains overexpressing different genes in the common isoprenoid pathway were constructed to examine the rate-controlling strength of each step (Table I). All these strains contain the astaxanthin synthesis genes to allow for convenient color screening. Without overexpressing any genes in the common isoprenoid pathway, no astaxanthin was observed. This result indicates that the production of GGPP in *E. coli* is indeed limiting the synthesis of isoprenoid compounds. Overexpressing *gps* showed the orange color characteristic of astaxanthin. Similarly, overexpressing *crtE* also showed the orange color. However, overexpressing *ispA* or *idi* alone or together did not show any color formation. Therefore, it can be concluded that the last step in GGPP synthesis is the first rate-controlling step. Its amplification is necessary for the production of astaxanthin.

Overexpression of *ispA* in addition to *crtE* did not increase the color intensity. However, overexpression of *idi* and *crtE* together increased the color formation. This result indicates that the isomerization reaction is the second rate-controlling step. In the presence of *idi* and *crtE,* overexpression of *ispA* further increased the color intensity. These results indicate the successive shift of rate-controlling steps from GGPP synthesis to IPP isomerization, and then to FPP formation. Since *gps* catalyzes the formation of GGPP directly from DMAPP, it does not need additional FPP synthase activity. However, amplification of the isomerase activity should increase the flux. Indeed, simultaneous overexpression of *gps* and *idi* significantly increased the color formation.

**Table I.** *E. coli* strain harboring the astaxanthin synthesis genes *crtBIYZW* was used as the color screening system to examine the rate-controlling strength of each step in the common isoprenoid pathway: –, no orange color observed; +, orange color intensity; ND, no detection.

| Genes overexpressed | Orange color | Astaxanthin yield (μg/g dry weight) |
|---|---|---|
| *crtBIYZW* | – | ND |
| *crtBIYZW, ispA* | – | ND |
| *crtBIYZW, idi* | – | ND |
| *crtBIYZW, idi, ispA* | – | ND |
| *crtBIYZW, crtE* | + | 32.8 |
| *crtBIYZW, crtE, ispA* | + | 35.3 |
| *crtBIYZW, crtE, idi* | ++ | 234.1 |
| *crtBIYZW, crtE, idi, ispA* | +++ | 390.3 |
| *crtBIYZW, gps* | + | 35.6 |
| *crtBIYZW, gps, idi* | +++ | 1418.8 |

### Astaxanthin Production

The proposed isoprenoid biosynthetic pathway in *E. coli* suggests that IPP comes from the glycolytic intermediates, pyruvate and G3P, although the genes in this pathway are not completely known (Horbach et al., 1993; Rohmer et al., 1993, 1996). Recently, the first putative gene *dxs,* which converts pyruvate and G3P to 1-deoxy-D-xylulose 5-phosphate in this unknown pathway, has just been cloned (Sprenger et al., 1997). This result suggests that the presence of glucose may enhance the production of astaxanthin. To demonstrate the effects of various factors that may influence the production of astaxanthin, we cultured the cells under different conditions. Results show that the strain overexpressing both *gps* and *idi* gave the highest astaxanthin yields in LB medium containing 0.2% glucose (Fig. 4). The highest yield is over 1.25 mg/g dry weight, which is 50-fold higher than the literature reported before (25 μg/g d.w.) (Breitenbach et al., 1996). The positive effect of glucose supports the notion that *E. coli* synthesizes IPP from glycolytic intermediates. The strain overexpressing only *gps* and *crtBIYZW* showed much lower astaxanthin yield, consistent with the plate screening. Furthermore, strains overexpressing *crtE, ispA,* and *idi* produced much lower astaxanthin than the *gps idi* combination, although the plate screen showed only a slight difference. Consistent with the rate controlling analysis, the strain overexpressing *crtE* and *idi* produced a lower amount of astaxanthin than the one overexpressing the *crtE ispA idi* combination.

## DISCUSSION

In order to reconstruct the isoprenoid pathway from DMAPP to GGPP in *E. coli,* we used two strategies: one

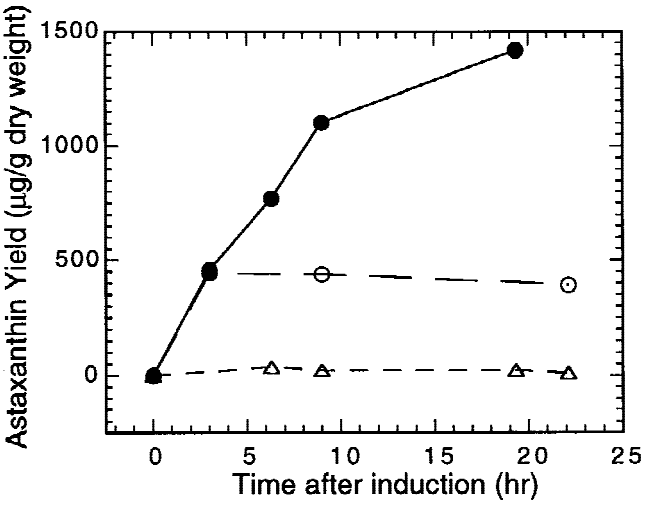


**Figure 4.** Time course of astaxanthin production. VJS676 was used as the host strain with plasmids that lead to astaxanthin production. The strains were cultured in shake-flasks at 37°C with a gyration rate of 225 rpm. To induce the expression of GPS on pCW3, 100 μ*M* IPTG was added at about 5 h after inoculation when the dry cell weight was about 0.1–0.12 g/L. The dry cell densities at the end of fermentation were 1, 0.33, and 1.5 g/L for the following strains, respectively. (●) pAK32/pCW3/pCW2 in LB medium containing 0.2% glucose; (○) pAK32/pCW3/pCW2 in LB medium; (△) pAK32/pCW3 in LB medium containing 0.2% glucose.

with direct synthesis from DMAPP to GGPP using a multifunctional GGPP synthase, the other with two separate enzymes (IspA and CrtE) with FPP being the intermediate. In either case, the isomerization between IPP and DMAPP was enhanced by overexpression of Idi. Furthermore, GGPP synthesized in vivo is directed to astaxanthin to avoid feedback inhibition and to allow easy screening and quantitation of flux. Apparently, astaxanthin produced is crystallized inside the cell, as the supernatant from lysed cells contained little astaxanthin.

Overexpression of different combinations of *idi*, *gps*, *ispA*, and *crtE* allows the identification of rate-controlling steps. Results show that the last step of GGPP synthesis is the first bottleneck. After overexpression of *crtE* or *gps*, the bottleneck shifts to IPP isomerization. After overexpression of *idi* in addition to *crtE*, then the final bottleneck is shifted to FPP synthesis. The *gps* gene product synthesizes GGPP directly from DMAPP, and it does not need additional FPP synthase. In deed, overexpression of *idi* and *gps* gave the highest astaxanthin yield.

Our newly identified GGPP synthase showed the special catalytic function of converting DMAPP directly to GGPP, and was utilized to enhance dramatically the production of carotenoids, specifically astaxanthin, combined with the native *E. coli* IPP isomerase. Most other GGPP synthases including several expressed from genes located on carotenogenic gene clusters are unable to use DMAPP as a substrate (Wiedemann et al., 1993). Compared to the previous efforts trying to increase the production of carotenoids (Becker-Hapak et al., 1997; Miura et al., 1998; Ruther et al., 1997), our work offers an easy way to achieve high levels of production and can be extended to synthesize any specific carotenoid by adjusting the carotenogenic gene cluster. At the same time, we also systematically investigated the metabolic flux controlling steps in the *E. coli* isoprenoid biosynthetic pathway. Furthermore, our study showed that glucose, rather than acetate, enhances the production of astaxanthin in *E. coli*. This result provides additional support that *E. coli* synthesizes IPP from glycolytic intermediates, rather than acetyl-CoA.

Since the overexpression of *gps* and *idi* significantly increased the GGPP production in vivo, such a strain can be used to synthesize other GGPP-derived isoprenoid compounds, such as casbene, taxadiene, abietadiene, gibberellins, and quinones. In vivo supply of isoprenoid precursors is an essential step in the production of any of these compounds. Because of the chemical variety of isoprenoids, it is desirable to construct an in vivo library of isoprenoids. The sufficient supply of GGPP is an essential step in constructing such a library. Once the specific isoprenoid is identified, the strain can readily be used in large scale production or for further characterization. Further improvement of the strain can be achieved by cloning *idi* and *gps* into the same plasmid, or integrating them into the chromosome with a strong promoter. In addition, the supply of GGPP may be further increased by amplifying the key genes in the IPP biosynthesis pathway. However, this pathway appears to be derepressed when the GGPP is directed to a downstream product. Therefore, without amplifying the pathway leading to IPP, the strains constructed here already show significant enhancement of astaxanthin production.

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