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Metabolic Engineering 8 (2006) 79–90
METABOLIC ENGINEERING
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# Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*

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Received 31 March 2005; received in revised form 24 August 2005; accepted 25 August 2005
Available online 28 October 2005

**Abstract**

For metabolic engineering it is advantageous in terms of stability, genetic regulation, and metabolic burden to modulate expression of relevant genes on the chromosome rather than relying on over-expression of the genes on multi-copy vectors. Here we have increased the production of β-carotene in *Escherichia coli* by replacing the native promoter of the chromosomal isoprenoid genes with the strong bacteriophage T5 promoter ($P_{T5}$). We recombined PCR fragments with the λ-Red recombinase to effect chromosomal promoter replacement, which allows direct integration of a promoter along with a selectable marker that can subsequently be excised by the Flp/FRT site-specific recombination system. The resulting promoter-engineered isoprenoid genes were combined by serial P1 transductions into a host strain harboring a reporter plasmid containing β-carotene biosynthesis genes allowing a visual screen for yellow color indicative of β-carotene accumulation. Construction of an *E. coli* $P_{T5}$-*dxs* $P_{T5}$-*ispDispF* $P_{T5}$-*idi* $P_{T5}$-*ispB* strain resulted in producing high titers (6 mg/g dry cell weight) of β-carotene. Surprisingly, over-expression of the *ispB* gene, which was expected to divert carbon flow from the isoprenoid pathway to quinone biosynthesis, resulted in increased β-carotene production. We thus demonstrated that chromosomal promoter engineering of the endogenous isoprenoid pathway yielded high levels of β-carotene in a non-carotenogenic *E. coli*. The high isoprenoid flux *E. coli* can be used as a starting strain to produce various carotenoids by introducing heterologous carotenoid genes.


*Keywords:* Chromosomal promoter replacement; Isoprenoid pathway; Carotenoids; Metabolic engineering

## 1. Introduction

Carotenoids are ubiquitous pigments synthesized by plants, fungi, algae and bacteria. Industrially carotenoids are used in pharmaceuticals, neutraceuticals, animal feed additives as well as colorants in cosmetics and food. In recent years, interest in production of natural carotenoids by microbial fermentation has increased and carotenogenic microbes such as *Phaffia*, *Haematococcus* alga, and *Blakeslea trispora* have been investigated for large-scale production (Jacobson et al., 2000; Olaizola, 2000; Mehta et al., 2003).

However, the use of recombinant carotenoid genes from carotenogenic microbes has made possible the synthesis of carotenoids in non-carotenogenic microbes, e.g. *Escherichia coli, Zymonmonas mobilis, Candida utilis,* and *Saccharomyces cerevisiae* (Misawa and Shimada, 1998). *E. coli* is a suitable host for carotenoid production because it readily expresses many carotenogenic genes to make various carotenoids such as lycopene, β-carotene, canthaxanthin, zeaxanthin, and astaxanthin (Ruther et al., 1997; Sandmann et al., 1999; Sandmann, 2002). Carotenoids are derived from two common building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized through either of two isoprenoid pathways, the mevalonate biosynthetic pathway in plants or the 1-deoxyxylulose-5-phosphate (DXP) pathway in plants plastids and bacteria (Rohmer et al., 1996; Lange et al., 2000; Lee and Schmidt-Dannert, 2002). *E. coli* contains the genes encoding all enzymes of the DXP pathway (Rohdich et al., 2003). The DXP pathway starts with the condensation of pyruvate with glyceraldehyde-3-phosphate (G3P) to form deoxy-D-xylulose via the enzyme

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doi:10.1016/j.ymben.2005.08.005

encoded by *dxs* (Fig. 1). A series of additional isoprenoid enzymes encoded by *ispC (dxr), ispD (ygbP), ispE (ychB), ispF (ygbB), ispG (gcpE), and ispH (lytB)*, are then used in subsequent sequential reactions, converting deoxy-D-xylulose to the C5 isoprene subunits, IPP and DMAPP, which are inter-converted via the enzyme encoded by *idi*. This reaction is followed by two sequential prenyltransferase reactions catalyzed via the enzyme encoded by *ispA*, leading to the formation of the farnesyl pyrophosphate (FPP). FPP is a common precursor and branch point for carotenoid biosynthesis and other isoprenoid molecules such as quinones and dolichols.

Carotenoid production can be improved by metabolic engineering of the isoprenoid pathway supplying the isoprenoid precursors (Matthews and Wurtzel, 2000; Lee and Schmidt-Dannert, 2002). Metabolic engineering for enhancing carotenoid production in *E. coli* has previously focused on plasmid overexpression of the several key isoprenoid genes (Lee and Schmidt-Dannert, 2002). This metabolic engineering approach has several drawbacks

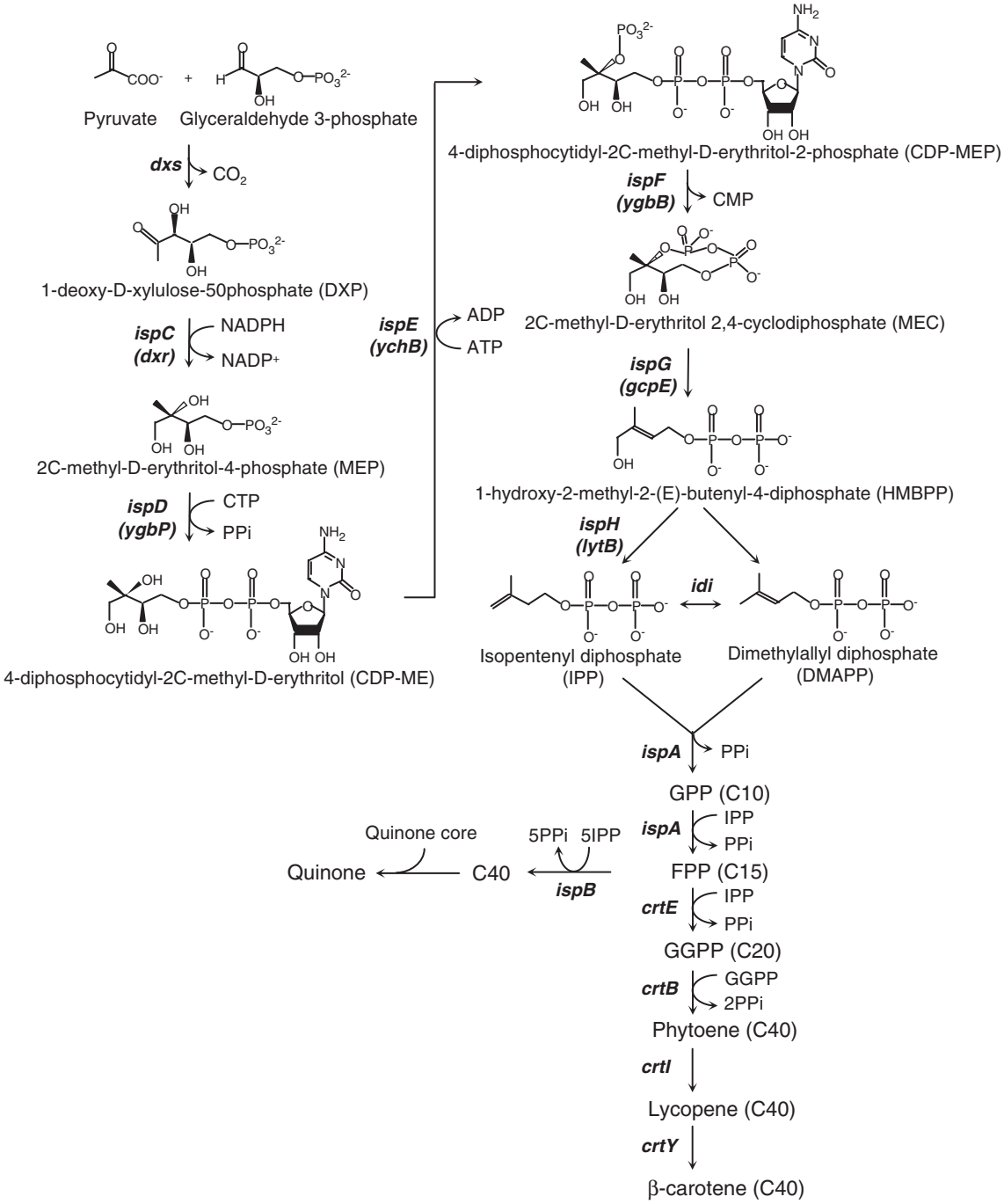


Fig. 1. 1-deoxyxylulose-5-phosphate (DXP) pathway for the isoprenoid biosynthesis in *E. coli* transformed with β-carotene biosynthetic genes from *Pantoea stewartii*.

associated with multi-copy plasmids such as vector burden, segregational instability, and difficulties in controlling gene expression (Noack et al., 1981; Bentley et al., 1990). Progress in genetic and genomic research, and an understanding of metabolic pathways and their regulation allow redesign of regulatory circuits to optimize metabolic flux through specific pathways. Recent technical advances in chromosomal engineering such as Red recombination system (Datsenko and Wanner, 2000) and gene gorging (Herring et al., 2003) enable efficient gene knockout and precise gene mutation. In this study, the Red recombination system in combination with P1 transduction has been used for chromosomal promoter engineering of the isoprenoid pathway to improve β-carotene production in *E. coli*. A successful strategy for redesigning the promoter region of the genes involved in the isoprenoid pathway was executed.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

The *E. coli* K-12 strain MC1061 ($F^-$ *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL*($Str^r$) *hsdR2*($r_K^- m_K^+$) *mcrA mcrB1*) (Casadaban and Cohen, 1980) was used as a host for chromosomal promoter replacement mediated by λ-Red homologous recombination, and MG1655 ($\lambda^-$, $F^-$, rph−1) (Bachmann, 1996) was used as a host for combining promoter-gene fusions.

The plasmids pPCB15 ($cam^R$) or pDCQ108 ($tet^R$) containing β-carotene biosynthesis genes *crtEXYIB* from *Pantoea stewartii* were used as a reporter plasmid for monitoring β-carotene production. The β-carotene biosynthetic genecluster *crtEXYIB* was PCR amplified from the *P. stewartii* genome with primers designed according to the related sequence of the *Erwinia herbicola* crt genes as demonstrated previously for the *crtE, crtB* and *crtI* genes (Scolnik and Bartley, 1995). The PCR fragment was cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) that is a low copy pACYC184-derived plasmid, generating pPCB15. The β-carotene biosynthetic genes *crtEXYIB* was subcloned from pPCB15 into *EcoR*I site of the broad host range medium copy pBHR1 vector (Mo Bi Tec, Inc., Australia), yielding pBHR1-crt ($kan^R$). pDCQ108 ($tet^R$) was a tetracycline resistant derivative of pBHR1-crt, where a tetracycline resistant gene was introduced by in vitro Tn5 insertion using Epicentre EZ-TN 〈TET〉 kit (Epicentre Technologies, Madison, WI) into the kanamycin resistant gene, inactivating the original kanamycin resistant gene on pBHR1-crt.

The λ-Red recombination plasmids, pDK46, pKD4, and pCP20, which were used for the chromosomal promoter replacement in this study, were obtained from Dr. Wanner (Datsenko and Wanner, 2000). Plasmid pSUH5 that was used as a template for PCR amplification of the fused kanamycin selectable marker-bacteriophage T5 promoter ($P_{T5}$), was constructed by cloning the 154-bp *Ndel-Ndel* fragment containing a phage T5 promoter, lacO and ribosome binding site into the *Ndel* site of pKD4. The 154-bp *Ndel-Ndel* fragment was synthesized by PCR from pQE30 (QIAGEN, Inc., Chatsworth, CA) with primer pairs introducing an NdeI site.

### 2.2. PCR-mediated λ-Red recombination

To integrate a bacteriophage T5 promoter ($P_{T5}$) along with a kanamycin selectable marker in the chromosome, we used PCR-mediated λ-Red recombination with either one PCR fragment containing fused kanamycin marker-$P_{T5}$ promoter or two PCR fragments one containing a kanamycin marker and the other the $P_{T5}$ promoter. Linear DNA fragments of the kanamycin marker, $P_{T5}$ promoter, or fused kanamycin marker-$P_{T5}$ promoter was generated by PCR from pKD4, pQE30 or pSUH5, respectively, with 5′- and 3′-primer pairs as shown in Table 1. The sequences underlined in Table 1 represent homology arms (40–50 bp), while the remainder is the priming sequence for hybridization to complementary nucleotide sequences on the template DNA. Standard PCR conditions were used to amplify the linear DNA fragments with AccuPrime™ pfx DNA polymerase (Invitrogen, Carlsbad, CA) or MasterAmp™ polymerase (Epicentre Technologies, Madison, WI). The PCR products were agarose gel-purified by using the QIAquick Gel Extraction Kit™ (QIAGEN, Inc., Chatsworth, CA) or Zymoclean™ Gel DNA Recovery kit (Zymo Research, Orange, CA).

Electro-competent *E. coli* MC1061 cells carrying both a λ-Red recombinase expression plasmid pKD46 ($amp^R$) and a reporter plasmid pPCB15 ($cam^R$) containing β-carotene biosynthesis genes were prepared as described (Datsenko and Wanner, 2000), and used as a host strain for transformation of PCR fragment(s) containing a kanamycin marker and promoter. Electro-competent cells (40 μl) were electroporated with 0.5–1 μg of one or two PCR fragment(s) using a Bio-Rad Gene Pulser set at 1.8 kV, 25 μF with the pulse controller set at 200 Ω. SOC medium (1 ml) (Invitrogen, Carlsbad, CA) was added after electroporation and incubated at 37 °C for 1 h. The cells were spread on Luria-Bertani (LB) plates containing both 25 μg/ml kanamycin and 25 μg/ml chloramphenicol and incubated at 37 °C overnight to select double antibiotic-resistant recombinants. The β-carotene production of the recombinants was monitored by introducing the reporter plasmid pPCB15 containing β-carotene biosynthesis genes. The λ-Red recombinase expression plasmid pKD46 has a temperature-sensitive replication origin and can be eliminated at 37 °C.

### 2.3. P1 transduction and marker removal

To combine the *kan*-$P_{T5}$-isoprenoid genes into a host strain *E. coli* MG1655 containing a reporter plasmid pPCB15 P1 transduction was used in combination with the Flp/FRT site-specific recombinase system for marker

Table 1
Primers for PCR amplification of *kan*, $P_{T5}$, and *kan*-$P_{T5}$ fragments

| PCR product | Template | Primer name | Primer sequence |
|---|---|---|---|
| *Kan* | pKD4 | 5′-*kan*(*dxs*) | TGGAAGCGCTAGCGGACTACATCATCCAGCGTAATAAATAA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*(*dxs*) | GAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA TATGAATATCCTCCTTAGTTCC[b] |
| | pKD4 | 5′-*kan*(*idi*) | TCTGATGCGCAAGCTGAAGAAAAATGAGCATGGAGAATAATATGA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*(*idi*) | GAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA TATGAATATCCTCCTTAGTTCC[b] |
| | pKD4 | 5′-*kan*(*ispC*) | GAAGCGGCGCTGGCAGACAAAGAAGCAGAACTGATGCAGTTCTGA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*(*ispC*) | GAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA TATGAATATCCTCCTTAGTTCC[b] |
| | pKD4 | 5′-*kan*(*ispDF*) | GACGCGTCGAAGCGCGCACAGTCTGCGGGGCAAAACAATCGATAA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*(*ispDF*) | GAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA TATGAATATCCTCCTTAGTTCC[b] |
| | pKD4 | 5′-*kan*(*ispH*) | TTTGATATTGAAGTGCTGGAAATCGATCCGGCACTGGAGGCGTAA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*(*ispH*) | GAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA TATGAATATCCTCCTTAGTTCC[b] |
| $P_{T5}$ | pQE30 | 5′-*T5*(*dxs*) | TAACCTATAAAAATAGGCGTATCACGAGGCCC |
| | | 3′-*T5*(*dxs*) | GGAGTCGACCAGTGCCAGGGTCGGGTATTTGGCAATATCAAAACTCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pQE30 | 5′-*T5*(*idi*) | TAACCTATAAAAATAGGCGTATCACGAGGCCC |
| | | 3′-*T5*(*idi*) | TGGGAACTCCCTGTGCATTCAATAAAATGACGTGTTCCGTTTGCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pQE30 | 5′-*T5*(*ispC*) | TAACCTATAAAAATAGGCGTATCACGAGGCCC |
| | | 3′-*T5*(*ispC*) | TGCAACCAATCGAGCCGGTCGAGCCCAGAATGGTGAGTTGCTTCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pQE30 | 5′-*T5*(*ispDF*) | TAACCTATAAAAATAGGCGTATCACGAGGCCC |
| | | 3′-*T5*(*ispDF*) | CGGCCGCCGGAACCACGGCGCAAACATCCAAATGAGTGGTTGCCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pQE30 | 5′-*T5*(*ispH*) | TAACCTATAAAAATAGGCGTATCACGAGGCCC |
| | | 3′-*T5*(*ispH*) | CTACCCCGGCACAAAAACCACGTGGGTTGGCCAACAGGATCTGCAT AGTTAATTTCTCCTCTTTAATG[c] |
| *kan*-$P_{T5}$ | pSUH5 | 5′-*kan*/*T5*(*ispE*) | GGTCAACGCATCAAGTTAAAAATGGATAACTGGATAGTGAAATAA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*/*T5*(*ispE*) | ATAAAAACAGATTAAGTTTTGCCGGAGAGGGCCACTGTGTCCGCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pSUH5 | 5′-*kan*/*T5*(*ispG*) | GTTGCGCGTCTGACCCTCAATGCCGAACAATCACCGGCGCAGTAA CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*/*T5*(*ispG*) | AAATACGTGTTGATTTTCTACGTTGAATTGGAGCCTGGTTATGCAT AGTTAATTTCTCCTCTTTAATG[c] |
| | pSUH5 | 5′-*kan*/*T5*(*ispB*) | ACCATAAACCCTAAGTTGCCTTTGTTCACAGTAAGGTAATCGGGG CGTCTTGAGCGATTGTGTAG[a] |
| | | 3′-*kan*/*T5*(*ispB*) | CGCCATATCTTGCGCGGTTAACTCATTGATTTTTTCTAAATTCAT AGTTAATTTCTCCTCTTTAATG[c] |

The underlined sequences illustrate homology patch chosen to match sequences in [a]the upstream region of the chromosomal integration site, [b]the 5′-end region of the $P_{T5}$ fragment, and [c]the downstream region of the chromosomal integration site, while the remainder is the priming sequence.

removal. P1 transduction using $P1_{vir}$ was performed as described (Miller, 1972). After P1 transduction, the cells were spread on LB plates containing both 25 μg/ml kanamycin and 25 μg/ml chloramphenicol, and incubated at 37 °C for 2 days to screen visually for darker color reflecting an increased level of β-carotene.

The transductants were electro-transformed with pCP20 (*amp*$^R$) containing Flp recombinase gene, and grown on LB plate containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 30 °C overnight. The Flp recombinase excises the kanamycin gene flanked by FRT sites from the chromosome. After growth at 42 °C for one day to cure the strain of the pCP20 plasmid that has a temperature-sensitive replication origin, ampicillin and kanamycin sensitivity were tested to verify loss of pCP20 and the kanamycin marker. The inserted $P_{T5}$ promoter DNA element is expected to remain in the chromosome.

### 2.4. Verification of chromosomal integration by PCR analysis

The correct integration of the PCR fragment(s) into the *E. coli* chromosome and subsequent elimination of the kanamycin marker were verified by colony PCR analysis. A colony was resuspended in 25 μl of PCR reaction mixture containing the appropriate primer pair, and run for 30 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for hybridization and 3 min at 72 °C for elongation. Test primers were chosen to amplify regions located either in the kanamycin or $P_{T5}$ promoter and the vicinity of the integration site. PCR products were run on 1% agarose gel in 1X TBE, 130 V for 40–50 min.

### 2.5. Measurement of carotenoid production

Production of β-carotene was quantified by measuring the absorption of the acetone-extracted β-carotene at 450 nm (Abe et al., 1998) and then normalizing it to the cell density ($OD_{600nm}$). The chromosomally engineered and control *E. coli* strains harboring a β-carotene biosynthesis expression plasmid, either pPCB15 or pDCQ108, were grown in 5 ml LB containing appropriate antibiotic at 37 °C for 24 h, and harvested by centrifugation at 4000 rpm for 10 min. β-carotene pigments were extracted from the cell pellet by resuspending in 300 μl of acetone and then homogenizing cells with glass beads in a Bead-Beater

(Biospec Products, Bartlesville, OK) for 30 s. After centrifuging the sample at 14,000 rpm for 1 min, the absorption spectrum of the acetone-soluble fraction containing β-carotene was measured by using an Ultrospec 3000 spectrophotomer (Amersham Biosciences, Piscataway, NJ).

## 3. Results

### 3.1. Chromosomal promoter replacement of the isoprenoid biosynthetic genes

Expression of the isoprenoid biosynthetic genes was analyzed from the *E. coli* 4290 open reading frames (ORFs) microarray experiments previously performed by Wei et al. (2001). The isoprenoid transcript profiles of cells growing exponentially in LB medium, growing exponentially in minimal medium, and transitioning from the exponential phase to the stationary phase in minimal medium were analyzed. The fractional expression of the isoprenoid genes as a function of the summed expression of total ORFs, which were normalized by genomic DNA as a standard probe, was expressed in percent (%) and ranked by the expression level in Table 2.

Microarray data indicated that most of the isoprenoid biosynthetic genes except *ispG* are expressed at low level under normal growth conditions. To increase the expression level of the chromosomal copy of the isoprenoid genes in *E. coli* we replaced the native promoter of the isoprenoid genes by the bacteriophage T5 strong promoter ($P_{T5}$) (Deuschle et al., 1986). The isoprenoid biosynthetic genes, *ispAdxs, ispC, ispDispF, ispE, ispG, ispH, idi,* or *ispB* are scattered over the chromosome in different transcriptional units. PCR-mediated λ-Red homologous recombination has been used for the chromosomal promoter replacement of each gene(s). We have extended the use of a gene disruption method (Datsenko and Wanner, 2000) to allow chromosomal promoter replacement using one or two PCR fragment(s) containing a kanamycin selectable marker and the $P_{T5}$ promoter. The kanamycin resistance gene is flanked by FRT site so that the marker can be excised by Flp recombinase.

Two isoprenoid genes *dxs* and *idi* in the isoprenoid pathway encode the rate-controlling enzymes in the isoprenoid pathway (Hacker and Bramley, 1999; Wang et al., 1999). Using the two PCR fragments integration method the upstream regions of *dxs* and, separately, *idi* was replaced by a $P_{T5}$ promoter containing a ribosomal binding site (rbs) as described in Fig. 2A, B. Primers for PCR of the kanamycin marker and $P_{T5}$ promoter fragments were designed to replace the upstream region (24 bp) of the *dxs* ORF and the putative promoter region (245 bp) of the *idi* ORF with the $P_{T5}$ promoter (Table 1). The two PCR fragments were co-transformed into *E. coli* MC1061 harboring both a λ-Red recombinase expression plasmid pKD46 and a reporter plasmid pPCB15 carrying β-carotene biosynthetic genes by electrophoresis. Successful recombinants having correctly integrated the kanamycin marker and $P_{T5}$ promoter were easily identified based on the increased color intensity imparted by β-carotene accumulation in cell. The increased production of β-carotene resulted from increased expression level of either *dxs* or *idi* genes under the control of $P_{T5}$ promoter. The co-integrated kanamycin marker and $P_{T5}$ promoter in the front of *dxs* or *idi* ORF was verified by colony PCR analysis with different sets of test primer (Fig. 2C, D). The strains were designated as MC1061 *kan*-$P_{T5}$-*dxs* or MC1061 *kan*-$P_{T5}$-*idi*. Likewise, the non-coding region upstream of *ispC*, *ispDispF*, or *ispH* also was replaced by $P_{T5}$ promoter along with the kanamycin selectable marker.

Table 2
Expression rank of the isoprenoid genes

| | LB medium Exponential phase[a] | | Minimal medium Exponential phase[a] | | Minimal medium Transition phase[b] | |
|---|---|---|---|---|---|---|
| | Rank[c] | %[d] | Rank | % | Rank | % |
| *dxs* | 995 | 0.0193 | 1619 | 0.0137 | 2214 | 0.0078 |
| *ispC (dxr)* | 2649 | 0.0079 | 2884 | 0.0062 | 2170 | 0.008 |
| *ispD (ygbP)* | 1412 | 0.0148 | 2521 | 0.0079 | 2049 | 0.0087 |
| *ispE (ychB)* | 1880 | 0.0114 | 741 | 0.0274 | 915 | 0.0215 |
| *ispF (ygbB)* | 2199 | 0.0099 | 2375 | 0.0087 | 1875 | 0.0099 |
| *ispG (gcpE)* | 395 | 0.0376 | 493 | 0.0363 | 315 | 0.0537 |
| *ispH (lytB)* | ND | ND | ND | ND | ND | ND |
| *idi* | 3238 | 0.0057 | 3668 | 0.003 | 1955 | 0.0093 |
| *ispA* | 1214 | 0.0165 | 921 | 0.0228 | 1533 | 0.0126 |

*E. coli* MG1655 was grown with aeration in either LB or minimal medium, M9 supplemented with 0.4% glucose at 37 °C.

[a]RNA transcripts obtained from cells growing exponentially in LB or minimal medium.

[b]RNA transcripts obtained from cells transitioning to the stationary phase in minimal medium.

[c]Expression rank of particular gene out of 4290 ORFs with 1 being the most highly expressed gene.

[d]Fraction of particular transcript/total transcripts hybridizing to all ORFs on the microarry expressed in percent (%). Note that average expression is estimated to be about 0.02% (Wei et al., 2001).

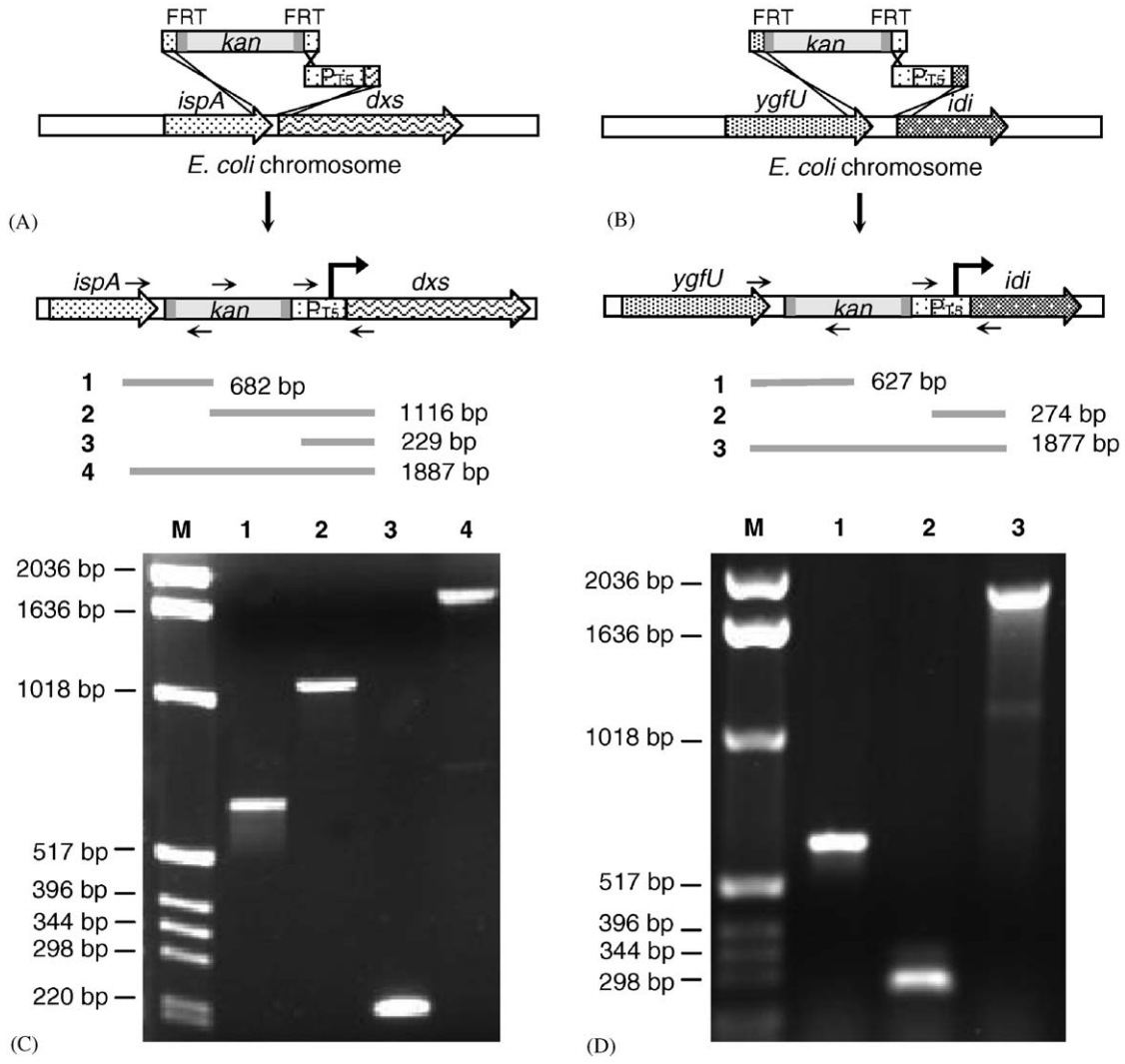


Fig. 2. Chromosomal promoter replacement of *dxs* or *idi* gene by PCR-based homologous recombination mediated by λ-Red recombinase. Kanamycin selectable marker and $P_{T5}$ promoter fragments were inserted to replace the non-coding region (24 bp) upstream of *dxs* (A) and the putative promoter region (245 bp) of *idi* (B). The co-integration of the kanamycin marker and $P_{T5}$ promoter in the front of *dxs* (C) or *idi* (D) was verified by colony PCR analysis with different sets of test primer. The bars represent an expected size of PCR fragments amplified using corresponding primer sets (shown by arrows).

The colony of MC1061 *kan*-$P_{T5}$-*ispDispF* showed slight increase in intensity of yellow color, while MC1061 *kan*-$P_{T5}$-*ispC* and MC1061 *kan*-$P_{T5}$-*ispH* showed no significant color change.

The two PCR fragment integration method yielded routinely 1–10 kanamycin resistant recombinants per transformation with 0.5–1 μg PCR fragments. The low efficiency of the recombination is because two PCR fragments integration requires triple homologous recombination events between the *E. coli* chromosome and homology arms of two linear PCR fragments (Fig. 2A, B). To achieve the high recombination efficiency for the chromosomal promoter replacement of other isoprenoid genes, we used the one PCR fragment variation containing a fused kanamycin marker-$P_{T5}$ promoter. The non-coding region upstream of *ispE*, *ispG*, *or ispB* was replaced with a fused kanamycin marker-$P_{T5}$ promoter fragment so that the gene expression is under the control of $P_{T5}$ promoter, generating MC1061 *kan*-$P_{T5}$-*ispE, kan*-$P_{T5}$-*ispG,* or *kan*-$P_{T5}$-*ispB.* The one PCR fragment method yielded 100–300 kanamycin resistant recombinants per transformation with 0.5–1 μg PCR fragment. The integration of the fused kanamycin marker-$P_{T5}$ promoter into a chromosomal target site was verified by PCR analysis.

β-carotene production of individual MC1061 *kan*-$P_{T5}$-isoprenoid gene(s) strain harboring pPCB15 was quantified by measuring the absobance of the acetone-extracted β-carotene at 450 nm (Table 3). Placement of the *dxs* or *idi* genes under the control of the $P_{T5}$ promoter resulted in 2.0- or 1.4-fold increases in β-carotene level, respectively. In addition, MC1061 *kan*-$P_{T5}$-*ispDF, kan*-$P_{T5}$-*ispE,* or *kan*-$P_{T5}$-*ispB* exhibited 1.4-, 1.2- or 1.3-fold increase in

Table 3
$\beta$-carotene production of chromosomal promoter engineered *E. coli*

| Strain | Plasmid | Chromosomal modifications | $OD_{453}/OD_{600}$ (average) | Relative $\beta$-carotene production |
|---|---|---|---|---|
| MC1061 | pPCB15 | Control | 0.14±0.00 | 1 |
| | | *kan*-$P_{T5}$-*dxs* | 0.28±0.05 | 2 |
| | | *kan*-$P_{T5}$-*idi* | 0.20±0.02 | 1.4 |
| | | *kan*-$P_{T5}$-*ispC* | 0.11±0.03 | 0.8 |
| | | *kan*-$P_{T5}$-*ispDispF* | 0.19±0.03 | 1.4 |
| | | *kan*-$P_{T5}$-*ispE* | 0.17±0.02 | 1.2 |
| | | *kan*-$P_{T5}$-*ispG* | 0.13±0.01 | 0.9 |
| | | *kan*-$P_{T5}$-*ispH* | 0.12±0.01 | 0.9 |
| | | *kan*-$P_{T5}$-*ispB* | 0.18±0.00 | 1.3 |
| MG1655 | pPCB15 | Control | 0.04±0.01 | 1 |
| | | $P_{T5}$-*dxs* | 0.13±0.02 | 3.3 |
| | | $P_{T5}$-*dxs* $P_{T5}$-*idi* | 0.16±0.01 | 4 |
| | | $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* | 0.18±0.01 | 4.5 |
| | | $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispDispF* | 0.18±0.01 | 4.5 |
| | | $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDispF* | 0.25±0.01 | 6.3 |
| | pDCQ108 | $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDispF* | 0.98±0.05 | 24.5 |

$\beta$-carotene level, respectively, while MC1061 *kan*-$P_{T5}$-*ispC, kan*-$P_{T5}$-*ispG,* or *kan*-$P_{T5}$-*ispH* showed no increase in $\beta$-carotene production when compared to the control strain.

### 3.2. Chromosomal stacking of kan-$P_{T5}$-isoprenoid gene(s) using P1 transduction in combination with Flp/FRT site-specific recombination system

P1 transduction in combination with Flp/FRT site-specific recombination system has been used to combine the multiple *kan*-$P_{T5}$-isoprenoid genes into one host strain. *E. coli* MG1655 harboring a $\beta$-carotene biosynthesis plasmid reporter pPCB15 ($cam^R$) was used as a host strain for stacking the various *kan*-$P_{T5}$-isoprenoid genes. First, we stacked the *kan*-$P_{T5}$-*dxs* genetic trait into MG1655 chromosome because it improved $\beta$-carotene production at highest level among the various *kan*-$P_{T5}$-isoprenoid genes. The *kan*-$P_{T5}$-*dxs* was transferred by P1 transduction from MC1061 *kan*-$P_{T5}$-*dxs* strain into MG1655 (pPCB15). The kanamycin resistance gene flanked by FRT sites was excised subsequently by transient transformation of the temperature-sensitive Flp expression plasmid pCP20 (Cherepanov and Wackernagel, 1995). This markerless, chromosomally modified strain was designated as MG1655 $P_{T5}$-*dxs,* which is ready for stacking other *kan*-$P_{T5}$-isoprenoid genes.

### 3.3. Combinatorial stacking of the $P_{T5}$-isoprenoid genes using pooled mixtures of P1 lysates

Conventional P1 transduction can transfer only one determined genetic trait at a time from one host to another host. We have developed combinatorial P1 transduction for transferring multiple genetic traits into an *E. coli* host in a parallel fashion using pooled mixtures of P1 lysates followed by screening the desired genetic trait. We took an advantage of easy yellow color-screening for selecting higher production of $\beta$-carotene than parental strain. The strategy for the combinatorial stacking of *kan*-$P_{T5}$-isoprenoid genes is shown in Fig. 3.

To identify other rate-limiting enzymes beside the *dxs* gene in the isoprenoid flux, we have taken the combinatorial approach for stacking other $P_{T5}$-isoprenoid genes in MG1655 $P_{T5}$-*dxs* chromosome. Equal amounts (pfu) of phage P1 lysates from MC1061 strains containing different $P_{T5}$-isoprenoid genes (*kan*-$P_{T5}$-*ispC, kan*-$P_{T5}$-*ispDF, kan*-$P_{T5}$-*ispE, kan*-$P_{T5}$-*ispG, kan*-$P_{T5}$-*ispH, kan*-$P_{T5}$-*idi,* or *kan*-$P_{T5}$-*ispB*) were mixed. The recipient MG1655 $P_{T5}$-*dxs* (pPCB15) was infected with the pooled P1 lysate, and kanamycin-resistant transductants were visually screened for increased yellow color. After incubation at 37 °C for 1–2 days, six colonies among those showing the deepest yellow color out of 430 kanamycin-resistant transductants were selected. The selected colonies accumulated higher $\beta$-carotene level than the parental MG1655 $P_{T5}$-*dxs* strain. The transferred *kan*-$P_{T5}$-isoprenoid gene(s) of the selected colonies was identified by PCR analysis with a 5′-primer complementary to the sequence within the kanamycin gene and a 3′-primer complementary to the sequence within each isoprenoid gene(s). PCR analysis showed that four colonies contained *kan*-$P_{T5}$-*idi,* one contained *kan*-$P_{T5}$-*ispB,* and one contained *kan*-$P_{T5}$-*ispG.* Among these, the colonies carrying *kan*-$P_{T5}$-*idi* exhibited the deepest yellow color on an LB plate containing chloramphenicol after growth at 37 °C for 2 days. The kanamycin selectable marker from the chromosome of MG1655 $P_{T5}$-*dxs kan*-$P_{T5}$-*idi* was eliminated by transient transformation of Flp site-specific recombinase expression pCP20 plasmid, yielding MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi*.

MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* (pPCB15) was used as a recipient cell for the second round of P1 transduction with the P1 lysate pool described above in order to further

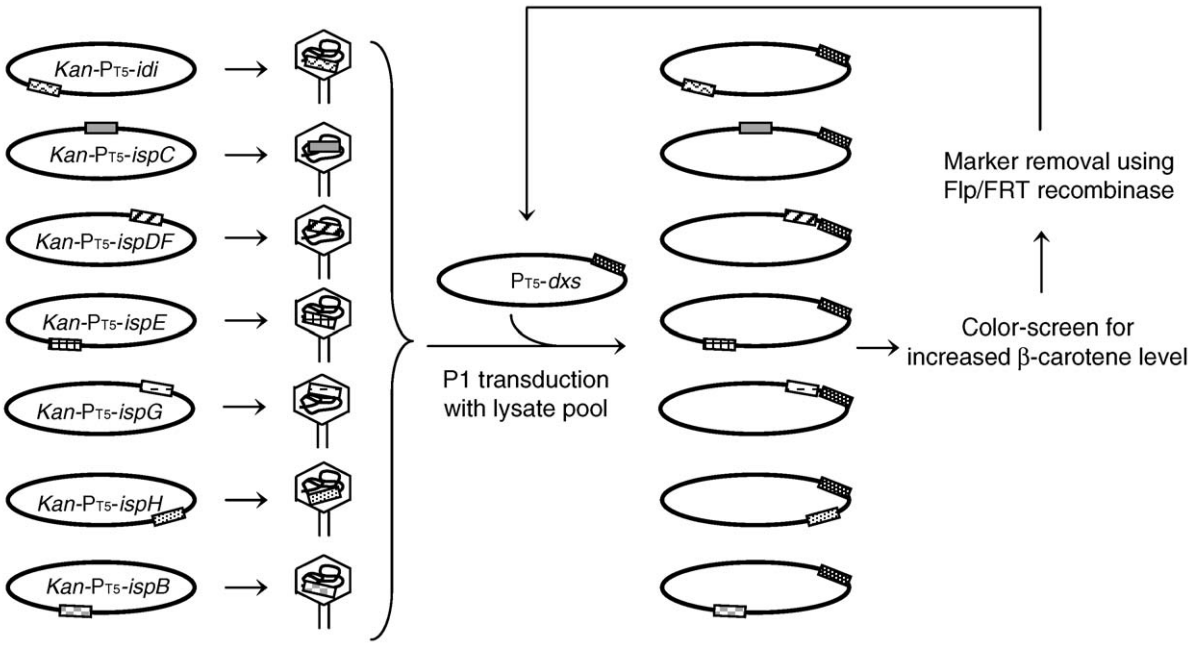


Fig. 3. In vivo combinatorial P1 transduction for transferring multiple *kan*-$P_{T5}$-isoprenoid genes into an *E. coli* host in a parallel fashion using pooled P1 lysate followed by color-screening with the characteristic yellow $\beta$-carotene color for selecting higher production of $\beta$-carotene than parental strain.

increase $\beta$-carotene production. Out of more than 1000 kanamycin and chloramphenicol resistance tranductants, ten colonies among those showing the deepest yellow color were picked, and PCR-analyzed to identify *kan*-$P_{T5}$-isoprenoid gene(s) stacked on the chromosome. Interestingly, all ten colonies contained *kan*-$P_{T5}$-*ispB,* and showed deeper yellow color than the parental MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* strain. The $\beta$-carotene production increase of the $P_{T5}$-promoter replacement of *ispB* gene is unexpected because the enzyme octaprenyl diphosphate synthase encoded by *ispB* gene was believed to divert the carbon flow from the isoprenoid pathway to quinone biosynthesis. We originally constructed $P_{T5}$-*ispB* to explore the impact of the over-expression of *ispB* gene, which is expected to drain FPP intermediate away from the isoprenoid pathway (Fig. 1), on the carotenoid biosynthesis. However, as MC1061 *kan*-$P_{T5}$-*ispB* exhibited an opposite effect, a slight increase in $\beta$-carotene level, P1 stock derived from the *kan*-$P_{T5}$-*ispB* strain was added to the lysate pool.

After marker removal, the resultant MC1061 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* (pPCB15) was used as a recipient for the third round of P1 transduction with the P1 lysate mixture pool obtained from MC1061 strains containing different $P_{T5}$-isoprenoid genes (*kan*-$P_{T5}$-*ispC, kan*-$P_{T5}$-*ispDF, kan*-$P_{T5}$-*ispE, kan*-$P_{T5}$-*ispG,* or *kan*-$P_{T5}$-*ispH*). Among ~900 kanamycin and chloramphenicol resistance tranductants four colonies among those showing the deeper-color than the parental strain MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* were picked. PCR analysis indicated that all four colonies carry *kan*-$P_{T5}$-*ispDF*. The kanamycin marker was removed, generating MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF*. Additional rounds of P1 transduction with the pooled lysate did not lead to further color-increase in comparison to MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF*.

The spectrophotometric quantification of $\beta$-carotene production showed that MG1655 $P_{T5}$-*dxs*, MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi*, MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB*, and MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* strains accumulated 3.3-, 4-, 4.5- and 6.3-fold higher $\beta$-carotene pigment in cell, respectively, than the control MG1655 strain (Table 3). We observed that the effect of $P_{T5}$-*dxs* fusion on $\beta$-carotene production is different between in MG1655 $P_{T5}$-*dxs* (3.3-fold) and MC1061 $P_{T5}$-*dxs* (2.0-fold). As shown in Table 3, the specific productivity ($OD_{453}$/$OD_{600}$) of $\beta$-carotene in MC1061 background generally was higher than that in MG1655. However, the cell density ($OD_{600}$) in MC1061 background was approximately 2-fold lower than that in MG1655. The combinatorial stacking of $P_{T5}$-isoprenoid gene(s) starting from MG1655 $P_{T5}$-*dxs* resulted in gradual increase in $\beta$-carotene accumulation in cells as the process was repeated. This approach allows for quickly mixing and matching of $P_{T5}$-isoprenoid genes for maximizing $\beta$-carotene production in *E. coli*. We also constructed MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispDF* by P1 transduction with a P1 lysate obtained from MC1061 *kan*-$P_{T5}$-*ispDF* to MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* (pPCB15) and subsequently eliminated the kanamycin marker. The $\beta$-carotene level accumulated by MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispDF* strain was 4.5-fold of that of the control strain (Table 3).

The enhanced $\beta$-carotene production in the engineered strains are expected due to the increased isoprenoid flux by the over-expression of the key isoprenoid genes under the control of $P_{T5}$ promoter. To examine how much the

expression level of the isoprenoid genes is increased under the control of $P_{T5}$ promoter, microarry experiments of MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* versus MG1655 control strains were performed with the cells harvested at log phase at 37 °C. Microarray data indicated that the expression levels of the *dxs, idi*, *ispD*, *ispF*, and *ispB* genes were 4-, 36-, 4-, 3-, and 40-fold, respectively, higher in the engineered strain than the control strain (Suh et al., unpublished data). The fold increase depends on the initial expression level of the genes under the control of its endogenous promoter. No quantitative correlation between increased expression level of the isoprenoid genes and increased β-carotene level may be due to the regulation at the level of translation and enzyme activity as well as transcription.

### 3.4. Effect of the copy number of the carotenoid gene expression plasmid on β-carotene production

In the previous section, we have focused on the metabolic engineering to increase carbon flux through the isoprenoid pathway. For the high isoprenoid flux strain, the downstream carotenoid pathway may become the bottleneck for β-carotene production because of the low-copy vector pPCB15 containing the β-carotene biosynthesis genes *crtEXYIB* from *P. stewartii*. We found that the transformation with a medium-copy plasmid pDCQ108, which also contains the *P. stewartii crtEXYIB,* into MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* resulted in an approximately 4-fold increase in β-carotene production when compared to MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* (pPCB15), an increase of 24.5-fold over initial levels of MG1655 (pPCB15) (Table 3). The titer of β-carotene in MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* (pDCQ108) was approximately 6 mg per gram of dry cell weight.

The growth rate was measured because the growth rate is an important factor for the specific productivity. The doubling time of the control strain MG1655 (pPCB15) and the high titer strain MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* (pDCQ108) was 40 and 65 min in LB medium, respectively, showing an inverse trend between the growth rate and β-carotene production. The final cell density of MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* (pPCB15) or (pDCQ108) after 24 h incubation at 37 °C was similar to that of the control strain MG1655 (pPCB15) or (pDCQ108), respectively. Stability of MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi* $P_{T5}$-*ispB* $P_{T5}$-*ispDF* (pPCB15) strain was confirmed by 5 times of serial transfers (1000-fold dilution) in liquid culture, followed by plating on LB containing 25 μg/ml chloramphenicol to test whether the colonies retain increased pigmentation (data not shown).

## 4. Discussion

All carotenoids, more than 600 carotenoids identified so far, are synthesized via an isoprenoid pathway. To develop a potential production host for various carotenoids we engineered *E. coli* to increase isoprenoid flux by chromosomal promoter engineering of the native isoprenoid pathway. Modulation of chromosomal gene expression is advantageous in terms of segregational instability, metabolic burden, and potential for gene rearrangements than over-expression of genes on multi-copy vectors. In addition, chromosomal engineering for increased isoprenoid flux has an advantage over plasmid-based engineering because the high isoprenoid flux *E. coli* can be used as a universal production platform for all isoprenoid compounds including carotenoids, terpenoids, and sterols just by introducing corresponding genes.

Our transcript analysis for the isoprenoid genes in *E. coli* suggested that the isoprenoid genes under normal growth condition are marginally expressed to provide minimal requirements of essential cellular isoprenoid compounds such as quinones and dolichols. To increase *E. coli* isoprenoid flux, which is naturally low, we replaced the native promoter of the isoprenoid genes with a strong promoter. The bacteriophage T5 promoter ($P_{T5}$) has been used as a strong promoter because in vivo, the strength of the $P_{T5}$ promoter was equivalent to that of phage $P_L$ promoter, and approximately 5-fold higher that that of lac promoter (Deuschle et al., 1986). To date, chromosomal engineering by controlling the expression level of endogenous gene(s) has not been used for metabolic engineering because of insufficient genetic regulatory knowledge and genetic tools. Recently, a new method for chromosomal promoter replacement using PCR-based homologous recombination mediated by λ-Red recombinase has been developed for metabolic pathway engineering (Meynial-Salles et al., 2005). In this method, the desired promoter(s) and regulatory element(s) was introduced into a linear DNA cassette by two steps of PCR amplification (1) with a primer containing synthetic promoters, and then (2) with a primer containing a mRNA-stabilizing element and ribosomal binding site. In parallel, we have developed a two PCR fragment integration method for chromosomal promoter replacement. Both methods enable chromosomal promoter modification in a one step process by direct integration of a desired promoter and regulatory element, but each has distinct limitations. The method of using two steps of PCR has a limitation in size of promoter(s) and regulatory element(s), while two PCR fragment chromosomal integration method has a lower efficiency in chromosomal integration than one PCR fragment integration method.

Our results demonstrate successful chromosomal promoter replacement of the isoprenoid pathway improving β-carotene production in *E. coli*. The *dxs* gene placed under the control of $P_{T5}$ promoter resulted in 2.0–3.3-fold increase in β-carotene production. The increased level is similar to the previously reported results that showed 2.4–2.9-fold increase in the production of lycopene when over-expressing *dxs* from a multi-copy plasmid (Jones et al., 2000). Promoter replacement of other isoprenoid genes with $P_{T5}$ promoter also affected β-carotene level.

However, $P_{T5}$-*dxs* among $P_{T5}$-isoprenoid genes has the biggest impact on β-carotene production, implying that D-1-deoxyxylulose 5-phosphate synthase encoded by the *dxs* gene is a primary rate-limiting step in the isoprenoid pathway. Considering that the basal expression of *dxs* is relatively higher than other isoprenoid genes (Table 2), enzymatic activity and/or regulation may also play a role in control of the isoprenoid flux. Interestingly, the *dxs* corresponding to the first step in the DXP pathway, which catalyzes the condensation reaction of pyruvate and D-glyceraldehyde 3-phosphate to D-1-deoxyxylulose 5-phosphate (DXP), is clustered with *ispA* of the last step to form the *ispAdxs* operon (Lois et al., 1998). We also constructed MG1655 $P_{T5}$-*ispAdxs* in which the $P_{T5}$ promoter drove the *ispAdxs* operon, and found an increased β-carotene level similar to that of MG1655 $P_{T5}$-*dxs* (data not shown).

The traditional strategy for metabolic engineering is to over-express enzymes(s) specified as the rate-limiting step in a multi-copy expression vector, which may cause a metabolic imbalance (Snoep et al., 1995). Metabolic control theory (Kacser and Burns, 1973) has shown that rate controlling steps can be distributed over many enzymes in a pathway. Increased expression of one rate-limiting enzyme may result in shifting the flux control to other steps in the pathway. Our P1 transduction using a pool of lysate for stacking the promoter engineered isoprenoid genes has been developed in view of such theory and subsequent experimental confirmation. The present approach enables us to identify sequential bottlenecks in isoprenoid flux.

From the first round of combinatorial stacking upon $P_{T5}$-*dxs,* the isopentenyl pyrophosphate isomerase encoded by *idi* gene has been suggested as a rate-controlling enzyme in the isoprenoid flux. The *idi* gene that encodes isopentenyl diphosphate isomerase that catalyzes the inter-conversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is nonessential in *E. coli* (Hahn et al., 1999). Consistently, genetic and biochemical studies demonstrated that IPP and DMAPP can be separately synthesized from HMBPP by the enzyme encoded by *ispH* (Rohdich et al., 2002; Adam et al., 2002). Microarray experiment showed that the hybridization signal of the *idi* gene was very low, not exceeding the noise by a factor of 2, suggesting that the *idi* gene is apparently not expressed or expressed at very low level under normal growth condition (Table 2). Although the *idi* gene apparently is not essential for the biosynthesis of IPP and DMAPP, our results indicate that *idi* gene plays an important role in control of the isoprenoid flux. The similar effect of *idi* gene on β-carotene level in *E. coli* has been previously observed (Misawa and Shimada, 1998). The expression of *idi* gene from *Phaffia rhodozyma, Haematococcus plucialis*, and *Saccharomyces cerevisiae* in *E. coli* resulted in 1.5–2.7-fold increase in β-carotene level. Transformation of *E. coli* with the genes for over-expression of *dxs, dxr*, and *idi* resulted in an increase production of carotenoids by a factor of 3.5 (Albrecht et al., 1999), which is similar to the increased level (3.5-fold) of β-carotene in MG1655 $P_{T5}$-*dxs* $P_{T5}$-*idi*.

We have subsequently observed that the increased expression of *ispB* gene under an exogenous strong promoter enhances β-carotene production. The *E. coli ispB* encoding octaprenyl-diphosphate synthase is responsible for the synthesis of the isoprenoid side chain of quinone by sequential addition of 5 IPP to FPP. The mechanism of how the increased expression of *ispB* gene under the control of $P_{T5}$ promoter increases accumulation of the β-carotene production is not clear yet. One possibility is that the increased expression of the *ispB* gene may enhance the flux from IPP and DMAPP to FPP and/or from FPP to GGPP that serves as a precursor of β-carotene. Sequence analysis of IspB revealed significant similarity to the FPP synthase encoded by *ispA* and GGPP synthase encoded by *crtE* (Fujisaki et al., 1990; Jeong et al., 1993). Misawa et al. (1990) demonstrated that *E. coli* is able to accumulate small amounts of carotenoids even in the absence of the geranylgeranyl pyrophosphate synthase gene (*crtE*), suggesting that *E. coli* has an enzyme capable of producing GGPP. Further work, however, will be necessary to understand the role of IspB and the relationship between the biosynthesis of an isoprenoid side chain of ubiquinone and carotenoids.

The enzymes encoded by *ispD* (*ygbP*) and *ispF* (*ygbB*) catalyze CTP-dependent cytidylation and cyclization with loss of CMP, respectively, during the steps of the conversion of MEP to MEC (Campos et al., 2001). In *E. coli* the *ispD* and *ispF* genes are adjacent and appear to be same operon. Our chromosomal promoter replacement study showed that both enzymes are rate-controlling steps in isoprenoid flux. Basal expression levels of *ispD* and *ispF* genes were not high at exponential phase in LB and minimal media (Table 2). Promoter replacement with $P_{T5}$ promoter resulted in a similar increase in the expression level of *ispD* (4-fold) and *ispF* (3-fold), suggesting that both genes are transcribed as one operon.

Recent in vivo and in vitro studies have shown that the enzymes encoded by *ispG* (*gcpE*) and *ispH* (*lytB*) catalyze the sequential reductive reactions for the conversion of MEC to IPP and DMAPP with a ratio of 6:1 (Rohdich et al., 2003). Both enzymes share structural and functional similarities, globular proteins, having three conserved cysteine residues, and catalyzing reduction of alcohol (McAteer et al., 2001). Transcript analysis revealed that the basal expression of *ispG* gene is highest among isoprenoid genes, which may explain why the promoter replacement with $P_{T5}$ promoter does not affect the isoprenoid flux.

The $P_{T5}$-*dxs,* $P_{T5}$-*idi,* $P_{T5}$-*ispB,* and $P_{T5}$-*ispDF* combined in the final strain were the genes that exhibited positive impact on β-carotene production by promoter replacement with T5 promoter in the single promoter replaced strain. Increased β-carotene production apparently results from the additive effect of each $P_{T5}$-isoprenoid gene. We also stacked other $P_{T5}$-isoprenoid genes in linear fashion, but

found that $P_{T5}$-*ispE,* $P_{T5}$-*ispG,* and $P_{T5}$-*ispH* have no effect on increasing β-carotene production.

It has been speculated that the maximum limits for carotenoid production in a non-carotenogenic *E. coli* may be around 1.6 mg/g cell dry weight due to limits of membranes storage capacity for lipophilic carotenoids (Albrecht et al., 1999; Sandmann et al., 1999). In previous study, a maximum lycopene content of 45 mg/g dry cell weight was obtained by overexpression of *dxs* and mutant *gps* encoding geranylgeranyl diphophate (GGPP) synthase (Wang et al., 2000). The reproducibility for such high titers, however, needs to be confirmed because the carotenoid levels in *E. coli* cells transformed with isoprenoid and carotenoid genes on multi-copy plasmids in all other reports have been typically a range of 1–2 mg/g dry cell weight (Kajiwara et al., 1995; Albrecht et al., 1999). Recently, high production (5 mg/g dry cell weight) of bixin, a dicarboxylic monomethyl ester apocarotenoid, in recombinant *E. coli* was attained (Bouvier et al., 2003). Bixin may be easier to accumulate in cell because it is a charged molecule.

In this work, we engineered chromosomal promoter of the isoprenoid biosynthetic genes, and then combined the promoter engineered isoprenoid genes in successive rounds of P1 transduction to sequentially relieve the rate controlling steps for the isoprenoid flux. This approach allows success in engineering an *E. coli* capable of producing β-carotene up to 6 mg/g of dry cell weight, which is a much higher level than the previously proposed maximum limit. This level is comparable to the carotenoid level accumulated in the carotenoid-producing strains; red yeast *Phaffia, Brevibacterium, Paracoccus* and algae *Harmatococcus. E. coli* offers advantages in terms of higher growth rate and ease of genetic manipulation for novel carotenoid production. The engineered *E. coli* having a high isoprenoid flux can be used as a potential production platform for many carotenoids including lycopene, β-carotene, cantaxanthin, astaxanthin and novel carotenoids.

## Acknowledgments

We thank B. Wanner (Purdue Univ.) for sharing λ-Red strains and plasmids used in this work. We also appreciate provision of plasmids and helpful comments by our DuPont colleagues, P.C. Brzostowicz, Q. Cheng, S. Picataggio, and D. J. DiCosimo.

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