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Investigation of factors influencing production of the monocyclic carotenoid torulene in metabolically engineered *Escherichia coli*

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Abstract Factors influencing production of the monocyclic carotenoid torulene in recombinant *Escherichia coli* were investigated by modulating enzyme expression level, culture conditions, and engineering of the isoprenoid precursor pathway. The gene dosage of in vitro evolved lycopene cyclase *crtY2* significantly changed the carotenoid profile. A culture temperature of 28°C showed better production of torulene than 37°C while initial culture pH had no significant effect on torulene production. Glucose-containing LB, 2×YT, TB and MR media significantly repressed the production of torulene, and the other carotenoids lycopene, tetradehydrolycopene, and β-carotene, in *E. coli*. In contrast, glycerol-containing LB, 2×YT, TB, and MR media enhanced torulene production. Overexpression of *dxs*, *dxr*, *idi* and/or *isp*A, individually and combinatorially, enhanced torulene production up to 3.1–3.3 fold. High torulene production was observed in a high dissolved oxygen level bioreactor in TB and MR media containing glycerol. Lycopene was efficiently converted into torulene during aerobic cultures, indicating that the engineered torulene synthesis pathway is well coordinated, and maintains the functionality and integrity of the carotenogenic enzyme complex.

Introduction

Carotenoids, produced by many microorganisms and plants, form an important class of natural pigments for biotechnology applications. These structurally diverse pigments have many different biological functions, such as species-specific coloration, photo protection, and light-harvesting, as well as serving as precursors for many hormones (Vershinin 1999). Carotenoids are commercially used as food colorants, animal feed supplements, in cosmetic and pharmaceutical applications and, more recently, as nutraceuticals. Presently, only a few carotenoids can be produced commercially by chemical synthesis, fermentation, or isolation, from a few abundant natural sources (Johnson and Schroeder 1995). The increasing industrial importance of carotenoids has led to renewed efforts to develop processes for the production of structurally diverse natural and non-natural carotenoids in useful quantities.

Carotenoids are derived from the general isoprenoid pathway (Fig. 1a). In *Escherichia coli*, the C15 isoprenoid farnesyl diphosphate (FPP) is extended with one C5 isoprene unit by a heterologous geranylgeranyl diphosphate (GGPP) synthase (*crtE*) to produce the C20 isoprenoid GGPP. Condensation of two molecules of GGPP by another heterologous enzyme, phytoene synthase crtB, yields the first colorless carotenoid structure phytoene. Further extension of this pathway with additional heterologous carotenoid-modifying genes yields different carotenoid structures in *E. coli* (Lee and Schmidt-Dannert 2002).

Recently we have developed an *E. coli* biosynthetic pathway for the fully conjugated acyclic carotenoid tetradehydrolycopene by gene shuffling of phytoene desaturase (*crtI*) (Fig. 1a). The evolved tetradehydrolycopene pathway was then extended with a shuffled lycopene cyclase gene (*crtY*) to establish a biosynthetic pathway for the monocyclic carotenoid torulene (Schmidt-Dannert et al. 2000). These in-vitro-evolved carotenoid pathways were further successfully extended with additional carotenogenic enzymes to produce a range of structurally novel acyclic oxocarotenoids and torulene derivatives (Lee et al. 2003). Torulene biosynthesis was selected as a model system for an investigation of factors governing recombinant production of novel carotenoids in *E. coli*. For efficient production of carotenoids in engineered *E. coli*, balanced expression of carotenogenic enzymes, optimized culture and/or production conditions are important (Lee and Schmidt-Dannert 2002). Efficient carotenoid produc-

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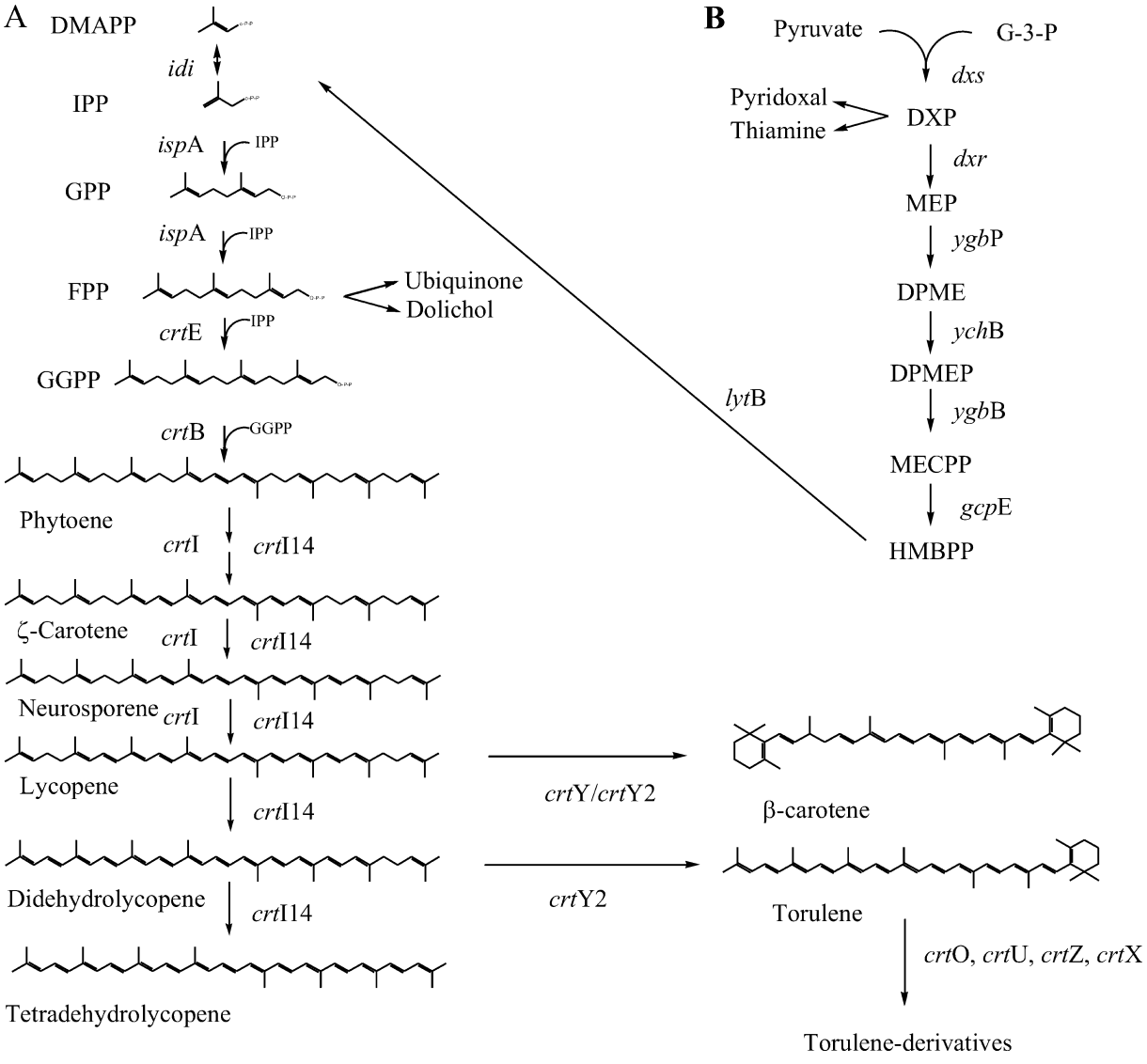

Fig. 1 In-vitro-evolved heterologous torulene (**a**) and native isoprenoid (**b**) biosynthetic pathways in *Escherichia coli*. **a** Biosynthetic pathway enzymes catalyzing individual reaction steps in torulene biosynthesis are shown sequentially: *CrtE* geranylgeranyl diphosphate (GGPP) synthase; *CrtB* phytoene synthase; *CrtI* phytoene desaturase; *CrtI14* mutant phytoene desaturase; *CrtY* lycopene β-cyclase; *CrtY2* mutant lycopene β-cyclase. *DMAPP* Dimethylallyl diphosphate. **b** Enzymes involved in isoprenoid biosynthesis in *E. coli* are shown sequentially: *dxs* 1-deoxy-D-xylose 5-phosphate (*DXP*) synthase; *dxr* 1-deoxy-D-xylose 5-phosphate reductase; *ygbP* 4-diphosphocytidyl-2C-methyl-D-erythritol (*DPME*) synthase; *ychB* 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; *ygbB* 2C-methyl-D-erythritol 2,4-cyclodiphosphate (*MECPP*) synthase; *gcpE* 1-hydroxy-2-methyl-2-butenyl-4-diphosphate (*HMBPP*) synthase; *lytB* lytB protein; *ispA* farnesyl diphosphate (FPP) synthase; *idi* isopentenyl diphosphate (*IPP*) isomerase

tion also requires sufficient supply and complete utilization of precursors without byproduct formation. In carotenoid-producing recombinant *E. coli*, the isoprenoid pathway supplies precursors for carotenoid formation (Fig. 1b).

In this article, we investigate torulene production in *E. coli* by (1) modulating the gene dosage of *crtY2*, (2) optimizing culture and/or medium conditions, and (3) metabolic engineering of the *E. coli* isoprenoid pathway. We also describe the first bioreactor study, to our knowledge, on recombinant carotenoid production in *E. coli*.

Materials and methods

Genes and plasmids

Genes encoding FPP synthase (*ispA*) (D00694), 1-deoxy-D-xylose 5-phosphate synthase (*dxs*) (D64771), 1-deoxy-D-xylose 5-phosphate reductase (*dxr*) (P45568), and isopentenyl diphosphate (IPP) isomerase (*idi*) (Q46822) were amplified from genomic DNA of *E. coli* JM109 (Stratagene, La Jolla, Calif.) using primers corresponding to the respective gene sequences. The 5′ PCR primer contained an *Xba*I or *Eco*RI site at its 5′ end, followed by an optimized Shine-Dalgarno sequence (underlined) and a translation start codon (bold) 5′-AGGAGGATTA-

CAAA**ATG**-3′, and the 3′ PCR primer contained a *Eco*RI or *Nco*I site at its 5′ end. PCR products were cloned into the corresponding sites of the constitutive expression plasmid pUCmod (Schmidt-Dannert et al. 2000), resulting in pUC-*ispA*, pUC-*dxs*, pUC-*dxr*, and pUC-*idi* (Table 1). The cloned *ispA*, *dxs*, *dxr* and *idi* were subcloned from pUCmod into the *Hin*dIII site, a *Sal*I site or a *Bam*HI site of pBBR1MCS-2 (Kovach et al. 1995) by amplification of the genes together with the modified constitutive *lac*-promoter using primers that introduce corresponding restriction enzyme sites at both sites to give pBBR-*ispA*, pBBR-*dxs*, pBBR-*dxr*, and pBBR-*idi*, respectively. The construction of plasmids expressing carotenoid biosynthetic genes, including lycopene (pAC-*crtE-crtB-crtI*), tetradehydrolycopene (pAC-*crtE-crtB-crtI14*) and β-carotene (pAC-*crtE-crtB-crtI14-crtY*), has been described previously (Schmidt-Dannert et al. 2000; Lee et al. 2003) (Table 1). *E. coli* JM109 was used to maintain plasmids and for carotenoid production.

DNA manipulations

Standard DNA manipulations were as described in Sambrook and Russell (2001). PCR products and DNA fragments were purified from agarose gels using Qiagen PCR purification and gel extraction kits (Qiagen, Hilden, Germany). Restriction enzymes, ligase, calf intestinal alkaline phosphatase and Vent DNA polymerase were purchased from New England Biolabs (Beverly, Mass.).

Media and culture conditions

Three different complex media, Luria Bertani (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter), 2× YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter) and Terrific Broth (TB) (tryptone 12 g, yeast extract 24 g, glycerol 4 ml, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4 per liter), were used (Sambrook and Russell 2001). Chemically defined Modified R (MR) medium (Wang and Lee 1997) was also used and contained the following per liter: K_2HPO_4, 22 g; $(NH_4)_2HPO_4$ 3 g; $MgSO_4{\cdot}7H_2O$, 0.7 g; citric acid, 0.8 g; trace metal solution, 5 ml. The trace metal solution contained the following per liter of 5 M HCl solution: $FeSO_4{\cdot}7H_2O$, 10 g; $CaCl_2$, 2 g; $ZnSO_4{\cdot}7H_2O$, 2.2 g; $MnSO_4{\cdot}4H_2O$, 0.5 g; $CuSO_4{\cdot}5H_2O$, 1 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.1 g; $Na_2B_4O_7{\cdot}10H_2O$, 0.02 g. Media were adjusted to pH 7.0 unless otherwise stated. Glucose, glycerol and thiamine were sterilized separately and aseptically added to media to final concentrations as indicated. Media were supplemented with appropriate antibiotics (100 μg/ml carbenicillin, 50 μg/ml kanamycin and/or 50 μg/ml chloramphenicol) to maintain plasmids. Flask cultures (100 ml) of recombinant *E. coli* JM109 in complex media (LB, TB, and 2× YT) and chemical defined MR medium were grown at 28°C (unless otherwise stated) for 48 and 65 h, respectively, to account for differences in growth rates causing maximum carotenoid accumulation after different cultivation times. Cell growth was monitored at OD=600 nm and dry cell weight (DCW) measured as described in Wang and Lee (1997).

Table 1 Plasmids used in this study. *Amp* Ampicillin, *Cm* chloramphenicol, *Km* kanamycin, *Tet* tetracycline

Plasmid	Relevant properties	Source or reference
pUCmod	Constitutive expression vector modified from pUC19, Amp^R	Schmidt-Dannert et al. (2000)
pACmod	Cloning vector modified from pACYC184, Tet^R, Cm^R	Schmidt-Dannert et al. (2000)
pBBR1MCS2 (pBBR)	Cloning vector, Km^R	Kovach et al. (1995)
pUC-*ispA*	*isp*A gene cloned into *Eco*RI, *Xba*I sites of pUCmod	This study
pUC-*dxs*	*dxs* gene cloned into *Eco*RI, *Xba*I sites of pUCmod	This study
pUC-*dxr*	*dxr* gene cloned into *Eco*RI, *Xba*I sites of pUCmod	This study
pUC-*idi*	*idi* gene cloned into *Eco*RI, *Nco*I sites of pUCmod	This study
pBBR-*ispA*	*isp*A gene cloned into *Hind*III site of pBBR1MCS22	This study
pBBR-*dxs*	*dxs* gene cloned into *Bam*HI site of pBBR1MCS2	This study
pBBR-*dxr*	*dxr* gene cloned into *Bam*HI site of pBBR1MCS2	This study
pBBR-*idi*	*idi* gene cloned into *Hind*III site of pBBR1MCS2	This study
pUC-*crtY2*	Evolved *crt*Y2 gene cloned into *Eco*RI, *Nco*I sites of pUCmod	Schmidt-Dannert et al. (2000)
pAC-*crtE-crtB-crtI1*	*crt*I gene along with constitutive *lac*-promoter cloned into *Hin*dIII site of pAC-*crtE-crtB*	Schmidt-Dannert et al. (2000)
pAC-*crtE-crtB-crtI14*	Evolved *crt*I14 gene along with constitutive *lac*-promoter cloned into *Hin*dIII site of pAC-*crtE-crtB*	Schmidt-Dannert et al. (2000)
pAC-*crtE-crtB-crtI14-crtY*	*crtY* gene along with constitutive *lac*-promoter cloned into *Sal*I site of pAC-*crtE-crtB-crtI14*	Lee et al. (2003)
pAC-*crtE-crtB-crtI14-crtY2*	Evolved *crtY2* gene along with constitutive *lac*-promoter cloned into *Sal*I site of pAC-*crtE-crtB-crtI14*	Lee et al. (2003)

Bioreactor studies

Batch cultures of torulene-producing *E. coli* JM109 cells having three compatible plasmids, pAC-*crtE-crtB-crtI14-crtY2*+pBBR-*idi*+pUC-*ispA*, were carried out in a bioreactor (5 l, New Brunswick Bioflo 3,000; Edison, N.J.) containing 3 l TB or MR medium supplemented with glycerol as a carbon source. Temperature and pH values were set and maintained at 28°C and 6.9±0.1, respectively, and foam was controlled by adding Antifoam 289 (Sigma, St. Louis, Mo.). Appropriate antibiotics (100 μg/ml carbenicillin, 50 μg/ml kanamycin and 50 μg/ml chloramphenicol) were added to selectively maintain the recombinant cells. The dissolved oxygen (DO) level was set at 70–40% saturation (5.6–3.2 ppm O_2) for fully aerobic conditions or 10% saturation (1.2–0.8 ppm O_2) for microaerobic conditions, respectively, and automatically controlled by changing the sparging rate (0.25–2.0 vvm) and agitation speed (100–500 rpm).

The concentrations of glycerol and fermentation products such as acetic acid, lactic acid and ethanol were measured with a high-performance liquid chromatography (HPLC) system [Waters 712 WISP (Millipore, Milford, Mass.), Altex 156 Refractive index (RI) detector, Applied Biosystems 757 Absorbance detector (UV at 214 nm) and Hewlett Packard HP3396A Integrator] equipped with an Aminex HPX-87H (300 mm ×7.8 mm, Bio-Rad, Richmond, Calif.) column. The detectors were connected in series for the quantification of glycerol and ethanol (RI detector) and organic acids (UV detector). The analysis was performed isocratically at a flow rate of 0.7 ml/min and at a temperature of 65°C with 0.013 *N* H_2SO_4 as a mobile phase.

Analysis of carotenoids

Well-drained cell pellets from 50 or 100 ml culture broth were repeatedly extracted with a total of 30 ml acetone in darkness for 1–2 h, with a sonifier (Branson Ultrasonics, Banbury, Conn.), until all visible pigments were extracted. Acetone extracts were separated from cell debris by centrifugation for 20 min at 4,000 *g*. The carotenoid extracts were partially evaporated by nitrogen gas and re-extracted with an equal volume of hexane after addition of one-fifth volume of saltwater (15% NaCl). The colored extracts were completely dried under nitrogen gas, dissolved in acetone or acetonitrile, and finally applied to silica gel thin layer chromatography (TLC) (Watman, Germany) as a preliminary test (Lee et al. 2003) and then, for quantification, to HPLC [Agilent Technologies 1,100 HPLC equipped with a Zorbax SB-C18 column (4.5×250 mm); Agilent Technologies, Palo Alto, Calif.]. Isocratic elution was used by mixing of 90% solvent A [acetonitrile/water, 99:1, (v/v)] and 10% solvent B [methanol/tetrahydrofuran, 80:20, (v/v)] at a flow rate of 1 ml/min. Absorbance spectra in the eluent were recorded on-line with a photodiode array detector. Carotenoids were identified by a combination of retention time on HPLC and absorption spectra with isolated or commercially obtained standards. The concentrations of lycopene and β-carotene were measured by integration of each corresponding peak in the chromatogram and calibration with known concentrations of authentic standards (Sigma). Since torulene is not commercially available, the absorption of torulene at 482 nm (λ_{max} of torulene) was measured and the concentration calculated by using a specific absorption coefficient of $A_{1\ cm}^{1\%}$ of 3,240 for torulene (Schiedt and Liaaen-Jensen 1995). Measurements were repeated on three independent cultures.

Results

Gene dosage effect of *crtY2*

We first examined the effects of the expression level of a mutant lycopene cyclase (encoded by *crt*Y2) (Schmidt-Dannert et al. 2000) on the carotenoid profile in *E. coli* by modulating its gene dosage. This was achieved by expressing *crt*Y2 on a low-copy number plasmid pAC-*crtE-crtB-crtI14-crtY2* (Lee et al. 2003) or on a high-copy number plasmid pUCmod as a combination of pAC-*crtE-crtB-crtI14*+pUC-*crtY2* in *E. coli*. Both expression systems carry all four genes for the synthesis of torulene (Fig. 1a) and each gene has the same constitutive *lac*-promoter and Shine-Dalgarno sequence.

Similar amounts of torulene and its precursor lycopene accumulated (250±11 μg lycopene/g-DCW and 240 ±21 μg torulene/g-DCW) with a small amount of β-carotene (48±3 μg β-carotene/g-DCW) as an undesired side-product in *E. coli* pAC-*crtE-crtB-crtI14-crtY2* +pUCmod grown for 48 h in LB medium at 28°C (see results below for choice of temperature). However, lycopene was completely converted into torulene and β-carotene in *E. coli* pAC-*crtE-crtB-crtI14*+pUC-*crtY2*. Even though the complete conversion of lycopene increased the amounts of torulene and β-carotene up to 330±19 μg torulene/g-DCW (1.4-fold) and 132±6 μg β-carotene/g-DCW (2.8-fold), the total amount of carotenoids was decreased by 20% (Fig. 2). The altered carotenoid profile had no influence on cell growth compared to the low gene dosage expression of *crtY2* (data not shown).

For enhanced production of torulene, less β-carotene formation is favored as it cannot be further transformed into torulene and thus depletes a precursor pool (Fig. 1a). Lycopene accumulated by *E. coli* pAC-*crtE-crtB-crtI14-crtY2*+pUCmod, however, can be further transformed into torulene in an optimized pathway. Thus, the low-copy number plasmid system, pAC-*crtE-crtB-crtI14-crtY2*, was chosen for subsequent experiments unless otherwise stated.

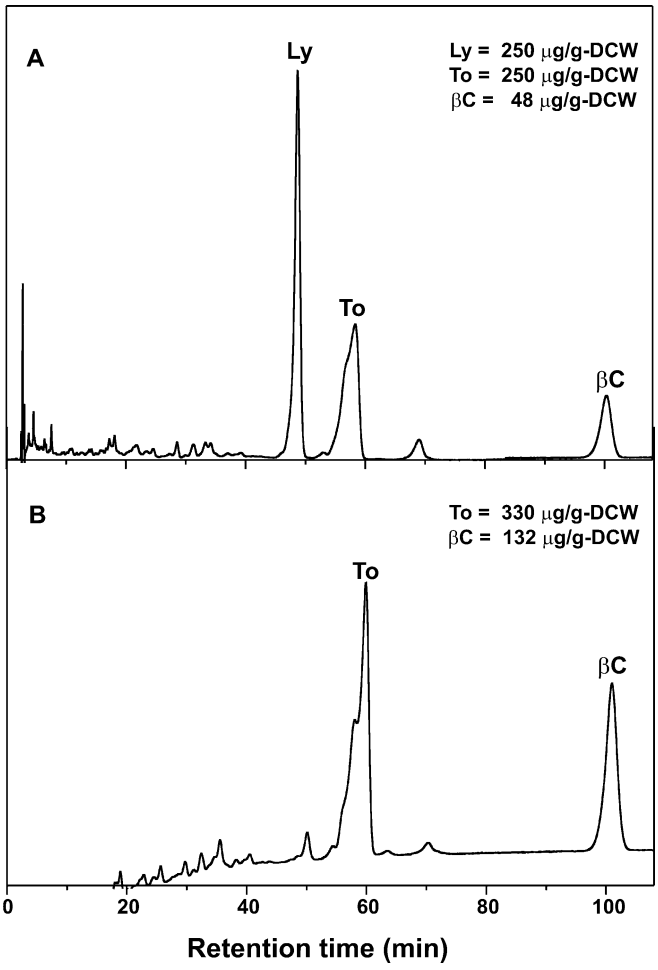

Fig. 2a,b Effect of gene dosage of *crtY2* on carotenoid accumulation. Torulene-producing *E. coli* strains containing plasmids pAC-*crtE-crtB-crtI14-crtY2*+pUCmod (low gene dosage of *crtY2*) or pAC-*crtE-crtB-crtI14*+pUC-*crtY2* (high gene dosage of *crtY2*) were grown in Luria-Bertani (LB) medium at 28°C for 48 h. Extracted carotenoids were analyzed by high performance liquid chromatography (HPLC) and UV-Vis spectrometry as described in Materials and methods. Carotenoid profiles of recombinant cells expressing *crtY2* at **a** low-gene dosage and **b** high-gene dosage. Peaks: *Ly* lycopene, *To* torulene, *βC*, β-carotene. Carotenoid quantities in μg/g-DCW are indicated. Absorbance was monitored at 480 nm

Investigation of culture conditions

We first examined the effect of culture temperature and initial pH of medium on torulene production. Recombinant *E. coli* cells were cultured in LB and MR media initially adjusted to pH values of 6.0, 6.5, 7.0, or 7.5, at temperatures of 28°C and 37°C. Because the carbon source in a medium can have a considerable influence on cell metabolism and/or regulation (Fang and Demain 1997; Lee et al. 2001; Martin et al. 2001), MR medium complemented with glucose (10 g/l) and glycerol (5 g/l) were tested as alternate carbon sources. It was found that initial pH had no significant influence on torulene production and cell growth in either medium (data not shown). However, the carbon source dramatically influenced torulene production: 280–300 μg-torulene/g-DCW from glycerol-grown cells (dark red cells) compared to 17–23 μg-torulene/g-DCW from glucose-grown cells (almost colorless cells). Cells grown in glycerol-containing MR medium had torulene production levels 20% greater than seen earlier in complex LB medium.

A number of native *E. coli* metabolic pathways, including those for thiamine and pyridoxal biosynthesis, branch from the isoprenoid pathway (Fig. 1b). Carbon flux towards the thiamine and pyridoxal biosynthesis pathways may deplete the isoprenoid precursor DXP pool in *E. coli* (Lopukhov et al. 2002; Carter et al. 2003) and therefore reduce torulene yield. In order to redirect the metabolic flux of DXP away from the thiamine pathway, the thiamine concentration in MR medium containing 5 g/l glycerol was increased from 10 to 20 mg/l. However, thiamine supplementation had no effect on torulene production in recombinant *E. coli* (data not shown).

Investigation of glucose suppression

To investigate the significant decrease in torulene production observed in glucose-containing MR medium, recombinant *E. coli* were grown in three complex media, LB, 2× YT, and TB, supplemented with glycerol (5 g/l) or glucose (10 g/l), respectively. Glycerol supplementation to LB medium slightly increased torulene production (approx. 15%, Table 2) to 285 μg-torulene/g-DCW. Torulene production was higher in the glycerol-containing nutrient rich media 2× YT and TB (310 and 343 μg-torulene/g-

Table 2 Effect of carbon source on carotenoid production in complex media. Recombinant *Escherichia coli* strains harboring plasmids directing torulene (pAC-*crtE-crtB-crtI14-crtY2*), lycopene (pAC-*crtE-crtB-crtI*), tetradehydrolycopene (pAC-*crtE-crtB-crtI14*) or β-carotene (pAC-*crtE-crtB-crtI14-crtY*) production were cultured in complex media (LB, 2× YT, TB; 48 h, 28 °C) supplemented with glycerol (5 g/l) or glucose (10 g/l) as a carbon source

	Torulene [μg/g-DCW][a]			Lycopene [μg/g-DCW][a]	Tetradehydrolycopene [μg/g-DCW][a]	β-carotene [μg/g-DCW][a]
	LB	2X YT	TB	LB	LB	LB
Glucose	5±0.2[a]	6±0.3	6±0.4	10±12	5±1.2	7±0.1
Glycerol	285±11	310±21	343±17	486±23	464±32	475±11

[a]Values are averages of triplicate experiments and are given as mean ± standard deviation

DCW, respectively). Cell growth was similar among the complex media tested, irrespective of torulene production levels (data not shown). In results similar to those observed for glucose-containing MR medium, all glucose-containing modified complex media demonstrated a loss of torulene production (Table 2). However, glucose-mediated inhibition of torulene production was more apparent in complex media than in the MR medium (approx. 50-fold vs 14-fold).

We next tested the effect of different glucose concentrations (0.5, 1, 2, 5, and 10 g/l) in LB medium on torulene production. However, regardless of the glucose concentration tested, torulene production was inhibited to similar levels (data not shown).

To examine the effectiveness of glycerol-containing media in torulene production, carotenoid profiles of recombinant *E. coli* cells grown on glycerol as a carbon source in TB, 2× YT, TB and MR were analyzed by HPLC. All four media showed similar carotenoid profiles: torulene > lycopene ≫ β-carotene (results not shown). Highest torulene accumulation was seen in TB followed by MR medium.

To analyze whether the recombinant production of other carotenoids in *E. coli* is also suppressed by glucose, three carotenoid-producing recombinant *E. coli* strains containing plasmids (Table 1) for lycopene (pAC-*crtE-crtB-crtI*), tetradehydrolycopene (pAC-*crtE-crtB-crtI14*) or β-carotene (pAC-*crtE-crtB-crtI14-crtY*) were grown in LB medium supplemented with 10 g/l glucose or 5 g/l glycerol, respectively. Similar to the results obtained with the torulene pathway, carotenoid production was strongly inhibited (about 68-fold) by the addition of glucose to the medium (Table 2). Production levels for lycopene, tetradehyrolycopene and β-carotene in glycerol-containing LB-medium were similar, about 475 μg/g-DCW; more than 1.5-fold the amount of torulene produced under the same conditions (Table 2).

Combinatorial expression of key isoprenoid pathway genes

A study on the combinatorial effect of all previously suggested key enzymes (*dxs*, *dxr*, *idi*, and/or *isp*A) involved in isoprenoid precursor biosynthesis (Fig. 1b) on recombinant carotenoid production has not been performed. Thus, we cloned these genes under the control of a constitutive *lac*-promoter into two compatible plasmids with different copy numbers: pUCmod and pBBR1MCS (pBBR) to achieve high and low gene dosages, respectively, in *E. coli*. Individual plasmids and plasmid combinations were transformed into torulene-producing *E. coli* pAC-*crtE-crtB-crtI14-crtY2* and after 48 h of growth (28°C, TB supplemented with 5 g/l glycerol) carotenoid accumulation was quantified (Table 3).

Overexpression of the individual genes *dxr*, *isp*A, or *idi* on pBBR or pUCmod enhanced both torulene and total carotenoid production up to 1.5 and 1.7-fold, respectively,

Table 3 Effect of isoprenoid gene overexpression on carotenoid production. Single plasmids (*top panel*) or plasmid combinations (*bottom panel*) expressing isoprenoid genes are transformed into torulene-producing *E. coli* pAC-*crtE-crtB-crtI14-crtY2* (grown for 48 h in TB at 28°C). Gene expression is driven by a constitutive *lac*-promoter on either a low copy number plasmid [pBBR1MCS (pBBR)] or a high copy number plasmid (pUCmod)

Carotenoid[a] [μg/g-DCW]	pUCmod	pUC-*dxs*	pUC-*dxr*	pUC-*idi*	pUC-*ispA*	pBBR	pBBR-*dxs*	pBBR-*dxr*	pBBR-*idi*	pBBR-*ispA*
Torulene	318±8[a]	137±7	404±42	547±23	489±36	337±21	256±11	358±32	502±36	422±33
Lycopene	112±6	40±3	133±9	170±12	168±10	108±11	75±5	121±12	164±13	143±9
β-Carotene	20±1.6	8±0.5	25±2.0	32±2.6	27±1.3	21±1.1	15±1.2	19±0.8	33±1.8	29±2.1
Fold change[b]	–	0.4	1.3	1.7	1.5	–	0.8	1.1	1.5	1.3

Carotenoid[a] [μg/g-DCW]	pBBR pUCmod	pBBR-*dxs*pUC-*dxr*	pBBR-*dxs*pUC-*idi*	pBBR-*dxs*pUC-ispA	pBBR-*dxr* pUC-*dxs*	pBBR-*dxr*pUC-*idi*	pBBR-*dxr*pUC-*ispA*	pBBR-*idi*pUC-*dxs*	pBBR-*idi*pUC-*dxr*	pBBR-*idi*pUC-*ispA*	pBBR-*ispA*pUC-*dxs*	pBBR-*ispA*pUC-*dxr*	pBBR-*ispA*pUC-*idi*
Torulene	331±12	356±22	706±23	401±10	155±7	928±60	651±19	270±17	1,111±63	1,082±18	178±18	653±21	1,019±22
Lycopene	101±9	107±7	252±17	132±12	49±5	290±30	210±13	77±6	317±28	318±21	50±3	225±18	302±10
β-Carotene	21±3.2	26±2.1	47±4.1	26±3.0	8.6±0.5	54±2.7	46±2.9	15±0.9	58±3.2	67±5.3	11±0.9	56±6.4	63±4.5
Fold change[b]	–	1.1	2.1	1.2	0.5	2.8	2.0	0.8	3.3	3.3	0.5	2.0	3.1

[a]Values are averages of triplicate experiments and are given as mean ± standard deviation
[b]Fold-change of torulene accumulation compared to control cultures; values also correspond to fold-change of total carotenoid accumulation

while the carotenoid profile in recombinant *E. coli* was unchanged compared to control cultures (Table 3). Thus, a higher gene dosage (pUCmod) of all three genes increased carotenoid production slightly (about 20%). Notably, overexpression of *idi* on both plasmids had the largest positive effect on carotenoid production. Overexpression of *dxs* on both pUCmod and pBBR plasmids, however, significantly inhibited carotenoid production (~60% less on pUCmod and ~20% less on pBBR, respectively) and cell growth (data not shown).

Next, the 12 possible two-gene combinations of the four different isoprenoid genes on pUC or pBBR were transformed into torulene-producing *E. coli* cells to investigate their synergic effects on carotenoid production (Table 3). The combinations of pBBR-*idi*+pUC-*dxr*, pBBR-*idi*+pUC-*ispA* or pBBR-*ispA*+pUC-*idi* increased carotenoid production more than 3-fold. The combination of pBBR-*dxs*+pUC-*idi* significantly reduced the negative effect seen with *dxs* alone and even enhanced torulene production. However, expression on pBBR of neither gene could alleviate the inhibitory effect of *dxs* when expressed on pUC-*dxs*. It is notable that negative combinations involved *dxs* overexpression while the positive combinations involved *idi* overexpression.

Bioreactor study

Bioreactors were run to acquire information on the kinetics of carotenoid formation as well as the effect of DO levels under better-controlled conditions. Two different DO levels were set and maintained: fully aerobic conditions and microaerobic conditions (Peng and Shimizu 2003), as described in Materials and methods. Strict anaerobic operation of bioreactors was omitted because anaerobically grown recombinant carotenogenic *E. coli* produced little or no carotenoids (data not shown). Therefore, the highest-torulene-producing *E. coli* strain containing plasmids pAC-*crtE-crtB-crtI14-crtY2*+pBBR-*idi*+pUC-*ispA* (Table 3) was chosen and cultured on glycerol-containing TB or MR medium under controlled conditions.

Carotenoid formation was highest at high DO levels in both media (Fig. 3). Quantitatively, the volumetric productivity of torulene was 2–4 times higher in aerobically grown *E. coli* than in microaerobically grown cells: 1,240 μg torulene/l (aerobic MR medium) vs 245 μg torulene/l (microaerobic MR medium); 1,431 μg torulene/l (aerobic TB medium) versus 500 μg torulene/l (microaerobic TB medium). No fermentative products were detectable in either aerobic or, notably, in microaerobic culture media. Complex TB medium favored cell growth

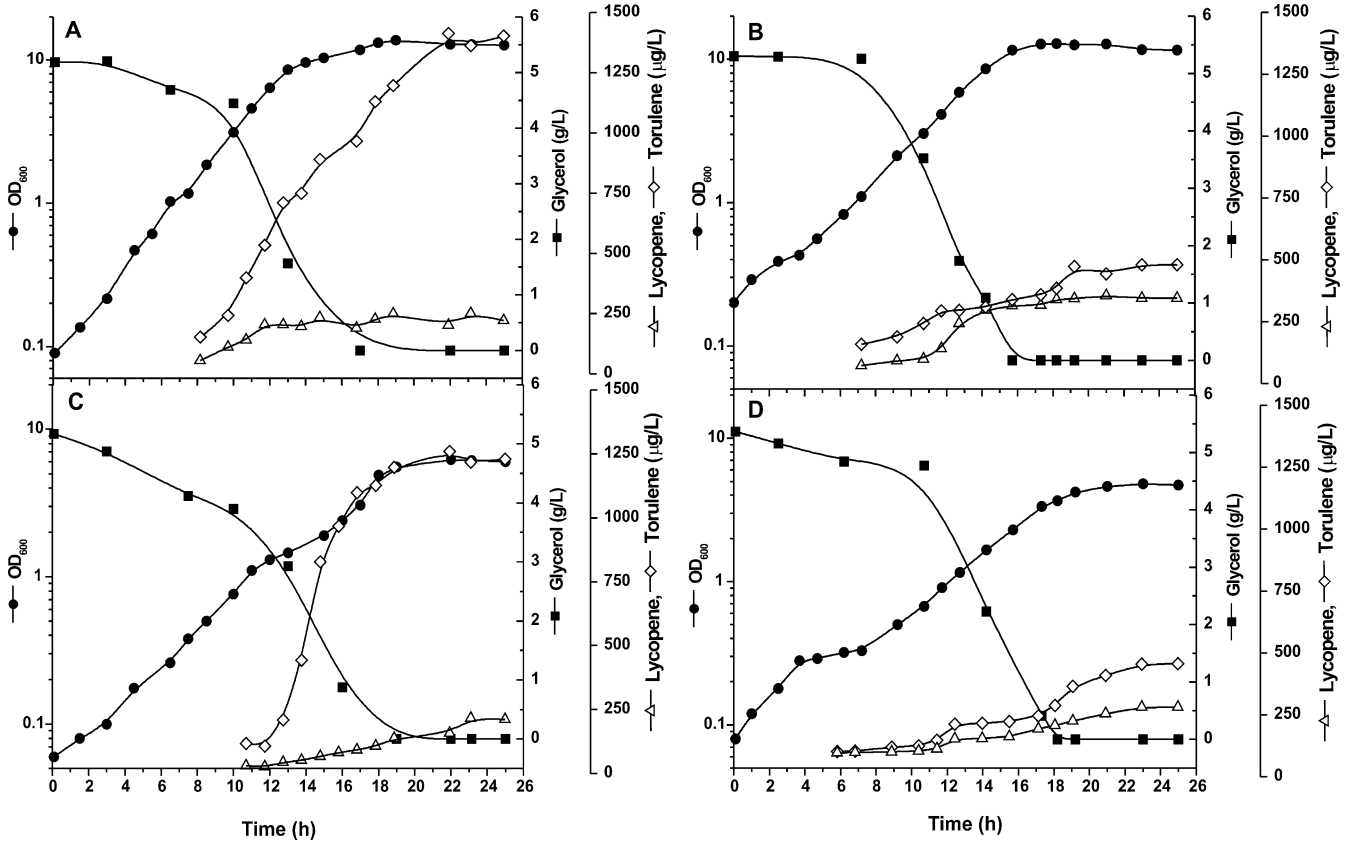

Fig. 3a–d Bioreactor study of torulene production in recombinant *E. coli* under aerobic and microaerobic conditions. Batch cultures of *E. coli* (pAC-*crtE-crtB-crtI14-crtY2*+pBBR-*idi*+pUC-*ispA*) were grown in TB (supplemented with 5 g/l glycerol) under **a** aerobic and **b** microaerobic conditions, and in MR medium (supplemented with 5 g/l glycerol) under **c** aerobic or **d** microaerobic conditions. Cell growth, glycerol consumption, carotenoid accumulation and accumulation of fermentation products (ethanol, acetic acid, lactic acid and formic acid not detected) were monitored

over torulene production under both aerobic and microaerobic conditions. The high cell mass obtained in bioreactors with TB reduced torulene yield compared to those obtained with MR medium: 398 μg torulene/g-DCW (aerobic TB) vs 645 μg torulene/g-DCW (aerobic MR); 143 μg torulene/g-DCW (microaerobic TB) vs 170 μg torulene/g-DCW (microaerobic MR).

The kinetics of carotenoid formation in *E. coli* cells suggests that carotenoid synthesis in *E. coli* is growth-associated and the highest amount of carotenoids was obtained in the late stationary growth phase. Prolonged cultivation decreased the carotenoid level in *E. coli* cells (data not shown). The precursor lycopene did not accumulate significantly and was efficiently converted into torulene during aerobic cultures, indicating that the engineered torulene synthetic pathway is well coordinated and maintains the functionality and integrity of the carotenogenic enzyme complex under aerobic conditions. In contrast, under microaerobic conditions, torulene production, as well as total carotenoid production, was significantly reduced in both TB and MR media and a relatively high amount of lycopene accumulated with slow conversion into torulene.

Discussion

Previously, we reported the creation of biosynthetic pathways for the synthesis of novel carotenoid structures in non-carotenogenic *E. coli* by using directed evolution and combinatorial strategies (Schmidt-Dannert et al. 2000; Lee et al. 2003). In an effort to increase yields of the novel structures produced in *E. coli*, we chose a new pathway leading to torulene as a model to investigate factors that govern its production in a recombinant host.

Controlled gene dosage via plasmids with different copy number has been regarded as especially effective in metabolic engineering for the production of various secondary metabolites (Kidwell et al. 1995), including lycopene (Jones et al. 2000). Our experiments show that the gene dosage of *crtY2* had a profound effect on the carotenoid profile in recombinant *E. coli*. Although a higher gene dosage of *crtY2* increased torulene accumulation, this increase was accompanied by an even larger accumulation of β-carotene, which is an undesired by-product. Thus, a low gene dosage of *crtY2* produced a more useful carotenoid profile for further optimization, in which torulene and its direct precursor lycopene accumulate in similar ratios.

Investigation of culture conditions indicated that production was enhanced approximately 2.5-fold at 28°C compared to 37°C. Previous studies on wild-type carotenogenic pathways also demonstrated a strong relationship between temperature and product yield (Albrecht et al. 1997; Ruther et al. 1997). In all culture media tested, the addition of glucose resulted in a significant reduction in torulene yield. Recombinant biosynthesis of β-carotene, tetradehydrolycopene and lycopene was also strongly inhibited by glucose. Unfortunately, the mechanism of glucose inhibition is not clear and little information is available about the regulation of carotenoid biosynthesis in carotenogenic bacteria and non-carotenogenic *E. coli* (Armstrong 1997). However, carotenoid biosynthesis in some carotenogenic *Erwinia herbicola* strains was reported to be repressed by glucose, and recombinant *Escherichia coli* carrying the *Erwinia herbicola* Eho 10 *crt* gene cluster even showed similar catabolic repression (Perry et al. 1986). Glycerol, on the other hand, showed a positive effect, and generally increased torulene production in complex media. This positive effect of glycerol is in contrast to a previous report by Farmer and Liao (2001), who observed that an external supply of glycerol had no effect on lycopene production, but agrees with the findings of Martin et al. (2001) who reported a positive effect of glycerol on sesquiterpene (another isoprenoid compound, Fig. 1) biosynthesis in *E. coli*.

Engineering of the carotenoid precursor pathway by overexpression of key isoprenoid pathway genes was utilized to improve torulene production. Overexpression of the *dxs* gene significantly reduced carotenoid yield. Previous reports have suggested that increased *dxs* expression can enhance recombinant carotenoid production (Wang et al. 2000; Matthews and Wurtzel 2000), which is in contrast to the results observed in our system. However, IPTG-induced overexpression of *dxs* has been shown by Jones et al. (2000) to reduce carotenoid production and cell growth in recombinant *E. coli*. The reason for these contradictory results is unclear but, as *dxs* directs the flux of the key metabolic intermediates pyruvate and glyceraldehyde-3-phosphate to the isoprenoid pathway, high-level *dxs* overexpression in recombinant *E. coli* may have metabolic consequences beyond isoprenoid production.

Highest production of torulene was achieved by overexpression of both the *ispA* (FPP synthase) and *idi* (IPP isomerase) genes. This suggests that isomerization of IPP and dimethylallyl diphosphate (DMAPP) and FPP biosynthesis (Fig. 1a) are important rate-limiting steps in the *E. coli* isoprenoid pathway. This is also supported by other reports on the effect of *idi* overexpression on the production of lycopene (Kajiwara et al. 1997), astaxanthin (Wang et al. 1999) and zeaxanthin (Albrecht et al. 1999).

To our knowledge, there are no bioreactor studies in the literature on recombinant carotenoid production in *E. coli*. Because of their biological function as antioxidants, it is widely accepted that high DO levels may have a negative effect on carotenoid formation due to the generation of highly reactive compounds (such as radicals or peroxides) as the result of the high overall metabolic rate of cells cultured under these conditions. However, volumetric productivity of torulene was 2–4 times higher under aerobic than microaerobic culture conditions. Aerobic conditions supported quick and efficient conversion of lycopene into torulene, while under lower oxygen saturation lycopene accumulated. Furthermore, carotenoid production was correlated with cell growth and decreased in late stationary phase, presumably due to the increased accumulation of harmful oxygen species in the cells'

starvation phase (Bridges and Timms 1998). Under microaerobic conditions, we expected competition for pyruvate as a precursor between mixed acid fermentative (organic acids and ethanol) and carotenoid pathways (Alexeeva et al. 2003). Surprisingly, no detectable fermentation products accumulated, which may be due to the low cultivation temperature (28°C) and a glycerol carbon source.

In summary, we optimized torulene production in recombinant *E. coli* by optimizing the gene dosage of *crtY2*, culture conditions and by metabolic engineering of the *E. coli* isoprenoid pathway. Thus, torulene production was increased up to 3-fold compared to the initial process, but is more than 1.5-fold lower than seen for β-carotene or lycopene under the same conditions. Furthermore, this study provides important data on the optimization of fermentation processes for the recombinant production of torulene and its derivatives as well as on future metabolic engineering of terpenoid production in recombinant *E. coli*.

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