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Biosynthesis of Structurally Novel Carotenoids in *Escherichia coli*

Pyung Cheon Lee, Abu Zafar Ruhul Momen, Benjamin N. Mijts, and Claudia Schmidt-Dannert*
Department of Biochemistry, Molecular Biology, and Biophysics
1479 Gortner Avenue
University of Minnesota
Saint Paul, Minnesota 55108

Summary

Previously, we utilized in vitro evolution to alter the catalytic functions of several carotenoid enzymes and produce the novel carotenoids tetradehydrolycopene and torulene in *Escherichia coli*. Here we report on the successful extension of these pathways and the C_{30} carotenoid diaponeurosporene pathway with additional carotenoid genes. Extension of the known acyclic C_{30} pathway with C_{40} carotenoid enzymes—spheroidene monooxygenase and lycopene cyclase—yielded new oxygenated acylic products and the unnatural cyclic C_{30} diapotorulene, respectively. Extension of acyclic C_{40} pathways with spheroidene monooxygenase generated novel oxygenated carotenoids including the violet phillipsiaxanthin. Extension of the torulene biosynthetic pathway with carotene hydroxylase, desaturase, glucosylase, and ketolase yielded new torulene derivatives. These results demonstrate the utility of extending an in vitro evolved central metabolic pathway with catalytically promiscuous downstream enzymes in order to generate structurally novel compounds.

Introduction

To explore the potential of in vitro evolution and metabolic engineering strategies to produce novel compounds in a microbial host, we chose carotenoids as a model system of biotechnological and medical importance [1]. Carotenoids constitute a structurally diverse class of natural pigments, which are produced as food colorants, feed supplements and, more recently, as nutraceuticals and for cosmetic and pharmaceutical purposes. Although microorganisms and plants synthesize more than 600 different carotenoids [2], only a handful can be produced in useful quantities [3]. The discovery that carotenoids exhibit significant anticarcinogenic activities and play an important role in the prevention of chronic diseases [4, 5] has triggered an increased interest in the synthesis of new carotenoid structures and the economic production of compounds in engineered cells.

The first committed step in C_{40} carotenoid biosynthesis is the extension of the general isoprenoid pathway by the enzymes geranyl geranyl disphosphate (GGDP) synthase (CrtE) and phytoene synthase (CrtB) to form the colorless carotenoid phytoene. The introduction of additional double bonds into phytoene by phytoene desaturase (CrtI) produces the colored carotenoids neurosporene (three desaturations) or lycopene (four desaturations) from which different acyclic and cyclic carotenoids are then synthesized [6] (Figure 1; see Table 1 for genes and plasmids used in this study). In previous work, we extended this central desaturation pathway by evolving a six-step phytoene desaturase (CrtI14) capable of synthesizing the fully conjugated 3,4,3′,4′-tetradehydrolycopene in *E. coli* [7]. Lycopene cyclase (CrtY) catalyzes the introduction of β-rings into either end of lycopene to synthesize β,β-carotene, which is then further modified (Figure 1) [6]. We previously modified CrtY by directed evolution to generate CrtY2, a variant that cyclizes didehydrolycopene, the precursor of tetradehydrolycopene, to produce the red carotenoid torulene [7].

Starting from C_{15} farnesyl diphosphate (FDP) instead of C_{20} GGDP as in C_{40} carotenoid biosynthesis, diverse acyclic C_{30} diapocarotenoids are synthesized by several nonphototrophic bacteria such as *Staphylococcus*, *Streptococcus*, and *Methylobacterium* species [8]. Only the genes encoding dehydrosqualene synthase (*crtM*) and dehydrosqualene desaturase (*crtN*) from *Staphylococcus aureus* have been cloned and functionally expressed in *E. coli*, resulting in the production of the yellow 4,4′-diaponeurosporene via diapophytoene and diapo-ζ-carotene [9] (Figure 1).

A number of genes encoding the enzymes for central carotenoid biosynthetic routes have been cloned, and genes from different species have been shown to function cooperatively when combined [10]. Enzymes catalyzing the synthesis and desaturation of acyclic C_{30} and C_{40} carotenoid backbones and initial cyclization reactions of C_{40} carotenoids appear well conserved in carotenoid-producing organisms. Less is known, however, about the enzymes responsible for the additional structural modifications that contribute to the tremendous diversity of natural carotenoids [2]. However, a number of these modifying enzymes have been identified and characterized from microbial sources including the monooxygenase from *Rhodobacter capsulatus* (CrtA) [11]; β-carotene ketolase from *Synechocystis* sp. (CrtO) [12]; β-carotene desaturase from *Streptomyces griseus*, *Brevibacterioum linens*, and *Mycobacterium aurum* (CrtU) [13–15]; and β-carotene hydroxylase (CrtZ) and zeaxanthin glycosylase (CrtX) from *Erwinia herbicola* [16].

We sought to probe the catalytic promiscuity of these carotenoid-modifying enzymes toward new carotenoid substrates synthesized by in vitro evolved biosynthetic routes. Here we report on the successful extension of the in vitro evolved torulene and tetradehydrolycopene pathways as well as the wild-type lycopene, β-carotene, and diaponeurosporene (a C_{30} carotenoid) pathways with carotenoid-modifying genes to produce structurally novel carotenoids in *E. coli* (Figure 1).

*Correspondence: schmi232@tc.umn.edu

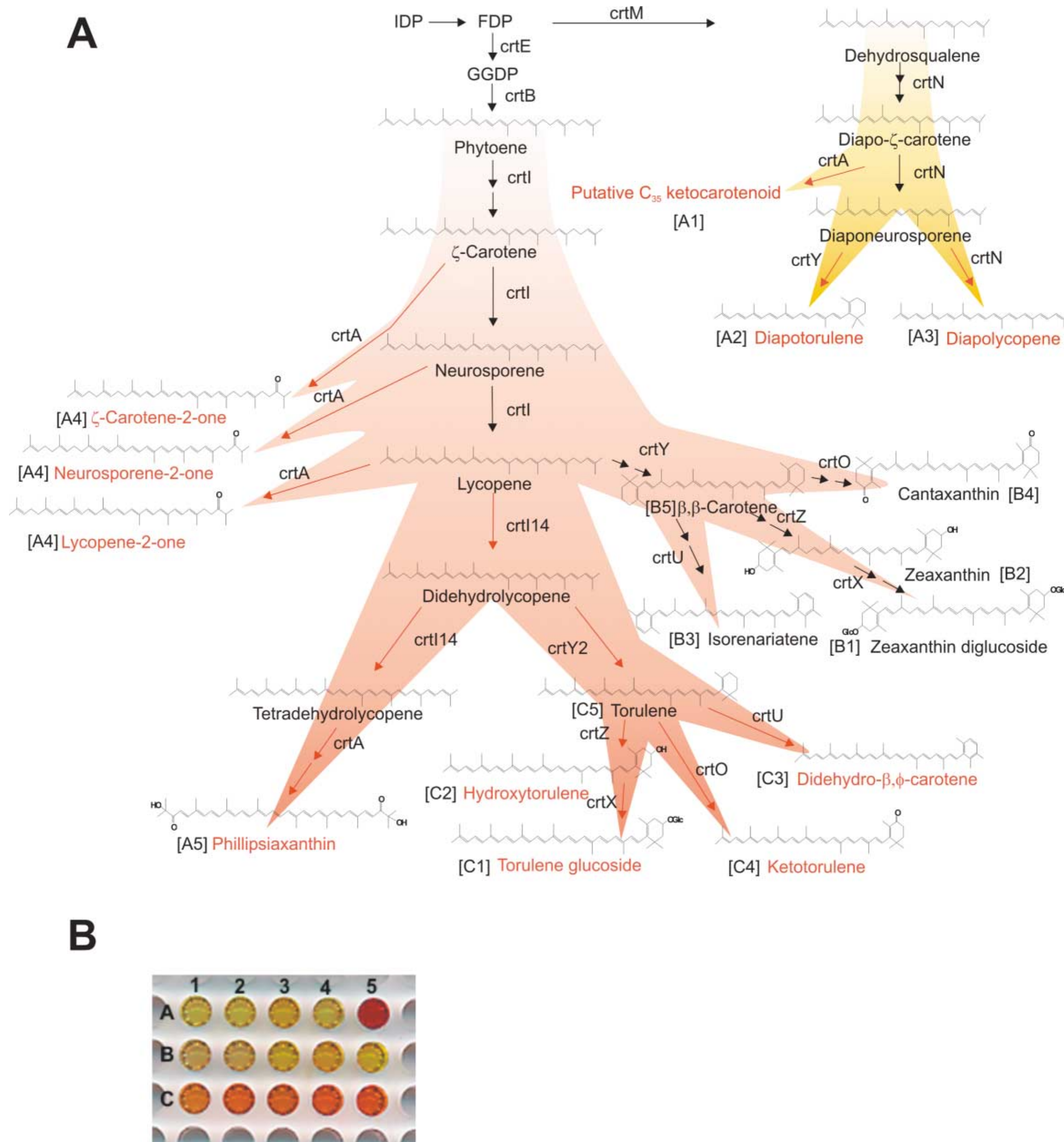

Figure 1. Carotenoids Produced in Recombinant *E. coli*

Biosynthetic routes to different acyclic and cyclic C_{40} and C_{30} carotenoids in engineered *E. coli* (A) and total carotenoid extracts of *E. coli* transformants carrying different recombinant carotenoid pathways (B). Red arrows indicate branching of the central desaturation pathways to the routes for the biosynthesis of novel carotenoid structures (red). The designation in brackets next to a final product of a carotenoid route indicates the position in the micro plate array of the corresponding extract from *E. coli* cells expressing the genes of this pathway. For enzyme names and functions, see Table 1.

Results and Discussion

Coexpression of Dehydrosqualene Synthase CrtM and Desaturase CrtN Produces the Fully Conjugated C_{30} Carotenoid Diapolycopene

To extend the isoprenoid pathway in *E. coli* for synthesis of C_{30} carotenoids, two expression cassettes comprising a constitutive *lac*-promoter upstream of either *crtM* or *crtN* were assembled to yield pAC-*crtM*-*crtN*. *E. coli* cells transformed with pAC-*crtM*-*crtN* developed a deep yellow-orange color, suggesting the production of diapocarotenoids. Analysis of the cell extracts by HPLC-mass spectrometry showed that, in our system, CrtN efficiently introduced four double bonds into dehydrosqualene to predominantly (90%) synthesize the fully conjugated 4,4′-diapolycopene in recombinant *E. coli* (Figure 2A). This is in contrast to earlier reports where CrtN was shown to catalyze efficiently the three-step desaturation of dehydrosqualene leading to the formation of 4,4′-diaponeurosporene in recombinant *E. coli* [9]. However, shortly before submission of this manuscript, Arnold and coworkers reported the accumulation of 30% diapolycopene in recombinant *E. coli* cells constructed for directed evolution studies aimed at evolving CrtM

Table 1. Genes and Plasmids Used in This Study

(A) Gene	Enzyme	Typical Reaction Catalyzed	Accession Number or Reference
crtM	Dehydrosqualene synthase	Head-to-head condensation of 2 FDP	X73889
crtN	Diapophytoene synthase	Introduction of 3 desaturations in dehydrosqualene	X73889
crtE	GGDP synthase	Head-to-head condensation of IDP+ FDP	D90087
crtB	Phytoene synthase	Head-to-head condensation of 2 GGDP	D90087
crtI	Phytoene desaturase	Introduction of 4 desaturations in phytoene	D90087
crtI14	In vitro evolved phytoene desaturase	Introduction of 6 desaturations in phytoene	[7]
crtY	Lycopene cyclase	Cyclization of ψ-end groups in lycopene to form β-rings	D90087
crtY2	In vitro evolved lycopene cyclase	Cyclization of ψ-end group in didehydrolycopene to form β-ring	[7]
crtA	Spheroidene monooxygenase	Oxygenation at C2 of spheroidene or hydroxysphroidene	Z11165
crtO	β-carotene oxygenase	Oxygenation at C4, C4′ of β-carotene	D64004
crtU	β-carotene desaturase	Desaturation/methyltransfer of β-rings in β-carotene	AF139916
crtZ	β-carotene hydroxylase	Hydroxylation of C3, C3′ of β-carotene	D90087
crtX	Zeaxanthin glucosylase	Glycosylation of C3, C3′ of zeaxanthin	D90087
(B) Plasmid		**Properties**	**Reference**
pUCmod		Constitutive expression vector modified from pUC19, Ap	[7]
pACmod		Cloning vector modified from pACYC184, Cm	[7]
pUC-crtM		pUCmod constitutively expressing *crtM*	This study
pUC-crtN		pUCmod constitutively expressing *crtN*	This study
pUC-crtY		pUCmod constitutively expressing *crtY*	[7]
pUC-crtY2		pUCmod constitutively expressing *crtY2*	[7]
pUC-crtA		pUCmod constitutively expressing *crtA*	This study
pUC-crtO		pUCmod constitutively expressing *crtO*	This study
pUC-crtU		pUCmod constitutively expressing *crtU*	This study
pUC-crtZ		pUCmod constitutively expressing *crtZ*	This study
pUC-crtX		pUCmod constitutively expressing *crtX*	This study
pAC-crtM-crtN		pACmod constitutively expressing *crtM* and *crtN* to produce diaponeurosporene	This study
pAC-crtE-crtB-crtI		pACmod constitutively expressing *crtE*, *crtB*, and *crtI* to produce lycopene	[7]
pAC-crtE-crtB-crtI14		pACmod constitutively expressing *crtE*, *crtB*, and mutant *crtI14* to produce tetradehydrolycopene	[7]
pAC-crtE-crtB-crtI14-crtY		pACmod constitutively expressing *crtE*, *crtB*, mutant *crtI14*, and *crtY* to produce β-carotene	This study
pAC-crtE-crtB-crtI14-crtY2		pACmod constitutively expressing *crtE*, *crtB*, mutant *crtI14*, and mutant *crtY2* to produce torulene	This study
pAC-crtE-crtB-crtI14-crtY-crtZ		pACmod constitutively expressing *crtE*, *crtB*, mutant *crtI14*, *crtY*, and *crtZ* to produce zeaxanthin	This study
pAC-crtE-crtB-crtI14-crtY2-crtZ		pACmod constitutively expressing *crtE*, *crtB*, mutant *crtI14*, mutant *crtY2*, and *crtZ* to produce monohydroxytorulene	This study

for function in a C_{40} pathway [17]. Unexpectedly, we observed that *E. coli* cells harboring pAC-*crtN*-*crtM* also accumulated significant amounts of polar carotenoids. Molecular masses and absorption spectra showed them to be various diapolycopene and diaponeurosporene derivatives carrying methoxy- and/or hydroxy-functional groups at one or both of their ends (data not shown). Acyclic end groups of bacterial C_{30} diapocarotenoids are frequently oxidized to hydroxy, aldehyde, or carboxy groups, which can be further acylated and/or glucosylated [18, 19]. Raisig et al. recently also reported formation of modified diapocarotenoids in recombinant *E. coli* [20]. Apparently, the diapocarotenoid end groups are prone to oxidation by free peroxyl-radicals (especially hydroperoxyl radicals) formed in lipid membranes during oxygen stress [21–23]. The observed methoxy groups may have formed from hydroperoxyl groups in the presence of methanol present during isolation and analysis [22]. We never observed significant modification of C_{40} carotenoids, suggesting that the orientation of the C_{30} carotenoids in the lipid membrane of *E. coli* may be different and thus increasing its reactivity with reactive oxygen species like peroxyl-radicals [23].

Lycopene Cyclase CrtY Cyclizes the C_{30} Carotenoid Diaponeuro-sporene

Cyclization of C_{30} diapocarotenoids, which is a common modification of C_{40} carotenoids, is so far unknown. Because lycopene cyclase CrtY acts on ψ-end groups [24], which are the same in acyclic C_{40} carotenoids (e.g., lycopene) and C_{30} carotenoids (e.g., diaponeurosporene or diapo-ζ-carotene), we reasoned that expression of *crtY* on pUC-*crtY* together with the genes for diapolycopene biosynthesis on pAC-*crtM-crtN* would produce novel unnatural cyclic diapocarotenoids in *E. coli*. Indeed, a novel cyclic carotenoid along with diaponeurosporene was detected in cell extracts of such cotransformed recombinant *E. coli* cells (Figure 2B). Absorption and mass spectrum confirmed it to be diapotorulene, the cyclic derivative of diaponeurosporene. Other possible monocyclic and dicyclic diapocarotenoids derived from diapo-ζ-carotene were not detected. As farnesyl diphos-

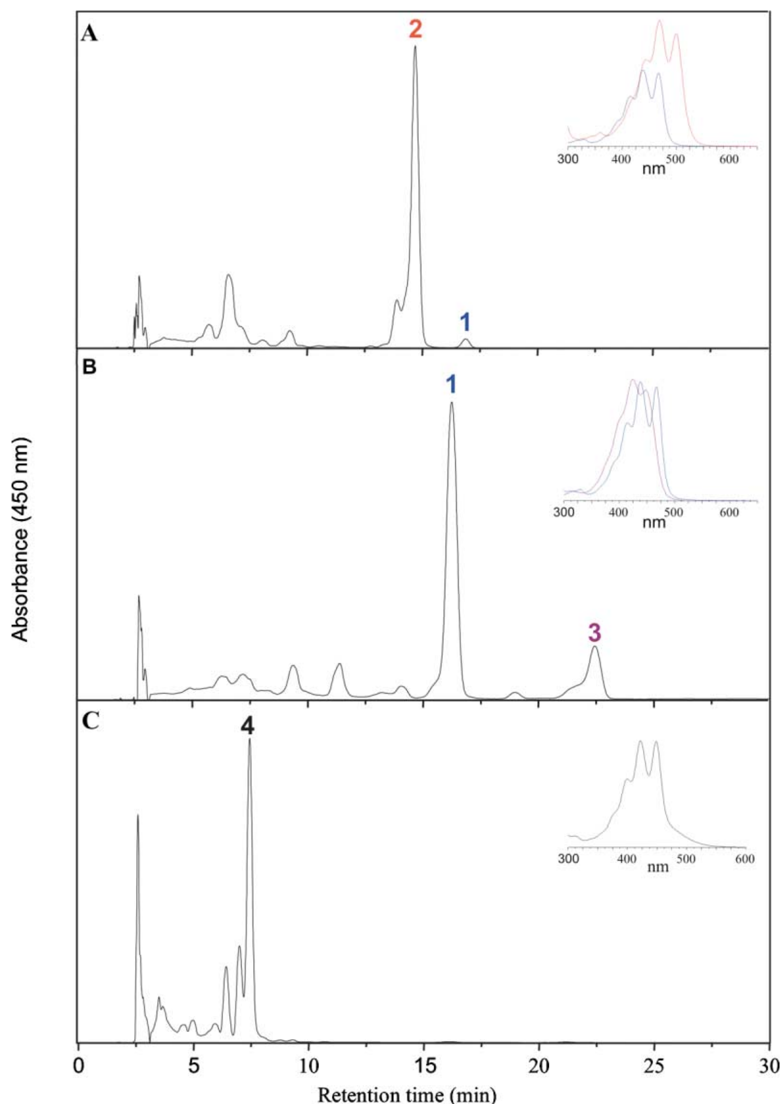

Figure 2. Analysis of *E. coli* Cells Producing C_{30} Carotenoids

HPLC analysis of carotenoid extracts of *E. coli* transformants expressing C_{30} carotenogenic enzymes (CrtM and CrtN) on pAC-*crtM-crtN* (A) together with lycopene cyclase pUC-*crtY* (B) or spheroidene monooxygenase pUC-*crtA* (C). The following diapocarotenoids were identified: peak 1, diaponeurosporene (λ_{max}: 415, 438, 467; M+ at m/e = 402.2); peak 2, diapolycopene (λ_{max}: 443, 468, 503; M+ at m/e = 400.1); peak 3, diapotorulene (λ_{max}: 425, 449; M+ at m/e = 402.1); peak 4, diaponeurosporene-derivative (λ_{max}: 399, 422, 449; M at m/e = 536.3). Double or triple peaks represent different geometrical isomers. Insets: recorded absorption spectra for individual peaks.

phate (FDP) is the precursor of the C_{30} biosynthetic pathway, the native *E. coli* FDP synthase (IspA) was overexpressed in order to increase the precursor pool and alter production levels. Expression of the resulting construct (pAC-*crtM-crtN-ispA*) in *E. coli* increased the diapotorulene to diaponeurosporene ratio 3- to 5-fold (data not shown).

Spheroidene Monooxygenase CrtA Oxygenizes Acyclic Intermediates of the Diapophytoene (C_{30}) Desaturation Pathway

Although many bacteria produce a large number of different acyclic xanthophylls (oxygenated carotenoids), only four genes encoding a hydratase (*crtC*), desaturase (*crtD*), methyl transferase (*crtF*), and a monooxygenase (*crtA*) have been cloned from *Rhodobacter* strains [11]. To obtain acyclic carotenoids with expanded chromophores, we chose CrtA as a possible enzyme for the introduction of keto groups into diapolycopene. In purple bacteria under aerobic conditions, CrtA catalyzes the asymmetrical introduction of one keto group at C2 as the terminal reaction of a sequence involving first hydroxylation at C1,C1′ (CrtC) of neurosporene or lycopene, followed by desaturation at C3,C4 (C3,C4′) (CrtD) and methoxylation at C1,C1′ (CrtF) [11, 25]. While CrtC and CrtD from *Rhodobacter* have recently been used to produce different hydroxylated lycopene and didehydrolycopene derivatives in *E. coli* [26], neither the enzymatic properties nor the substrate specificity of CrtA has yet been investigated.

To produce acyclic C_{30} xanthophylls in engineered *E. coli* cells, we extended the diapolycopene pathway in *E. coli* pAC-*crtM-crtN* with *crtA* on pUC-*crtA*. The cotransformed cells appeared more yellow than *E. coli* pAC-*crtM-crtN*. HPLC analysis of the cell extract showed three new very polar peaks (Figure 2C). The absorption maxima and spectral fine structure of the major carotenoid corresponds to an acyclic carotenoid without conjugated carbonyl functions and with eight conjugated double bonds as opposed to the nine conjugated double bonds in diaponeurosporene (Figure 1). The two minor peaks showed spectral properties similar

to diapo-ζ-carotene and diapophytoene (data not shown). Further structural analysis of the yellow carotenoid by HPLC-mass spectrometry showed an unexpected molecular mass of *m/z* 536.3 along with the prominent $[M-18]^+$ (loss of a hydroxy group) and $[M-58]^+$, $[M-87]^+$ ions (loss of an end group adjacent to a keto group), indicating a putative C_{35} backbone structure rather than C_{30}. Further fragmentation of the parent ion by MS/MS analysis gave additional unique $[M-18-16]^+$ (loss of oxygen from carbonyl group) and $[M-18-28]^+$ ions (loss of carbonyl group). Although these fragmentation patterns are consistent with expected CrtA end group monooxygenase activity, the high overall mass suggests a nonspecific activity or unknown biocatalytic function of CrtA. More detailed structural analysis of this compound is currently underway.

Spheroidene Monooxygenase CrtA Oxygenizes Acyclic Intermediates of the Phytoene (C_{40}) Desaturation Pathway

In order to generate new, acyclic, purple C_{40} xanthophylls in *E. coli* from the wild-type lycopene and in vitro evolved tetradehydrolycopene biosynthetic pathways, we applied CrtA to introduce keto groups and thus extend the chromophore of these products.

When lycopene- or tetradehydrolycopene-accumulating *E. coli* cells harboring pAC-*crtE-crtB-crtI* (orange-red cells) or pAC-*crtE-crtB-crtI14* (pink cells) [7] were cotransformed with pUC-*crtA*, the cell color changed to yellow and deep red, respectively (see colored cell extracts in Figure 1). All carotenoid extracts were separated by high-performance thin layer chromatography (HP-TLC) and high-pressure liquid chromatography (HPLC) (Figure 3), and structural identification was achieved by considering their polarity, absorption properties, and mass fragmentation patterns (compared to fragmentation patterns of known carotenoid end groups [27–29]). Extension of the lycopene pathway by coexpression of pUC-*crtA* with pAC-*crtE-crtB-crtI* in *E. coli* resulted in the synthesis of three novel acyclic xanthophylls: ζ-carotene-2-one (7,8,7′,8′-tetrahydro-1,2-dihydro-ψ,ψ-caroten-2-one), neurosporene-2-one (7,8-dihydro-1,2-dihydro-ψ,ψ-caroten-2-one), and lycopene-2-one (1,2-dihydro-ψ,ψ-caroten-2-one) (Figure 3A). Unexpectedly, the yellow carotenoids ζ-carotene and neurosporene, undetectable intermediates in lycopene producing *E. coli* pAC-*crtE-crtB-crtI* [7], also accumulated, indicating that CrtA uncouples the desaturation sequence catalyzed by CrtI. In addition, several minor more polar compound peaks were observed after HPLC separation. These compounds showed absorption characteristics of lycopene and neurosporene but with masses corresponding to the respective diketo- and dihydroxy-diketo-derivatives (data not shown). A deep purple dihydroxy-diketo-derivative of tetradehydrolycopene identified as phillipsiaxanthin (chemical synthesis and mass fragmentation described in [27]) and lycopene constitute the major carotenoids synthesized by *E. coli* pAC-*crtE-crtB-crtI14* coexpressing pUC-*crtA*. Phillipsiaxanthin is the first reported deep purple carotenoid produced in recombinant cells. Lycopene-2-one was accumulated as a minor product along with other polar xanthophylls that could not be identified unequivocally (Figure 3B).

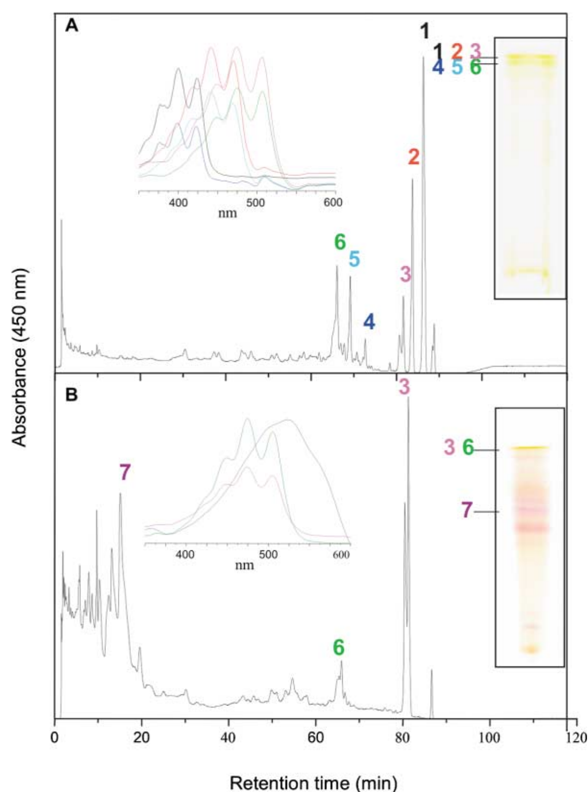

Figure 3. Analysis of *E. coli* Cells Producing Acyclic Oxygenated C_{40} Carotenoids

HPLC and HP-TLC analysis of carotenoid extracts of *E. coli* pAC-*crtE-crtB-crtI* (A) and *E. coli* pAC-*crtE-crtB-crtI14* (B) both coexpressing spheroidene monooxygenase (pUC-*crtA*). The following carotenoids were identified: peak 1, ζ-carotene (λ_{max}: 377, 400, 424; M+ at m/e = 540.4); peak 2, neurosporene (λ_{max}: 419, 442, 470; M+ at m/e = 538.4); peak 3, lycopene (λ_{max}: 449, 475, 507; M+ at m/e = 536.4); peak 4, ζ-carotene-2-one (λ_{max}: 377, 400, 424; M+ at m/e = 556.4); peak 5, neurosporene-2-one (λ_{max}: 419, 442, 470; M+ at m/e = 554.4); peak 6, lycopene-2-one (λ_{max}: 449, 475, 507; M+ at m/e = 552.4); peak 7, phillipsiaxanthin (λ_{max}: 516, 524; M+ at m/e = 596.3). Double or triple peaks represent different geometrical isomers. Insets: recorded absorption spectra for individual peaks and HP-TLC separations.

Our results show that coexpression of CrtA with acyclic C_{40} carotenoid pathways can introduce a keto group at the C(2,2′) position of unnatural substrates that do not exhibit a C(3,4) double bond as was previously thought to be necessary [6]. In addition, the complete conversion of tetradehydrolycopene to phillipsiaxanthin observed (Figure 3B) suggests it is a favorable substrate for CrtA activity when compared to the incomplete conversion of lycopene to lycopene-2-one in the presence of CrtA.

β-Carotene Oxygenase CrtO Introduces Keto Groups in Torulene and β,β-Carotene

Consequently, the next step was to probe the catalytic promiscuity of different cloned β,β-carotene-modifying enzymes toward torulene for the production of novel cyclic carotenoids. To extend the evolved torulene and, as a control, the wild-type β,β-carotene pathway, with different carotenoid genes in *E. coli,* we cloned lycopene

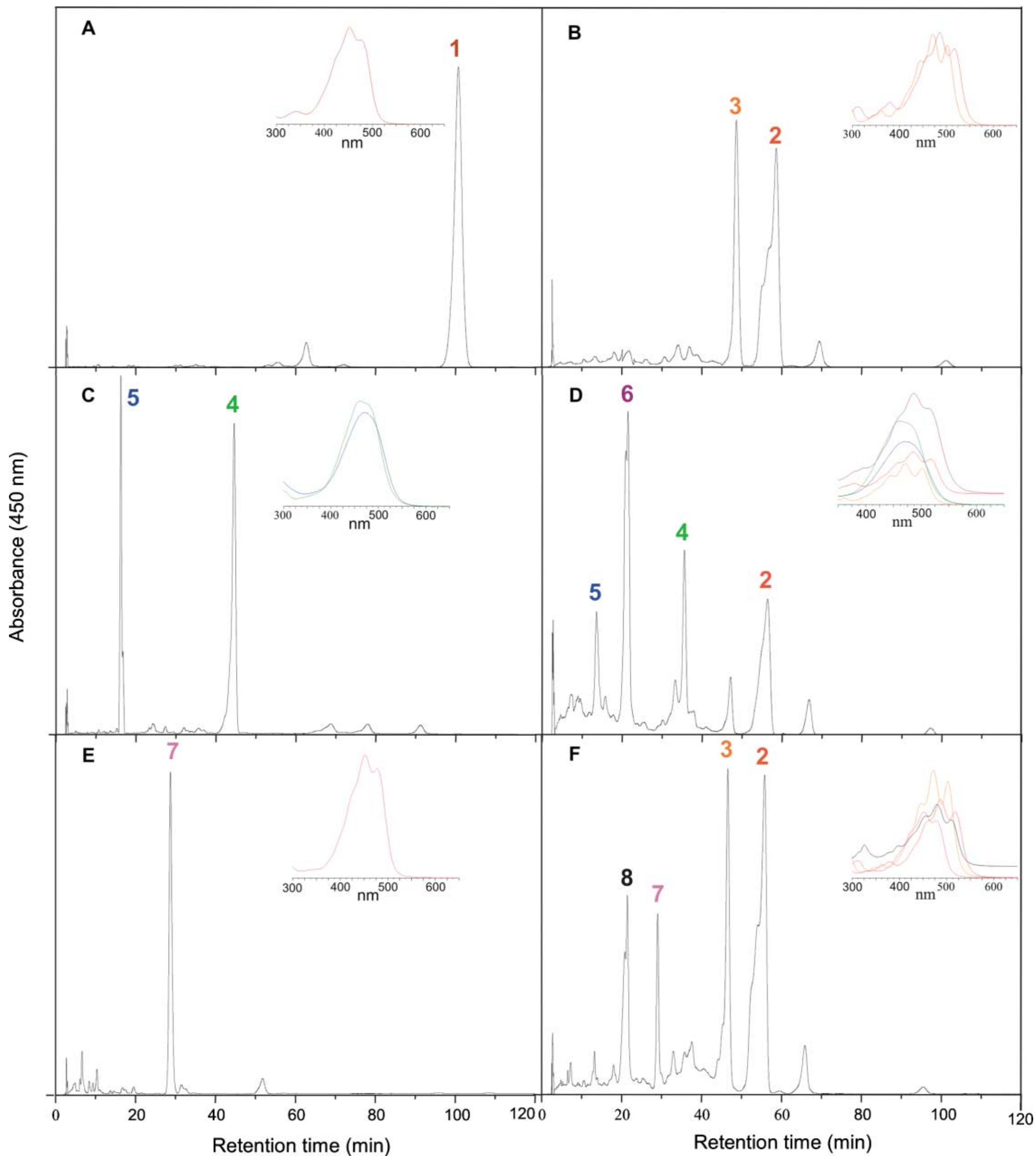

Figure 4. Analysis of Carotenoids Produced by Carotene Oxygenase and Desaturase

HPLC analysis of carotenoid extracts of *E. coli* transformants expressing: (A) pAC-crtE-crtB-crtI14-crtY (β,β-carotene pathway); (B) pAC-*crtE-crtB-crtI14-crtY2* (evolved torulene pathway); (C) pAC-*crtE-crtB-crtI14-crtY*; and (D) pAC-*crtE-crtB-crtI14-crtY2*, extended with carotene oxygenase CrtO on pUC-*crtO*; and (E) pAC-*crtE-crtB-crtI14-crtY* and (F) pAC-*crtE-crtB-crtI14-crtY2*, extended with carotene desaturase CrtU on pUC-*crtU*. The following carotenoids were identified: peak 1, β,β-carotene (λ_{max}: 425, 451, 478; M+ at m/e = 536.4); peak 2, torulene (λ_{max}: 454, 481, 514; M+ at m/e = 534.4); peak 3, lycopene (λ_{max}: 449, 475, 507; M+ at m/e = 536.4); peak 4, echinenone (λ_{max}: 457; M+ at m/e = 550.4); peak 5, canthaxanthin (λ_{max}: 463; M+ at m/e = 564.4); peak 6, Ketotorulene (λ_{max}: 454, 481, 514; M+ at m/e = 548.3); peak 7, isoreniaratene (λ_{max}: 425, 451, 478; M+ at m/e = 528.3); peak 8, didehydro-β,ϕ-carotene (λ_{max}: 454, 481, 514; M+ at m/e = 530.2). Double or triple peaks represent different geometrical isomers. Insets: recorded absorption spectra for individual peaks.

cyclase *crtY* or evolved cyclase *crtY2* into pAC-*crtE-crtB-crtI14* to yield pAC-*crtE-crtB-crtI14-crtY* and pAC-*crtE-crtB-crtI14-crtY2*. *E. coli* cells harboring pAC-*crtE-crtB-crtI14-crtY* developed a bright orange color due to the synthesis of β,β-carotene, while *E. coli* cells transformed with pAC-*crtE-crtB-crtI14-crtY2* turned bright red due to the production of torulene and lycopene (Figures 4A and 4B).

The introduction of keto groups at position C4(4′) of one or both rings of β,β-carotene is catalyzed by β-carotene oxygenases or ketolases. Most β-carotene oxygenases show homology to fatty acid desaturases and introduce keto groups at both β-rings to synthesize canthaxanthin, the precursor of the biotechnologically important carotenoid astaxanthin [30, 31] (Figure 1). However, β-carotene oxygenase CrtO from *Synechocystis* sp. is unique as it shows high homology to phytoene dehydrogenases and has been reported to intro-

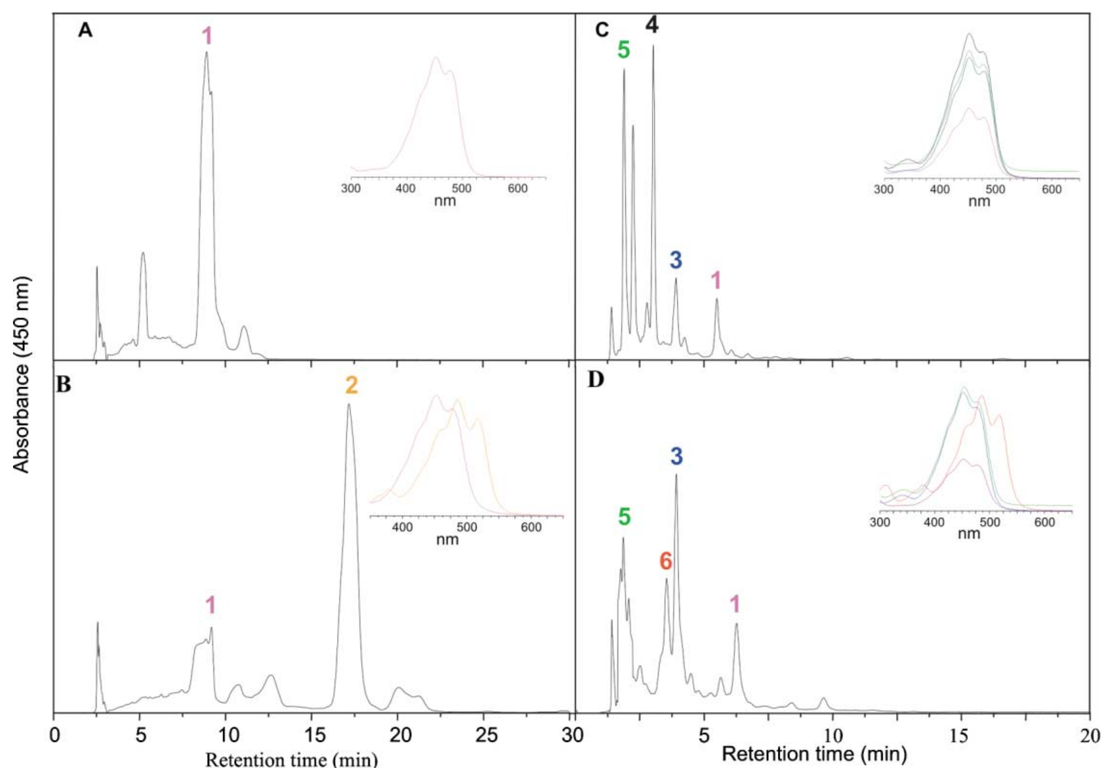

Figure 5. Analysis of Hydroxylated and Glucosylated Carotenoids Produced by Engineered *E. coli* Cells

HPLC analysis of carotenoid extracts of *E. coli* cells carrying: (A) pAC-*crtE-crtB-crtI14-crtY* (β,β-carotene pathway) and (B) pAC-*crtE-crtB-crtI14-crtY2* (evolved torulene pathway), together with β-carotene hydroxylase (*crtZ*); and (C) pAC-*crtE-crtB-crtI14-crtY-crtZ* and (D) pAC-*crtE-crtB-crtI14-crtY2-crtZ*, together with zeaxanthin glucosylase (*crtX*). The following carotenoids were identified: peak 1, zeaxanthin (λ_{max}: 425, 451, 478; M+ at m/e = 568.3); peak 2, hydroxy-torulene (λ_{max}: 454, 481, 514; M+ at m/e = 550.3); peak 3, β-cryptoxanthin-monoglucoside (λ_{max}: 425, 451, 478; M+ at m/e = 714.5); peak 4, zeaxanthin-monoglucoside (λ_{max}: 425, 451, 478; M+ at m/e = 730.5); peak 5, zeaxanthin-diglucoside (λ_{max}: 425, 451, 478; M+ at m/e = 892.5); peak 6, torulene-monoglucoside (λ_{max}: 454, 481, 514; M+ at m/e = 712.4).

duce only one keto group at C4 of one β-ring, as present in torulene, to synthesize echinenone [12]. We therefore cotransformed *E. coli* pAC-*crtE-crtB-crtI14-crtY* or pAC-*crtE-crtB-crtI14-crtY2* expressing the β,β-carotene or torulene pathways, respectively, with pUC-*crtO*. Surprisingly, in our system where each carotenoid enzyme is individually expressed under the control of a constitutive *lac*-promoter, CrtO introduced keto groups efficiently at both rings of β,β-carotene to yield canthaxanthin in a similar ratio to the mono-keto product echinenone (Figure 4C). The symmetrical activity of CrtO on β,β-carotene was not related to the gene copy number of *crtO* on pUC-*crtO* as similar ratios of canthaxanthin and echinenone were produced by *E. coli* with the single plasmid system pAC-*crtE-crtB-crtI14-crtY-crtO* (data not shown). Analysis of extracts from cells expressing the CrtO extended torulene pathway, however, revealed synthesis of a new, major carotenoid in addition to smaller amounts of echinenone, canthaxanthin, torulene, and lycopene (Figure 4D). Absorption maxima, polarity, and mass fragmentation spectrum of this new carotenoid identified it as 4-keto-torulene (Figure 1).

Aromatic Carotenoids Are Produced from β,β-Carotene and Torulene by CrtU

Aromatic carotenoids have been isolated from several bacteria, and three bacterial β-carotene desaturases (CrtU) have recently been cloned and characterized in their homologous hosts [13–15]. The symmetrical aromatization of β,β-carotene to isorenieratene (ϕ,ϕ-carotene) by CrtU involves the introduction of two double bonds and a concurrent methyl group shift for each β-ring (Figure 1). Because, to our knowledge, successful expression of CrtU in engineered *E. coli* cells for the production of aromatic carotenoids such as isorenariatene has not yet been reported, we first examined whether CrtU can function cooperatively with other heterologous carotenoid enzymes in engineered *E. coli*.

The exclusive formation of isorenariatene by *E. coli* pAC-*crtE-crtB-crtI14-crtY* coexpressed with pUC-*crtU* proved, therefore, that CrtU functions cooperatively with other carotenoid enzymes assembled from different organisms (Figure 4E). When *E. coli* cells harboring pAC-*crtE-crtB-crtI14-crtY2* were cotransformed with pUC-*crtU,* a new, more polar major carotenoid accumulated along with isoreniaratene, lycopene, and torulene (Figure 4F) and was identified by adsorption maxima, polarity, and mass fragmentation spectrum as aromatic torulene (didehydro-β,ϕ-carotene).

β-Carotene Hydroxylase CrtZ and Zeaxanthin Glucosylase CrtX Produce Novel Torulene Derivatives

The catalytic promiscuity observed for CrtO and CrtU with torulene suggested that β-carotene hydroxylase

CrtZ and zeaxanthin glucosylase CrtX, which converts β,β-carotene to the highly polar zeaxanthin-diglucoside in, e.g., *Erwinia* strains [16] (Figure 1), may exhibit similar broad substrate specificities and therefore enable synthesis of a novel polar torulene-glucoside in *E. coli*. To extend the torulene and, as a control, the β,β-carotene biosynthesis pathway in *E. coli* with the two enzymes (CrtZ and CrtX) necessary for β-ring glucosylation, we first cloned *crtZ* into pAC-*crtE-crtB-crtI14-crtY* (β,β-carotene) and pAC-*crtE-crtB-crtI14-crtY2* (torulene) to create pAC-*crtE-crtB-crtI14-crtY-crtZ* and pAC-*crtE-crtB-crtI14-crtY2-crtZ*. Pathway extension with CrtZ resulted in the symmetrical hydroxylation of β,β-carotene to zeaxanthin, which was formed as the only product in *E. coli* pAC-*crtE-crtB-crtI14-crtY-crtZ* (Figure 5A). However, a new polar carotenoid, with an absorption spectrum similar to torulene but with a mass spectrum expected for hydroxytorulene, accumulated as the main product in *E. coli* pAC-*crtE-crtB-crtI14-crtY2-crtZ* (Figure 5B), suggesting that torulene and β,β-carotene are equally good substrates for CrtZ. Subsequent combination in *E. coli* of pAC-*crtE-crtB-crtI14-crtY2/crtY-crtZ*, together with the terminal enzyme CrtX of the glucosylation pathway expressed on pUC-*crtX,* gave rise to a number of very polar carotenoid structures in *E. coli*. The assembled β,β-carotene glucosylation pathway in *E. coli* harboring pAC-*crtE-crtB-crtI14-crtY-crtZ* and pUC-*crtX* produced zeaxanthin-diglucoside as a major product. Other biosynthesis intermediates such as zeaxanthin, zeaxanthin-monoglucoside, and β-cryptoxanthin-monoglucoside (one β-ring of β,β-carotene glucosylated) were also produced (Figure 5C). Neither hydroxytorulene nor its precursor torulene accumulated in *E. coli* cells carrying the assembled torulene glycosylation pathway, but a new carotenoid identified as torulene glucoside is synthesized in addition to different hydroxylated and glucosylated β,β-carotene derivatives (Figure 5D). The formation of carotenoids where only one β-ring is hydroxylated or glucosylated indicates that CrtZ and CrtX catalyze β-ring modification irrespective of the other end structure present in a carotenoid molecule.

Significance

Extension of an in vitro evolved metabolic pathway with a functionally diverse array of modifying enzymes allowed us to engineer pathways for the recombinant production of nine novel carotenoid structures in *E. coli*. More importantly, several of the novel carotenoids have never before been isolated in nature (e.g., diapotorulene) or synthesized in engineered cells (e.g., the first deep purple carotenoid phillipsiaxanthin). These results therefore represent the most extensive example of combinatorial biosynthesis outside the polyketide field. Furthermore, this study shows that modifying genes located later in a biosynthetic pathway can exhibit a higher catalytic promiscuity than those earlier in the pathway, allowing them to accept unnatural substrates. Therefore, we believe that the combination of directed evolution to diverge natural pathways toward new possible metabolic routes and extension of these pathways with additional genes is a powerful approach to discover novel natural and unnatural compounds and produce these compounds in microbial hosts.

Experimental Procedures

Cloning and Culture Growth

Genes encoding dehydrosqualene synthase (*crtM*), diapophytoene synthase (*crtN*) from *Staphylococcus aureus* (ATCC 35556D), spheroidene monooxygenase (*crtA*) from *Rhodobacter capsulatus* (DSMZ 1710), β-carotene oxygenase (*crtO*) from *Synechocystis* sp. (ATCC 27184), β-carotene desaturase (*crtU*) from *Brevibacterium linens* (DSMZ 20426), and β-carotene hydroxylase (*crtZ*) and zeaxanthin glucosylase (*crtX*) from *Erwinia uredovora* (*Pantoea ananatis* DSMZ 30080) were amplified from genomic DNA using a 5′ primer containing at its 5′ end a *Xba*I or *Eco*RI site followed by an optimized Shine-Dalgarno sequence (underlined) and a start codon (bold) (5′-AGGAGGATTACAAA**ATG**-3′) and a 3′ primer containing at its 5′ end a *Eco*RI or *Nco*I site (Table 1A). PCR products were then digested with restriction enzymes and cloned into the corresponding sites of plasmid pUCmod [7] to facilitate constitutive expression from a modified *lac*-promoter.

For C_{30} carotenoid pathway assembly, *crtM* and *crtN* were subcloned from pUCmod into the *Sal*I (*crtM*) or a *Bam*HI (*crtN*) site of pACmod [7] by amplification of the genes together with the modified constitutive *lac*-promoter, using primers that introduce the corresponding restriction enzyme sites at both ends, to give pAC-*crtM-crtN* where *crtM* and *crtN* have the same orientation as the disrupted tetracycline resistance gene. Likewise, for assembly of the β-carotene and torulene pathways, genes encoding wild-type (*crtY*) or mutant lycopene cyclase (*crtY2*) were subcloned from pUCmod into the *Sal*I site of pAC-*crtE-crtB-crtI14* [7] to give pAC-*crtE-crtB-crtI14-crtY* and pAC-*crtE-crtB-crtI14-crtY2*, respectively (*crtY/Y2* have the same orientation as *crtE* and *crtI14*). To assemble the glucosylation pathways, *crtZ* was subcloned similarly into the *Ppm*UI site of pAC-*crtE-crtB-crtI14-crtY* and pAC-*crtE-crtB-crtI14-crtY2* to produce pAC-*crtE-crtB-crtI14-crtY-crtZ* and pAC-*crtE-crtB-crtI14-crtY2-crtZ*, respectively (*crtZ* has the same orientation as *crtY/Y2*). These plasmids and the carotenoids biosynthetic pathways expressed are described in Table 1B.

For carotenoid production, recombinant *E. coli* JM109 were cultivated for 48 hr in the dark at 28°C in Luria-Bertani (LB) medium (200 ml medium in a 500 ml flask or 1 l medium in a 3 l flask) supplemented with the appropriate selective antibiotics chloramphenicol (50 μg/ml) and/or carbenicillin (100 μg/ml).

Isolation of Carotenoids

Wet cells from a 200 ml (~500 mg) or 4 l culture (~10 g) were repeatedly extracted at 4°C with a total volume of 30 ml or 400 ml methanol or acetone until all visible pigments were extracted. After centrifugation (4°C, 6000 rpm), the colored supernatants were pooled and combined supernatants were centrifuged again, filtrated (nylon membrane 0.2 μm, Whatman) to remove fine particles, evaporated in a vacuum to dryness, and finally resuspended with 30–50 ml acetone. The acetone extract was kept at –80°C for 1 day to form a white precipitate and filtrated with 0.2 μm nylon membrane to remove the precipitate. The resulting pigment extracts were re-extracted with an equal volume of ethyl acetate or hexane after addition of 1/2 volume of saltwater (15% NaCl). The organic phase that contained carotenoids was collected and washed with water. The collected organic phase was completely evaporated in a vacuum to dryness at room temperature, resuspended with 0.5–1 ml hexane, applied to silica gel chromatography (25 × 120 cm), and eluted stepwise with increasing amount of acetone in hexane (0% acetone to 30% acetone in hexane basis). The color fractions were then dried under nitrogen gas or in a vacuum and dissolved in 1–2 ml hexane. A 1–3 μl aliquot of the fractions and the crude extracts were subjected to high-performance TLC separation for initial analysis of the crude extract and the color fractions composition on Whatman silica gel 60 Å plates (4.5 μm particle size, 200 μm thickness) using the following solvent systems: (1) acetone:hexane

(40:60) for acyclic C_{30} and C_{40} xanthophylls, (2) hexane:chloroform:acetone (85:15:20) for diapocarotenoids and cyclic xanthophylls, (3) hexane:chloroform (85:15) for cyclic aromatic carotenoids, (4) hexane:chloroform (100:5) for cyclic C_{40} carotenoids, (5) hexane:acetone (80:20) for hydroxylated cyclic C_{40} carotenoids, and (6) chloroform:methanol (80:20) for glucosylated cyclic C_{40} carotenoids. For the further purification of carotenoids, a preparative TLC and HPLC were used. The preparative TLC was performed under the same conditions as the above and carotenoids were eluted with acetone or methanol. The preparative HPLC, if needed, was carried out with a semipreparative Zorbax SB-C18 column (9.6 $\times$ 250 mm, 5 μm; Agilent Technologies, Palo Alto, CA), and eluted under isocratic conditions with two solvent systems (A, 90% acetonitrile and 10% methanol, and B, 90% [acetonitrile:water, 100:15] and 10% methanol) at a flow rate of 1.5 ml min^{-1}, which were optimized based on peak resolution, using an Agilent 1100 HPLC system equipped with an photodiode array detector.

Analysis of Carotenoids

For the analysis of carotenoids, 10–20 μl of the crude extract and the collected color fractions were applied to a Zorbax SB-C18 column (4.6 $\times$ 250 mm, 5 μm; Agilent Technologies, Palo Alto, CA) and typically eluted under isocratic conditions with a solvent system containing 90% (acetonitrile:H_2O, 99:1) and 10% (methanol:tetrahydrofurane, 8:2) at a flow rate of 1 ml min^{-1} using an Agilent 1100 HPLC system equipped with an photodiode array detector. Gradient conditions with solvent A (acetonitrile:H_2O, 85:15) and solvent B (methanol:tetrahydrofurane, 8:2) were used for the elution of acyclic C_{40} xanthophylls (0–30 min, A:B 95:5; 30–60 min, A:B 88:12; 60–90 min, A:B 1:1; 90–120 min, A:B 1:9). For structural elucidation, carotenoids were identified by a combination of HPLC retention times, absorption spectra, and mass fragmentation spectra [27–29]. Authentic standards for comparison were isolated from recombinant *E. coli* containing plasmids for lycopene, tetradehydrolycopene, torulene, and β,β-carotene biosynthesis. Mass fragmentation spectra were monitored in a mass range of m/z 200–800 or 1000 on a LCQ mass spectrophotometer equipped with an electron spray ionization (ESI) or atmosphere pressure chemical ionization (APCI) interface (Thermo Finnigan, USA). Parent molecular ions were further fragmented by MS/MS analysis using an APCI interface at optimal collision-induced dissociation energy (28%–30%).

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