Applied and Environmental Microbiology, Aug. 2010, p. 5199–5206 Vol. 76, No. 15
0099-2240/10/$12.00 doi:10.1128/AEM.00263-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Redesign, Reconstruction, and Directed Extension of the *Brevibacterium linens* C_{40} Carotenoid Pathway in *Escherichia coli*▽†

Se Hyeuk Kim,[1] Yun Hee Park,[1] Claudia Schmidt-Dannert,[2] and Pyung Cheon Lee[1]*

Department of Molecular Science and Technology and Department of Biotechnology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea,[1] and Department of Biochemistry, Molecular Biology and Biophysics, 1479 Gortner Avenue, University of Minnesota, Saint Paul, Minnesota 55108[2]

Received 1 February 2010/Accepted 26 May 2010

In this study, the carotenoid biosynthetic pathways of *Brevibacterium linens* DSMZ 20426 were reconstructed, redesigned, and extended with additional carotenoid-modifying enzymes of other sources in a heterologous host *Escherichia coli*. The modular lycopene pathway synthesized an unexpected carotenoid structure, 3,4-didehydrolycopene, as well as lycopene. Extension of the novel 3,4-didehydrolycopene pathway with the mutant *Pantoea* lycopene cyclase $CrtY_2$ and the *Rhodobacter* spheroidene monooxygenase CrtA generated monocyclic torulene and acyclic oxocarotenoids, respectively. The reconstructed β-carotene pathway synthesized an unexpected 7,8-dihydro-β-carotene in addition to β-carotene. Extension of the β-carotene pathway with the *B. linens* β-ring desaturase CrtU and *Pantoea* β-carotene hydroxylase CrtZ generated asymmetric carotenoid agelaxanthin A, which had one aromatic ring at the one end of carotene backbone and one hydroxyl group at the other end, as well as aromatic carotenoid isorenieratene and dihydroxy carotenoid zeaxanthin. These results demonstrate that reconstruction of the biosynthetic pathways and extension with promiscuous enzymes in a heterologous host holds promise as a rational strategy for generating structurally diverse compounds that are hardly accessible in nature.

Carotenoids, which are produced by many microorganisms and plants, belong to a class of pigment chemicals found in nature. These structurally diverse pigments have different biological functions such as coloration, photo protection, light-harvesting, and precursors for many hormones (3, 22). Carotenoids are commercially used as food colorants, animal feed supplements and, more recently, as nutraceuticals and as cosmetic and pharmaceutical compounds (19). Currently, only a few carotenoids can be produced commercially by chemical synthesis, fermentation, or isolation from a few abundant natural sources (13). The increasing industrial importance of carotenoids has led to renewed efforts to develop bioprocesses for large-scale production of a range of carotenoids, including lycopene, β-carotene, and more structurally diverse carotenoids (17, 21, 30, 31, 34). Interestingly, a recent study showed that carotenoids with more diverse structures tend to have higher biological activity than simple structures (1).

Previously, *in vitro* evolution altered the catalytic functions of the carotenoid enzymes phytoene desaturase CrtI and lycopene cyclase CrtY (Fig. 1) and produced novel carotenoid structures of tetradehydrolycopene and torulene in *Escherichia coli* (27). Furthermore, these *in vitro* evolved pathways and redesigned C_{30} carotenoid biosynthetic pathways were successfully extended with additional, wild-type carotenoid modifying enzymes and evolved enzymes (21), generating novel carotenoid structures (26).

Beside *in vitro* evolution (23, 34), combinatorial biosynthesis with carotenoid-modifying enzymes in a heterologous host has often been used to generate structurally novel carotenoids (24, 32). This combinatorial biosynthetic approach basically relies on the functional coordination of pathway enzymes from different sources in a heterologous host (5, 19, 35). Carotenogenic enzymes tend to be promiscuous in their substrate specificity (33) and show unexpected/hidden activities (20) when expressed in heterologous host microorganisms. One example is the unusual activity of diapophytoene desaturase CrtN in *E. coli*, which resulted in structurally novel compounds (20). Therefore, utilizing the promiscuity of carotenogenic enzymes makes combinatorial biosynthesis one of the most powerful strategies to generate structurally novel carotenoids that cannot be accessed in nature.

Yellow colored *Brevibacterium linens* is commonly used as a food colorant by the cheese industry (15). Interestingly, *B. linens* is known to synthesize aromatic ring-containing carotenoids, isorenieratene and its hydroxy derivatives (6, 7, 16). They are produced by seven carotenogenic enzymes expressed in *B. linens*: GGPP synthase CrtE, phytoene synthase CrtE, phytoene desaturase CrtI, lycopene cyclase CrtYcYd, β-carotene desaturase CrtU, and the cytochrome P450 (Fig. 1). Even though the carotenoid biosynthetic pathways of *B. linens* have been recently studied (6, 10), there have been no systematic functional study of downstream enzymes such as lycopene cyclase CrtYcYd in the biosynthetic pathway of *B. linens* in a heterologous environment.

Therefore, in the present study, for the first time we reconstructed, redesigned, and rationally extended the *B. linens*

* Corresponding author. Mailing address: Department of Molecular Science and Technology and Department of Biotechnology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea. Phone: 82-31-219-2461. Fax: 82-31-219-1610. E-mail: pclee@ajou.ac.kr.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 4 June 2010.

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

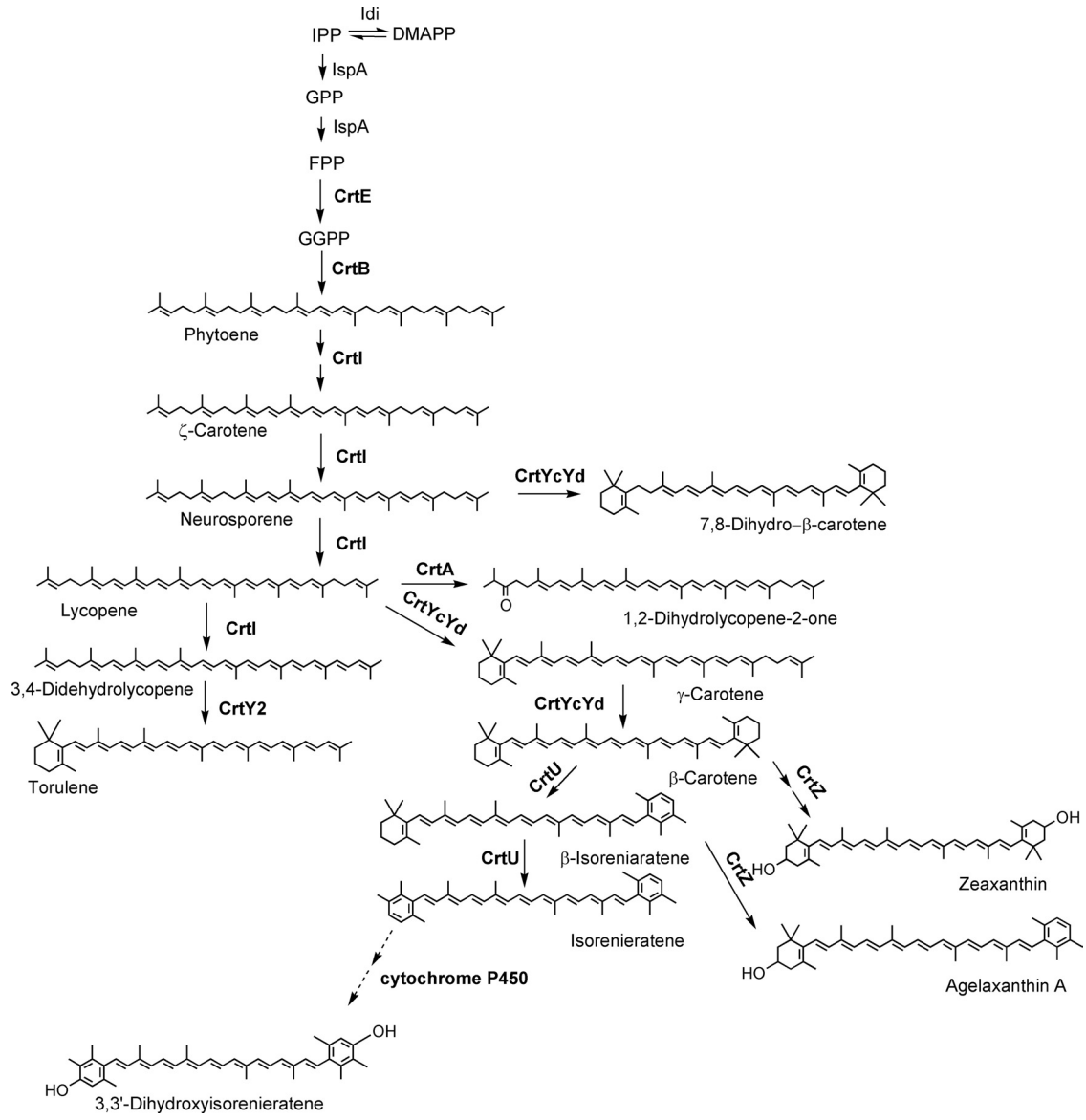

FIG. 1. Reconstructed and redesigned *B. linens* carotenoid biosynthetic pathway in the heterologous host *E. coli*. Carotenogenic enzymes of *B. linens*, *P. ananatis*, and *R. capsulatus*, which were used for the biosynthetic pathway reconstruction, are indicated by boldface letters. Idi (IPP isomerase), IspA (FPP synthase), CrtE (GGPP synthase), CrtB (phytoene synthase), CrtI (phytoene desaturase), CrtYcYd (lycopene cyclase), CrtU (β-carotene desaturase), CrtZ (β-carotene hydrolase), $CrtY_2$ (mutant lycopene cyclase), and CrtA (spheroidene monooxygenase). *B. linens* 3,3′-dihydroxyisorenieratene biosynthesis is indicated by dashed arrows.

carotenoids biosynthetic pathway in *E. coli* to investigate the flexibility of the pathway enzymes in a heterologous host. Using this approach, we obtained an unexpected structure 3,4-didehydrolycopene, 7,8-dihydro-β-carotene, torulene, and the asymmetric carotenoid, agelaxanthin A, from engineered *B. linens* carotenoid pathways in *E. coli*.

MATERIALS AND METHODS

Gene cloning. *crtE*, *crtB*, *crtI*, *crtYcYd*, *crtU*, ORF10 encoding cytochrome P450 of *B. linens* DSMZ 20426, *crtA* of *Rhodobacter capsulatus* DSMZ 1710, and *crtZ* of *Pantoea ananatis* (formerly *Erwinia uredovora*) DSMZ 30080 were amplified by PCR using a 5′ primer containing a XbaI restriction enzyme site followed by an optimized Shine-Dalgarno sequence (underlined) and a start codon (indicated in boldface; 5′-AGGAGGATTACAAA**ATG**-3′), and a 3′ primer containing a EcoRI or NotI restriction enzyme site at its 5′ end (see the supplemental material). PCR products were purified by using a PCR product purification kit (Intron, Korea) or a gel extraction kit (Intron, Korea) and digested with the corresponding restriction enzymes (New England Biolabs). Purified insert DNAs were ligated into corresponding restriction enzyme sites of the plasmid pUCM, which had a modified constitutive *lac* promoter and new restriction enzyme sites (XbaI, AvaI, XmaI, SmaI, EcoRI, NcoI, NotI, and ApaII). pUCM is a high-copy-number plasmid pUC19 derivative that is devoid of the *lacZ* fragment region using PCR primers (5′-CCG GAA TTC CCA TGG GCG GCC GC TGC GGT ATT TTC TCC-3′ and 5′-CCG GAA TTC CCC GGG CGC TCT AGA CGC TCA CAA TTC CAC ACA-3′). To assemble a lycopene biosynthetic pathway in *Escherichia coli*, *crtE* was subcloned into the BamHI and HindIII sites of pACYC184, resulting in $pACM\text{-}E_{BL}$; *crtB* was subcloned into the BamHI site of pACYC184, resulting in $pACM\text{-}B_{BL}$; and *crtI* was subcloned into the HindIII site of pACYC184, resulting in $pACM\text{-}I_{BL}$ by amplification of the genes together with the modified constitutive *lac*-promoter, using primers that introduce corre-

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference[a]
Strains		
B. linens	C_{40} carotenoid pathway	DSMZ 20426
P. ananatis	C_{40} carotenoid pathway	DSMZ 30080
R. capsulatus	C_{40} carotenoid pathway	DSMZ 1710
Plasmids		
pUC19	Cloning vector; inducible *lac* promoter, Ap	NEB
pUCM	Cloning vector modified from pUC19; constitutive *lac* promoter, Ap	This study
pACYC184	Cloning vector, Cm	NEB
pUCM-E_{BL}	Constitutively expressed *crtE* gene of *B. linens*	This study
pUCM-B_{BL}	Constitutively expressed *crtB* gene of *B. linens*	This study
pUCM-I_{BL}	Constitutively expressed *crtI* gene of *B. linens*	This study
pUCM-Y_{BL}	Constitutively expressed *crtY* gene of *B. linens*	This study
pUCM-U_{BL}	Constitutively expressed *crtU* gene of *B. linens*	This study
pUCM-$P450_{BL}$	Constitutively expressed cytochrome P450 gene of *B. linens*	This study
pUCM-Z_{PAN}	Constitutively expressed *crtZ* gene of *P. ananatis*	This study
pUCM-A_{RC}	Constitutively expressed *crtA* gene of *R. capsulatus*	This study
pUC-crtY_2	Constitutively expressed *in vitro* evolved *crtY2* gene	27
pUC19-U_{BL}-P450	Inducibly expressed both *crtU* and cytochrome P450 gene of *B. linens*	This study
pUC19-U_{BL}-Z_{PAN}	Inducibly expressed both *crtU* of *B. linens* and *crtZ* gene of *P. ananatis*	This study
pACM-E_{BL}	Constitutively expressed *crtE* gene of *B. linens* on pACYC184	This study
pACM-B_{BL}	Constitutively expressed *crtB* gene of *B. linens* on pACYC184	This study
pACM-I_{BL}	Constitutively expressed *crtI* gene of *B. linens* on pACYC184	This study
pACM-E_{BL}-B_{BL}	Constitutively expressed *crtE* and *crtB* genes of *B. linens* on pACYC184	This study
pACM-E_{BL}-I_{BL}	Constitutively expressed *crtE* and *crtI* genes of *B. linens* on pACYC184	This study
pACM-B_{BL}-I_{BL}	Constitutively expressed *crtB* and *crtI* genes of *B. linens* on pACYC184	This study
pAC-crtE-crtB-crtI14	Constitutively expressed *crtE*, *crtB*, and *crtI14* genes of *P. ananatis* produce 3,4,3′,4′-tetradehydrolycopene, as well as lycopene	27
pAC-crtE-crtB-crtI	Constitutively expressed *crtE*, *crtB*, and *crtI* genes of *P. ananatis* to produce lycopene	27
pACM-E_{BL}-B_{BL}-I_{BL}	Constitutively expressed *crtE*, *crtB*, and *crtI* genes of *B. linens* on pACYC184 to produce lycopene	This study
pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}	Constitutively expressed *crtE*, *crtB*, *crtI*, and *crtY* genes of *B. linens* on pACYC184 to produce β-carotene	This study

[a] NEB, New England Biolabs.

sponding restriction enzyme sites at both ends (Table 1). Next, the *crtB* module from pUCM-B_{BL} was subcloned into pACM-E_{BL} to generate plasmid pACM-E_{BL}-B_{BL}, which express CrtE and CrtB together. Similarly, the *crtI* module from pUCM-I_{BL} and the *crtB* module from pUCM-B_{BL} were subcloned into pACM-E_{BL} and pACM-I_{BL} to generate two plasmids: pACM-E_{BL}-I_{BL} expressing CrtE and CrtI together and pACM-B_{BL}-I_{BL} expressing CrtB and CrtI together. The functionality of the resulting synthetic modules consisting of two genes was examined by complementation with the third gene, for example, complementing CrtE on pUCM-E_{BL} with a synthetic module of CrtB and CrtI on pACM-B_{BL}-I_{BL}. Finally, the third *crtI* gene was functionally assembled into pACM-E_{BL}-B_{BL}, resulting in the plasmid pACM-E_{BL}-B_{BL}-I_{BL}. To reconstruct the β-carotene biosynthesis pathway, *crtYcYd* was subcloned into the *Ppu*MI site of pACM-E_{BL}-B_{BL}-I_{BL}, resulting in pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}. Amplification of genes was performed by using the *Vent* polymerase (New England Biolabs), and the T4 DNA ligase (New England Biolabs) was used for the ligation reaction.

Culture growth and isolation of carotenoids. For carotenoid production, recombinant *E. coli* SURE cells harboring the carotenogenic plasmids were cultivated in 100 ml of Terrific Broth (TB) medium supplemented with the appropriate selective antibiotics chloramphenicol (50 μg/ml) and/or ampicillin (100 μg/ml) for 48 h at 30°C with 250 rpm. Cells were pelleted by centrifugation (4°C, 4,000 rpm) and extracted repeatedly with a total volume of 15 ml of acetone until all visible pigments were extracted. After centrifugation (4°C, 4,000 rpm), the colored supernatants were pooled and reextracted with an equal volume of hexane after the addition of an equal volume of double-distilled water. All extracts were passed through sodium sulfate (anhydrous; BioBasic) for dehydration, subjected to silica gel chromatography, and eluted with 100% hexane. The color fractions were then dried under nitrogen gas and resuspended with 0.5 ml of acetone. After centrifugation (13,000 rpm, 20 min), extracts were filtered (0.45-μm-pore-size GHP membrane; Pall) to remove fine particles.

Analysis of carotenoids. Thin-layer chromatography (TLC) analysis was performed for the initial analysis under a 100% hexane solvent system (17). For oxygenated carotenes, a acetone-hexane (40:60) solvent system was used. A total of 20 μl of the collected color fractions were applied to a Zorbax eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent Technologies) and typically eluted under isocratic conditions with a solvent system containing 80% acetonitrile, 15% methanol, and 5% isopropanol at a flow rate of 1 ml min^{-1} using an Agilent 1200 high-pressure liquid chromatography (HPLC) system equipped with a photodiode array detector. For the elution of oxygenated carotenes, the mobile phase consisted of acetonitrile-H_2O (90:10) for 20 min, followed by a gradient to acetonitrile-methanol-isopropanol (80:15:5) at 50 min. For structural elucidation, carotenoids were identified using a combination of HPLC retention times, absorption spectra, and mass fragmentation spectra. Mass fragmentation spectra were monitored in a mass range of *m/z* 200 to 800 on a liquid chromatography-mass spectrometry (LC/MS; Waters) equipped with an electron spray ionization (ESI) interface.

RESULTS AND DISCUSSION

Functional assembly of *B. linens* lycopene and β-carotene synthetic modules in *E. coli*. When plasmid pACM-E_{BL}-B_{BL}-I_{BL} was transformed into *E. coli* cells, the transformants turned reddish, indicating successful formation of reddish lycopene *in vivo*. When crude carotenoid extracts of the reddish cells were analyzed by TLC and HPLC, another small peak in addition to lycopene was detected in the HPLC chromatogram (Fig. 2A). This new peak corresponded to the reddish spot on the TLC plate (Fig. 2C). Based on its polarity, UV/Vis absorption property (λ_{max} = 460, 491, and 523 nm) and relative molecular mass (M^+ at *m/z* = 534.69), the new carotenoid was identified as

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

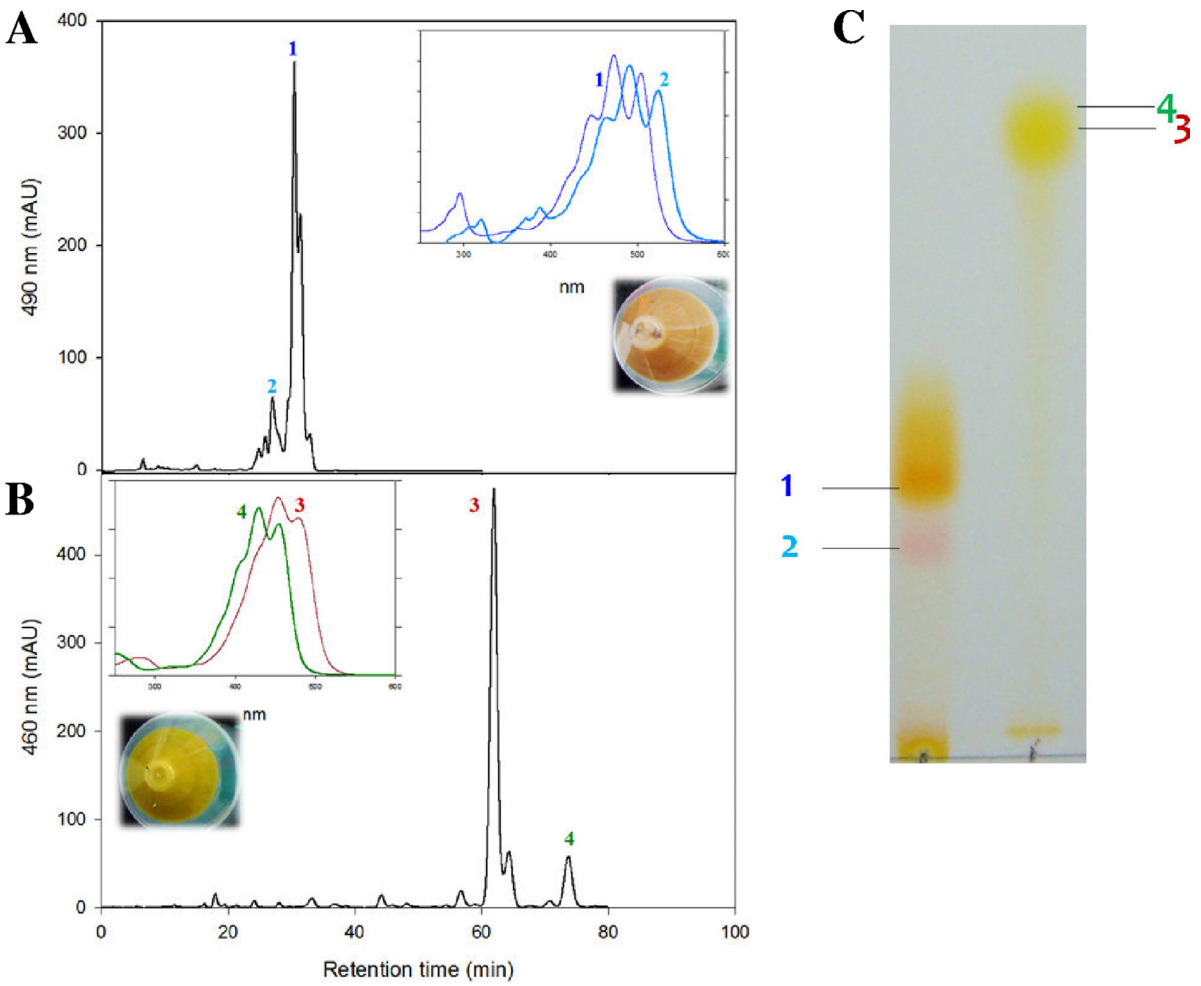

FIG. 2. Analysis of recombinant *E. coli* cells engineered with reconstructed *B. linens* carotenoid biosynthetic pathways. HPLC analyses of crude extracts of *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}) (A) and *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}) (B) were carried out, as well as TLC analysis (C). The following carotenoids were identified: peak 1, lycopene (λ_{max} = 447, 473, and 503; $[M+H]^+$ at *m/z* = 537.75); peak 2, 3,4-didehydrolycopene (λ_{max} = 460, 491, and 523; M^+ at *m/z* = 534.69); peak 3, β-carotene (λ_{max} = 427, 453, and 481; M^+ at *m/z* = 536.86); peak 4, 7,8-dihydro-β-carotene (λ_{max} = 403, 429, and 453; M^+ at *m/z* = 539). Insets show the recorded absorption spectra for individual peaks and pelleted cells of recombinant *E. coli*.

3,4-didehydrolycopene, which has 13 conjugated double bonds (CDB) in its backbone. The ratio of 3,4-didehydrolycopene to lycopene was 1:5.6 based on the peak area calculated by using Agilent ChemStation software. Formation of 3,4-didehydrolycopene was further confirmed by complementing two dissected lycopene pathway modules. All *E. coli* cells with a combination of pACM-E_{BL}-B_{BL} + pUCM-I_{BL}, pACM-B_{BL}-I_{BL} + pUCM-E_{BL}, or pACM-E_{BL}-I_{BL} + pUCM-B_{BL} produced 3,4-didehydrolycopene, as did *E. coli* (pACM-E_{BL}-B_{BL}-I_{BL}) (data not shown). These results indicate that 3,4-didehydrolycopene is one of the end products of the reconstructed *B. linens* lycopene pathway.

The bacterial phytoene desaturase CrtI introduces CDBs into colorless phytoene to neurosporene (9 CDBs), lycopene (11 CDBs), or 3,4-didehydrolycopene (13 CDBs) with three-, four-, or five-step desaturation reactions, respectively (25). Wild-type five-step-desaturase CrtI, which is capable of producing 3,4-didehydrolycopene, is very rare in nature, whereas four-step -desaturase CrtI, which is capable of producing lycopene, such as *Pantoea*-derived CrtI, and three-step-desaturase CrtI, which is capable of producing neurosporene, such as *Rhodobacter*-derived CrtI or *Staphylococcus*-derived CrtN, are common in nature (18). It is known that fungi *Neurospora crassa* phytoene desaturase Al-1 has the ability to catalyze the stepwise introduction of up to five double bonds into phytoene and produce 3,4-didehydrolycopene, which is further transformed into torulene (11). Formation of the rare 3,4-didehydrolycopene was also reported as an intermediate in an *in vitro* evolved 3,4,3′,4′-tetradehydrolycopene pathway utilizing the mutant *Pantoea* $CrtI_{14}$ even though 3,4-didehydrolycopene was not directly detected (27). Interestingly, purified *Pantoea* CrtI could produce a small amount of fully conjugated 3,4,3′,4′-tetradehydrolycopene under certain *in vitro* conditions (8). The major carotenoids synthesized in *B. linens* are isorenieratene and its hydroxy compounds, which came from lycopene containing 11 CDBs in its backbone (12, 15). However, in a heterologous *E. coli*, *B. linens* CrtI introduces 13 CDBs in phytoene unlike the original host strain *B. linens* (15). This result suggests that heterologously expressed *B. linens* CrtI may show altered activity than expected. This has been previously sug-

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

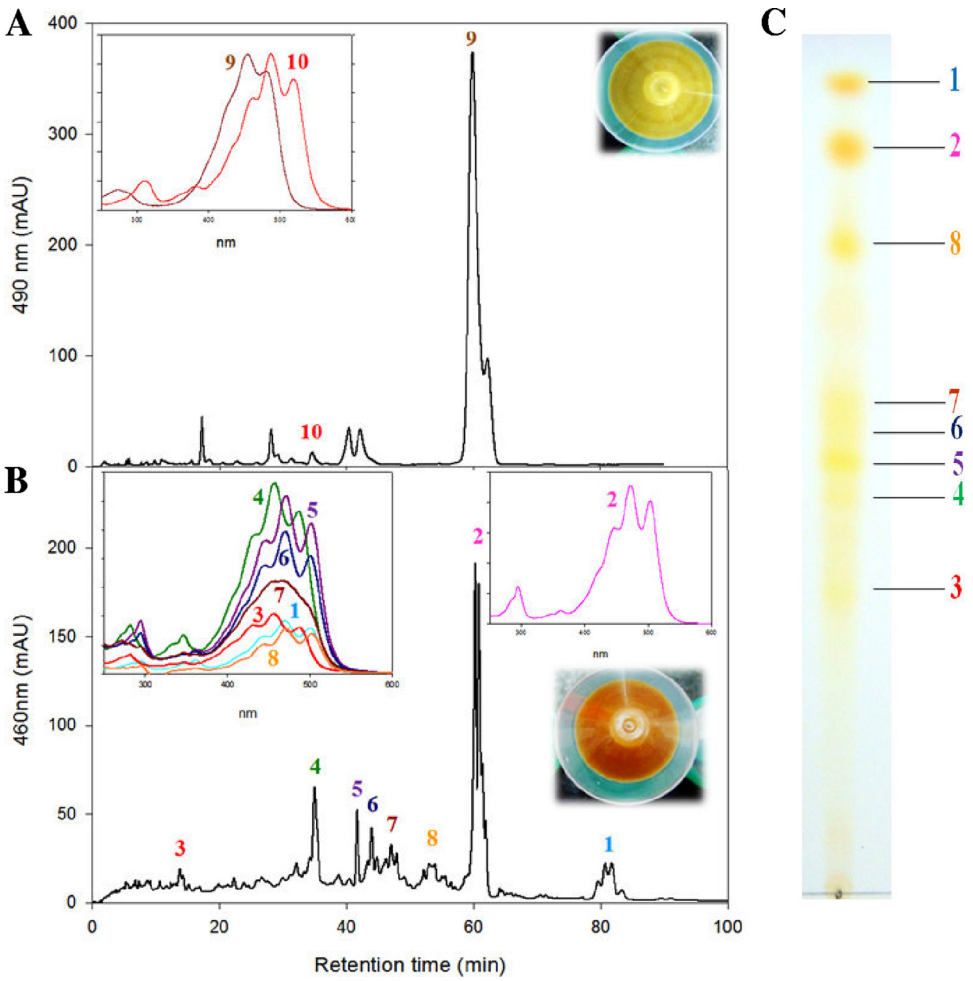

FIG. 3. Analysis of extension of the reconstructed lycopene/3,4-didehydrolycopene pathway with a mutant $CrtY_2$ or $CrtA_{RC}$. HPLC analysis of the reconstructed lycopene/didehydrolycopene pathway with a mutant lycopene cyclase $CrtY_2$ (A) and a spheroidene monooxygenase $CrtA_{RC}$ (B) in *E. coli* was carried out, as well as TLC (C). The following carotenoids were identified: peak 1, lycopene (λ_{max} = 447, 473, and 503; $[M+H]^+$ at m/z = 537.75); peak 2, 1,2-dihydrolycopene-2-one (λ_{max} = 443, 469, and 500; $[M+H]^+$ at m/z = 553.74); peak 9, β-carotene (λ_{max} = 427, 453, and 481; M^+ at m/z = 536.86); peak 10, torulene (λ_{max} = 460, 488, and 520 nm; $[M+H]^+$ at m/z = 535.86); peaks 3, 4, 5, 6, 7, and 8 were unidentified polar carotenoids. Insets show the recorded absorption spectra for individual peaks and pelleted cells of recombinant *E. coli*.

gested, however, until now, there has only been limited experimental data supporting this hypothesis.

TLC, HPLC, and LC/MS analysis of the crude extracts of β-carotene producing *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}) were performed. As expected, β-carotene (λ_{max} = 427, 453, and 481 nm; M^+ at m/z = 536.69) was the dominant compound, and there was no accumulation of lycopene (Fig. 2B), indicating that four heterologously expressed enzymes—CrtE, CrtB, CrtI, and CrtYcYd—were functionally assembled in *E. coli*. Interestingly, in addition to the main peak corresponding to β-carotene, a small peak was also detected in the HPLC chromatogram, corresponding to a compound that was lighter yellow than β-carotene on the TLC plate (Fig. 2C). This compound (λ_{max} = 403, 429, and 453 nm; $[M+H]^+$ m/z = 539) was found to be 7,8-dihydro-β-carotene, which is a neurosporene-derivative that contains a β-ionone ring at both ends (Fig. 1). CrtY from *P. ananatis* and LCY from the plant *Capsicum annuum* are reported to be able to produce 7,8-dihydro-β-carotene from neurosporene (29). Unlike common lycopene cyclase, heterodimeric *B. linens* CrtYcYd consisting of two genes, *crtYc* and *crtYd*, showed low amino acid sequence homology with other lycopene cyclases (16).

Extension of 3,4-didehydrolycopene pathway with a mutant lycopene cyclase $CrtY_2$ and a spheroidene monooxygenase CrtA in *E. coli*. As shown in Fig. 2B, no 3,4-didehydrolycopene-derivative torulene was detected, indicating that 3,4-didehydrolycopene was not a substrate for wild-type *B. linens* lycopene cyclase CrtYcYd. Thus, the engineered 3,4-didehydrolycopene synthetic pathway was extended by incorporating the mutant *Pantoea* lycopene cyclase $CrtY_2$ in *E. coli*. The mutant *Pantoea* $CrtY_2$ was generated by *in vitro* evolution and used to produce torulene in 3,4,3′,4′-tetradehydrolycopene/lycopene-producing *E. coli* (27). Coexpression of mutant $CrtY_2$ on pUC-$CrtY_2$ in 3,4-didehydrolycopene/lycopene-producing *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}) resulted in the formation of torulene (λ_{max} = 460, 488, and 520 nm; $[M+H]^+$ at m/z = 535.86) as a minor product and β-carotene as a major product (Fig. 3A).

The spheroidene monooxygenase CrtA from *Rhodobacter*

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

strains had been known to catalyze the asymmetrical introduction of one keto-group at the C2 position of spheroidene (2) and CrtA from *Rubrivivax gelatinosus* known to produce symmetrical 2,2′-diketospirilloxanthin (14). However, our recent study showed that CrtA is more promiscuous and able to introduce one or two keto-groups into acyclic carotenoid structures such as lycopene and 3,4,3′,4′-tetradehydrolycopene (17). However, extension of the wild-type or engineered 3,4-didehydrolycopene pathway has never been examined using CrtA. Therefore, the catalytic promiscuity of CrtA in the engineered 3,4-didehydrolycopene pathway in *E. coli* was examined. Coexpression of CrtA on pUCM-A_{RC} in 3,4-didehydrolycopene/lycopene-accumulating *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}) produced acyclic xanthophylls 1,2-dihydrolycopene-2-one (λ_{max} = 443, 469, and 500 nm; $[M+H]^+$ at *m/z* = 553.74) (16) as a main product and several polar compounds (Fig. 3B). The minor polar compounds had a very similar UV/Vis absorption spectra to lycopene and neurosporene (see insets in Fig. 3B), indicating that they may be oxolycopene or neurosporene derivatives. However, this could not be conclusively verified because only low amounts of the compounds were observed in the cell extracts, which made the structural analysis difficult. A similar profiling pattern was observed in our previous study (17), where extension of the 3,4,3′,4′-tetradehydrolycopene/lycopene pathway with CrtA produced 1,2-dihydrolycopene-2-one and phillipsiaxanthin, which is a dihydroxy-diketo derivative of 3,4,3′,4′-tetradehydrolycopene, as main products.

Extension of *B. linens* β-carotene synthetic module in *E. coli*. *P. ananatis* carotene hydroxylase CrtZ was used for directed extension of the engineered *B. linens* β-carotene pathway in *E. coli*. When *P. ananatis* $CrtZ_{PAN}$ was coexpressed in *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}), hydroxylated β-carotene zeaxanthin (λ_{max} = 424, 453, and 480; $[M+H]^+$ at *m/z* = 569.8) was produced without the accumulation of β-carotene (Fig. 4A). It has been reported that most P450 monooxygenases are not active in prokaryotes, but CYP175A1 (P450 monooxygenase) from *Thermus thermophilus* was shown to be functional in *E. coli*, and this enzyme introduced hydroxyl groups into the β-ionone rings of β-carotene, producing zeaxanthin (4).

B. linens was reported to have a P450-dependent cytochrome monooxygenase, which catalyzes the hydroxylation of aromatic rings of isorenieratene, resulting in 3-hydroxyisorenieratene and 3,3′-dihydroxyisorenieratene (6). We therefore examined whether *B. linens* P450 monooxygenase was active on a nonnatural substrate β-carotene in heterologous *E. coli*. When *B. linens* P450 monooxygenase was constitutively expressed on pUCM-$P450_{BL}$ in *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}), hydroxylated β-carotene zeaxanthin was not detected (Fig. 4B), indicating that *B. linens* P450 monooxygenase could not use β-carotene as a substrate. It might be due to the lack of a cofactor. We then examined whether *B. linens* P450 monooxygenase could use a natural substrate isorenieratene to produce hydroxy isorenieratene in a heterologous *E. coli*.

To test this, the reconstructed β-carotene pathway was further extended by coexpressing *B. linens* carotene desaturase CrtU on pUCM-U_{BL} in *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}). CrtU is known to desaturate β-ionone rings of β-carotene and simultaneously transfer a methyl group to form an aryl carotenoid, isorenieratene, in *B. linens*. As expected, β-carotene

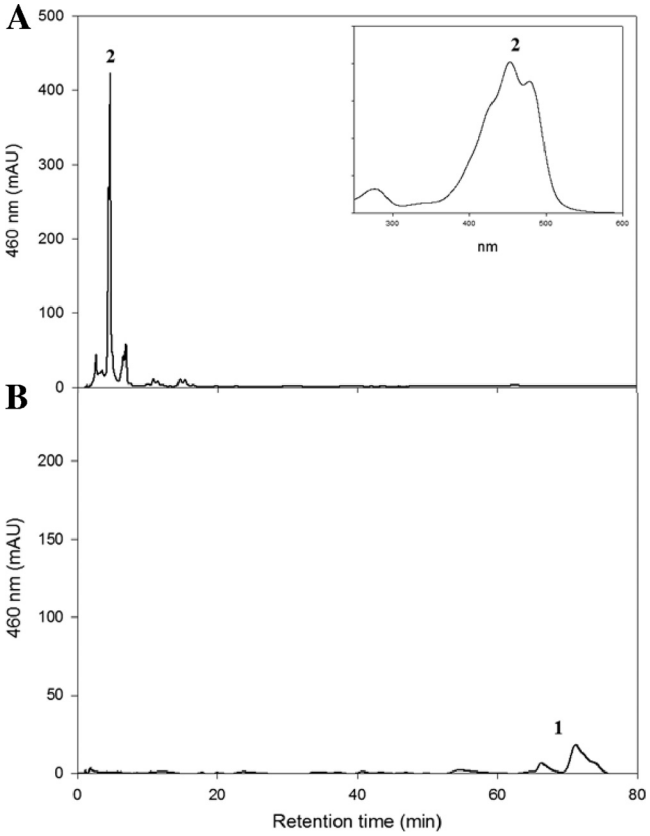

FIG. 4. Functional analysis of cytochrome P450 in *E. coli* cells producing β-carotene. Carotenoid profiles of β-carotene-producing recombinant *E. coli* cells expressing the background plasmid pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL} with pUCM-Z_{PAN} (A) and pUCM-P450 (B) are shown. The following carotenoids were identified: peak 1, β-carotene (λ_{max} = 427, 453, and 481; M^+ at *m/z* = 536.69); and peak 2, zeaxanthin (λ_{max} = 424, 453, and 480; $[M+H]^+$ at *m/z* = 569.8). The inset shows the recorded absorption spectra for individual peaks.

produced in *E. coli* was converted to isorenieratene (λ_{max} = 427, 453, and 481 nm; M^+ at *m/z* = 528.91) as the major product and a small amount of a β-isorenieratene-like compound (λ_{max} = 427, 453, and 481 nm) was also produced (Fig. 5A). Next, the isorenieratene synthetic pathway was further extended by exploiting the *B. linens* P450 monooxygenase to generate 3-hydroxyisorenieratene and 3,3′-dihydroxyisorenieratene. When P450 monooxygenase and CrtU on pUCM-U_{BL}-$P450_{BL}$ were simultaneously coexpressed in β-carotene-producing *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}), isorenieratene and β-isorenieratene-like compounds were produced in addition to a large amount of β-carotene (Fig. 5B). Incomplete conversion of β-carotene into isorenieratene indicates that P450 monooxygenase expression negatively affected the function of CrtU or decoupled the optimal coordination of CrtU with other carotenogenic enzymes. To reduce the metabolic burden caused by the high level of expression of P450 monooxygenase and CrtU, the inducible *lac* promoter-containing pUC19 was used instead of the constitutive *lac* promoter-containing pUCM. Without induction, the expression of carotenogenic enzymes on pUC19 is enough to produce carotenoids in *E. coli*.

When P450 monooxygenase and CrtU on pUC19-U_{BL}-

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

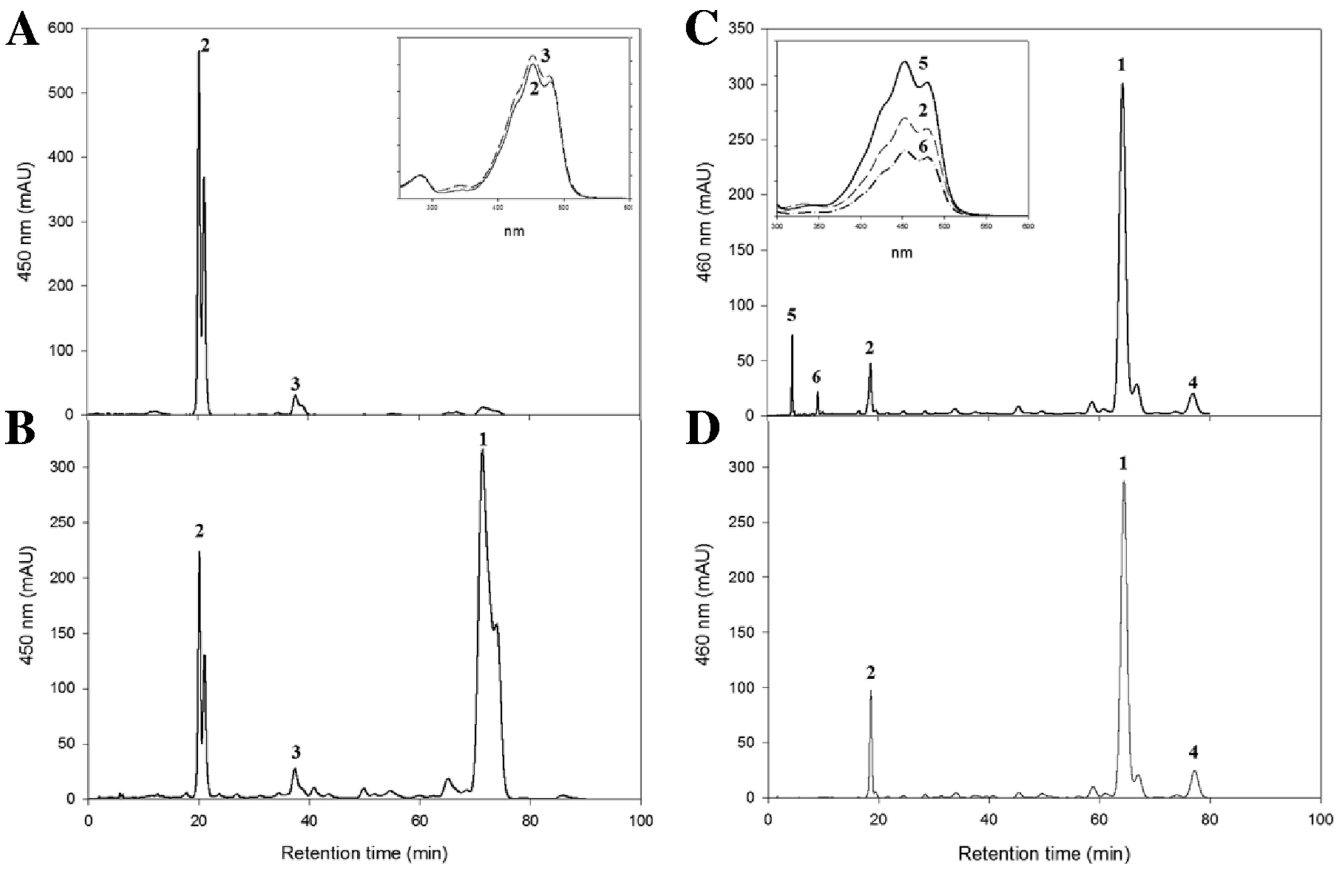

FIG. 5. HPLC analysis of β-carotene and its derivatives produced by engineered *E. coli*. HPLC analysis of reconstructed β-carotene and its derivative pathways in *E. coli*(pUCM-U_{BL}) (A), *E. coli*(pUCM-U_{BL}-P450) (B), *E. coli*(pUC19-U_{BL}-Z_{PAN}) (C), or *E. coli*(pUC19-U_{BL}-P450) (D) was performed. The following carotenoids were identified: peak 1, β-carotene (λ_{max} = 427, 453, and 481; M^+ at *m/z* = 536.69); peak 2, isorenieratene (λ_{max} = 427, 453, and 481; M^+ at *m/z* = 528.91); peak 3, β-isorenieratene (λ_{max} = 427, 453, and 481); peak 4, 7,8-dihydro-β-carotene (λ_{max} = 403, 429, and 453; $[M+H]^+$ at *m/z* = 539); peak 5, zeaxanthin (λ_{max} = 424, 453, and 480; $[M+H]^+$ at *m/z* = 569.8); and peak 6, agelaxanthin A (λ_{max} = 424, 453, and 480; $[M+H]^+$ at *m/z* = 548.77). Insets show the recorded absorption spectra for individual peaks.

$P450_{BL}$ was simultaneously coexpressed without induction in β-carotene-producing *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}), isorenieratene was produced as a major product, accumulating a large amount of β-carotene (Fig. 5C). The carotenoid profile observed in pUC19-U_{BL}-$P450_{BL}$ + pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL} was similar to the profile observed in pUCM-U_{BL}-$P450_{BL}$ + pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}. Next, active $CrtZ_{PAN}$ was coexpressed instead of P450 monooxygenase with CrtU because P450 monooxygenase appeared to be inactive in *E. coli*. When $CrtZ_{PAN}$ and CrtU on pUC19-U_{BL}-Z_{PAN} were simultaneously coexpressed without induction in β-carotene-producing *E. coli*(pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}), zeaxanthin, isorenieratene, and a new compound were detected with the accumulation of β-carotene (Fig. 5D). The new compound was identified as agelaxanthin A (λ_{max} = 427, 453, and 481 nm; M^+ at *m/z* = 548.77), which has one aromatic ring and a hydroxy group in the β-ionone ring (Fig. 1). One of the most interesting results was the formation of agelaxanthin A because it is a very rare structure with asymmetric ends (28) and is primarily present in marine sponges (9). The asymmetric structure of agelaxanthin A could be generated by a sequential or simultaneous reaction of CrtU and $CrtZ_{PAN}$ in *E. coli*(pUC19-U_{BL}-Z_{PAN} + pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}). If agelaxanthin A was generated by a simultaneous reaction between both CrtU and $CrtZ_{PAN}$, CrtU and $CrtZ_{PAN}$ would have to recognize less than half molecule of β-carotene, and then hold one end of β-carotene at the active site of each enzyme until the reaction was finished. In the case of a sequential reaction for agelaxanthin A generation, CrtU or $CrtZ_{PAN}$ would have to form functional multimers and release its intermediate compound, for example, β-isorenieratene for CrtU or β-cryptoxanthin for $CrtZ_{PAN}$, from the active site of each enzyme and take up a non-natural intermediate compound, for example, β-isorenieratene for $CrtZ_{PAN}$. Although these are a probable mechanism, it is very difficult to conclusively know the reaction sequence for agelaxanthin A. However, agelaxanthin A formation using CrtU and $CrtZ_{PAN}$ demonstrates that combinatorial biosynthesis can generate asymmetric carotenoid structures that are hardly accessible without directed evolution.

In conclusion, the present study showed for the first time that the *B. linen* lycopene, β-carotene, and isorenieratene pathways were functionally expressed in a synthetic module in *E. coli*. Interestingly, *B. linen* phytoene desaturase (CrtI) unexpectedly produced 3,4-didehydrolycopene in addition to lycopene in *E. coli*. Using the combinatorial biosynthesis, 7,8-dihydro-β-carotene, torulene and asymmetric dicyclic carotenoid

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest

agelaxanthin A were obtained. These results demonstrate that reconstruction of biosynthetic pathways and extension with promiscuous enzymes in a heterologous host holds promise as a powerful strategy for generating structurally diverse compounds.

ACKNOWLEDGMENTS

This study was supported by National Research Foundation of Korea grants funded by the Korean Government (2009-0071135 and NRF-2009-C1AAA001-2009-0093062). This study was also supported by the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2009-0093826) and by an Ajou University research fellowship of 2009.

REFERENCES

1. **Albrecht, M., S. Takaichi, S. Steiger, Z. Y. Wang, and G. Sandmann.** 2000. Novel hydroxycarotenoids with improved antioxidative properties produced by gene combination in *Escherichia coli*. Nat. Biotechnol. **18:**843–846.
2. **Armstrong, G. A., M. Alberti, F. Leach, and J. E. Hearst.** 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol. Gen. Genet. **216:** 254–268.
3. **Armstrong, G. A., and J. E. Hearst.** 1996. Genetics and molecular biology of carotenoid pigment biosynthesis. FASEB J. **10:**228–237.
4. **Blasco, F., I. Kauffmann, and R. D. Schmid.** 2004. CYP175A1 from *Thermus thermophilus* HB27, the first β-carotene hydroxylase of the P450 superfamily. Appl. Microbiol. Biotechnol. **64:**671–674.
5. **De Boer, A. L., and C. Schmidt-Dannert.** 2003. Recent efforts in engineering microbial cells to produce new chemical compounds. Curr. Opin. Chem. Biol. **7:**273–278.
6. **Dufosse, L., and M. C. De Echanove.** 2005. The last step in the biosynthesis of aryl carotenoids in the cheese ripening bacteria *Brevibacterium linens* ATCC 9175 (*Brevibacterium aurantiacum* sp. nov.) involves a cytochrome P450-dependent monooxygenase. Food Res. Int. **38:**967–973.
7. **Dufosse, L., P. Mabon, and A. Binet.** 2001. Assessment of the coloring strength of *Brevibacterium linens* strains: spectrocolorimetry versus total carotenoid extraction/quantification. J. Dairy Sci. **84:**354–360.
8. **Fraser, P. D., N. Misawa, H. Linden, S. Yamano, K. Kobayashi, and G. Sandmann.** 1992. Expression in *Escherichia coli*, purification, and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase. J. Biol. Chem. **267:**19891–19895.
9. **Garson, M. J., V. Partali, S. e. Liaaen-Jensen, and I. L. Stoilov.** 1988. Isoprenoid biosynthesis in a marine sponge of the *Amphimedon* genus: incorporation studies with [1-^{14}C]acetate, [4-^{14}C]cholesterol, and [2-^{14}C]mevalonate. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **91:**293–300.
10. **Guyomarc'h, F., A. Binet, and L. Dufosse.** 2000. Production of carotenoids by *Brevibacterium linens*: variation among strains, kinetic aspects and HPLC profiles. J. Ind. Microbiol. Biotechnol. **24:**64–70.
11. **Hausmann, A., and G. Sandmann.** 2000. A single five-step desaturase is involved in the carotenoid biosynthesis pathway to beta-carotene and torulene in *Neurospora crassa*. Fungal Genet. Biol. **30:**147–153.
12. **Hsieh, L. K., T. C. Lee, C. O. Chichester, and K. L. Simpson.** 1974. Biosynthesis of carotenoids in *Brevibacterium* sp. KY 4313. J. Bacteriol. **118:**385–393.
13. **Johnson, E., and W. Schroeder.** 1995. Microbial Carotenoids. Adv. Biochem. Eng. Biotechnol. **53:**119–178.
14. **Kakitani, Y., R. Fujii, Y. Hayakawa, M. Kurahashi, Y. Koyama, J. Harada, and K. Shimada.** 2007. Selective binding of carotenoids with a shorter conjugated chain to the LH2 antenna complex and those with a longer conjugated chain to the reaction center from *Rubrivivax gelatinosus*. Biochemistry **46:**7302–7313.
15. **Kohl, W., H. Achenbach, and H. Reichenbach.** 1983. The pigments of *Brevibacterium linens*: aromatic carotenoids. Phytochemistry **22:**207–210.
16. **Krubasik, P., and G. Sandmann.** 2000. A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. Mol. Gen. Genet. **263:**423–432.
17. **Lee, P. C., A. Z. R. Momen, B. N. Mijts, and C. Schmidt-Dannert.** 2003. Biosynthesis of structurally novel carotenoids in *Escherichia coli*. Chem. Biol. **10:**453–462.
18. **Lee, P. C., and C. Schmidt-Dannert.** 2002. Metabolic engineering toward biotechnological production of carotenoids in microorganisms. Appl. Microbiol. Biotechnol. **60:**1–11.
19. **Lee, P. C., Y. G. Yoon, and C. Schmidt-Dannert.** 2009. Investigation of cellular targeting of carotenoid pathway enzymes in *Pichia pastoris*. J. Biotechnol. **140:**227–233.
20. **Lee, P. C., C. Salomon, B. Mijts, and C. Schmidt-Dannert.** 2008. Biosynthesis of ubiquinone compounds with conjugated prenyl side chains. Appl. Environ. Microbiol. **74:**6908–6917.
21. **Mijts, B. N., P. C. Lee, and C. Schmidt-Dannert.** 2005. Identification of a carotenoid oxygenase synthesizing acyclic xanthophylls: combinatorial biosynthesis and directed evolution. Chem. Biol. **12:**453–460.
22. **Mijts, B. N., P. C. Lee, and C. Schmidt-Dannert.** 2004. Engineering carotenoid biosynthetic pathways. Methods Enzymol. **388:**315–329.
23. **Romero, P. A., and F. H. Arnold.** 2009. Exploring protein fitness landscapes by directed evolution. Nat. Rev. Mol. Cell. Biol. **10:**866–876.
24. **Sandmann, G.** 2002. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chembiochem **3:**629–635.
25. **Sandmann, G.** 2009. Evolution of carotene desaturation: the complication of a simple pathway. Arch. Biochem. Biophys. **483:**169–174.
26. **Schmidt-Dannert, C., P. C. Lee, and B. N. Mijts.** 2006. Creating carotenoid diversity in *Escherichia coli* cells using combinatorial and directed evolution strategies. Phytochem. Rev. **5:**67–74.
27. **Schmidt-Dannert, C., D. Umeno, and F. H. Arnold.** 2000. Molecular breeding of carotenoid biosynthetic pathways. Nat. Biotechnol. **18:**750–753.
28. **Shimada, A., Y. Ezaki, J. Inanaga, T. Katsuki, and M. Yamaguchi.** 1981. Partial synthesis of aromatic carotenoids, tedanin, agelaxanthin *a*, tethyatene, and renieratene. Tetrahedron Lett. **22:**773–774.
29. **Takaichi, S., G. Sandmann, G. Schnurr, Y. Satomi, A. Suzuki, and N. Misawa.** 1996. The carotenoid 7,8-dihydro-Ψ end group can be cyclized by the lycopene cyclases from the bacterium *Erwinia uredovora* and the higher plant *Capsicum annuum*. Eur. J. Biochem. **241:**291–296.
30. **Tao, L., S. Picataggio, P. E. Rouvière, and Q. Cheng.** 2004. Asymmetrically acting lycopene β-cyclases (CrtLm) from non-photosynthetic bacteria. Mol. Genet. Genomics **271:**180–188.
31. **Tao, L., H. Yao, and Q. Cheng.** 2007. Genes from a *Dietzia* sp. for synthesis of C_{40} and C_{50} β-cyclic carotenoids. Gene **386:**90–97.
32. **Tobias, A. V., and F. H. Arnold.** 2006. Biosynthesis of novel carotenoid families based on unnatural carbon backbones: a model for diversification of natural. Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids. **1761:**235–246.
33. **Umeno, D., and F. H. Arnold.** 2004. Evolution of a pathway to novel long-chain carotenoids. J. Bacteriol. **186:**1531–1536.
34. **Umeno, D., A. V. Tobias, and F. H. Arnold.** 2005. Diversifying carotenoid biosynthetic pathways by directed evolution. Microbiol. Mol. Biol. Rev. **69:** 51–78.
35. **Ye, R. W., H. Yao, K. Stead, T. Wang, L. Tao, Q. Cheng, P. L. Sharpe, W. Suh, E. Nagel, D. Arcilla, D. Dragotta, and E. S. Miller.** 2007. Construction of the astaxanthin biosynthetic pathway in a methanotrophic bacterium *Methylomonas* sp. strain 16a. J. Ind. Microbiol. Biotechnol. **34:**289–299.

Downloaded from http://aem.asm.org/ on April 12, 2015 by guest