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Heterologous Carotenoid-Biosynthetic Enzymes: Functional Complementation and Effects on Carotenoid Profiles in *Escherichia coli*

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A limited number of carotenoid pathway genes from microbial sources have been studied for analyzing the pathway complementation in the heterologous host *Escherichia coli*. In order to systematically investigate the functionality of carotenoid pathway enzymes in *E. coli*, the pathway genes of carotenogenic microorganisms (*Brevibacterium linens*, *Corynebacterium glutamicum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodopirellula baltica*, and *Pantoea ananatis*) were modified to form synthetic expression modules and then were complemented with *Pantoea agglomerans* pathway enzymes (CrtE, CrtB, CrtI, CrtY, and CrtZ). The carotenogenic pathway enzymes in the synthetic modules showed unusual activities when complemented with *E. coli*. For example, the expression of heterologous CrtEs of *B. linens*, *C. glutamicum*, and *R. baltica* influenced *P. agglomerans* CrtI to convert its substrate phytoene into a rare product—3,4,3′,4′-tetradehydrolycopene—along with lycopene, which was an expected product, indicating that CrtE, the first enzyme in the carotenoid biosynthesis pathway, can influence carotenoid profiles. In addition, CrtIs of *R. sphaeroides* and *R. capsulatus* converted phytoene into an unusual lycopene as well as into neurosporene. Thus, this study shows that the functional complementation of pathway enzymes from different sources is a useful methodology for diversifying biosynthesis as nature does.

Carotenoids, which are isoprenoids 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 serving as precursors for many hormones (1). Carotenoids are commercially used as food colorants and animal feed supplements, and more recently, they are being used as nutraceuticals and cosmetic and pharmaceutical compounds (2). The increasing industrial importance of carotenoids has led to renewed efforts in developing bioprocesses for large-scale production of a range of carotenoids, including lycopene, β-carotene, and other more structurally diverse carotenoids (3–6).

In addition to *in vitro* evolution (6, 27), combinatorial biosynthesis with carotenoid-modifying enzymes in a heterologous host has often been used to generate structurally diverse carotenoids (7). This combinatorial approach basically relies on the functional coordination/complementation of pathway enzymes from different sources in a heterologous host (2, 8, 9). Among the carotenogenic microorganisms (2, 8), *Pantoea* (formerly *Enterobacter*) is a well-known carotenogenic bacterium that produces zeaxanthin and its derivatives (8). The carotenogenic pathway consists of 6 enzymes: geranylgeranyl diphosphate (GGPP) synthase CrtE, phytoene synthase CrtB, phytoene desaturase CrtI, lycopene cyclase CrtY, β-carotene hydroxylase CrtZ, and the zeaxanthin glucosyltransferase CrtX (Fig. 1). The carotenoid biosynthetic pathways of *Pantoea* have been successfully used as basic pathways for many carotenoid studies (11–13). The pathway enzymes from different sources have occasionally shown unusual activities or substrate affinities in a heterologous host (14–16). Carotenogenic enzymes, especially carotenoid-modifying enzymes, tend to be promiscuous in their substrate specificity (14) and show unexpected/hidden activities (3, 16) when expressed in heterologous host microorganisms. Therefore, the activities of carotenoid-modifying enzymes (e.g., spheroidene monooxygenase CrtA and CrtZ) or important pathway branch point enzymes (e.g., CrtY) in heterologous hosts have been the focus of study. In contrast, little attention has been paid to carotenoid backbone-generating enzymes such as CrtE, CrtB, and CrtI.

In this study, in order to systematically investigate the activity and functionality of carotenoid-backbone-generating pathway enzymes, as well as important pathway branch point enzymes, the pathway enzymes of carotenogenic microorganisms (*Brevibacterium linens*, *Corynebacterium glutamicum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodopirellula baltica*, and *Pantoea ananatis*) were complemented with the *Pantoea agglomerans* pathway enzymes in synthetic expression modules in *Escherichia coli*. In particular, we showed that CrtE, the first upstream enzyme in the carotenoid biosynthesis pathway, can influence carotenoid profiles in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium (28) at 37°C for gene cloning and in Terrific broth (TB) medium (28) at 30°C for carotenoid production on a rotary

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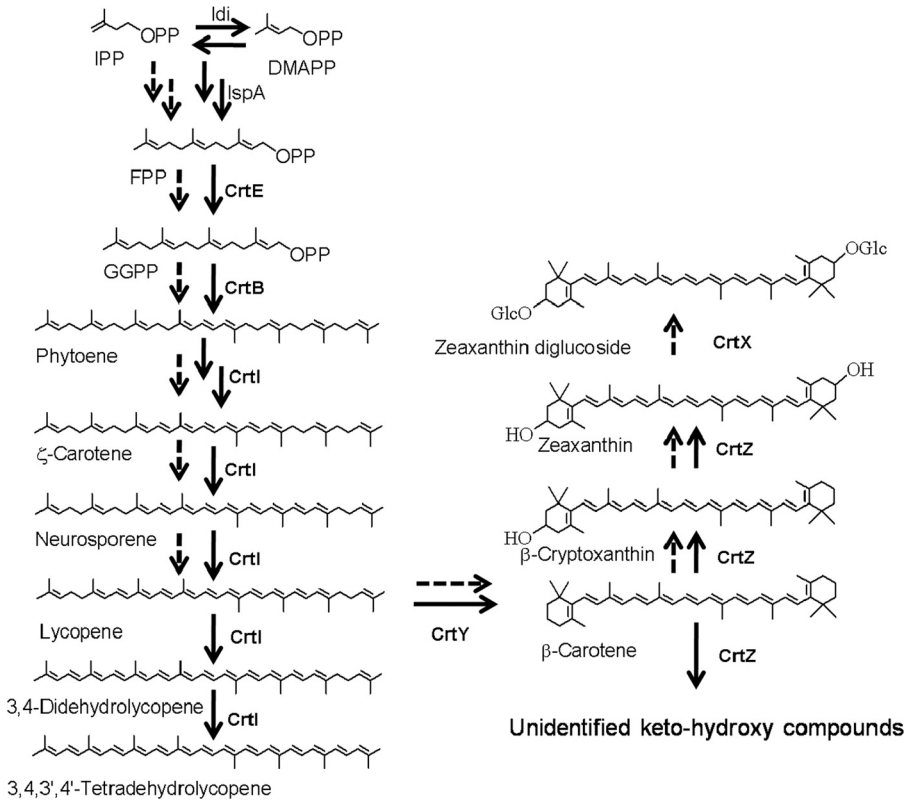

FIG 1 Carotenoid pathways of *Pantoea agglomerans* wild-type and reconstructed pathways in *E. coli*. Carotenogenic enzymes of *P. agglomerans* are indicated by bold letters. Shown are IDI (IPP isomerase), IspA (FPP synthase), CrtE (GGPP synthase), CrtB (phytoene synthase), CrtI (phytoene desaturase), CrtI (lycopene cyclase), CrtZ (β-carotene hydrolase), and CrtX (zeaxanthin glucosyltransferase). Solid arrows refer to reconstructed carotenoid pathways in *E. coli*, and dotted arrows refer to wild-type carotenoid pathways in *P. agglomerans*.

shaker at 250 rpm. Chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), and/or kanamycin (30 μg/ml) (Sigma) were added as required. *Pantoea agglomerans* KCTC 2479 was grown in LB medium at 37°C.

Recombinant DNA techniques. Restriction enzymes, polymerase, and DNA ligase were purchased from New England BioLabs, TaKaRa, or Stratagene. Genomic DNA of *P. agglomerans* was isolated using a genomic DNA (gDNA) extraction kit (GeneAll, South Korea). Plasmid DNA was prepared with the DNA-spin Plasmid DNA Purification kit (Intron, South Korea). PCR was carried out using a DNA Engine Thermal Cycler (Bio-Rad) with Vent DNA polymerase or PfuUltra II Fusion HS DNA polymerase, according to the manufacturer's instructions.

Gene cloning. *P. agglomerans* carotenoid pathway genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* were amplified from gDNA of *P. agglomerans* KCTC 2479 by PCR using primers that were designed according to the corresponding gene sequences from *P. agglomerans* pv. millettiae (AB076662) and *Pantoea* sp. C1B1Y (AY876938) (see Table S1 in the supplemental material). The PCR products were then cloned into pUCM (15), which has been modified to facilitate the constitutive expression of a cloned gene, resulting in pUCM-X_{PAG} (where X is a pathway gene) (Table 1).

Functional assembly of carotenoid biosynthetic genes. To assemble the carotenogenic pathway genes of *P. agglomerans*, genes together with a modified constitutive *lac*-promoter and a ribosome binding site were sequentially subcloned from pUCM-X (where X is a pathway gene) into pACYC184 vector as described previously (15). Briefly, a CrtE expression module was subcloned from pUCM-E_{PAG} into the HindIII site of pACYC184, resulting in pACM-E_{PAG}; a CrtB expression module was subcloned from pUCM-B_{PAG} into the SalI site of pACYC184, resulting in pACM-B_{PAG}; and a CrtI expression module was subcloned from pUCM-I_{PAG} into the BamHI site of pACYC184, resulting in pACM-I_{PAG} (Table 1). Next, the CrtI expression module was subcloned from pUCM-I_{PAG} into pACM-E_{PAG} to generate the plasmid pACM-E_{PAG}-I_{PAG}, which expresses CrtE and CrtI together. Similarly, the CrtE expression module from pUCM-E_{PAG} and the CrtB expression module from pUCM-B_{PAG} were subcloned into pACM-B_{PAG} and pACM-I_{PAG}, respectively, to generate 2 expression plasmids: pACM-E_{PAG}-B_{PAG}, expressing CrtE and CrtB together, and pACM-B_{PAG}-I_{PAG}, expressing CrtB and CrtI together. The functionality of the resulting synthetic modules expressing 2 pathway enzymes was confirmed by complementation with a third gene: for example, complementing CrtB on pUCM-B_{PAG} with a synthetic module of CrtE and CrtI on pACM-E_{PAG}-I_{PAG}. Finally, the lycopene expression module was constructed by assembling the CrtB expression module into pACM-E_{PAG}-I_{PAG}, resulting in the plasmid pACM-E_{PAG}-B_{PAG}-I_{PAG}. Similarly, to reconstruct the β-carotene biosynthetic pathway, the CrtY expression module was subcloned from pUCM-Y_{PAG} into the XmnI site of pACM-E_{PAG}-B_{PAG}-I_{PAG}, resulting in pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}. A zeaxanthin expression module was constructed by assembling a CrtZ expression module into pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}, resulting in pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}-Z_{PAG} (Table 1).

Phylogenetic tree. The amino acid sequences of carotenoid genes were aligned using the BioEdit program (20). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (21).

Extraction of carotenoids from recombinant *E. coli*. For carotenoid production, recombinant *E. coli* cells harboring carotenogenic plasmids were cultivated in 100 ml of TB medium supplemented with the appropriate selective antibiotics for 48 h at 30°C and 250 rpm. The cells were pelleted by centrifugation (4°C, 4,000 rpm) and extracted repeatedly with a total volume of 15 ml acetone until all visible pigments were extracted. After centrifugation (4°C, 4,000 rpm), the colored supernatants were

TABLE 1 Strains and plasmids used in this study

Strains and plasmids	Relevant properties	Source or reference
Strains		
Pantoea agglomerans	C_{40} carotenoid pathway	KCTC 2479
Pantoea ananatis	C_{40} carotenoid pathway	DSM 30080
Brevibacterium linens	C_{40} carotenoid pathway	DSM 20426
Rhodobacter sphaeroides	C_{40} carotenoid pathway	KCTC 12085
Rhodobacter capsulatus	C_{40} carotenoid pathway	KCTC 2583
Rhodopirellula baltica	C_{40} carotenoid pathway	DSM 10527
Corynebacterium glutamicum	C_{50} carotenoid pathway	KCTC 1445
E. coli SURE	*endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5 uvrC e14-* Δ(*mcrCB-hsdSMR-mrr*)171 F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*]	Stratagene
E. coli XL1-Blue	*endA1 gyrA96*(Nal^r) *thi-1 recA1 relA1 lac glnV44* F′[::Tn*10 proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR17*(r_K^- m_K^+)	Stratagene
Plasmids		
pUC19	Cloning vector, *lac* promoter, Ap	NEB
pUCM	Cloning vector modified from pUC19; constitutive *lac* promoter, Ap	15
pACYC184	Expression vector that is compatible with pMB1 or ColE1-related plasmids	NEB
pBBR1MCS-2	Cloning vector, Km	17
pUCM-E_{PAG}	Constitutively expressed *crtE* gene from *P. agglomerans*	This study
pUCM-B_{PAG}	Constitutively expressed *crtB* gene from *P. agglomerans*	This study
pUCM-I_{PAG}	Constitutively expressed *crtI* gene from *P. agglomerans*	This study
pUCM-Y_{PAG}	Constitutively expressed *crtY* gene from *P. agglomerans*	This study
pUCM-Z_{PAG}	Constitutively expressed *crtZ* gene from *P. agglomerans*	This study
pACM-E_{PAG}	Constitutively expressed *crtE* gene from *P. agglomerans*	This study
pACM-B_{PAG}	Constitutively expressed *crtB* gene from *P. agglomerans*	This study
pACM-I_{PAG}	Constitutively expressed *crtI* genes from *P. agglomerans*	This study
pACM-E_{PAG}-B_{PAG}	Constitutively expressed *crtE* and *crtB* genes from *P. agglomerans*	This study
pACM-E_{PAG}-I_{PAG}	Constitutively expressed *crtE* and *crtI* genes from *P. agglomerans*	This study
pACM-B_{PAG}-I_{PAG}	Constitutively expressed *crtB* and *crtI* genes from *P. agglomerans*	This study
pACM-E_{PAG}-B_{PAG}-I_{PAG}	Constitutively expressed *crtE*, *crtB*, and *crtI* genes from *P. agglomerans* to produce lycopene	This study
pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}	Constitutively expressed *crtE*, *crtB*, *crtI*, and *crtY* genes from *P. agglomerans* to produce β-carotene	This study
pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}-Z_{PAG}	Constitutively expressed *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes from *P. agglomerans* to produce zeaxanthin	This study
pUCM-E_{BL}	Constitutively expressed *crtE* gene from *B. linens*	15
pUCM-E_{RB}	Constitutively expressed *crtE* gene from *R. baltica*	Unpublished
pUCM-E_{GG}	Constitutively expressed *crtE* gene from *C. glutamicum*	Unpublished
pUCM-E_{RS}	Constitutively expressed *crtE* gene from *R. sphaeroides*	Unpublished
pUCM-E_{RC}	Constitutively expressed *crtE* gene from *R. capsulatus*	Unpublished
pUCM-B_{BL}	Constitutively expressed *crtB* gene from *B. linens*	15
pUCM-B_{RB}	Constitutively expressed *crtB* gene from *R. baltica*	Unpublished
pUCM-$B1_{CG}$	Constitutively expressed *crtB1* gene from *C. glutamicum*	Unpublished
pUCM-B_{RS}	Constitutively expressed *crtB* gene from *R. sphaeroides*	Unpublished
pUCM-B_{RC}	Constitutively expressed *crtB* gene from *R. capsulatus*	Unpublished
pUCM-I_{BL}	Constitutively expressed *crtI* gene from *B. linens*	15
pUCM-$I2_{CG}$	Constitutively expressed *crtI2* gene from *C. glutamicum*	Unpublished
pUCM-I_{RS}	Constitutively expressed *crtI* gene from *R. sphaeroides*	Unpublished
pUCM-I_{RC}	Constitutively expressed *crtI* gene from *R. capsulatus*	Unpublished
pUCM-I_{RB}	Constitutively expressed *crtI* gene from *R. baltica*	Unpublished
pUCM-Y_{PAN}	Constitutively expressed *crtY* gene from *P. ananatis*	18
pUCM-$YcYd_{BL}$	Constitutively expressed *crtYcYd* gene from *B. linens*	15
pUCM-$YeYf_{CG}$	Constitutively expressed *crtYeYf* gene from *C. glutamicum*	Unpublished
pUCM-Z_{PAN}	Constitutively expressed *crtZ* gene from *P. ananatis*	4

pooled and reextracted with an equal volume of hexane after the addition of an equal volume of double-distilled water. The collected color fractions were dried completely using an EZ-2 Plus centrifugal evaporator (Genevac Inc., NY), and resuspended in 0.5 ml of acetone. After centrifugation (13,000 rpm, 20 min), the extracts were filtered (0.45-μm GHP membrane; Pall) to remove the fine particles.

Analysis of carotenoids. Thin-layer chromatography (TLC) analysis was performed using a 100% hexane solvent system for the initial analysis (4). High-pressure liquid chromatography (HPLC) analysis was carried out with a Zorbax Eclipse XDB-C18 column (4.6 by 150 mm, 5 μm; Agilent Technologies); the elution was performed under isocratic conditions with a solvent system containing 80% acetonitrile, 15% methanol,

and 5% isopropanol at a flow rate of 1 ml/min by using an Agilent 1200 system equipped with a photodiode array detector. For structural elucidation, carotenoids were identified using a combination of HPLC retention times, UV-visible (UV-Vis) absorption spectra, and mass fragmentation spectra. Mass fragmentation spectra were monitored in the mass range of *m/z* 300 to 800 on a liquid chromatography-mass spectrometer (LC/MS) (Agilent 6150) equipped with an atmospheric pressure chemical ionization (APCI) or an electrospray ionization (ESI) ion source (Agilent Technologies, CA). For the reduction of carotenoids with $NaBH_4$, carotenoids were dried and resuspended in 100% ethanol and then incubated for 1 h in the dark at 30°C after a few crystals of $NaBH_4$ (22) were added.

***In vitro* CrtZ activity assay.** *In vitro* CrtZ activity was assayed by the method of Hundle et al. (23) with some modifications. *E. coli* cells containing the plasmid pUCM (as the negative control) and pUCM-Z_{PAG} were grown in LB medium (300 ml) at 30°C for 12 h. The cells were pelleted, washed, resuspended in 300 μl sodium phosphate buffer (100 mM, pH 7.0), and incubated for 15 min at 25°C after the addition of 3 μl of lysozyme (100 mg/ml). The cells were then sonicated on ice (65% power with a cycle of 5 s pulse-on and 10 s pulse-off for 75 s), and the cell debris was removed by centrifugation at 13,000 rpm for 30 min at 4°C. The crude protein extracts were collected, quantified using a Bio-Rad protein assay kit (Bio-Rad), and used directly for *in vitro* assays. The reactions were started at 30°C for 14 h by adding 300 μl of protein extract (~5 μg) into a 200-μl mixture of sodium phosphate buffer (100 mM, pH 7.0) containing 3 μl of $FeCl_2$ (20 mM), 0.3 μl of Tween 80, and 1.2 nmol of β-carotene.

Nucleotide sequence accession numbers. The GenBank accession numbers of carotenogenic enzymes of *P. agglomerans* KCTC 2479 are as follows: CrtE, JX871355; CrtB, JX871356; CrtI, JX871357; CrtY, JX871358; CrtZ, JX871359.

RESULTS AND DISCUSSION

Construction of platform modules expressing *P. agglomerans* carotenoid pathway enzymes. For the construction of basic carotenoid expression modules for functional complementation, *P. agglomerans* carotenoid biosynthetic genes were chosen because these pathway genes have been extensively used as a model system in bacteria and yeasts (8). Unidentified carotenoid pathway genes of yellow *P. agglomerans* KCTC 2479 were obtained by performing PCR on the basis of the corresponding gene sequences of *P. agglomerans* pv. millettiae and *Pantoea* sp. C1B1Y (Table 2). In sequence analysis, 5 cloned genes from *P. agglomerans* showed a high homology to those from *P. agglomerans* pv. millettiae (97 to 99%), *Pantoea* sp. C1B1Y (98 to 99%), and *P. ananatis* LMG20103 (73 to 87%) (see Table S1 in the supplemental material). Each gene encoding a carotenoid pathway enzyme ($CrtE_{PAG}$, $CrtB_{PAG}$, $CrtI_{PAG}$, $CrtY_{PAG}$, or $CrtZ_{PAG}$) was individually modulated and then sequentially assembled into the pACYC184 vector to generate pACM-E_{PAG}-B_{PAG}, pACM-B_{PAG}-I_{PAG}, pACM-E_{PAG}-I_{PAG}, pACM-E_{PAG}-B_{PAG}-I_{PAG}, pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}, and pACM-E_{PAG}-B_{PAG}-I_{PAG}-Y_{PAG}-Z_{PAG}. To investigate the functionality of the heterologous carotenoid biosynthetic enzymes, we selected 15 heterogeneous lycopene biosynthetic enzymes (CrtE, CrtB, and CrtI) from 5 carotenogenic microorganisms (*Brevibacterium linens*, *Corynebacterium glutamicum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodopirellula baltica*) and 3 carotene cyclases (CrtYs) from 3 carotenogenic microorganisms (*Brevibacterium linens*, *Corynebacterium glutamicum*, and *P. ananatis*) (Table 1).

Functional complementation of heterogeneous phytoene desaturases (CrtIs) from 5 carotenogenic microorganisms. First, we investigated the heterologous complementations of 5 CrtIs from 5 carotenogenic microorganisms (BLI, CrtI of *B. linens*; CGI, CrtI of *C. glutamicum*; RSI, CrtI of *R. sphaeroides*; RCI, CrtI of *R. capsulatus*; and RBI, CrtI of *R. baltica*) and the homologous complementation of CrtI from *P. agglomerans* (PAI) with the *P. agglomerans* carotenogenic module expressing $CrtE_{PAG}$ + $CrtB_{PAG}$. Figure 2A shows the phylogenetic distances between the 6 CrtIs (PAI, BLI, CGI, RSI, RCI, and RBI). Coexpression of each CrtI with the module expressing $CrtE_{PAG}$ + $CrtB_{PAG}$ changed the color densities of the recombinant *E. coli* cells (Fig. 2B). HPLC and LC/MS analysis detected carotenoid peaks in the extracts of 5 recombinant *E. coli* cells but not in a complementation of RBI + $CrtE_{PAG}$ + $CrtB_{PAG}$ or in a negative control expressing empty vectors (Fig. 2C). Interestingly, RSI and RCI produced neurosporene and lycopene at a similar ratio in this study (Fig. 2D), a result that conflicts with the reported activity of CrtI (RCI) from *R. capsulatus*, which is known to produce only neurosporene as an end product (29). Unexpectedly, the carotenoids 3,4,3′,4′-tetradehydrolycopene and 3,4-didehydrolycopene were detected in the homologous complementation PAI + $CrtE_{PAG}$ + $CrtB_{PAG}$ and the heterologous complementation CGI + $CrtE_{PAG}$ + $CrtB_{PAG}$, respectively. In other studies, the rare, fully saturated 3,4,3′,4′-tetradehydrolycopene was produced when a CrtI generated by directed evolution was involved (18) or wild-type CrtI of *P. ananatis* was expressed at high levels (11). BLI, CGI, and RBI, which belong to an outgroup of the phylogenetic tree, produced a far smaller amount of carotenoids, suggesting that these heterologous complementations were less efficient in converting phytoene into lycopene. This hypothesis is supported by the fact that the 3 enzymes were able to produce more lycopene with their own pathway enzymes (unpublished data for CGI and RBI; see reference 15 for BLI). There were no distinguishable differences in the expression observed by performing SDS-PAGE (see the supplemental material).

TABLE 2 Primers used for cloning *Pantoea agglomerans* in this study

Gene	Sequence[a]	Enzyme site
crtE	F: 5′-GCTCTAGAAGGAGGATTACAAAATGATG GTCTGCGCA-3′	XbaI
	R: 5′-CCGGAATTCTTAACTGACGGCAGC-3′	EcoRI
crtB	F: 5′-GCTCTAGAAGGAGGATTACAAAATG G**M**GGTKGG**M**TCG-3′	XbaI
	R: 5′-CCGGAATTCTTAGATGGGGCGCTG-3′	EcoRI
crtI	F: 5′-GC TCTAGAAGGAGGATTACAAAAT GAA**WMS**AACTAC**R**-3′	XbaI
	R: 5′-CCGGAATTCTCA**WRY**CAG**R**TCC**Y**C-3′	EcoRI
crtX	F: 5′-GCTCTAGAAGGAGGATTACAAAATG AGCCACTTTGCGGTC-3′	XbaI
	R: 5′-CCGGAATTCTCATACCGCGGCATAGT-3′	EcoRI
crtY	F: 5′-GCTCTAGAAGGAGGATTACAAAATGC CGCGGTATGATCTG-3′	XbaI
	R: 5′-CCGGAATTCTCATTGCATCGCCTGTTG-3′	EcoRI
crtZ	F: 5′-GCTCTAGAAGGAGGATTACAAAATGTT GTGGATTTGGAATGC-3′	XbaI
	R: 5′-CCGGAATTCTTACTTCCCGGGTGGCG-3′	EcoRI

[a] Underlining indicates restriction enzyme sites, and bold indicates mixed nucleotides (M = A and C; R = A and G; W = A and T; S = C and G; Y = C and T). F, forward; R, reverse.

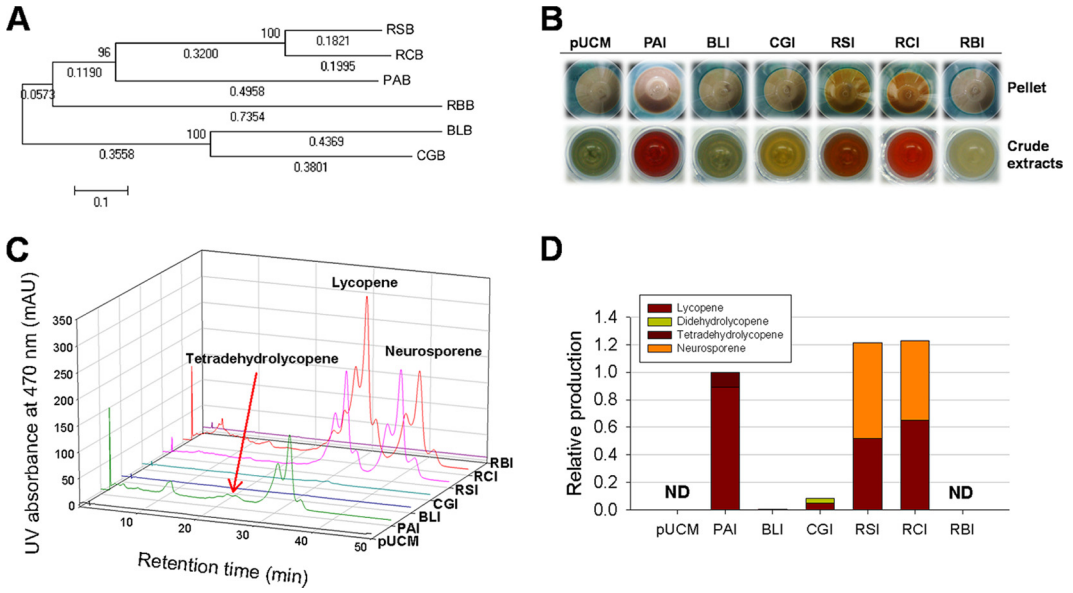

FIG 2 Functional complementations of 6 CrtIs from carotenogenic microorganisms in *E. coli* expressing CrtE and CrtB from *Pantoea agglomerans*. (A) Phylogenetic tree of 6 CrtIs from 6 different carotenogenic microorganisms. The neighbor-joining tree is based on the CrtI protein sequence. Bootstrap values expressed as a percentage of 1,000 replications are shown next to each node. (B) Pellets (upper row) and extracts (lower row) of recombinant *E. coli* expressing lycopene biosynthetic enzymes (CrtE, CrtB, and CrtI). (C) HPLC analysis of the carotenoid profiles of recombinant *E. coli* expressing lycopene biosynthetic enzymes. (D) Relative production of carotenoids in recombinant *E. coli* expressing lycopene biosynthetic enzymes. PAI, CrtI of *P. agglomerans*; BLI, CrtI of *B. linens*; CGI, CrtI of *C. glutamicum*; RSI, CrtI of *R. sphaeroides*; RCI, CrtI of *R. capsulatus*; RBI, CrtI of *R. baltica*. The following carotenoids were identified: 3,4,3′,4′-tetradehydrolycopene (λ_{max}, 493, 516, and 540); 3,4-didehydrolycopene (λ_{max}, 460, 491, and 523); lycopene (λ_{max}, 447, 473, and 503); and neurosporene (λ_{max}, 419, 442, and 470). ND, not detected.

Functional complementation of heterogeneous phytoene synthases (CrtBs) from 5 carotenogenic microorganisms. Next, the heterologous complementations of 5 CrtBs (BLB, CrtB of *B. linens*; CGB, CrtB of *C. glutamicum*; RSB, CrtB of *R. sphaeroides*; RCB, CrtB of *R. capsulatus*; and RBB, CrtB of *R. baltica*) and the homologous complementation of CrtB (PAB) were investigated in *E. coli* cells expressing $CrtE_{PAG}$ + $CrtI_{PAG}$. Phylogenetic distances between the 6 CrtBs (PAB, BLB, CGB, RSB, RCB, and RBB)

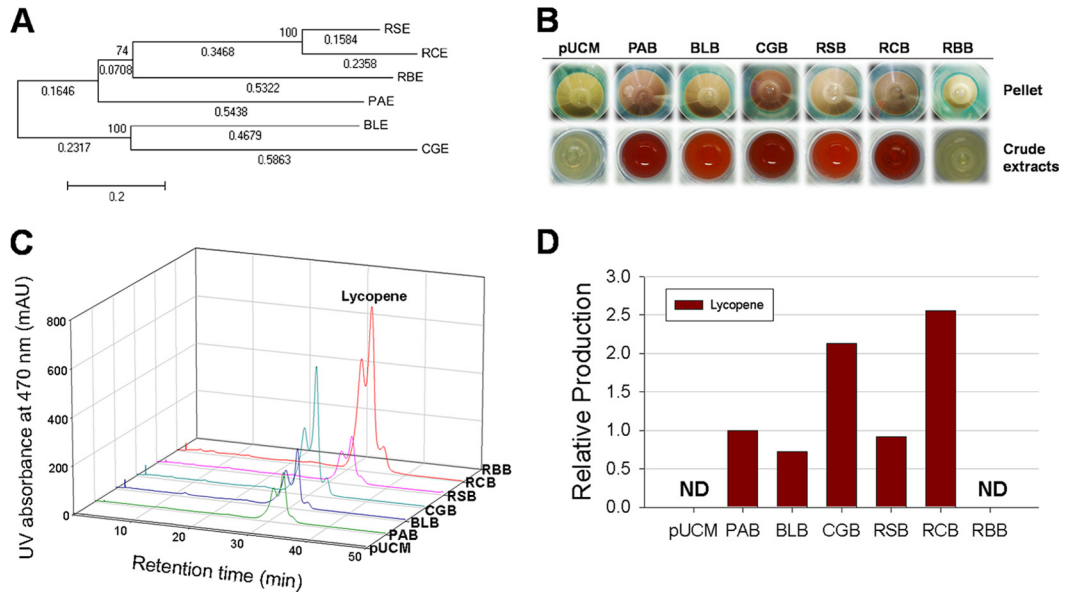

FIG 3 Functional complementations of 6 CrtBs from carotenogenic microorganisms in *E. coli* expressing CrtE and CrtI from *Pantoea agglomerans*. (A) Phylogenetic tree of 6 CrtBs from 6 different carotenogenic microorganisms. The neighbor-joining tree is based on the CrtB protein sequence. Bootstrap values expressed as a percentage of 1,000 replications are shown next to each node. (B) Pellets (upper row) and extracts (lower row) of recombinant *E. coli* expressing lycopene biosynthetic enzymes (CrtE, CrtB, and CrtI). (C) HPLC analysis of the carotenoid profiles of recombinant *E. coli* expressing lycopene biosynthetic enzymes. (D) Relative production of carotenoids in recombinant *E. coli* expressing lycopene biosynthetic enzymes. PAB, CrtB of *P. agglomerans*; BLB, CrtB of *B. linens*; CGB, CrtB of *C. glutamicum*; RSB, CrtB of *R. sphaeroides*; RCB, CrtB of *R. capsulatus*; RBB, CrtB of *R. baltica*. The following carotenoid was identified: lycopene (λ_{max}, 447, 473, and 503). ND, not detected.

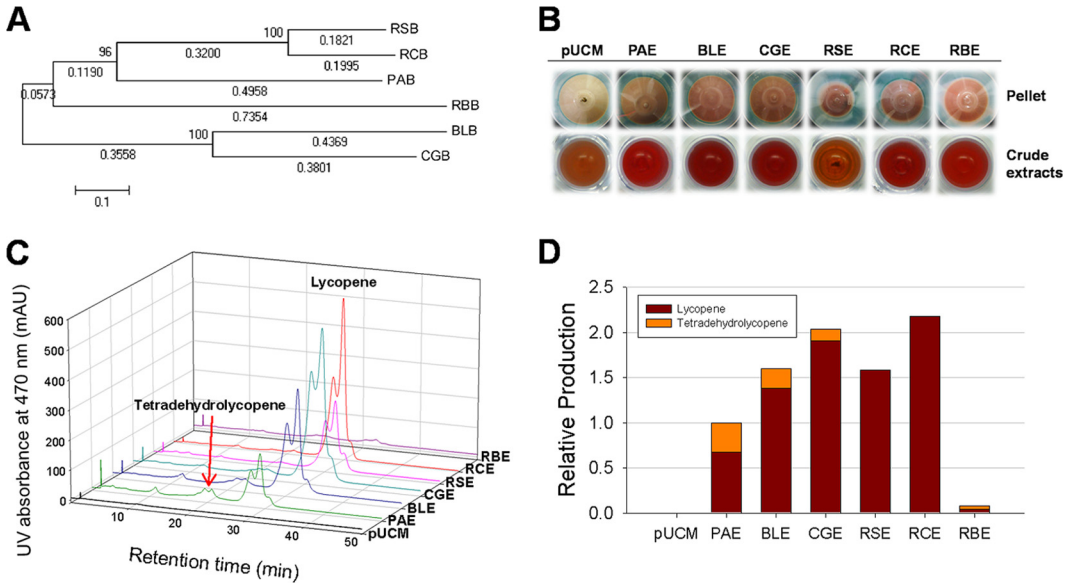

FIG 4 Functional complementations of 6 CrtEs from carotenogenic microorganisms in *E. coli* expressing CrtB and CrtI from *Pantoea agglomerans*. (A) Phylogenetic tree of 6 CrtEs from 6 different carotenogenic microorganisms. The neighbor-joining tree is based on the CrtE protein sequence. Bootstrap values expressed as a percentage of 1,000 replications are shown next to each node. (B) Pellets (upper row) and extracts (lower row) of recombinant *E. coli* expressing lycopene biosynthetic enzymes (CrtE, CrtB, and CrtI). (C) HPLC analysis of the carotenoid profiles of recombinant *E. coli* expressing lycopene biosynthetic enzymes. (D) Relative production of carotenoids in recombinant *E. coli* expressing lycopene biosynthetic enzymes. PAE, CrtE of *P. agglomerans*; BLE, CrtE of *B. linens*; CGE, CrtE of *C. glutamicum*; RSE, CrtE of *R. sphaeroides*; RCE, CrtE of *R. capsulatus*; RBE, CrtE of *R. baltica*. The following carotenoids were identified: 3,4,3′,4′-tetradehydrolycopene (λ_{max}, 493, 516, and 540) and lycopene (λ_{max}, 447, 473, and 503).

were similar to those between the CrtIs (Fig. 3A). However, when each CrtB was complemented with the module expressing $CrtE_{PAG}$ + $CrtI_{PAG}$, recombinant *E. coli* cells showed a deeper color (Fig. 3B) than that observed in the case of *E. coli* expressing CrtI from different sources. Unlike the carotenoid profiles observed in the CrtI complementations, only lycopene was detected in 5 of the CrtB complementations. No carotenoids were detected for the RBB complementation (Fig. 3C). Notably, RSB and RCB produced only lycopene without neurosporene (8, 19), unlike RSI and RCI (compare Fig. 2D and Fig. 3D). Furthermore, complementations of BLB and CGB, which belong to an outgroup of the phylogenetic tree, produced more lycopene than the complementations of BLI and CGI did (compare Fig. 2D and 3D). This suggests that BLB and CGB are better fitted than BLI and CGI to assemble the heterologous carotenogenic machinery in *E. coli* (4). The highest production of lycopene was observed with RCB, followed by CGB, PAB, RSB, and BLB. Interestingly, even though RCB and RSB come from the same genus (*Rhodobacter*) and are separated by a short phylogenetic distance (Fig. 3A), they showed a different heterologous complementation pattern in *E. coli*, as seen in a different pattern of carotenogenic enzymes from *P. agglomerans* and *P. ananatis* (24).

Functional complementation of heterogeneous GGPP synthases (CrtEs) from 5 carotenogenic microorganisms. The heterologous complementations of 5 CrtEs (BLE, CrtE of *B. linens*; CGE, CrtE of *C. glutamicum*; RSE, CrtE of *R. sphaeroides*; RCE, CrtE of *R. capsulatus*; and RBE, CrtE of *R. baltica*) and the homologous complementation of CrtE of *P. agglomerans* (PAE) were investigated with a module expressing $CrtB_{PAG}$ + $CrtI_{PAG}$ (Fig. 4A). Unlike the results obtained with CrtI and CrtB complementations, all CrtE complementations, including RBE, turned recombinant *E. coli* cells a deep reddish color (Fig. 4B). HPLC and LC/MS analysis showed that PAE, BLE, CGE, and RBE produced 3,4,3′,4′-tetradehydrolycopene as well as lycopene (Fig. 4C). Detection of 3,4,3′,4′-tetradehydrolycopene in CrtE complementations is very unusual because so far only CrtI has been reported to be capable of synthesizing 3,4,3′,4′-tetradehydrolycopene or 3,4-didehydrolycopene (2). Therefore, most research has focused on CrtI to understand the desaturation of phytoene (19). It is obvious that, as shown in Fig. 1, CrtE is not directly responsible for the biosynthesis of 3,4,3′,4′-tetradehydrolycopene and lycopene. However, for the first time, to our knowledge, our results clearly show that CrtE induces, through an unknown mechanism, the

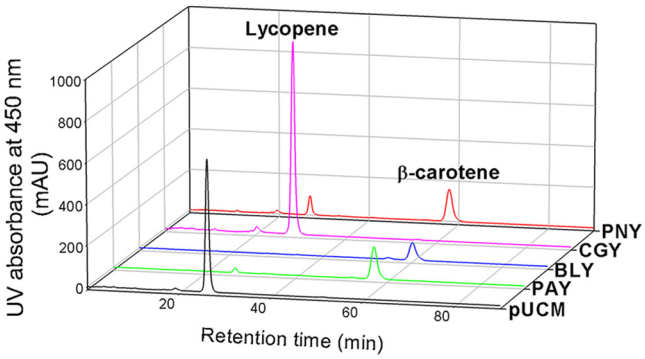

FIG 5 Functional complementations of 4 CrtYs from carotenogenic microorganisms in *E. coli* expressing CrtE, CrtB, and CrtI from *Pantoea agglomerans*. HPLC analysis of the carotenoid profiles of recombinant *E. coli* expressing a lycopene cyclase with the lycopene biosynthetic enzymes. PAY, CrtY of *P. agglomerans*; BLY, CrtY of *B. linens*; CGY, CrtY of *C. glutamicum*; PNY, CrtY of *P. ananatis*. The following carotenoids were identified: β-carotene (λ_{max}, 427, 453, and 481) and lycopene (λ_{max}, 447, 473, and 503).

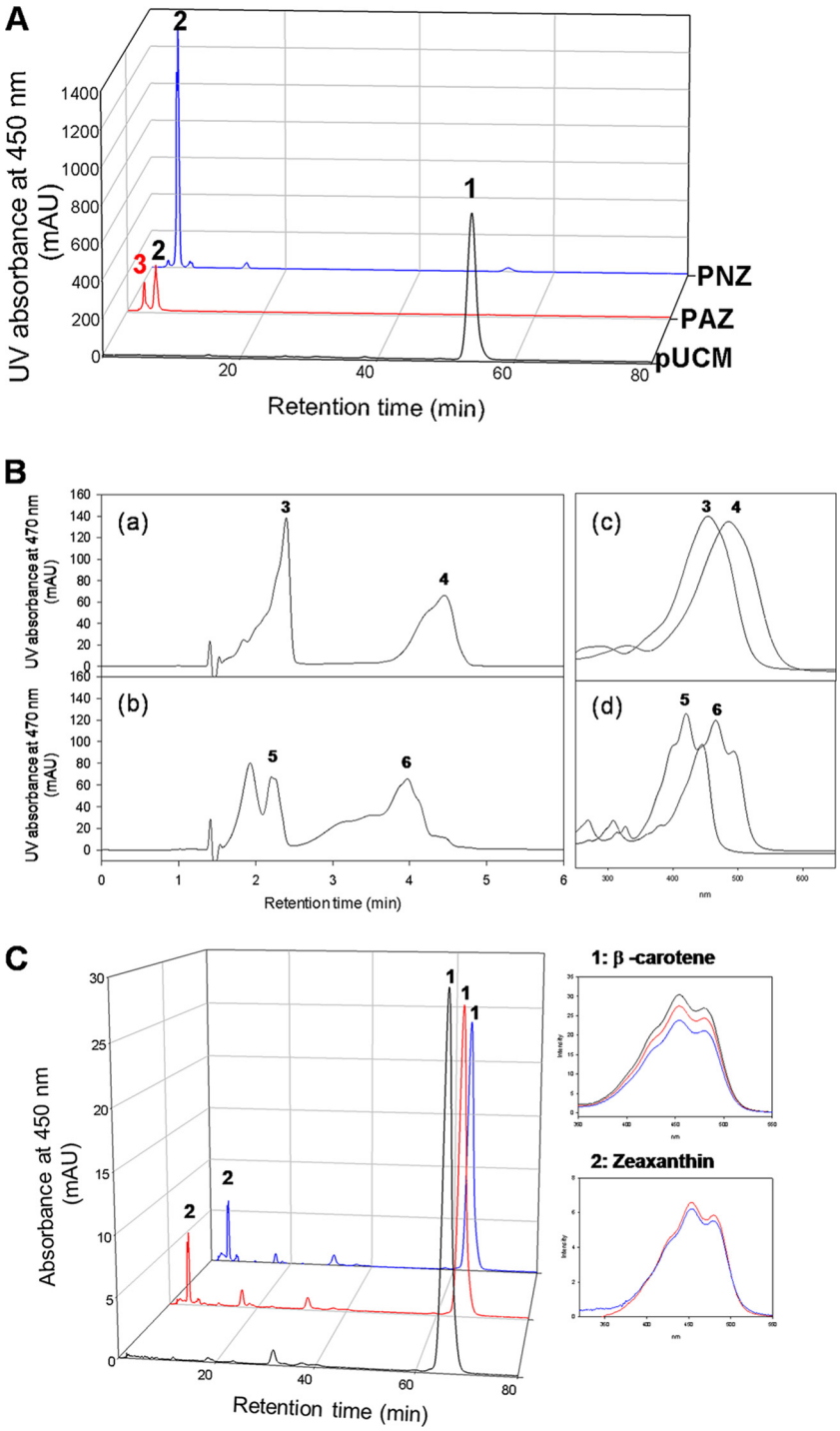

FIG 6 Functional complementations of 2 CrtZs from carotenogenic microorganisms in *E. coli* expressing CrtE, CrtB, CrtI, and CrtY from *Pantoea agglomerans*. (A) HPLC analysis of the carotenoid profiles of recombinant *E. coli* expressing a β-carotene hydroxylase with the β-carotene biosynthetic enzymes. PAZ, CrtZ of *P. agglomerans*; PNZ, CrtZ of *P. ananatis*. The following carotenoids were identified: peak 1, β-carotene (λ_{max}, 427, 453, and 481); peak 2, zeaxanthin (λ_{max}, 427, 453, and 481); peak 3, unidentified keto-hydroxy compounds. (B) HPLC analysis of unidentified keto-hydroxy compounds before (a) and after (b) reduction with $NaBH_4$. UV-Vis spectra of peaks 3 and 4 (c) and peaks 5 and 6 (d). Peaks 3 and 4, oxidized unidentified keto-hydroxy compounds; peaks 5 and 6, reduced unidentified keto-hydroxy compounds. (C) HPLC analysis of the *in vitro* activity of CrtZ with β-carotene.

carotenogenic machinery to synthesize 3,4,3′,4′-tetradehydrolycopene in the heterologous host *E. coli*. Similar to RSB and RCB, RSE and RCE produced only lycopene without neurosporene accumulation (compare Fig. 3D and 4D). Interestingly, with the exception of RBE, heterologous complementation of CrtE (PAE, BLE, CGE, and RBE) produced more total carotenoid (3,4,3′,4′-tetradehydrolycopene + lycopene) than did homologous complementation of PAE (Fig. 4D). CrtE has been considered one of the

rate-limiting enzymes in carotenoid biosynthesis (24). Therefore, the enhancement of carotenoid production can be explained by CrtEs with different affinities for farnesyl pyrophosphate (FPP) having flexibility in the heterologous carotenogenic machinery assembly. How CrtE influenced the activity of CrtI to produce the unusual 3,4,3′,4′-tetradehydrolycopene product as well as lycopene, however, remains to be answered.

Functional complementation of heterogeneous lycopene cyclases (CrtYs) from 3 carotenogenic microorganisms. Next, we further extended our complementation study to the β-carotene pathway in *E. coli*. Because the microbial sources for lycopene cyclase CrtY were relatively limited, 3 lycopene cyclases (PNY, CrtY of *P. ananatis*; BLY, $CrtY_cY_d$ of *B. linens*; and PAY, CrtY of *P. agglomerans*) and 1 carotene cyclase (CGY, $CrtY_eY_f$ of *C. glutamicum*) were chosen and then complemented with a lycopene module expressing PAE + PAB + PAI in *E. coli*. Both heterologous BLY and homologous PAY completely converted lycopene into β-carotene, while heterologous PNY did not completely convert lycopene into β-carotene (Fig. 5). As expected, CGY was not functionally complemented with a lycopene module because CGY has a substrate specificity for carotenoid backbones with C_{45} and C_{50} rather than C_{40} lycopene (25). CGY was functionally complemented with homologous upstream pathway enzymes in *E. coli* (data not shown).

Functional complementation of β-carotene hydroxylase CrtZ. Two CrtZ enzymes (PNZ, CrtZ of *P. ananatis*; and PAZ, CrtZ of *P. agglomerans*) were further complemented with a β-carotene module expressing PAE + PAB + PAI + PAY in *E. coli*. Both heterologous PNZ and homologous PAZ produced zeaxanthin without accumulation of an intermediate β-cryptoxanthin derivative (Fig. 6A). However, unlike PNZ, PAZ complementation produced additional polar compounds (Fig. 6A). The polar compound could be a carotenoid with a keto group(s) in its structure, which has been deduced on the basis of the reduction of the new polar compound with $NaBH_4$ and the UV-Vis spectra of the reduced compounds (Fig. 6B). Interestingly, the conversion of β-carotene to zeaxanthin without formation of polar compounds was observed only *in vitro* (Fig. 6C). Therefore, we assume that a specific *in vivo* environment may account for the unusual activity of PAZ in *E. coli*. It is worth noting that CrtZ has a relatively broad substrate preference (26) and is bifunctional as a hydroxylase/ketolase (9). Detailed structural analysis of these compounds is under way.

In conclusion, microbial carotenogenic pathway enzymes in synthetic expression modules showed unusual activities that differed from those in previous reports when complemented with *P. agglomerans* pathway enzymes in the heterologous host *E. coli*. In particular, the expression of heterologous CrtEs from *B. linens*, *C. glutamicum*, and *R. baltica* and the homologous CrtE from *P. agglomerans* induced, by an unknown mechanism, the *P. agglomerans* CrtI to convert its substrate phytoene into a rare 3,4,3′,4′-tetradehydrolycopene as well as the expected lycopene. This indicates that single expression of CrtE, the first upstream enzyme in the carotenoid biosynthesis pathway (Fig. 1), can influence carotenoid profiles in *E. coli*. Additionally, heterologous CrtIs (from *R. sphaeroides*, *R. capsulatus*, and *C. glutamicum*) and homologous CrtI (from *P. agglomerans*) converted phytoene into unusual carotenoids (for example, lycopene by CrtIs from *R. sphaeroides* and *R. capsulatus*) as well as into the expected end product carotenoids. Therefore, the functional complementation of carotenoid pathway enzymes from different sources is a useful methodology for diversifying biosynthesis as nature does (8).

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