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Nonnatural biosynthetic pathway for 2-hydroxylated xanthophylls with C_{50}-carotenoid backbone

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Carotenoids are structurally diverse pigments with various important biological functions. There has been a large interest in the search for novel carotenoid structures, since only a slight structural changes can result in a drastic difference in their biological functions. Carotenoid-modifying enzymes show remarkable substrate promiscuity, allowing rapid access to a vast set of novel carotenoids by combinatorial biosynthesis. We previously constructed a nonnatural carotenoid biosynthetic pathway in *Escherichia coli* that can produce C_{50} carotenoids having a longer chain than their natural C_{40} counterparts. In this study, a carotenoid 2,2′-hydroxylase (*crtG*) from *Brevundimonas* sp. SD212 was coexpressed together with our laboratory-engineered C_{50}-zeaxanthin and C_{50}-astaxanthin biosynthetic pathways. We identified six novel nonnatural C_{50}-xanthophylls, namely, C_{50}-nostoxanthin, C_{50}-caloxanthin, C_{50}-adonixanthin, C_{50}-4-ketonostoxanthin, C_{50}-2-hydroxyastaxanthin, and C_{50}-2,2′-dihydroxyastaxanthin.

[**Key words:** Synthetic biology; Biosynthesis; Hydroxylase; Xanthophylls; Isoprenoids]

Carotenoids are natural pigments that are widespread in nature, being produced by a diverse range of microorganisms and plants. More than 1000 different kinds of carotenoids have been identified to date (1). Carotenoids possess a range of important natural functions, including involvement in animal coloration, light harvesting and photoprotection, antioxidant activity, controlling membrane fluidity, and acting as precursors to hormones (2). They are also commercially important as food colorants, cosmetic and pharmaceutical compounds, and nutraceutical agents (3,4).

It is widely accepted that slight changes in the structures of carotenoids can result in the drastic change in their biological functions, and this has inspired researchers to search for novel carotenoid structures for years. Different numbers of conjugated double bonds within carotenoid backbones can result in different colors and potency as antioxidants (5,6), while the presence or absence of a ring structure can alter their stability (7). The hydroxylation, glycosylation, and esterification of carotenoids have marked effects on the polarity of carotenoids, influencing their antioxidant activity (8) and their distribution and orientation in cell membrane (9,10). One of the notable examples of nonnatural carotenoids created by combinatorial biosynthesis, 2,2′-dihydroxycanthaxanthin (11), turned out to possess superior antioxidant activity compared with natural carotenoids with similar structures such as canthaxanthin and β-carotene.

Carotenoids with a 3 or 3′-hydroxylated β-ring, such as zeaxanthin, lutein, and astaxanthin, are widespread in natural plants. In contrast, carotenoids with a 2 or 2′-hydroxylated β-ring, such as nostoxanthin and caloxanthin, are identified in limited species (12,13). Interestingly, this 2- and 2′-modification to the β-ring are known to be notoriously difficult to chemically modify this end structure (11). A 2,2′-β-ring hydroxylase, encoded by *crtG*, has been reported from bacteria such as cyanobacteria (12,13), *Brevundimonas* (11,14), and *Sphingomonas* (15). This enzyme has low activity toward β-carotene, but is highly active toward zeaxanthin (3,3′-hydroxyl-β-ends), canthaxanthin (4,4′-carbonyl-β-ends), and astaxanthin (3,3′-hydroxyl-4,4′-carbonyl-β-ends) (11,14,16,17).

Recently, our group created a nonnatural C_{50}-backbone carotenoid pathway in *Escherichia coli*, including C_{50}-lycopene, C_{50}-β-carotene, C_{50}-zeaxanthin, C_{50}-canthaxanthin, or C_{50}-astaxanthin (18), by performing several rounds of directed evolution to the natural C_{30}- or C_{40}-carotenoid biosynthetic enzymes. Since there are numerous carotenoid-modifying enzymes that functionalize the end-ring structures or hydroxyl groups, we can use these biosynthetic pathways as a starting point to further expand the C_{50}-carotenoid family.

In this study, we coexpressed a carotenoid 2,2′-hydroxylase *crtG* from *Brevundimonas* sp. SD212 (11) with the engineered C_{50}-zeaxanthin and C_{50}-astaxanthin biosynthetic pathway in *E. coli* (Fig. 1). We identified six novel C_{50}-xanthophylls: C_{50}-nostoxanthin, C_{50}-caloxanthin, C_{50}-adonixanthin, C_{50}-4-ketonostoxanthin, C_{50}-2-hydroxyastaxanthin, and C_{50}-2,2′-dihydroxyastaxanthin. Our results demonstrate the functional plasticity of carotenoid-modifying enzymes: the biosynthetic genes in carotenoid pathways are

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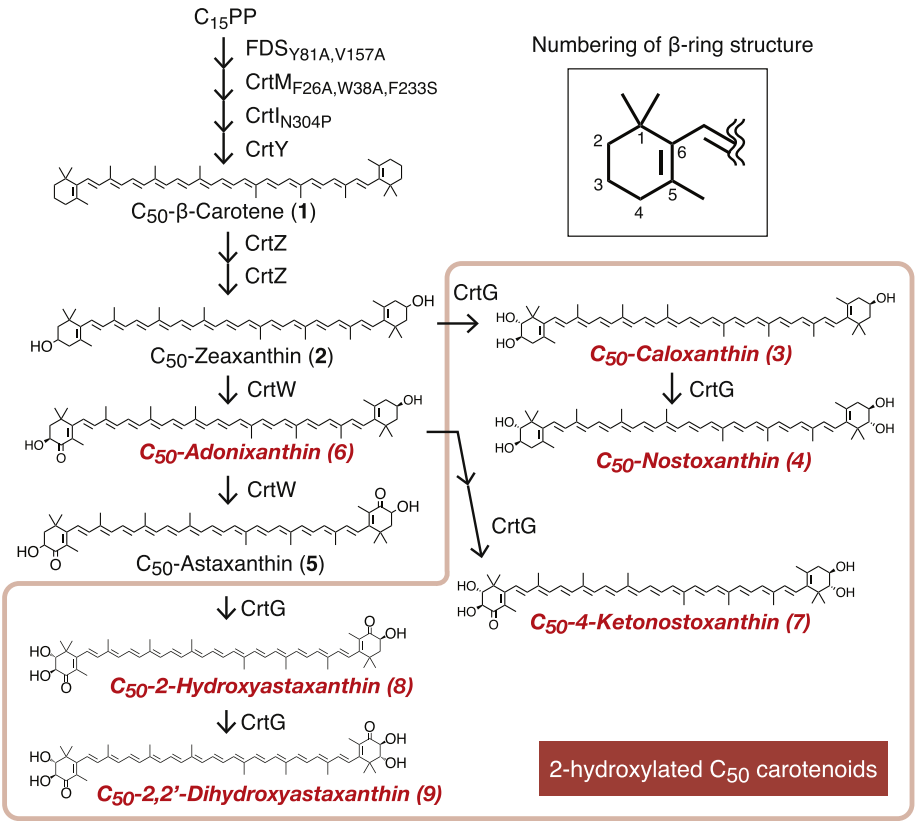

FIG. 1. Nonnatural C_{50}-xanthophyll pathway expanded using CrtG, a carotenoid 2,2'-hydroxylase from *Brevundimonas* sp. SD212. The novel carotenoid structures identified in this study are indicated in red.

known to be promiscuous, or locally specific (19), which means they tend not to recognize the whole molecule, but only a local part of the structure. Various nonnatural carotenoids with novel structures and functions have been reported using this strategy (19–22). In addition, our results show that laboratory-engineered C_{50}-carotenoid pathways could provide an abundance of new structures, providing candidates to search for novel and interesting biological activities.

MATERIALS AND METHODS

Strains and reagents *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) was used for all experiments. The reagents used were 50 mg/mL carbenicillin (carb), 30 mg/mL chloramphenicol (cm), and 20% (w/v) L-arabinose, which were purchased from Nacalai Tesque (Kyoto, Japan). LB-Lennox broth was purchased from Invitrogen (Carlsbad, CA, USA) or Nacalai Tesque. Terrific Broth was purchased from BD Biosciences (San Jose, CA, USA).

Plasmid construction The plasmids and genes used in this study are listed in Tables S1 and S2, respectively. The plasmids pAC-*fds*$_{Y81A,V157A}$-*crtM*$_{F26A,W38A,F233S}$ and pAC-*fds*$_{Y81M}$-*crtM*$_{F26A,W38A}$ are derived from a previous study (18). The plasmids for the downstream enzymes (*crtI*, *crtY*, *crtW*, *crtZ*, *and crtG*) and their derivatives are based on pUCara vectors (18) with arabinose promoter. For pUCara-*crtI*$_{N304P}$-*crtYGWZ* and pUCara-*crtI*$_{N304P}$-*crtYGZ*, the first *crtG* gene was amplified using p5Bre2-15 (11) as a template by adding *Spe*I site and ribosomal binding site (RBS) sequence (actagtaggaggattacaa) to the 5′ end and *Xba*I site to the 3′ end. The amplified *crtG* fragment was digested using *Spe*I and *Xba*I, and then inserted into the *Spe*I site of pUCara-*crtI*$_{N304P}$-*crtYWZ* or pUCara-*crtI*$_{N304P}$-*crtYZ* (18), respectively.

Culture medium and cultivation conditions Plasmids were transformed into XL1-Blue cells, which were then plated on LB-Lennox agar (carb-cm) plates covered with a nitrocellulose membrane (Pall, Port Washington, NY, USA) and incubated at 37°C for 24 h. For the colony color development, colonies on the nitrocellulose membrane were transferred to LB-Lennox agar (carb-cm) plates containing 0.2% (w/v) L-arabinose and incubated for an additional 48 h at room temperature. For liquid culture experiments, fresh colonies were inoculated with 2 mL of LB-Lennox carb-cm medium in test tubes and shaken at 37°C for 16 h. The cultures were then diluted 100-fold into 40 mL of fresh Terrific Broth carb-cm medium in 200 mL flasks and shaken at 30°C and 200 rpm. The cultures were shaken for 8 h, followed by the addition of L-arabinose to a final concentration of 0.2% (w/v) and an additional 40 h of shaking, unless otherwise indicated.

Carotenoid extraction and high performance liquid chromatography analysis Thirty milliliters of each cell culture were centrifuged at 3300 ×*g* and 4°C for 15 min. The cell pellets were washed with 10 mL of 0.9% (w/v) NaCl and then repelleted by centrifugation. Carotenoids were extracted by adding 10 mL of acetone, followed by vigorous shaking. One milliliter of chloroform and 35 mL of 1% (w/v) NaCl were added, the samples were centrifuged at 3300 ×*g* for 15 min, and the carotenoid-containing chloroform phase was collected. The chloroform was then evaporated with vacuum drying.

The dried samples were dissolved in 100 μL of hexane, chloroform, or (6:4) methanol/tetrahydrofuran (THF) just before high performance liquid chromatography (HPLC) analysis. A 25 μL aliquot of the final extract was analyzed by an HPLC-MS system (Prominence; Shimadzu LC/MS-2020, Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector and MS detector.

The analysis of xanthophylls (Figs. 2A, B and 3A, B) was performed in accordance with the conditions reported by Nishida et al. (11). This was performed using a TSK gel ODS-80T column (4.6 × 150 mm) (TOSOH Co., Tokyo, Japan), and the carotenoids were eluted at a flow rate of 1.0 mL min^{-1} with solvent A (methanol:water, 95:5) for 5 min, then by a linear gradient from solvent A to solvent B (methanol:THF, 7:3) for 5 min, and finally by isocratic elution with solvent B for 8 min. All solvents were of HPLC grade and purchased from Nacalai Tesque.

Individual carotenoids were quantified by their peak areas using a calibration curve generated with known amounts of β-carotene (quantified by absorbance), then multiplying by the molar extinction coefficient (ε) of β-carotene (138,900 M^{-1} cm^{-1} at 450 nm) and dividing by the ε value for the carotenoids. The production weights of carotenoids were then normalized to the dry cell weight (DCW) of each culture. The DCW was calculated using an OD600-DCW calibration curve.

Pigment identification To identify the pigment structure, the cell extract was further analyzed using the following methods. The ultraviolet–visible spectroscopy (UV-VIS) spectra were recorded with a Hitachi U-2001 spectrophotometer (Hitachi Field Navigator, Tokyo, Japan) in ether. The LC/MS analysis of carotenoids was carried out using a Waters Xevo G2S Q TOF mass spectrometer (Waters Corporation, Milford, CT, USA) equipped with an Acquity UPLC system. The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z* 100 to 1500 with a capillary voltage of 3.2 kV, cone voltage of

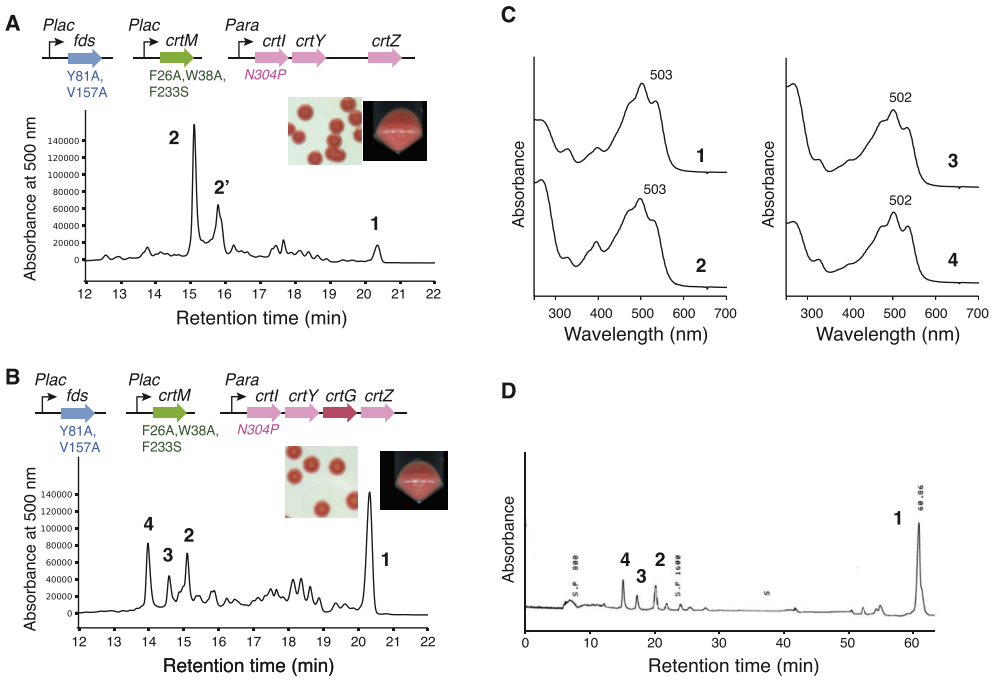

FIG. 2. Production of 2-hydroxylated C_{50}-zeaxanthin. (A) Absorbance chromatogram of carotenoid extract from cells expressing C_{50}-zeaxanthin pathway genes. Compound **2**′ indicates a cis/trans isomer of **2**. (B) Absorbance chromatogram of carotenoid extract from cells expressing CrtG in addition to C_{50}-zeaxanthin pathway genes. (C) Absorbance spectrum of indicated peaks in the HPLC chromatogram in panels A and B. (D) Absorbance chromatogram of carotenoid extract from the same strain in panel C for large-scale carotenoid purification and isolation using a different mobile phase and elution conditions (see Materials and methods).

40 eV, and source temperature of 120°C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra in Figs. S1 and S3 were measured with a quadrupole-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V. UV-VIS absorption spectra were recorded from 200 to 600 nm using a PDA. An Acquity 1.7 μm BEH UPLC C18 column (Waters Corporation) was used as a stationary phase and UPLC ODS 80 % MeOH-100 % MeOH as a mobile phase, at a flow rate of 0.4 mL/min for the HPLC system (for Figs. 2D and 3C). The ^{1}H NMR (500 MHz) spectrum was measured with a Varian UNITY INOVA 500 spectrometer (Varian Corporation, Palo Alto, CA, USA) in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. Preparative HPLC was performed with a Hitachi L-6000 intelligent pump and an L-4250 UV-VIS detector (Hitachi Field Navigator) set at 450 nm. The column used was a 250 × 4.6 mm i.d., 5 μm Cosmosil 5SL-II (Nacalai Tesque) with acetone:hexane (2:8, v/v) as a solvent at a flow rate of 1.0 mL/min and a 250 × 4.6 mm i.d., 5 μm Cosmosil 5C18-MS-II (Nacalai Tesque).

RESULTS

CrtG coexpression with C_{50}-zeaxanthin biosynthetic genes results in C_{50}-nostoxanthin and C_{50}-caloxanthin production in *E. coli* In our previous study, we constructed a construct (two plasmids) to produce C_{50}-zeaxanthin in *E. coli*: one plasmid for C_{50}-phytoene biosynthesis containing two genes (pAC-$fds_{Y81A,V157A}$-$crtM_{F26A,W38A,F233S}$), and another that expresses three genes encoding desaturase, cyclase, and 3,3′-hydroxylase (pUCara-$crtI_{N304P}$-*crtYZ*). In this study, we cloned the *crtG* gene, encoding β-carotene 2,2′-hydroxylase, from *Brevundimonas* sp. SD212 in the latter plasmid to construct pUCara-$crtI_{N304P}$-*crtYGZ*. We coexpressed this plasmid with pAC-$fds_{Y81A,V157A}$-$crtM_{F26A,W38A,F233S}$ to determine whether we could detect 2,2′-hydroxylated C_{50}-carotenoids.

The transformed cells formed colonies with a similar purple color, much like those producing C_{50}-zeaxanthin (Fig. 2A, B). The cells were grown in a culture flask for 48 h, followed by cell harvesting and carotenoid extraction. HPLC analysis of the cell extract showed a decrease in the intensity of C_{50}-zeaxanthin peaks, along with the appearance of two new peaks **3** and **4** with shorter retention time than that of C_{50}-zeaxanthin (Fig. 2B), thus indicating greater hydrophilicity than for C_{50}-zeaxanthin.

The absorption spectra of these compounds were nearly identical to that of C_{50}-zeaxanthin (Fig. 2C), indicating that they possess the same chromophores. Carotenoid from peak **3** showed absorption maximum at 502 and 534 nm indicating the presence of C_{50}-β-carotene chromophore system (18). The compound was purified by HPLC (Fig. 2D) and the molecular formula of this compound was determined to $C_{50}H_{68}O_3$ (*m/z* 716.5165 M^+, calcd for $C_{50}H_{68}O_3$, 716.5182) by positive ion electrospray ionisation time-of-flight mass spectrometry (ESI-TOF-MS) spectral data (Fig. S1 and Table 1). Characteristic ^{1}H-NMR of H_3-20 (δ 1.01), H_3-21 (δ 1.13), H_3-22 (δ 1.72), H-2 (δ 3.33), H-3 (δ 3.83), and H-4 (δ 2.49) indicated the presence of 2,3-dihydroxy-β-end group (Table 2). Furthermore, the coupling constant of H2−H3 (J = 11.3 Hz) indicated that hydroxy groups at C-2 and C-3 were located *trans* configuration (23). On the other hand, ^{1}H-NMR of H_3-20′ (δ 1.07), H_3-21′ (δ 1.07), H_3-22′ (δ 1.74), and H-3′ (δ 4.00) indicated the presence of 3-hydroxy-β-end group in the molecule (23). The ^{1}H-NMR signals of polyene parts (H-7 to H-19, H_3-23 to H_3-25, H-7′ to H-19′, H_3-23′ to H_3-25′) were assigned as shown in Table 2 (18). From these spectral data, the structure of this compound was determined to be C_{50}-β,β-carotene-3,4,3′-triol and this compound was named C_{50}-caloxanthin.

Carotenoid from peak **4** showed the same absorption spectrum as that of C_{50}-caloxanthin. The molecular formula was determined to be $C_{50}H_{68}O_4$ (*m/z* 732.5099 M^+, calcd for $C_{50}H_{68}O_4$, 732.5118) by positive ion ESI-TOF-MS spectral data (Fig. S1 and Table 1). The ^{1}H-NMR of this compound showed that this compound had symmetrical structure. Characteristic ^{1}H-NMR (Table 2) of H_3-20, 20′ (δ 1.01), H_3-21, 21′ (δ 1.13), H_3-22, 22′ (δ 1.72), H-2, 2′ (δ 3.33), H-3, 3′ (δ 3.83), and H-4, 4′ (δ 2.49) indicated the presence of 2,3-dihydroxy-β-end group (23) both side of polyene chain. Furthermore, the hydroxy groups at C-2 and C-3 (C-2′ and C-3′) were located at trans

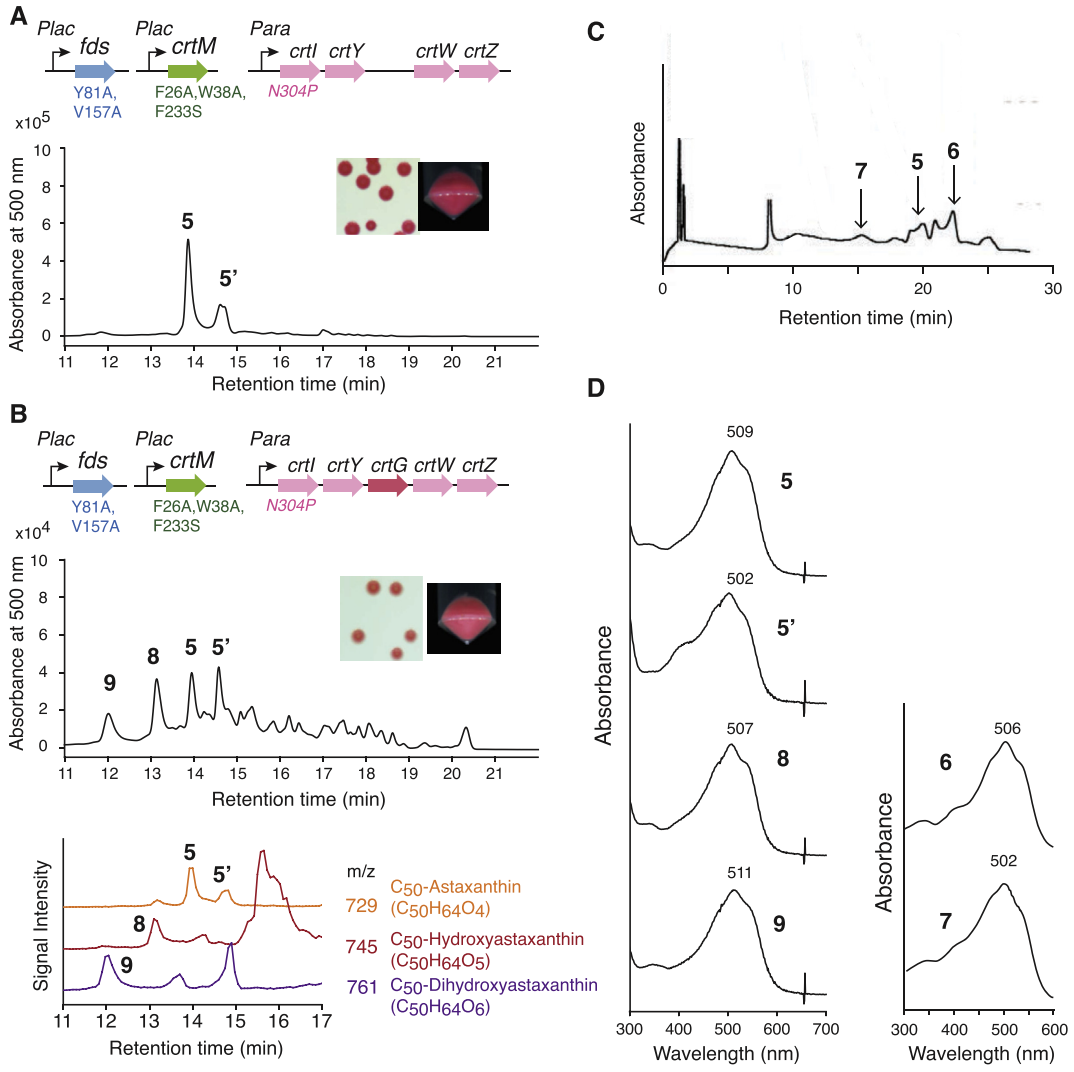

FIG. 3. Production of 2-hydroxylated carotenoids by coexpressing CrtG with the C_{50}-astaxanthin biosynthetic pathway. (A) Absorbance chromatogram of carotenoid extract from cells expressing C_{50}-astaxanthin pathway genes. Compound **5**′ indicates a cis/trans isomer of **5**. (B) Absorbance (top) and selected mass (bottom) chromatogram of carotenoid extract from cells expressing CrtG in addition to C_{50}-astaxanthin pathway genes. (C) Absorbance chromatogram of carotenoid extracts from the same strain in panel C from a large-scale culture for carotenoid isolation. The mobile phase and elution conditions were different from those in panels A and B (see Materials and methods). (D) Absorbance spectrum of indicated peaks in the HPLC chromatograms.

configuration by coupling constant of H2—H3 (H2′-H3′) (J = 11.3 Hz). Therefore, the structure of this compound was determined to be C_{50}-β,β-carotene-3,4,3′,4′-tetrol and this compound was named C_{50}-nostoxanthin.

The fraction of C_{50}-β-carotene (peak **1**) in Fig. 2B is higher compared to the modified carotenoids (peaks **2**–**4**). This indicates that the cellular total activity of CrtZ is not as high as in Fig. 2A, which might have derived from the lower expression of CrtZ resulted from the operon construct change. Further optimizing the culture condition or increasing the expression of CrtZG should result in better production of C_{50}-nostoxanthin and C_{50}-caloxanthin.

CrtG coexpression with C_{50}-astaxanthin biosynthetic genes We previously constructed two plasmids to produce nonnatural C_{50}-astaxanthin biosynthesis in *E. coli* (pAC-*fds*$_{Y81A,V157A}$,-*crtM*$_{F26A,W38A,F233S}$, and pUCara-*crtI*$_{N304P}$-*crtYWZ*). We cloned the *crtG* gene between *crtY* and *crtW*, and named this plasmid pUCara-*crtI*$_{N304P}$-*crtYGWZ*.

TABLE 1. ESI-TOF-MS positive of C_{50} carotenoids.

	C_{50}-Nostoxanthin	C_{50}-Caloxanthin	C_{50}-4-Ketonostoxanthin	C_{50}-Adonixanthin	C_{50}-Astaxanthin	C_{50}-β-Carotene
M^+						
Formula	C50H68O4	C50H68O3	C50H66O5	C50H66O3	C50H66O3	C50H68
Calc	732.5118	716.5182	746.4910	714.5012	728.4805	668.5321
Found	732.5099	716.5165	746.4891	714.5047	728.4852	668.5311
$M+Na^+$						
Formula	C50H68O4Na	C50H68O3Na	C50H66O5Na	C50H66O3Na	C50H66O4Na	
Calc	755.5015	739.5066	769.4808	737.4910	751.4702	N.D.
Found	755.5003	739.5055	769.4807	737.4911	751.4700	N.D.

TABLE 2. ^{1}H NMR of C_{50}-nostoxanthin and C_{50}-caloxanthin in $CDCl_3$.

C_{50}-Nostoxanthin				C_{50}-Caloxanthin			
Position	δ	Mult.	J (Hz)	Position	δ	Mult.	J (Hz)
H-2	3.33	dd	11, 3	H-2	3.33	dd	11, 3
H-3	3.83	m		H-3	3.83	m	
H-4	2.49	dd	17, 6	H-4	2.49	dd	17,6
H-7	6.06	d	15.5	H-7	6.06	d	15.5
H-8	6.13	d	15.5	H-8	6.13	d	15.5
H-10	6.17	d	11	H-10	6.17	d	11
H-11	6.64	dd	15, 11	H-11	6.64	dd	15, 11
H-12	6.38	d	15	H-12	6.38	d	15
H-14	6.23	d	11	H-14	6.23	d	11
H-15	6.66	dd	15, 11	H-15	6.66	dd	15, 11
H-16	6.39	d	15	H-16	6.39	d	15
H-18	6.28	br. d	10	H-18	6.28	br. d	10
H-19	6.64	m		H-19	6.64	m	
H_3-20	1.01	s		H_3-20	1.01	s	
H_3-21	1.13	s		H_3-21	1.13	s	
H_3-22	1.72	s		H_3-22	1.72	s	
H_3-23	1.97	s		H_3-23	1.97	s	
H_3-24	1.98	s		H_3-24	1.98	s	
H_3-25	1.99	s		H_3-25	1.99	s	
H-2	3.33	dd	11, 3	H-2	Not assigned Not assigned		
H-3′	3.83	m		H-3′	4.00	m	
H-4′	2.49	dd	17,6	H-4′	2.04 2.39	dd dd	16, 10 16, 6
H-7′	6.06	d	15.5	H-7′	6.1	d	15.5
H-8′	6.13	d	15.5	H-8′	6.16	d	15.5
H-10′	6.17	d	11	H-10′	6.17	d	11
H-11′	6.64	dd	15, 11	H-11′	6.64	dd	15, 11
H-12′	6.38	d	15	H-12′	6.38	d	15
H-14′	6.23	d	11	H-14′	6.23	d	11
H-15′	6.66	dd	15, 11	H-15′	6.66	dd	15, 11
H-16′	6.39	d	15	H-16′	6.39	d	15
H-18′	6.28	br. d	10	H-18′	6.28	br. d	10
H-19′	6.64	m		H-19′	6.64	m	
H_3-20′	1.01	s		H_3-20′	1.07	s	
H_3-21′	1.13	s		H_3-21′	1.07	s	
H_3-22′	1.72	s		H_3-22′	1.74	s	
H_3-23′	1.97	s		H_3-23′	1.97	s	
H_3-24′	1.98	s		H_3-24′	1.98	s	
H_3-25′	1.99	s		H_3-25′	1.99	s	

By co-transformation with these plasmids, *E. coli* acquired a deep purple hue, similar to the color of cells synthesizing C_{50}-astaxanthin (Fig. 3A, B). We cultured the cells for 48 h, after which we collected the carotenoids from the cell pellet by acetoene extraction. Additional expression of CrtG resulted in the modest (~30%) decrease in the total carotenoid production (Fig. S2). HPLC analysis revealed large decrease in the intensity of the C_{50}-astaxanthin (peak **5**), while numerous new carotenoid peaks emerged. Two new peaks, peak **8** and peak **9**, with shorter retention times (indicating that the associated compounds are more hydrophilic) than that of C_{50}-astaxanthin (Fig. 3B). Both peak **8** and peak **9** had almost identical absorption maxima near 510 nm, indicating the possession of chromophore system identical to C_{50}-astaxanthin (18). Molecular formula of compounds **8** and **9** were determined to be $C_{50}H_{64}O_5$ and $C_{50}H_{64}O_6$, respectively, from the HPLC-MS spectrum (m/z = 745 and 761). From the UV-VIS and MS spectral data, compounds **8** and **9** were assumed to be mono- and di-hydroxyl substituted derivatives of C_{50}-astaxanthin. Considering the native function of CrtG is 2- and 2′-hydroxylase in natural C_{40}-context (11), the structure of compounds **8** and **9** were proposed to be C_{50}-2-hydroxyastaxanthin and C_{50}-2,2′-dihydroxyastaxanthin, respectively. We failed to obtain a high-resolution MS/MS or NMR spectrum of these molecules, but the retention time and APCI-MS strongly indicated that peaks **8** and **9** represent C_{50}-2-hydroxyastaxanthin and C_{50}-2,2′-dihydroxyastaxanthin.

We re-analyzed and purified the carotenoids from the cell extract using HPLC with 40% $CHCl_3$/MeOH (Fig. 3C). With this elution condition, compounds **8** and **9** failed to make distinct peaks, but new peaks **6** and **7** emerged instead. Carotenoid from peak **6** showed the absorption maximum at 503 nm. The molecular formula was determined to be $C_{50}H_{66}O_3$ (m/z 714.5047 M^+, calcd for $C_{50}H_{66}O_3$, calcd for 714.5012) by positive ion ESI-TOF-MS spectral data (Fig. S3A and Table 1). Characteristic ^{1}H-NMR (Table 3) of H_3-20 (δ 1.32), H_3-21 (δ 1.21), H_3-22 (δ 1.96), and H-3 (δ 4.32) indicated the presence of 4-keto-3-hydroxy-β-end group (23) in the molecule. On the other hand, ^{1}H-NMR of H_3-20′ (δ 1.07), H_3-21′ (δ 1.07), H_3-22′ (δ 1.74), and H-3′ (δ 4.00) indicated the presence of 3-hydroxy-β-end group in the molecule (23). The ^{1}H-NMR signals of polyene parts (H-7 to H-19, H_3-23 to H_3-25, H-7′ to H-19′, H_3-23′ to H_3-25′) were assigned as shown in Table 3 (18). Therefore, the structure of this compound was determined to 3,3′-dihydroxy-C_{50}-β,β-caroten-4-one. This structure corresponds to C_{50} carotenoid analog of adonixanthin. Therefore, this compound was named C_{50}-adonixanthin.

Carotenoid from peak **7** showed the absorption maximum at 503 nm. The molecular formula was determined to be $C_{50}H_{66}O_5$ (m/z 746.4891 M^+, calcd for $C_{50}H_{66}O_5$, 746.4910) by positive ion ESI-TOF-MS spectral data (Fig. S3B and Table 1). Characteristic ^{1}H-NMR of H_3-20 (δ 1.29), H_3-21 (δ 1.26), H_3-22 (δ 1.96), H-2 (δ 3.54), and H-3 (δ 4.17) indicated the presence of 4-keto-2,3-dihydroxy-β-end group (24). The visinal proton coupling constant (H2−H3) of 11 Hz indicated that hydroxy group at C-2 and C-3 were located at *trans* configuration (23). ^{1}H-NMR of H_3-20 (δ 1.01), H_3-21 (δ 1.13), H_3-22 (δ 1.72), H-2 (δ 3.33), H-3 (δ 3.83), and H-4 (δ 2.49) indicated the presence of 2,3-dihydroxy-β-end group with 2,3-trans configuration (23). The ^{1}H-NMR signals of polyene parts (H-7 to H-19, H_3-23 to H_3-25, H-7′ to H-19′, H_3-23′ to H_3-25′) were assigned as shown in Table 3. From these spectral data, the structure of this compound was determined to be 3,4,3′,4′-trihydroxy-C_{50}-β,β-caroten-4-one and this compound was named C_{50}-4-ketonostoxanthin.

DISCUSSION

Carotenoids are biosynthesized in all three domains of life, showing remarkable structural and functional diversity. The range of known enzymes for carotenoid biosynthesis is continuing to expand as the amount of sequence information in biological databases grows (1,4). Mixing and matching or engineering the carotenoid biosynthetic enzymes has enabled us to produce numbers of novel carotenoids never explored by nature (19,21,22,25,26).

In this study, we coexpressed 2-hydroxylase *crtG* from *Brevundimonas* with our recently engineered pathways for nonnatural C_{50}-zeaxanthin and C_{50}-astaxanthin (18). Without any engineering or expression tuning, we were able to access six novel C_{50}-xanthophylls: C_{50}-nostoxanthin (**4**), C_{50}-caloxanthin (**3**), C_{50}-adonixanthin (**6**), C_{50}-4-ketonostoxanthin (**7**), C_{50}-2-hydroxyastaxanthin (**8**), and C_{50}-2,2′-dihydroxyastaxanthin (**9**). The structures of the first four compounds were confirmed by the absorbance spectra, ESI-TOF-MS/MS, and ^{1}H-NMR spectroscopy, while those of the latter two were deduced via absorption spectra, retention time in reverse-phase HPLC, and their molecular mass.

In our previous study, we reported that CrtW (β-ring 4-ketolase) and CrtZ (β-ring 3-hydroxylase), both enzymes isolated from the natural C_{40} carotenoid pathway, can accommodate β-rings on the C_{50} carotenoids (C_{50}-β-carotene), exhibiting their locally specific nature shared by many carotenoid-modifying enzymes. Similarly, this paper demonstrates that the CrtG (β-ring 2-hydroxylase) is also locally specific, enabling rapid access to novel C_{50} carotenoids.

TABLE 3. ^{1}H NMR of C_{50}-4-ketonoxtoxanthin and C_{50}-adonixanthin in $CDCl_3$.

C_{50}-4-Ketonostoxanthin				C_{50}-Adonixanthin			
Position	δ	Mult.	J (Hz)	Position	δ	Mult.	J (Hz)
H-2	3.54	d	11	H-2	1.82	dd	12.5, 12.5
					2.15	dd	12.5, 5.5
H-3	4.17	d	11	H-3	4.32	m	
H-4				H-4	Not assigned		
					Not assigned		
H-7	6.22	d	15.5	H-7	6.22	d	15.5
H-8	6.43	d	15.5	H-8	6.43	d	15.5
H-10	6.30	d	11	H-10	6.30	d	11
H-11	6.66	dd	15, 11	H-11	6.66	dd	15, 11
H-12	6.45	d	15	H-12	6.45	d	15
H-14	6.30	d	11	H-14	6.30	d	11
H-15	6.66	dd	15, 11	H-15	6.66	dd	15, 11
H-16	6.39		15	H-16	6.39		15
H-18	6.28	br. d	10	H-18	6.28	br. d	10
H-19	6.64	m		H-19	6.64	m	
H_3-20	1.29	s		H_3-20	1.32	s	
H_3-21	1.26	s		H_3-21	1.21	s	
H_3-22	1.96	s		H_3-22	1.96	s	
H_3-23	2.00	s		H_3-23	2.00	s	
H_3-24	2.00/1.99	s		H_3-24	1.99	s	
H_3-25	2.00/1.99	s		H_3-25	1.99	s	
H-2	3.33	dd	11, 3	H-2	Not assigned		11, 3
					Not assigned		
H-3′	3.83	m		H-3′	4.00	m	
H-4′	2.49	dd	17, 6	H-4′	Not assigned		17, 6
					~2.39		
H-7′	6.06	d	15.5	H-7′	6.10	d	15.5
H-8′	6.13	d	15.5	H-8′	6.15	d	15.5
H-10′	6.17	d	11	H-10′	6.15	d	11
H-11′	6.64	dd	15, 11	H-11′	6.64	dd	15, 11
H-12′	6.38	d	15	H-12′	6.37	d	15
H-14′	6.23	d	11	H-14′	6.23	d	11
H-15′	6.66	dd	15, 11	H-15′	6.66	dd	15, 11
H-16′	6.39	d	15	H-16′	6.39	d	15
H-18′	6.28	br. d	10	H-18′	6.28	br. d	10
H-19′	6.64	m		H-19′	6.64	m	
H_3-20′	1.01	s		H_3-20′	1.07	s	
H_3-21′	1.13	s		H_3-21′	1.07	s	
H_3-22′	1.72	s		H_3-22′	1.74	s	
H_3-23′	1.97	s		H_3-23′	1.97	s	
H_3-24′	1.98	s		H_3-24′	1.98	s	
H_3-25′	1.99	s		H_3-25′	1.99	s	

Taking these findings together, it is now apparent that laboratory-invented C_{50} carotenoid pathways can be expanded, as their natural C_{40} counterparts were, to those harboring hundreds or even thousands of unique structures, simply by adding and matching carotenogenic genes.

Among the six compounds that we identified, four were easily predictable from our pathway design: C_{50}-caloxanthin (**3**) and C_{50}-nostoxanthin (**4**) were one- or two-step 2-hydroxylated products of C_{50}-zeaxanthin (**2**), and C_{50}-2-hydroxyastaxanthin (**8**) and C_{50}-2,2′-dihydroxyastaxanthin (**9**) were one- or two-step hydroxylated products of C_{50}-astaxanthin (**5**). Two other compounds, C_{50}-adonixanthin (**6**) and C_{50}-4-ketonostoxanthin (**7**), identified in the carotenoid extract from the C_{50}-astaxanthin plus CrtG pathway (Fig. 3C), were unexpected. The former, C_{50}-adonixanthin, is an intermediate toward C_{50}-astaxanthin (Fig. 1), one side of which remains untreated by CrtW. The latter, C_{50}-4-ketonostoxanthin (**7**), is a two-step 2-hydroxylation product of C_{50}-adonixanthin (**6**). We speculate that expressing an additional enzyme (*crtG*) lowered the expression levels of other enzymes in the cells, such as CrtW and CrtZ, resulting in the accumulation of the carotenoid intermediate C_{50}-adonixanthin (**6**) in the cell, and then it became the substrate of CrtG. Owing to their broad substrate specificities, CrtW and CrtZ are known to form a typical "*matrix*" pathway harboring ten unique compounds. Our results indicate that CrtG could participate to this matrix pathway to form an even larger matrix. It would be of great interest to test whether we could explore other carotenoid structures by systematically tuning the expression level or three β-end decoration enzymes CrtW, CrtZ, and CrtG in C_{50} pathways.

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