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Elucidation of a Carotenoid Biosynthesis Gene Cluster Encoding a Novel Enzyme, 2,2′-β-Hydroxylase, from *Brevundimonas* sp. Strain SD212 and Combinatorial Biosynthesis of New or Rare Xanthophylls

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A carotenoid biosynthesis gene cluster mediating the production of 2-hydroxyastaxanthin was isolated from the marine bacterium *Brevundimonas* sp. strain SD212 by using a common *crtI* sequence as the probe DNA. A sequence analysis revealed this cluster to contain 12 open reading frames (ORFs), including the 7 known genes, *crtW*, *crtY*, *crtI*, *crtB*, *crtE*, *idi*, and *crtZ*. The individual ORFs were functionally analyzed by complementation studies using *Escherichia coli* that accumulated various carotenoid precursors due to the presence of other bacterial *crt* genes. In addition to functionally identifying the known *crt* genes, we found that one (ORF11, named *crtG*) coded for a novel enzyme, carotenoid 2,2′-β-hydroxylase, which showed intriguingly partial homology with animal sterol-C5-desaturase. When this *crtG* gene was introduced into *E. coli* accumulating zeaxanthin and canthaxanthin, the resulting transformants produced their 2-hydroxylated and 2,2′-dihydroxylated products which were structurally novel or rare xanthophylls, as determined by their nuclear magnetic resonance and high-performance liquid chromatography/photodiode array detector/atmospheric pressure chemical ionization mass spectrometry spectral data. The new carotenoid produced was suggested to have a strong inhibitory effect on lipid peroxidation.

Carotenoid pigments, which are included in a majority of vegetables, a variety of fruits, and a certain part of edible fish and shellfish, have attracted strong attention due to their beneficial effects on human health, e.g., their very likely prevention of chronic diseases such as cancer, cardiovascular ailments, and age-related macular degeneration (7, 12, 27, 36). A number of studies on carotenoids in relation to their health benefits, ranging from basic studies using experimental animals to clinical and epidemiological studies, have revealed that each carotenoid has characteristic individuality. For example, recent epidemiological studies have shown that β-cryptoxanthin (3-hydroxy-β-carotene), among dietary carotenoids, is associated with a reduced risk of lung cancer (24, 56), whereas supplemental β-carotene has been observed to have either no effect or even a harmful effect on lung cancer risk (2, 24). Epidemiological and clinical studies have also shown that only lycopene, among dietary carotenoids, was inversely associated with prostate cancer risk (16, 51). Supplemental lutein and zeaxanthin are thought to protect against the development or progression of age-related macular degeneration and other eye diseases from the results of many studies (28, 45). Astaxanthin has been shown to inhibit the oxidation of low-density lipoprotein (20).

Xanthophylls are modified carotenes with various oxygen-containing functional groups, which constitute a major part of structurally and functionally diverse carotenoid pigments. More than 700 carotenoids have now been isolated from natural sources (8), and evaluating the pharmaceutical potential of various carotenoids with different structures could be an exciting field for medical researchers. However, the carotenoid species studied for this purpose so far have been restricted to a small number, including β-carotene, α-carotene, and lycopene (carotenes) and lutein, zeaxanthin, β-cryptoxanthin, canthaxanthin, and astaxanthin (xanthophylls). With the exception of those carotenoids, which can be isolated from a species of higher plants or be chemically synthesized, it has been difficult to find natural sources for supplying sufficient amounts of carotenoids. Metabolic engineering (combinatorial biosynthesis), using a variety of carotenoid biosynthesis genes, should be one of the most powerful methods to generate plenty of structurally diverse carotenoids (xanthophylls).

Since the first finding in 1990 that the catalytic functions of carotenoid biosynthetic gene products were able to be assigned by complementation analysis using *Escherichia coli* (30), a considerable number of carotenogenic genes have been cloned and functionally assigned (5, 32, 39, 42). Twenty-five carotenogenic genes, whose gene products have different catalytic functions, have been identified so far as necessary to synthesize different carotenoids, including *crtU* for β-carotene desaturase, *crtZ* for β-carotene hydoroxylase (3,3′-β-hydroxylase), *crtW* (*bkt*) for β-carotene ketolase (4,4′-β-ketolase; 4,4′-β-oxygenase), *crtO* (from *Synechocystis* sp. strain PCC6803) for β-carotene mono-ketolase, and *crtX* for zeaxanthin glucosyltransferase. Functional expression of such genes in heterologous hosts such as *E. coli* has enabled us to engineer new pathways for producing carotenoids that are novel or rare in nature (40,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristic(s)[b]	Reference and/or source
Strains		
Brevundimonas sp. strain SD212	2-Hydroxyastaxanthin-producing bacterium	55, MBIC03018
Escherichia coli XL1-Blue MR	Host strain of cosmid vector SuperCos 1	Stratagene
E. coli DH5α	Host strain for DNA manipulation	Toyobo
E. coli JM109	Host strain for heterologous expression of *crt* genes	Toyobo
Plasmids		
pACCAR25ΔcrtE[c]	Cm^r, plasmid carrying *crtB, crtI, crtY, crtZ*, and *crtX*	41
pACCAR25ΔcrtB[c]	Cm^r, plasmid carrying *crtE, crtI, crtY, crtZ*, and *crtX*	9
pACCRT-EB[c]	Cm^r, plasmid carrying *crtE* and *crtB*	31
pACCRT-EIB[c]	Cm^r, plasmid carrying *crtE, crtB*, and *crtI*	31
pACCAR16ΔcrtX[c]	Cm^r, plasmid carrying *crtE, crtB, crtI*, and *crtY*	31
pACCAR25ΔcrtX[c]	Cm^r, plasmid carrying *crtE, crtB, crtI, crtY*, and *crtZ*	31
pAC-Cantha[d]	Cm^r, plasmid carrying *crtE, crtB, crtI, crtY*, and *crtW*	This study
pAC-Asta[d]	Cm^r, plasmid carrying *crtE, crB, crtI, crtY, crtZ*, and *crtW*	This study
SuperCos 1	Ap^r, cosmid vector	Stratagene
pBluescript II KS(−)	Ap^r, cloning vector for *E. coli*	Toyobo
pGEM-T Easy	Ap^r, cloning vector for *E. coli*	Promega
pUC18	Ap^r, cloning vector for *E. coli*	Toyobo
pCos5-2	Ap^r, 47-kb fragment (partial Sau3AI digest) of strain SD212 inserted into the BamHI site of SuperCos 1	This study
pCRTI-SD212	Ap^r, 1.1-kb fragment containing *crtI* amplified by PCR inserted into pGEM-T Easy	This study
p5Bre2-15	Ap^r, 12-kb EcoRI fragment derived from pCos5-2 inserted into pBluescript II KS(−)	This study
pUCBre-O11	Ap^r, 0.77-kb fragment containing ORF11 amplified by PCR derived from p5Bre2-15 inserted into pUC18	This study

[a] Plasmids for the expression of individual genes or ORFs from *Brevundimonas* sp. strain SD212 in *E. coli* are not included in this table, except for pUCBre-O11.
[b] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.
[c] The plasmids contain the *crt* genes from *Pantoea ananatis* (*Erwinia uredovora* 20D3).
[d] The plasmids contain the *crtW* gene from *Paracoccus* sp. strain N81106 in addition to the *crt* genes from *P. ananatis*.

42). Further acquisition of new carotenoid biosynthesis genes, in particular a gene encoding a β-ionone ring (β-ring)-modifying enzyme, should contribute to the biotechnological synthesis of a variety of novel or rare xanthophylls.

The Marine Biotechnology Institute has isolated a number of marine bacteria which produce astaxanthin and its relevant ketocarotenoids (53–55) (http://www.mbio.jp/mbic). We have taxonomically reevaluated these bacteria and found that they fall into three genera of α-*Proteobacteria*, *Brevundimonas* sp., *Paracoccus* sp., and *Erythrobacter* sp. Two genes encoding β-ring-modifying oxygenase enzymes, *crtZ* and *crtW*, have been isolated from *Paracoccus* sp. strain N81106 (MBIC01143), which was formerly classified as *Agrobacterium aurantiacum* (21), and *Paracoccus* sp. strain PC1 (MBIC03024), formerly classified as *Alacaligenes* sp. strain PC-1 (29), and the catalytic functions of the gene products, CrtZ and CrtW, have been identified (13, 15, 29, 31). However, there has been no report on the cloning of a carotenoid oxygenase gene from *Brevundimonas* sp. or *Erythrobacter* sp., except for one patent describing the cloning of *crtW* from *Brevundimonas aurantiaca* (PCT WO/02/079395). *Brevundimonas* sp. strain SD212 (MBIC03018), *Paracoccus* sp. strain N81106 (MBIC01143), and *Erythrobacter* sp. strain PC6 (MBIC02351) are, respectively, able to produce (2*R*,3*S*,3′*S*)-2-hydroxyastaxanthin (55), astaxanthin β-D-glucoside (53), and (2*R*,3*S*,2′*R*,3′*R*)-4-ketonostoxanthin 3′-sulfate (54) in addition to astaxanthin. Judging from their chemical structures, the two bacterial genera *Brevundimonas* and *Erythrobacter* may possess a novel oxygenase gene encoding 2,2′-β-hydroxylase in addition to two known oxygenase genes, *crtZ* and *crtW*. We report here the cloning and functional identification of a carotenoid biosynthesis gene cluster including the 2,2′-β-hydroxylase gene that is structurally and functionally novel. We further show that combinatorial biosynthesis with the new gene enables the synthesis of a novel or rare carotenoid(s) with pharmaceutical potential which would otherwise be very difficult to chemically synthesize.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and the majority of plasmids used in this study are listed in Table 1. *Brevundimonas* sp. strain SD212 (Marine Biotechnology Institute culture collection number MBIC03018) was grown on marine broth 2216 (Difco) with shaking at 25°C. The *Escherichia coli* strains used were grown on the Luria-Bertani (LB) medium (38) with shaking at 37°C or 30°C. The media were supplemented when required with the following antibiotics at the indicated concentrations: ampicillin, 100 to 150 μg ml^{-1}; chloramphenicol, 20 to 30 μg ml^{-1}. A 1 mM concentration of IPTG (isopropyl-β-D-thiogalactopyranoside) was used for inducing the *E. coli* JM109 transformants as required. The *E. coli* XL1-Blue MR and DH5α transformants were grown overnight at 37°C for the preparation of cosmid and plasmid DNA, respectively. The *E. coli* JM109 transformants were grown for 2 days at 30°C for pigment analysis.

Recombinant DNA techniques. The restriction enzymes, alkaline phosphatase (from *E. coli* C75), and DNA ligation kit were purchased from New England BioLabs, Takara Biochemicals, and Toyobo, respectively. DNA manipulation was conducted by standard methods (38) or as instructed by the suppliers. Plasmid or cosmid DNA was prepared with the QIAEX II purification kit (QIAGEN) or the automatic DNA isolation system PI-200 (Kurabo). PCR assay was carried out with an automated thermal cycler (Techne) using LA *Taq* polymerase (Takara Biochemicals). Colony hybridization and Southern hybridization were effected with Hybond N+ membranes and the Alkphos direct labeling and detection system (Amersham Pharmacia). The 1.1-kb *crtI* fragment

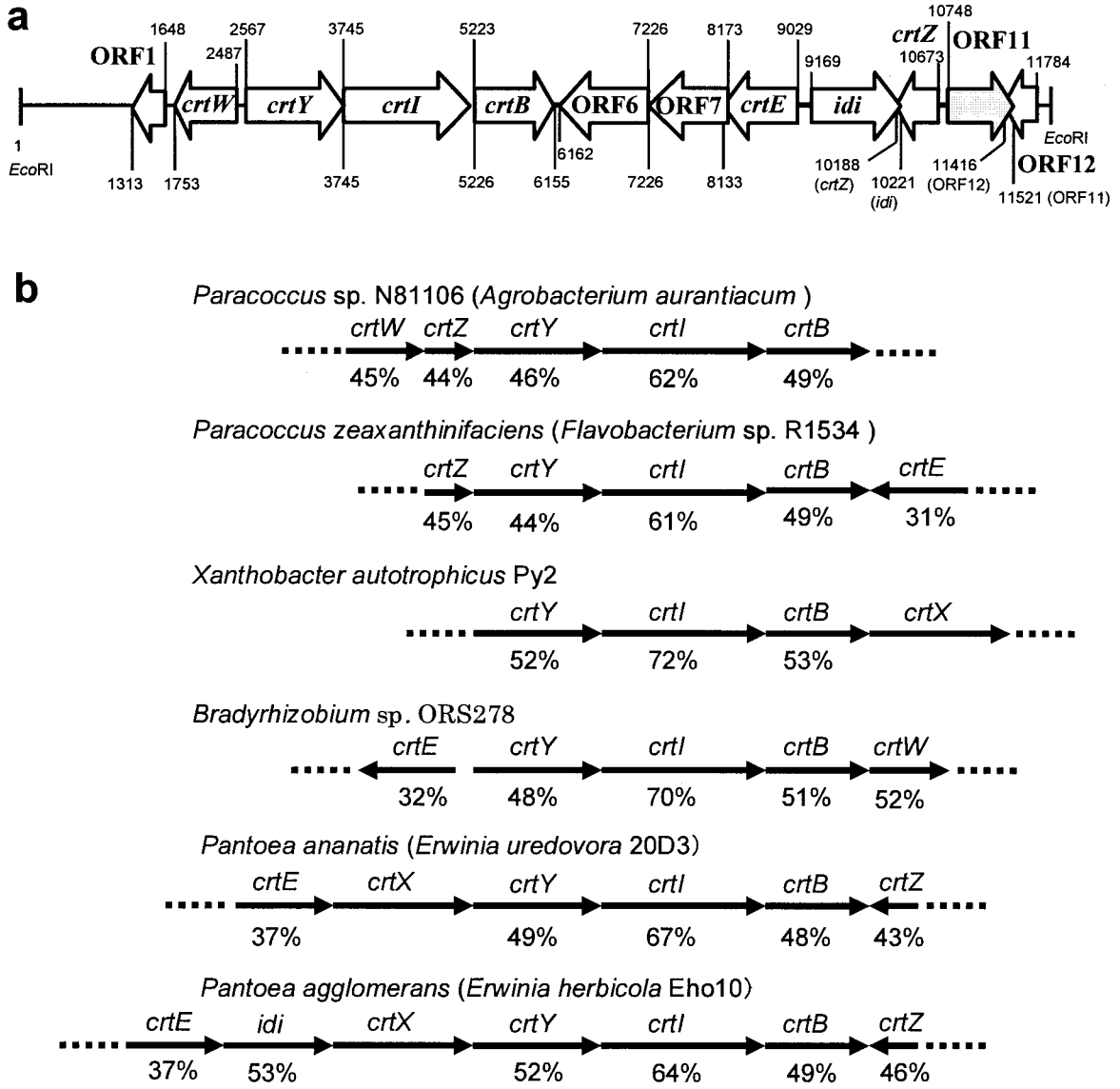

FIG. 1. Organization of the carotenoid biosynthesis gene clusters isolated from *Brevundimonas* sp. strain SD212 and from other cyclic carotenoid-generating bacteria. (a) Organization of the *Brevundimonas* sp. strain SD212 carotenoid biosynthesis gene cluster composed of an 11,991-bp EcoRI fragment. Numbers represent nucleotide positions numbered from the 5′ end of the EcoRI site. The numbers over and under the gene map, respectively, represent the positions of the start and termination of individual genes or ORFs. ORF11 was identified as a 2,2′-β-hydroxylase gene in this study and is designated *crtG*. (b) Comparison of the organization of the cyclic carotenoid gene clusters of *Paracoccus* sp. strain N81106 (GenBank accession number D58420) (31), *Paracoccus zeaxanthinifaciens* (U62808) (37), *Xanthobacter autotrophicus* Py2 (AF408848) (23), *Bradyrhizobium* sp. strain ORS278 (AF218415) (17), *Pantoea ananatis* (formerly classified as *Erwinia uredovora* 20D3, D90087) (30), and *Pantoea agglomerans* (M87280) (19). Arrows represent the orientations of genes. The values shown below the genes indicate the percentages of amino acid identity compared with *Brevundimonas* sp. strain SD212, which were calculated by using Clustal W (18) and GeneDoc (35). The zeaxanthin-hyperproducing bacterium that was formerly classified as *Flavobacterium* sp. strain R1534 has recently been reported to be the mutant of *P. zeaxanthinifaciens* ATCC 21588, as taxonomically reevaluated by Berry et al. (6). Note that all of the other bacteria, except for the genus *Pantoea* of the γ-*Proteobacteria*, belong to the α-*Proteobacteria*.

of pCRTI-SD212 was used as the probe DNA for these hybridization experiments.

Construction of plasmids for accumulation of canthaxanthin and astaxanthin in *E. coli*. Plasmids for the biosynthesis of canthaxanthin, pAC-Cantha, and astaxanthin, pAC-Asta, were constructed as follows. The *Paracoccus* sp. strain N81106 *crtW* gene, which was modified according to the codon usage of the *Candida utilis GAP* gene to reduce the GC content of the original gene (65%) (33), was used as the template for PCR amplification with primers H1437 (5′-GTC CCG AGA AGG AGG CTA GAT ATG TCC GCT CAC GCT TTG C-3′; the AvaI site is underlined) and H1438 (5′-CGG CGG CCG CCC GGG ACT AAG CGG TGT CAC CCT TGG TTC T-3′; the NotI site is underlined). An amplified 0.76-kb fragment was digested with AvaI and NotI. The *Pantoea ananatis crtE* gene (GenBank accession number D90087) was amplified from plasmid pCAR16 (30) by PCR using primers H1431 (5′-ATG CGG CCG CTT ATA AGG ACA GCC CGA ATG-3′; the NotI site is underlined) and H1432 (5′-CAG TCG ACA TCC TTA ACT GA CGG CAG CGA G-3′; the SalI site is underlined). An amplified 1.1-kb fragment was digested with NotI and SalI. The above 0.76-kb-AvaI-*crtW*-NotI and 1.1-kb NotI-*crtE*-SalI fragments were ligated with the larger fragment carrying *P. ananatis crtB*, *crtI*, and *crtY*, which had been obtained by digestion with AvaI and SalI from plasmid pACCAR16ΔcrtX, to

TABLE 2. Gene products of individual ORFs in the 12-kb fragment of *Brevundimonas* sp. strain SD212 and homology with other proteins

Gene	Amino acid residues encoded	Assigned gene product	% Identity with other protein[a]	GenBank accession no.
ORF1	111	Unknown		
crtW	244	β-Carotene 4,4′-oxygenase	96, CrtW, *Brevundimonas aurantiaca*	AY166610
crtY	392	Lycopene cyclase	52, CrtY, *Xanthobacter autotrophicus* Py2	AF408848
crtI	493	Phytoene desaturase	72, CrtI, *Xanthobacter autotrophicus* Py2	AF408848
crtB	310	Phytoene synthase	53, CrtB, *Xanthobacter autotrophicus* Py2	AF408848
ORF6	354	Unknown		
ORF7	315	Unknown		
crtE	298	GGPP synthase	39, CrtE, *Xanthobacter autotrophicus* Py2	AF408847
idi	350	Type 2 IPP isomerase	53, IPP isomerase, *Pantoea agglomerans*	M87280
crtZ	161	β-Carotene 3,3′-hydroxylase	46, CrtZ, *Pantoea agglomerans*	M87280
ORF11	257	Unknown		
ORF12	122	Unknown		

[a] Percent identity at the amino acid level with the most homologous protein according to the National Center for Biotechnology Information Blast (tblastn).

construct plasmid pAC-Cantha. Furthermore, both the 0.76-kb and 1.1-kb fragments were ligated with the larger fragment carrying *P. ananatis crtB*, *crtI*, *crtY*, and *crtZ*, which had been obtained by digestion with AvaI and SalI from plasmid pACCAR25ΔcrtX, to construct plasmid pAC-Asta.

Preparation of genomic DNA and construction of cosmid library. Total DNA was extracted from *Brevundimonas* sp. strain SD212 as previously described (49), partially digested with Sau3AI, and ligated into the BamHI site of SuperCos 1 (Stratagene). The ligates were packaged into bacteriophage λ with a Lambda Inn packaging extract (Wako). *E. coli* XL1-Blue MR was infected with the resulting phages.

PCR amplification of *crtI*. Well-conserved domains in the N-terminal region (FDAGPTV) and C-terminal region (GAGIPG) in the *crtI* genes were chosen for designing degenerate primers crtI-f (5′-TTY GAY GCI GGI CCI ACI GT-3′) and crtI-r (5′-CCI GGR TGI GTI CCI GCI CC-3′). PCR amplification was performed in a 25-μl reaction mixture containing 100 ng of strain SD212 genomic DNA, each deoxynucleoside triphosphate (200 μM), primers (2.5 μM each), $MgCl_2$ (2.5 mM), 1.0 U of LA *Taq* polymerase, and the buffer supplied with the enzyme. PCR assay was conducted as follows: preincubation at 95°C for 5 min; a total of 35 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; and a final elongation step of 5 min at 72°C. An amplified 1.1-kb fragment was ligated into a pGEM-T Easy vector to construct plasmid pCRTI-SD212.

Nucleotide sequencing and computer analysis. The nucleotide sequences of the inserted fragments of plasmids pCRTI-SD212, p5Bre2-15, and the others derived from p5Bre2-15 were determined in both orientations by a DNA sequencing kit (Big dye terminator cycle sequencing ready reaction kit, version 2; Perkin-Elmer) and a model 3700 DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions. Homologous protein sequences in the protein sequence database were retrieved using version 2.0 of the BLAST program (3) and aligned with Clustal W, version 1.8 (18) and GeneDoc (35) (http://www.psc.edu/biomed/genedoc).

Construction of plasmids for expression of the individual SD212 *crt* genes or ORFs. Plasmids for the expression of the individual *crt* genes or open reading frames (ORFs) of *Brevundimonas* sp. strain SD212 were constructed using plasmid p5Bre2-15 as the template for PCR amplification. Primers were designed to have the EcoRI site at the 5′ end and the BamHI or XbaI site at the 3′ end. The amplified fragments were digested with EcoRI and BamHI or XbaI and ligated with the EcoRI- and BamHI- or XbaI-digested pUC18. The primers for the construction of plasmid pUCBre-O11 were BO11-f (5′-TACGAA TTC GAT GTT GAG GGA TCT GCT CAT CA-3′; the EcoRI site is underlined) and BO11-r (5′-TAGAGG ATC CTC ACC GAA GAG GCG CTG AG-3′; the BamHI site is underlined).

HPLC analysis of carotenoid pigments accumulated in *E. coli*. The cultured cells of *E. coli* JM109 transformants, which were obtained from a 2-ml culture, were extracted with 200 μl of acetone. The supernatant of the acetone extract was analyzed by high-performance liquid chromatography (HPLC) (Alliance; Waters) with a photodiode array detector (PDA) (model 2996; Waters). This was performed using a TSK gel ODS-80Ts column (4.6-mm inner diameter by 150 mm; Tosoh Co.), 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-tetrahydrofuran, 7:3) for 5 min, and finally by isocratic elution with solvent B for 8 min. HPLC analysis to identify lycopene and β-carotene used an octadecyl silica reverse-phase Nova-pak HR 6-μm C_{18} column (3.9 by 300 mm; Waters) developed at a flow rate of 1 ml/min with acetonitrile–methanol–2-propanol (45:3:2 [vol/vol/vol]).

Purification and spectroscopic analysis of the carotenoid pigments. The cultured cells of *E. coli* JM109 transformants were extracted with acetone-methanol (1:1). The concentrated extract was applied to a silica gel 60 column and progressively eluted with mixtures of hexane-ethyl acetate (8:2, 7:3, 6:4, and 1:1). Pigments 1 (0.05 mg), 2 (0.1 mg), and 3 (0.1 mg) were obtained from a 2-liter culture of *E. coli*(pUCBre-O11, pAC-Asta). Pigments 4 (0.5 mg), 5 (0.6 mg), and 6 (0.8 mg) were obtained from a 10-liter culture of *E. coli*(pUCBre-O11, pACCAR25ΔcrtX). Pigments 7 (0.2 mg), 8 (0.4 mg), and 9 (0.4 mg) were

TABLE 3. Analysis of carotenoids accumulated in *E. coli* transformants carrying various combinations of *crt* genes from *Brevundimonas* sp. strain SD212 and other bacterial strains

Plasmid carried by *E. coli* strain[a]	*crt* genes	Precursor accumulated[b]	*E. coli* transformant characteristics after complementation	
			Plasmid for expression of SD212 *crt* gene	Carotenoid(s) accumulated
pACCAR25ΔcrtE	*crtB, -I, -Y, -Z, -X*	FPP	pUCBre-E (*lacZ*::*crtE*)	Zeaxanthin β-D-diglucoside,[c] zeaxanthin
pACCAR25ΔcrtB	*crtE, -I, -Y, -Z, -X*	GGPP	pUCBre-B (*lacZ*::*crtB*)	Zeaxanthin β-D-diglucoside,[c] zeaxanthin
pACCRT-EB	*crtE, -B*	Phytoene	pUCBre-I (*lacZ*::*crtI*)	Lycopene
pACCRT-EIB	*crtE, -B, -I*	Lycopene	pUCBre-Y (*lacZ*::*crtY*)	β-Carotene
pACCAR16ΔcrtX	*crtE, -B, -I, -Y*	β-Carotene	pUCBre-Z (*lacZ*::*crtZ*)	Zeaxanthin
pAC-Cantha	*crtE, -B, -I, -Y, -W*	Canthaxanthin	pUCBre-Z (*lacZ*::*crtZ*)	Astaxanthin, adonixanthin[d]
pACCAR16ΔcrtX	*crtE, -B, -I, -Y*	β-Carotene	pUCBre-W (*lacZ*::*crtW*)	Canthaxanthin
pACCAR25ΔcrtX	*crtE, -B, -I, -Y, -Z*	Zeaxanthin	pUCBre-W (*lacZ*::*crtW*)	Astaxanthin, adonirubin[d]

[a] A more detailed explanation is presented in Table 1.
[b] Accumulated carotenoid precursors are confirmed in the references listed in Table 1.
[c] Zeaxanthin β-D-diglucoside produced by an *E. coli* transformant was identified (34).
[d] Syntheses of astaxanthin, adonixanthin, and adonirubin by *E. coli* transformants were described previously (31).

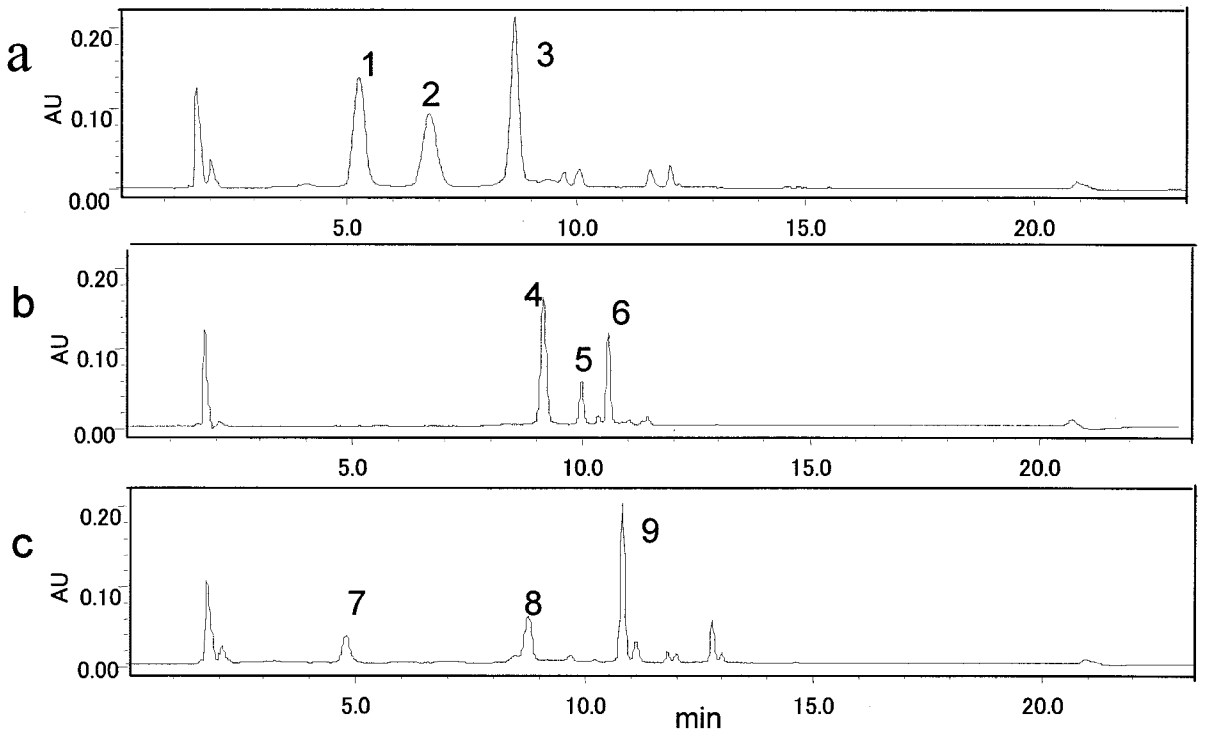

FIG. 2. Carotenoid products from *E. coli* carrying pUCBre-O11 and pAC-Asta (a), pUCBre-O11 and pACCAR25ΔcrtX (b), and pUCBre-O11 and pAC-Cantha (c). HPLC/PDA was performed using a TSK gel ODS-80Ts column, as described in Materials and Methods. Detection was carried out at 470 nm. The numbers of carotenoids correspond to those discussed in "Spectral data for individual carotenoids" in Materials and Methods.

obtained from a 10-liter culture of *E. coli* (pUCBre-O11, pAC-Cantha). Each pigment fraction was analyzed by HPLC/PDA/atmospheric pressure chemical ionization mass spectrometry (APCI-MS), nuclear magnetic resonance spectrometry (NMR), and high-resolution fast atom bombardment mass spectrometry (HRFABMS).

HPLC/PDA/APCI-MS was carried out with an LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) connected to a NanoSpace SI-2 HPLC/PDA instrument (Shiseido, Tokyo, Japan). The positive-ion APCI mass spectra were acquired by scanning from *m/z* 200 to 800. HPLC separation was conducted in a Develosil C30-UG-3 column (1.0-mm inner diameter by 150 mm; Nomura Chemical Co., Seto, Japan) with gradient elution by a binary system consisting of solvent A (methanol-water, 96:4) and solvent B (*tert*-butyl methyl ether) at a flow rate of 0.1 ml/min. Isocratic elution by solvent A was used for the initial 12 min, followed by linear gradient elution to 60% (vol/vol) of solvent B for 60 min and then by isocratic elution with 60% of solvent B for a further 10 min.

NMR spectra were obtained with a Unity INOVA 750 instrument (Varian, Palo Alto, CA), using $CDCl_3$ as the solvent. HRFABMS data were measured by a JMS700 instrument (Jeol, Tokyo, Japan), using *m*-nitro benzyl alcohol as a matrix.

Authentic samples of carotenoids were purchased from Sigma or Funakoshi or purified from *Brevundimonas* sp. strain SD212 or *E. coli* transformants expressing other bacterial *crt* genes.

Spectral data for individual carotenoids. For pigment 1, (2*R*)-hydroxyastaxanthin: visible (VIS) λ_{max} 474 nm; LC/APCI-MS *m/z* 613 [$M^+ + H$]; ^{1}H NMR (750 MHz) δ 1.21 (3H, s), 1.26 (3H, s), 1.29 (3H, s), 1.32 (3H, s), 1.81 (1H, t, J = 13.0 Hz), 1.95 (6H, s), 1.98 to 2.00 (12H, s), 2.16 (1H, dd, J = 13.0, 5.5 Hz), 3.52 (1H, dd, J = 11.5, 2.0 Hz), 4.18 (1H, dd, J = 11.5, 2.5 Hz), 4.32 (1H, ddd, J = 13.0, 5.5, 2.0 Hz), 6.2 to 6.7 (14H, m). For pigment 2, astaxanthin: VIS λ_{max} 473 nm; LC/APCI-MS *m/z* 597 [$M^+ + H$]. For pigment 3, adonixanthin: VIS λ_{max} 461 nm; LC/APCI-MS *m/z* 583 [$M^+ + H$]. For pigment 4, (2*R*, 2′*R*)-dihydroxyzeaxanthin (nostoxanthin): VIS λ_{max} 449, 475 nm; LC/APCI-MS *m/z* 601 [$M^+ + H$], 583 [$M^+ + H - H_2O$], 565 [$M^+ + H - 2H_2O$] (see Table 4 for ^{1}H NMR data). For pigment 5, (2*R*)-hydroxyzeaxanthin (caloxanthin): VIS λ_{max} 450, 476 nm; LC/APCI-MS *m/z* 585 [$M^+ + H$], 567 [$M^+ + H - H_2O$] (see Table 4 for ^{1}H NMR data). For pigment 6, zeaxanthin: VIS λ_{max} 450, 476 nm; LC/APCI-MS *m/z* 569 [$M^+ + H$]. For pigment 7, (2*R*, 2′*R*)-dihydroxycanthaxanthin [(2*R*, 2′*R*)-2, 2′-dihydroxy-β,β-carotene-4,4′-dione]: VIS λ_{max} 472 nm; LC/APCI-MS *m/z* 597 [$M^+ + H$], 579 [$M^+ + H - H_2O$], 561 [$M^+ + H - 2H_2O$]; HRFABMS *m/z* [M^+] (see Table 4 for ^{1}H NMR data). Calculated: $C_{40}H_{32}O_4$, 596.3866. Found: 596.3863. For pigment 8, (2*R*)-hydroxycanthaxanthin [(2*R*)-2-hydroxy-β,β-carotene-4,4′-dione]: VIS λ_{max} 474 nm; LC/APCI-MS *m/z* 581 [$M^+ + H$], 563 [$M^+ + H - H_2O$]; HRFABMS *m/z* [M^+] (see Table 4 for ^{1}H NMR data). Calculated: $C_{40}H_{32}O_3$, 580.3916. Found: 580.3900. For pigment 9, canthaxanthin: VIS λ_{max} 475 nm; LC/APCI-MS *m/z* 565 [$M^+ + H$].

Inhibitory activity against lipid peroxidation. In vitro inhibitory activity of the carotenoids against lipid peroxidation was measured by the oxygen radical-generating reaction, which was initiated with sodium ascorbate in a rat brain homogenate, as described previously (48).

Nucleotide sequence accession number. The nucleotide sequence of the 12.0-kb EcoRI region of p5Bre2-15 has been deposited in DDBJ/EMBL/GenBank under accession number AB181388.

RESULTS

Isolation of a DNA fragment including *crtI*. The *crtI* gene encoding phytoene desaturase has been isolated and characterized for a variety of organisms. A comparison of the deduced amino acid sequences of CrtI proteins from *Paracoccus* sp. strain N81106 (MBIC01143) (31), *Paracoccus zeaxanthinifaciens* ATCC 21588 (formerly classified as *Flavobacterium* sp. strain R1534) (6, 37), *Pantoea agglomerans* (formerly classified as *Erwinia herbicola* strain Eho10) (4), *Bradyrhizobium* sp. strain ORS278 (17), and *Erythrobacter longus* strain Och101 (25) has revealed well-conserved domains in the N-terminal region (FDAGPTV) and C-terminal region (GAGIPG). Genomic DNA was isolated from the cells of *Brevundimonas*

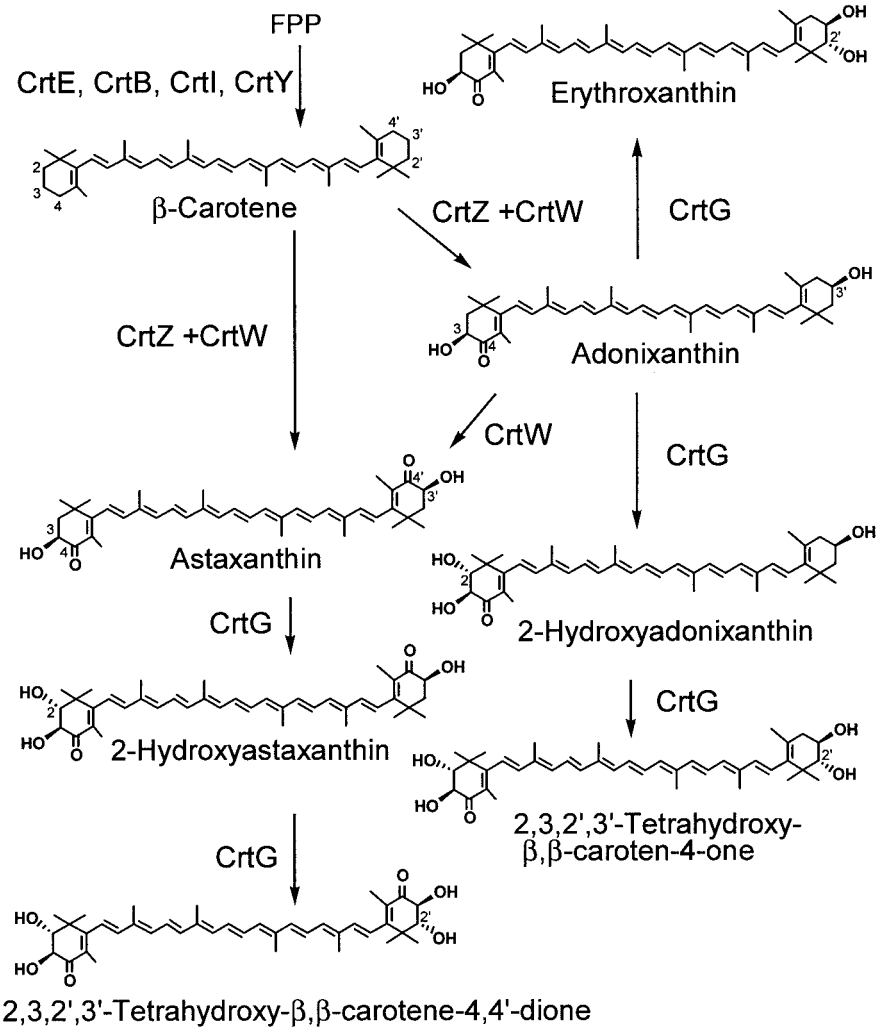

FIG. 3. Chemical structures of the carotenoid pigments produced by *Brevundimonas* sp. strain SD212 (55) and proposed functions of the carotenoid biosynthesis gene products. The functions of CrtE, CrtB, CrtI, and CrtY are shown in Fig. 4. FPP, farnesyl diphosphate (pyrophosphate).

sp. strain SD212 (MBIC03018), whose 16S rRNA gene and *gyrB* sequences are available in DDBJ/EMBL/GenBank under accession numbers AB016849 and AB014993, respectively. Based on the degenerate primers designed from the above-mentioned motifs, PCR amplification was done to isolate a 1.1-kb fragment from the SD212 genomic DNA and ligated into pGEM-T Easy to yield plasmid pCRTI-SD212 (Table 1). The nucleotide sequence of this 1.1-kb inserted fragment was similar to the known CrtI sequences at the amino acid level.

A cosmid library of the genomic DNA from *Brevundimonas* sp. strain SD212, which comprised 1,000 colonies, was constructed using cosmid vector SuperCos 1 (Table 1). Colony hybridization experiments on 500 of these colonies were conducted with the DNA probe of the 1.1-kb fragment from pCRTI-SD212. As a result, six colonies showing positive signals were obtained. Cosmid DNA was prepared from these colonies and subjected to Southern hybridization experiments after being digested with appropriate restriction enzymes; common fragments, i.e., 12-kb EcoRI, 9-kb BamHI, and 8.2 kb-EcoRI/BamHI, showed positive hybridization signals. These signals were also detected in the digested fragments that had been derived from the SD212 genomic DNA. One cosmid (named pCos5-2) was used for further experiments. A 12-kb EcoRI fragment was isolated from pCos5-2 and inserted into the EcoRI site of vector pBluescript II KS(−) to yield plasmid p5Bre2-15 (Table 1).

Structural analysis of the carotenoid biosynthesis gene cluster. The nucleotide sequence of the 12-kb EcoRI fragment of plasmid p5Bre2-15 revealed this fragment, which was 11,991 bp in length and had a high GC content (69%), to contain 12 ORFs. Seven ORFs were similar to the known carotenogenic genes, *crtW*, *crtY*, *crtI*, *crtB*, *crtE*, *idi*, and *crtZ*, and the same designations were assigned (Fig. 1a and Table 2). The translated sequences of the other five ORFs showed no overall homology with any other proteins. The *crtY*, *crtI*, and *crtB* genes occurred in this order and were oriented in the same direction, and these characteristics have been observed in all of the cyclic (dicyclic) carotenoid biosynthesis gene clusters pre-

TABLE 4. ^{1}H NMR data in $CDCl_3$

Proton	Result (multiplied, *J*) for pigment:			
	4	5	7	8
H-2_{ax}	3.33 (d, 10.0)	3.33 (d, 10.0)	3.90 (dd, 9.0, 4.5)	3.90 (dd, 9.0, 4.5)
H-2_{eq}				
H-3_{ax}	3.84 (dt, 6.7, 10.0)	3.84 (dt, 6.7, 10.0)	2.62 (dd, 17.4, 9.0)	2.62 (dd, 17.4, 9.0)
H-3_{eq}			2.8 (dd, 17.4, 4.5)	2.8 (dd, 17.4, 4.5)
H-4_{ax}	2.15 (dd, 17.4, 10.0)	2.15 (dd, 17.4, 10.0)		
H-4_{eq}	2.49 (dd, 17.4, 6.7)	2.49 (dd, 17.4, 6.7)		
H_3-16, 17	1.01 (s)	1.01 (s)	1.22 (s)	1.22 (s)
	1.14 (s)	1.14 (s)	1.26 (s)	1.26 (s)
H_3-18	1.72 (s)	1.72 (s)	1.89 (s)	1.89 (s)
H-$2'_{ax}$	3.33 (d, 10.0)	1.49 (t, 12.0)	3.90 (dd, 9.0, 4.5)	1.85 (t, 7.0)
H-$2'_{eq}$		1.80 (m)		1.85 (t, 7.0)
H-$3'_{ax}$	3.84 (dt, 6.7, 10.0)	4.01 (m)	2.62 (dd, 17.4, 9.0)	2.51 (t,7.0)
H-$3'_{eq}$			2.8 (dd, 17.4, 4.5)	2.51 (t,7.0)
H-$4'_{ax}$	2.15 (dd, 17.4, 10.0)	2.05 (dd, 17.4, 10.5)		
H-$4'_{eq}$	2.49 (dd, 17.4, 6.7)	2.40 (dd, 17.4, 6.3)		
H_3-16′, 17′	1.01 (s)	1.08 (s)	1.22 (s)	1.21 (s)
	1.14 (s)	1.08 (s)	1.26 (s)	1.21 (s)
H_3-18′	1.72 (s)	1.75 (s)	1.89 (s)	1.90 (s)
H_3-19, 20, 19′, 20′	1.98–1.99 (s)	1.98–1.99 (s)	2.00–2.02 (s)	2.00–2.02 (s)
Olefinic-H(14H)	6.0–6.7 (m)	6.0–6.7 (m)	6.2–6.7 (m)	6.2–6.7 (m)

viously reported (Fig. 1b). The organization of the SD212 carotenoid biosynthesis gene cluster did, however, have several unique characteristics. (i) The sequence for type 2 isopentenyl diphosphate (IDP or IPP) isomerase (*idi*) (22) was present in this cluster. Interestingly, this *idi* sequence has only previously been found in the carotenoid biosynthesis gene cluster from *P. agglomerans* (*E. herbicola* Eho10), the gene of which exists between the *crtE* and *crtX* genes (19), and had remained unidentified for a decade. (ii) The *crtW* and *crtZ* genes, which mediate the oxygenation reaction from β-carotene to astaxanthin and exist adjacently in the *Paracoccus* strains, were present at a distance from each other in *Brevundimonas* sp. strain SD212. (iii) Five transcription units are likely to be present in this gene cluster for the expression of all seven *crt* genes and five ORFs. (iv) The individual SD212 carotenogenic gene products showed low or medium levels of amino acid homology to the corresponding proteins derived from the other genera of bacteria reported (31 to 72%) (Fig. 1b). (v) No substantial difference has been observed in the degree of amino acid homology with the *Brevundimonas* Crt proteins between those derived from the *Pantoea* species of γ-*Proteobacteria* and the other bacterial species of α-*Proteobacteria* (Fig. 1b).

Functional analysis of individual carotenoid biosynthesis genes. Functional analysis of many carotenoid biosynthesis genes has successfully been carried out in *E. coli*. However, both *E. coli* transformants carrying the entire *crt* gene cluster of *Brevundimonas* sp. strain SD212 (plasmid p5Bre2-15) and carrying plasmid pACCRT-EB for phytoene production in addition to p5Bre2-15 did not produce any pigmented carotenoids. Moreover, p5Bre2-15 was not able to complement *E. coli* carrying pACCAR25ΔcrtE, pACCAR25ΔcrtB, pACCRT-EIB, pACCAR16ΔcrtX, or pACCAR25ΔcrtX to produce the additional carotenoid pigments, suggesting that these genes are not easily expressed in *E. coli*. It is possible that translational signals such as the ribosomal binding sites that are included in the gene cluster are not functional in *E. coli*.

Thus, to analyze the functions of the individual *crt* genes or ORFs of SD212, we devised a plasmid construct for their expression in *E. coli* which would be able to synthesize each gene product fused to a lead sequence of β-galactosidase (LacZ) under transcription from the *lac* promoter by vector pUC18. The lead sequence in these plasmids, with the seven amino acid residues Met-Thr-Met-Ile-Thr-Asn-Ser derived from LacZ, was designed to be added to the Met of the original start of the individual genes. These hybrid genes are expected to be efficiently translated using the original ribosomal binding site and start codon of the *lacZ* gene. These plasmids were pUCBre-E, pUCBre-B, pUCBre-I, pUCBre-Y, pUCBre-Z, and pUCBre-W for the expression of the individual SD212 *crt* genes (Table 3) and pUCBre-O1, pUCBre-O6, pUCBre-O7, pUC-Bre-O11, and pUCBre-O12 for the respective expression of the unknown SD212 ORFs, ORF1, ORF6, ORF7, ORF11, and ORF12. These plasmids (Ap^r) were introduced into *E. coli* transformants, accumulating the desired carotenoid precursors due to the presence of the pACYC184-derived plasmids with Cm^r that carried the various combinations of bacterial *crt* genes for complementation studies (Table 3). The identification of pigments produced in the resulting *E. coli* transformants with Cm^r and Ap^r exhibited the individual *crt* genes of strain SD212 coding for proteins with the predicted catalytic functions (Table 3).

Since *Brevundimona*s sp. strain SD212 produced the ketocarotenoids with the 2 (2′)-hydroxy group, it was possible to include a novel oxygenase gene encoding 2,2′-β-hydroxylase among ORF1, ORF6, ORF7, ORF11, and ORF12. Therefore, the plasmids for expressing these ORFs, i.e., pUCBre-O1, pUCBre-O6, pUCBre-O7, pUCBre-O11, and pUCBre-O12, were introduced into *E. coli*, accumulating astaxanthin and the

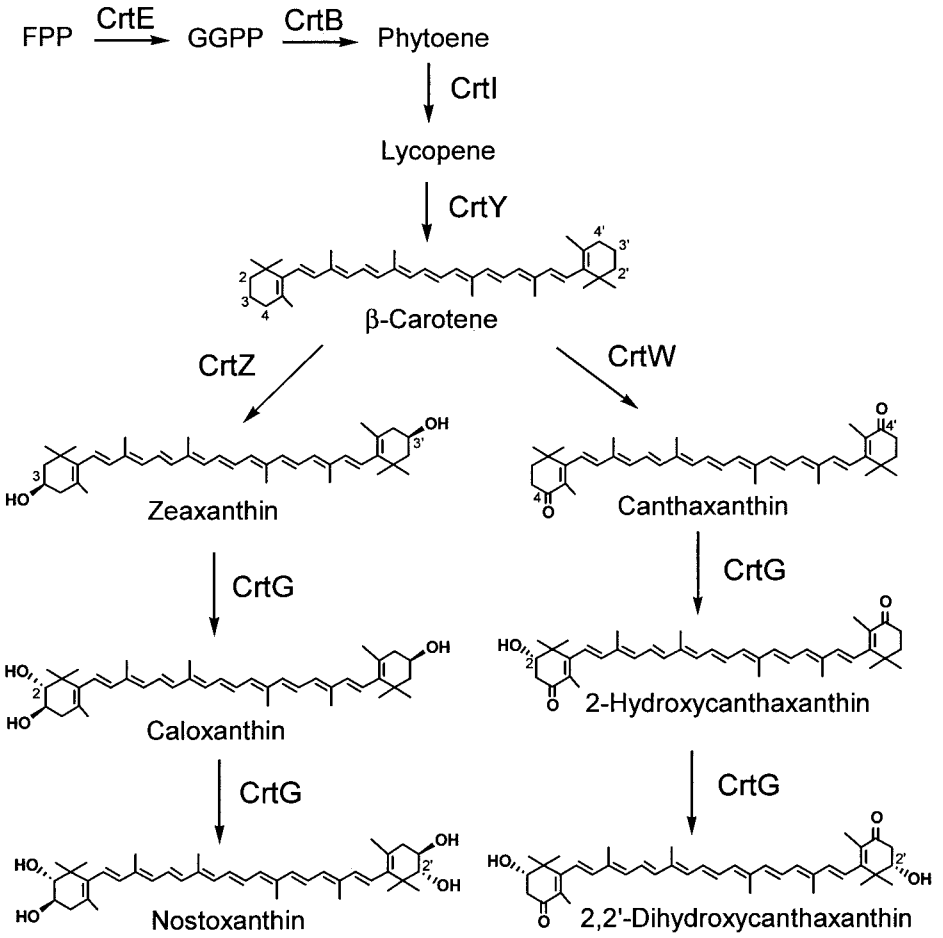

FIG. 4. Combinatorial biosynthesis of novel and rare carotenoids by using *E. coli* carrying plasmids pUCBre-O11 and pACCAR25ΔcrtX or pAC-Cantha.

intermediates such as adonixanthin due to the presence of plasmid pAC-Asta, with these accumulated pigments analyzed by HPLC. It was found that only *E. coli* carrying pUCBre-O11 and pAC-Asta could produce an additional polar carotenoid besides the carotenoids accumulated in a control *E. coli* strain carrying only one plasmid, pAC-Asta. The HPLC profile is shown in Fig. 2a. Major carotenoid pigments 1, 2, and 3 in the extract from the cells were purified, and various instrumental analyses were conducted to determine the structures. Pigments 2 and 3, which also appeared in the control strain, were confirmed to be astaxanthin and adonixanthin by their LC/APCI-MS and UV-VIS spectral data. Pigment 1 was identified as 2-hydroxyastaxanthin by its LC/APCI-MS, UV-VIS, and ^{1}H NMR spectral data (55). The stereostructure of the 2-hydroxy group was assigned to be *R* according to the circular dichroism spectral data of the carotenoids identified in strain SD212 (55). It is therefore clear that ORF11 coded for the novel carotenoid biosynthesis enzyme, carotenoid 2,2′-β-hydroxylase (β-ring C2-hydroxylase). This gene was designated *crtG*. *crtG* nomenclature was used only once by Schnurr et al. in 1991 (44), instead of the *crtX* gene coding for zeaxanthin glucosyltransferase (30), and has never been used since then, since *crtX* has been in common use.

The carotenoid pigments that *Brevundimonas* sp. strain SD212 produced had already been identified (55), and their structures are shown in Fig. 3. Since we seem to have identified the functions of all of the SD212 carotenoid biosynthesis gene products, the carotenoid biosynthetic pathway was proposed at the gene level as shown in Fig. 3. Except for (2*R*)-2-hydroxyastaxanthin, we could not detect the ketocarotenoids with a 2(2′)-hydroxyl group in the extract from *E. coli* cells expressing *crtG* as well as the genes required for astaxanthin synthesis. However, it should be feasible to detect these ketocarotenoids by enhancing the expression level of the carotenoid biosynthesis-related genes in a heterologous host such as *E. coli*, as discussed in Discussion.

TABLE 5. Inhibitory effects of several carotenoids on lipid peroxidation in a rat brain homogenate

Carotenoid	IC_{50} (μM)[a]
β-Carotene	>100
Canthaxanthin	50
2-Hydroxycanthaxanthin	8.1
2,2′-Dihydroxycanthaxanthin	4.8

[a] IC_{50}, 50% inhibitory concentration.

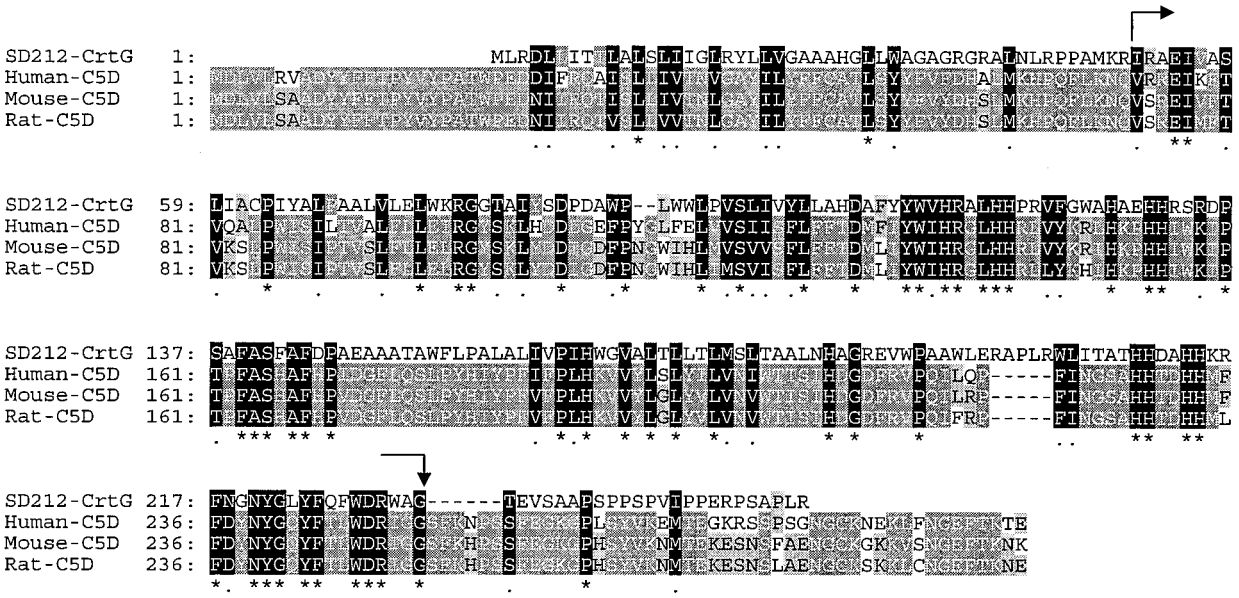

FIG. 5. Alignment of the deduced amino acid sequences of CrtG from *Brevundimonas* sp. strain SD212 and sterol-C5-desaturases (C5D) derived from human (*Homo sapiens*) (GenBank accession no. AB016247), mouse (*Mus musculus*) (GenBank accession no. BC024132), and rat (*Rattus norvegicus*) (GenBank accession no. AB052846) origins. Asterisks and dots indicate amino acid residues identical and similar, respectively, for all of the aligned sequences. Identical or similar residues among all aligned sequences are shown with a black background. A gray background indicates partially aligned sequences. Arrows indicate the homologous region (the amino acids shown for CrtG are 51 to 233).

Combinatorial biosynthesis of new and rare carotenoids using the new 2,2′-β-hydroxylase gene. The acquisition of a new class of carotenoid biosynthesis gene should make it possible to achieve combinatorial biosynthesis of various new or rare carotenoids by using the gene. Thus, plasmid pUCBre-O11 was introduced into *E. coli* carrying pACCAR25ΔcrtX that accumulates zeaxanthin, and the yellow cells of the resulting *E. coli* transformant were subjected to pigment analysis. The HPLC profile is shown in Fig. 2b. Pigments 4, 5, and 6 produced by this *E. coli* transformant (pUCBre-O11 and pACCAR25ΔcrtX) were predicted to be carotenoids involving the 3-hydroxy-β-ring as the end groups corresponding to those of zeaxanthin. Pigment 6 was found to be zeaxanthin from its LC/APCI-MS analysis and by comparison with an authentic sample. Pigment 4, which displayed *m/z* 601 [$M^+ + H$], 583 [$M^+ + H - H_2O$], and 565 [$M^+ + H - 2H_2O$], and pigment 5, which displayed *m/z* 585 [$M^+ + H$] and 567 [$M^+ + H - H_2O$] by LC/APCI-MS analysis, were identical to 2, 2′-dihydroxyzeaxanthin and 2′-hydroxyzeaxanthin, respectively. Their ^{1}H NMR data are shown in Table 4. The coupling constant between H-2 (H-2′) and H-3 (H-3′) of 10 Hz predicted that the H-2 (H-2′) proton and the 2 (2′)-hydroxyl group would have axial and equatorial confirmations, respectively. Since zeaxanthin synthesized with an *E. coli* transformant carrying the *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes of *P. ananatis* (*Erwinia uredovora* 20D3) has shown the 3*R*,3′*R* configuration (15), the stereostructure of C-2,2′ in pigment 4 was determined to be *R*. Pigment 5 was found to have the same 2,3-dihydroxy-β ring as that of pigment 4 and the same 3-hydroxy-β-ring as that of zeaxanthin from its ^{1}H NMR data (Table 4). These results enabled pigments 4 and 5 to be identified as (2*R*, 2′*R*)-dihydroxyzeaxanthin (nostoxanthin) and (2*R*)-hydroxyzeaxanthin (caloxanthin), respectively, which are rarely found in nature. The biosynthetic pathway to caloxanthin and nostoxanthin by the recombinant *E. coli* is shown in Fig. 4.

Furthermore, plasmid pUCBre-O11 was introduced into *E. coli* carrying pAC-Cantha, which accumulates canthaxanthin, and the orange cells of the resultant *E. coli* transformant were subjected to pigment analysis. The HPLC profile is shown in Fig. 2c. Pigments 7, 8, and 9 produced by this *E. coli* transformant (pUCBre-O11 and pAC-Cantha) were carotenoids showing a ^{1}H NMR signal of 4-keto-β type. Pigment 9 was found to be canthaxanthin from its LC/APCI-MS analysis and by comparison with an authentic sample. Pigment 7 was predicted to have a symmetrical structure from its ^{1}H NMR data (Table 4), and its COSY, HSQC, and HMBC spectral data showed that pigment 7 had hydroxyl groups at the 2 and 2′ positions. The correlation of H-16 (16′) and H-17 (17′) with C-2 (2′) of ca. 75 ppm supported the 2,2′-dihydroxyl groups. Taking the results of HRFABMS and LC/APCI-MS into consideration, pigment 7 was identified as the novel carotenoid 2,2′-dihydroxycanthaxanthin (2, 2′-dihydroxy-β,β-carotene-4,4′-dione). Since the respective coupling constants between H-2 and H-3_{ax} (H-2′ and H-$3'_{ax}$) and H-2 and H-3_{eq} (H-2′ and H-$3'_{eq}$) were 9 Hz and 5 Hz, the H-2 (2′) proton and the 2 (2′)-hydroxyl group had axial and equatorial conformations, respectively.

Pigment 8 was found to have the 2-hydroxy-4-keto-β-ring like pigment 7 and the 4-keto-β-ring like canthaxanthin from its ^{1}H NMR data (Table 4). Thus, pigment 8 was identified as 2-hydroxycanthaxanthin [2-hydroxy-β,β-carotene-4,4′-dione] from its HRFABMS, LC/APCI-MS, and ^{1}H NMR data. This pigment has only previously been isolated from a marine animal (*Crustacea*) (11). The *R* chirality at C-2 of pigment 8 was confirmed by the coincidence with the spectral data of (2*R*)-2-hydroxycanthaxanthin described in this paper. Therefore, the stereostructure of C-2, 2′ in novel pigment 7 was determined to

be of *R* configuration. The biosynthetic pathway to (2*R*)-2-hydroxy-β,β-carotene-4,4′-dione and (2*R*, 2′*R*)-2,2′-dihydroxy-β,β-carotene-4,4′-dione by the recombinant *E. coli* is shown in Fig. 4.

When the *crtG* gene (plasmid pUCBre-O11) was introduced into β-carotene-synthesizing *E. coli* carrying plasmid pACCAR16ΔcrtX, the resulting *E. coli* transformant was not able to produce detectable 2-hydroxy carotenoids. Therefore, CrtG is likely to require an oxygenated β-end group, i.e., a 3-hydroxy- or 4-keto-β-end group as a substrate for the 2-hydroxylation reaction.

Antioxidative activity of new and rare ketocarotenoids. We examined the in vitro inhibitory effects of several carotenoids, including the new xanthophyll, 2,2′-dihydroxycanthaxanthin, toward the lipid peroxidation induced by free radicals in a rat brain homogenate, as shown in Table 5. It was consequently suggested that the 2-hydroxy and 2,2′-dihydroxy products had much stronger antioxidative activity than the precursor canthaxanthin.

DISCUSSION

The present study has enabled us to elucidate for the first time the structures and functions of a gene cluster encoding carotenoid biosynthetic enzymes from the genus *Brevundimonas* belonging to α-*Proteobacteria*. This cluster contained the seven known genes *crtW*, *crtY*, *crtI*, *crtB*, *crtE*, *idi*, and *crtZ* in addition to a new gene (designated *crtG*), which was found to encode a novel enzyme, carotenoid 2,2′-β-hydroxylase. This enzyme, CrtG, composed of 257 amino acid residues showed no overall homology with any other proteins. However, this enzyme surprisingly exhibited intriguingly partial homology with the middle region of animal sterol-C5-desaturase (Δ7-sterol 5-desaturase), which catalyzes the oxidation reaction from lathosterol to 7-dehydrocholesterol in the pathway for cholesterol biosynthesis (Fig. 5). The degree of identity of CrtG (amino acids 51 to 233) with the middle region of sterol-C5-desaturases was 28%. It is difficult to speculate on the function of the homologous sequences between the two enzymes, but they may be involved in their binding to the isoprenoid substrates and/or in the common oxidation reactions.

This study has also demonstrated that we can produce a variety of structurally novel or rare carotenoids with a 2-hydroxy group and 2,2′-dihydroxy groups by using *crtG* in various combinations with other known *crt* genes. This successful combinatorial biosynthesis of such xanthophylls with a new functional group(s), which are very difficult to synthesize chemically, seems to make it feasible to evaluate their pharmaceutical potential, as we have here suggested their potential as superior antioxidants. It should be feasible to enhance the productivity of these carotenoids by using *E. coli* that has an improved isoprenoid central pathway due to the introduction of such key biosynthesis genes as DXP (1-deoxy-D-xylose 5-phosphate) synthase and IDP (IPP) isomerase genes (1, 10, 26, 52) or by using other efficient heterologous hosts such as the yeast *Candida utilis* (47) and higher plants (14, 46). Directed evolution, such as DNA shuffling (43, 50), may also be applicable to enhancing or changing the substrate affinity or preference of the CrtG enzyme for the 2 (2′)-hydroxylation reaction; this technique may make it possible to produce a CrtG enzyme which can catalyze the efficient conversion from β-carotene to 2,2′-dihydroxy-β-carotene by way of 2-hydroxy-β-carotene.

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