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Characterization of bacterial β-carotene 3,3′-hydroxylases, CrtZ, and P450 in astaxanthin biosynthetic pathway and adonirubin production by gene combination in *Escherichia coli*

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Abstract β-Carotene hydroxylase (CrtZ) is one of rate-limiting enzymes for astaxanthin production. A complementation analysis was conducted using *Escherichia coli* transformants to compare the catalytic efficiency of bacterial CrtZ from *Brevundimonas* sp. SD212, *Paracoccus* sp. PC1 (formerly known as *Alcaligenes* sp. PC-1), *Paracoccus* sp. N81106 (*Agrobacterium aurantiacum*), *Pantoea ananatis* (*Erwinia uredovora* 20D3), marine bacterium P99-3, and P450 monooxygenase (CYP175A1) from *Thermus thermophilus* HB27. Each *crtZ* or CYP175A1 gene was expressed in *E. coli* transformants synthesizing canthaxanthin and β-carotene due to the respective presence of plasmids pAC-Cantha and pACCAR16ΔcrtX. The carotenoids that accumulated in the resulting recombinant *E. coli* cells were examined by chromatographic and spectroscopic analyses. *E. coli* carrying *Brevundimonas* sp. SD212 *crtZ* showed the highest astaxanthin production efficiency among the transformants examined, while there was no significant difference in the catalytic efficiency for conversion from β-carotene to zeaxanthin. Recombinant *E. coli* expressing the CYP175A1 gene, in addition to the genes for canthaxanthin synthesis, surprisingly accumulated adonirubin (phoenicoxanthin) as the main product, although the other recombinant *E. coli* did not accumulate any adonirubin. The present results suggest that the *Brevundimonas* sp. SD212 *crtZ* and *T. thermophilus* HB27 CYP175A1 genes could, respectively, be used for the efficient production of astaxanthin and adonirubin in heterologous hosts.

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Introduction

Carotenoid pigments are synthesized in all of the photosynthetic bacteria, cyanobacteria, algae, higher plants, and also in some nonphotosynthetic bacteria, yeast, and fungi. Carotenoids have attracted strong attention due to their beneficial effects on human health as well as their usefulness in food colorants and animal feed because animals are unable to synthesize them de novo. In particular, dicyclic ketocarotenoids such as astaxanthin [(3*S*,3′*S*)-3,3′-dihydroxy-β,β-carotene-4,4′-dione] and canthaxanthin are high-value carotenoids used industrially as colorants and feed supplements (Meyers 1994). Furthermore, astaxanthin has been shown to have anticancer activity (Chen et al. 1999), inhibitory effect toward oxidation of low-density lipoprotein (Iwamoto et al. 2000), singlet oxygen-quenching activity (Tatsuzawa et al. 2000), and enhancement of immune responses (Chen and Park 2004).

Metabolic engineering (combinatorial biosynthesis), using a variety of carotenoid biosynthesis genes should be one of the most powerful methods to produce such ketocarotenoids for industrial and nutritional purposes because organisms which are capable of synthesizing dicyclic ketocarotenoids are not common in nature; for example, astaxanthin and its dicyclic-carotenoid intermediates are synthesized in some marine eubacteria (Yokoyama et al. 1994, 1996), in the yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) (Andrewes et al. 1976), in the green algae *Haematococcus pluvialis* (Boussiba and Vonshak 1991), and in the petal of *Adonis* plants (Renstrom et al. 1981). The astaxanthin biosynthetic pathway has been elucidated at enzyme and

gene levels by in vitro (Fraser et al. 1997, 1998) and in vivo (Misawa et al. 1995) studies. Astaxanthin can be synthesized from β-carotene (β,β-carotene) with the introduction of keto and hydroxyl moieties at the C-4,4′ and C-3,3′ positions into the β-ionone rings by CrtW, β-carotene 4,4′-ketolase (carotenoid 4,4′-oxygenase; 4,4′-β-oxygenase), and CrtZ, β-carotene 3,3′-hydroxylase (carotenoid 3,3′-hydroxylase; 3,3′-β-hydroxylase), via many hydroxylated or ketolated carotenoid intermediates, as shown in Fig. 1.

Some of studies have suggested that the cooperation of appropriate hydroxylase and ketolase enzymes or their conversion capacity toward various substrates may be crucial for the efficient production of astaxanthin in a heterologous host (Yokoyama and Miki 1995b; Misawa et al. 1995; Fraser et al. 1997; Ralley et al. 2004; Papp et al. 2005). It is therefore necessary to find and identify such hydroxylase and ketolase that have high conversion efficiency from β-carotene to astaxanthin. We have recently compared for the first time the astaxanthin-synthesizing efficiency of the CrtW enzymes, which were derived from *Brevundimonas* sp. strain SD212 (MBIC03018), *Paracoccus* sp. strain PC1 (MBIC03024) (see http://www.mbio.jp/mbic/ and Berry et al. 2003), formerly classed as *Alcaligenes* sp. PC-1, and *Paracoccus* sp. strain N81106 (MBIC01143), formerly classed as *Agrobacterium aurantiacum* N81106, in a recombinant *E. coli* host synthesizing zeaxanthin, and elucidated that the CrtW of *Brevundimonas* sp. SD212 produced a high level of astaxanthin due to the efficient conversion of adonixanthin [(3*S*,3′*R*)-3,3′-dihydroxy-β,β-caroten-4-one] to astaxanthin (Choi et al. 2005).

Various genes encoding β-carotene 3,3′-hydroxylase, which are responsible for the conversion from β-carotene to zeaxanthin via β-cryptoxanthin, have been isolated from eubacteria and cyanobacteria, and functionally confirmed by complementation analysis using a recombinant *E. coli*. The *crtZ*-type of genes comprise the predominant group, including those derived from α-*Proteobacteria* such as *Paracoccus* sp. (Misawa et al. 1995) and *Brevundimonas* sp. (Nishida et al. 2005), and from γ-*Proteobacteria* *Pantoea* sp. (Misawa et al. 1990). A *crtZ* homolog has recently been isolated from the unique marine bacterium, strain P99-3 (previously called as *Flavobacterium* sp.) (Yokoyama and Miki 1995a) belonging to the *Cytophaga–Flavobacterium–Bacterioides* complex, which produced myxol (a γ-carotene derivative), although no functional identification has yet been carried out (Teramoto et al. 2003). A *crtR*-type of gene has also been found in the cyanobacterium, *Synechocystis* sp. PCC6803, and functionally assigned (Masamoto et al. 1998). The gene encoding P450 (CYP175A1), the heme enzyme, has been isolated from *Thermus thermophilus* HB27 and shown to have the same function as the above-mentioned genes encoding nonheme β-carotene hydroxylase (Blasco et al. 2004).

We reveal in this article that the CrtZ homolog from marine bacterial strain P99-3 represents β-carotene 3,3′-hydroxylase activity, and compare the catalytic efficiency of the CrtZ enzymes from *Brevundimonas* sp. SD212, *Paracoccus* sp. PC1, *Paracoccus* sp. N81106, *Pantoea ananatis* (formerly known as *Erwinia uredovora* 20D3), marine bacterial strain P99-3, and CYP175A1 from *T.*

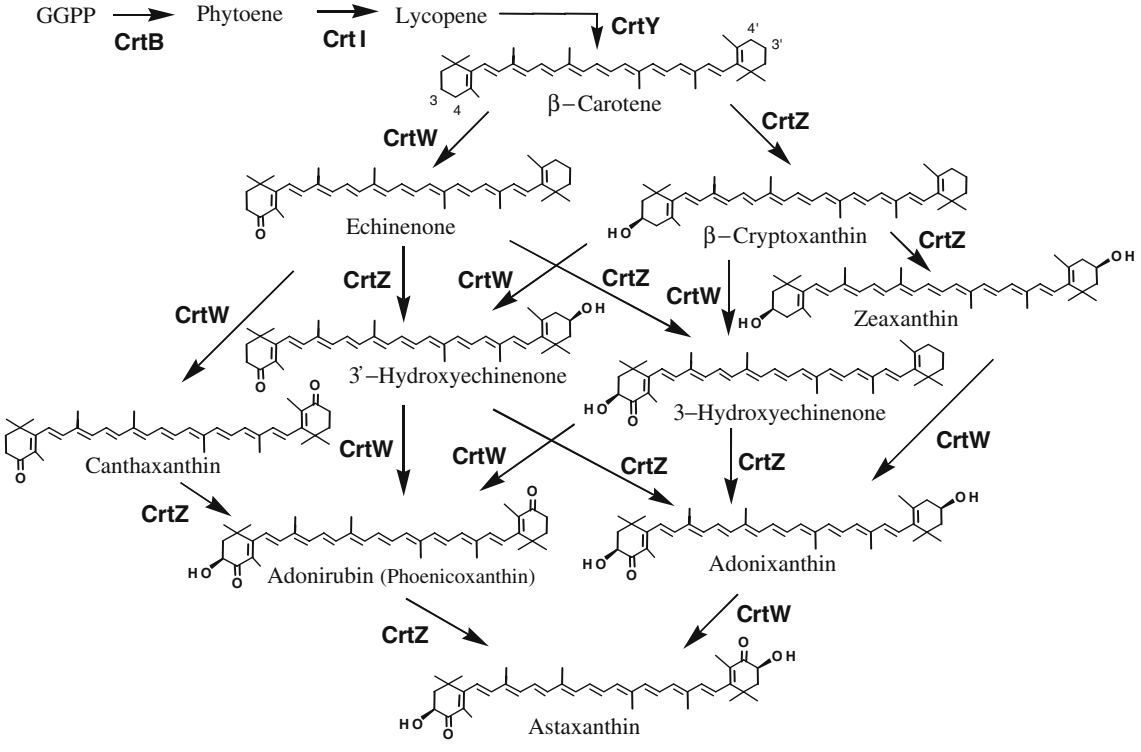

Fig. 1 Astaxanthin biosynthetic pathway in astaxanthin-producing bacteria and the catalytic functions of CrtZ and CrtW. Astaxanthin is synthesized by way of many dicyclic-ketocarotenoid intermediates

Table 1 Oligonucleotides used for amplifying the *crtZ*, *crtZ* homolog and CYP175A1 genes

Constructed plasmid	Primer
pUCBre-Z	F: 5'-TAC GAA TTC GAT GGC CTG GCT GAC GT-3'
	R: 5'-TAG AGG ATC CTC AGG CGC CGC TGC TGG-3'
pUCPan-Z	F: 5'-TAC GAA TTC GAT GTT GTG GAT TTG GA-3'
	R: 5'-TAG AGG ATC CTT ACT TCC CGG ATG CGG-3'
pUCParaPC1-Z	F: 5'-TAC GAA TTC GAT GAC GCA ATT CCT CA-3'
	R: 5'-TAG AGG ATC CTC ACG ACG GAC GCT CGT-3'
pUCParaN8-Z	F: 5'-TAC GAA TTC GAT GAC CAA TTT CCT GA-3'
	R: 5'-TAG AGG ATC CTC ACG TGC GCT CCT GCG-3'
pUCP99-Z	F: 5'-TTC CCG GGG AAT GGA AAT AGT TCT CT-3'
	R: 5'-TGC CAA GCT TTT ATT TGA AAT ACT TGA-3'
pUCHB27-Z	F: 5'-TAC GAA TTC GAT GAA GCG CCT TTC CC-3'
	R: 5'-TAG ATC TAG ATC ACG CCC GCA CCT CCT-3'
pUC19-HB27-CYP175	F: 5'-GGG AAG CTT GAA GTG GGT GGC GGA GCT CC-3'
	R: 5'-GGG GAA TTC CTT AGG CGC CTC AGG GCC TC-3'

F and R respectively indicate the forward and reverse primers
The *Eco*RI, *Bam*HI, *Sma*I, *Hin*dIII or *Xba*I sites are underlined

thermophilus HB27 for their ability to, respectively, produce astaxanthin and zeaxanthin by complementation analysis in recombinant *E. coli* synthesizing canthaxanthin and β-carotene. We also propose the *Brevundimonas* sp. SD212 *crtZ* and *T. thermophilus* HB27 CYP175A1 genes as good respective candidates for the efficient production of astaxanthin and adonirubin [(3*S*)-3-hydroxy-β,β-carotene-4,4′-dione] in a heterologous host.

Materials and methods

Recombinant DNA techniques

The restriction enzymes and DNA ligation kit were respectively purchased from New England BioLabs (Beverly, CA, USA) and Toyobo (Osaka, Japan). Plasmid DNA was prepared with a Miniprep DNA purification kit (Takara, Shiga, Japan). The polymerase chain reaction (PCR) was carried out by an automated thermal cycler (Takara) with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). All recombinant DNA procedures were conducted by the standard molecular biology technique (Sambrook et al. 1989) or as instructed by the suppliers.

Construction of the expression plasmids

The *crtZ* genes of *Brevundimonas* sp. strain SD212 (AB181388), *P.ananatis* (D90087), *Paracoccus* sp. strain PC1 (D58422) and *Paracoccus* sp. strain N81106 (D58420), and the *crtZ* homolog of marine bacterial strain P99-3 (AB097813) were, respectively, amplified by PCR from plasmids p5Bre2-15 (Nishida et al. 2005), pCAR25 (Misawa et al. 1990), pPC17 (Fraser et al. 1997), pAK96K (Misawa et al. 1995), and pBS606 (Teramoto et al. 2003), using the top 10 synthetic oligonucleotide primers described in Table 1. The P450 CYP175A1 gene (AE017222) was isolated from *T. thermophilus* HB27 using a primer pair to construct plasmid pUC19-HB27-CYP175 (Table 1), this being used as the template for PCR amplification with the primer pair for pUCHB27-Z. The PCR products were, respectively, digested with the proper restriction endonucleases, and then inserted into the corresponding sites of vector pUC18 (or pUC8 in the case of the P99-3 *crtZ* homolog) to construct pUCBre-Z (for *Brevundimonas* sp. SD212 *crtZ*), pUCPan-Z (for *P. ananatis crtZ*), pUCParaPC1-Z (for *Paracoccus* sp. PC1 *crtZ*), pUCParaN8-Z (for *Paracoccus* sp. N81106 *crtZ*), pUCP99-Z (for the marine bacterial strain P99-3 *crtZ* homolog), and pUCHB27-Z (for the *T. thermophilus* HB27 CYP175 gene), where ATG of the original start of each *crtZ*, *crtZ* homolog, or CYP175A1 gene was placed next to the *Eco*RI site (pUCBre-Z, pUCPan-Z, pUCParaPC1-Z, pUCParaN8-Z, or pUCHB27-Z), or next to the *Sma*I site (pUCP99-Z) to form the CrtZ, CrtZ homolog, or CYP175A1 protein fused to the beginning of the β-glucosidase (LacZ). The nucleotide sequences of the inserted fragments of the plasmids were confirmed using a DNA sequencing kit (Bigdye terminator cycle sequencing ready reaction kit version 3.1; Applied Biosystems, Foster City, CA, USA) and a 3730 DNA analyzer (Applied Biosystems) according to the manufacturer's instructions.

Cultures of recombinant bacteria

Escherichia coli JM109 (Takara) carrying plasmid pAC-Cantha with the five carotenoid biosysthesis genes, *crtE*, *crtB*, *crtI*, and *crtY* from *P. ananatis* and *crtW* from *Paracoccus* sp. N81106 (Nishida et al. 2005), and plasmid pACCAR16ΔcrtX with *crtE*, *crtB*, *crtI*, and *crtY* from *P. ananatis* (Misawa et al. 1995) were, respectively, used as the hosts for producing astaxanthin and zeaxanthin. A Luria–Bertani medium (4 ml) containing appropriate antibiotics was inoculated with 40 μl of a fully grown

culture of *E. coli* transformants, and incubated at 30 °C while shaking. *E. coli* containing pAC-Cantha or pAC-CAR16ΔcrtX required chloramphenicol (Cm) at a final concentration of 30 μg/ml, and *E. coli* carrying plasmid pUCBre-Z, pUCPan-Z, pUCParaPC1-Z, pUCParaN8-Z, pUCP99-Z, or pUCHB27-Z, as well as plasmid pAC-Cantha or pACCAR16ΔcrtX, each required Cm and ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM after the OD_{600} value of the culture had reached about 0.5. After cultivating for 12–48 h, the cells were harvested by centrifugation at 4 °C and stored at −70 °C.

Analysis of accumulated carotenoids in the recombinant bacteria

HPLC-PDA and HPLC-PDA-APCI-MS analyses of the carotenoids from the *E. coli* transformants were performed as previously described (Choi et al. 2005). The HPLC-PDA analysis was carried out on a TSK ODS-80Ts column (4.6×150 nm, Tosoh). The crude extract was eluted at a rate of 1 ml/min with solvent A (water/methanol, 5:95, *v*/*v*) for 5 min, followed by a linear gradient from solvent A to solvent B (tetrahydrofuran/methanol, 3:7, *v*/*v*) for 5 min, solvent B alone for 8 min, and then back to solvent A. The relative percentage of each carotenoid was determined by comparing the HPLC peak area at 470 nm. The HPLC-PDA-APCI-MS analysis was conducted in a Develosil C30-UG-3 column (1.0 mm i.d.×150 nm, Nomura), with a Develosil C30-UG-S used as a precolumn. The crude extract was eluted at a rate of 0.09 ml/min with solvent A for 15 min, followed by a linear gradient from solvent A to solvent C (methanol/*tert*-butyl methyl ether, 3:7, *v*/*v*) for 100 min, solvent C alone for 20 min, and then back to solvent A. Authentic samples of the carotenoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) or purified from *E. coli* transformants expressing the *crt* genes derived from *Paracoccus* sp. N81106 and *Brevundimonas* sp. SD212 (Choi et al. 2005).

Spectral data for the individual carotenoids

Astaxanthin (1)
: HPLC-PDA, retention time (RT) 5.7 min, λ_{max} 478 nm; HPLC-PDA-APCI-MS, RT 10.1 min, λ_{max} 476 nm, m/z 597 $[M+H]^+$

Adonixanthin (2)
: HPLC-PDA, RT 7.2 min, λ_{max} 464 nm; HPLC-PDA-APCI-MS, RT 13.2 min, λ_{max} 463 nm, m/z 583 $[M+H]^+$

Adonirubin (3)
: HPLC-PDA, RT 8.7 min, λ_{max} 475 nm; HPLC-PDA-APCI-MS, RT 16.1 min, λ_{max} 470 nm, m/z 581 $[M+H]^+$

Zeaxanthin (4)
: HPLC-PDA, RT 9.1 min, λ_{max} 451, 478 nm

3′-Hydroxyechinenone (3′-hydroxy-β,β-carotene-4-one) (5)
: HPLC-PDA, RT 9.7 min, λ_{max} 465 nm; HPLC-PDA-APCI-MS, RT 20.9 min, λ_{max} 470 nm, m/z 567 $[M+H]^+$

Canthaxanthin (6)
: HPLC-PDA, RT 10.2, λ_{max} 478 nm; HPLC-PDA-APCI-MS, RT 25.3 min, λ_{max} 470 nm, m/z 565 $[M+H]^+$

β-Carotene (7)
: HPLC-PDA, RT 14.2 min, λ_{max} 452, 481 nm.

Phylogenetic tree analysis

The amino acid sequences having significant homology to CrtZ of *Brevundimonas* sp. SD212 (AB181388) were retrieved from the GenBank database with the BLAST program (Altschul et al. 1997) and aligned by Clustal W (DDBJ version; http://www.ddbj.nig.ac.jp/search/ex_clustalw-j.html). A phylogenetic tree was constructed by using the Clustal X program (Thompson et al. 1997). The evolutionary distances were computed with the Kimura 2-parameter model (Kimura 1980), and the phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei 1987).

Results

The CrtZ homolog from marine bacterium P99-3 represents dicyclic carotenoid 3,3′-hydroxylase activity

A *crtZ* gene homolog has recently been isolated from a bacterium P99-3, which is able to produce myxol (Teramoto et al. 2003). Thus, the CrtZ homolog was thought to be a γ-carotene β-ring hydroxylase that hydroxylates at C-3 on the β-ring of γ-carotene in myxol biosynthesis. However, there was no direct evidence of its function because myxol could not be detected in the cells of *E. coli* that contained both plasmids carrying the isolated carotenoid biosynthetic gene cluster and carrying the *crtE*, *crtB*, and *crtI* genes for lycopene biosynthesis (pACCART-EIB) (Teramoto et al. 2003). We examined whether the CrtZ homolog shows β-carotene 3,3′-hydroxylase activity, because it has low homology with the known hydroxylases, e.g., CrtZ of *P. ananatis* (39 % similarity) and CrtZ of *Paracoccus* sp. N81106 (32 %) as described in "Materials and methods" section. Consequently, the *E. coli* transformant carrying pAC-Cantha and pUCP99-Z produced astaxanthin and adonixanthin (Fig. 2f), while the transformant carrying pACCAR16ΔcrtX and pUCP99-Z produced zeaxanthin (Fig. 4f). These results clearly indicate that the P99-3 CrtZ homolog exhibits dicyclic carotenoid 3,3′-hydroxylase activity to produce astaxanthin and zeaxanthin, including β-carotene 3,3′-hydroxylase activity to produce the latter carotenoid, although the biosynthetic pathway for these carotenoids does not exist in the P99-3 bacterium.

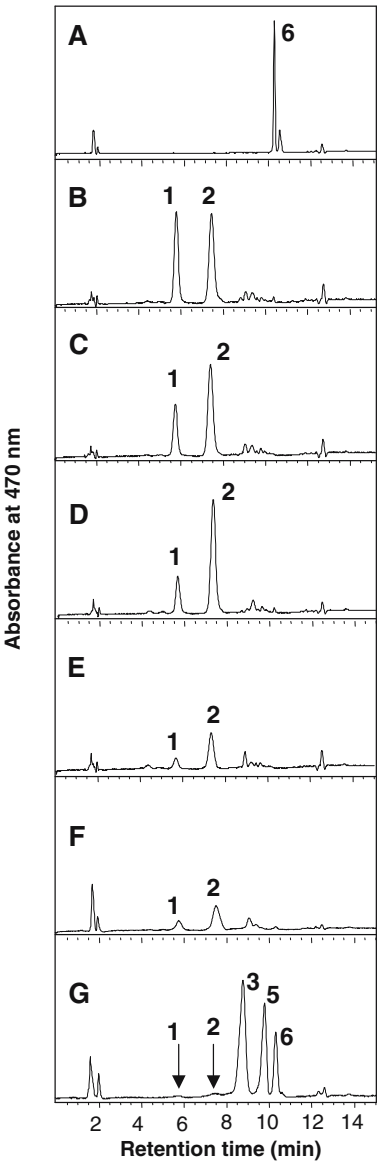

Fig. 2 HPLC traces of the carotenoids accumulated in a 48-h culture of *E. coli* carrying appropriate plasmids for astaxanthin production. **a** pAC-Cantha; **b** pAC-Cantha and pUCBre-Z; **c** pAC-Cantha and pUCPan-Z; **d** pAC-Cantha and pUCParaPC1-Z; **e** pAC-Cantha and pUCParaN8-Z; **f** pAC-Cantha and pUCP99-Z; and **g** pAC-Cantha and pUCHB27-Z. **1** astaxanthin; **2** adonixanthin; **3** adonirubin; **5** 3′-hydroxyechinenone; **6** canthaxanthin

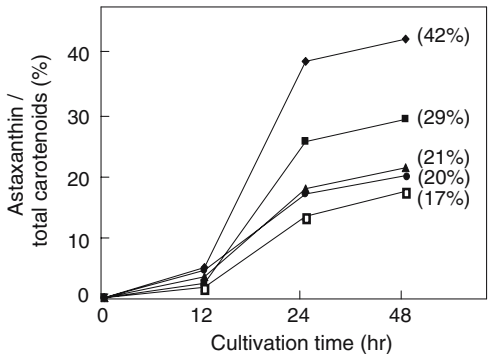

Fig. 3 Astaxanthin production by *E. coli* carrying the *crtZ* genes from *Brevundimonas* sp. strain SD212, *P. ananatis*, *Paracoccus* sp. strain PC1, *Paracoccus* sp. strain N81106, and marine bacterial strain P99-3. The ratios of produced astaxanthin to the total carotenoids produced are shown. ◆ *E. coli* (pAC-Cantha and pUCBre-Z); ▪ *E. coli* (pAC-Cantha and pUCPan-Z); ▲ *E. coli* (pAC-Cantha and pUCParaPC1-Z); ● *E. coli* (pAC-Cantha and pUCParaN8-Z); □ *E. coli* (pAC-Cantha and pUCP99-Z). Three independent experiments were done, and the average values are shown

Comparison of the astaxanthin- and zeaxanthin-producing efficiency with the respective CrtZ and CYP175A1

Astaxanthin production The respective *E. coli* transformants were harvested after 12, 24, or 48 h of cultivation, and the content of astaxanthin and its intermediates in each was measured by HPLC-PDA and HPLC-PDA-APCI-MS. Typical results are shown in Figs. 2 and 3. The *E. coli* control strain carrying only plasmid pAC-Cantha synthesized canthaxanthin (**6**) (Fig. 2a). When plasmids pUCBre-Z, pUCPan-Z, pUCParaPC1-Z, pUCParaN8-Z, pUCP99-Z, and pUCHB27-Z were introduced into *E. coli* carrying pAC-Cantha, the resulting transformants produced astaxanthin (**1**) and adonixanthin (**2**) as the predominant pigments (Fig. 2b–f), while the *E. coli* transformant with pUCHB27-Z accumulated adonirubin (**3**), 3′-hydroxyechinenone (**5**) and canthaxanthin (**6**) (Fig. 2g). Throughout the growth phase, the *E. coli* transformant with pAC-Cantha and pUCBre-Z, which carried the *Brevundimonas* sp. SD212 *crtZ* gene, showed the highest level of the astaxanthin-producing efficiency, compared with those carrying the *crtZ* gene from *P. ananatis*, *Paracoccus* sp. PC1, *Paracoccus* sp. N81106, or marine bacterial strain P99-3 (Fig. 3). The level of astaxanthin appears to be inversely associated with that of adonixanthin because there was no significant difference in the accumulation of the other intermediates except for the case of *T. thermophilus* HB27 CYP175A1. It is interesting to note that the *E. coli* transformant with the CYP175A1 gene could accumulate a high level of adonirubin (57 %) as the main product in addition to 3′-hydroxyechinenone (27 %), canthaxanthin (15 %), and traces of astaxanthin and adonixanthin (Fig. 2g). This indicates that the production of adonirubin would be feasible in a heterologous host by gene combination, as indicated in the "Discussion" section.

Zeaxanthin production We also evaluated the conversion efficiency from β-carotene to zeaxanthin by expressing each *crtZ* or CYP175A1 gene in β-carotene-synthesizing *E. coli* resulting from the presence of plasmid pACCAR16ΔcrtX. All CrtZ and CYP175A1 enzymes clearly converted β-carotene into zeaxanthin (**4**), whereas the *E. coli* control with plasmid pACCAR16ΔcrtX produced β-carotene (**7**) (Fig. 4). No difference was apparent in their substrate preference or conversion efficiency for β-carotene or β-cryptoxanthin (a monohydroxylated β-carotene).

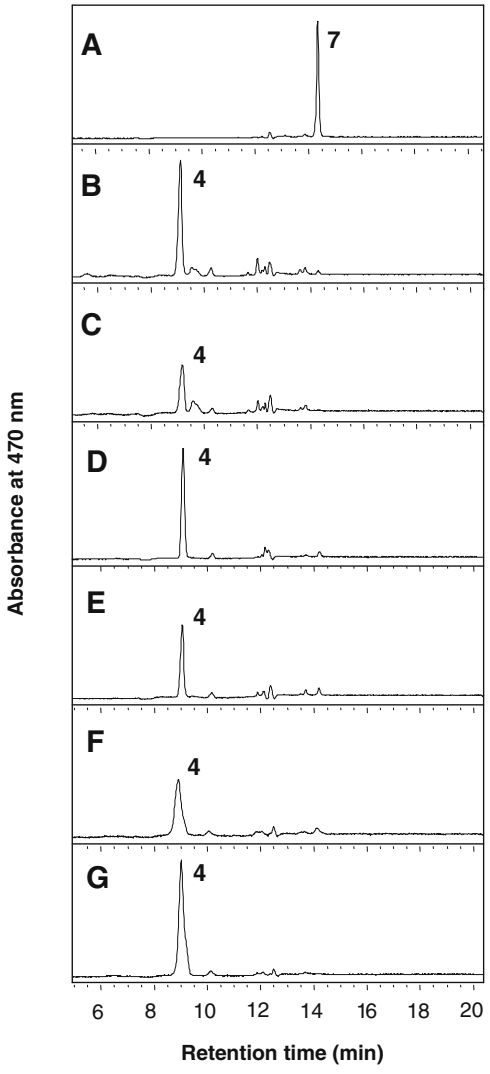

Fig. 4 HPLC traces of the carotenoids accumulated in a 48-h culture of *E. coli* carrying appropriate plasmids for zeaxanthin production. **a** pACCAR16ΔcrtX; **b** pACCAR16ΔcrtX and pUC-Bre-Z; **c** pACCAR16ΔcrtX and pUCPan-Z; **d** pACCAR16ΔcrtX and pUCParaPC1-Z; **e** pACCAR16ΔcrtX and pUCParaN8-Z; **f** pACCAR16ΔcrtX and pUCP99-Z; **g** pAC-Cantha and pUCHB27-Z. **4** zeaxanthin; **7** β-carotene

Discussion

The rRNA sequence of marine bacterium strain P99-3 (MBIC03313; http://www.mbio.jp/mbic/), which is the only myxol-producer among the bacteria reported (Yokoyama and Miki 1995a), suggests that this strain belongs to a new genus in the *Cytophaga–Flavobacterium–Bacterioides* complex. A gene cluster containing carotenoid biosynthetic gene homologs (*crtI*, *crtB*, *crtZ*, *crtY*, *crtD*, and *crtA*) has recently been isolated from this bacterium P99-3, and the *crtY* homolog was identified as the lycopene β-monocyclase gene which metabolized lycopene to γ-carotene (Teramoto et al. 2003), and the *crtD* homolog was identified as the 1-Hydroxy monocyclic carotenoid 3,4-dehydrogenase (Teramoto et al. 2004). Figure 2 clearly indicates that the P99-3 CrtZ homolog exhibits dicyclic carotenoid 3,3′-hydroxylase activity to produce astaxanthin [(3*S*,3′*S*)-3,3′-dihydroxy-β,β-carotene-4,4′-dione] and zeaxanthin. It will be interesting whether CrtZ homolog from P99-3 shows a γ-carotene β-ring hydroxylase that hydroxylates at C-3 on the β-ring of γ-carotene in myxol biosynthesis.

We compared the catalytic capability of various CrtZ enzymes by complementation analysis using the *E. coli* transformants accumulating a carotenoid substrate. The level of astaxanthin accumulated in the *E. coli* cells carrying each *crtZ* appears to be inversely associated with that of adonixanthin [(3*S*,3′*R*)-3,3′-dihydroxy-β,β-caroten-4-one] (Fig. 2). The conversion efficiency of adonirubin [(3*S*)-3-hydroxy-β,β-carotene-4,4′-dione] to astaxanthin by the various CrtZs appears to be similar because no accumulation of adonirubin was detected in the *E. coli* transformants except for that synthesizing P450 CYP175A1 (Fig. 2). The conversion efficiency of adonixanthin to astaxanthin by CrtW is expected to be the same because we used the same *Paracoccus* sp. N81106 *crtW* gene in this complementation study, although this conversion efficiency was in a low level in vitro (Fraser et al. 1998). We, therefore, have to consider that the conversion from adonixanthin to astaxanthin may be the largest rate-limiting step and that the difference in the accumulated levels of astaxanthin and adonixanthin was

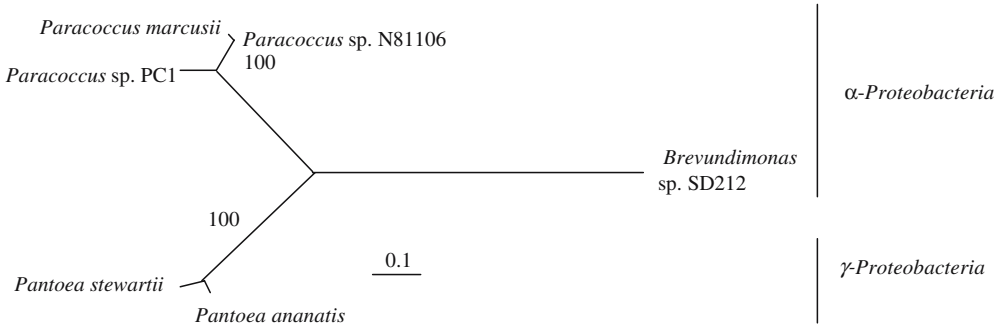

Fig. 5 Neighbor-joining tree based on the CrtZ (β-carotene hydroxylase) sequences between α-*Proteobacteria* and γ-*Proteobacteria*. The *number* shown next to each node indicates the bootstrap value of 100 replicates (Felsenstein 1985), and the *scale bar* indicates a genetic distance of 0.2 (*Knuc*). The DDBJ/EMBL/GenBank accession numbers are as follows: AB181388, *Brevundimonas* sp. strain SD212; D58422, *Paracoccus* sp. strain PC1; Y15112, *Paracoccus marcussi*; D58420, *Paracoccus* sp. strain N81106; D90089, *Pantoea ananatis*; AY166713, *Pantoea stewartii*

caused by the characteristic difference in the respective CrtZ enzymes, and probably their difference in affinity toward the dicyclic-carotenoid intermediates (e.g., echinenone, 3-hydroxyechinenone and 3′-hydroxyechinenone). An in vitro study on the substrate affinity of the CrtZ enzymes for each intermediate in microenvironments should provide more information. On the other hand, the cooperation of the CrtZ and CrtW enzymes in vivo may be very important to produce astaxanthin from β-carotene in a heterologous host. The results obtained in this study (Figs. 2 and 3) indicate the CrtZ enzyme derived from *Brevundimonas* sp. SD212 to be the better candidate for the efficient production of astaxanthin in a heterologous host.

CrtZ proteins derived from α-*Proteobacteria*, γ-*Proteobacteria*, and cyanobacteria have so far been reported. The CrtR enzyme from cyanobacterium *Synechocystis* sp. strain PCC6803, which had the same catalytic function as that of CrtZ (Masamoto et al. 1998), had significant homology with CrtW, rather than with CrtZ (Choi et al. 2005). *P. ananatis*, belonging to γ-*Proteobacteria*, produced zeaxanthin and zeaxanthin-β-diglucoside as the main carotenoids (Misawa et al. 1990). *Brevundimonas* sp. SD212, belonging to α-*Proteobacteria*, produced astaxanthin and could further use it as the substrate to produce (2*R*,3*S*,3′*S*)-2-hydroxyastaxanthin (Yokoyama et al. 1996), and a novel hydroxylase gene (*crtG*) encoding carotenoid 2,2′-β-hydroxylase was recently identified (Nishida et al. 2005). *Paracoccus* sp. N81106 produced astaxanthin and adonixanthin in addition to their glucosides (Yokoyama et al. 1994, 1995). The CrtW enzyme of *Brevundimonas* sp. SD212 produced a high level of astaxanthin due to the more efficient conversion of adonixanthin to astaxanthin than that with *Paracoccus* sp. strains, and was in an independent phylogenetic position from the other genera producing dicyclic ketocarotenoids (Choi et al. 2005). Figure 5 shows the phylogenetic positions of proteins which have a degree of homology with CrtZ from *Brevundimonas* sp. SD212 among *Proteobacteria*, indicating that the phylogenetic position of the CrtZ protein of *Brevundimonas* sp. SD212 is also independent from those of the other CrtZ proteins among *Proteobacteria*.

A new CrtO-type of β-carotene ketolase (4,4′-oxygenase) has recently been reported (Mochimaru et al. 2005; Tao and Cheng 2004). The CrtO enzyme could catalyze β-carotene to canthaxanthin via echinenone, whereas this does not share significant amino acid sequence homology with CrtW. It would be interesting to examine whether the CrtO enzyme can catalyze the reaction from zeaxanthin to astaxanthin in a heterologous host.

Metabolic engineering has been applied to transgenic higher plants to enhance the production of β-carotene (Romer et al. 2000; Shewmaker et al. 1999; Ye et al. 2000). No higher plant sources of free astaxanthin or its intermediates exist, except for the petals of *Adonis* plants. To synthesize astaxanthin from β-carotene, the two genes, *crtW* and *crtZ*, or their corresponding genes, are needed. Metabolic engineering has also been applied to produce astaxanthin in higher plants (Mann et al. 2000; Stalberg et al. 2003). A β-carotene ketolase gene from the green alga, *H. pluvialis* has been expressed in tobacco and *Arabidopsis*, resulting in the formation of such ketocarotenoids as canthaxanthin, adonixanthin, adonirubin, and 4-keto-lutein, rather than astaxanthin. We have also expressed the green-algal ketolase gene (*bkt*) (Kajiwara et al. 1995) in tobacco and confirmed ketocarotenoid formation in tobacco seeds, but no astaxanthin was detected (Choi et al. unpublished data). These studies suggested that green-algal ketolase and endogenous hydroxylase of plants did not work cooperatively. In an attempt to eliminate this problem, Ralley et al. (2004) have expressed the *crtZ* and *crtW* genes simultaneously from *Paracoccus* sp. N81106 as a polyprotein in both tobacco and tomato. However, the most abundant carotenoid accumulated in flower nectary tissue was esterified adonixanthin, rather than astaxanthin. These results indicate that efficient conversion from the ketocarotenoid intermediates to astaxanthin appears to be very important for producing a high level of astaxanthin in a heterologous host. On the other hand, Ravanello et al. (2003) have expressed multiple bacterial carotenoid genes and plant lycopene β-cyclase in canola, and proposed that multiple enzymes from the same species seems to be well work in transgenic plants, although there is an argument for complex interactions between bacterial and plant proteins. We propose from the results of our present and previous study that the genes encoding the CrtZ and CrtW enzymes of *Brevundimonas* sp. SD212 should be introduced into higher plants to examine their efficiency for producing astaxanthin. It will be interesting to see the difference of bacterial and plant CrtZs when coexpressed with the *crtW* gene in higher plants.

This study has for the first time demonstrated that adonirubin production is feasible in a heterologous host by gene combination with the P450 CYP175A1 gene. The accumulation of adonirubin was very low in the marine bacterium, *Paracoccus* sp. N81106, producing astaxanthin (Yokoyama and Miki 1995b). As adonirubin was not detected in the *E. coli* transformants including the *Paracoccus* sp. N81106 *crtZ* gene (Fig. 2), adonirubin may easily be converted into astaxanthin by bacterial CrtZ. Cytochrome P450 monooxygenases are widespread enzymes in such organisms as bacteria, algae, fungi, plants, and animals. P450s in bacteria play an essential role in the biosynthesis of such secondary metabolites as antibiotics, and in the utilization of carbon compounds as an energy source. Blasco et al. (2004) have recently reported that P450 monooxygenase CYP175A1 from *T. thermophilus* HB27 represented a new type of β-carotene 3,3′-hydroxylase, and that the CYP175A1 gene was functionally expressed in *E. coli*. They assumed that the catalytic activity of CYP175A1 was functionally supported by an endogenous redox parter in *E. coli*; e.g., the soluble flavodoxin and flavodoxin reductase (Fpr-Fld) from *E. coli* may work to support cytochrome P450 activity. The *E. coli* transformant carrying a plasmid, which expressed the *crtE*, *crtB*, *crtI*, and *crtY* genes from *P. ananatis*, the β-carotene ketolase gene (*bkt*) from *H. pluvialis*, and the CYP175A1 gene from *T. thermophilus* HB27, accumulated zeaxanthin, 3′-hydroxyechinenone and canthaxanthin, and they con-

cluded that CYP175A1 is a β-carotene-specific enzyme and does not accept canthaxanthin as a substrate. In the present study, the *E. coli* transformant carrying plasmid pAC-Cantha with the *crtE*, *crtB*, *crtI*, and *crtY* genes from *P. ananatis*, the *crtW* gene from *Paracoccus* sp. N81106, and plasmid pUCHB27-Z for CYP75A1 accumulated adonirubin as the main product. In both cases, the used carotenogenic genes differ only in the ketolase genes, *crtW* and *bkt*. It is therefore likely that the difference in the accumulated carotenoids resulted from that of the substrate affinity in the CrtW and BKT enzymes. Adonirubin accumulation in our *E. coli* transformant seems to be due to the action of the *Paracoccus* sp. N81106 CrtW, which can accept both β-rings unsubstituted and 3-hydroxylated as substrates because CYP175A1 cannot utilize a 4,4′-diketolated carotenoid as the substrate. It is likely that the BKT enzyme preferentially accept an unsubstituted β-ionone ring as the substrate; a hydroxyl group at C-3 prevents the formation of a keto group at position C-4 in vivo (Breitenbach et al. 1996), whereas not only CrtW from *Paracoccus* sp. N81106, but also BKT from *H. pluvialis* converted a little of the 3-and 3′-hydroxyechinenone to adonirubin in vitro (Fraser et al. 1998).

Bacterial P450 CYP175A1 appears to be related to a group of P450s in *Arabidopsis* that contains CYP97C1 (an ε-ring hydroxylase) and CYP97A3 (hypothesized as a likely candidate for β-ring hydroxylase) (Blasco et al. 2004). The results of these studies and the absence of homology between CYP175A1 and the known *crtZ* genes in their amino acid sequence could explain its different catalytic mechanism in the astaxanthin biosynthetic pathway in *E. coli*.

Rare and novel carotenoids could be produced in a heterologous host by gene combination or direct enzyme evolution (Lee and Schmidt-Dannert 2002; Umeno et al. 2005). We have revealed in this study that CYP175A1 did not accept adonirubin as the substrate by complementation analysis in *E. coli*. The results obtained indicate that adonirubin production is feasible in a heterologous host by gene combination from different organisms, and this will facilitate the investigation of its beneficial effects on human health.

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