MARINE BIOTECHNOLOGY

Characterization of β-Carotene Ketolases, CrtW, from Marine Bacteria by Complementation Analysis in *Escherichia coli*

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Abstract

A complementation analysis was performed in *Escherichia coli* to evaluate the efficiency of β-carotene ketolases (CrtW) from the marine bacteria *Brevundimonas* sp. SD212, *Paracoccus* sp. PC1 (*Alcaligenes* PC-1), and *Paracoccus* sp. N81106 (*Agrobacterium aurantiacum*), for astaxanthin production. Each *crtW* gene was expressed in *Escherichia coli* synthesizing zeaxanthin due to the presence of plasmid pACCAR25ΔcrtX. Carotenoids that accumulated in the resulting *E. coli* transformants were examined by chromatographic and spectroscopic analyses. The transformant carrying the *Paracoccus* sp. PC1 or N81106 *crtW* gene accumulated high levels of adonixanthin, which is the final astaxanthin precursor for CrtW, and astaxanthin, while the *E. coli* transformant with *crtW* from *Brevundimonas* sp. SD212 did not accumulate any adonixanthin and produced a high level of astaxanthin. These results show efficient conversion by CrtW of *Brevundimonas* sp. SD212 from adonixanthin to astaxanthin, which is a new-found characteristic of a bacterial CrtW enzyme. The phylogenetic positions between CrtW of the two genera, *Brevundimonas* and *Paracoccus*, are distant, although they fall into α-*Proteobacteria.*

Key words: β-carotene ketolase, β-carotene oxygenase — *Brevundimonas* sp., *Paracoccus* sp., astaxanthin — adonixanthin

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Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is the most commonly found carotenoid pigment in marine animal tissues (Miki et al., 1982). Astaxanthin is being industrially exploited as a food dye, and particularly as a feed supplement in poultry farming and aquaculture. For example, sales of astaxanthin as a pigmentation source in salmon aquaculture in the United States amount to about U.S. $200 million per year (Lorenz and Cysewski, 2000). The diverse biological functions of astaxanthin include involvement in the anti-oxidative activity of low-density lipoprotein (Iwamoto et al., 2000), anticancer activity (Tanaka et al., 1994; Chen et al., 1999), enhancement of immune responses (Chen and Park, 2004), and singlet oxygen-quenching activity (Tatsuzawa et al., 2000). Therefore, its use in the pharmaceutical and food industries is expected to increase dramatically in the near future.

Organisms that are capable of synthesizing astaxanthin are limited, but include some marine eubacteria (Yokoyama et al., 1994, 1996), the yeast *Xanthophyllomyces dendrorhous* (renamed from *Phaffia rhodozyma*; Andrewes et al., 1976), and the green algae *Haematococcus pluvialis* (Boussiba and Vonshak, 1991). The most comprehensive study on astaxanthin biosynthesis has been conducted with the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* sp. strain PC-1, which were respectively reclassified as *Paracoccus* sp. strain N81106 (MBIC01143) and *Paracoccus* sp. strain PC1 (MBIC03024) (see *http://www.mbio.jp/mbic/* and Berry et al., 2003). The astaxanthin biosynthetic pathway has been elucidated at enzyme and gene levels by in vitro studies (Fraser et al., 1997) and in vivo studies (Misawa et al., 1995). Astaxanthin can be synthesized from β-carotene (β,β-carotene) with the

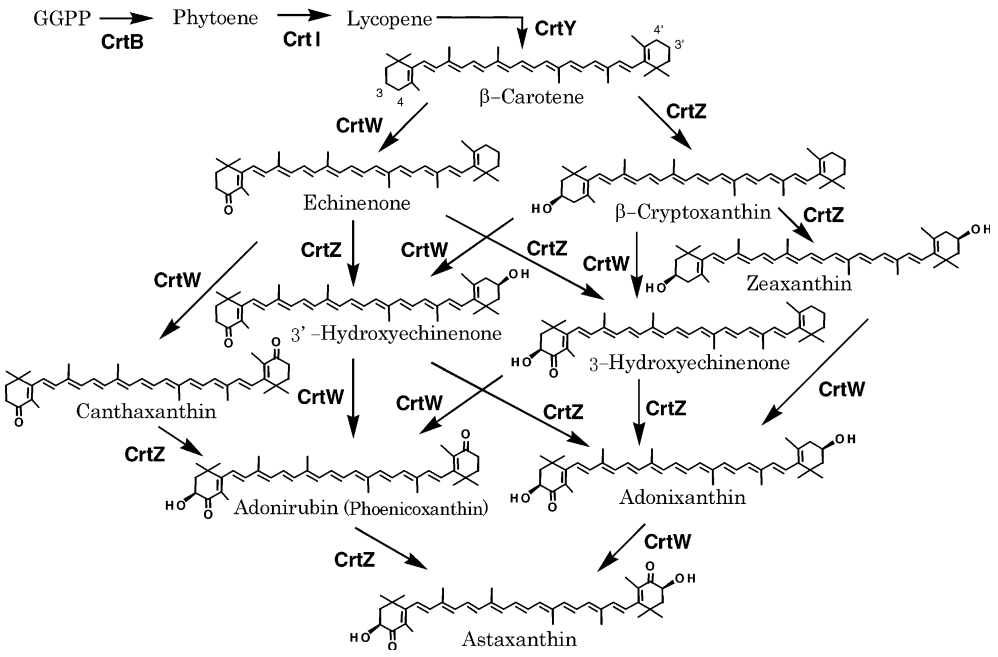

Fig. 1. Astaxanthin biosynthetic pathway in astaxanthin-producing bacteria and the catalytic functions of CrtZ and CrtW.

introduction of keto and hydroxyl moieties at the 4,4′ and 3,3′ positions of the (β-ionone rings by CrtW, β-carotene ketolase (β-carotene oxygenase; β-C4-oxygenase), and CrtZ, β-carotene hydroxylase (β-C3-hydroxylase), and is finally formed from the final astaxanthin precursors, adonixanthin (3,3′ -dihydroxy-β,β-caroten-4-one) and adonirubin (phoenicoxanthin; 3-hydroxy-β,β-carotene-4, 4′-dione), respectively, as shown in Figure 1. The in vitro enzymatic study has shown that for the CrtW enzymes of *Paracoccus* sp. PCI and N81106 and β-carotene ketolase (BKT) of *H. pluvialis*, the conversion of adonixanthin to astaxanthin seems to be an important limiting step (Fraser et al., 1997). The in vivo study by a complementation analysis using *E. coli* has shown that adonixanthin accumulated up to 47% of the total carotenoids in recombinant *E. coli* strains carrying the *crt* genes required for zeaxanthin (3,3′-dihydroxy-β,β-carotene) synthesis, in addition to the *Paracoccus* PC1 or N81106 *crtW* gene (Misawa et al., 1995), and that adonixanthin was also often more predominant than astaxanthin in *Paracoccus* sp. N81106 (Yokoyama et al., 1994) and other marine bacteria (A. Yokoyama and W. Miki, unpublished data). These results suggest that the conversion efficiency with CrtW of adonixanthin to astaxanthin may be an important step, not only for more efficient production in the marine bacteria, but also for artificial production of astaxanthin in transgenic organisms.

The astaxanthin-producing marine bacterium *Brevundimonas* sp., strain SD212, has been isolated from seawater around the Iwo islands in southern Japan (Yokoyama et al., 1996). A *crtW* gene has more recently been isolated from this *Brevundimonas* strain (Y. Nishida et al., unpublished data). The CrtW protein from *Brevundimonas* sp. SD212 possessed 45% identity to that of the *Paracoccus* sp. PC1 or *Paracoccus* sp. N81106, whereas 75% identity existed between the two *Paracoccus* strains. We compare astaxanthin production with the CrtW enzymes from *Brevundimonas* sp. SD212, *Paracoccus* sp. PCI, and *Paracoccus* sp. N81106 by using the *E. coli* complementation system and discuss the phylogenetic positions of the known CrtW proteins, including these 3 β-carotene ketolases.

Materials and Methods

Recombinant DNA Techniques. The restriction enzymes and DNA ligation kit were respectively purchased from New England BioLabs and Toyobo. DNA manipulation was conducted by the standard methods (Sambrook et al., 1989) or as instructed by the suppliers. Plasmid DNA was prepared with the Miniprep DNA Purification Kit (Takara). The polymerase chain reaction (PCR) was carried out by an automated thermal cycler (Techne) with pfu turbo DNA polymerase (Stratagene).

Construction of Expression Plasmids. The *crtW* genes of *Brevundimonas* sp. SD212, *Paracoccus* sp. PC1, and *Paracoccus* sp. N81106 were respectively amplified by PCR from plasmids p5Bre2-15, which contained a 12-kb carotenogenic fragment derived from *Brevundimonas* sp. SD212 genomic DNA (DDBJ/EMBL/GenBank accession number.

Table 1. Oligonucleotides Used for Amplifying *crtW* Genes encoding β-Carotene Ketolases

Constructed plasmid	*Primer*[a]
pUCBre-W	F: 5′-TAC GAA TTC GAT GAG CGC CGC CGT CG-3′
	R: 5′-TAG AGG ATC CTC AAG ACT CGC CGC GCC ACA A-3′
pUCParaPC1-W	F: 5′-TAC GAA TTC GAT GTC CGG ACG GAA GC-3′
	R: 5′-TAG AGG ATC CTC ATG CGC GGC CTC CGG-3′
pUCParaN8-W	F: 5′-TAC GAA TTC GAT GAG CGC ACA TGC CC-3′
	R: 5′-TAG AGG ATC CTC ATG CGG TGT CCC CCT-3′

[a]F and R respectively indicate forward and reverse primers. The *Eco*RI and *Bam*HI sites are underlined.

AB181388), pPC17 (Misawa et al., 1995), and pAK96K (Misawa et al., 1995), using the 6 synthetic oligonucleotide primers described in Table 1. The PCR products were digested with *Eco*RI and *Bam*HI, and then inserted into the corresponding sites of pUC18 (Toyobo) to respectively construct pUCBre-W (for *Brevundimonas* sp. SD212 *crtW*), pUCParaPCl-W (for *Paracoccus* sp. PC1 *crtW*), and pUCParaN8-W (for *Paracoccus* sp. N81106 *crtW*), where ATG for the original starts of the respective *crtW* genes were placed next to the *Eco*RI site (underlined) to form the CrtW proteins fused with the additional 7-amino-acid terminus of β-glucosidase (LacZ) as follows (start codon of the *crtW* gene):

-ATG ACC ATG ATT *ACG AAT TCG* **ATG-**
-Met Thr Met Ile Thr Asn Ser **Met-**

The nucleotide sequences of the inserted fragments of the 3 plasmids were confirmed using a DNA sequencing kit (Big dye terminator cycle sequencing ready reaction kit version 2, PerkinElmer) and a model 3700 DNA sequencer (PerkinElmer) according to the manufacturer's instructions.

Cultures of E. coli. *E. coli* JM109 (Sambrook et al., 1989) carrying plasmid pACCAR25ΔcrtX that contained the 5 carotenoid biosysthesis genes, *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ*, from *Pantoea ananatis* (D90087; renamed from *Erwinia uredovora*; Misawa et al., 1995) was used as the host for producing astaxanthin. A Luria-Bertani medium (4 ml; Sambrook et al., 1989) 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 pACCAR25ΔcrtX required chloramphenicol (Cm) at a final concentration of 30 μg/ml, and *E. coli* carrying plasmid pACCAR25ΔcrtX, as well as pUCBre-W, pUCParaPC1-W, and pUCParaN8-W, each required Cm and ampicillin (100 μg/ml). When OD at 600 nm of the culture had reached about 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After cultivating for 6 to 48 hours, the cells were harvested by centrifugation at 4°C and stored at −70°C.

Analysis of Accumulated Carotenoids in E. coli. Frozen cells were vigorously shaken for 30 minutes after adding a volume of acetone sufficient to extract the carotenoid pigments. The extract was centrifuged at 14,000*g* for 20 minutes and at 4°C to remove the cell debris. The carotenoid pigments were analyzed by high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection (Waters 2695–Waters 2996) or by HPLC–PDA–APCI (atmospheric pressure chemical ionization)–MS (mass spectrometry) with a Shiseido Nano Space SI-ThermoQuest UV600LP-ThermoQuest LCQ Advantage system.

The HPLC-PDA analysis was carried out on a TSK ODS-80Ts column (4.6 × 150 nm, Tosoh) as previously described (Yokoyama and Miki, 1995). The crude extract was eluted at a rate of 1 ml/min with solvent A (water-methanol, 5:95, v/v) for 5 minutes, followed by a linear gradient from solvent A to solvent B (tetrahydrofuran-methanol, 3:7, v/v) for 5 minutes, solvent B alone for 8 minutes, 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 minutes, followed by a linear gradient from solvent A to solvent C (methanol–*tert*-butyl methyl ether, 3:7, v/v) for 100 minutes, solvent C alone for 20 minutes, and then back to solvent A. Mass spectra were monitored in the mass range of *m/z* 200–1200 by the LCQ Advantage instrument. The capillary temperature was set to 150°C, the APCI vaporizer temperature was held at 400°C, the capillary voltage was optimized to 23 V, and the sheath nitrogen gas flow was set to 28 (arbitrary units).

Authentic samples of carotenoids were purchased from Sigma or purified from the *E. coli* transformants expressing the *crt* genes derived from *P. ananatis* and *Paracoccus* sp. N81106 (Misawa et al., 1995).

Spectral Data for the Individual Carotenoids. Astaxanthin (**1**): HPLC-PDA, retention time (RT) 5.8 minutes, λmax 473 nm; HPLC-PDA-APCI-MS, RT 13.3 minutes, λmax 476 nm, *m/z* 597 $[M+H]^+$.

Adonixanthin (**2**): HPLC-PDA, RT 7.6 minutes, λmax 462 nm; HPLC-PDA-APCI-MS: RT 17.5 minutes, λmax 463 nm, *m/z* 583 $[M+H]^+$.

Adonirubin (**3**): HPLC-PDA, RT 8.1 minutes, λmax 472 nm; HPLC-PDA-APCI-MS, RT 20.9 minutes, λmax 470 nm, *m/z* 581$[M+H]^+$.

Zeaxanthin (**4**): HPLC-PDA, RT 8.5 minutes, λmax 450, 479 nm; HPLC-PDA-APCI-MS, RT 25.6 minutes, λmax 450, 476 nm, *m/z* 569 $[M+H]^+$.

3-Hydroxyechinenone, 3-hydroxy-β,β-caroten-4-one (**5**): HPLC-PDA, RT 11.5 minutes, λmax 463 nm; HPLC-PDA-APCI-MS, RT 62.1 minutes, λmax 464 nm, *m/z* 567 $[M+H]^+$.

Lycopene, Ψ,Ψ-carotene (**6**): HPLC-PDA, RT 13.4 minutes, λmax 446, 473, 505 nm; HPLC-PDA-APCI-MS, RT 90.3 minutes, λmax 444, 471, 501 nm, *m/z* 537 $[M+H]^+$.

Phylogenetic Tree Analysis. The amino acid sequences having significant homology to CrtW 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).

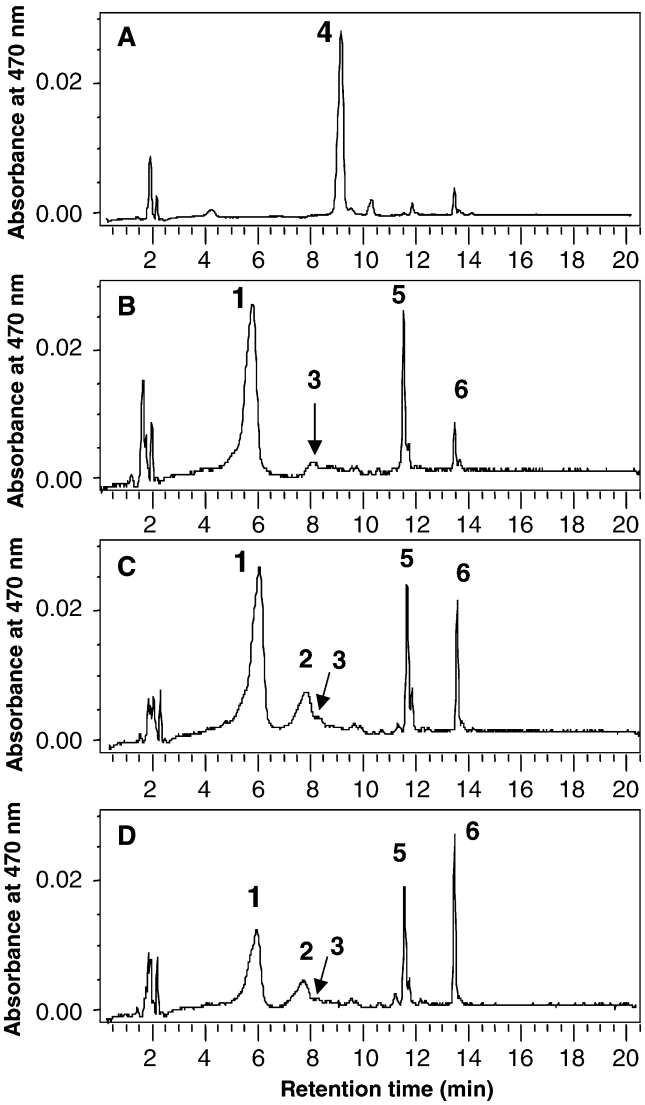

Fig. 2. HPLC traces of the carotenoids accumulated in a 48-hour culture of *E. coli* carrying appropriate plasmids: **A**, pACCAR25ΔcrtX; **B**, pACCAR25ΔcrtX and pUCBre-W; **C**, pACCAR25ΔcrtX and pUCParaPCl-W; and **D**, pAC CAR25ΔcrtX and pUCParaN8-W. **1**, astaxanthin; **2**, adonixanthin; **3**, adonirubin; **4**, zeaxanthin; **5**, 3-hydroxyechinenone; **6**, lycopene.

Results and Discussion

Identification of Accumulated Carotenids in E. coli. Plasmids pUCBre-W, which contained *crtW* from *Brevundimonas* sp. SD212 (AB181388), pUC-ParaPCl-W, which contained *crtW* from *Paracoccus* sp. PC1 (D58422), and pUCParaN8-W, which contained *crtW* from *Paracoccus* sp. N81106 (D58420), were constructed as shown in the ''Materials and methods'' section. The expression levels of the individual *crtW* genes in these plasmids were expected to be identical, as they utilized the same transcription and translation signals derived from vector pUC18, and the codon usage and GC content of the 3 *crtW* genes were similar (GC content: 70% SD212 *crtW*; 64% PC1 *crtW*; 65% N81106 *crtW*). The *E. coli* control strain carrying pACCAR25ΔcrtX synthesized zeaxanthin (**4**) after a 48-hour cultivation (Figure 2, A). When plasmids pUCBre-W, pUC-ParaPCl-W, and pUCParaN8-W were introduced into this *E. coli* strain, the resulting transformants produced astaxanthin (**1**) as the predominant pigment after a 48-hour cultivation (Figures 2, B, C and D). Adonirubin (phoenicoxanthin, **3**), 3-hydroxyechine-

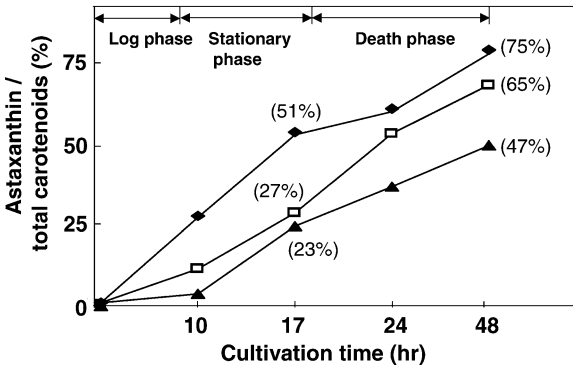

Fig. 3. Astaxanthin production by *E. coli* carrying the *crtW* genes from *Brevundimonas sp.* SD212, *Paracoccus* sp. PC1, and *Paracoccus* sp. N81106. The ratios of produced astaxanthin to the total carotenoids produced are shown. Symbols: ♦, *E. coli* (pACCAR25ΔcrtX and pUCBre-W); □, *E. coli* (pACCAR25ΔcrtX and pUCParaPC1-W); ▲, *E. coli* (pACCAR25ΔcrtX and pUCParaN8-W).

none (**5**), and lycopene (**6**) were detected in *E. coli* (pACCAR25ΔcrtX and pUCBre-W) as the intermediates for astaxanthin synthesis (Figure 2, B). In contrast, *E. coli* (pACCAR25ΔcrtX and pUCParaPC1-W) and *E. coli* (pACCAR25ΔcrtX and pUCParaN8-W) accumulated high levels of adonixanthin (**2**), in addition to the carotenoids detected in *E. coli* (pACCAR25ΔcrtX and pUCBre-W) (Figure 2, C and D). Except for adonixanthin, the levels of the astaxanthin intermediates were similar among the *E. coli* transformants containing pACCAR25ΔcrtX, as well as the plasmids for expression of the *crtW* genes (Figures 2, B, C and D). The production level of total carotenoids in these *E. coli* transformants was approximately 0.25 mg/g dry weight.

Comparison of Astaxanthin Production Efficiency of Respective CrtWs. The respective *E. coli* transformants that expressed the *crtW* genes from *Brevundimonas* sp. SD212, *Paracoccus* sp. PC1, and *Paracoccus* sp. N81106 were harvested after 10, 17, 24, or 48 hours of cultivation, and the content of astaxanthin and its intermediates of each were measured by HPLC-PDA to compare their astaxanthin-producing ability. A result is shown in Figure 3. Throughout the growth phase, *E. coli* (pACCAR25ΔcrtX and pUCBre-W), which carried the *Brevundimonas* sp. SD212 *crtW* gene, showed the highest level of the astaxanthin production efficiency, compared with that by *E. coli* carrying *Paracoccus* sp. PC1 and *Paracoccus* sp. N81106*crtW* (Figure 3). In particular, there was a large difference in their astaxanthin production efficiency up to stationary phase (0–19 hours). These results indicate

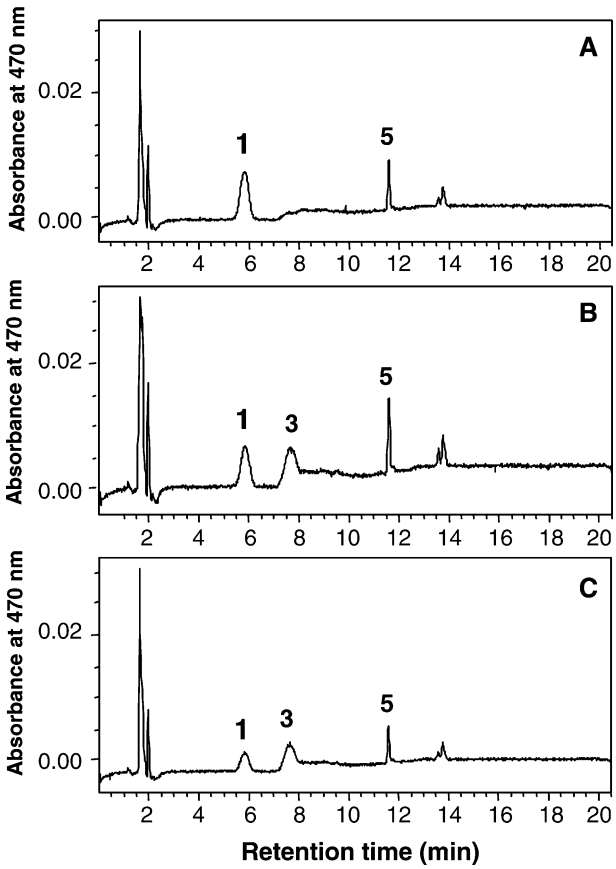

Fig. 4. HPLC traces of the carotenoids accumulated in a 6-hour culture of *E. coli* carrying appropriate plasmids: **A**, pACCAR25ΔcrtX and pUCBre-W; **B**, pACCAR25ΔcrtX and pUCParaPC1-W; **C**, pACCAR25ΔcrtX and pUCParaN8-W. **1**, astaxanthin; **2**, adonixanthin; **5**, 3-hydroxyechinenone.

that the CrtW enzyme derived from *Brevundimonas* sp. SD212 was likely to have been the most efficient for astaxanthin production. Astaxanthin was formed from the final precursors, adonirubin and adonixanthin, by CrtZ and CrtW, respectively, as shown in Figure 1. High levels of adonixanthin were also detected in *E. coli* (pACCAR25ΔcrtX and pUCParaPC1-W) and *E. coli* (pACCAR25ΔcrtX and pUCParaN8-W), which included *crtW* from *Paracoccus* sp., whereas no adonixanthin was detected in *E. coli* (pACCAR25ΔcrtX and pUCBre-W) (Figure 2). We also analyzed the accumulated carotenoids in the *E. coli* transformants after a 6-hour cultivation as shown in Figure 4. No adonixanthin was detected in *E. coli* expressing the *Brevundimonas* sp. SD212 *crtW* gene (Figure 4, A), although a large amount of adonixanthin had accumulated; accordingly, the astaxanthin contents were lower in *E. coli* expressing *crtW* from *Paracoccus* sp. PC1 and *Paracoccus* sp. N81106 (B and C). The level of astaxanthin seems to have been reversely associated with that of adoni-

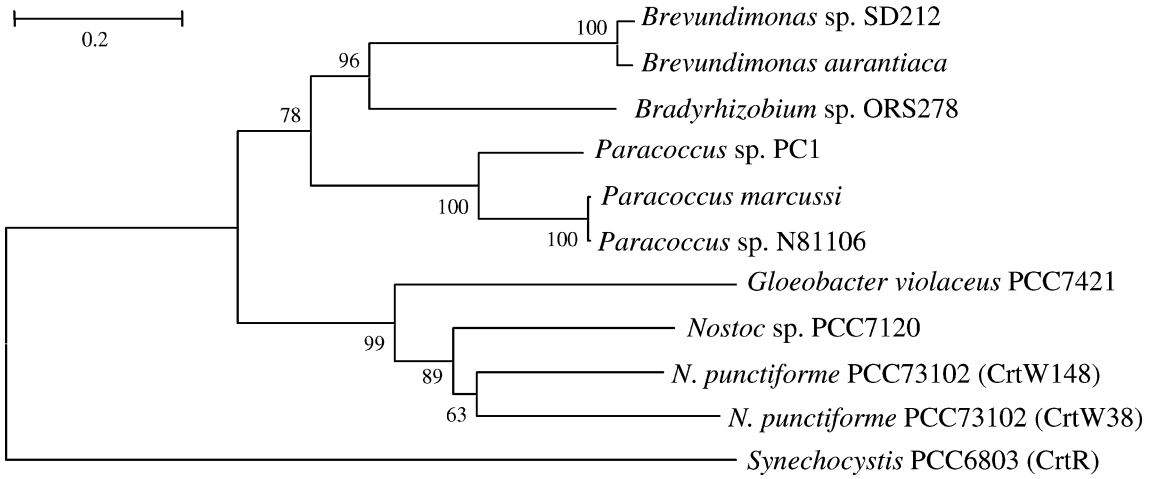

Fig. 5. Neighbor-joining tree based on the CrtW (β-carotene ketolase) sequences. The phylogenetic position of CrtR (β-carotene hydroxylase) from *Synechocystis* sp. PCC6803 is also shown. The number shown next to each node indicates the bootstrap value of 100 replicates (Felsenstein, 1985), and the scale bar indicates a genetic distance of 0.2 (*Knuc*). The DDBJ/EMBL/GenBank accession numbers are as follows: AB181388, *Brevundimonas* sp. SD212; AY166610, *Brevundimonas aurantiaca*; AF218415, *Bradyrihizobium* sp. ORS278; AP006574, *Gloeobacter vilaceus* PCC7421; AP003592, *Nostoc* sp. PCC7120; D58422, *Paracoccus* sp. PC1; Y15112, *Paracoccus marcussi*; D58420, *Paracoccus* sp. N81106; D90906, *Synechocystis* sp. PCC6803. The nucleotide sequences of the two genes *crtW148* and *crtW38*, which were derived from *Nostoc punctiforme* PCC73102, were obtained from *www.jgi.doe.gov*

xanthin. This is the first report showing the introduction of a bacterial CrtW capable of efficiently converting adonixanthin to astaxanthin.

Phylogenetic Positions of Respective CrtWs. Figure 5 shows the phylogenetic positions of proteins that have a degree of homology with CrtW from *Brevundimonas* sp. SD212. The phylogenetic positions of the CrtW proteins from *Brevundimonas* sp. and *Paracoccus* sp. were the most distant among the known CrtWs derived from α-*Proteobacteria*. The CrtW enzyme from the photosynthetic bacterium *Bradyrhizobium* sp. ORS278 (α-*Proteobacteria*), which is responsible for the conversion from β-carotene to canthaxanthin (Hannibal et al., 2000), was relatively close to *Brevundimonas* CrtW. Several cyanobacteria, *Gloeobacter vilaceus* PCC7421, *Nostoc* sp. PCC7120, and *Nostoc punctiforme* PCC73102, whose genome sequences are available, possessed proteins homologous to CrtWs from α-*Proteobacteria*, and formed a group independent from the others. Steiger and Sandmann (2004) showed that *N. punctiforme* PCC73102 possessed 2 *crtW* genes, *crtW148* and *crtW38*, and that they both mediated the conversion from β-carotene to canthaxanthin. It was also reported that CrtW148 protein was able to introduce 4-keto groups into zeaxanthin, yielding astaxanthin, whereas CrtW38 was unable to catalyze this reaction (Steiger and Sandmann, 2004). However, it is difficult to explain the difference in their catalytic functions from their phylogenetic positions. It should be interesting to examine whether CrtW from *G. violaceus* PCC7421 or *Nostoc* sp. PCC7120 can catalyze the reaction from zeaxanthin to astaxanthin, or not. The CrtR enzyme from cyanobacterium *Synechocystis* PCC6803, which had the same catalytic function as that of CrtZ, β-C3-hydroxylase (Masamoto et al., 1998), had significant homology with CrtW, rather than with CrtZ (Figure 5).

Possibility for Use of Brevundimonas crtW. Several attempts at metabolic engineering have been made to produce astaxanthin by using *E. coli* (Misawa et al., 1995; Wang et al., 1999) and the food yeast *Candida utilis* (Miura et al., 1998), and the higher plants tobacco and tomato (Mann et al., 2000; Ralley et al., 2004). The *crtW* gene of *Paracoccus* sp. N81106 (MBIC01143) was used in many of these studies. It seems likely that the level of astaxanthin production can be improved by using the *crtW* gene of *Brevundimonas* sp. SD212 instead of the foregoing gene. Metabolic engineering has also been applied to transgenic higher plants for enhancing the production of β-carotene; for example, transgenic tomato plants with the phytoene desaturase gene (*crtI*) from *Pantoea ananatis* (*Erwinia uredovora*) produced 3-fold the amount of β-carotene in the fruits (Romer et al., 2000). Overexpression of the phytoene synthase gene (*crtB*) of *P. ananatis* in transgenic rape seeds increased the carotenoid content of mature seed by up to 50-fold (Shewmaker et al., 1999). Rice grains are devoid of carotenoids, but the accumulation of β-carotene in the rice endosperm has proved successful by the expression of the plant phytoene synthase

(*psy*) and *P. ananatis* phytoene desaturase (*crtI*) genes (Ye et al., 2000). In order to synthesize astaxanthin from β-carotene, the 2 genes *crtW* and *crtZ* are needed. The *crtZ* gene of *P. ananatis* has been functionally expressed in tobacco (Gotz et al., 2002). The *Brevundimonas crtW* gene should be a promising candidate for expression in the higher plants to produce a high level of astaxanthin, as in the case of the *P. ananatis crtZ* gene.

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