

## Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*

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### Abstract

We succeeded in isolating a novel cDNA involved in astaxanthin biosynthesis from the green alga *Haematococcus pluvialis*, by an expression cloning method using an *Escherichia coli* transformant as a host that synthesizes  $\beta$ -carotene due to the *Erwinia uredovora* carotenoid biosynthesis genes. The cloned cDNA was shown to encode a novel enzyme,  $\beta$ -carotene ketolase ( $\beta$ -carotene oxygenase), which converted  $\beta$ -carotene to canthaxanthin via echinenone, through chromatographic and spectroscopic analysis of the pigments accumulated in an *E. coli* transformant. This indicates that the encoded enzyme is responsible for the direct conversion of methylene to keto groups, a mechanism that usually requires two different enzymatic reactions proceeding via a hydroxy intermediate. Northern blot analysis showed that the mRNA was synthesized only in the cyst cells of *H. pluvialis*. *E. coli* carrying the *H. pluvialis* cDNA and the *E. uredovora* genes required for zeaxanthin biosynthesis was also found to synthesize astaxanthin (3S, 3'S), which was identified after purification by a variety of spectroscopic methods.

### Introduction

Carotenoids are ubiquitous pigments in nature, which usually consist of 40 carbon atoms, and play diverse roles in many organisms. In plants and other photosynthetic organisms, carotenoids protect the tissues or cells against deleterious ef-

fects caused by photosensitized oxidations in addition to their function as accessory pigments in light harvesting [6, 15]. Astaxanthin (3,3'-dihydroxy- $\beta$ ,  $\beta$ -carotene-4, 4'-dione) is a widely distributed carotenoid in the animal kingdom, especially in marine animals; the red or pinkish color in salmonoids, crustaceans and birds is attributed

to astaxanthin [11, 15]. Recently, its diverse biological functions have been revealed as a vitamin A precursor [16], a scavenger and/or quencher against free radicals and active oxygen species [18], an anti-cancer agent [15, 31], and an enhancer of the immune response [12]. Since animals cannot synthesize carotenoids *de novo*, carotenoids that are biosynthesized by microorganisms or plants must be transferred to animals as ingredients in feed or food [15, 19]. Astaxanthin has been developed for industrial use as a supplement for pigmentation of cultured fish and shellfish [11, 17].

Some microorganisms have been shown to synthesize astaxanthin [2, 10, 33]. A unicellular green alga, *Haematococcus pluvialis*, and a red yeast, *Phaffia rhodozyma*, have been reported to be promising microorganisms as producers of astaxanthin [4, 11, 25]. Some researchers have tried to clone genes mediating astaxanthin biosynthesis from these microorganisms, since this pigment possesses such biologically and commercially important characteristics. However, no relevant paper has been published so far. This coincides with the fact that only very few reports have been published with respect to the genes or enzymes involved in the biosynthesis of oxygenated carotenoids, called xanthophylls [3, 5, 22, 29]. Presumably such a lack of progress is due to the fact that most of these enzymes are membrane-integrated, and readily lose activity on solubilization, thus hampering their purification and subsequent cloning of the genes encoding them.

In *H. pluvialis*, green motile vegetative cells change to thick-walled red imotile cyst cells that accumulate astaxanthin, under unfavorable culture conditions such as nitrogen deficiency and high irradiation [4, 13, 14]. The cyst cells accumulate the carotenoid in the cytoplasm [10, 14]. In this paper, we have isolated a novel cDNA involved in astaxanthin biosynthesis from the *H. pluvialis* cyst cells, by an expression cloning method using an *Escherichia coli* transformant as a host that synthesizes  $\beta$ -carotene due to the introduction of the *Erwinia uredovora* carotenoid biosynthesis genes [22, 30]. We have further determined the function of the cloned cDNA

through chromatographic and spectroscopic analysis of the pigments accumulated in several *E. coli* transformants carrying the cDNA and the *E. uredovora* carotenogenic genes that are required for  $\beta$ -carotene or zeaxanthin biosynthesis. The cloned cDNA has been shown to encode a novel enzyme, which is responsible for the conversion of  $\beta$ -carotene to canthaxanthin via echinenone. Moreover, we have succeeded in synthesizing astaxanthin in *E. coli* carrying the cDNA from *H. pluvialis* and the zeaxanthin biosynthesis genes from *E. uredovora*.

## Materials and methods

### *Strains and growth conditions*

*Haematococcus pluvialis* Flotow NIES-144 was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. A basal medium for this green alga consisted of 0.2 g of yeast extract, 0.4 g of L-asparagine, 1.2 g of  $\text{CH}_3\text{COONa}$ , 0.2 g of  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$ , 0.01 g of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.02 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  per liter (pH 6.8) [13]. *H. pluvialis* was subcultured in the basal medium at 20 °C under conditions of 12 h light (20  $\mu\text{E}/\text{m}^2\text{s}$ , fluorescent light) and 12 h dark for 4 days. To induce cyst formation for astaxanthin biosynthesis from the vegetative cells,  $\text{CH}_3\text{COONa}$  and  $\text{FeSO}_4$  were added to the above culture at a final concentration of 45 mM and 450  $\mu\text{M}$ , respectively, and the light conditions were changed to 125  $\mu\text{E}/\text{m}^2\text{s}$  under continuous illumination [14].

*Escherichia coli* JM101 was used as a host for construction of the cDNA library. *E. coli* DH5 and DH5 $\alpha$  were also used [28].

### *Extraction of mRNAs from H. pluvialis*

Vegetative and cyst cells of *H. pluvialis* were frozen with liquid nitrogen, and ground to fine powders in a mortar and pestle. They were suspended in Isogen-LS (Nippon Gene), which is a reagent for isolating RNA [7] and stored for 5 min. The

suspension was mixed with chloroform and centrifuged at  $12000 \times g$  for 15 min at 4 °C. The aqueous phase was transferred and precipitated with 2-propanol. The pellet consisting of total RNAs was washed with 70% ethanol and dissolved in TE buffer (pH 8.0). Poly(A)-RNAs were isolated from this solution using Oligotex-dT 30 Super (Takara).

#### *Construction of plasmids and cDNA library*

Recombinant DNA techniques were performed using standard methods [28].

The 6.0 kb and 6.5 kb *Asp718 (KpnI)-EcoRI* fragments of pCAR16deIB and pCAR25deIB [22], which contain the *Erwinia uredovora* carotenoid biosynthesis genes, were isolated, filled in with Klenow enzyme, and ligated into the *EcoRV* site of pACYC184 [27]. As a result, plasmids pACCAR16ΔcrtX [20] and pACCAR25ΔcrtX were constructed, which carried the *crtE*, *crtB*, *crtI* and *crtY* genes for β-carotene biosynthesis and the *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* genes for zeaxanthin biosynthesis, respectively, and conferred a yellow pigmentation on *E. coli*.

cDNAs were synthesized from the poly(A)-RNAs that were prepared from the cyst cells of *H. phuvialis* with the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco BRL). The synthesized cDNAs were ligated into the *SalI-NotI* site of vector pSPORT1 (Gibco BRL), and transformed into *E. coli* JM101 (pACCAR16ΔcrtX) which consequently accumulated β-carotene. The transformants were cultured on LB plates including 50 μg/ml ampicillin (Ap), 30 μg/ml chloramphenicol (Cm) and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 20 °C for 3 days.

#### *DNA sequencing*

A cDNA fragment was isolated after digestion with *SalI* and *XbaI*, and ligated into the *SalI-XbaI* site of pBluescriptII KS + or pBluescriptII SK + (Stratagene). For DNA sequencing or deletion

analysis, plasmids containing a nested series of deletions of the cDNA fragment were constructed using a Kilo-sequence Deletion Kit (Takara) according to the supplier's instructions. Double-stranded DNAs were sequenced using a *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems) on an ABI 373A DNA sequencer.

#### *Extraction and purification of carotenoids accumulated in E. coli*

*E. coli* JM101 carrying a *H. phuvialis* cDNA along with pACCAR16ΔcrtX or pACCAR25ΔcrtX was grown to stationary phase in  $2 \times$  YT medium [28] including 28 μg/ml  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 37 μg/ml EDTA, 150 μg/ml Ap, 30 μg/ml Cm and 0.1 mM IPTG at 28 °C. Carotenoid pigments were extracted from the cells with acetone. The acetone extracts were dried *in vacuo*, further extracted with chloroform/methanol (9:1), and concentrated to a small volume. Red pigments obtained were subjected to high-performance liquid chromatography (HPLC); the column (3.9 mm by 300 mm; Nova-pak HR 6 μ C<sub>18</sub> [Waters]) was developed with acetonitrile/methanol/2-propanol (90:6:4) at a flow rate of 1 ml/min, and the absorbance of the eluate recorded with a Waters photodiode array detector 996. Pigments were purified by silica gel (60F<sub>254</sub>) thin-layer chromatography (TLC) developed with chloroform/methanol (15:1 or 50:1), and further purified by Sephadex LH-20 column chromatography developed with methanol or chloroform/methanol (1:1).

#### *Identification of purified carotenoids*

Purified carotenoid pigments were identified by spectroscopic methods. Visible absorption spectra (VIS) were measured with a Beckman DU-65 spectrophotometer. FD-MS spectra were recorded with a JEOL SX-102A mass spectrometer, and 500 MHz <sup>1</sup>H-NMR spectra were obtained in CDCl<sub>3</sub> solution on a JEOL GX-500 spectrometer. A circular dichroism (CD) spectrum was recorded with a Jasco J-720 using di-

ethyl ether/2-propanol/ethanol (5:5:2) as solvent. Synthetic canthaxanthin and astaxanthin were purchased from Carl Roth and Hoffman-La Roche, respectively, and used as reference samples.

## Results

### *Cloning of a cDNA involved in astaxanthin biosynthesis*

Astaxanthin accumulated in the cyst cells of *H. phuvialis* that are induced by addition of acetate and  $\text{Fe}^{2+}$  [14]. Total RNAs (4.1 mg) were isolated from 400 ml of the harvested cells, and ca. 14  $\mu\text{g}$  of poly(A)-RNAs was then purified from 1 mg of the total RNAs. cDNAs were synthesized from 5  $\mu\text{g}$  of the poly(A)-RNAs, and inserted in the *SalI-NotI* site of the *E. coli* expression vector pSPORT1 (Gibco-BRL). A cDNA library corresponding to 40 000 clones was constructed after amplification in *E. coli* DH5. Plasmid DNAs from the cDNA library were introduced into *E. coli* JM101 carrying plasmid pACCAR16 $\Delta$ crtX that produces  $\beta$ -carotene and displays a yellow phenotype. About 40 000 colonies were incubated on LB agar plates containing Ap, Cm and 1 mM IPTG. By inspecting the color of the colonies on the LB agar plates, one colony was found to have changed to an orange phenotype. Plasmid DNA, designated pHP5, was isolated from this orange colony and found to include a 1.7 kb fragment (Fig. 1). This 1.7 kb fragment was isolated after *SalI* and *XbaI* digestion and ligated into the vector pBluescriptII KS+. The resulting plasmid, designated

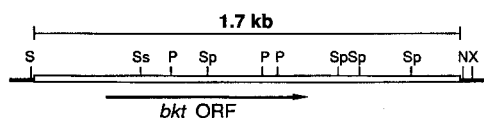


Fig. 1. Structure of plasmids pHP5 and pHP51. In pHP5, a 1.7 kb cDNA fragment is inserted into the *SalI-NotI* site of the *E. coli* expression vector pSPORT1. In pHP51, A 1.7 kb *SalI-XbaI* fragment isolated from pHP5 is inserted into the *SalI-XbaI* site of the vector pBluescriptII KS+. S, *SalI*; Ss, *SspI*; P, *PstI*; Sp, *SphI*; N, *NotI*; X, *XbaI*.

pHP51, was used to retransform *E. coli* (pACCAR16 $\Delta$ crtX), resulting in a yellow to orange color change in phenotype.

### *Sequence analysis*

Plasmid pHP52 was constructed by inserting the 1.7 kb *SalI-XbaI* cDNA fragment into the *SalI-XbaI* site of pBluescriptII SK+. Plasmids pHP51 and pHP52 were used to determine the nucleotide sequence of the 1.7 kb cDNA. The sequence comprised 1662 bp nucleotides and a 15 bp poly(A) tail. The open reading frame (ORF) encoded a polypeptide consisting of 320 amino acids, which showed no amino acid homology to any protein from data bases. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2.

### *Northern blot analysis*

Poly(A)-RNAs (1.5  $\mu\text{g}$ ) were prepared from the vegetative and cyst cells of *H. phuvialis*, and subjected to northern blot analysis using the 1.7 kb *SalI-XbaI* cDNA fragment as a probe (Fig. 3). A signal was detected in the RNA prepared from cyst cells at a position of 1.7 kb, while no signal was detected in the RNA preparation from the vegetative cells. This result shows that transcripts from the cloned gene only accumulate in the cyst cells of *H. phuvialis*.

### *Analysis of carotenoids synthesized in E. coli*

Carotenoid pigments were extracted from the cells of *E. coli* JM101 carrying the two plasmids, pHP51 and pACCAR16 $\Delta$ crtX, and subjected to HPLC analysis (Fig. 4). Pigments extracted from *E. coli* harboring the vector pBluescriptII SK+ and pACCAR16 $\Delta$ crtX were also subjected to HPLC analysis as a control (Fig. 4B). The *E. coli* transformant carrying pHP51 and pACCAR16 $\Delta$ crtX (Fig. 4A) accumulated red pigments 1 and 2 in addition to  $\beta$ -carotene (pig-

CGGGCAACTCAAGAAATCAACAGCTGCAAGCGCCCCAGCCTCA	47
CAGCGCCAAGTGAGCTATCGACGTGGTTGTGAGCGCTCGACGTGGTCCACTGACGGGCCT	107
GTGAGCCTCTGCGCTCCGTCTCTGCCAAATCTCGCGTCGGGGCCTGCCTAAGTCGAAGA	167
ATGCACGTGCGCATCGGCACTAATGGTCGAGCAGAAAGGCAGTGAGGCAGCTGCTTCCAGC	227
M H V A S A L M V E Q K G S E A A A S S	20
CCAGACGTCTTGAGAGCGTGGCGACACAGTATC <u>ATGCCATCCGAGTCGTGACAGC</u> GCA	287
P D V L R A W A T Q Y H M P S E S S D A	40
GCTCGTCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCAG	347
A R P A L K H A Y K P P A S D A K G I T	60
ATGGCGCTGACCATCATGGCACCTGGACCGCAGTGTFTTTACACGCAATATTTCAAATC	407
M A L T I I G T W T A V F L H A I F Q I	80
AGGCTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAG	467
R L P T S M D Q L H W L P V S E A T A Q	100
CTTTTGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTC	527
L L G G S S S L L H I A A V F I V L E F	120
CTGTACTACTGGTCTATTCATCACCACACATGACGCAATGCATGGCACCATAGCTTTGAGG	587
L Y T G L F I T T H D A M H G T I A L R	140
CACAGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGAC	647
H R Q L N D L L G N I C I S L Y A W F D	160
TACAGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAA	707
Y S M L H R K H W E H H N H T G E V G K	180
GACCCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCTGGTTCGCCAGCTTCATGTCC	767
D P D F H K G N P G L V P W F A S F M S	200
AGCTACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGCAGTGGTGATGCAAATG	827
S Y M S L W Q F A R L A W W A V V M Q M	220
CTGGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTGAGCA	887
L G A P M A N L L V F M A A A P I L S A	240
TTCCGCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCA	947
F R L F Y F G T Y L P H K P E P G P A A	260
GGCTCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGT	1007
G S Q V M A W F R A K T S E A S D V M S	280
TTCTTGACATGCTACCACTTTGACCTGCCTGGGAGCACCACAGTGGCCCTTTGCCCCC	1067
F L T C Y H F D L H W E H H R W P F A P	300
TGGTGGCAGCTGCCCCACTGCCCGCCTGTCCGGGCTGGCCTGGTGCCTGCCTGGCA	1127
W W Q L P H C R R L S G R G L V P A L A	320
TGACCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTATGCTACAGGGTGC	1187
*	
TGCGGCCAGTGGCAGCGCAGTGCCTCTCAGCCTGTATGGGGCTACCGCTGTGCCACTGA	1247
GCACTGGGCATGCCACTGAGCACTGGGCGTCTACTGAGCAATGGCGTGTACTGAGCA	1307
ATGGCGTGTACTGACAATGGGCGTCTACTGGGGTCTGGCAGTGGCTAGGATGGAGTT	1367
TGATGCATTAGTACGGTGGCCAAACGTATGTGGATGGTGAAGTGTGAGGGGTTTAG	1427
GCAGCCGGCATTGAGAGGGCTAAGTTATAAATCGCATGTCTCATCGGCACATATCTG	1487
CACACAGCCAGGGAAATCCCTTCGAGAGTGATTATGGGACACTTGTATTGGTTTCGTGCT	1547
ATTGTTTATTACAGCAGCAGTACTTAGTGAGGGTGAAGCAGGGTGGTGAAGTGGAGTG	1607
AGTGAGTATGAACCTGGTCAGCGAGGTGAACAGCCTGTAATGAATGACTCTGTCTAAAAA	1667
AAAAAAAAA	1677

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the 1677 bp cDNA isolated from *Haematococcus pluvialis*. Amino acid sequence encoded by the ORF that starts with the first ATG codon is shown under the DNA sequence. The ORF that starts with ATG at nucleotide position 264 (double-underlined) is also capable of encoding a polypeptide with the enzymatic activity in *Escherichia coli*, as described later.

ment 3) [22]. Pigment 1 was purified and identified as canthaxanthin ( $\beta,\beta$ -carotene-4,4'-dione) (Fig. 7) by its VIS, FD-MS and  $^1\text{H-NMR}$  spec-

tral data. Pigment 2 was also shown to be identical to echinenone ( $\beta,\beta$ -caroten-4-one) by its VIS, FD-MS and the retention time in HPLC. *E. coli*

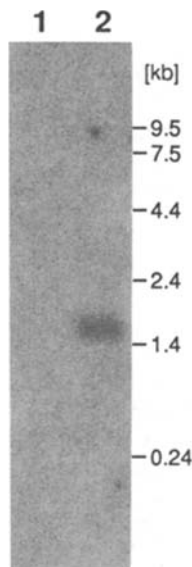


Fig. 3. Northern blot analysis of RNAs prepared from vegetative cells (lane 1) and cyst cells (lane 2) of *H. phuvialis* using the 1.7 kb pHP51 cDNA fragment as a probe. The cyst cells were harvested 6 hrs after the addition of  $\text{CH}_3\text{COONa}$  and  $\text{FeSO}_4$  to the medium.

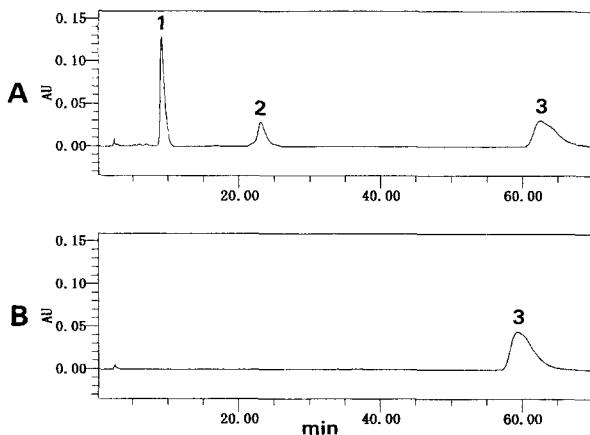


Fig. 4. HPLC analysis of carotenoid pigments extracted from *E. coli* cells carrying plasmids pHP51 and pACCAR16 $\Delta$ crtX. (A) and pBluescriptII SK + and pACCAR16 $\Delta$ crtX (B). Peaks with retention times of 9 min (1), 23 min (2) and 59–62 min (3) were identified as canthaxanthin, echinenone, and  $\beta$ -carotene, respectively.

(pHP51, pACCAR16 $\Delta$ crtX) produced 150  $\mu\text{g}$  of canthaxanthin and 70  $\mu\text{g}$  of echinenone per gram of dry weight.

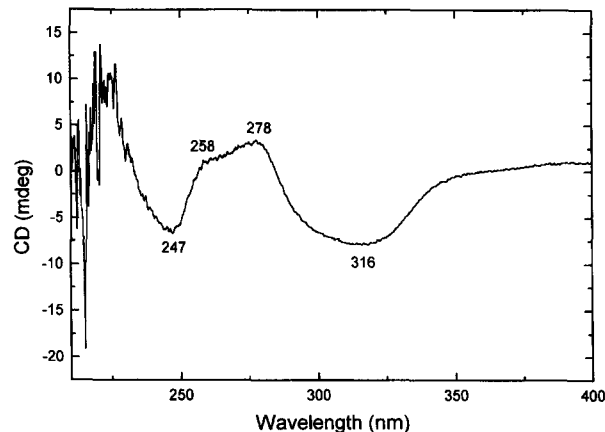


Fig. 5. CD spectrum of astaxanthin purified from *E. coli* cells carrying plasmids pHP51 and pACCAR25 $\Delta$ crtX.

Pigments were also extracted from *E. coli* JM101 cells carrying the two plasmids, pHP51 and pACCAR25 $\Delta$ crtX that contained the *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* genes from *E. uredoovora* to produce zeaxanthin. The carotenoids were separated as pigments (Rf 0.91, red; 0.72, red; 0.54, red; and 0.42, yellow) on silica gel by TLC developed with chloroform/methanol (15:1). The red pigment of Rf 0.72 (30% of the total carotenoids, 120  $\mu\text{g}/\text{g}$  dry weight) was purified and shown to be identical to astaxanthin (3,3'-dihydroxy- $\beta$ , $\beta$ -carotene-4,4'-dione) (Fig. 7) by its VIS, FD-MS and  $^1\text{H-NMR}$  spectral data. The CD spectrum of this pigment (Fig. 5) coincided with that of (3S, 3'S)-astaxanthin reported by Andrewes *et al.* [1], indicating that the stereochemistry of the obtained astaxanthin is 3S, 3'S (Fig. 7). The red pigment of Rf 0.91 and yellow pigment of Rf 0.42 were determined to be canthaxanthin and zeaxanthin [22], respectively. The carotenoid whose Rf value was 0.54 is awaiting verification by  $^1\text{H-NMR}$ .

#### Determination of the minimum catalytic region

In addition to the first initiation codon, there are four in-frame ATG codons near the first ATG (Fig. 6). In order to determine the minimum catalytic region of the cloned cDNA that is functional

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                    50
CGGGGCAACTCAAGAAATCAACAGCTGCAAGCGCGCCCGCCAGCCTCACAGGCCCAAGTGA
                    100
GCTATCGACGTGGTGTGAGCGCTCGACGTGGTCCACTGACGGGCTGTGAGCCTCTGCGC
                    150
CTCGCTCCCTCTGCCAAATCTCGCGTCGGGGCCTGCCTAAGTCGAAGAATGCACGTGCGCAT
pHP511                200
CGGCACTAATGGTCGAGCAGAAAGGCAGTGAGGCAGCTGCTTCCAGCCCAGACGTCTTGA
                    250                264                300
GAGCGTGGGCGACACAGTATCACATGCCATCCGAGTCGTAGACGCAGCTCGTCTGCGCGC
pHP512                350
TAAAGCAGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATGGCGGTGACCA
                    pHP513                400
TCATTGGCACCTGGACCGCAGTGTTTTACAGGCAATATTCAAATCAGGCTACCGACAT
                    450 pHP514
CCATGGACCAGCTTCACTGGTTGCTGTGCCAAGCCACAGCCAGCTTTGGGCGGAA

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Fig. 6. Structure of deletion plasmids pHP511, pHP512, pHP513 and pHP514. In-frame ATG codons are underlined. ATG at nucleotide position 264 is double-underlined.

in *E. coli*, four different deletion plasmids, named pHP511, pHP512, pHP513 and pHP514, were constructed from pHP51 (Fig. 6). These plasmids were introduced into *E. coli* JM101 harboring pACCAR16 $\Delta$ crtX.

Consequently, transformants carrying pHP511 or pHP512 were able to synthesize canthaxanthin from  $\beta$ -carotene, while transformants carrying pHP513 or pHP514 were not able to convert  $\beta$ -carotene to canthaxanthin. This result reveals that a protein starting with the ATG at nucleotide position 264 (Fig. 6) is an enzyme with the minimum length to maintain the catalytic activity in *E. coli*.

## Discussion

*E. coli* (pHP51, pACCAR16 $\Delta$ crtX) synthesized canthaxanthin and echinenone, while *E. coli*

(pBluescriptII SK+, pACCAR16 $\Delta$ crtX), in which only the *H. pluvialis* cDNA was absent, accumulated  $\beta$ -carotene. It is therefore evident that the cloned cDNA encodes  $\beta$ -carotene ketolase ( $\beta$ -carotene oxygenase), which is capable of converting the  $\beta$ -ionone rings of  $\beta$ -carotene into 4(4')-keto- $\beta$ -ionone rings to produce canthaxanthin via echinenone (Fig. 7). Thus, we have designated the gene *bkt*. This indicates that only one gene product is responsible for the direct conversion of methylene to keto groups, a mechanism that has usually been reported to require two different enzymatic reactions proceeding via a hydroxy intermediate [20, 26]. Northern blot analysis has also shown that the *bkt* mRNA of *H. pluvialis* accumulates only in the cyst cells, which are induced under unfavorable culture conditions.

It is surprising that, besides canthaxanthin and zeaxanthin, *E. coli* (pHP51, pACCAR25 $\Delta$ crtX) was able to synthesize astaxanthin. The absolute configuration (3S, 3'S) of astaxanthin was determined by spectroscopic methods, and found to be identical to that of the astaxanthin produced in *H. pluvialis* [10]. The two genes *bkt* and *E. uredo**vara crtZ* are maintained in the *E. coli* transformant, in addition to the  $\beta$ -carotene biosynthesis genes *crtE*, *crtB*, *crtI* and *crtY*. This indicates that only *bkt* and *crtZ* are responsible for the conversion of  $\beta$ -carotene to astaxanthin. The *E. uredo**vara crtZ* gene was shown to encode  $\beta$ -carotene hydroxylase, which catalyzes the reaction from  $\beta$ -carotene to zeaxanthin via  $\beta$ -cryptoxanthin (Fig. 7) [22]. At least one of the

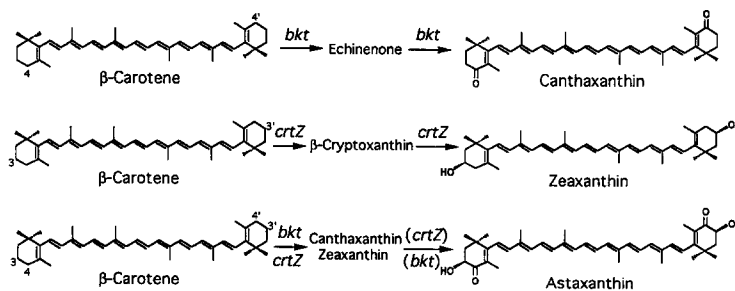


Fig. 7. Chemical structures of various carotenoid pigments and functions of the *H. pluvialis bkt* gene and the *Erwinia uredo**vara crtZ* gene.

gene products *crtZ* or BKT appears to be a bifunctional enzyme and to be able to utilize canthaxanthin or zeaxanthin as a substrate to form astaxanthin (Fig. 7). A more detailed analysis is currently under way.

We have also succeeded in isolating the corresponding genes, *crtW*, from the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* PC-1 [33] by a similar expression cloning method [20]. Figure 8 shows the amino acid sequences of the *bkt* and two *crtW* gene products. Significant homology has been found between the *H. pluvialis* BKT and the marine bacterial *CrtW* (36–37% identities over their entire length). The alignment of these three  $\beta$ -carotene ketolases reveals four highly conserved regions (Fig. 8), indicating that a green alga and marine bacteria share significant sequence homology in an enzyme involved in the late steps of carotenoid biosynthesis. The early carotenogenic enzymes, geranylgeranyl pyrophosphate synthase and phytoene synthase, seem

to be conserved in their amino acid homologies beyond species [3, 24]. In contrast, phytoene desaturases between plants and bacteria are different enzymes with respect to their function and amino acid homology [9, 21, 29]. Cunningham *et al.* have also found that a cyanobacterial lycopene cyclase bears little sequence resemblance to the corresponding bacterial enzymes *CrtY*, although their function is identical [8]. It has therefore been postulated that the late enzymatic steps of carotenoid biosynthesis in plants are distinct from those of bacteria [3]. Our present results offer a view contrary to this assumption.

The alignment of the three  $\beta$ -carotene ketolases also shows that BKT includes an extended non-homologous N-terminal region with 64 amino acid residues (Fig. 8), which may not be needed for the catalytic function. This region is unlikely to be a transit peptide sequence, since astaxanthin accumulates in the cytoplasm of the cyst cells [10, 14]. There are four in-frame ATG codons in

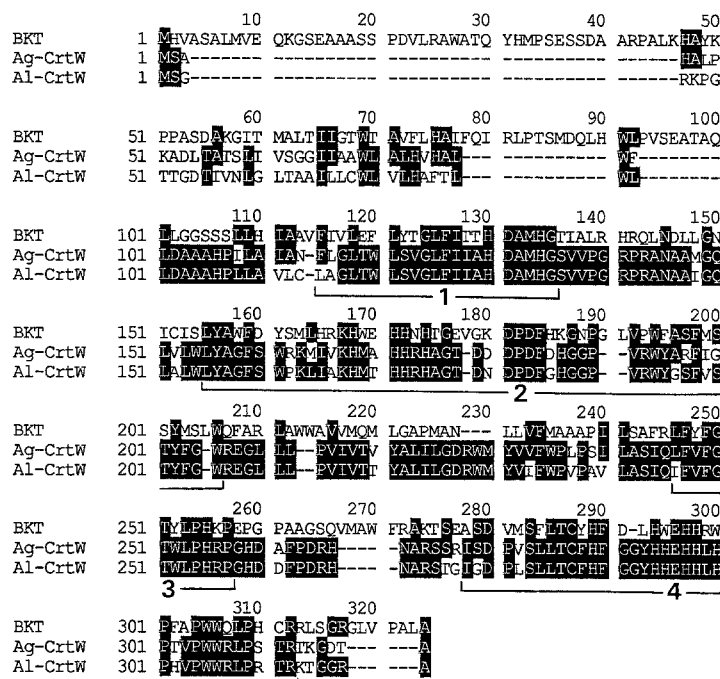


Fig. 8. Amino acid sequences of the *crtW* gene products (*CrtW*) in *Agrobacterium aurantiacum* (Ag) and *Alcaligenes* PC-1 (Al) and the *H. pluvialis* *bkt* gene product (BKT), and amino acid sequence homology among them. Four highly conserved regions are underlined.



the non-homologous coding region (Fig. 6). Therefore, we have determined the minimum catalytic region by deletion analysis. This has shown that a protein starting with the ATG at nucleotide position 264 (Figs. 2 and 6) is a functional enzyme with the minimum length, corresponding to the deletion of 32 N-terminal amino acid residues.

Synthetic astaxanthin is widely used in fish feeds primarily for economic reasons. However, the synthetic forms may include the unnatural configurations or carotenoid-like compounds in the preparations. There is also a growing trend towards using natural sources in ingredients of feeds and foods [11]. *H. pluvialis* appears to be able to produce the highest level of astaxanthin (0.5–2% dry weight) among all organisms [4, 11]. However, most of the astaxanthin synthesized by this green alga is esterified [10], which may reduce its bioavailability to fish [11]. Moreover, this alga needs high light levels for astaxanthin formation [14]. In order to compete economically with the synthetic chemical, it may be necessary to improve *H. pluvialis* by genetic manipulation. However, no report is available on genes or enzymes involved in carotenoid biosynthesis in this green alga so far. The present study would therefore be the first step toward its genetic manipulation. Furthermore, we have initially succeeded in synthesizing astaxanthin in *E. coli*, which cannot naturally synthesize any carotenoid, using the *bkt* gene and the *E. uredothora* carotenogenic genes *crtE*, *crtB*, *crtI*, *crtY* and *crtZ*. We have also shown that an ethanol-producing bacterium *Zymomonas mobilis* and the yeast *Saccharomyces cerevisiae*, which possess no carotenoid biosynthesis genes, acquire the ability to produce  $\beta$ -carotene by the introduction of the *E. uredothora* carotenoid genes [23, 32]. These results reveal the feasibility of astaxanthin production by appropriate microorganism species, which may be safer as feeds or foods, whose growth rates are faster, or whose bioavailability is more favorable to fish and other animals, regardless of their natural ability to produce carotenoids.

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## References

1. Andrewes AG, Borch G, Liaaen-Jensen S, Snatzke G: Animal carotenoids. 9. On the absolute configuration of astaxanthin and actinoerythrin. *Acta Chem Scand B* 28: 730–736 (1974).
2. Andrewes AG, Phaff HJ, Starr MP: Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. *Phytochemistry* 15: 1003–1007 (1976).
3. Armstrong GA: Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. *J Bact* 176: 4795–4802 (1994).
4. Boussiba S, Fan L, Vonshak A: Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. *Meth Enzymol* 213: 386–391 (1992).
5. Bouvier F, Huguency P, d'Harlingue A, Kuntz M, Camara B: Xanthophyll biosynthesis in chromoplasts: isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. *Plant J* 6: 45–54 (1994).
6. Britton G: Biosynthesis of carotenoids. In: Goodwin TW (eds) *Plant Pigments*, pp. 133–182. Academic Press, London (1988).
7. Chomczynski P: A reagent for single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* 15: 532–536 (1993).
8. Cunningham FX Jr, Sun Z, Chamovitz D, Hirschberg J, Gantt E: Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. *Plant Cell* 6: 1107–1121 (1994).
9. Fraser PD, Misawa N, Linden H, Yamano S, Kobayashi K, Sandmann G: Expression in *E. coli*, purification and reactivation of the recombinant *Erwinia uredothora* phytoene desaturase. *J Biol Chem* 267: 19891–19895 (1992).
10. Grung M, D'Souza FML, Borowitzka M, Liaaen-Jensen S: Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3'S)-astaxanthin esters. *J Appl Phycol* 4: 165–171 (1992).
11. Johnson EA, An G-H: Astaxanthin from microbial sources. *Crit Rev Biotechnol* 11: 297–326 (1991).
12. Jyonouchi H, Zhang L, Tomita Y: Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances *in vivo* antibody production to T-dependent anti-

- gens without facilitating polyclonal B-cell activation. *Nutr Cancer* 19: 269–280 (1993).
13. Kakizono T, Kobayashi M, Nagai S: Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in green alga, *Haematococcus pluvialis*. *J Ferment Bioeng* 74: 403–405 (1992).
  14. Kobayashi M, Kakizono T, Nagai S: Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. *Appl Environ Microbiol* 59: 867–873 (1993).
  15. Krinsky NI, Mathews-Roth MM, Taylor RF (eds): Carotenoids: Chemistry and Biology. Plenum Press, New York (1989).
  16. Matsuno T: Xanthophylls as precursors of retinoids. *Pure Appl Chem* 63: 81–88 (1991).
  17. Meyers SP: Developments in world aquaculture, feed formulations, and role of carotenoids. *Pure Appl Chem* 66: 1069–1076 (1994).
  18. Miki W: Biological functions and activities of animal carotenoids. *Pure Appl Chem* 63: 141–146 (1991).
  19. Miki W, Otaki N, Yokoyama A, Izumida H, Shimidzu N: Okadaxanthin, a novel C<sub>50</sub>-carotenoid from a bacterium, *Pseudomonas* sp. KK10206C associated with marine sponge, *Halichondria okadai*. *Experientia* 50: 684–686 (1994).
  20. Misawa N, Kajiwara S, Kondo K, Yokoyama A, Satomi Y, Saito T, Miki W, Ohtani T: Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon  $\beta$ -carotene by a single gene. *Biochem Biophys Res Com* 209: 867–876 (1995).
  21. Misawa N, Masamoto K, Hori T, Ohtani T, Böger P, Sandmann G: Expression of an *Erwinia* phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. *Plant J* 6: 481–489 (1994).
  22. Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izaawa Y, Nakamura K, Harashima K: Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J Bacteriol* 172: 6704–6712 (1990).
  23. Misawa N, Yamano S, Ikenaga H: Production of  $\beta$ -carotene in *Zymomonas mobilis* and *Agrobacterium tumefaciens* by introduction of the biosynthesis genes from *Erwinia uredovora*. *Appl Environ Microbiol* 57: 1847–1849 (1991).
  24. Misawa N, Yamano S, Linden H, de Felipe MR, Lucas M, Ikenaga H, Sandmann G: Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of  $\beta$ -carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J* 4: 833–840 (1993).
  25. Nelis HJ, Leenheer APD: Microbial sources of carotenoid pigments used in foods and feeds. *J Appl Bact* 70: 181–191 (1991).
  26. Reddy CC, Hamilton GA, Madyastha KM: Biological Oxidation Systems, vol 1. Academic Press, San Diego (1990).
  27. Rose RE: The nucleotide sequence of pACYC184. *Nucl Acids Res* 16: 355 (1988).
  28. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
  29. Sandmann G: Carotenoid biosynthesis in microorganisms and plants. *Eur J Biochem* 223: 7–24 (1994).
  30. Sandmann G, Misawa N: New functional assignment of the carotenogenic genes *crtB* and *crtE* with constructs of these genes from *Erwinia* species. *FEMS Microbiol Lett* 90: 253–258 (1992).
  31. Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, Mori H: Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. *Carcinogenesis* 15: 15–19 (1994).
  32. Yamano S, Ishii T, Nakagawa M, Ikenaga H, Misawa N: Metabolic engineering for production of  $\beta$ -carotene and lycopene in *Saccharomyces cerevisiae*. *Biosci Biotech Biochem* 58: 1112–1114 (1994).
  33. Yokoyama A, Izumida H, Miki W: Production of astaxanthin and 4-ketozeaxanthin by the marine bacterium, *Agrobacterium aurantiacum*. *Biosci Biotech Biochem* 58: 1842–1844 (1994).