Functional Analysis of Combinations in Astaxanthin Biosynthesis Genes from *Paracoccus haeundaensis*

Jae Hyung Lee[1,2], Yong Bae Seo[1], Seong-Yun Jeong[3], Soo-Wan Nam[2,4], and Young Tae Kim[1]*

[1] Department of Microbiology, Pukyong National University, Busan 608-737, Korea
[2] Department of Biomaterial Control, Dong-Eui University (BK 21 program), Busan 614-714, Korea
[3] Korea BIO-IT Foundry Center, Pusan National University, Busan 609-735, Korea
[4] Department of Biotechnology and Bioengineering, Dong-Eui University, Busan 614-714, Korea

Abstract Carotenoids are important natural pigments produced by many microorganisms and plants. We have previously reported the isolation of a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, mainly in the form of astaxanthin. The astaxanthin biosynthesis gene cluster, consisting of six carotenogenic genes, was cloned and characterized from this organism. Individual genes of the carotenoid biosynthesis gene cluster were functionally expressed in *Escherichia coli* and each gene product was purified to homogeneity. Their molecular characteristics, including enzymatic activities, were previously reported. Here, we report cloning the genes for crtE, crtEB, crtEBI, crtEBIY, crtEBIYZ, and crtEBIYZW of the *P. haeundaensis* carotenoid biosynthesis genes in *E. coli* and verifying the production of the corresponding pathway intermediates. The carotenoids that accumulated in the transformed cells carrying these gene combinations were analyzed by chromatographic and spectroscopic methods. © KSBB

Keywords: astaxanthin, *carotenoid*, *expression*, Paracoccus haeundaensis, *pigment*

Carotenoids are natural lipid-soluble pigments, produced primarily within bacteria, algae and plants, that have recently attracted increased attention because of their beneficial effects on human health, including their function as antioxidants [1,2], which are involved in cancer prevention [3-5], and enhancing immune responses [6-8]. The area of carotenoid research is therefore extremely important from both fundamental and applied perspectives, and extensive studies have been conducted on the general aspects of the chemical structures, physical and biochemical properties, biosynthetic and molecular genetics, and biotechnological applications of carotenoids [9-11].

Many carotenoid biosynthesis genes have been cloned and characterized from various organisms and the functions of the gene products have been determined [12-19]. In recent years, the individual carotenoid biosynthesis genes have most frequently been used to study carotenoid biosynthesis [9,20-22], and the carotenoid biosynthetic pathways have been elucidated through analyses of the pigments accumulated in *E. coli* cells carrying various combinations of the carotenoid biosynthetic genes [17,23] or through *in vitro* assays of the gene products synthesized in *E. coli* [24-27]. *E. coli* is one of the most popular host organisms for the high-level production of recombinant proteins [28,29]. The functions of many carotenoid biosynthesis genes from a variety of carotenogenic organisms such as bacteria [30] as well as higher plants [31-33] have been studied using transformed *E. coli*. These genes are easily expressed in *E. coli* cells and their recombinant proteins utilize suitable substrates for carotenoid biosynthesis within the cells.

The functions of the individual gene products involved in the astaxanthin biosynthesis pathways have been extensively studied. Biosynthesis of carotenoids includes the formation of geranyl geranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP) by a GGPP synthase (CrtE; encoded by a *crtE* gene), the formation of phytoene from GGPP by a phytoene synthase (CrtB; *crtB* gene), the formation of lycopene from phytoene by a phytoene dehydrogenase (CrtI; *crtI* gene), and the formation of β-carotene by a lycopene β-cyclase (CrtY; *crtY* gene). In the final stages, β-carotene can

***Corresponding author**
Tel: +82-51-620-6366 Fax: +82-51-611-6358
e-mail: ytkim@pknu.ac.kr

Table 1. Oligonucleotides used in this study

Name	Nucleotide sequence	Remarks
CrtW-1	5′-CCATATGAGCGCACATGCCCTG-3′	Primer for *crtW*, Forward (*Nde*I site)
CrtZ-1	5′-GCATATGACCAATTTCCTGATC -3′	Primer for *crtZ*, Forward (*Nde*I site)
CrtY-1	5′-GCATATGACCATGACGTGCTG-3′	Primer for *crtY*, Forward (*Nde*I site)
CrtI-1	5′-TCATATGAACGCCCATTCGC-3′	Primer for *crtI*, Forward (*Nde*I site)
CrtB-1	5′-GCATATGAGCGATCTGGTCCTG-3′	Primer for *crtB*, Forward (*Nde*I site)
CrtB-3	5′- CTCGAGCCTAGACGTGATGCGG-3′	Primer for *crtB*, Reverse (*Xho*I site)
CrtE-1	5′-CCATATGAGACGAGACGTCAA-3′	Primer for *crtE*, Forward (*Nde*I site)
CrtE-3	5′-GCCGCCTCGAGTCTAGGCGC-3′	Primer for *crtE*, Reverse (*Xho*I site)
T7-1	5′-CTCGAGTAATACGACTCACTATA-3′	Primer for *crtE*, Forward (*Xho*I site)

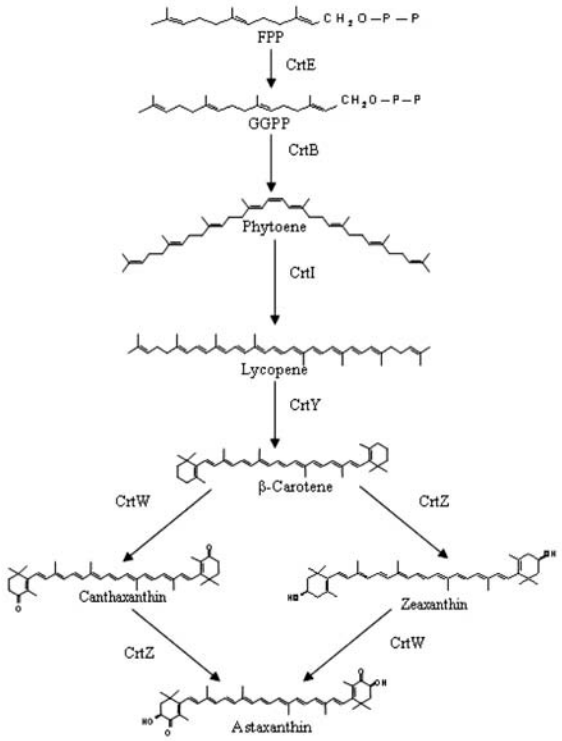

Fig. 1. Schematic of the carotenoid biosynthesis pathways from farnesyl pyrophosphate (FPP) to astaxanthin.

be converted to astaxanthin using only two enzymes: a β-carotene ketolase (CrtW; *crtW* gene) and a β-carotene hydroxylase (CrtZ; *crtZ* gene). The astaxanthin biosynthesis pathway is summarized in Fig. 1.

We previously reported the isolation of a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, mainly in the form of astaxanthin [34] and described the cloning and sequence analysis of genes encoding the astaxanthin biosynthetic enzymes from this organism [35]. All six genes of the astaxanthin biosynthesis gene cluster of *P. haeundaensis* (*crtW*, *crtZ*, *crtY*, *crtI*, *crtB*, and *crtE*, which contain 726, 486, 1158, 1503, 912, and 879 base pairs, respectively) are necessary for astaxanthin biosynthesis, and those genes were cloned and characterized [36]. Individual carotenoid biosynthesis genes of *P. haeundaensis* were functionally expressed in *E. coli* and each gene product was purified to homogeneity. Their molecular characteristics, including enzymatic activities, were reported [36]. Detailed studies of the involvement of the astaxanthin biosynthetic enzymes on carotenoid biosynthesis, however, have not yet been conducted. In order to elucidate the mechanism responsible for controlling the astaxanthin biosynthetic pathway and the intracellular carotenoid concentration, it would therefore be necessary to conduct a comparative analysis of the structure, expression, and function of the astaxanthin biosynthesis genes through various combinations of these genes.

To create the expression plasmid for the genes containing both the *crtB* and *crtE* genes, each gene was amplified by PCR using a pair of gene-specific oligonucleotides (Table 1) with the plasmid pCR-XL-TOPO-Crt-full as a template, which carries the full-length astaxanthin biosynthesis gene cluster [35]. The amplified fragment was subcloned into the vector pGEM-T (Promega, Madison, WI, USA). The subcloned plasmid was digested with *Nde*I and *Xho*I restriction enzymes, and the excised fragment was then ligated into the expression plasmid pET44-a(+) vector. The resulting plasmids carrying an individual gene of the carotenoid biosynthesis enzymes were designated pET-44a(+)-CrtB and pET-44a(+)-CrtE (Fig. 2), respectively. Next, PCR amplification was performed using a pair of oligonucleotides with T7-1 and CrtE-3 primers (Table 1) using pET-44(a)-CrtE (Fig. 2) as a template, and ligated into the pGEM-T-easy vector. The resulting plasmid, pGEM-T-easy-CrtE, was digested with *Xho*I enzyme and ligated into the pET-44(a)-CrtB plasmid previously digested with *Xho*I enzyme and treated with calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation. The resulting plasmid, pET-44(a)-CrtEB (Fig. 2), was transformed into *E. coli* BL21(DE3) Codon Plus. Also, the plasmids, pET-44(a)-CrtEBI, pET-44(a)-CrtEBIY, pET-44(a)-CrtEBIYZ, and pET-44(a)-CrtEBIYZW (Fig. 2), were constructed by the methods described as above. The resulting plasmids were transformed into *E. coli* BL21(DE3) Codon Plus. The cells harboring the various carotenoid genes were cultured in LB medium (containing 50 μg/mL ampicillin) and induced by adding final concentration of 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at a

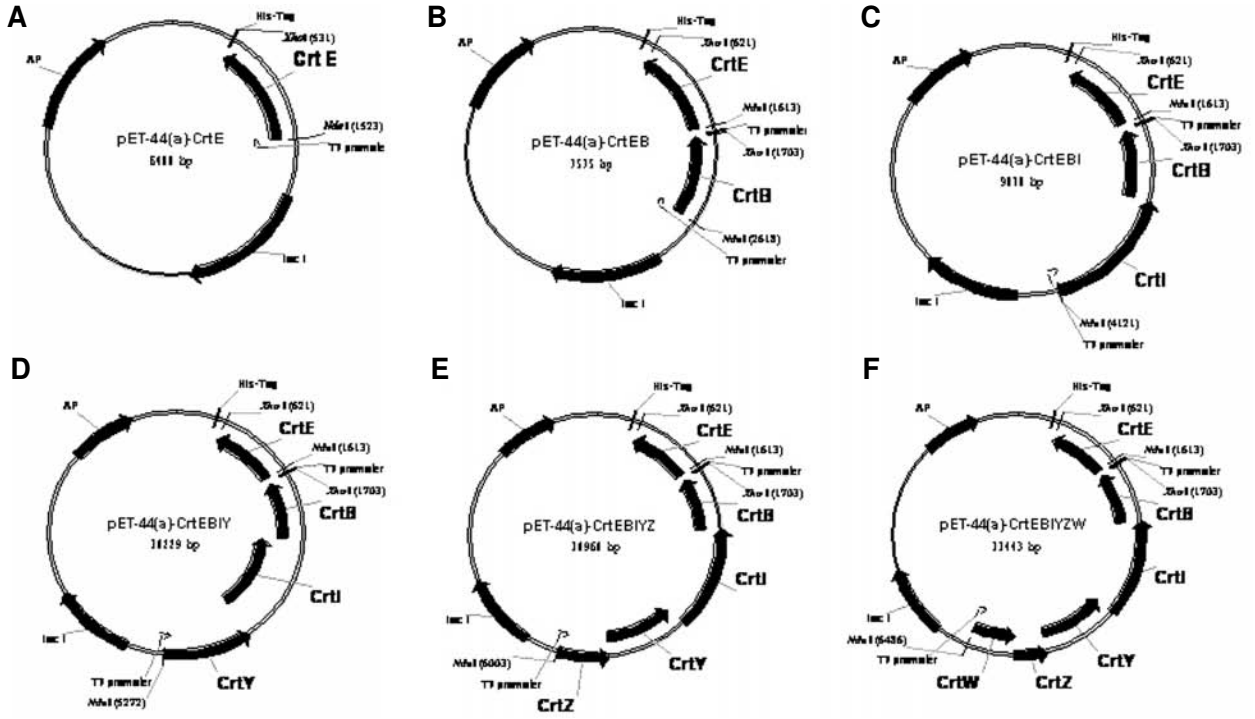

Fig. 2. Construction of the expression plasmids carrying various combinations of the *Paracoccus haeundaensis* carotenoid biosynthesis genes. A: pET-44(a)-CrtE, B: pET-44(a)-CrtEB, C: pET-44(a)-CrtEBI, D: pET-44(a)-CrtEBIY, E: pET-44(a)-CrtEBIYZ, F: pET-44(a)-CrtEBIYZW plasmids.

cell density corresponding to OD_{600} = 0.5. Induced cells were incubated in a rotary shaking incubator at 37°C and 150 rpm for 12 h.

To test the functions of the astaxanthin biosynthesis enzymes, we performed chromatographic and spectroscopic analyses of pigments produced from the transformed *E. coli* with the combinations of the carotenoid biosynthesis genes. The pigments produced in the *E. coli* transformants carrying various combinational constructs of the carotenoid biosynthesis genes of *P. haeundaensis* were analyzed with chromatographic and spectroscopic methods. Ten grams of the lyophilized cells of *E. coli* BL21(DE3) Codon Plus carrying a plasmid were resuspended in 10 mL acetone and incubated overnight at 4°C. The acetone was then evaporated and the pellet was dissolved in 10 mL *n*-hexane-ethanol (1:1, v/v). The extract was diluted to one-half with distilled water, and the two phases were divided using a separating funnel. The organic phase (*n*-hexane phase) was washed with 30% aqueous ethanol until colorless and near neutral pH. After separation, the organic phase was blown to dryness under a stream of nitrogen, and the residue was stored in a refrigerator.

The carotenoid extract was dissolved in 2-propanol and subjected to high-performance liquid chromatography (HPLC). Chromatography was performed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a temperature-controlled autosampler and a diode array detector and the column was a YMC carotenoid C_{30} column (5 micron, steel, 250 mm long × 4.6 mm i.d.; Waters Corp., Milford, MA, USA). The guard column was a Pelliguard LC-18 cartridge (20 mm; SUPELCO, Bellefonte, PA, USA). The mobile phase was a methanol/methyl *tert*-butyl ether (A/B) gradient having the following parameters (all percentages expressed as v/v): start, 80% A/20% B; 10 min, 65% A/35% B; 20 min, 10% A/90% B. A flow rate of 1.0 mL/min was used. The injection volume and column temperature were 10 μL and 15°C, respectively. Carotenoids were detected by absorbance at 470 nm (except for a phytoene, which was detected at 286 nm). Astaxanthin, β-carotene, and lycopene were purchased from Sigma (St. Louis, MO, USA), and phytoene and zeaxanthin were purchased from Carl Roth GmbH (Karlsruhe, Germany) as authentic samples.

To calculate the amount of the accumulated carotenoids from the transformed cells, the following equation was used [37].

$$\text{Total carotenoid (g)} = \frac{\text{mL of solvent} \times A_{\lambda max}}{E^{1\%}_{1cm} \times 100} \quad (1)$$

The specific absorbance coefficient, $A^{1\%}_{1cm}$ (= specific extinction coefficient, $E^{1\%}_{1cm}$), where representing the absorbance of 1% (w/v) solution (1 g/100 mL) in a 1-cm path cuvette at the appropriate wavelength, was applied for the determination of the concentrations of carotenoids.

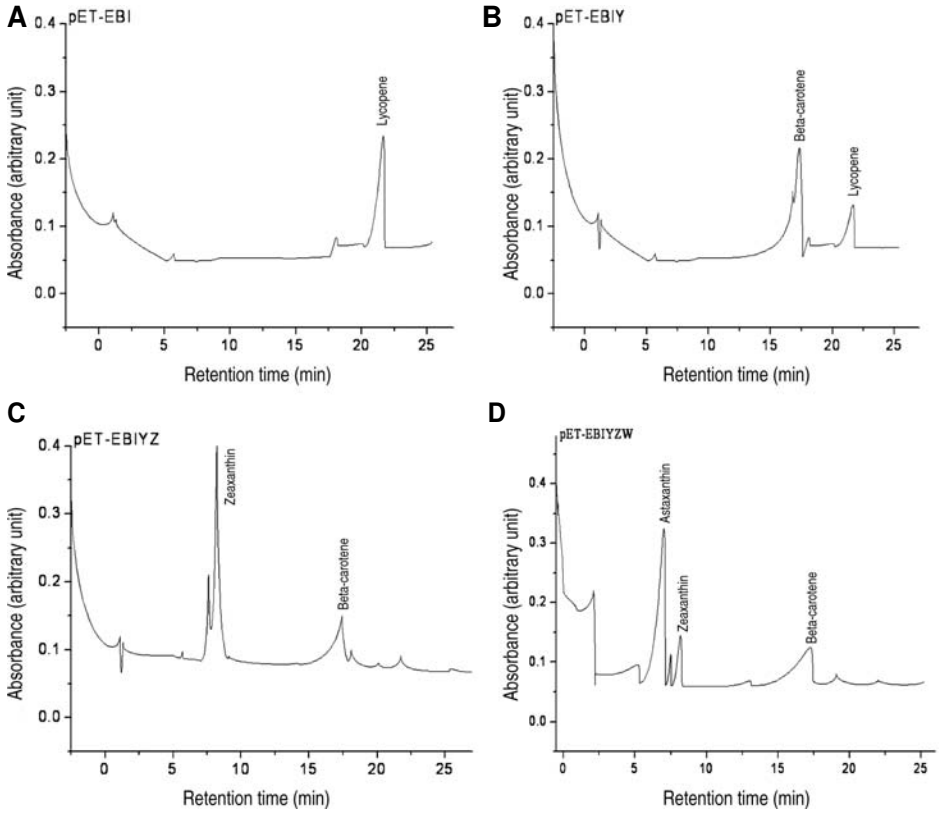

Fig. 3. HPLC analysis of the products synthesized in *Escherichia coli* cells carrying (A) pET-44(a)-CrtEBI, (B) pET-44(a)-CrtEBIY, (C) pET-44(a)-CrtEBIYZ, and (D) pET-44(a)-CrtEBIYZW plasmids.

The maximal wavelength and absorbance coefficient ($E^{1\%}_{1cm}$) of a phytoene are 286 nm and 1,250, respectively. A colorless main peak of the extracts prepared from the cells transformed with pET-44(a)-CrtEB was absorbed at 286 nm, eluted at a retention time of 25.4 min from the HPLC, and determined to be phytoene by comparing it to the standard (data not shown). From the calculation using the above Eq. (1), the concentration of the phytoene produced was 1.4 mg/g DCW (dry cell weight).

The main peak of the HPLC analysis from the cell extracts transformed with pET-44(a)-CrtEBI was found to be lycopene by comparing it to the standard. This peak was eluted at a retention time of 21.7 min and absorbed at maximal wavelength of 470 nm (Fig. 3A). The absorbance coefficient ($E^{1\%}_{1cm}$) of a lycopene is 3,450. The amount of lycopene calculated with the above Eq. (1) was 1.1 mg/g DCW.

The elution peaks of the HPLC analysis from the cell extracts transformed with pET-44(a)-CrtEBIY were found to be two pigments corresponding to β-carotene and lycopene when these peaks were compared with standards. These peaks were eluted at a retention time of 17.4 min for β-carotene and 21.7 min for lycopene, respectively (Fig. 3B). The maximal wavelength and absorbance coefficient ($E^{1\%}_{1cm}$) of a β-carotene are 450 nm and 2,592, respectively. The amounts of β-carotene and lycopene using the above formula (1) were calculated to be 0.8 and 0.1 mg/g DCW, respectively.

The HPLC elution profile of the cell extracts transformed with pET-CrtEBIYZ was shown in Fig. 3C. The peaks were turned out to be three pigments corresponding to zeaxanthin, β-carotene, and lycopene by comparing them to the standard pigments. These peaks were eluted at retention times of 8.2 min for zeaxanthin, 17.4 min for β-carotene, and 21.7 min for lycopene, respectively. The maximal wavelength and absorbance coefficient ($E^{1\%}_{1cm}$) of a zeaxanthin are 451 nm and 2,348, respectively. The amounts of zeaxanthin, β-carotene, and lycopene calculated using the above formula (1) were 0.7 mg/g DCW, 0.2 mg/g DCW, and 50 μg/g DCW, respectively.

The result of the HPLC analysis from the cells transformed with pET-44(a)-CrtEBIYZW was shown in Fig. 3D. The main peaks were turned out to be three pigments corresponding to astaxanthin, zeaxanthin, and β-carotene, when these peaks were compared with standard pigments. These peaks were eluted at retention time at 6.9 min for astaxanthin, at 8.2 min for zeaxanthin, and at 17.4 min for β-carotene, respectively. The maximal wavelength of astaxanthin is at 470 nm and the absorbance coefficient (E) of astaxanthin is 2,500. The amounts of astaxanthin, zeaxanthin, and β-carotene using the above formula (1) were calculated to 0.4 mg/g DCW, 0.2 mg/g DCW, and 80 μg/g DCW, respectively.

In this study, we have conducted a comparative analysis of the structure, expression, and function of the astaxanthin biosynthesis genes of *P. haeundaensis* through various combinations of these genes. We have further studied their expression, organization, and characteristics of the carotenoid biosynthesis enzymes using chromatographic and spectroscopic analyses. The observations and genetic manipulations of the astaxanthin biosynthesis enzymes from *P. haeundaensis* make this species a very useful model in which to study the mechanism of astaxanthin biosynthesis. In addition, the results of this study can be used to enhance the production of astaxanthin through the manipulation of carotenoid biosynthesis genes in *P. haeundaensis*, an important application since astaxanthin is a pigment of high economic value. These data will provide a wider base of knowledge on the primary structure of the astaxanthin biosynthesis gene cluster at the molecular level as well as further the biotechnological applications of carotenoids.

Acknowledgements This research was supported by a grant (M2007-05) from the Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs and Fisheries, Republic of Korea.

Received December 7, 2006; accepted March 13, 2007

REFERENCES

1. Murtaugh, M. A., K. N. Ma, J. Benson, K. Curtin, B. Caan, and M. L. Slattery (2004) Antioxidants, carotenoids, and risk of rectal cancer. *Am. J. Epidemiol.* 159: 32-41.
2. Neuman, I., H. Nahum, and A. Ben-Amotz (2000) Reduction of exercise-induced asthma oxidative stress by lycopene, a natural antioxidant. *Allergy* 55: 1184-1189.
3. Bertram, J. S. and A. L. Vine (2005) Cancer prevention by retinoids and carotenoids: independent action on a common target. *Biochim. Biophys. Acta* 1740: 170-178.
4. Giovannucci, E., A. Ascherio, E. B. Rimm, M. J. Stampfer, G. A. Colditz, and W. C. Willett (1995) Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer Inst*. 87: 1767-1776.
5. Kurihara, H., H. Koda, S. Asami, Y. Kiso, and T. Tanaka (2002) Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. *Life Sci*. 70: 2509-2520.
6. Amar, E. C., V. Kiron, S. Satoh, and T. Watanabe (2004) Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish Shellfish Immunol*. 16: 527-537.
7. Chew, B. P. and J. S. Park (2004) Carotenoid action on the immune response. *J. Nutr*. 134: 257S-261S.
8. Hix, L. M., S. F. Lockwood, and J. S. Bertram (2004) Bioactive carotenoids: potent antioxidants and regulators of gene expression. *Redox Rep*. 9: 181-191.
9. Armstrong, G. A. (1997) Genetics of eubacterial carotenoid biosynthesis: a colorful tale. *Annu. Rev. Microbiol*. 51: 629-659.
10. Johnson, E. A. (2003) *Phaffia rhodozyma*: colorful odyssey. *Int. Microbiol*. 6: 169-174.
11. Sandmann, G. (2001) Carotenoid biosynthesis and biotechnological application. *Arch. Biochem. Biophys*. 385: 4-12.
12. Hannibal, L., J. Lorquin, N. A. D'Ortoli, N. Garcia, C. Chaintreuil, C. Masson-Boivin, B. Dreyfus, and E. Giraud (2000) Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic bacterium *Bradyrhizobium* sp. strain ORS278. *J. Bacteriol*. 182: 3850-3853.
13. Harker, M. and J. Hirschberg (1997) Biosynthesis of ketocarotenoids in transgenic cyanobacteria expressing the algal gene for beta-C-4-oxygenase, crtO. *FEBS Lett*. 404: 129-134.
14. Kajiwara, S., T. Kakizono, T. Saito, K. Kondo, T. Ohtani, N. Nishio, S. Nagai, and N. Misawa (1995) Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. *Plant Mol. Biol*. 29: 343-352.
15. Krubasik, P. and G. Sandmann (2000) A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. *Mol. Gen. Genet*. 263: 423-432.
16. Misawa, N., S. Kajiwara, K. Kondo, A. Yokoyama, Y. Satomi, T. Saito, W. Miki, and T. Ohtani (1995) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon beta-carotene by a single gene. *Biochem. Biophys. Res. Commun*. 209: 867-876.
17. Misawa, N., M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol*. 172: 6704-6712.
18. Pasamontes, L., D. Hug, M. Tessier, H. P. Hohmann, J. Schierle, and A. P. van Loon (1997) Isolation and characterization of the carotenoid biosynthesis genes of *Flavobacterium* sp. strain R1534. *Gene* 185: 35-41.
19. Verdoes, J. C., N. Misawa, and A. J. van Ooyen (1999) Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous*. *Biotechnol. Bioeng*. 63: 750-755.
20. Hundle, B., M. Alberti, V. Nievelstein, P. Beyer, H. Kleinig, G. A. Armstrong, D. H. Burke, and J. E. Hearst (1994) Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. *Mol. Gen. Genet*. 245: 406-416.
21. Nishida, Y., K. Adachi, H. Kasai, Y. Shizuri, K. Shindo,

A. Sawabe, S. Komemushi, W. Miki, and N. Misawa (2005) 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. *Appl. Environ. Microbiol.* 71: 4286-4296.
22. To, K. Y., E. M. Lai, L. Y. Lee, T. P. Lin, C. H. Hung, C. L. Chen, Y. S. Chang, and S. T. Liu (1994) Analysis of the gene cluster encoding carotenoid biosynthesis in *Erwinia herbicola* Eho13. *Microbiology* 140 (Pt 2): 331-339.
23. Hundle, B. S., P. Beyer, H. Kleinig, G. Englert, and J. E. Hearst (1991) Carotenoids of *Erwinia herbicola* and an *Escherichia coli* HB101 strain carrying the *Erwinia herbicola* carotenoid gene cluster. *Photochem. Photobiol*. 54: 89-93.
24. Fraser, P. D., N. Misawa, H. Linden, S. Yamano, K. Kobayashi, and G. Sandmann (1992) Expression in *Escherichia coli*, purification, and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase. *J. Biol. Chem*. 267: 19891-19895.
25. Hundle, B. S., D. A. O'Brien, M. Alberti, P. Beyer, and J. E. Hearst (1992) Functional expression of zeaxanthin glucosyltransferase from *Erwinia herbicola* and a proposed uridine diphosphate binding site. *Proc. Natl. Acad. Sci. USA* 89: 9321-9325.
26. Hundle, B. S., D. A. O′Brien, P. Beyer, H. Kleinig, and J. E. Hearst (1993) *In vitro* expression and activity of lycopene cyclase and beta-carotene hydroxylase from *Erwinia herbicola. FEBS Lett*. 315: 329-334.
27. Math, S. K., J. E. Hearst, and C. D. Poulter (1992) The crtE gene in *Erwinia herbicola* encodes geranylgeranyl diphosphate synthase. *Proc. Natl. Acad. Sci. USA* 89: 6761-6764.
28. Kim, J. E., E. J. Kim, W. J. Rhee, and T. H. Park (2005) Enhanced production of recombinant protein in *Escherichia coli* using silkworm hemolyph. *Biotechnol. Bioprocess Eng.* 10: 353-356.
29. Jang, M. Y., W. Y. Ryu, and M. H. Cho (2006) Enhanced production of laccase from *Trametes* sp. by combination of various inducers. *Biotechnol. Bioprocess Eng*. 11: 96-99.
30. Cunningham, Jr., F. X., D. Chamovitz, N. Misawa, E. Gantt, and J. Hirschberg (1993) Cloning and functional expression in *Escherichia coli* of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of beta-carotene. *FEBS Lett*. 328: 130-138.
31. Misawa, N., M. R. Truesdale, G. Sandmann, P. D. Fraser, C. Bird, W. Schuch, and P. M. Bramley (1994) Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation *in vivo* and *in vitro*, and functional analysis of the various truncated gene products. *J. Biochem.* 116: 980-985.
32. Pecker, I., D. Chamovitz, H. Linden, G. Sandmann, and J. Hirschberg (1992) A single polypeptide catalyzing the conversion of phytoene to zeta-carotene is transcriptionally regulated during tomato fruit ripening. *Proc. Natl. Acad. Sci. USA* 89: 4962-4966.
33. Jin, E. and A. Melis (2003) Microalgal biotechnology: Carotenoid production by the green algae *Dunaliella salina. Biotechnol. Bioprocess Eng*. 8: 331-337.
34. Lee, J. H., Y. S. Kim, T. J. Choi, W. J. Lee, and Y. T. Kim (2004) *Paracoccus haeundaensis* sp. nov., a Gram-negative, halophilic, astaxanthin-producing bacterium. *Int. J. Syst. Evol. Microbiol*. 54: 1699-1702.
35. Lee, J. H. and Y. T. Kim (2006) Cloning and characterization of the astaxanthin biosynthesis gene cluster from the marine bacterium *Paracoccus haeundaensis. Gene* 370: 86-95.
36. Lee, J. H. and Y. T. Kim (2006) Functional expression of the astaxanthin biosynthesis genes from a marine bacterium, *Paracoccus haeundaensis*. *Biotechnol. Lett.* 28: 1167-1173.
37. An, G. H., D. B. Schuman, and E. A. Johnson (1989) Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Environ. Microbiol*. 55: 116-124.