Thesis for the Degree Master of Science

Cloning and characterization of zeaxanthin glucosyltransferase gene (*crtX*) from the astaxanthin-producing marine bacterium, *Paracoccus haeundaensis*

by

Seong Seok Choi

Department of Microbiology

The Graduate School

Pukyong National University

February 2009

Cloning and characterization of zeaxanthin glucosyltransferase gene (*crtX*) from the astaxanthin-producing marine bacterium, *Paracoccus haeundaensis*

Astaxanthin 생산 해양 미생물 *Paracoccus haeundaensis*에서
유래된 Zeaxanthine glucosylase(CrtX)의
클로닝 및 특성 연구

Advisor: Young Tae Kim

by

Seong Seok Choi

A thesis submitted in partial fulfillment of the requirement
for the degree of

Master of Science

in the Department of Microbiology, The Graduate School,
Pukyong National University

February 2009

Cloning and characterization of zeaxanthin glucosyltransferase gene (*crtX*) from the astaxanthin-producing marine bacterium, *Paracoccus haeundaensis*

A dissertation

by

Seong Seok Choi

Approved as to style and content by :

_____

(Chairman) Gun Do Kim

_____ _____

(Member) Myung Suk Lee (Member) Young Tae Kim

February 2009

CONTENTS

LISTS OF FIGURES

Cloning and characterization of zeaxanthine glucosyltransferase (CrtX) from the astaxanthin-producing marine bacterium, *Paracoccus haeundaensis*

Seong Seok Choi

Department of Microbiology, The Graduate School,
Pukyong National University

ABSTRACT

Zeaxanthin, a member of the carotenoid pigment family, is most abundant in dark, leafy green vegetables and is crucial to the good health of the eyes. Previously, we reported the isolation of a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, mainly astaxanthin. Zeaxanthin glucosyltransferase (CrtX) mediates the formation of zeaxanthin to zeaxanthin diglucoside. Here, I report cloning of the *crtX* gene responsible for zeaxanthin diglucoside biosynthesis from *P. haeundaensis* and the production of the corresponding carotenoids in the transformed cells carrying this gene. The *crtX*

was isolated from the marine bacterium *P. haeundaensis* and shown to consist of 1248 bp encoding 415 amino acids residues. The nucleotide sequence of *crtX* was compared with those of other species and was shown by phylogenetic analysis to be well conserved during evolution. The expression plasmid containing *crtX* gene (pSTCRT-X) was constructed, and *Escherichia coli* cells containing this plasmid produced the recombinant protein of approximately 46 kDa, corresponding to the molecular weight of zeaxanthin glucosyltransferase. Biosynthesis of zeaxanthin diglucoside was obtained when the plasmid pSTCRT-X was co-transformed into *E. coli* containing the pET-44a(+)-CrtEBIYZ carrying *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes required for zeaxanthin β-D-diglucoside biosynthesis.

INTRODUCTION

The carotenoids are a widely distributed class of structurally and functionally diverse yellow, orange, and red natural pigments. These pigments are synthesized in bacteria, algae, fungi and plants, and have been widely used as a feed supplement from poultry rearing to aquaculture. Carotenoids also exhibit diverse biological properties, such as strong antioxidant activity [1, 2], and antitumor activities [3, 4], and enhancement of immune responses [5, 6].

Zeaxanthin [(3R,3'R)-β,β-carotene-3,3'diol] is one of the xanthophylls, which are most abundantly in dark, leafy green vegetables, and are crucial to the good health of the eyes. Zeaxanthin helps protect the eye from damage by ultra-violet (UV) irradiation, and prevents free-radical damage to the retina and the lens of the eye associated with diabetic retinopathy, macular degeneration, cataracts, and glaucoma. Zeaxanthine diglucosides are considered to protect cells against photooxidative damage from activated oxygen plus visible light [7] or to stabilize a membrane environment in a manner similar to the function of sterols in eukaryotic cells [8].

Carotenoids are essential for organisms with oxygenic photosynthesis (plants, algae, cyanobacteria) because of their protective role as antioxidants [9, 10]. The genus *Paracoccus* consists of Gram-negative cocci or short rods bacteria that show substantial metabolic versatility. Nearly, 20 species have been described to date [11]. Some species produce carotenoids: adonixanthin diglucoside from *Paracoccus schoinia* (=*marinus*) NBRC 100637 [12],

astaxanthin and canthaxanthin from *Paracoccus marcusii* [13], astaxanthin from *Paracoccus* sp. MBIC 03024 [14] zeaxanthin from *Paracoccus zeaxanthinifaciens* R-1534 [15], and astaxanthin from *Paracoccus carotinifaciens* [16] and *Paracoccus haeundaensis* [17].

Lee clarified the biosynthetic pathway of astaxanthin in *Paracoccus haeundaensis* as reported previously [18, 19]. A simplified carotenoids biosynthetic pathway is shown Figure 1. Carotenoids are characterized by a large 40-carbon atom polyene chain and sometimes terminated by rings. The first steps in the biosynthesis of carotenoids include the formation of geranyl geranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP) by GGPP synthase, an enzyme encoded by the *crtE* genes. The formation of phytoene from GGPP, a step that is well conserved in all carotenogenic organisms, is catalyzed by a phytoene synthase encoded by the *crtB* genes. Phytoene is then dehydrated by a phytoene dehydrogenase (c*rtI*). In many carotenogenic organisms, the process is completed with the cyclization of lycopene β-cyclase (c*rtY*). β-carotene ketolase (c*rtW*) initially converts β-carotene to canthaxanthin via echinenone, and β-carotene hydroxylase (c*rtZ*) initially mediates the conversion of β-carotene to zeaxanthin via β-cryptoxanthin. Finally, through a few more intermediates, the *crtW* and *crtZ* gene products act in combination to produce astaxanthin [18]. Zeaxanthin glucosyltransferase (CrtX) converts zeaxanthin to zeaxanthin diglucoside.

In a previous study, I isolated and characterized a marine bacterium, *P. haeundaensis*, which produces astaxanthin [17] and the cloning and characterization of genes encoding the astaxanthin biosynthetic enzymes [18, 19]. Here, to clarify the organization and expression of the

carotenoid biosynthesis genes in this organism, I describe the cloning and sequencing of gene encoding the zeaxanthin diglcoside biosynthesis enzyme

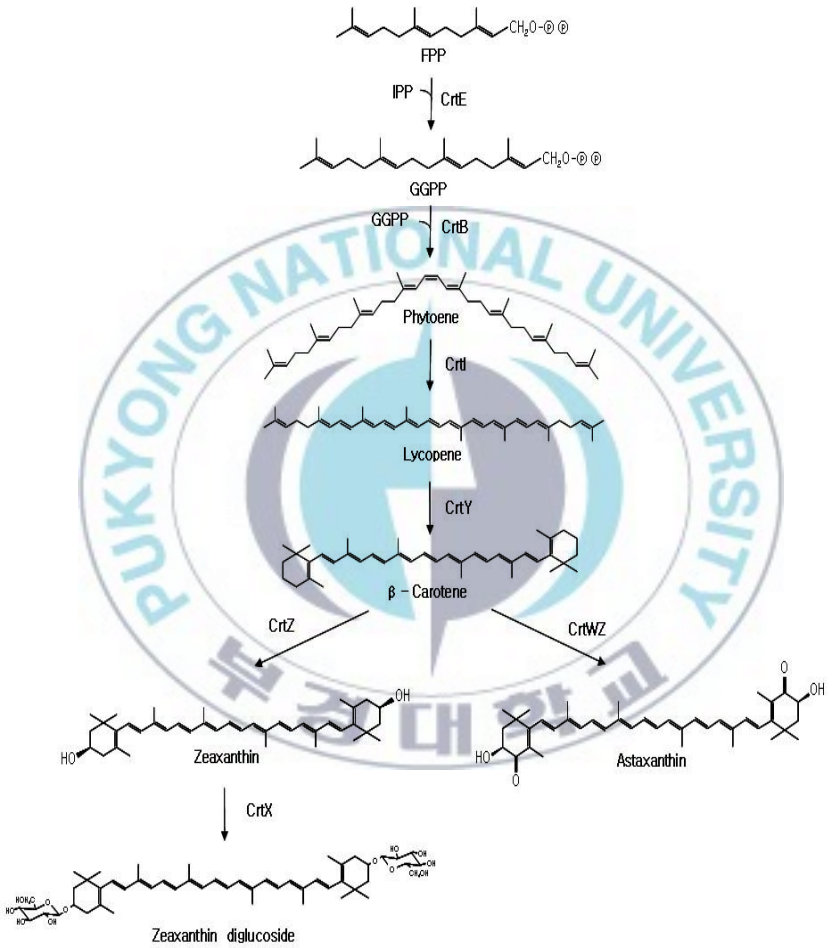


Fig. 1. Scheme of the carotenoid biosynthesis pathways from farnesyl pyrophosphate (FPP) to zeaxanthin diglucoside.

(GenBank accession number ; EU431079). I also discuss the molecular characteristics and expression of our newly identified zeaxanthin diglucoside biosynthesis gene, the function of which was determined by chromatographic analyses of the pigments produced in *E. coli* transformed with the cloned gene. These data provide insight into the primary structure of the zeaxanthin diglucoside biosynthesis gene and will facilitate the biotechnological applications of carotenoids.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli strain XL1-blue [F′::Tn10 proA+B+ lacIq Δ(lacZ) M15/reaA1 endA1 gyrA96(Nalr) thi hsdR17 (rK-mK+) supE44 relA1 lac] was used for gene cloning expreiments. BL21 (DE3) [F- ompT hsdSβ(rβ-mβ-) dcm gal (DE3) tonA] was used for expression and production of zeaxanthin diglucoside.

E. coli strain BL21 (DE3) harboring the pSTCRT-X and pET-44(a)-EZ plasmid (contains crtEBIYZ gene) were cultured at 37℃ in DYT medium containing chloramphenicol (170 ㎍/ml) and kanamycine (50 ㎍/ml).

Preparation of genomic and plasmid DNA

Chromosomal DNA was prepared from cells of *P. haeundaensis* grown in marine broth medium (Difco). Genomic DNA of *P. haeundaensis* in the late-exponential growth phase was isolated by a modification of the procedure of Murray and Thompson [20]. Late-exponential phase cells were harvested by centrifugation at 5,100 x g for 10 min at room temperature and washed with 50 ml of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM ethylenediamine tetraacetic acid). The cells were collected by centrifugation at 11,000 x g for 10 min at room temperature, resuspended in 9 ml of TE buffer containing 5 mg/ml lysozyme and 100 μg/ml RNase A (Sigma), and incubated at 37°C for 1 h. After adding 0.5 ml of 10% (w/v) SDS and 0.1 ml of 10 mg/ml of

proteinase K, the suspension was incubated at 37°C with shaking for several hours to complete lysis. The lysate together with 1.8 ml of 5 M NaCl and 1.5 ml of 10% (w/v) acetyl trimenthyl ammonium bromide (CTAB) in 0.7 M NaCl was incubated at 65°C for 20 min. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added, and the mixture was mixed gently by inverting the tube at room temperature for 10 min. The phases were separated by centrifugation at 11,000 x g for 20 min at room temperature. After two extractions with chloroform/isoamyl alcohol, the aqueous phase was extracted once with an equal volume of phenol saturated with 1 M Tris-HCl (pH 7.5) and again with chloroform/isoamyl alcohol. Two volumes of cold ethanol (- 20°C) was added, and the precipitated DNA was collected using a glass rod and was rinsed with 70% (v/v) ethanol. The ethanol was evaporated under vacuum, and the DNA was suspended in 3 ml of TE buffer. The DNA concentration was determined by measuring the absorbance of a 20-fold dilution at 260 and 280 nm.

The purified chromosomal DNA of *P. haeundaensis* was partially digested by incubation at 37°C for 1 h with 5 kU Sau3AI/g genomic DNA. A genomic DNA library of *P. haeundaensis* was constructed using a ZAP Express® BamHI/Gigapack® III cloning kit (Stratagene) as described previously [18]. The resulting library contained approximately 1×10^5 clones. The library was amplified to 3×10^9 clones/ml. Plasmid DNA was prepared from cells of *E. coli* transformants using the Wizard plus SV minipreps DNA purification system (Promega).

Screening and sequencing of the zeaxanthin glucosyltransferase (*crtX*) gene

The conserved nucleotide sequences of previously identified zeaxanthin glucosyltransferase (*crtX*) genes were obtained from the National Center for Biotechnology Information (NCBI) nucleotide and protein sequence database. Degenerative oligonucleotides designed based on the conserved sequences and synthesized by GenoTech were used as probes to screen for the *crtX* genes; the sequences of the oligonucleotides used in this study are shown in Table 1.

The probes for the *crtX* genes were amplified by PCR using upstream (Crt-X-F) and downstream (CrtX-R) primers. The main PCR program consisted of 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The amplified probes were labeled using a digoxigenin (DIG) oligonucleotide 3'-end labeling kit (Roche). The plaque hybridization method was used to screen the *P. haeundaensis* genomic DNA library for *crtX* genes. Approximately 1×10^5 plaques were screened. Positive plaques recovered from the first screening were confirmed with a second screening [21, 22]. The positive plaques were recovered from the second screen, and the phagemids containing inserts were excised [23]. The excised inserts were sequenced using an ABI Prism DNA sequencing kit and an ABI 377 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Table 1. Oligonucleotide primers used for the study

Name	Nucleotide sequence	Remarks
CrtX-F	5'- CGACCTGCACCGCCCCGACC-3'	Forward primer for *crtX* gene cloning
CrtX-F1	5′- ATGACGGTCGCCGCATCCGTG- 3′	Primer for full-length zeaxanthin glucosylase, Forward
CrtX-F2	5′- AAGCTTATGACGGTCGCCGC- 3′	Forward primer for *crtX* gene expression (*Hind*IIIsite)
CrtX-R	5'- TTCGCGCACCGGGGCGGTGT -3'	Primer for full-length zeaxanthin glucosylase, Reverse
CrtX-R1	5′- TCAGTGGACCGGCGCGCCGAT- 3′	Reverse primer for *crtX* gene cloning
CrtX-R2	5′- GAATTCGTGGTGGTGGTGGTGGTG - 3′	Reverse primer for *crtX* gene expression (*EcoR*Isite)

Comparative sequence analysis of zeaxanthin glucosyltransferase (*crtX*) gene from *Paracoccus haeundaensis*

To examine the molecular evolution of zeaxanthin glucosyltransferase (*crtX*) from *P. haeundaensis*, the following *crtX* sequences were imported from SwissPort/GenBank: *Acaryochloris marina* MBIC11017 (YP001519359), *Lyngbya* sp. PCC 8106 (ZP01619956), *Microcystis aeruginosa* (CAO89015), *Methylobacterium nodulans* (ZP02119601), *Chlorobium limicola* DSM (ZP00513474), *Gluconobacter oxydans* (YP191143), and *P. haeundaensis* (EU431079). The nucleotide sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A multiple sequence alignment was performed using Clustal W2 (http://www.ebi.ac.uk/clustalw2), and sequence identities were calculated using GeneDoc (http://www.psc.edu/biomed /genedoc). A phylogenetic tree was constructed by the neighbor-joining (NJ) method with the program Treecon [24] using the amino acid sequences of the zeaxanthin glucosyltransferase encoded by *crtX* gene.

Expression of zeaxanthin glucosyltransferase (*crtX*) in *E. coli*

To express the *crtX* gene in *E. coli*, the coding region of the cloned *crtX* gene was amplified by PCR using a pair of oligonucleotides (CrtX-F1 as the forward and Crt-X-R1 as the reverse primers for the full-length zeaxanthin glucosylase gene; Table 1). The amplified fragment was subcloned into pET-44a(+) expression vector (Promega), which allows expression of a recombinant protein with a C-terminal fusion His-tag, and the resulting

construct was designated as pET-44(a)-CrtX. Subcloning of the carotenoid biosynthesis genes into the pET-44a(+) vector produces a fusion protein tagged with histidine residues in the carboxyl-terminus, resulting in a CRT–His fusion protein. In the next step, PCR was performed using a pair of oligonucleotides primers, CrtX-F2 and CrtX-R2, containing restriction sites HindIII and EcoRI, respectively (Table 1), with pET-44(a)-CrtX as a template, and ligated into pGEM-T-easy vector (Promega). The resulting plasmid, pGEM-T-easy-CrtX-His tag, was digested with HindIII and EcoRI, and ligated into the expression vector pSTV29. The resulting plasmid, pSTCRT-X (Fig. 2A), was transformed into *E. coli* BL21(DE3) cells.

Cultured cells expressing the zeaxanthin glucosyltransferase protein were harvested by centrifugation, and the cell pellets were resuspended in extraction buffer (20 mM Tris-HCl, pH 8.0) then sonicated with an ultrasonicator (VC130; Sonics and Materials Inc.). The samples were centrifuged for 10 min at 12,000 x g and the precipitates were resuspended in isolation buffer (20 mM Tris-HCl, 2 M urea, 0.5 M NaCl, 2% Triton X-100, pH 8.0), sonicated again, and harvested by centrifugation for 10 min at 12,000 x g. Prepared samples were subjected to 12% SDS–PAGE. Immunoblotting analysis was performed according to standard procedures [25]. The blots were incubated for 1 h with 3% (w/v) gelatin in Tris-Tween buffered saline (TTBS; 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS. Subsequently, a goat polyclonal anti-6-histidine antibody (diluted 1:500) was added and incubated for 1 h at room temperature. After rinsing three times with TTBS, the membrane was incubated with alkaline phosphatase-conjugated anti-goat IgG (diluted 1:2000 in TTBS containing 1%

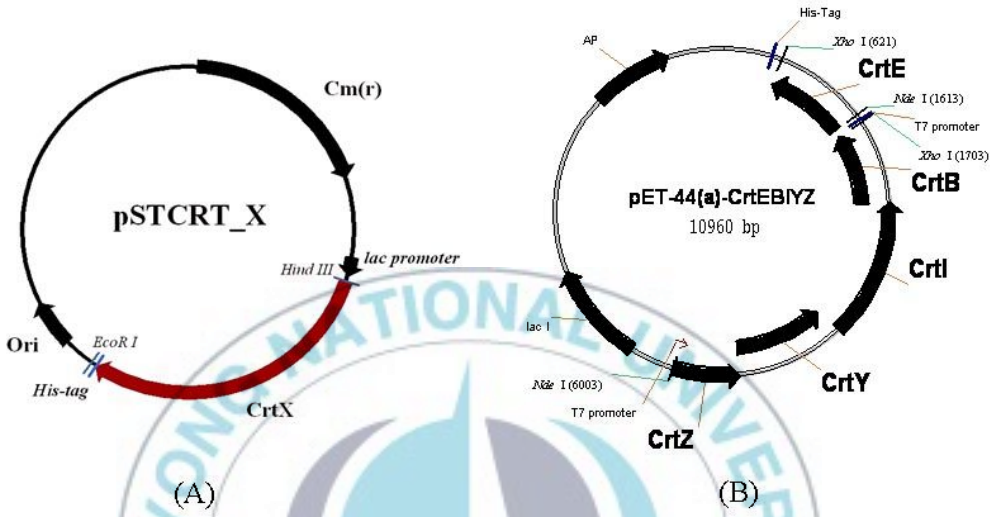


Fig. 2. Construction of the expression plasmids of the *Paracoccus haeundaensis* zeaxanthin diglucoside biosynthesis genes. pSTCRT-X (A), pET-44(a)-CrtEBIYZ (B)

gelatin; Sigma) as the secondary antibody at room temperature for 30 min. The membrane was then rinsed three times with TTBS and developed at room temperature.

Extraction and purification of zeaxanthin diglucoside produced in transformed *E. coli*

Acetone was used to extract the zeaxanthin diglucoside from the transformants carrying the zeaxanthin diglucoside biosynthesis genes. The acetone extracts were evaporated to dry and further extracted with chloroform/methanol (9:1, v/v). After dissolving in a small volume of n-hexane, the carotenoids were analyzed by HPLC using a Nova-Pak HR 6U C_{18} column (3.9 mm x 300 mm) with acetonitrile/methanol/2-propanol (90:6:4, by vol.) at 1 ml/min, and the elution profiles were recorded with a photodiode array detector (Model 996; Waters Corp.). Synthetic zeaxanthin, lycopene, β-carotene, and zeaxanthin diglucoside (Sigma) were used as references.

RESULTS

Cloning of the zeaxanthin diglucoside biosynthesis gene

To identify the zeaxanthin diglucoside biosynthesis gene from *P. haeundaensis*, a genomic library was constructed from chromosomal *P. haeundaensis* DNA partially digested with Sau3AI and fractionated to give fragments of 6 to 10 kb. The DNA was ligated into the BamHI site of the vector λZAP in a ZAP Express® BamHI/Gigapack® III cloning kit (Stratagene). The resulting library contained approximately 1×10^5 individual clones.

Upstream (CrtX-F) and downstream (CrtX-R) degenerate oligonucleotide primers, designed according to the conserved nucleotide sequences of previously identified *crtX* genes, were synthesized, amplified by PCR, and end-labeled for use as probes to screen for zeaxanthin diglucoside biosynthesis gene using the plaque hybridization method. Eight positive plaques containing *crtX* genes were isolated, and the phagemids containing the inserts were excised and analyzed using an ABI 377 XL upgrade DNA sequencer [23]. The full-length zeaxanthin diglucoside biosynthesis gene (*crtX*) was isolated from *P. haeundaensis* with CrtX-F1 and CrtX R1 oligonucleotide primer in PCR reaction (Fig. 3). The *crtX* gene of *P. haeundaensis* consisted of 1248 bp encoding a polypeptide of 415 amino acid residues (Fig. 4).

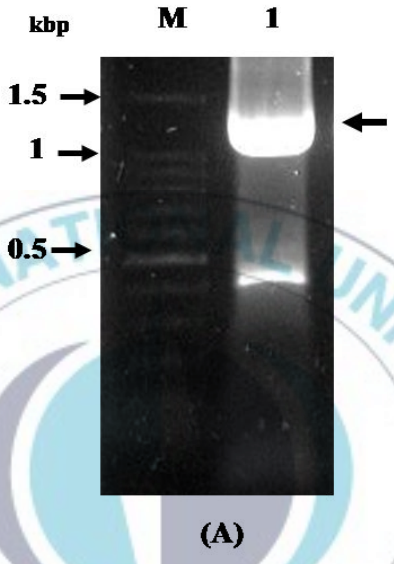


Fig. 3. PCR product amplified with CrtX-F1 and CrtX-R1 (A), The lane M 100 bp DNA ladder (Takara); lane 1, amplified zeaxanthin glucosylase

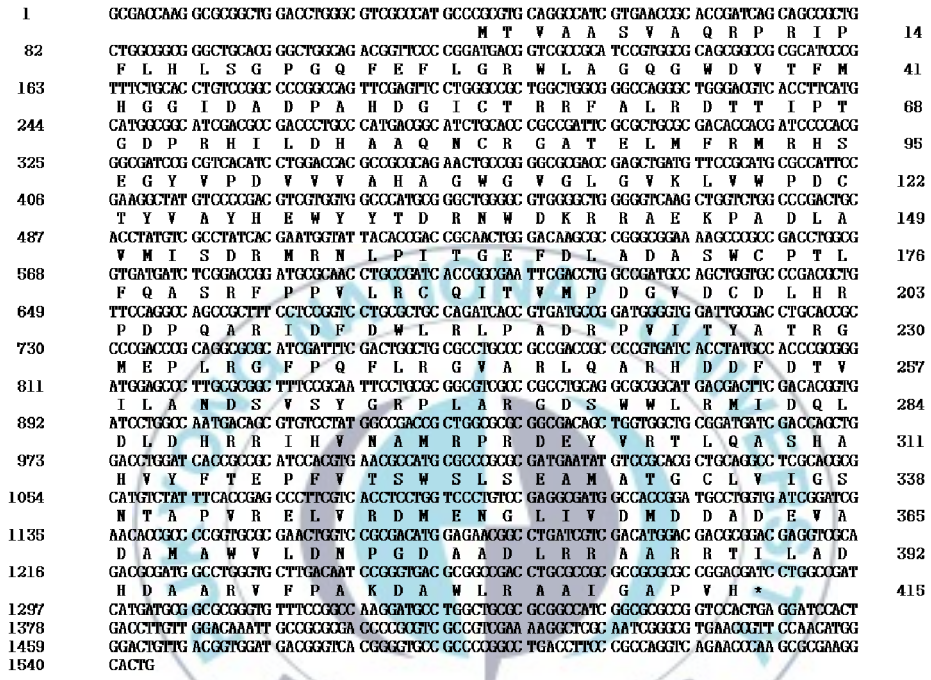


Fig. 4. Nucleotide and deduced amino acid sequences of zeaxanthin diglucoside biosynthesis gene in *Paracoccus haeundaensis*.

Sequence analysis of zeaxanthin diglucoside biosynthesis gene

The nucleotide and deduced amino acid sequences of the zeaxanthin diglucoside biosynthesis gene from *P. haeundaensis* were analyzed (Fig. 4). Amino acid sequence identity was calculated using the Genedoc program. The deduced amino acid sequence of the *P. haeundaensis crtX* gene shown homology and a high degree of sequence conservation with previously identified zeaxanthin diglucoside biosynthesis enzymes from other species (Figure 5). Molecular phylogenetic analysis revealed the evolutionary relationship among the zeaxanthin diglucoside biosynthesis genes of various organisms (Fig. 6). The results indicated evolutionary divergence of the *crtX* genes of *A. marina* MBIC11017, *Lyngbya* sp. PCC 8106, *M. aeruginosa*, *M. nodulans*, *C. limicola* DSM, *G. oxydans*.

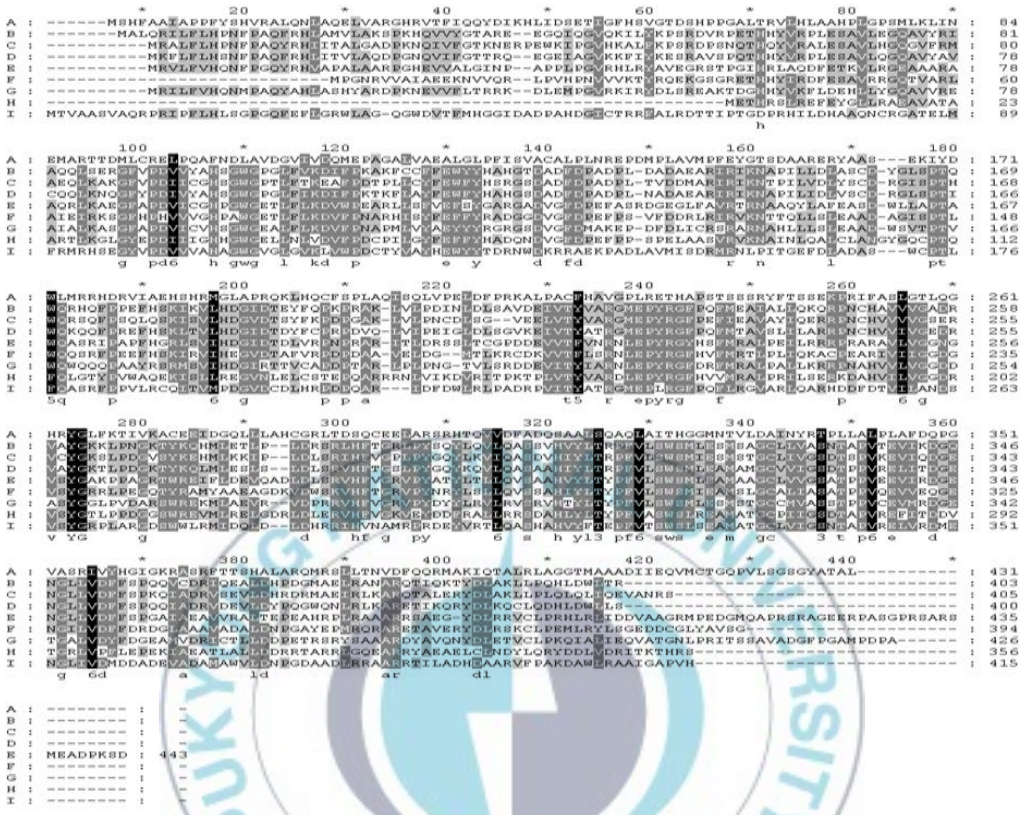


Fig. 5. Multiple alignments of the deduced amino acid sequences of the *P. haeundaensis* zeaxanthin glucosyltransferase with those of other species obtained from GenBank. The accession numbers are given in the text. A, *Pantoea ananatis*; B, *Acaryochloris marina* MBIC11017; C, *Lyngbya* sp. PCC 8106; D, *Microcystis aeruginosa*; E, *Methylobacterium nodulans*; F, *Chlorobium limicola* DSM; G, *Proteobacterium* BAL199; H, *Gluconobacter oxydans*; I, *P. haeundaensis*.

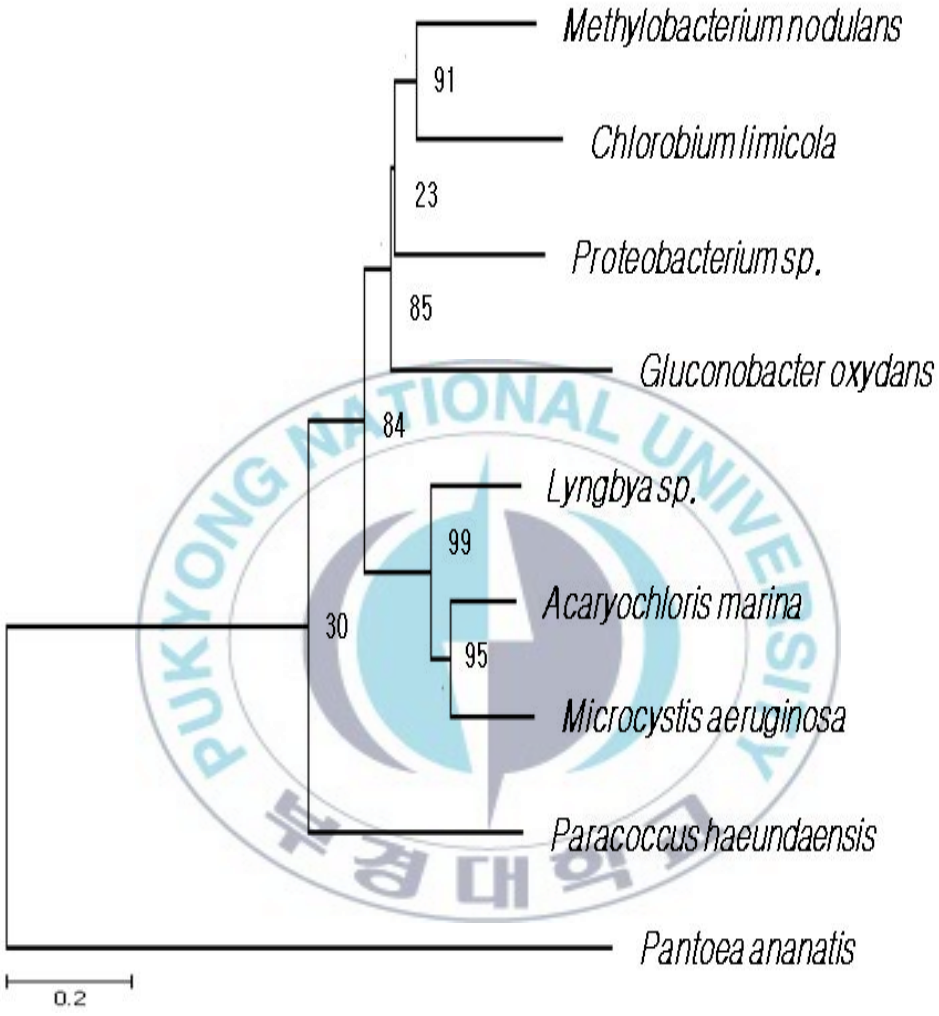
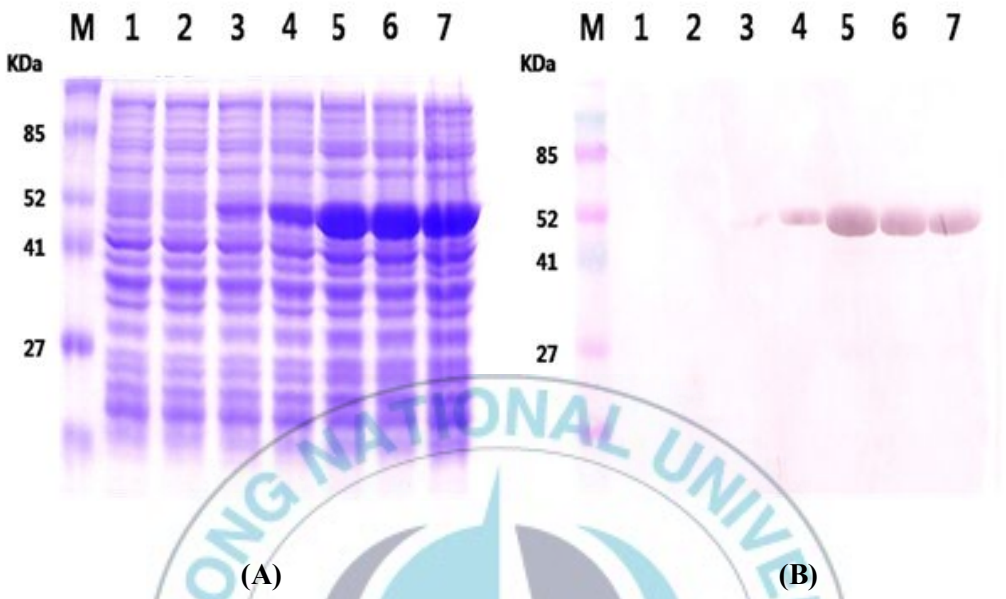


Fig. 6. Molecular phylogenetic tree of zeaxanthin glucosyltransferases based on the NJ method. The values shown on each internal branch are the percent support determined from a bootstrap analysis with 1,000 replications.

Zeaxanthin diglucoside biosynthesis gene expression in *E. coli*

To express the zeaxanthin glucosyltransferase in a prokaryotic system, the *crtX* gene was subcloned into the pSTV29 expression vector to yield pSTCRT-X, which produced the recombinant protein with C-terminal fusion His-tag (Fig. 2A). Expression of the recombinant CrtX protein was induced by adding isopropyl-beta-D-thiogalactopyranoside(IPTG) and incubating at 37°C for various times; production of the recombinant protein was analyzed by 12% SDS-PAGE (Fig. 7A). As shown in Figure 7A, the optimal induction time of recombinant CrtX protein was 5 h. The recombinant CrtX protein had a molecular weight of approximately 46 kDa, as confirmed by Western blotting analysis, which corresponded to the predicted size (Fig. 7B).



(A) **(B)**

Fig. 7. Analysis of the expressed proteins using SDS-PAGE (A) and Western blot (B).

The lane M indicates molecular weight marker; lane 1, proteins from uninduced cell extracts; lane 2-7, proteins from induced cell extracts at 0, 1, 3, 5, 7 hours and overnight after IPTG induction, respectively. (B) Western blot analysis of expressed proteins. Lanes 1-7, proteins used the same order as loaded (A).

Production of zeaxanthin diglucoside in *E. coli*

To produce zeaxanthin diglucoside, pSTCRT-X carrying the *crtX* gene was transformed into the *E. coli* BL21 (DE3) cells containing the plasmid pET-44a(+)-CrtEBIYZ (Fig. 2B), which carried the zeaxanthin biosynthesis gene cluster composed of five structural genes (*crtE*, *crtB*, *crtI*, *crtY* and *crtZ* genes) from *P. haeundaensis*. *E. coli* BL21 (DE3) cell containing the plasmid pET-44a(+)-CrtEBIYZ produced zeaxanthin required for zeaxanthin β -D-diglucoside biosynthesis. The amount of zeaxanthin diglucoside accumulated in the transformed cells by the zeaxanthin diglucoside biosynthesis enzyme were examined using chromatographic and spectrophotometric methods. Biosynthesis of zeaxanthin diglucosides was identified by HPLC analysis of the pigments produced by *E. coli* BL21 (DE3) cells co-transformed with both pSTCRT-X and pET-44a(+)-CrtEBIYZ, as shown in Figure 8. Four main pigment peaks corresponded to zeaxanthin diglucoside, zeaxanthin, β-carotene, and lycopene, as determined by comparison with standard pigment samples. These peaks were eluted at retention time of 3.2 min for zeaxanthin diglucoside, 7.2 min for zeaxanthin, 16.5 min for β-carotene, and 23.4 min for lycopene.

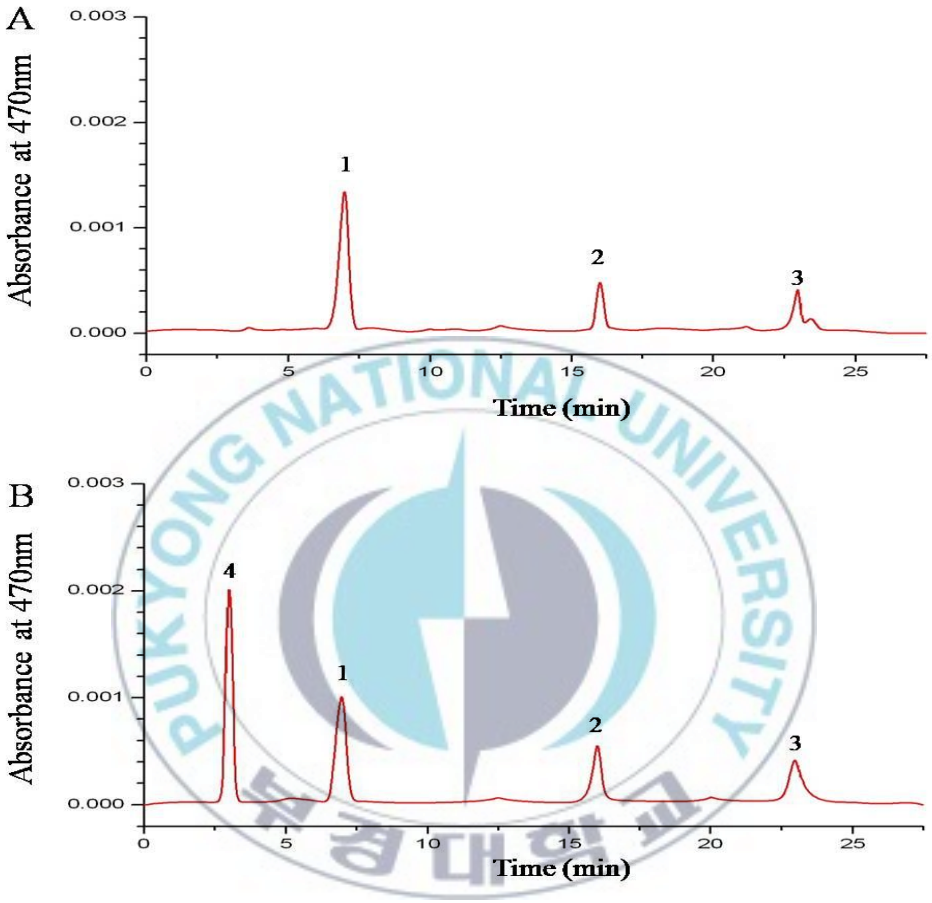


Fig. 8. HPLC analysis of the pigments produced in the *E. coli* strain BL21(DE3) carrying the pET-44a(+)-CrtEBIYZ vector (A) and pSTCRT_X (B). Peak 1, zeaxanthin; peak 2, β-carotene; peak 3, lycopen; peak 4, zeaxanthin diglucoside.

DISCUSSION

The previous study, I described the cloning and sequence analysis of genes encoding the astaxanthin biosynthetic enzymes from this organism [13, 16]. All six genes of the astaxanthin biosynthesis gene cluster of *P. haeundaensis* are required for the production of astaxanthin and encoded ß-carotene ketolase (CrtW), ß-carotene hydroxylase (CrtZ), lycopene cyclase (CrtY), phytoene dehydrogenase (CrtI), phytoene synthase (CrtB) and geranylgeranyl diphosphate synthase (CrtE) [13, 16]. In order to elucidate the mechanism responsible for controlling the astaxanthin biosynthetic pathway and the intracellular carotenoid concentration, it would be necessary to conduct a comparative analysis of the structure, expression, and function of the carotenoid biosynthesis genes.

In the present study, I have identified zeaxanthin diglucoside biosynthesis gene (*crtX*) from the *P. haeundaensis*. The comparison of the amino acid sequence of bacteria zeaxanthin diglucoside indicated that the *P. haeundaensis* zeaxanthin glucosylase are conserved with those of other species. The deduced *P. haeundaensis* zeaxanthin glucosylase amino acid sequence was about 99.0% identical with *Paracoccus* sp. N81106. The molecular phylogenetic tree analysis revealed the evolutionary relationship among the carotenoid biosynthesis genes of various organisms.

Zeaxanthin glucosylase (*crtX*) catalyzes the synthesis of zeaxanthin diglucoside from zeaxanthin in the carotenoid biosynthetic pathway. Zeaxanthine diglucosides are considered to protect cells against photooxidative damage by activated oxygen plus visible light [3] or to stabilize a membrane

environment, similarly to the function of sterols in eucaryotic cells [4].

The *crtX* gene isolated from *P. haeundaensis* was composed of 1248 bp encoding a polypeptide of 415 amino acid residues. The CrtX protein was induced by adding IPTG and induced cells were incubated at 37°C for various incubation times. The optimal induction time for the production of CrtX protein was achieved at 5 hours after induction. The expressed proteins were confirmed by western blot analysis.

In order to produce zeaxanthin diglucoside, I have constructed pET-44a(+)-CrtEBIYZ and pSTCRT_X plasmid DNA. And then two plamid DNAs were co-transformed into *E. coli* BL21 (DE3) and the expression of each plasmid was accomplished by the addition of IPTG. The transformant was cultured at 37℃, 18 hours in DYT medium containing chloramphenicol (170 ㎍/ml) and kanamycine. After 18 hrs culture, the cells were harvested by centrifugation, and sonicated three times for 10 sec with Ultra sonicator, and precipitated by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded. The cell pellets were resuspended in 10 ml acetone and incubated overnight at 4℃. Acetone was evaporated and the pellet was dissolved in 10 ml of n-hexane-ethanol (1:1, v/v). Then the extract was diluted to 1/2 with distilled water, and two phases were separated with a separatory funnel. 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 dry under a stream of nitrogen, and then, the residue was stored in a refrigerator.

The result of the HPLC analysis from the cell transformed with pET-CrtEBIYZ and pSTCRT-X were shown in Figure 8. The main peaks were

turned out to be four pigments corresponding to zeaxanthin diglucoside, zeaxanthin, β-carotene, and lycopene, when these peaks were compared with standard pigments. These peaks were eluted at retention time at 3.2 min for zeaxanthin diglucoside, at 7.2 min for zeaxanthin, at 16.5 min for β-carotene, and at 23.4 min for lycopene, respectively.

In the study, I were cloned and expressed zeaxanthin glucosyltransferase gene (*crtX*) from *P. haeundanesis* zeaxanthin glucosylase gene (*crtX*). Also, The pET-44a(+)-CrtEBIYZ and pSTCRT-X plasmid DNA were co-transformed in BL21(DE3) cell. Using the transformant, I produced zeaxanthine diglcucoside in *E. coli*. The results of this study can be used to enhance the production of zeaxanthin diglucoside through the manipulation of carotenoid biosynthesis genes in *P. haeundaensis*.

국문초록

Zeaxanthin은 carotenoid의 한 종류로, 녹색 식물의 잎에 풍부하게 존재하고, 눈의 건강에 중요하게 작용하는 물질이다.

새로운 해양세균인 *Paracoccus haeundaensis*의 분리 및 동정은 이미 보고되었고, 이는 carotenoid 중 astaxanthin을 주로 생산하는 균주이다. 6개의 유전자로 구성된 astaxanthin 생합성 유전자가 cloning되어 효소 활성을 포함한 그들의 분자적 특징이 이미 보고되었다.

Zeaxanthin glucosyltransferase (CrtX)는 zeaxanthin에서 zeaxanthin diglucoside로의 전환을 매개한다 *Paracoccus haeundaensis*로부터 분리된 zeaxanthin glucosyltransferase 유전자(*crtX*)는, 1248개의 염기쌍으로 구성되며, 415개의 아미노산을 암호화한다. 유전자 *crtX*의 염기 서열은 다른 종의 *crtX* 서열과 함께 분석되어졌고, 확립된 계통도로부터 진화하는 동안 잘 보존되어진 것으로 나타났다. CrtX gene을 포함하는 plasmid (pSTCRT-X)는 단백질 발현을 위해 구성하였고. 이 plasmid가 포함된 *E. coli*에서 약 46 KDa의 zeaxanthin glucosyltransferase 재조합 단백질이 생산되었다. Zeaxanthin β-D-diglucoside의 생합성은 zeaxanthin의 생합성을 위해 요구되는 *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* 유전자들을 운반하는 pET-44a(+)-CrtEBIYZ plasmid를 가지고 있는 *E. coli* 에 pSTCRT-X plasmid를 co-transformation 함으로써 이루어진다. 이 실험은 *Paracoccus haeundaensis*에서 기존에 cloning한 6개의 carotenoid 생합성 유전자 이외에 새로운 유전자가 존재한다는 것을 밝힌 실험으로 그 의미가 있다.

본 논문은 *P. haeundaensis*에서 zeaxanthin diglucoside 생합성 기능이 있는 *crtX* 유전자의 cloning과, 이 유전자를 가진 형질전환 된 *E.coli* 에서 같은 종류의 carotenoid의 생산을 다루었다.

ACKNOWLEDGEMENT

학부 2학년 때 실험실에 들어와서 석사를 졸업할 때까지 5년이라는 시간이 흘렀습니다. 5년이라는 시간동안 좋은 일도 많았고, 힘들었던 순간도 있었습니다. 힘들고 어렵던 순간마다 저를 도와준 많은 사람들이 있었기에 제가 여기까지 올 수 있었습니다. 학부 3년, 석사 2년 모두 5년이라는 시간동안 많은 지도와 격려를 해주신 김영태 지도교수님께 감사의 말씀을 전합니다. 이 논문이 나오기까지 끊임없는 조언과 격려를 해 주신 이명숙 교수님, 김군도 교수님께 고개숙여 감가의 말씀을 드립니다. 또한 모르는 것이 있을 때마다 아낌없는 지도와 조언을 해 주신 이원재 교수님, 김진상 교수님, 이훈구 교수님, 송영환 교수님, 최태진 교수님께 감사드립니다.

처음 실험실에 들어와서 아무것도 모르던 저에게 실험과 이론을 가르쳐 주신 이재형 박사님, '실험이란 이런 것이다.' 라는 것을 보여주신 서용배 선배님께 진심으로 감사드립니다. 모르는 것이 있을 때마다 자상하게 가르쳐주신 전정민 박사님, 이대성 선배님, 정인영 선배님, 김윤숙 선배님, 지근호 형께도 감사하다고 말하고 싶습니다. 학부 2학년 때부터 같이 수업을 들으며, 같이 대학원에 진학하면서 나에게 힘이 되어준 철우 형과 경용이 형에게도 고맙다는 말을 하고 싶습니다. 실험실 후배이면서 나랑 같이 동고동락한 태혁이, 지원이에게도 고마운 마음 뿐 입니다. 또한 내가 실험할 때 옆에서 많이 도와주며 무뚝뚝한 나와도 친하게 지내준 후배들, 은주, 혜경이, 현수, 명석이, 승우, 현택이, 나영이에게도 고마운 마음이 듭니다.

언제나 나에게 큰 힘이 되어준 믿음직한 큰 형 최경석 형과, 언제나 함께인 쌍둥이 형 최강석 형에게도 감사의 말을 전합니다. 마지막으로 제가 이 자리까지 올 수 있었던 가장 큰 원동력이 된 저의 부모님께 감사의 말씀을 드립니다. 가족이라는 큰 힘이 저에게 있었기 때문에 이 자리까지 흔들림 없이 올 수 있었습니다.

저에게 큰 힘이 되어준 많은 분들이 있었기 때문에 오늘의 제가 있을 수 있었고, 그들 모두에게 이 작은 결실을 바치고 싶습니다.

REFERENCES

1. Bartley, G.E. and Scolnik, P.A. 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human heath. Plant Cell 7, 1027-1038.

2. Kurihara, H., Koda, H., Asami, S., Kiso, Y., and Tanaka, T., 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.

3. Bertram, J.S. and Vine, A.L., 2005. Cancer prevention by retinoids and carotenoids: independent action on a common target. Biochim. Biophys. Acta 1740, 170–178.

4. Mayne, S.T. 1996. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J 10, 690-701.

5. Chew, B.P. and Park, J.S., 2004. Carotenoid action on the immune response. J. Nutr. 134, 257S–261S.

6. Amar, E.C., Kiron, V., Satoh, S. and Watanabe, T. 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. Tuveson, R.W., Akyon, R. A. L. and Kagan, J. 1988. Role of cloned carotenoid genes expressed in *Escherichia coli* in protecting against inactivation by near-UV light and specific phototoxic molecules. J. Bacteriol. 170, 4675-4680.

8. Rohmer, M., Bouvier, P. and Ourisson. G. 1979. Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. Proc. Natl. Acad. Sci. U.S.A. 76, 847-51.

9. Hannibal, L., Lorquin, J., D'ortoli, N. A., Garcia, N., Chaintreuil, C., Masson-Boivin, C., Dreyfus, B. and Giraud, E. 2000. Isolation and characterization of canthaxanthin biosynthesis genes from the photosynthetic bacterium *Bradyrhizobium* sp. strain ORS278. J. Bacteriol 182, 3850-3853.

10. Sieiro, C., Poza, M., de Miguel, T. and Villa, T.G. 2003. Genetic basis of microbial carotenogenesis. Int. Microbiol. 6, 11-16.

11. van Spanning, R.J.M., Stouthamer, A.H., Baker, S.C. and van Verseveld, H.W. 2005. In Bergey's Manual of Systematic Bacteriology, Second edition, Volume 2, Part C; Brenner, D.J., Krieg, N.R., Staley, J.T., Eds; Springer, New York, pp197-204.

12. Takaichi, S., Maoka, T., Akimoto, N., Khan, S. T. and Harayama, S. 2006. Major Carotenoid isolated from Paracoccus schoinia NBRC 100637 is adonixanthin diglucoside. J. Nat. Prod. 69, 1823-1825.

13. Harker, M., Hirschberg, J. and Oren,A. 1998. Paracoccus marcusii sp. nov., an orange Gram-negative coccus, Int. J. Syst. Bacteriol. 48, 543-548.

14. Misawa, N., Kajiwara, S., Kondo, K., Yokoyama, A., Satomi, Y., Saito, T., Miki, W. and Ohtani, T. 1995. Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon β-carotene by a single gene. Biochem. Biophys. Res. Commun. 209, 867-876.

15. Pasamontes, L., Hug, D., Tessier, M., Hohmann, H-P., Schierle, J., and A. P. G. M van Loon., 1997. Isolation and characterization of the carotenoid biosynthesis genes of Flavobacterium sp. strain R1534. Gene, 185, 35-41.

16. Berry, A., Janssens, D., Hümbelin, M., Jore, J.P.M., Hoste, B., Cleenwerck, I., Vancanneyt, M., Bretze,l W., Mayer, A.F., Lopez-Ulibarri, R., Shanmugam, B., Swings, J. and Pasamontes, L. 2003. Paracoccus zeaxanthinifaciens sp. nov., a zeaxanthin-producing bacterium. Int. J. Syst. Evol. Microbiol. 53, 231-238.

17. Lee, J.H., Kim, Y.S., Choi, T.J., Lee, W.J. and Kim, Y.T. 2004. Paracoccus haeundaensis sp. nov., a gram-negative, halophilic, astaxanthin-producing bacterium. Int. J. Syst. Evol. Microbiol., 54, 1699-1702.

18. Lee, J.H. and Kim, Y.T. 2006a. Cloning and characterization of the astaxanthin biosynthesis gene cluster from the marine bacterium *Paracoccus*

haeundaensis. Gene 370, 86-95.

19. Lee, J.H. and Kim, Y.T. 2006b. Functional expression of the astaxanthin biosynthesis genes from a marine bacterium, *Paracoccus haeundaensis*. Biotechnol. Lett. 28, 1167-1173.

20. Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321–4325.

21. Kim, Y.T. and Richardson, C.C. 1994. Acidic carboxyl-terminal domain of gene 2.5 protein of bacteriophage T7 is essential for protein-protein interactions. J. Biol. Chem. 269, 5270–5278.

22. Cho, J.J., Lee, J.H., Kim, S.K., Choi, T.J. and Kim, Y.T. 1999. Complementary DNA encoding nm23/NDP kinase gene from the Korean tiger shark *Scyliorhinus torazame*. Mar. Biotechnol. (NY) 1, 131–136.

23. Lee, J.H., Nam, S.W. and Kim, Y.T. 2003. Isolation of cDNAs for gonadotropin-II of flounder (*Paralichthys olivaceus*) and its expressions in adult tissues. J. Microbiol. Biotechnol. 13, 710–716.

24. Van de Peer, Y. and De Wachter, R. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. 10, 569-570.

25. Gershoni JM, Palade GE (1983) Protein blotting: principles and applications. Anal. Biochem. 131: 1-15.