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Molecular Characterization of Carotenoid Biosynthesis Gene Cluster from the Marine Bacterium, *Paracoccus haeundaensis*

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Carotenoids are the most widely distributed class of pigments in nature, displaying yellow, orange, and red color. These pigments are synthesized in bacteria, algae, fungi, and plants and play essential roles as protective agents against oxidative damage [2] and anti-tumor activites [9]. Also, carotenoids are precursors of phytohormones [3] and responsible for the color of many plants and animals. They typically consist of a C_{40} hydrocarbon backbone often modified by different oxygen-containing functional groups, to yield cyclic or acyclic xanthophylls. In the microbial world, carotenoids are present in both anoxygenic and oxygenic photosynthetic bacteria and algae and in many fungi [1, 4, 5, 8]. However, only a few carotenoids (β -carotene, lycopene, astaxanthin, canthaxanthin, lutein, β-apo-8-carotenal, and β-apo-8-carotenal-ester) can be produced commercially by chemical synthesis, fermentation or isolation from the small number of abundant natural sources [6]. Their demands are rapidly increased but their supplies are not enough. The objectives of this study, therefore, are mainly focused on the isolation of new microorganisms that produce carotenoids, the cloning and genetic manipulations of carotenoid biosynthesis genes from the isolated organisms, and the characterization of carotenoids produced from the enzymes overexpressed in transformed *Escherichia coli* with carotenoid biosynthesis genes.

Paracoccus haeundaensis sp. nov., a Gram-negative halophilic astaxanthin-producing bacterium

A new species of aerobic, non-motile, Gram-negative, orange-pigmented, rod-shaped, astaxanthin-producing marine bacterium was isolated from the Haeundae Coast, Korea [7]. This strain, BC74171^T, produced carotenoids, mainly astaxanthin. The optimal temperature and pH for growth is 25℃ and pH 8, respectively. This strain can grow 1-6% (w/v) NaCl concentration. All the type strains of the genus *Paracoccus* were compared with strain BC74171^T using 16S rDNA sequence analysis, fatty acid patterns, and physiological reaction profiles. Based on these results, it is proposed that strain BC74171^T can be classified as a new species, *Paracoccus haeundaensis*. The type strain is BC74171^T (= KCCM 10460^T= LMG P-21903^T).

Isolation of astaxanthin biosynthesis gene cluster from the marine bacterium *Paracoccus haeundaensis*

The strain BC74171^T, *Paracoccus haeundaensis*, produced carotenoids, mainly accumulated with astaxanthin from biosynthesis gene cluster. The carotenoid biosynthesis gene cluster have been cloned and

this gene cluster composed of six genes that identified as β-carotene ketolase (CrtW), ß-carotene hydroxylase (CrtZ), lycopene cyclase (CrtY), phytoene dehydrogenase (CrtI), phytoene synthase (CrtB), and geranylgeranyl diphosphate synthase (CrtE).

The β-carotene ketolase gene (*crtW*) isolated from *Paracoccus haeundaensis* consisted of 726 bp encoding 242 amino acid residues. The deduced amino acid sequence from CrtW of *Paracoccus haeundaensis* showed 98%, 81%, and 58% sequence identity to that of *Paracoccus* sp. MBIC1143, to *Alcaligenes* sp. strain PC1, and to *Bradyrhizobium* sp. ORS278, respectively.

Moreover, the domain of *Histidine-rich-region* from 213 to 224 amino acids (HFGG YHHEHHLH) of *Paracoccus haeundaensis* is well conserved with those of other species. The product of the ß-carotene hydroxylase gene (*crtZ*) isolated from *Paracoccus haeundaensis* consisted of 486 bp encoding a polypeptide of 162 amino acid residues. The deduced amino acid sequence from CrtZ of *Paracoccus haeundaensis* showed 99%, 88%, 65%, and 51% sequence identity to that of *Paracoccus* sp. MBIC1143, to *Alcaligenes* sp. strain PC1, to *Flavobacterium* sp. ATCC21588, and to *Erwinia uredovora,* respectively. It also contains a domain of *Histidine-rich-region* from 22 to 44 amino acids (HRWIMHGPLGWGWHKSHHEEHDH) and this region is well conserved with those of other species.

The products of *crtW* and *crtZ* genes are sufficient for the transformation of β-carotene into astaxanthin for both prokaryotes and eukaryotes, using different keto-carotenoids as intermediates, depending on the microorganisms.

The product of the lycopene cyclase gene (*crtY*) isolated from *Paracoccus haeundaensis* consisted of 1158 bp encoding a polypeptide of 386 amino acid residues. The deduced amino acid sequence from CrtY of *Paracoccus haeundaensis* showed 98%, 71%, 42%, 41%, 44%, 41%, and 19% sequence identity to that of *Paracoccus* sp. MBIC1143, to *Flavobacterium* ATCC21588, to *Erwinia uredovora,* to *Pantoea agglomerans,* to *Xanthobacter* sp. Py2, to *Bradyrhizobium* sp. ORS278, and to *Streptomyces avermitilis,* respectively. These results show different sequence similarities between the species from 98% to 19% of sequence identitity. It contains the domain of *Arginine-rich-region* from 296 to 338 amino acids (RGAIRDYAIDRARRDRFLRLLNRMLFRGCAPDRRYTLLQRFYR) and the sequence of binding to cofactors (DX4GXGXAX4A: from 4 to 18 amino acids). These sequences are well conserved with these of other species and showing a convergent evolution of different types of enzyme that need certain domains to carry out essential functions.

The product of the phytoene dehydrogenase gene (*crtI*) isolated from *Paracoccus haeundaensis* consisted of 1503 bp encoding a polypeptide of 501 amino acid residues. The deduced amino acid sequence from the phytoene dehydrogenase (CrtI) of *Paracoccus haeundaensis* showed 96%, 77%, 62%, 62%, 63%, 61%, 40%, 39%, 30%, and 9% sequence identity to that of *Paracoccus* sp. MBIC1143, to *Flavobacterium* ATCC21588, to *Erwinia uredovora,* to *Pantoea agglomerans,* to *Bradyrhizobium* sp. ORS278, to *Xanthobacter* sp. Py2, to *Rhodobacter capsulatus,* to *Rhodobacter sphaeroides,* to *Streptomyces avermitilis*-1, and to *Streptomyces avermitilis*-2, respectively. There is a high similarity between the CrtI of *Paracoccus haeundaensis* and *Paracoccus* sp. MBIC1143. Phytoene dehydrogenase signature region from 466 to 486 amino acids (NFYLVGAGTHPGAGLPGVVGS) of *Paracoccus haeundaensis* and this region

shows a great similarity with other species.

The product of the phytoene synthase gene (*crtB*) isolated from *Paracoccus haeundaensis* consisted of 912 bp encoding a polypeptide of 304 amino acid residues. The deduced amino acid sequence from CrtB of *Paracoccus haeundaensis* showed 92%, 79%, 46%, 45%, 50%, 40%, 30%, 29%, and 31% sequence identity to that of *Paracoccus* sp. MBIC1143, to *Flavobacterium* sp. ATCC21588, to *Erwinia uredovora,* to *Pantoea agglomerans,* to *Xanthobacter* sp. Py2, to *Bradyrhizobium* sp. ORS278, to *Rhodobacter capsulatus,* to *Rubrivivax gelatinosus,* to *Rhodobacter sphaeroides,* respectively. Phytoene synthases have been known to have sequence homology with the diapophytoene synthase, and the squalene synthases, which condense two molecules of farnesyl pyrophosphate to form diapophytoene and squalene, respectively. The domain consisted of Y-[CSAM]-x(2)-[VSG]-A-[GSA]-[LIVAT]-[IV]-G-x(2)-[LMSC]-x(2)-[LIV] is typical conserved regions of the squalene and phytoene synthases. In the amino acid sequence of CrtB, there are two signature regions of squalene and phytoene synthases from 130 to 145 amino acids (YSYHVAGIVGVMMARV) and from 160 to 185 amino acids (LGLAFQLTNIARDVIDARIGRCYIP). These regions are well conserved with those of other species.

The product of the geranylgeranyl diphosphate synthase gene (*crtE*) isolated from *Paracoccus haeundaensis* consisted of 879 bp encoding a polypeptide of 293 amino acid residues. The deduced amino acid sequence from CrtE of *Paracoccus haeundaensis* showed 64%, 31%, 29%, 26%, 33%, 29%, and 16% sequence identity to that of *Flavobacterium* sp. ATCC 21588, to *Erwinia uredovora,* to *Pantoea agglomerans,* to *Rhodobacter capsulatus,* to *Bradyrhizobium* sp. ORS278, to *Rhodobacter sphaeroides,* and to *Streptomyces avermitilis,* respectively. A typical signature domain of polyprenyl synthetases was found from 80 to 96 (LIFDDLPCMDDAGLRRG) amino acids and these regions are well conserved with those of other species.

Characterization of proteins from the carotenoid biosynthesis gene cluster in *E. coli*

The carotenoid biosynthesis gene cluster was transformed into *E. coli* BL21(DE3) codon plus, and the transformed cell produced carotenoids, mainly astaxanthin. The production of astaxanthin from the transformed cell was corresponded to the amount of 488 μg (astaxanthin)/g (dry cell weight).

In order to characterize the enzymatic properties of each gene product of carotenoid biosynthesis gene cluster from *Paracoccus haeundaensis*, each gene was subcloned into the expression vector, pET-44(a)+ vector. The result showed that the plasmid containing *crtW, crtZ, crtY, crtI, crtB*, and *crtE* genes produced the recombinant proteins of 27kDa, 18kDa, 42kDa, 55kDa, 33kDa, and 30kDa, respectively. Each protein was purified to homogeneity and the purified proteins were enzymatically active. The enzymatic properties of each gene product of carotenoid biosynthesis gene cluster from *Paracoccus haeundaensis*, were characterized and analyzed.

Functional studies on the carotenoid biosynthesis gene cluster

E. coli BL21(DE3) codon plus has been engineered, to confer a novel biosynthetic pathway for the astaxanthin, zeaxanthin, β-carotene, lycopene, and phytoene by introducing the *Paracoccus haeundaensis*

carotenoid biosynthesis genes. The colony transformed with either pET-CrtE or pET-CrtEB plasmid did not show any colors. However the colony transformed with each of pET-CrtEBI, pET-CrtEBIY, pET-CrtEBIYZ, and pET-CrtEBIYZW plasmid (Fig. 1) showed red, yellow, orange, and red color, respectively. The carotenoids accumulated in the cells produced by the combinations of the carotenoid biosynthesis genes from *Paracoccus haeundaensis* were analyzed by chromatographic and spectroscopic methods.

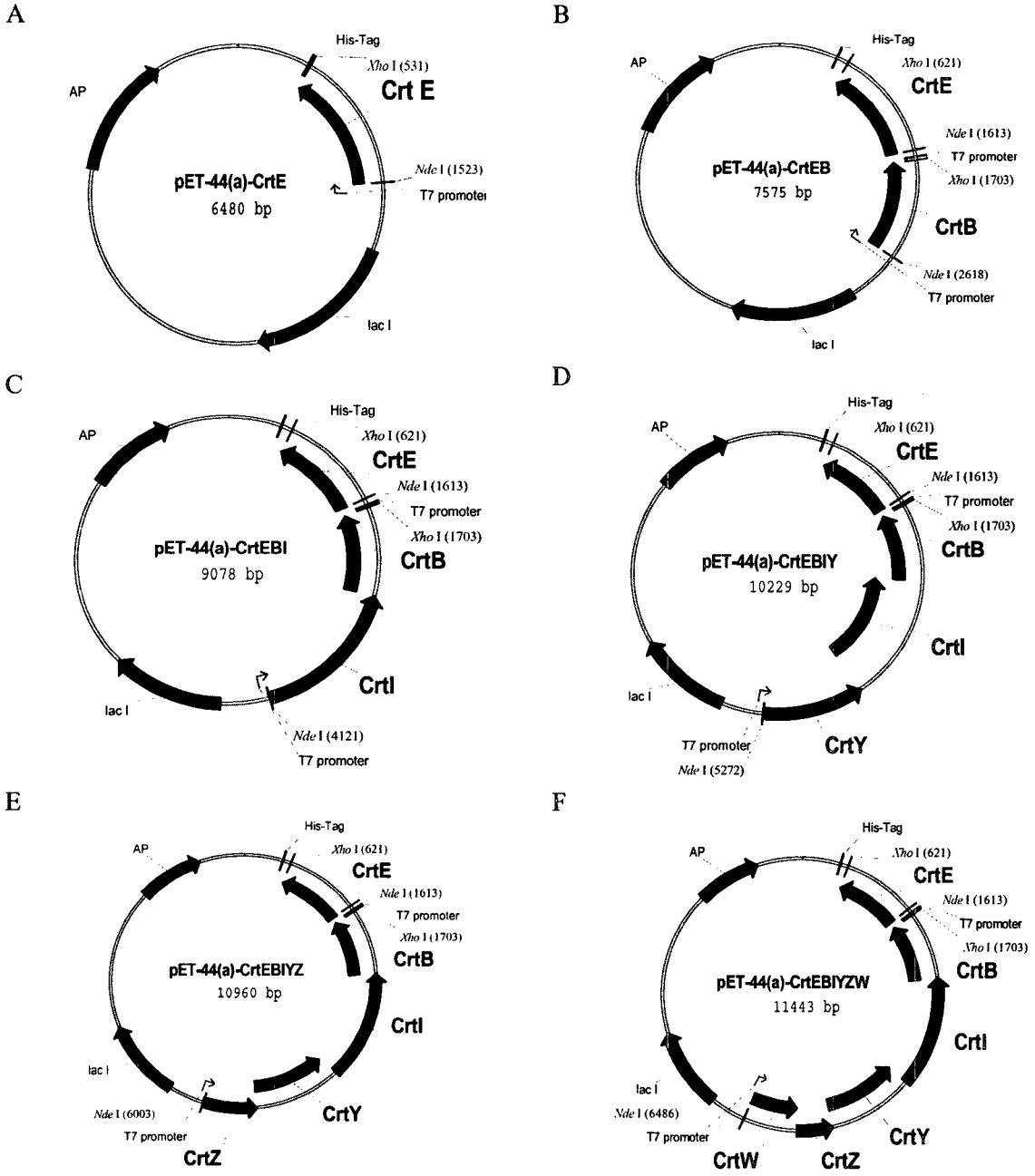

Figure 1. Construction of expression plasmids for crt genes. pET-44(a)-CrtE (A), pET-44(a)-CrtEB (B), pET-44(a)-CrtEBI (C), pET-44(a)-CrtEBIY (D), pET-44(a)-CrtEBIYZ (E), and pET-44(a)-CrtEBIYZW (F) plasmid.

Conclusion

This is the first report on the isolation and identification of the marine bacteria strain producing carotenoids in Korea and the molecular cloning of the genes required in the carotenoid biosynthesis. We characterized the enzymatic properties of the recombinant proteins manipulated from the carotenoid biosynthesis genes. The carotenoid biosynthesis gene cluster is organized in the order of *crtE-B-I-Y-Z-W* genes. The comparative data of the amino acid sequences of carotenoid biosynthesis gene products indicate that the products of carotenoid biosynthesis gene cluster from *Paracoccus haeundaensis* are highly conserved with those of other species.

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