When Carotenoid Biosynthesis Genes Met *Escherichia coli* : The Early Days and These Days

15

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Abstract

Nowadays, carotenoid biosynthetic pathways are sufficiently elucidated at gene levels in bacteria, fungi, and higher plants. Also, in pathway engineering for isoprenoid (terpene) production, carotenoids have been one of the most studied targets. However, in 1988 when the author started carotenoid research, almost no carotenoid biosynthesis genes were identified. It was because carotenogenic enzymes are easily inactivated when extracted from their organism sources, indicating that their purification and the subsequent cloning of the corresponding genes were infeasible or difficult. On the other hand, natural product chemistry of carotenoids had advanced a great deal. Thus, those days, carotenoid biosynthetic pathways had been proposed based mainly on the chemical structures of carotenoids without findings on relevant enzymes and genes. This chapter shows what happened on carotenoid research, when carotenoid biosynthesis genes met non-carotenogenic *Escherichia coli* around 1990, followed by subsequent developments.

Keywords

Carotenoid biosynthetic pathway · *Escherichia coli* · *Pantoea ananatis* · *Erwinia uredovora* · Land plants · β-Carotene ketolase

15.1 Introduction

Carotenoids are biosynthesized in all photosynthetic prokaryotes that contain photosynthetic bacteria and cyanobacteria, in all photosynthetic eukaryotes including algae and land plants, and further in some non-photosynthetic bacteria and fungi. Nowadays, carotenoid biosynthetic pathways are sufficiently elucidated at gene levels in bacteria, fungi, and higher plants. Also, in pathway engineering for isoprenoid (terpene) production, carotenoids have been one of the most studied targets (Misawa 2011). However, almost no knowledge about enzymes and genes involved in carotenoid biosynthesis had been available up to 32 years ago. The present chapter shows what happened on carotenoid research, when carotenoid biosynthesis genes met *Escherichia coli* those days, followed by subsequent developments.

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15.2 The Early Days When Carotenoid Biosynthesis Genes Met *Escherichia coli*

In 1988 when I started carotenoid research as an employee of Kirin Brewery Co. Ltd., no carotenoid biosynthesis genes were identified. It was because carotenogenic enzymes are easily inactivated when extracted from their organism sources, indicating that their purification and the subsequent cloning of the corresponding genes were infeasible or difficult. On the other hand, natural product chemistry of carotenoids had advanced a great deal (Goodwin and Britton 1988). Thus, those days, carotenoid biosynthetic pathways had been proposed based on the chemical structures of carotenoids or metabolic analysis without findings on relevant enzymes and genes (Britton 1988). Carotenoids were also the first group of compounds among isoprenoids, which were synthesized from foreign genes in non-carotenogenic *E. coli* as a heterologous host (Sandmann et al. 1999). The first report was the cloning of a 12.4-kb carotenogenic gene cluster in *E. coli*, resulting in yellow pigmentation (Perry et al. 1986; Tuveson et al. 1988). This gene cluster was derived from *Erwinia herbicola* (reclassified as *Pantoea agglomerans*) that belongs to the same *γ-Proteobacteria* class as *E. coli*. This result represented that genes for the yellow pigment were functionally expressed in *E. coli*. Up to then, molecular biologists of carotenoids had noted carotenogenic genes from photosynthetic bacteria such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, which belong to the *α-Proteobacteria* class. DNA sequence of a 11-kb *R. capsulatus* carotenoid biosynthesis gene cluster was determined in 1989, which was the first reports on nucleotide sequence of carotenogenic genes (Armstrong et al. 1989; Bartley and Scolnik 1989), while its gene functions had remained unclear.

We were able to isolate a yellow pigment-generating gene cluster from *Erwinia uredovora* (reclassified as *Pantoea ananatis*) as a 6.9-kb fragment using *E. coli* (Misawa et al. 1990). This gene cluster was sequenced and found to contain six open reading frames (ORFs) (Misawa et al. 1990). Its three ORFs were found to exhibit significant homology to the *crtE*, *crtI*, and *crtB* genes in the carotenogenic gene cluster of *R. capsulatus*. Thus, the same designation was applied to the corresponding three ORFs, and the other *P. ananatis* three ORFs (novel genes) were newly designated *crtX*, *crtY*, and *crtZ*. *E. coli* cells carrying the six genes of *P. ananatis* were found to produce zeaxanthin 3,3′-β-D-diglucoside as a main carotenoid by chromatographic and spectroscopic analysis (Nakagawa and Misawa 1991). Next, each ORF was disrupted using a unique restriction endonuclease site, and *E. coli* cells carrying the remaining five ORFs or *E. coli* cells that carried various combinations among the six ORFs were analyzed by chromatographic and spectroscopic methods. Consequently, the *crtI*, *crtY*, *crtZ*, and *crtX* genes were found to be responsible for the conversion of phytoene (15,15′-*cis*) to lycopene (all-*trans*), lycopene to β-carotene, β-carotene to zeaxanthin, and zeaxanthin to zeaxanthin 3,3′-β-D-diglucoside, respectively (Fig. 15.1) (Misawa et al. 1990). The *crtE* and *crtB* genes were shown to encode geranylgeranyl diphosphate (GGPP) synthase [its substrates: farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP)] and phytoene synthase, respectively, by metabolic analysis using crude enzyme extracts (Fig. 15.1) (Math et al. 1992; Sandmann and Misawa 1992).

Characters of the gene products (enzymes), CrtE, CrtI, CrtY, and CrtX, were further examined using the respective proteins synthesized in recombinant *E. coli* cells (Fraser et al. 1992; Hundle et al. 1992; Schnurr et al. 1996; Wiedemann et al. 1993). The above-mentioned results also demonstrated the suggestion, made some years earlier, that carotenogenic enzymes typically recognize a particular half-molecule, end group, or structural feature rather than a specific whole molecule (Britton et al. 2017), e.g., CrtY catalyzes reactions for converting lycopene and γ-carotene into γ-carotene and β-carotene, respectively, and CrtZ for converting β-carotene and β-cryptoxanthin into β-cryptoxanthin and zeaxanthin, respectively, as shown in Fig. 15.1.

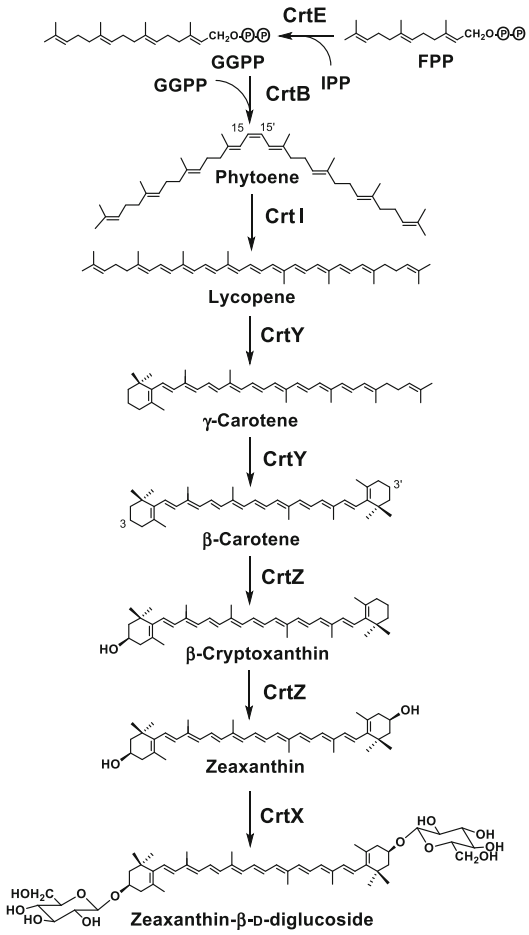

Fig. 15.1 Carotenoid biosynthetic pathway of the *Pantoea* genus containing *Pantoea ananatis* and *Pantoea agglomerans* and functions of the gene products (the enzymes encoded by the genes)

15.3 Subsequent Rapid Advance

The above-described results significantly indicated that we obtained a new and powerful tool to analyze the functions of carotenoid biosynthesis genes, since we became able to synthesize in *E. coli* basic carotenoids that contain phytoene, lycopene, β-carotene, and zeaxanthin and GGPP, as substrates. Since 1992, numerous carotenoid biosynthesis genes have been isolated from bacteria including cyanobacteria and other *Proteobacteria,* fungi including yeasts, algae, and land plants that contained higher plants and bryophytes, and the great majority has been functionally analyzed using the recombinant *E. coli* strains, which has resulted in their functional assignments (Fraser and Bramley 2004; Misawa 2010; Nishida et al. 2005). For example, as for carotenogenic genes from cyanobacteria and higher plants, phytoene synthase genes [*pys* and *PSY* (pTOM5)] that exhibit homology to the *crtB* gene were first isolated from *Synechococcus* PCC7942 and tomato, respectively (Chamovitz et al. 1992; Ray et al. 1987), and functionally confirmed using GGPP-accumulating *E. coli* cells due to the presence of the *P. ananatis crtE* gene (Chamovitz et al. 1992; Misawa et al. 1994). Phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*) genes from higher plants were cloned and functionally analyzed in *E. coli* (Bartley et al. 1999; Linden et al. 1994; Pecker et al. 1992).

Figure 15.2 shows carotenoid biosynthetic pathway common to land plants, which has been elucidated at gene levels (Giuliano 2014; Moise et al. 2014; Takemura et al. 2014; Zhu et al. 2003). LCYb (lycopene β-cyclase) and BHY (-β-carotene 3,3′-hydroxylase; also called CHYb and BCH) of land plants show homology as well as the same functions to CrtY and CrtZ, respectively. On the other hand, the route from phytoene to lycopene requires four enzymes, PDS, Z-ISO (ζ-carotene isomerase), ZDS, and CRTISO (carotene isomerase) in higher plants, which is comparable to one enzyme reaction with CrtI. Cyanobacteria also retain the four enzyme-mediated desaturation reactions same to higher plants, with the exception that CrtI is used in *Gloeobacter violaceus* which partially retains ancestral properties of cyanobacteria (Tsuchiya et al. 2005).

Cytochromes P450 typically require redox partner proteins such as NADPH-P450 reductase to exert their catalytic activity (Chang et al. 2007; Hannemann et al. 2007; Nodate et al. 2006). It is thus worth noting that the *CYP97A* and *CYP97C* genes were functionally expressed without a heterologous redox partner gene in *E. coli* that naturally does not possess any P450 and catalyzed the synthesis of zeinoxanthin and lutein there, respectively (Fig. 15.2) (Kim and Della Penna 2006; Quinlan et al. 2007). It was also confirmed using

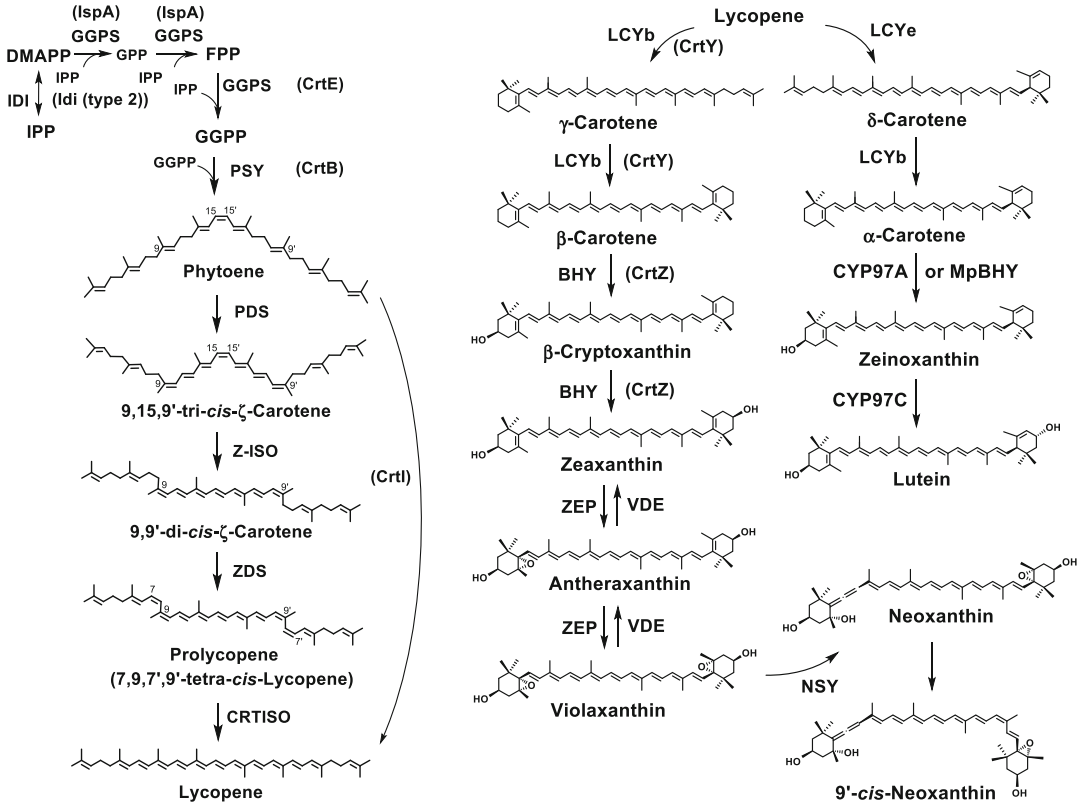

Fig. 15.2 Carotenoid biosynthetic pathway of land plants containing higher plants and liverworts and functions of the gene products
Gene product names from bacteria are enclosed in parentheses and shown as reference. Idi (type 2) and IspA were described by Kaneda et al. (2001) and Fujisaki et al. (1990), respectively.
DMAPP dimethylallyl diphosphate, *GPP* geranyl diphosphate, *GGPS* GGPP synthase

α-carotene-accumulating *E. coli* that in liverwort *Marchantia polymorpha*, nonheme β-carotene 3,3′-hydroxylase (MpBHY) can bifunctionally convert α-carotene into zeinoxanthin instead of CYP97A (Fig. 15.2) (Takemura et al. 2015).

Nowadays, all of the carotenoids shown in Fig. 15.2, except for neoxanthin (and its 9′-*cis* form), can be synthesized in *E. coli* (Takemura et al. 2019). IPP isomerase (Idi) is known as one of rate-limiting step enzymes for the biosynthesis of isoprenoids including carotenoids in *E. coli* (Harada and Misawa 2009). We first showed that further expression of an exogenous *IDI* gene in recombinant *E. coli* cells, that synthesized carotenoids, resulted in two to threefold increase of carotenoid content (Kajiwara et al. 1997).

15.4 The Early Days When Astaxanthin Biosynthesis Genes Met *Escherichia coli*

Recently, astaxanthin, one of commercialized carotenoids, attracts a lot of attention because of its diverse clinical benefits against age-related functional decline and muscle or eye fatigue (Guerin et al. 2003; Kidd 2011; Yamashita 2006). However, astaxanthin had only been noted as the red pigment used for aquaculture, until its strong antioxidant activity was suggested (Miki 1991). Marine Biotechnology Institute (MBI) isolated some marine bacteria that produced astaxanthin (Yokoyama et al. 1995, 1996). Independently in almost the same time, Hebrew University and JX Nippon Oil & Energy

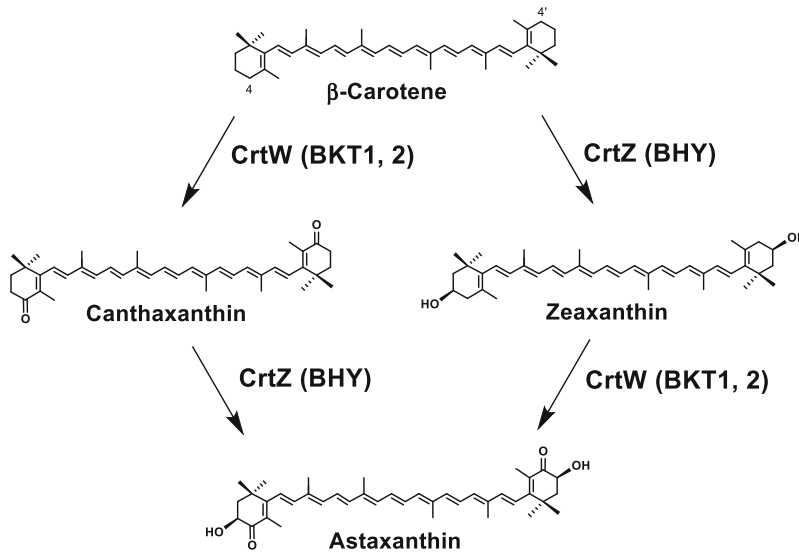

Fig. 15.3 Astaxanthin biosynthetic pathway of the *Paracoccus* genus including *Paracoccus* sp. strain N81106 and green alga *Haematococcus pluvialis* and functions of the gene products
The biosynthetic pathway to β-carotene in the *Paracoccus* genus is the same as that shown in Fig. 15.1. Gene product names from *H. pluvialis* are enclosed in parentheses

Corporation (now, ENEOS Corporation) isolated a soil bacterium (named *Paracoccus marcussi*) and a river bacterium (named *Paracoccus carotinifaciens*), respectively, as astaxanthin producers (Harker et al. 1998; Tsubokura et al. 1999). These bacteria belonged to the *α-Proteobacteria* class.

According to color change in *E. coli*, we first isolated an astaxanthin biosynthesis gene cluster (Misawa et al. 1995a, b) from a marine bacterium *Agrobacterium aurantiacum* (Yokoyama et al. 1995), which was later renamed to *Paracoccus* sp. strain N81106. The functions of the individual genes were identified by the same methods as those of the *Pantoea* genes (Misawa et al. 1995b). A novel gene, named *crtW*, was found to encode β-carotene (β,β-carotenoid) 4,4′-ketolase (Misawa et al. 1995a). Figure 15.3 shows the biosynthetic pathway of astaxanthin from β-carotene.

A gene named *bkt* [renamed *BKT2* by Huang et al. (2006)] that shares homology to *crtW* was isolated from green alga *Haematococcus pluvialis* (Kajiwara et al. 1995). Separately from us, Lotan and Hirschberg (1995) isolated a similar gene from this alga and named *crtO* [renamed *BKT1* by Huang et al. (2006)]. In *H. pluvialis*, β-carotene is converted to astaxanthin with the same biosynthetic routes to Fig. 15.3 by BKT1 or BKT2 (CrtW homolog) and by β-carotene (β,β-carotenoid) 3,3′-hydroxylase (BHY; CrtZ homolog). We also carried out enzyme characterizations of ketolases, CrtW and BKT2, along with CrtZ (Fraser et al. 1997, 1998).

Postscript and Acknowledgment This chapter was written intending to reveal or recall what happened on carotenoid research, when carotenoid biosynthesis genes met *Escherichia coli* in the beginning, as a witness of such exciting events of those days.
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