Chapter 7

Engineering *Escherichia coli* for Canthaxanthin and Astaxanthin Biosynthesis

Qiong Cheng and Luan Tao

Abstract

Escherichia coli is a non-carotenogenic bacterium that could synthesize farnesyl pyrophosphate precursor through the isoprenoid pathway. Carotenoid production in *E. coli* requires heterologous expression of carotenoid synthesis genes. The carotenoid synthesis operons are assembled from genes isolated from carotenogenic bacterial sources. Expression of the different operons yields different carotenoid titers. The operons containing the *idi* gene give more than fivefold higher carotenoid titers than the operons lacking the *idi* gene. The carotenoid modification genes encoding ketolases and hydroxylases are incorporated into the operons for canthaxanthin and astaxanthin production. The ketolases and hydroxylases from different bacterial sources produce astaxanthin of different purity relative to the total carotenoids. Expression of the ketolases and hydroxylases closer to the promoter appears to give higher astaxanthin purity than expression farther from the promoter at the end of the operons. Balanced expression of ketolases and hydroxylases is critical to achieve high astaxanthin purity. Here, we describe methods to assemble carotenoid biosynthesis operons from carotenogenic gene clusters isolated from different bacterial sources and evaluate canthaxanthin or astaxanthin production in *E. coli*.

Key words: Carotenoid biosynthesis, Overlapping PCR, Gene cluster, Astaxanthin, Canthaxanthin

1. Introduction

Astaxanthin and canthaxanthin are high value carotenoids that are widely used in aquaculture and poultry industries. They have also recently been introduced into nutraceutical and personal care markets. Study synthesis of astaxanthin and canthaxanthin in *E. coli* provides valuable insights for biological production of these carotenoids in microbes.

Carotenoid biosynthesis starts with the common isoprenoid pathway (1) to generate farnesyl pyrophosphate precursor (Fig. 1). The *idi* gene encoding isopentenyl pyrophosphate isomerase is one of the genes in the isoprenoid pathway that is responsible for

José-Luis Barredo (ed.), *Microbial Carotenoids from Bacteria and Microalgae: Methods and Protocols*, Methods in Molecular Biology, vol. 892, DOI 10.1007/978-1-61779-879-5_7, © Springer Science+Business Media, LLC 2012

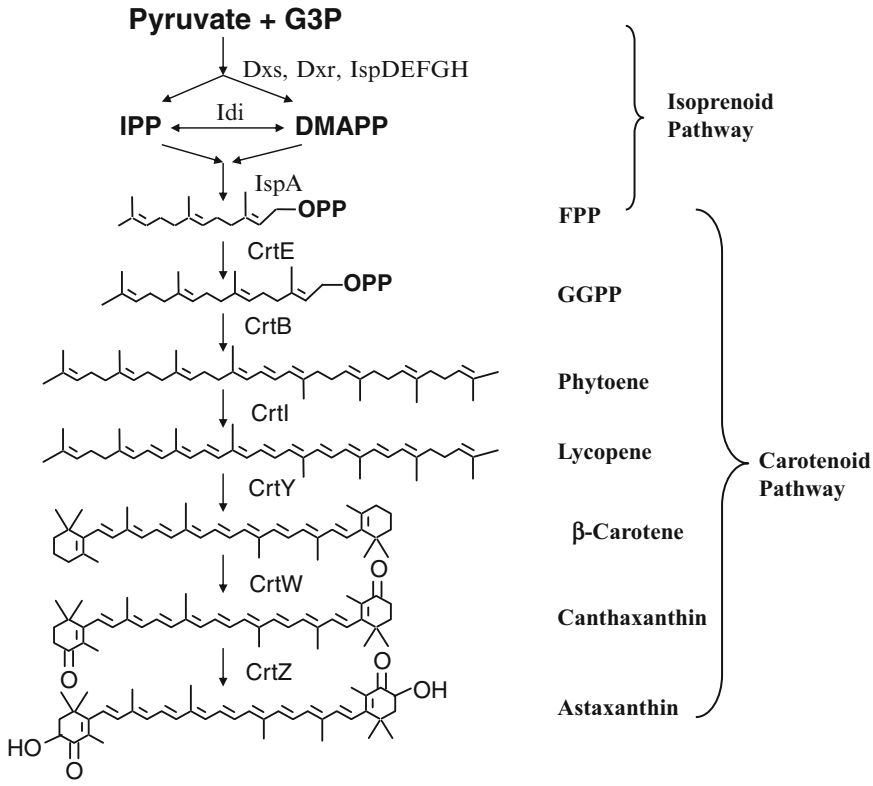


Fig. 1. Pathway for synthesis of canthaxanthin and astaxathin. Multiple steps in the non-mevalonate isoprenoid pathway catalyzed by Dxs, Dxr, IspD, IspE, IspF, IspG, and IspH are not shown in detail. Idi: isopentenyl pyrophosphate isomerase; IspA: fanesyl pyrophosphate synthase; CrtE: geranylgeranyl pyrophosphate synthase; CrtB: phytoene synthase; CrtI: phytoene desaturase; CrtY: lycopene β-cyclase; CrtW: 4, (4′)-β-ionone ring ketolase; CrtZ: 3, (3′)-β-ionone ring hydroxylase. The abbreviations for the compounds are as follows. *G3P* glyceraldehyde 3-phosphate, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophophate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate.

the intramolecular conversion of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, both of which are the substrates for synthesis of farnesyl pyrophosphate. The carotenoid pathway (2) enzymes (CrtEBIY) present in common carotenogenic organisms are involved in subsequent synthesizing C_{40} carotenes, such as β-carotene.

Overexpression of endogenous or exogenous *idi* gene not linked to the carotenogenic gene cluster was reported to increase carotenoid titer (3–5). The ketolase and hydroxylase (CrtWZ) modifies the β-carotene ring to create oxygenated derivatives, such as canthaxanthin and astaxanthin. The oxygenation is the rate-limiting step for astaxanthin synthesis which could generate multiple intermediates that decrease astaxanthin purity as measured by the percentage of astaxanthin produced relative to the total carotenoid content. Previous in vitro (6) and in vivo (7, 8) studies showed that the CrtW and CrtZ enzymes from different bacterial sources had different activities and substrate specificities.

Here, we show the method to assemble β-carotene synthesis operons from carotenogenic gene clusters isolated from different bacterial sources. We also describe how to incorporate different *crtWZ* gene combinations into the operon for canthaxanthin and astaxanthin synthesis. Balanced high expression of ketolases and hydroxylases is critical to achieve high astaxanthin purity.

2. Materials

2.1. Sources of β-Carotene Synthesis Gene Clusters

1. *crtEXYIBZ* gene cluster from *Enterobacteriaceae* bacterium DC260 (GenBank accession number DQ090833).
2. *crtEidiYIBZ* gene cluster from *Enterobacteriaceae* bacterium DC404 (GenBank accession number DQ090834).
3. *crtEXYIBZ* gene cluster from *Enterobacteriaceae* bacterium DC416 (GenBank accession number DQ090835).
4. *crtEidiXYIBZ* gene cluster from *Enterobacteriaceae* bacterium DC413 (GenBank accession number DQ090836).

2.2. Sources of Carotenoid Modification Genes

1. *crtW* from *Sphingomonas* sp. DC18 (GenBank accession number DQ400932).
2. *crtW* from *Brevundimonas vesicularis* DC263 (GenBank accession number DQ309446).
3. *crtW* synthetic gene from *Paracoccus* sp. MBIC1143 (formerly classified as *Agrobacterium aurantiacum*) (GenBank accession number D58420).
4. *crtW* from *Algoriphagus* sp. KK10202C (GenBank accession number DQ286432).
5. *crtZ* from *Brevundimonas vesicularis* DC263 (GenBank accession number DQ309446).
6. *crtZ* from *Novosphingobium aromaticivorans* ATCC700278 (Accession number AAAV01000142.1).
7. *crtZ* synthetic gene from *Paracoccus* sp. MBIC1143 (formerly classified as *Agrobacterium aurantiacum*) (GenBank accession number D58420).
8. *crtZ* from *Enterobacteriaceae* strain DC260 (GenBank accession number DQ090833).
9. *crtZ* from *Enterobacteriaceae* strain DC404 (GenBank accession number DQ090834).

2.3. DNA Oligonucleotide Primers

1. Forward primer 1: 5′-tataGAATTC**CCTAGG**TGCAAGTAA GGACTGCCATTATGAC-3′.
2. Reverse primer 1: 5′-GATGTCATAGTACTTCTCCGCGCG CTGTACATCATAGCGTTTCTCCGGCGCGAT-3′.

3. Forward primer 2: 5′-ATCGCGCCGGAGAAACGCTATGA TGTACAGCGCGCGGAGAAGTACTATGACATC-3′.
4. Reverse primer 2: 5′-tataGAATTCACTAGTTAACGCGGA CGCTGCCAGAG-3′.
5. Sequencing primer 1: 5′-AACCAGACCGTTCAGCTGGA-3′.
6. Sequencing primer 2: 5′-GGTGTAACAAGGGTGAACAC-3′.
7. crtW forward primer: 5′-tataTCTAGA**AAGGAGGAAT AAACC**ATGCGGCAAGCGAACAGGATG-3′.
8. crtW reverse primer: 5′-tataCCTAGGCTAGCTGAACAAA CTCCACCAG -3′.
9. crtZ forward primer: 5′-tataTCTAGA**AAGGAGGAAT AAACC**ATGTCCTGGCCGACGATGATC-3′.
10. crtZ reverse primer: 5′-tataACTAGTCAGGCGCC GTTGCTGGATGA-3′.

2.4. Reagents and Equipment

1. *PfuUltra*® II Fusion HS DNA Polymerase (Stratagene, Santa Clara, CA, USA).
2. GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA).
3. DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA, USA).
4. Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA).
5. Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
6. Reliant® Gel System 1.0% Seakem® Gold agarose gel (Cambrex BioSciences, Rockland, ME, USA).
7. High DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA).
8. 10× BlueJuice™ Gel Loading Buffer (Invitrogen, Carlsbad, CA, USA).
9. 10× TBE buffer (10× electrophoresis TBE buffer stock solution pH 8.3): Weight 86.4 g of Tris, 44 g boric acid and 7.36 g of EDTA. Add water and dissolve. Bring to 800 mL with water. For 1× working solution, dilute stock solution 1:10 with sterilized water.
10. pBHR1 plasmid (Mobitec, Marco Island, FL, USA).
11. Restriction enzymes: *Eco*RI, *Spe*I, *Xba*I, *Avr*II.
12. T4 DNA ligase.
13. *E. cloni* 10G electrocompetent cells (Lucigen, Middletown, WI, USA).
14. Gene Pulser® (Bio-Rad, Hercules, CA, USA).
15. Gene Pulser® Cuvette, 1 mm electrode gap (Bio-Rad, Hercules, CA, USA).

16. Recovery medium (Lucigen, Middletown, WI, USA).
17. LB: 5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl. Adjust pH to 7.5 with 1 N NaOH. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
18. LB agar: LB and 15 g/L agar before autoclaving. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
19. Kanamycin (Sigma, St. Louis, MO, USA).
20. QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).
21. Agilent Series 1100 LC/MSD SI (Agilent Technologies, Santa Clara, CA, USA).
22. 150 mm×4.6 mm ZORBAX C18 (3.5 μm particles) HPLC column (Agilent Technologies, Santa Clara, CA, USA).
23. HPLC Buffer A: 95% acetonitrile and 5% dH_2O; Buffer B: 100% tetradehydrofuran.
24. β-carotene (Sigma, St. Louis, MO, USA).
25. Canthaxanthin (CaroteNature, Lupsingen, Swizerland).
26. Zeaxanthin (CaroteNature, Lupsingen, Swizerland).
27. Astaxanthin (CaroteNature, Lupsingen, Swizerland).

3. Methods

3.1. Overlapping PCR Amplification of crtEidiYIB Genes from Strain DC413

The native carotenoid synthesis gene clusters isolated from *Enterobacteriaceae* strains are usually organized as *crtEXYIBZ*; whereas the *crtEXYIB* genes are transcribed in one direction as an operon, the downstream *crtZ* gene is transcribed in the opposite direction (9). The genes required for β-carotene synthesis are assembled as the *crtE crtY crtI crtB* operon by overlapping PCR. We remove the *crtX* gene encoding zeaxanthin glucosyl transferase, which converts zeaxanthin to zeaxanthin-β-diglucoside. The *idi* gene that is naturally present in several clusters is kept intact and in these cases the genes in the operon are *crtE idi crtY crtI crtB*. Following is an example on how to perform overlapping PCR to assemble the *crtEidiYIB* genes together as an operon and to clone the operon into pBHR1 vector to be expressed under the control of the chloramphenicol resistance gene promoter (see Note 1), resulting in construct pDCQ380.

3.1.1. Set up First Round PCR Reactions to Amplify crtEidi and crtYIB, Respectively

1. Set up PCR mixture for each PCR reaction as following: 40.6 μL of distilled water (dH_2O), 5.0 μL of 10× PfuUltra® II Fusion HS DNA polymerase buffer, 0.4 μL of dNTPs (25 mM each dNTP), 1.0 μL of DNA template (~10 ng/μL), 1.0 μL of forward primer (100 ng/μL), 1.0 μL of reverse primer

(100 ng/μL), and 1.0 μL of PfuUltra® II Fusion HS DNA polymerase (2.5 U/μL) in a total reaction volume of 50.0 μL (see Note 2).

2. Add the components in order while mixing gently into a thin-wall PCR tube.
3. Cover the PCR tube with a PCR tube cap or an aluminum foil.
4. Put the tube into a PCR cycler with a preheated lid, and close the lid.
5. Perform the PCR using the following cycling conditions: (I) 95°C for 2 min. (II) 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min (for <3 kb PCR product) or 3 min (for ≥3 kb PCR product). (III) 72°C for 5 min.
6. To analyze PCR amplified crtEidi and crtYIB products on 1.0% Seakem® Gold agarose gel, mix 9 μL of PCR amplification product with 1 μL 10× BlueJuice™ gel loading buffer. Additionally, mix 4.5 μL High DNA Mass Ladder with 0.5 μL 10× BlueJuice™ gel loading buffer.
7. Load the sample and DNA marker into different wells of a 1.0% Seakem® Gold agarose gel.
8. Run the gel in 1× TBE buffer at 90 V for about 30 min.
9. Transfer the gel onto an UV transilluminator. Turn on the power and check the size and purity of the amplified PCR product (see Note 3).

3.1.2. Purification of PCR Amplified crtEidi and crtYIB Products Using DNA Clean & Concentrator™-5 Kit

1. Add DNA-binding buffer to the tube containing the DNA sample. The amount of buffer added is two times the volume of DNA sample to be purified.
2. Mix by vortex briefly.
3. Load the solution into a Zymo-Spin™ Column in a Collection Tube.
4. Centrifuge at maximal speed in the microcentrifuge for 30 s. Discard the flow-through.
5. Add 200 μL of DNA Wash buffer to the column and centrifuge for 30 s. Discard the flow-through. Repeat the wash step once.
6. Add 10 μL of distilled water to the column matrix. Place column into a new 1.5 mL microcentrifuge tube and centrifuge for 60 s to elute DNA.
7. Measure DNA concentration using Nanodrop 1000 spectrophotometer (see Subheading 3.1.4).

3.1.3. Purification of PCR Amplified crtEidi and crtYIB Products from Agarose Gels Using Zymoclean™ Gel DNA Recovery Kit

1. Put the agarose gel on a UV transilluminator, turn on the UV light to see the DNA bands.
2. Locate the position of the DNA band with correct size.
3. Cut the correct gel band containing the needed DNA using a razor blade and put it in a 1.5 mL microcentrifuge tube.

4. Add agarose gel dissolving buffer to the tube. The amount of buffer added is three times the volume of the gel slice in the tube.
5. Incubate at 55°C for 10 min until gel slice is completely dissolved.
6. Load the solution into a Zymo-Spin™ Column in a collection tube.
7. Centrifuge at maximal speed in the microcentrifuge for 30 s. Discard the flow-through.
8. Add 200 μL of DNA Wash buffer to the column and centrifuge for 30 s. Discard the flow-through. Repeat the wash step once.
9. Add 10 μL of distilled water to the column matrix. Place column into a new 1.5 mL microcentrifuge tube and centrifuge for 60 s to elute DNA.
10. Measure DNA concentration using Nanodrop 1000 spectrophotometer (see Subheading 3.1.4).

3.1.4. Measuring DNA Concentrations of Purified PCR Amplified crtEidi and crtYIB Products Using Nanodrop 1000 Spectrophotometer

1. Turn on the Nanodrop 1000 spectrophotometer and its computer software.
2. Choose "DNA-50" for the sample type selection column on the computer screen.
3. Open the arm of the spectrophotometer.
4. Pipet 1 μL distilled water onto the sample pedestal. Close the arm.
5. Press "blank" button on the screen. Wait until the calibration completes.
6. Open the arm and dry the surface of the pedestal with a lint-free lab tissue wiper.
7. Pipet 1 μL DNA sample onto the sample pedestal. Close the arm.
8. Press "measure" button on the screen. Wait until the measurement completes. Record the concentration reading of the DNA sample.

3.1.5. Set up Second Round Overlapping PCR to Amplify crtEidiYIBgene Cluster

1. The PCR mixture (see Note 4) and running condition is the same as described in Subheading 3.1.1. The DNA template used here is 5 ng each of the purified PCR products of *crtEidi* and *crtYIB* from Subheading 3.1.4.
2. The PCR amplified *crtEidiYIB* product is purified and quantified using the same protocols described from Subheadings 3.1.1 to 3.1.4.

3.2. Cloning of the Overlapping PCR Product of the Assembled crtEidiYIB Genes into the EcoRI Site in pBHR1 Vector to Create Construct pDCQ380

3.2.1. Restriction Enzyme EcoRI Digestion of pBHR1 or Purified PCR Product of crtEidiYIB

1. Set up *EcoR*I digestion mixture as following: 7 μL of distilled water (dH_2O), 2 μL of 10× NEBuffer 4, 10 μL of pBHR1 plasmid DNA (vector) or purified crtEidiYIB PCR product (50–100 ng/μL) from Subheading 3.1.5, and 1 μL of *EcoR*I (20 U/μL) in a total reaction volume of 20 μL.
2. Add the components in order while mixing gently into a 1.5 mL microcentrifuge tube.
3. Incubate at 37°C for 1 h.
4. Purify the *EcoR*I-digested *pBHR1* and the *EcoR*I-digested PCR products of crtEidiYIB with DNA Clean & Concentrator™-5 Kit (see Subheading 3.1.2).
5. Measure DNA concentration using Nanodrop 1000 spectrophotometer (see Subheading 3.1.4).

3.2.2. Ligation of Purified EcoRI-Digested pBHR1 and Purified EcoRI-Digested PCR Product of crtEidiYIB

1. Setting up ligation mixture as following: 1 μL of distilled water (dH_2O), 2 μL of 5×T4 DNA ligase buffer, 1 μL of *EcoR*I-digested pBHR1 (diluted to 10 ng/μL with dH_2O) from Subheading 3.2.1, 5 μL of *EcoR*I-digested *crtEidiYIB* PCR product (diluted to 10 ng/μL with dH_2O) from Subheading 3.2.1, and 1 μL of T4 DNA ligase (1 U/μL) in a total reaction volume of 10 μL.
2. Add the components in order while mixing gently into a 1.5 mL microcentrifuge tube.
3. Incubate at room temperature for 1 h. The DNA mixture is ready for transformation.

3.2.3. Transformation of Ligation Reaction Mixture into E. cloni 10G Electrocompetent Cells

1. Thaw one tube of competent cells on ice.
2. Put a Gene Pulser® cuvette (1 mm) and a 1.5 mL microcentrifuge tube on ice for 10 min.
3. Add 0.5 μL DNA ligation reaction mixture from Subheading 3.2.2 into the tube.
4. Aliquot 20 μL competent cells into the tube. Mix gently. Make sure there are no bubbles.
5. Transfer mixture of DNA and cells into the ice cold cuvette.
6. Set the electroporator parameters: voltage = 1.8 kV, resistance = 200 Ω, and capacitance = 25 microFaradays (μF).
7. Electroporate the cells.
8. Immediately after electroporation, add 1 mL recovery medium into the electroporation cuvette. Mix by pipetting.
9. Transfer cells into a Falcon 2059 tube, incubate at 250 rpm, 37°C for 45 min.
10. Spread 1 μL to 100 μL of the cells onto LB plates with 50 μg/mL kanamycin.
11. Incubate the plates at 30°C for 2 days or longer until some colonies turn yellow.

3.2.4. Preparation and Confirmation of pDCQ380 Plasmid DNA

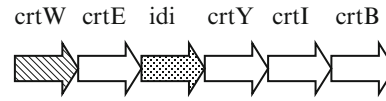
1. Pick several yellow colored clones from the LB plate with 50 μg/mL kanamycin in Subheading 3.2.3 and inoculate each in 3 mL LB medium with 50 μg/mL kanamycin in a Falcon 2059 tube. Incubate at 250 rpm, 37°C for 16 h.
2. Transfer 1.5 mL cell culture into a 1.5 mL microcentrifuge tube, centrifuge in the microcentrifuge at maximal speed for 1 min. Discard the supernatant completely.
3. Add 0.25 mL ice-cold buffer P1 in QIAprep Spin Miniprep Kit. Resuspend the cell pellet completely.
4. Add 0.25 mL buffer P2, invert the tube five times, let stay for 4 min at room temperature to lyse the cells.
5. Add 0.35 mL buffer N3, vortex for 10 s. Centrifuge at maximal speed for 10 min.
6. Transfer the supernatant to QIAprep Spin column. Centrifuge at maximal speed for 30 s. Discard the flow-through.
7. Add 0.8 mL buffer PE to the column. Centrifuge at maximal speed for 30 s. Discard the flow-through. Centrifuge at maximal speed for another 30 s to dry the column.
8. Add 50 μL buffer EB onto the matrix of the column. Centrifuge at maximal speed for 30 s to elute plasmid DNA.
9. The pDCQ380 plasmid DNA concentration is measured using Nanodrop 1000 spectrophotometer (see Subheading 3.1.4).
10. Plasmid is confirmed by sequencing (see Note 5).

3.3. Assembly of Gene Operons for Canthaxanthin Synthesis

The β-carotene ketolase gene (*crtW*) was added to the β-carotene synthesis operon for canthaxanthin synthesis. Typical cathaxanthin synthesis gene operons (Fig. 2) we assembled contained the *crtW* gene added as the first gene in the operon. Many *crtW* genes can fulfill this reaction and achieve almost complete (>90%) conversion of β-carotene to canthaxanthin. Materials section (see Subheading 2) listed several sources of *crtW* genes we used. More can be found in the databases from different bacterial sources: *Paracoccus marcusii* (accession number Y15112), *Paracoccus haeundaensis* (accession number AY957386), *Alcaligenes* sp. (accession number D58422), *Bradyrhizobium* sp. (accession number AF218415), *Brevundimonas aurantiaca* (accession number AY166610), *Brevundimonas* sp. SD212 (accession number AB181388). Following is an example of how to assemble the *crtWEidiYIB* cluster on pBHR1 vector, resulting in canthaxanthin expression construct pDCQ392W.

1. Set up PCR reaction as described in Subheading 3.1.1 to amplify a *crtW* gene (see Note 6).
2. Purify the PCR product as described in Subheading 3.1.2. Measure the DNA concentration as described in Subheading 3.1.4.
3. Double digest the purified *crtW* PCR product with *Xba*I *and Avr*II as following: 22.5 μL of distilled water (dH_2O), 4.0 μL

1. typical assembled canthaxanthin synthesis gene cluster



2. typical assembled astaxanthin synthesis gene cluster

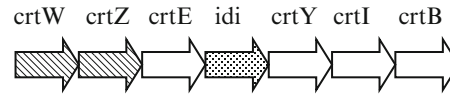


Fig. 2. Assembled typical gene clusters for canthaxanthin and astaxanthin synthesis. The genes required for β-carotene synthesis (*crtEYIB*) were shown as *open arrows*. The genes required for β-carotene modification (*crtWZ*) were shown as *striped arrows*. The *idi* gene in the isoprenoid pathway is shown as the *dotted arrow* and increases carotenoid titers.

of 10× NEBuffer 4, 10.0 μL of purified *crtW* PCR product (50–100 ng/μL), 1.0 μL of *Xba*I (20 U/μL), and 2.5 μL of *Avr*II (4 U/μL) in a total reaction volume of 40.0 μL; incubate at 37°C for 1 h.

4. Digest pDCQ380 plasmid DNA with *Avr*II as following: 10.0 μL of distilled water (dH_2O), 2.5 μL of 10× NEBuffer 4, 10.0 of μL purified *pDCQ380* plasmid DNA (50–100 ng/μL) from Subheading 3.2.4, and 2.5 μL of *Avr*II (4 U/μL) in a total reaction volume of 25.0 μL; incubate at 37°C for 1 h.
5. Purify the digested DNA products as described in Subheading 3.1.2. Measure the DNA concentration as described in Subheading 3.1.4.
6. Setting up ligation mixture as following: 3 μL of distilled water (dH_2O), 2 μL of 5×T4 DNA ligase buffer, 2 μL of *Avr*II digested pDCQ380 (diluted to 10 ng/μL with dH_2O), 2 μL of *Xba*I/*Avr*II double-digested *crtW* PCR product (diluted to 10 ng/μL with dH_2O), and 1 μL of T4 DNA ligase (1 U/μL) in a total reaction volume of 10 μL; incubate at room temperature for 1 h.
7. Transform the ligation mixture into *E. cloni* 10G electrocompetent cells following the same procedure in Subheading 3.2.3. Incubate the plates at 30°C for 2 days or longer until some colonies turn orange.
8. Pick several orange colored clones and perform plasmid DNA preparation of pDCQ392W following the same procedure as described in Subheading 3.2.4.
9. Confirm plasmid pDCQ392W by sequencing (see Note 7).

3.4. Assembly of Gene Operons for Astaxanthin Synthesis

Addition of *crtZ* gene to the canthaxanthin gene cluster could result in astaxanthin synthesis. Typical astaxanthin synthesis gene clusters we assembled (Fig. 2) contain the *crtWZ* genes upstream of the *crtEYIB* genes in the same operon (see Note 8). Several sources of *crtZ* genes we used are listed in Materials (see Subheading 2). More can be found in the databases from different bacterial sources: *Paracoccus marcusii* (accession number Y15112), *Paracoccus haeundaensis* (accession number AY957386), *Alcaligenes* sp. (accession number D58422), *Brevundimonas* sp. SD212 (accession number AB181388), *Erwinia uredovora* (reclassified as *Pantoea ananatis*, accession number D90087), *Pantoea agglomerans* (accession number M87280), *Pantoea stewartii* (AY166713), *Enterobacteriaceae* strains DC416 (accession number DQ090835) and DC413 (accession number DQ090836). Different combinations of *crtW* and *crtZ* genes from different bacterial sources (see Note 9) are used to improve astaxanthin purity (Fig. 3). Following is an example of how to assemble the *crtWZEidiYIB* cluster on pBHR1 vector, resulting in astaxanthin expression construct pDCQ392.

1. Set up PCR reaction as described in Subheading 3.1.1 to amplify a *crtZ* gene (see Note 10).
2. Purify the PCR product as described in Subheading 3.1.2. Measure the DNA concentration as described in Subheading 3.1.4.
3. Double digest the purified *crtZ* PCR product with *Xba*I *and Spe*I (see Note 11) as following: 6 μL of distilled water (dH_2O), 2 μL of 10× NEBuffer 4, 10 μL of purified *crtZ* PCR product (50–100 ng/μL), 1 μL of *Xba*I (20 U/μL), and 1 μL of *Spe*I (10 U/μL) in a total reaction volume of 20 μL; incubate at 37°C for 1 h.

Plasmid	Ast%	Genes expressed on plasmid
pDCQ334:	75%	$W_{agro}\ Z_{agro}$ EiYIB$_{404}$
pDCQ337:	29%	$Z_{404}\ W_{agro}\ Z_{agro}$ EiYIB$_{404}$
pDCQ339:	81%	$W_{202C}\ W_{agro}\ Z_{agro}$ EiYIB$_{404}$
pDCQ338:	85%	$W_{202C}\ Z_{404}\ W_{agro}\ Z_{agro}$ EiYIB$_{404}$
pDCQ343:	64%	$W_{18}\ Z_{263}$ E YIB$_{260}$
pDCQ378:	45%	$W_{18}\ Z_{260}$ E YIB$_{260}$
pDCQ344:	86%	$Z_{263}\ W_{263}$ E YIB$_{260}$
pDCQ377:	85%	$W_{263}\ Z_{263}$ EiYIB$_{404}$
pDCQ381:	81%	EiYIB$_{413}\ W_{263}\ Z_{263}$
pDCQ392:	90%	$W_{263}\ Z_{263}$ EiYIB$_{413}$

Fig. 3. Astaxanthin purity from *E. coli* containing different astaxanthin synthesis plasmids. The *subscript letters* refer to the bacterial sources where the genes were isolated.

4. Digest pDCQ392W plasmid DNA with *Avr*II as following: 10.0 μL of distilled water (dH_2O), 2.5 μL of 10× NEBuffer 4, 10.0 μL of purified pDCQ392W plasmid DNA (50–100 ng/μL) from Subheading 3.3, and 2.5 μL of *Avr*II (4 U/μL) in a total reaction volume of 25.0 μL; incubate at 37°C for 1 h.
5. Purify the digested DNA products as described in Subheading 3.1.2.
6. Setting up ligation mixture as following: 3 μL of distilled water (dH_2O), 2 μL of 5×T4 DNA ligase buffer, 2 μL of *Avr*II digested pDCQ392W (diluted to 10 ng/μL with dH_2O), 2 μL of *Xba*I/*Spe*I double-digested *crtZ* PCR product (diluted to 10 ng/μL with dH_2O), and 1 μL of T4 DNA ligase (1 U/μL) in a total reaction volume of 10 μL; incubate at room temperature for 1 h.
7. Transform the ligation mixture into *E. cloni* 10G electrocompetent cells following the same procedure in Subheading 3.2.3. Incubate the plates at 30°C for 2 days or longer until some colonies turn red.
8. Pick several red colored clones and perform plasmid DNA preparation of pDCQ392 following the same procedure as described in Subheading 3.2.4.
9. Confirm plasmid pDCQ392 by sequencing (see Note 12).

3.5. Analysis of Canthaxanthin and Astaxanthin in E. coli

3.5.1. Growing of the Carotenoids Producing E. coli Cultures in Shake Flasks

1. Transfer cells from streaked single colony on the kanamycin plate to 100 mL LB medium in a 500 mL flask (see Note 13) and culture at 30°C and 220 rpm for 2 days.
2. Aliquot 50 mL cell cultures each into two 50 mL conical polypropylene tubes for carotenoid extraction and dry cell weight measurement.

3.5.2. Extraction of Carotenoids from Cells

1. Pellet cells by centrifugation at 4,000 × *g* for 15 min. Remove supernatant completely.
2. Add 5 mL of an acetone–methanol mixture (50:50, v/v) to the cell pellet, vortex for 10 min to extract carotenoids.
3. Centrifuge at 4,000 × *g* for 15 min. Transfer supernatant to a new 50 mL conical tube.
4. Repeat steps 2 and 3 once. Combine the extracted solution.
5. Dry the extracted solution under nitrogen completely.
6. Add 0.5 mL of acetone–methanol mixture to redissolve the carotenoids.
7. Filter the solution into an autosampler vial with a 0.2 μm pore size Teflon filter and a 1 mL syringe.

3.5.3. Analysis of Carotenoids by HPLC

1. The high performance liquid chromatography (HPLC) system used has an autosampler and a DAD detector. A 150 mm × 4.6 mm ZORBAX C18 (3.5 μm particles) column is used for the separation of carotenoids.
2. Load the sample vials into the autosampler of the HPLC system.
3. Program the HPLC system as following: Keep the column temperature at 40°C. The solvent flow rate is 1 mL/min. The solvent running program used is: 0–2 min: 95% Buffer A and 5% Buffer B; 2–10 min: linear gradient from 95% Buffer A and 5% Buffer B to 60% Buffer A and 40% Buffer B; 10–12 min: linear gradient from 60% Buffer A and 40% Buffer B to 50% Buffer A and 50% Buffer B; 12–18 min: 50% Buffer A and 50% Buffer B; and, 18–20 min: 95% Buffer A and 5% Buffer B.
4. Pre-run the HPLC with the starting solvent (95% Buffer A and 5% Buffer B) at 1 mL/min until the DAD detector shows stable baseline.
5. Start the HPLC and inject 10 μL sample for each run.
6. After the run, analyze data by comparing the peak area value of the sample with that of the standard on the standard curve (see Note 14).

3.5.4. Carotenoids Standard Preparation

The elution time of the standard under the above condition is: β-carotene, 13.2 min; zeaxanthin, 5.9 min; canthaxanthin, 7.5 min; astaxanthin, 4.8 min. The spectra data and molecular weight of the carotenoids are: β-carotene, λ_{max}: 456, 484 nm; MH^+: 537; zeaxanthin, λ_{max}: 456, 480 nm; MH^+: 569; canthaxanthin, λ_{max}: 475 nm; MH^+: 565; astaxanthin, λ_{max}: 480 nm; MH^+: 597.

1. Dissolve 2 mg carotenoid standard in 5 mL acetone first, then add 5 mL of methanol to the mixture.
2. Filter this 200 μg/mL carotenoid stock solution into a 15 mL conical tube with a 0.2 μm pore size Teflon filter and a 10 mL syringe.
3. Transfer 1 mL of the 200 μg/mL carotenoid stock solution into 9 mL acetone–methanol mixture (50:50, v/v) to make 20 μg/mL standard solution.
4. Dilute this 20 μg/mL standard solution with acetone–methanol mixture (50:50, v/v) to make 15, 10, 5 μg/mL standard solutions. Transfer 1 mL of each standard solution into an autosampler vial.
5. Run 20 μL of each of the 5, 10, 15, 20 μg/mL standard solutions on LC/MS.
6. Identify the carotenoid peak position and record the peak area value for each concentration of standard.
7. A standard curve can be plotted using the peak area value against the concentration of each standard.

3.5.5. Determination of Carotenoid Titer

The titer of canthaxanthin or astaxanthin produced could be calculated as μg of canthaxanthin or astaxanthin per g of dry cell weight (ppm). Dry cell weight of the cultures could be determined as following.

1. Pre-weight an aluminum weight boat and 47-mm 0.2 μm pore size polycarbonate Whatman Nuclepore membrane filter on an analytic balance using tweezers.
2. Put the filter into vacuum filter unit, load the cell culture and turn on house vacuum.
3. Let medium pass the filter and the retain cells on the filter under vacuum.
4. When filter become dry, remove it from the unit using tweezers and put into the same weight boat.
5. Place weight boat and filter into 105°C oven for 24 h.
6. Calculate the dry cell weight by subtracting the weight of the filter and weight boat alone from the weight of the filter and weight boat plus the cells.

The carotenoid produced from the *idi*-containing gene clusters in the native *E. coli* host (no host modification) is approximately 1,400–1,800 ppm. The carotenoid produced from the *idi*-lacking gene clusters in the native *E. coli* host (no host modification) is approximately 200–300 ppm. With host modifications including increasing isoprenoid flux, it is possible to increase carotenoid titer in *E. coli* to about 6,000–10,000 ppm (9, 10).

4. Notes

1. It is possible to use other expression vectors of different origins with different promoters to express the carotenoid biosynthesis genes.
2. The DNA template used here is pWEB413 (11), a cosmid derived from strain DC413 that contains the caroteinoid gene cluster. To amplify the *crtEidi* genes (~2 kb), the forward primer 1 and the reverse primer 1 are used. To amplify the *crtYIB* genes (~3.6 kb), the forward primer 2 and the reverse primer 2 are used. The underlined primer sequences are *Eco*RI sites, and the bold primer sequence is an *Avr*II site. These sites are added for cloning purposes.
3. If a single correct sized PCR band is observed, purify the rest of the PCR amplification product with DNA Clean & Concentrator™-5 Kit (see Subheading 3.1.2). If other by product

bands are present, load the rest of the PCR amplification product into a 1.0% agarose gel and purify the correct size DNA band with Zymoclean™ Gel DNA Recovery Kit (see Subheading 3.1.3).

4. To amplify the *crtEidiYIB* gene cluster (~5.6 kb), forward primer 1 and reverse primer 2 are used. The underlined primer sequences are *Eco*RI sites, and the bold primer sequence is an *Avr*II site. These sites are added for cloning purposes.
5. Plasmid is sequenced using sequencing primer 1 and sequencing primer 2. These two primers target the region on the pBHR1 vector flanking the *Eco*RI-cloning site. Sequencing results should confirm the existence and orientation of the *crtEidiYIB* gene cluster in the pDCQ380 plasmid. The yellow color of the colonies is also a good indication of expression of the cloned β-carotene synthesis genes.
6. The DNA template used here is pWEB263, a cosmid derived from strain DC263 that contains the *crtW* gene (12). To amplify the *crtW* gene (~0.8 kb), crtW forward primer and crtW reverse primer are used. The underlined primer sequence is an *Xba*I or *Avr*II site. These sites are added for cloning purposes. The bold primer sequence is an incorporated ribosome-binding site.
7. The pDCQ392W plasmid is sequenced using sequencing primer 1, which binds to the region flanking the *Eco*RI-cloning site on the pBHR1 vector. The sequencing result should confirm the existence and orientation of the *crtW* gene in the pDCQ392W construct. The orange color of the colonies is also a good indication of expression of the cloned β-carotene ketolase.
8. Balanced expression of ketolases and hydroxylases is critical to achieve high astaxanthin purity. Stronger expression of ketolases and hydroxylases closer to the promoter appears to give higher astaxanthin purity than expression farther from the promoter at the end of the operons.
9. The ketolases and hydroxylases from different bacterial sources have different activities and substrate specificities. Some mutant enzymes with altered activity/substrate specificity (13) may be useful for production of astaxanthin at high purity.
10. The DNA template used here is pWEB263 (12), a cosmid derived from strain DC263 that contains the *crtZ* gene. To amplify the *crtZ* gene (~0.5 kb), crtZ forward primer and crtZ reverse primer are used. The underlined primer sequence is an *Xba*I or *Spe*I site. These sites are added for cloning purposes. The bold primer sequence is an incorporated ribosome-binding site.

11. Restriction enzymes *Avr*II/*Spe*I/*Xba*I have compatible ends and can be used for successive cloning of different fragments into one construct.
12. The pDCQ392 plasmid is sequenced using the *crtZ* reverse primer. The sequencing result should confirm the existence and orientation of the *crtZ* gene in the pDCQ392 construct. The red color of the colonies is also a good indication of astaxanthin biosynthesis.
13. Astaxanthin purity could also be affected by growth conditions, especially oxygen concentration in the medium. Make sure there is plenty of aeration in shake flasks and maintain dissolved oxygen >20% in fermentors.
14. Canthaxanthin can be usually produced with high purity (>90%). Purity of astaxanthin may be compromised by accumulation of less oxygenated intermediates, such as adonixanthin, adonirubin, canthaxanthin, and echinenone.

References

1. Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sahm H (1996) Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. J Am Chem Soc 118:2564–2566
2. Sandmann G (2001) Carotenoid biosynthesis and biotechnological application. Arch Biochem Biophys 385:4–12
3. Albrecht M, Misawa N, Sandmann G (1999) Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. Biotechnol Lett 21:791–795
4. Wang CW, Oh MK, Liao JC (1999) Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. Biotechnol Bioeng 62:235–241
5. Kajiwara S, Fraser PD, Kondo K, Misawa N (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem J 324:421–426
6. Fraser PD, Miura Y, Misawa N (1997) In vitro characterization of astaxanthin biosynthetic enzymes. J Biol Chem 272:6128–6135
7. Choi SK, Nishida Y, Matsuda S, Adachi K, Kasai H, Peng X, Komemushi S, Miki W, Misawa N (2005) Characterization of beta -carotene ketolases, CrtW, from marine bacteria by complementation analysis in *Escherichia coli*. Mar Biotechnol (NY) 7:515–522
8. Choi SK, Matsuda S, Hoshino T, Peng X, Misawa N (2006) Characterization of bacterial beta-carotene 3,3'-hydroxylases, CrtZ, and P450 in astaxanthin biosynthetic pathway and adonirubin production by gene combination in *Escherichia coli*. Appl Microbiol Biotechnol 72:1238–1246
9. Yuan LZ, Rouvière PE, LaRossa RA, Suh W (2006) Chromosomal promoter replacement of isoprenoid pathway for enhancing carotenoid production in *E. coli*. Metab Eng 1:79–90
10. Alper H, Stephanopoulos G (2008) Uncovering the gene knockout landscape for improved lycopene production in *E. coli*. Appl Microbiol Biotechnol 78:801–810
11. Sedkova N, Tao L, Rouvière EP, Cheng Q (2005) Diversity of carotenoid synthesis gene clusters from environmental *Enterobacteriaceae* strains. Appl Environ Microbiol 71:8141–8146
12. Tao L, Rouvière PE, Cheng Q (2006) A carotenoid synthesis gene cluster from a non-marine *Brevundimonas* that synthesizes hydroxylated astaxanthin. Gene 379:101–108
13. Tao L, Wilczek J, Odom JM, Cheng Q (2006) Engineering a β-carotene ketolase for astaxanthin production. Metab Eng 8:523–531