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Production of New Carotenoids, Astaxanthin Glucosides, by *Escherichia coli* Transformants Carrying Carotenoid Biosynthetic Genes

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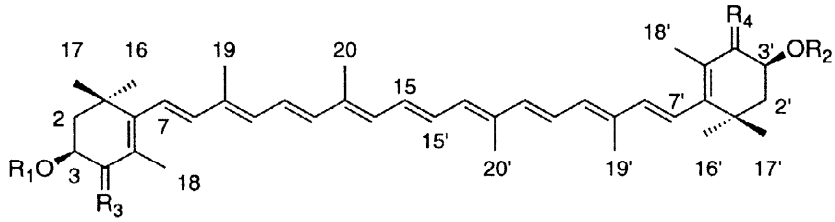
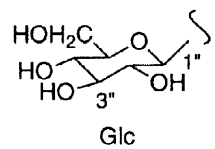
Abstract: For the purpose of production of astaxanthin glucosides, which is expected to be useful polar carotenoids, construction of plasmids having seven carotenoid biosynthetic genes and expression in transformed *E. coli* were carried out through the approach of metabolic engineering. From two transformed *E. coli* strains, two new carotenoid glucoside, astaxanthin β-D-diglucoside (**1**) and adonixanthin 3'-β-D-glucoside (**2**), and known astaxanthin glucoside (**3**) were isolated. These structures were determined by spectral means. © 1998 Elsevier Science Ltd. All rights reserved.

Astaxanthin (3,3'-dihydroxy-β,β–carotene-4,4'-dione, **4**) is a highly oxidized carotenoid, which is commonly found in marine animal tissues.[1] This carotenoid has been successfully employed to enhance pigmentation of cultured fish and shellfish. Further biological functions of **4** have been revealed as a vitamin A precursor,[2] a scavenger and/or quencher of active oxygen species,[3] a preventative against cancer,[4] and an enhancer of the immune response.[5] Recently, we reported the first astaxanthin-producing bacterium *Agrobacterium aurantiacum* isolated from seawater.[6] New carotenoid glucosides, astaxanthin β-D-glucoside (**3**) and adonixanthin 3-β-D-glucoside (**5**) were isolated from this bacterium.[7] Future studies on the biological function of such hydrophilic analogs of **4** should prove interesting because of their polarity and water solubility. In the last 8 years, some carotenoid biosynthetic genes have been cloned, and the biosynthetic pathways like that leading to **4** have been elucidated at the levels of genes and enzymes.[8-10] Here we describe seven carotenoid biosynthetic genes isolated from two different bacterial strains simultaneously expressed in *Escherichia coli* to produce **3** and the new diglucoside, astaxanthin diglucoside (**1**).



1: R_1=Glc, R_2=Glc, R_3=O, R_4=O
2: R_1=H, R_2=Glc, R_3=O, R_4=H_2
3: R_1=Glc, R_2=H, R_3=O, R_4=O
4: R_1=H, R_2=H, R_3=O, R_4=O
5: R_1=Glc, R_2=H, R_3=O, R_4=H_2
6: R_1=Glc, R_2=Glc, R_3=H_2, R_4=H_2



Glc

PII: S0040-4039(98)00542-5

The biosynthetic pathways of zeaxanthin β-D-diglucoside (**6**) and **4** have been elucidated through isolation of the biosynthetic genes from a soil bacterium *Erwinia uredovora* and a marine bacterium *A. aurantiacum*, and by *in vivo* and *in vitro* functional analysis of *E. coli* expressing these genes (Fig. 1).[8-10] β-Carotene is synthesized by *crtE*, *crtB*, *crtI*, and *crtY* genes through the formation of a C40 carbon chain, desaturation, and cyclization reactions. Carotenoid **6** is synthesized by *crtZ* and *crtX* genes through hydroxylation and glucosylation of β-carotene in *Erwinia* species.[8] Carotenoid **4** is synthesized by *crtZ* and *crtW* genes through hydroxylation and oxidation reactions of β-carotene in *A. aurantiacum*.[9] These results suggested that astaxanthin mono- and diglucoside may be produced by expression of all seven *crt* genes in a single microorganism such as *E. coli*.

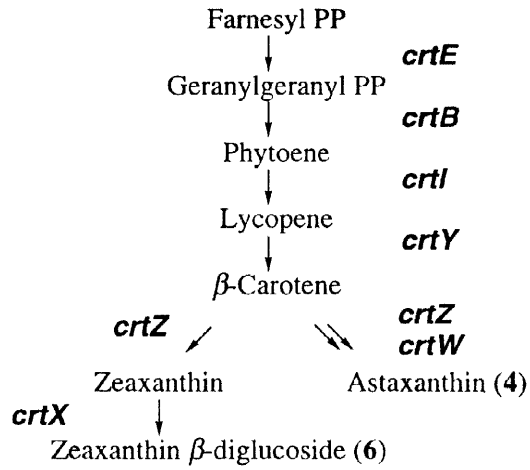


Fig. 1. Functions of Carotenoid Biosynthesis Genes for zeaxanthin β-diglucoside (**6**) in *E. uredovora* and astaxanthin (**4**) in *A. aurantiacum*.

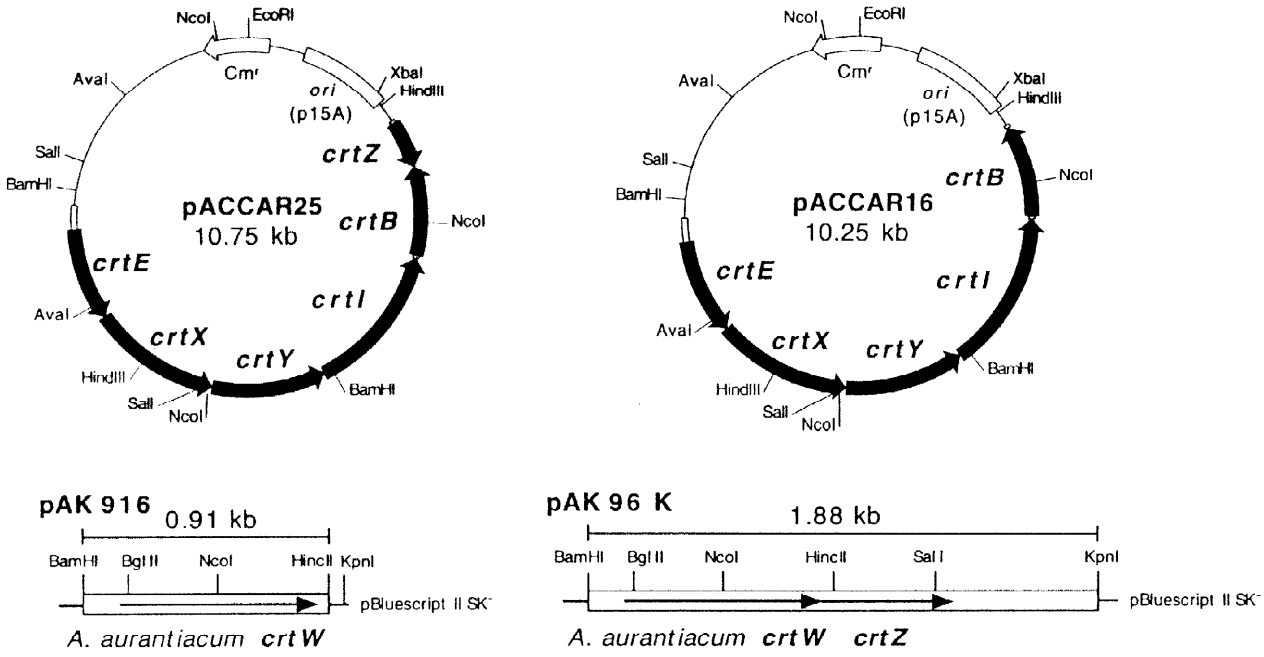


Fig. 2. Structures of Plasmids Containing the *E. uredovora crt* genes (pACCAR25 and pACCAR16) and the *A. aurantiacum crt* gene(s) (pAK916 and pAK96K).

Cmr: chloramphenicol resistance gene, ori: replication origin.

Four different constructed plasmids are shown in Fig. 2. The 6.50 kb fragment carrying all six *crt* genes from *E. uredovora* was isolated by digestion with *Asp* 718 (*Kpn*I)-*Eco*RI from pCAR25,[8] treatment with Klenow enzyme, and ligation into the *Eco*RV site of pACYC184, to produce plasmid pACCAR25. Similarly, the 6.01 kb fragment carrying the five *Erwinia crt* genes without *crtZ* was isolated from pCAR16,[8] and inserted into pACYC184, to produce plasmid pACCAR16. The previously described plasmid pAK916 carries the *A. aurantiacum crtW* gene on the *E. coli* vector pBluescript II SK$^-$, which also has the ampicillin resistance gene.[9] Similarly, plasmid pAK96K has both the *crtW* and *crtZ* genes derived from *A. aurantiacum*. Plasmids pACCAR25 and pACCAR16 were introduced into an *E. coli* JM109 strain along with plasmids pAK916 and pAK96K, respectively.

E. coli transformant JM109 (pACCAR25, pAK916) was cultured at 30°C for 48 hours with LB medium[11] containing chloramphenicol (30 mg/L), ampicillin (150 mg/L), and isopropyl β-D-thiogalacto-pyranoside (0.2 mM). The collected cells from a 12 liters culture were extracted with acetone and acetone-MeOH (7:3). The extracts were combined, concentrated and partitioned between *n*-hexane and H_2O-MeOH (2:8). The methanolic fraction was purified by silica gel column with $CHCl_3$-MeOH (8:2). Compound **3** (49% of total peak area in HPLC analysis[12] at 470nm, 4.2mg) was further purified by reversed-phase HPLC (TSK gel ODS 80Ts) with H_2O-MeOH (5:95). Compound **1** (10% of total peak area, 2.3mg) was purified by reversed-phase HPLC with H_2O-MeOH (1:9).

Compound **3** was identified as astaxanthin β-glucoside on the basis of TLC, HPLC, and ^{1}H-NMR data comparison with authentic material isolated from *A. aurantiacum*.[7] The absorption maximum in the visible spectrum of **1** was 492 nm in $CHCl_3$-MeOH (2:1) and high-resolution FABMS of **1** gave an $[M+H]^+$ ion peak at 921.4992, corresponding to the formula $C_{52}H_{73}O_{14}$ (Δ -0.8 mmu), suggesting astaxanthin diglucoside. Integration of the ^{1}H-NMR spectrum of **1** indicated one symmetric carotenoid and two single hexoses.[13] Comparison of **1** with authentic **3** by ^{1}H-NMR analysis revealed 3-glucopyranosyl-4-keto β -ionone type at both ends of **1**. β-Linkage of glucoside was established by $J_{1''2''}$=8 Hz. Absolute configuration of hydroxyl groups at C-3 and 3' is presumed 3*S*, 3'*S* form by the character of a hydroxylase encoded *crtZ* gene.[8,9] Thus **1** was determined to be a new carotenoid glucoside, (3*S*, 3'*S*)-astaxanthin β -D-diglucoside.

Another transformed *E. coli* JM109 (pACCAR16, pAK96K) was cultured in 12 liters of medium by the same technique. By HPLC analysis, compound **4** (54% of total peak area in HPLC) and an unknown peak (45%, compound **2**) were detected. Compound **2** was extracted, partitioned, and purified by silica gel column chromatography in the similar manner as above. By using reversed-phase HPLC with H_2O-MeOH (1:9), **2** (less than 1 mg) was isolated. The visible spectrum [λ_{max} 479nm in $CHCl_3$-MeOH (2:1)] and HRFABMS $[M+H]^+$ ion peak at 745.4677 ($C_{46}H_{65}O_8$, Δ -0.2 mmu) were compatible with those of **5**, however the ^{1}H-NMR data was different from that of **5**.[7] The portion of ^{1}H-NMR spectrum for each end group of **2** was superimposable with those of **4** and **6** on the basis of direct comparison with authentic samples in the same solvent.[14] Therefore, **2** was determined to be (3*S*, 3'*R*)-adonixanthin 3'-β-D-glucoside. The reason why two transformants gave unequal metabolites was expected to be the deference of the activities of hydroxylase encoded *crtZ* genes, although two *crtZ* genes from *E. uredovora* and *A. aurantiacum* have 56% amino acid homology.[9,10]

As described above, natural known carotenoid **3** and unnatural new carotenoid **1** were successfully produced by extensive use of metabolic engineering (introduction of seven kinds of carotenoid biosynthetic genes and expression of these gene in *E. coli*), without using organic synthesis. This result clearly showed that

metabolic engineering using recombinant DNA techniques is a powerful tool for the production of new useful fine chemicals.[15] Antioxidation or cancer prevention activities of these hydrophilic astaxanthin glucosides will be investigated.

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11. 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 L of water.
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13. ^{1}H-NMR data for **1** (400MHz, $CDCl_3$-CD_3OD 2:1) δ 1.19 (6H, s, 17 and 17'-H_3), 1.31 (6H, s, 16 and 16'-H_3), 1.86 (6H, s, 18 and 18'-H_3), 1.94 (2H, m, 2 and 2'-H), 1.95-1.97 (12H, s, 19, 20, 19', and 20'-H_3), 2.18 (2H, m, 2 and 2'-H), 3.27 (2H, m, 5"-H), 3.36 (2H, dd, J=8 and 9Hz, 2"-H), 3.41 (2H, t, J=9Hz, 4"-H), 3.43 (2H, t, J=9Hz, 3"-H), 3.70 (2H, dd, J=5 and 12Hz, 6"-H), 3.83 (2H, dd, J=3 and 12Hz, 6"-H), 4.49 (2H, d, J=8Hz, 1"-H), 4.77 (2H, m, 3 and 3'-H), 6.18-6.67 (14H, olefinic protons).
14. ^{1}H-NMR data for **2** (400MHz, $CDCl_3$-CD_3OD 2:1) δ 1.03 (6H, s, 16' and 17'-H_3), 1.18 (3H, s, 17-H_3), 1.29 (3H, s, 16-H_3), 1.52 (1H, t, J=12Hz, 2'-H), 1.69 (3H, s, 18'-H_3), 1.79 (1H, m, 2-H), 1.83 (1H, m, 2'-H), 1.87 (3H, s, 18-H_3), 1.92-1.96 (12H, s, 19, 20, 19', and 20'-H_3), 2.06 (1H, m, 2-H), 2.08 (1H, m, 4'-H), 2.42 (1H, dd, J=4 and 17Hz, 4'-H), 3.19 (1H, m, 2"-H), 3.27 (1H, m, 5"-H), 3.38 (2H, m, 3" and 4"-H), 3.72 (1H, dd, J=5 and 12Hz, 6"-H), 3.83 (1H, dd, J=3 and 12Hz, 6"-H), 4.05 (1H, m, 3'-H), 4.28 (1H, dd, J=6 and 14Hz, 3-H), 4.42 (1H, d, J=8Hz, 1"-H), 6.05-6.70 (14H, olefinic protons).
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