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The astaxanthin dideoxyglycoside biosynthesis pathway in *Sphingomonas* sp. PB304

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Abstract A major carotenoid in *Sphingomonas* sp. PB304, originally isolated from a river in Daejon City, South Korea, was identified as astaxanthin dideoxyglycoside. Gene clusters encoding the astaxanthin dideoxyglycoside biosynthetic enzymes were identified by screening *Sphingomonas* sp. PB304 fosmid libraries using degenerate probes that harbor highly conserved sequences from the *Sphigomonas elodea*-derived *crtI* and *Nostoc* sp. PCC 7120-dervied *crtW* genes. Selected positive gene clusters were fully sequenced and annotated, revealing genes encoding six putative carotenogenic enzymes: phytoene synthase (CrtB), phytoene desaturase (CrtI), lycopene cyclase (CrtY), carotene hydroxylase (CrtZ), carotene ketolase (CrtW), and glycosyltransferase (CrtX). All of the carotenogenic enzymes, except for CrtX, were functional in the recombinant host *Escherichia coli* expressing synthetic carotenogenic modules from *Pantoea agglomerans*. CrtX did not take up UDP-glucose or GDP-fucose as sugar substrates during the in vitro reaction. Although no direct experimental evidence was obtained for the function of *Sphingomonas* sp. PB304 CrtX, it can be categorized as a putative deoxyglycosyltransferase based on the presence of astaxanthin dideoxyglycoside in *Sphingomonas* sp. PB304, a putative corresponding gene in the carotenoid biosynthetic gene cluster, and high amino acid sequence homology to the existing glycosyltransferases. Therefore, we propose that astaxanthin dideoxyglycoside can be synthesized in *Sphingomonas* sp. PB304 via sequential reactions of six pathway enzymes, including CrtX on the phytoene intermediate.

Keywords Carotenoid · *Sphingomonas* · Astaxanthin dideoxyglycoside · Glycosyltransferase

Introduction

Carotenoids are the most common natural pigments and isoprenoid derivatives that are biosynthesized in various organisms (Cazzonelli 2011). To date, more than 700 structurally different carotenoids have been reported from various sources (Walter and Strack 2011). Carotenoids play various roles in nature, including acting as antioxidants, participating in light-harvesting and energy-transfer reactions, regulating membrane fluidity, and acting as provitamins (e.g., provitamin A) (Walter and Strack 2011). Biotechnological interest in carotenoids has focused on their antioxidative, anticancer, and anti-inflammatory activities, both for human health applications, as well as for food or feed additives (Nishino et al. 2009). The colors associated with carotenoids are derived from the unique presence of conjugated double bonds (CDBs) found in their chromophore backbones (Britton 1995). Varying numbers of CDBs confer carotenoid-unique colors ranging from yellow to violet.

The C_{40} carotenoids such as lycopene and β-carotene are the most abundant carotenoids. However, C_{30} or C_{50} carotenoids are also present in limited amounts (Krubasik et al. 2001; Kim and Lee 2012). The biosynthesis pathways for diverse types of carotenoids in various organisms have been widely studied, but limited carotenogenic enzymes have been studied in vitro and in vivo (Walter and Strack 2011; Cazzonelli 2011; Shumskaya and Wurtzel 2013). Carotenoid backbones can be

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synthesized from the condensation of two farnesyl diphosphate (FPP) molecules or two geranylgeranyl diphosphate (GGPP) molecules to generate 4,4′-diapophytoene (C_{30}) or phytoene (C_{40}), by the action of 4,4′-diapophytoene/phytoene synthase (CrtM/CrtB), respectively. Next, CDBs are sequentially introduced into 4,4′-diapophytoene/phytoene by diapophytoene/phytoene desaturase (CrtN or CrtI in bacteria, and CrtP plus CrtQ in plants and cyanobacteria), respectively. Various carotenoid-modifying enzymes, including lycopene cyclase (CrtY), carotene ketolase (CrtW), carotene hydroxylase (CrtZ), lycopene elongase (CrtEb), glucosyltransferase (CrtX), and acyl transferase (CruD), confer diverse functional groups onto a desaturated carotene backbone, thereby provoking diverse biological properties and functions (Lee and Schmidt-Dannert 2002).

Recently, the structurally notable carotenoid astaxanthin dirhamnoside was identified from a radiotolerant bacterium, *Sphingomonas astaxanthinifaciens* TDMA-17^T (Asker et al. 2009). Astaxanthin dirhamnoside has a unique structure in which a rhamnose moiety is attached to each β-ionone ring. However, the details of the astaxanthin dirhamnoside biosynthesis pathway have not yet been elucidated (Asker et al. 2009). The genus *Sphingomonas* belongs to α-proteobacteria that have yellow or orange pigmentation (Yabuuchi et al. 1990). Although *Sphingomonas* strains are well known to produce structurally diverse carotenoids, only the nostoxanthin pathway from the gellan gum-producing bacterium *Sphigomonas elodea* ATCC 31461 (Asker et al. 2009; Zhu et al. 2012) has been elucidated to date.

In this study, we identified gene clusters that included *crtYIBZWX* and encoded astaxanthin dideoxyglycoside biosynthesis enzymes in *Sphingomonas* sp. PB304, a strain isolated from a river in Daejon City, South Korea. Subsequent complementation analysis confirmed the functionality and pathway reactions of these *Sphingomonas* sp. PB304 carotenogenic gene products in *Escherichia coli*. Phylogenetic analysis of a putative deoxyglycosyltransferase (CrtX), a rare enzyme in the carotenoid pathway, was performed using sequences from various carotenoid glycosyltransferases. Based on complementation studies, identification of pathway products, and phylogenetic analysis, an astaxanthin dideoxyglycoside biosynthesis pathway in *Sphingomonas* sp. PB304 is hereby proposed.

Materials and methods

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this study are listed in Table 1. For gene cloning, the *E. coli* XL1-Blue strain was grown in Luria-Bertani (LB) medium at 37 °C; Terrific Broth (TB) medium was used for carotenoid production, using a rotary shaker at 250 rpm and 30 °C. Chloramphenicol (50 μg/mL), ampicillin (100 μg/mL), and/or kanamycin (30 μg/mL; St. Louis, MO, USA, Sigma) were added as required. The PB304 strain with orange pigmentation was isolated from a river in Daejon City, South Korea, and grown on Reasoner's 2A agar plates at 30 °C for 3–4 days. Based on the 16S rRNA sequence analysis (see next section), the strain PB304 was found to belong to the genus *Sphingomonas*, and designated as *Sphingomonas* sp. PB304. Detailed taxonomic analysis, including a DNA-DNA hybridization experiment (unpublished data), indicated that the strain PB304 could be considered to be a novel species strain. *Sphingomonas* sp. PB304 was deposited in the Korean Collection for Type Cultures (KCTC 32458) and in the Spanish Type Culture Collection (CECT 8383).

Phylogenetic analysis of 16S rRNA The 16S rRNA gene of *Sphingomonas* sp. PB304 was amplified with a polymerase chain reaction (PCR), using the purified genomic DNA and the universal bacterial primer pair 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1512R (5′-AGAAAGGAGGTGATCCAGCC-3′). The complete 16S rRNA gene sequence was determined (GenBank accession number KF214258) and aligned with the reference sequences obtained from the EzTaxon-e server (Kim et al. 2012) by using the CLUSTALW software (Larkin et al. 2007). Evolutionary distances were calculated using the model of Jukes and Cantor (1969), and a phylogenetic tree (Fig. 1) was generated using the neighbor-joining method (Saitou and Nei 1987). The resulting tree was subjected to bootstrap analyses (Felsenstein 1985) based on 1,000 re-samplings.

Recombinant DNA techniques Restriction enzymes, DNA polymerase, and DNA ligase were all purchased from New England Biolabs (Beverly, MA, USA). Genomic DNA was isolated from *Sphingomonas* sp. PB304 using an MG™ Genomic DNA Purification kit (Macrogen; Seoul, Korea). Plasmid DNA was prepared with the MG™ plasmid SV mini-prep kit (Macrogen; Seoul, Korea). PCR was carried out using a DNA Engine Thermal Cycler (Bio-Rad; Hercules, CA, USA) with Vent DNA Polymerase according to the manufacturer's instructions.

Fosmid library construction A fosmid library was constructed using the Copy Control™ Fosmid Library Production kit (EPICENTRE® Biotechnologies; Madison, WI, USA) according to the manufacturer's instructions. Briefly, 10–15 μg of genomic DNA was sheared by passing the DNA through a syringe (1 cc) equipped with a 26-G needle. After the sheared DNA fragments were end-repaired, DNA fragments that were 35–40 kb in size were isolated from a low-melt agarose (Bio-Rad; Hercules, CA, USA) gel. The recovered DNA fragments were ligated into the pCC2FOS vector, packaged with phage particles, and then transfected into EPI300-T1^R *E. coli* cells.

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant properties	Source or reference
Strains		
Sphingomonas sp. PB304	Astaxanthin dideoxyglycoside biosynthesis pathway	This study
Nostoc sp. PCC 7120	C_{40} carotenoid biosynthesis pathway	UTEX 2576
E. coli XL1-Blue	*endA*1 *gyrA*96(nal^R) *thi*-1 *recA*1 *relA*1 *lac glnV*44 F′[::Tn10 *proAB*$^+$*lacI*^{q} (Δ*lacZ*)M15] *hsdR*17($r_K^-m_K^+$)	Agilent Technologies (Palo Alto, CA, USA)
E. coli EPI300™	[F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) (StrR) φ80d*lacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *galU galK* λ$^-$ *rpsL nupG trfA tonA dhfr*]	EPICENTRE® Biotechnologies (Madison, WI, USA)
Plasmids		
pUCM	Cloning vector modified from pUC19; constitutive *lac* promoter, Ap	Kim et al. (2010)
pCC2FOS™	Linearized vector for construction of fosmid library, Cm	EPICENTRE® Biotechnologies (Madison, WI, USA)
pUCM-304B	Constitutively expressed *crtB* gene from *Sphingomonas* sp. PB304	This study
pUCM-PAB	Constitutively expressed *crtB* gene from *P. agglomerans*	Song et al. (2013)
pUCM-304I	Constitutively expressed *crtI* gene from *Sphingomonas* sp. PB304	This study
pUCM-PAI	Constitutively expressed *crtI* gene from *P. agglomerans*	Song et al. (2013)
pUCM-304Y	Constitutively expressed *crtY* gene from *Sphingomonas* sp. PB304	This study
pUCM-PAY	Constitutively expressed *crtY* gene from *P. agglomerans*	Song et al. (2013)
pUCM-304Z	Constitutively expressed *crtZ* gene from *Sphingomonas* sp. PB304	This study
pUCM-PAZ	Constitutively expressed *crtZ* gene from *P. agglomerans*	Song et al. (2013)
pUCM-304W	Constitutively expressed *crtW* gene from *Sphingomonas* sp. PB304	This study
pUCM-NOW	Constitutively expressed *crtW* gene from *Nostoc* sp. PCC 7120	This study
pUCM-304X	Constitutively expressed *crtX* gene from *Sphingomonas* sp. PB304	This study
pUCM-PAX	Constitutively expressed *crtX* gene from *P. agglomerans*	Song et al. (2013)
pACM-EB$_{PA}$	Constitutively expressed *crtE* and *crtB* genes from *P. agglomerans*	Song et al. (2013)
pACM-EI$_{PA}$	Constitutively expressed *crtE* and *crtI* genes from *P. agglomerans*	Song et al. (2013)
pACM-EBI$_{PA}$	Constitutively expressed *crtE*, *crtB*, and *crtI* genes from *P. agglomerans* to produce lycopene	Song et al. (2013)
pACM-EBIY$_{PA}$	Constitutively expressed *crtE*, *crtB*, *crtI*, and *crtY* genes from *P. agglomerans* to produce β-carotene	Song et al. (2013)
pACM-EBIYZ$_{PA}$	Constitutively expressed *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes from *P. agglomerans* to produce zeaxanthin	Song et al. (2013)

Titers were calculated using LB plates (150 mm) containing 12.5 μg/mL of chloramphenicol.

Colony hybridization A *crtI* probe for hybridization was generated by PCR using primers (Online Resource, Table S1) previously designed by Zhu et al. (2011), from the genomic DNA of *Sphingomonas* sp. PB304. A *crtW* probe for hybridization was obtained by PCR from the genomic DNA of *Sphingomonas* sp. PB304, using the following degenerate primers: forward 5′-GGS CTK TTC ATC GTC GCG-3′ and reverse 5′-RCC GAA RTG RAA RCA GGT-3′. The amplified PCR products were purified, ligated into a pGEM T-easy vector (Promega; Madison, WI, USA), and confirmed through sequence analysis (Macrogen; Seoul, Korea). After amplification with specific primers for optimal hybridization, the purified PCR product was randomly labeled using digoxigenin-dUTP (Roche Applied Science; Mannheim, Germany). Colony hybridization was performed using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science; Mannheim, Germany), as per the manufacturer's instructions. Briefly, DNA was cross-linked to nylon membrane disks (132 mm Φ; Roche Applied Science; Mannheim, Germany) after denaturing and neutralizing the lifted colonies. Then, the membranes were hybridized with the appropriate probe (25–30 ng/mL) for 5 h, followed by stringent washing, blocking, and antibody (anti-digoxigenin-AP) treatment (dilution ratio 1:10,000). Chemiluminescent signals were visualized using ImageQuant™ LAS 4000 mini (GE Healthcare; Buckinghamshire, UK) after CSPD (Roche; Applied Science; Mannheim, Germany) was applied to the membranes. Positive spots were screened by PCR, and each clone was sequenced after plasmid preparation of the fosmid clones, for which the

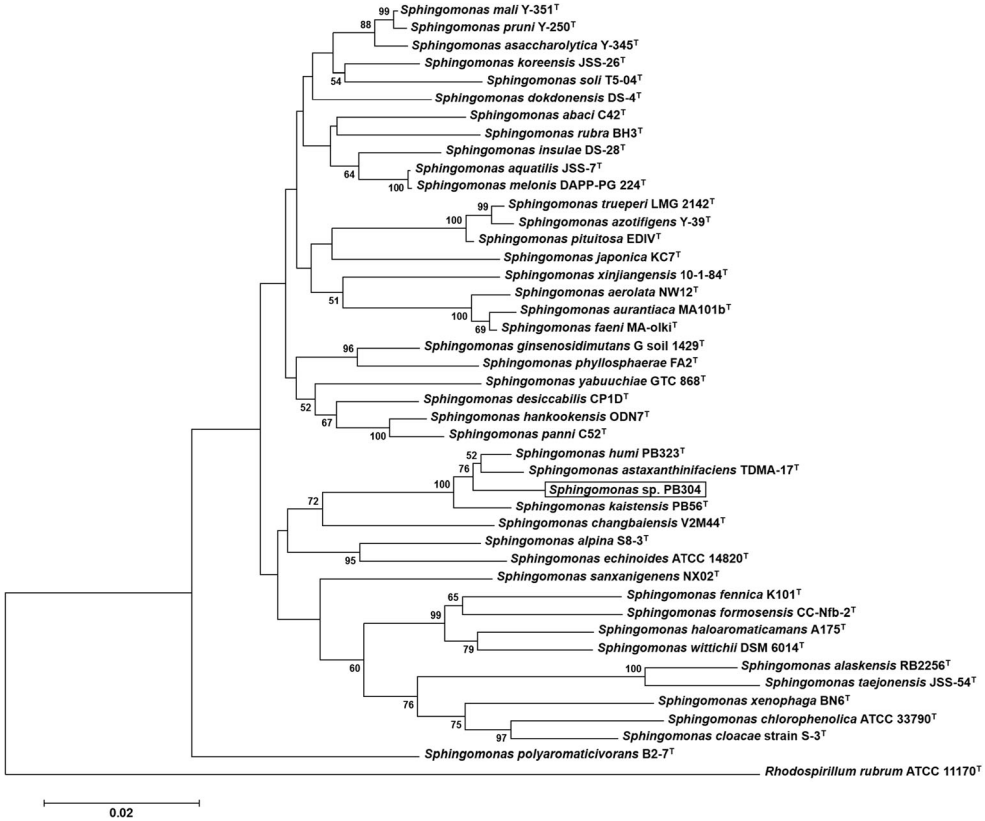

Fig. 1 The phylogenetic position of strain PB304 within the genus *Sphingomonas*, based on the 16S rRNA gene sequences. The tree was created using the neighbor-joining method; percentages at the nodes are the levels of bootstrap support (>50 %) from 1,000 re-sampled datasets. *Rhodospirillum rubrum* ATCC 11170^T was used as an outgroup

copy numbers were enhanced by using an induction solution. The GenBank accession numbers of the two positive *Sphingomonas* sp. PB304 fosmid clones are KF724895 (cluster 1) and KF724896 (cluster 2).

Gene cloning The genes encoding CrtB, CrtI, CrtY, CrtZ, CrtW, and CrtX were amplified from *Sphingomonas* sp. PB304 genomic DNA with primers designed using the sequences obtained from the fosmid clones (Online Resource, Table S1). The PCR products were then cloned into pUCM (Kim et al. 2010), which was modified to facilitate the constitutive expression of a cloned gene, resulting in pUCM-304X (where pUCM indicates the plasmid used; 304 represents the gene source microorganism such as *Sphingomonas* sp. PB304; X represents a pathway gene name; see Table 1).

Carotenoid extraction and purification Carotenoids were repeatedly extracted from cell pellets with methanol until all the visible pigments disappeared from the pellet. Colored supernatants were pooled after centrifugation (4 °C and 3,000×*g*) and concentrated to 5–10 mL using an EZ-2 *Plus* centrifugal evaporator (Genevac Inc.; Vally Center, NY, USA). An equal volume of ethyl acetate (EtOAc) was added to the concentrated solution and re-extracted after adding 5 mL of 5 N NaCl solution for the salting out process. The upper phase containing carotenoids was collected, washed with distilled water, and completely dried using the EZ-2 *Plus* evaporator. The dried samples were stored at −80 °C until further use. For the purification of astaxanthin and astaxanthin dideoxyglycoside, the isolated carotenoids were dissolved in a small volume of methanol and loaded onto a silica gel column, which was pre-equilibrated with a 9:1 hexane/EtOAc solvent system. Carotenoids were eluted using the same solvent system with a gradient of an increasing portion of methanol. Each of the eluted carotenoids was concentrated into a small volume, loaded onto a thin-layer chromatography (TLC) silica gel plate (Merck Millipore, Billerica, MA, USA), and developed with a solvent system comprising hexane/EtOAc/methanol (9:1:3, *v*/*v*/*v*). Each carotenoid band was scraped from the TLC plate, followed by elution with methanol. Glassware was used for each step, and stray light was blocked with aluminum foil. Astaxanthin was quantified using a spectrophotometer (SpectraMax Plus^{384}; Molecular Devices, Sunnyvale, CA, USA) using the extinction coefficient of astaxanthin ($A^{1\%}_{1cm}$ in hexane=2,100) (An et al. 1989). The purified astaxanthin was used as a substrate for the in vitro CrtX assay.

Structural analysis of carotenoids An aliquot (10–20 μL) of the collected fractions or crude extracts was applied to a ZORBAX Eclipse XDB-C18 column (4.6 mm×150 mm or 4.6 mm×250 mm, 5.0 μm; Agilent Technologies, Inc.; Palo Alto, CA, USA) and eluted under isocratic conditions with a solvent (80:15:5, acetonitrile/methanol/isopropanol) at a flow rate of 1 mL/min using an Agilent 1200 high-performance

liquid chromatography (HPLC) system equipped with a photodiode array detector (Agilent Technologies, Inc.; Palo Alto, CA, USA). The mass fragmentation spectra were monitored using both negative and positive ion modes in the mass range of *m/z* 200 to 1,000 on a liquid chromatography-mass spectrometer (LC/MS; Agilent 6150) equipped with an atmospheric pressure chemical ionization ion source (Agilent Technologies; Palo Alto, CA, USA). For the nuclear magnetic resonance (NMR) analysis of astaxanthin dideoxyglycoside, the purified astaxanthin dideoxyglycoside was dissolved in CD_3OD and the ^1H NMR (600 MHz) spectra were recorded on a Bruker Avance 600 system. The HPLC retention times, the UV/Vis absorption spectra, the mass fragmentation spectra, and the NMR spectra were all combined for the structural identification of astaxanthin dideoxyglycoside.

In vitro reaction of CrtX To obtain the crude enzyme extracts from the *E. coli* expressing *Pantoea agglomerans* CrtX (Song et al. 2013) (PAX), *Sphingomonas* sp. PB304 CrtX (304X), or the empty plasmid pUCM, each plasmid (pUCM-PAX, pUCM-304X, or pUCM) was transformed into *E. coli* XL1-Blue. The resulting single colonies were inoculated in 5 mL LB medium containing 100 μg/mL of ampicillin at 37 °C for 12 h, diluted in 50 mL TB medium to an optical density of 0.05 at 600 nm, and cultivated at 30 °C for 12 h. The cells were harvested by centrifugation (13,500×*g*), washed in phosphate-buffered saline, resuspended in 1 mL of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM 2-mercaptoethanol, and then lysed by sonication (Sonics & Materials Inc.; Newtown, CT, USA—5 s on/15 s off, 15 cycles). After centrifugation (13,500×*g*, 4 °C) for 30 min, each supernatant was collected and quantified using the Bradford reagent (Bio-Rad; Hercules, CA, USA). The in vitro assay mixture (100 μL) was prepared by mixing 80 μL of the enzyme extract (1.5 mg), 8.5 μM of astaxanthin in 5 μL of acetone, and 15 μL of 100 nmol of UDP-glucose (Sigma-Aldrich; St. Louis, MO, USA) or GDP-fucose (Sigma-Aldrich; St. Louis, MO, USA) in 50 mM Tris–HCl (pH 7.5) containing 1 mM 2-mercaptoethanol. The reaction mixtures were incubated at 37 °C for 5 h in the dark, and the reaction was stopped by adding 200 μL of methanol into the mixture, and then extracted using 300 μL of EtOAc after adding a 5 N NaCl solution. The upper phase was collected and dried using the EZ-2 *Plus* evaporator. The samples were resuspended in 50 μL of methanol, and a 20-μL aliquot was injected into the Agilent 6150 HPLC/MS analysis system.

Results

Taxonomic analysis of the isolated strain PB304 For taxonomic analysis, the 16S rRNA gene sequence of the strain PB304 was amplified and aligned with the reference sequences. The analysis of 16S rRNA sequence similarities (Fig. 1) showed that PB304 is most closely related to *Sphingomonas astaxanthinifaciens* TDMA-17^T (97.78 %), *Sphingomonas humi* PB323^T (98.94 %), and *Sphingomonas kaistensis* PB56^T (98.24 %), which are all known to produce orange to deep red carotenoid pigments (Yi et al. 2010). Based on the 16S rRNA sequence analysis, the strain PB304 belongs to the genus *Sphingomonas* and was therefore named *Sphingomonas* sp. PB304.

Carotenoid profile of Sphingomonas sp. PB304 and structural assignment of the major Sphingomonas sp. PB304 carotenoid As pigmented *Sphingomonas* strains are known to produce polar carotenoids, we analyzed the carotenoid profile of *Sphingomonas* sp. PB304 using HPLC. The astaxanthin-producing recombinant *E. coli* strain XL1-Blue [pACM-EBIYZ$_{PA}$ + pUCM-NOW] (Table 1) was used for comparison. One major peak (peak 1 in Fig. 2a) in the crude

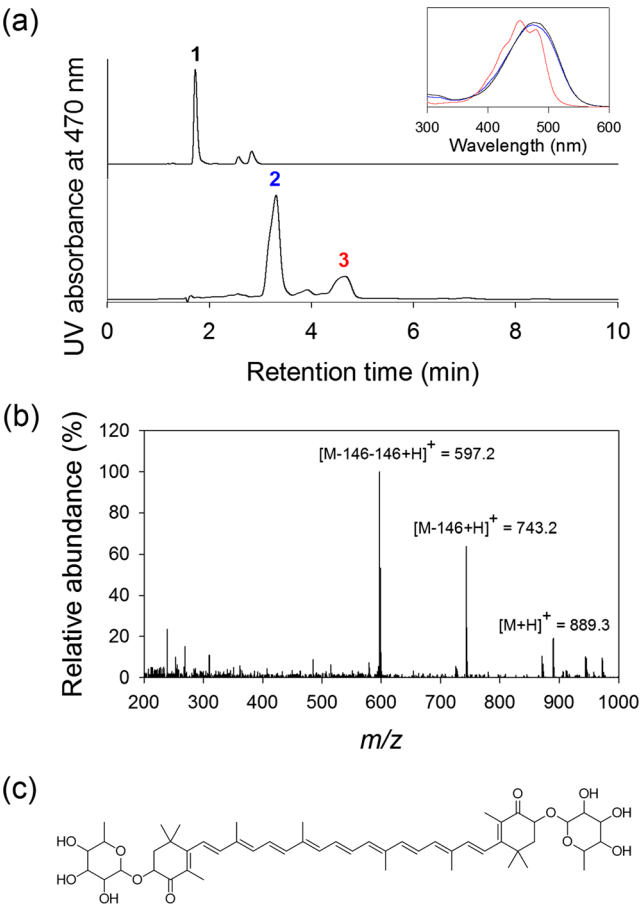

Fig. 2 **a** Carotenoid profiles for *Sphingomonas* sp. PB304 (*top*) and recombinant *E. coli* expressing astaxanthin and zeaxanthin (*bottom*). The *inset* shows the UV/Vis spectra for each peak recorded in the HPLC chromatograms. Each peak was identified as follows: *peak 1*, astaxanthin dideoxyglycoside; *peak 2*, astaxanthin; and *peak 3*, zeaxanthin. **b** Mass spectrum of astaxanthin dideoxyglycoside. **c** Structure of astaxanthin dideoxyglycoside

methanol extract from *Sphingomonas* sp. PB304 had a shorter retention time than that of astaxanthin (peak 2 in Fig. 2a), but its UV/Vis spectrum (λ_{max}=478 nm) was almost identical to that of astaxanthin (inset in Fig. 2a). HPLC/MS and ^{1}H NMR analyses were performed for characterizing the major carotenoid of *Sphingomonas* sp. PB304. MS analysis revealed that the major carotenoid has a molecular ion with $[M+H]^+$=889.3 and two dominant daughter ions with $[M-146+H]^+$=743.2 and $[M-146-146+H]^+$=597.2 (Fig. 2b). This fragmentation pattern suggests that the major carotenoid has two deoxysugar moieties such as dideoxyglycoside (Asker et al. 2009). ^{1}H NMR analysis supported the MS results and indicated that the major carotenoid has two deoxysugar moieties at each of the β-ionone rings (Online Resource Fig. S1, Online Resource Table S2). Based on the retention time, UV/Vis spectrum, MS fragmentation pattern, and ^{1}H NMR spectrum, we propose that the major carotenoid of *Sphingomonas* sp. PB304 is astaxanthin 3,3′-dideoxyglycoside (Fig. 2c), whose structure is similar to that identified in *S. astaxanthinifaciens* (Asker et al. 2009).

Identification of the crtZ, crtB, crtI, and crtY genes of Sphingomonas sp. PB304 We tried to isolate the gene clusters involved in astaxanthin dideoxyglycoside biosynthesis in *Sphingomonas* sp. PB304. To identify the gene clusters encoding the astaxanthin dideoxyglycoside biosynthetic enzymes, a *Sphingomonas* sp. PB304 fosmid library (containing ≈3,000 clones) was visually screened based on the expected phenotypic change (i.e., the appearance of color in recombinant *E. coli* clones due to the accumulation of carotenoids) (Krubasik and Sandmann 2000). However, as it took several months to obtain positive color clones, as an alternative, we designed degenerate PCR primers (Online Resource Table S1) that harbored highly conserved CrtI amino acid sequences from *S. elodea* ATCC 31461 (Zhu et al. 2011) and used them to screen the fosmid library clones. Following colony hybridization of the fosmid library, five positive clones were selected and fully sequenced. Four putative carotenogenic genes (*crtZ*, *crtB*, *crtI*, and *crtY*) were found in cluster 1 using the National Center for Biotechnology Information (NCBI) Blast program (blastp; Fig. 3a, cluster 1). Of the putative *Sphingomonas* sp. PB304 enzymes, CrtZ showed the highest amino acid similarity to *Sphingobium indicum* CrtZ (60 %); CrtB showed 57 % similarity to *S. elodea* CrtB, CrtI showed 70 % similarity to *Blastomonas* sp. AAP53 CrtI; and CrtY showed 57 % similarity to *Sphingobium chlorophenolicum* L-1 CrtY. The other open-reading frames (ORFs) showed very low amino acid similarities to proteins involved in carotenoid biosynthesis. Similar to the other carotenoid biosynthetic gene clusters, the arrangements of carotenogenic genes were highly conserved in the gene cluster of *Sphingomonas* sp. PB304 (Online Resource Fig. S2).

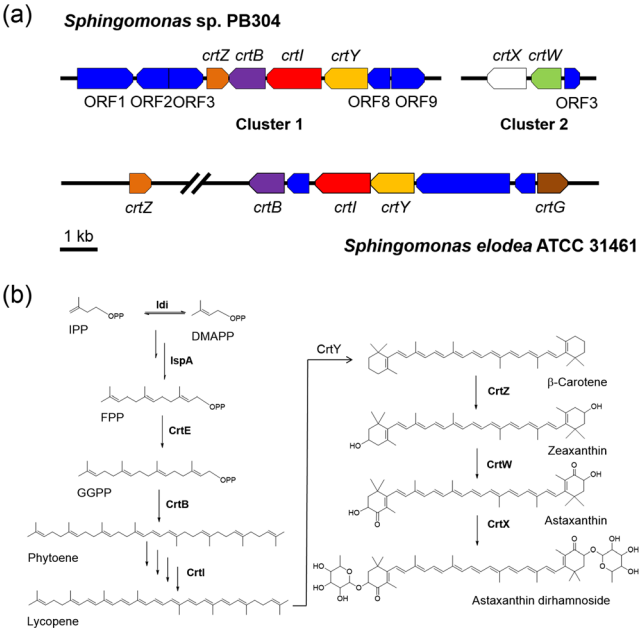

Fig. 3 **a** Carotenogenic gene clusters in *Sphingomonas* sp. PB304 (*top*) and *S. elodea* ATCC 31461 (*bottom*). A 1-kb scale bar is shown. **b** Proposed biosynthetic pathway of astaxanthin dideoxyglycoside in *Sphingomonas* sp. PB304. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *Idi* isopentenyl pyrophosphate isomerase, *IspA* FPP synthase

Identification of the crtW and crtX genes of Sphingomonas sp. PB304 Cluster 1, which encodes the putative enzymes CrtZ, CrtB, CrtI, and CrtY, could only lead to the production of hydroxylated carotenoids such as zeaxanthin, which does not correlate with the observed carotenoid profile for *Sphingomonas* sp. PB304 and the presence of astaxanthin dideoxyglycoside (Fig. 2). The carotenoid profile and the unique UV/Vis absorption spectrum of astaxanthin dideoxyglycoside, which is similar to that of ketocarotenoids (Lee et al. 2003), strongly indicate that additional genes, such as those encoding β-carotene ketolase, should be present. Therefore, we designed a second group of degenerate PCR primers (Online Resource Table S1), which contained highly conserved CrtW amino acid sequences from *Nostoc* sp. PCC7120 (Lee et al. 2003), and used them as probes to screen for sequences encoding β-carotene ketolase. From the fosmid library of 3,000 clones, five positive clones were selected and fully sequenced. Two genes encoding putative carotenogenic enzymes (CrtX, CrtW) were identified in cluster 2 using the NCBI Blast program (blastp; Fig. 3a, cluster 2). *Sphingomonas* sp. PB304 CrtX showed the highest amino acid similarity to a putative glucosyltransferase from *Aurantimonas manganoxydans* (44 %) and a zeaxanthin glucosyltransferase from *Pseudomonas stutzeri* (43 %). *Sphingomonas* sp. PB304 CrtW showed the highest amino acid similarity to a fatty acid desaturase from *Parvularcula bermudensis* (55 %). ORF3 in cluster 2 showed very low amino acid similarities to the proteins involved in carotenoid

biosynthesis. Based on the structural information of the major carotenoid, astaxanthin dideoxyglycoside, and six putative carotenogenic enzymes, the biosynthesis pathway for astaxanthin dideoxyglycoside in *Sphingomonas* sp. PB304 is proposed as shown in Fig. 3b. Unfortunately, we could not find a gene encoding a putative GGPP synthase (CrtE) although we sequenced clusters 1 and 2 completely. It is notable that a few other carotenoid biosynthesis clusters, such as those in *P. bermudensis* (Oh et al. 2011) and *S. elodea* (Zhu et al. 2012), also lack a gene encoding CrtE (Online Resource Fig. S2).

Functional analysis of the putative carotenogenic enzymes from Sphingomonas sp. PB304 For the functional analysis of the six astaxanthin dideoxyglycoside biosynthetic enzymes from *Sphingomonas* sp. PB304, each gene was modified for constitutive expression in a strain of *E. coli* (Kim et al. 2010) designed to express carotenoid biosynthetic modules from *P. agglomerans* (Song et al. 2013). Each gene product functionally catalyzed its expected reaction of converting a substrate to a product in the heterologous host *E. coli* (Fig. 4), except for CrtX. CrtB and CrtI produced lycopene (peak 1 in Fig. 4a, b) when complemented with *P. agglomerans* CrtE-CrtI and CrtE-CrtB, respectively, in *E. coli*. The accumulating lycopene turned the *E. coli* cells reddish in color (inserts in Fig. 4a, b). CrtY converted lycopene to β-carotene (peak 2 in Fig. 4c) when complemented with *P. agglomerans* CrtE-CrtB-CrtI, resulting in yellow *E. coli* (insert in Fig. 4c). CrtZ converted β-carotene to zeaxanthin (peak 3 in Fig. 4d) when complemented with *P. agglomerans* CrtE-CrtB-CrtI-CrtY, resulting in deep yellow *E. coli* (insert in Fig. 4d). CrtW converted zeaxanthin to astaxanthin (peak 4 in Fig. 4e) when complemented with *P. agglomerans* CrtE-CrtB-CrtI-CrtY-CrtZ, resulting in deep yellow *E. coli* (insert in Fig. 4e). As inferred from the astaxanthin dideoxyglycoside biosynthesis pathway (Fig. 3b), *Sphingomonas* sp. PB304 CrtX did not produce any glycosylated carotenoids in *E. coli* (Fig. 4f). One possible explanation for this result is that the dideoxyglycoside moieties in astaxanthin dideoxyglycoside could be rhamnose, as described for *S. astaxanthinifaciens* TDMA-17^T (Asker et al. 2009), but *E. coli* might not harbor an available GDP-D-rhamnose pool for *Sphingomonas* sp. PB304 CrtX unlike *Sphingomonas* sp. PB304. Because no authentic GDP-D-rhamnose is commercially available, an alternative in vitro assay for *Sphingomonas* sp. PB304 CrtX in a crude extract form was carried out with the deoxy sugar GDP-fucose, UDP-glucose, and astaxanthin as the substrates, instead of the natural deoxy sugar GDP-D-rhamnose. Crude extracts of *E. coli* expressing *P. agglomerans* CrtX or empty plasmid were also prepared as a positive and negative control, respectively. HPLC/MS analysis of the in vitro reaction mixtures showed that *Sphingomonas* sp. PB304 CrtX possessed the same carotenoid profile as that of the negative control, indicating that *Sphingomonas* sp. PB304 CrtX took up neither the deoxy sugar GDP-fucose nor the UDP-glucose (Fig. 5a). However, *P. agglomerans* CrtX took up UDP-glucose as a substrate and functionally attached the glucose moiety to astaxanthin (peak 1 in Fig. 5a), leading to the production of astaxanthin glucoside (peak 2 in Fig. 5a), as indicated by the recorded UV/Vis and MS spectra (Fig. 5b, c). Unexpectedly, *P. agglomerans* CrtX seemed to attach GDP-fucose to astaxanthin, leading to the production of astaxanthin

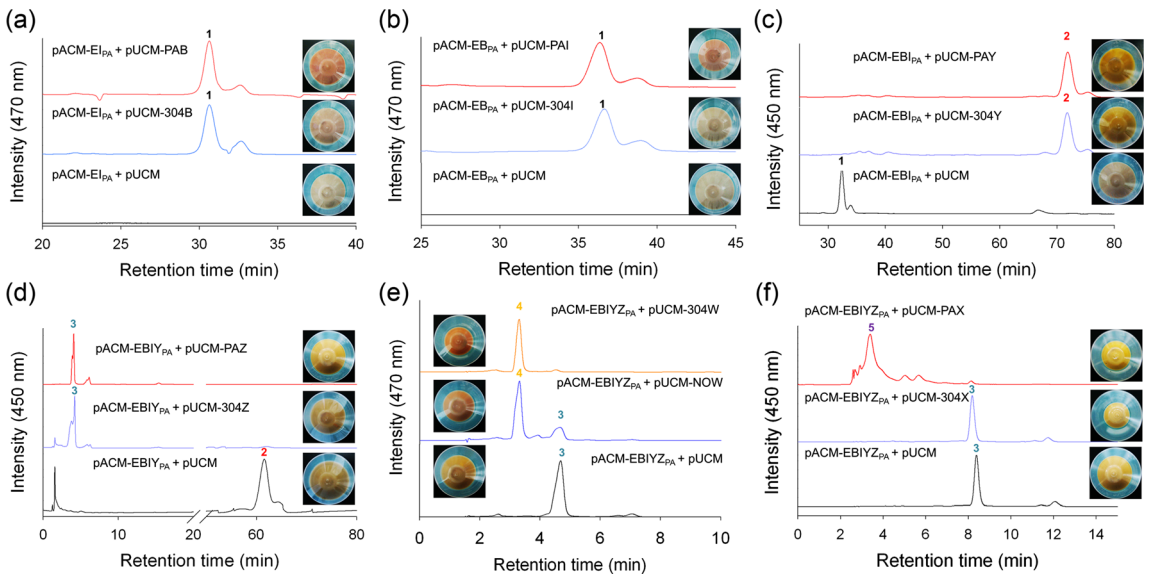

Fig. 4 Functional complementation of putative carotenogenic enzymes from *Sphingomonas* sp. PB304. CrtB (**a**), CrtI (**b**), CrtY (**c**), CrtZ (**d**), CrtW (**e**), and CrtX (**f**) were coexpressed with the complementary gene modules (Table 1) of *P. agglomerans* in *E. coli*. Each peak was identified as follows: *peak 1*, lycopene; *peak 2*, β-carotene; *peak 3*, zeaxanthin; *peak 4*, astaxanthin; and *peak 5*, zeaxanthin diglucoside. *Inserts* are cell pellets expressing carotenoids

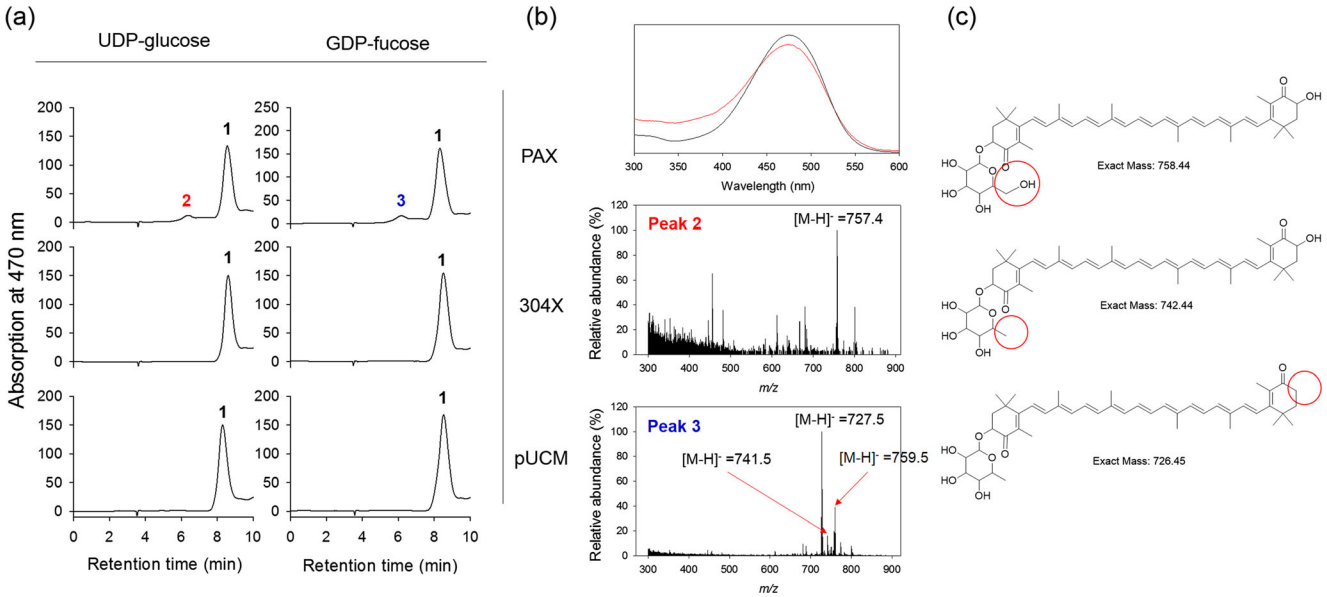

Fig. 5 In vitro activity of *Sphingomonas* sp. PB304 CrtX (304X) and *P. agglomerans* CrtX (PAX). Crude extracts of *E. coli* expressing 304X, PAX, or the empty vector (pUCM) were prepared and used for an in vitro assay with GDP-L-fucose, GDP-glucose, and astaxanthin as substrates. **a** HPLC profiles of each in vitro reaction mixture catalyzed by 304X and PAX. *pUCM* indicates the negative control. **b** UV/Vis and MS spectra of *peaks 2* and *3*. **c** Expected structures of the glycosylated carotenoids. Each peak was identified as follows: peak 1, astaxanthin; peak 2, astaxanthin glucoside; and peak 3, astaxanthin monofucoside

monofucoside (peak 3 in Fig. 5a). This interpretation was based on the UV/Vis spectrum and on the masses of the fragment ions (Fig. 5b, c), although the mass of the parent ion was not detected in the MS spectrum.

Phylogenetic analysis of Sphingomonas sp. PB304 CrtX In nature, most cyclic or acyclic carotenoids that contain sugar(s) in their structures possess a glucose moiety, the transfer reaction of which is catalyzed by a glucosyltransferase (CrtX) (Dembitsky 2005). However, less common sugars such as fucose, rhamnose, and chinovose can be incorporated into carotenoids as an alternative sugar moiety in some cyanobacteria and bacteria (Takaichi and Mochimaru 2007; Takaichi et al. 2010). As described above, although *Sphingomonas* sp. PB304 CrtX is proposed to transfer deoxyglycoside rather than glucose, as shown in Fig. 2, experimental evidence for this mechanism is limited. Therefore, a phylogenetic analysis was performed with selected orthologous bacterial glycosyltransferases (Fig. 6), whose function was analyzed to determine the phylogenetic position of *Sphingomonas* sp. PB304 CrtX. Despite the substrate specificity of *Sphingomonas* sp. PB304 CrtX for deoxyglycoside, in the phylogenetic tree, it is located within the glucosyltransferase group, which catalyzes the attachment of glucose to the β-ionone rings in zeaxanthin to form zeaxanthin diglucoside (*Pantoea ananatis*, Misawa et al. 1990; *P. agglomerans*, Hundle et al. 1992; *Paracoccus haeundaensis*, Seo et al. 2009). The other group includes glycosyltransferases, which catalyze the transfer of glycoside to the hydroxyl group(s) in a linear carotene backbone (rather than in a β-ionone ring) to produce carotenoid glycosides such as myxoxanthophyll, staphyloxanthin, and decaprenoxanthin diglucoside (*Staphylococcus aureus*, Pelz et al. 2005; *Chlorobium tepidum*, Maresca and Bryant 2006; *Dietzia* sp., Tao et al. 2007; *Synechococcus* sp. PCC 7002, Graham and Bryant 2009; *Micrococcus luteus*, Netzer et al. 2010; *Corynebacterium glutamicum*, Heider et al. 2014). The phylogenetic position of *Sphingomonas* sp. PB304 CrtX indicates that bacterial sugar transferases in carotenoid pathways distinguish the carotenoid backbone structures, especially the end-groups, more strictly than the sugar moieties.

Discussion

Sphingomonas strains have been isolated from various sources including soil, ponds, and radioactive sites (Takeuchi et al. 2001; Asker et al. 2007b). They produce yellow to orange carotenoids as one of the resistant factors to combat the oxidative stress in unfavorable environments. The species *Sphingomonas* sp. PB304, isolated from a river in Daejon City, South Korea, produces astaxanthin dideoxyglycoside, a carotenoid containing deoxyglycoside at each end of its β-ionone rings (Fig. 2c). This unique structure was first identified in *S. astaxanthinifaciens*, which was isolated from a highly radioactive site in Japan (Asker et al. 2007a, 2009). Although both the strains produce astaxanthin dideoxyglycoside, *Sphingomonas* sp. PB304 produces it as its major carotenoid (Fig. 2a), whereas in *S. astaxanthinifaciens*,

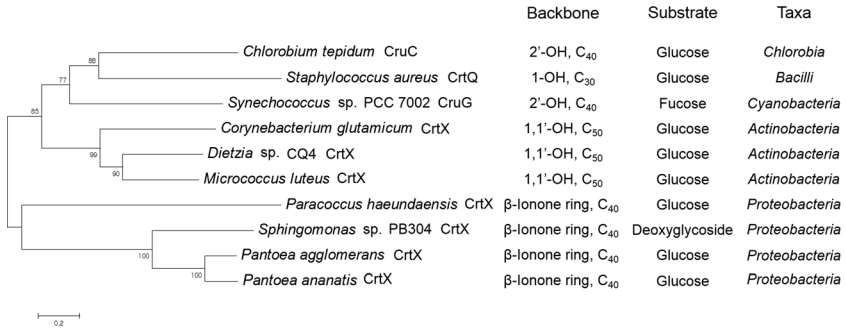

Fig. 6 Neighbor-joining phylogenetic tree constructed using the MEGA6 software (Tamura et al. 2013) with CrtX protein sequences showing homology to *Sphingomonas* sp. PB304 CrtX. Bootstrap values expressed as a percentage of 1,000 replications determined using the MEGA6 program are shown next to each node

it constitutes only 58.4 % of the total carotenoids produced (Asker et al. 2009). In spite of a significant amount of research work on astaxanthin dideoxyglycoside biosynthesis, little is known about the biosynthesis of astaxanthin dideoxyglycoside in the genus *Sphingomonas*.

To elucidate the biosynthesis pathway of astaxanthin dideoxyglycoside in *Sphingomonas* sp. PB304, a fosmid library was constructed using the *Sphingomonas* sp. PB304 genomic DNA, which was then screened with *crtI* and *crtW* gene-specific probes. Although next-generation sequencing technology is available for the identification of specific pathways, a fosmid library covering the whole genome of a specific microorganism is also a valuable source for elucidating gene clusters under 30–40 kb in size (Schmidt et al. 2005) or for screening novel enzymes from the metagenomic samples (Kakirde et al. 2010). Six ORFs involved in the biosynthesis of astaxanthin dideoxyglycoside were identified from two separate gene clusters (clusters 1 and 2; Fig. 3a). Cluster 1 has four genes encoding the putative enzymes CrtZ, CrtB, CrtI, and CrtY for the biosynthesis of zeaxanthin (Fig. 3b), and cluster 2 has two genes encoding the putative enzymes CrtW and CrtX, which catalyze two reactions for the biosynthesis of astaxanthin dideoxyglycoside from zeaxanthin (Fig. 3b). Notably, a gene encoding CrtE could not be found in either gene cluster, which was also observed in the case of the carotenogenic gene cluster of *S. elodea* ATCC 31461 (Zhu et al. 2012). Another notable characteristic of the *Sphingomonas* sp. PB304 astaxanthin dideoxyglycoside gene cluster is the order of the genes on the cluster (*crtY*, *crtI*, *crtB*). Many different carotenogenic gene clusters found in bacteria show the same order of these genes (Online Resource Fig. S2). It is possible that the gene cluster encoding putative CrtY, CrtI, and CrtB has been conserved during evolution, because β-carotene, which is produced via sequential reactions with CrtB, CrtI, and CrtY, serves as a substrate for the structurally more diverse carotenoids such as zeaxanthin, astaxanthin, and nostoxanthin. All five putative carotenogenic enzymes except for CrtX were functional in the heterologous host *E. coli* (Fig. 5). It seems that the lack of cellular GDP-rhamnose in *E. coli* prevents the formation of a glycosylated carotenoid when CrtX is coexpressed with the zeaxanthin biosynthesis pathway enzymes (Fig. 5f). Unlike *P. agglomerans* CrtX, which functionally attaches a sugar moiety of GDP-fucose and a UDP-glucose moiety to astaxanthin, *Sphingomonas* sp. PB304 CrtX did not take up either the deoxy sugar GDP-fucose or the UDP-glucose as substrates during the in vitro reaction (Fig. 6), thereby suggesting that it has narrower substrate specificity than *P. agglomerans* CrtX.

The cellular functions for the glycoside moieties of carotenoids have not yet been identified but are suspected to influence membrane rigidity, fluidity, and stability, especially under harsh conditions (Britton 1995; Mohamed et al. 2005). In terms of industrial applications, glycosylated carotenoids have the advantage of higher water solubility compared to other carotenoids. For example, crocin, a C_{20} glycosylated carotenoid with four glucose moieties (Moraga et al. 2004), is a water-soluble carotenoid of commercial importance. Like crocin, astaxanthin dirhamnoside also shows higher solubility than astaxanthin (Asker et al. 2009), although it exhibits a slightly lower antioxidant activity. Phylogenetic analysis of *Sphingomonas* sp. PB304 CrtX showed that it is included in a clade of carotenoid glucosyltransferases such as zeaxanthin glucosyltransferase, which catalyze the transfer of glucose onto the β-ionone ring(s) in the carotene backbones. This is presumably the first description of a CrtX that transfers GDP-deoxyglycoside instead of a UDP-glucose onto the β-ionone rings, although further studies, such as the mutational analyses of the *crtX* gene in *Sphingomonas* sp. PB304, are needed to confirm this function. Therefore, CrtX could serve to be a good model protein for studying the differences between the catalytic preferences of carotenoid glycosyltransferases.

In conclusion, the astaxanthin dideoxyglycoside pathway of *Sphingomonas* sp. PB304 proposed here helps to elucidate the evolutionary history and relationships of carotenogenesis in the genus *Sphingomonas*. Carotenogenic genes from *Sphingomonas* sp. PB304, such as *crtW* and *crtX*, can be used for producing diverse carotenoid structures of industrial significance. Moreover, further elucidation of the proposed

function of CrtX, i.e., its ability to transfer GDP-deoxyglycoside onto the β-ionone ring(s), will offer additional insights into the carotenoid glycosyltransferase protein family.

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Conflict of interest The authors have no conflict of interest to declare.

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