The cyanobacterium *Gloeobacter violaceus* PCC 7421 uses bacterial-type phytoene desaturase in carotenoid biosynthesis

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Abstract Carotenoid composition and its biosynthetic pathway in the cyanobacterium *Gloeobacter violaceus* PCC 7421 were investigated. β-Carotene and (2*S*,2′*S*)-oscillol 2,2′-di(α-L-fucoside), and echinenone were major and minor carotenoids, respectively. We identified two unique genes for carotenoid biosynthesis using in vivo functional complementation experiments. In *Gloeobacter*, a bacterial-type phytoene desaturase (CrtI), rather than plant-type desaturases (CrtP and CrtQ), produced lycopene. This is the first demonstration of an oxygenic photosynthetic organism utilizing bacterial-type phytoene desaturase. We also revealed that echinenone synthesis is catalyzed by CrtW rather than CrtO. These findings indicated that *Gloeobacter* retains ancestral properties of carotenoid biosynthesis.
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1. Introduction

Carotenoids are ubiquitously found in photosynthetic organisms. Their functions are light harvesting, photo-protection by quenching of the chlorophyll (Chl) triplet state, scavenging of singlet oxygen and harmful radicals, and assembly of functional photosynthetic apparatus by stabilization of pigment–protein complexes [1].

Carotenoid biosynthetic pathways have been studied in various species. The initial step is condensation of two geranylgeranyl diphosphate molecules to yield phytoene through the isoprenoid biosynthetic pathway. This pathway is widely distributed, and phytoene synthase genes (*crtB*/*pys*) are commonly conserved among all organisms. Phytoene is converted to lycopene by four-step desaturation; however, the enzyme(s) involved in this reaction varies depending on the species [2]. Oxygenic photosynthetic organisms (cyanobacteria and higher plants) use two structurally related enzymes, phytoene desaturase (CrtP/Pds) and ζ-carotene desaturase (CrtQ/Zds), whereas most bacteria and fungi use only one enzyme, phytoene desaturase (CrtI), which catalyzes four-step desaturation of phytoene [2].

Gloeobacter violaceus PCC 7421 (hereafter referred to as *Gloeobacter*) is a unicellular cyanobacterium isolated from calcareous rock in the mountainous regions of Switzerland [3]. Its most remarkable morphological characteristic is the lack of intracellular membranes. Phylogenetic analysis of 16S rRNA sequences revealed that *Gloeobacter* branched off at an early stage within the cyanobacterial clade [4]; therefore, this organism is thought to retain traces of the ancestral properties of cyanobacteria. Recently, the complete genome sequence of *Gloeobacter* was determined [5], enabling systematic studies on this organism. In this study, we determined the carotenoid composition and identified carotenoid biosynthetic genes of *Gloeobacter* by in vivo complementation experiments. We found that the biosynthetic pathway of carotenoids in *Gloeobacter* was unique in cyanobacteria, reflecting the evolutionary position of this species in the cyanobacterial clade.

2. Materials and methods

2.1. Growth of the cyanobacterium and carotenoid analysis

Gloeobacter violaceus PCC 7421 was grown photoautotrophically in BG-11 medium as described previously [6]. Pigments were extracted using an acetone/methanol mixture (7:2, v/v) and analyzed by high performance liquid chromatography (HPLC) equipped with a μBondapak C_{18} column (8 × 100 mm, RCM type, Waters, USA) eluted with methanol (2.0 ml/min); carotenes were analyzed with a Novapak C_{18} column (8 × 100 mm, RCM type) eluted with acetonitrile/methanol/tetrahydrofuran (58:35:7, by vol.). Elution patterns were monitored with a MCPD-3600 photodiode array detector (Otsuka Electronics, Japan). The circular dichroism (CD) spectrum was measured with a J-820 spectropolarimeter (JASCO, Japan) in diethyl ether/2-pentane/ethanol (5:5:2, by vol.). Relative molecular masses were measured with a M-2500 double-focusing gas chromatograph-mass spectrometer

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Abbreviations: CD, circular dichroism; Chl, chlorophyll; FD-MS, field desorption mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance

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(Hitachi, Japan) equipped with field-desorption apparatus (FD-MS). Nuclear magnetic resonance (NMR) spectra in $CDCl_3$ were measured with a UNITY INOVA-500 system (500 MHz; Varian, USA), as described previously [7].

2.2. Plasmid construction

We constructed a new expression vector compatible with p15A replicon to avoid additional amino acid residues fusing at the N-terminus of recombinant proteins. The broad host range plasmid RSF1010 [8] was digested with *Xmn*I and *Pst*I, and ligated to the *Sma*I–*Pst*I fragment of pBSL130 (streptomycin-resistance gene cassette) [9] to yield pKUT101. The *tac* promoter, which was modified by introducing *Kpn*I sites at both ends and a *Nde*I site at the start codon, was then produced by PCR and introduced into the *Kpn*I site of pKUT101 (pKUT111). Three genes (*glr0867*, *gll0394* and *gll1728*) with a *Nde*I site at the 5′ end and *Hin*dIII site at the 3′ end were generated by PCR and subcloned into pKUT111 (pKUTG-I, pKUTG-O, and pKUTG-W, respectively). The sequence integrity of all constructs generated by PCR was confirmed by nucleotide sequencing.

2.3. Carotenoid production in E. coli

Three plasmids (pKUTG-I, pKUTG-O, and pKUTG-W) were transformed into the phytoene-producing *E. coli* JM101 (pACCRT-EB) [10] and β-carotene-producing transformant JM101 (pAC-CAR16ΔcrtX) [11]. Resultant transformants were cultured in Luria–Bertani medium containing chloramphenicol (30 μg/ml), streptomycin (20 μg/ml) and 0.1 mM isopropyl β-D-thiogalactopyranoside at 25 °C for 48 h in the dark. Cells were then harvested by centrifugation (3000 × *g*, 10 min), and pigments were extracted and analyzed as described above.

3. Results

3.1. Carotenoid composition of Gloeobacter

First, we determined the carotenoid composition of *Gloeobacter* (Fig. 1). The major carotenoids were β-carotene and oscillol glycoside, and a trace amount of echinenone was also detected; no other carotenoids were found. β-Carotene and echinenone were further confirmed by FD-MS. The relative molecular mass of oscillol glycoside was 892. ^{1}H NMR spectra revealed that the carotenoid moiety was oscillol and two molecules of α-L-fucose were attached to the C-2 and C-2′ hydroxyl groups of oscillol. The stereochemistry was determined by a CD spectrum, and conclusively the structure was determined as (2*S*,2′*S*)-oscillol 2,2′-di(α-L-fucoside) (Fig. 3).

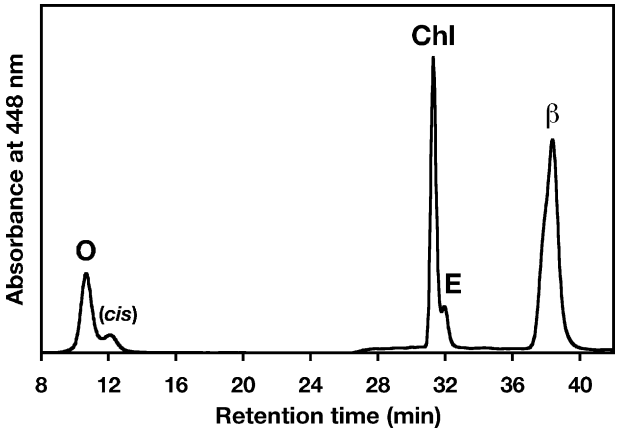

Fig. 1. The HPLC elution profile of pigments extracted from *Gloeobacter violaceus*. Pigments were analyzed with μBondapak C_{18} column. The eluent was a methanol–water (9:1) mixture for the first 20 min, and then absolute methanol. O, oscillol difucoside; Chl, Chl *a*; E, echinenone; β, β-carotene.

3.2. Carotenogenic gene candidates in the genome sequence of Gloeobacter

BLAST was used to search for carotenogenic genes in the genome sequence of *Gloeobacter* [12]. Seven genes homologous to functionally identified genes were found as summarized in Table 1. One gene, *gll0416*, showed 62% identity to the geranylgeranyl diphosphate synthase (CrtE) gene of *Thermosynechococcus elongatus* at the amino acid level [13], and another, *glr1744*, had 51% identity to phytoene synthase (CrtB) of *Synechocystis* sp. PCC 6803 [14]. Two plant-type desaturase genes (*crtP* and *crtQ*) conserved in cyanobacteria and higher plants were not found in *Gloeobacter*; CrtP and CrtQ catalyze desaturation steps sequentially from phytoene to lycopene via ζ-carotene as the intermediate. However, a gene, *glr0867*, homologous to a bacterial-type phytoene desaturase gene (*crtI*) was found. CrtI catalyzes lycopene synthesis through four-step desaturation of phytoene in (photosynthetic) bacteria and fungi. Thus, we hypothesized that *glr0867* is involved in carotenoid biosynthesis. CrtH is an isomerase that converts poly-*cis* lycopene produced by CrtQ into all-*trans* lycopene [15,16]. Although a *crtH*-like gene (*gll2133*) was discovered, it showed relatively low identity (33%) to that in *Synechocystis*. CrtL is the only known cyanobacterial lycopene β-cyclase to have been reported in *Synechococcus* and *Prochlorococcus* [17,18]; however, *crtL*-like genes were not found in *Gloeobacter*, unlike most other cyanobacteria. In *Synechocystis*, echinenone is produced by β-carotene ketolase (CrtO) [19]; in *Gloeobacter*, *gll0394* had 58% identity to *Synechocystis* CrtO. Moreover, one gene, *gll1728*, was homologous to another β-carotene ketolase (CrtW) [11]. β-Carotene hydroxylase genes (*crtR* and *crtZ*) were absent in the genome sequence of *Gloeobacter*, which is consistent with the lack of β-cryptoxanthin and zeaxanthin.

3.3. Identification of Gloeobacter carotenogenic genes by functional complementation in E. coli

The functions of probable carotenogenic genes in *Gloeobacter* were identified by functional complementation experiments using carotenoid-producing *E. coli*. First, *glr0867* (*crtI*-like gene) was expressed in phytoene-producing *E. coli* and carotenoid composition was analyzed (Fig. 2A). Lycopene accumulated as the major pigment, and intermediates (asymmetric-ζ-carotene and neurosporene) were also detected. This result shows that Glr0867 produced lycopene without CrtH. The presence of asymmetric-ζ-carotene suggests that the catalytic property of Glr0867 is similar to that of bacterial-type phytoene desaturases in some photosynthetic bacteria [20]. Thus, *glr0867* in *Gloeobacter* was identified as *crtI*.

We also found two genes in *Gloeobacter* homologous to structurally unrelated β-carotene ketolase genes (*crtO* and *crtW*) (Table 1). Each gene was independently introduced into β-carotene-producing *E. coli*. While no novel carotenoid was found in the *gll0394* (*crtO*-like gene)-expressing cells (data not shown), *gll1728* (*crtW*-like gene)-expressing cells produced echinenone and a small amount of canthaxanthin (Fig. 2B). Therefore, CrtW, not CrtO, catalyzed echinenone biosynthesis. Based on these results, we proposed the carotenoid biosynthetic pathway in *Gloeobacter* (Fig. 3).

Table 1
Candidates for carotenogenic enzymes in *Gloeobacter*

Identifier (residue)	Possible function	Identity with designated gene products (%)
Gll0416 (295)	Geranylgeranyl diphosphate synthase	CrtE of *Thermosynechococcus elongatus* (62)
Glr1744 (317)	Phytoene synthase	CrtB of *Synechocystis* sp. PCC 6803 (51)
Glr0867 (560)	Phytoene desaturase	CrtI of *Erwinia uredovora* (43)
Gll2133 (503)	Carotene isomerase	CrtH of *Synechocystis* sp. PCC 6803 (33)
Gll0394 (573)	β-Carotene ketolase	CrtO of *Synechocystis* sp. PCC 6803 (58)
Gll1728 (257)	β-Carotene ketolase	CrtW38 of *Nostoc punctiforme* PCC 73102 (41)
Gll2874 (507)	3,4-Desaturase	CrtD of *Synechocystis* sp. PCC 6803 (59)
Not found	Phytoene desaturase	CrtP
Not found	ζ-Carotene desaturase	CrtQ
Not found	Lycopene β-cyclase	CrtL
Not found	β-Carotene hydroxylase	CrtR
Not found	β-Carotene hydroxylase	CrtZ

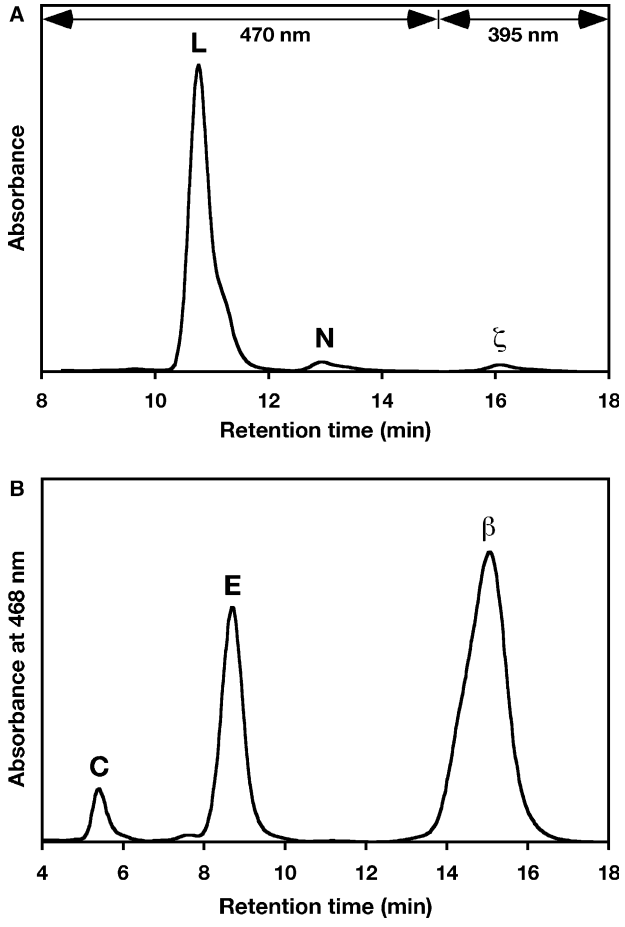

Fig. 2. HPLC elution profiles of carotenoids extracted from *E. coli* cells carrying plasmids pACCRT-EB and pKUTG-I (A), and pACCAR16ΔcrtX and pKUTG-W (B). Novapak C_{18} and μBondapak C_{18} columns were used for (A) and (B), respectively. Lycopene and neurosporene were detected at 470 nm (8–15 min), and asymmetric-ζ-carotene was detected at 395 nm (15–18 min) (A). L, lycopene; N, neurosporene; ζ, asymmetric-ζ-carotene; C, canthaxanthin; E, echinenone; β, β-carotene.

4. Discussion

Rippka et al. [3] were the first to determine the pigment composition of *Gloeobacter*; that is, Chl *a*, β-carotene, echinone (echinenone) and oscillaxanthin (oscillol glycoside(s)), using R_f values on thin layer chromatography and absorption maxima. We confirmed the presence of three carotenoid species and determined the stereochemistry and attached glycoside of oscillol difucoside. The carotenoid composition of *Gloeobacter* was very simple compared to other cyanobacteria, and seems to be a characteristic feature of this organism. Myxol glycoside, a widely distributed carotenoid glycoside in cyanobacteria, was absent in *Gloeobacter*, probably as a result of differences in the substrate specificity of lycopene cyclase and the following enzymes.

In photosynthetic organisms, there are two kinds of structurally unrelated phytoene desaturases [2], CrtP and CrtI. Cyanobacterial/plant-type phytoene desaturase (CrtP/Pds) converts phytoene to ζ-carotene, and ζ-carotene is further converted to all-*trans* lycopene by ζ-carotene desaturase (CrtQ/Zds) and carotene isomerase (CrtH). Although *Anabaena* sp. PCC 7120 contains CrtI-like desaturase, it shows ζ-carotene desaturase (CrtQ) activity [21]. *Gloeobacter* used bacterial-type phytoene desaturase (CrtI) to convert phytoene to lycopene. Thus, *Gloeobacter* is the first oxygenic photosynthetic organism that has been shown to use bacterial-type phytoene desaturase. These observations led us to hypothesize the following evolutionary scheme of this reaction step: desaturation of phytoene was carried out by CrtI in ancestral cyanobacteria then *crtP* and related desaturase genes were acquired; replacement of *crtI* by *crtP* occurred later.

In *Gloeobacter*, we found two genes (*gll0394* and *gll1728*) homologous to known β-carotene ketolases (Table 1). Since CrtO catalyzes echinenone biosynthesis in *Synechocystis*, the *crtO*-like gene (*gll0394*) is the most probable candidate for echinenone synthesis in *Gloeobacter*. However, Gll0394 exhibited no β-carotene ketolase activity, and on the contrary, echinenone accumulated by introduction of the *crtW*-like gene (*gll1728*) in *E. coli* (Fig. 2B). We examined expression levels of introduced genes in all transformants; no obvious accumulation of gene products was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). This means that accumulation of a large amount of gene products was not necessary for functional complementation. Although, we cannot exclude the possibility that Gll0394 is expressed at a much lower level than CrtI and CrtW in *E. coli*, we consider Gll0394 does not have β-carotene ketolase activity, because there have been no reports of organisms that use both CrtW and CrtO as β-carotene ketolase.

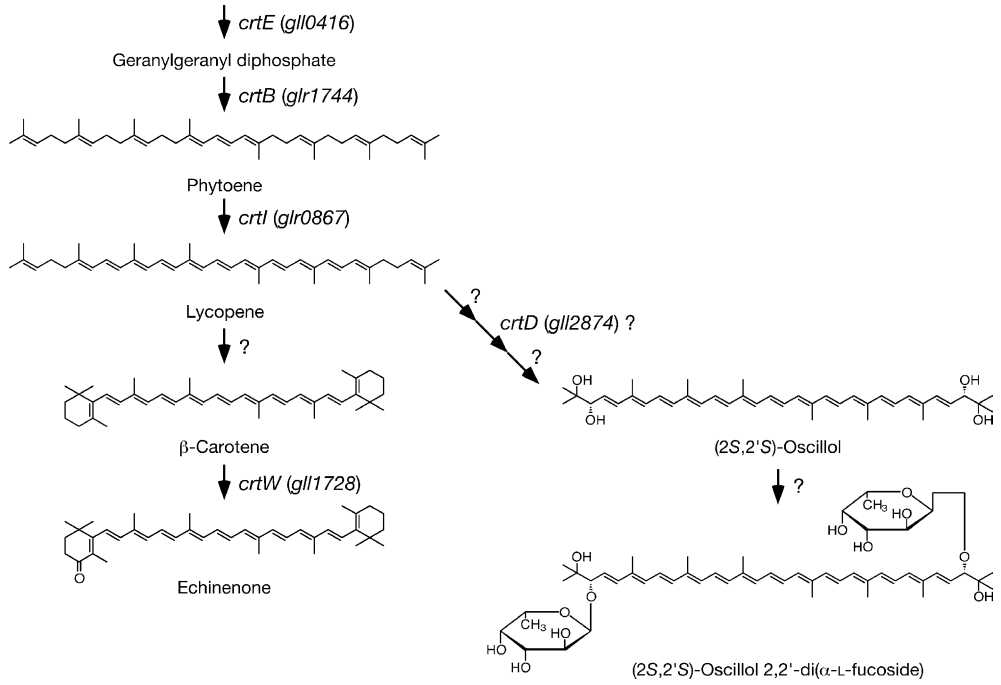

Fig. 3. Structures of carotenoids and the proposed carotenoid biosynthetic pathway in *Gloeobacter* with corresponding genes. Functions of *crtI* and *crtW* were confirmed in this study.

Recently, it was reported that two β-carotene ketolases (CrtW38 and CrtW148) in *Nostoc punctiforme* PCC 73102 catalyze conversion of β-carotene to canthaxanthin similar to other bacterial and algal CrtW [22]. Gll1728 produced a small amount of canthaxanthin in *E. coli* (Fig. 2B) even though *Gloeobacter* cells showed no canthaxanthin synthesis (Fig. 1A); this might have been caused by the effect of heterologous overexpression in *E. coli*, because the same result was observed with *Synechocystis* CrtO [19]. In addition, it was reported that CrtO from *Rhodococcus erythropolis* and *Deinococcus radiodurans* produced canthaxanthin by overexpression in β-carotene-producing *E. coli* [23]. Thus, both CrtO- and CrtW-type β-carotene ketolases can mediate echinenone and canthaxanthin formation. The accumulation of echinenone in *Gloeobacter* might have been caused by the substrate specificity of CrtW.

Although we demonstrated the presence of oscillol difucoside in *Gloeobacter* (Figs. 1 and 3), its biosynthetic pathway and related genes remain largely unknown. At least four enzymes (1,2-hydratase, 3,4-desaturase, 2-hydroxylase and fucose transferase) are necessary for production of oscillol difucoside from lycopene. Recently, a cyanobacterial 3′,4′-desaturase gene (*crtD*) involved in myxol biosynthesis was identified in *Synechocystis* sp. PCC 6803 [24]. Because *Gloeobacter* Gll2874 is homologous to *Synechocystis* CrtD, Gll2874 might function as 3,4-desaturase for oscillol biosynthesis. Other biosynthetic genes, even though there are a few candidates, for example, *gll0394*, remain unknown. Further studies to elucidate this unsolved pathway are therefore necessary. In conclusion, the findings of this study show that the carotenoid biosynthetic pathway(s) in *Gloeobacter* is quite unique in cyanobacteria, possibly reflecting the phylogenetic position of this organism.

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