

A New Type of Asymmetrically Acting β -Carotene Ketolase Is Required for the Synthesis of Echinenone in the Cyanobacterium *Synechocystis* sp. PCC 6803*

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Blanca Fernández-González, Gerhard Sandmann‡, and Agustín Vioque§

From the Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Americo Vespucio s/n, 41092 Sevilla, Spain and the ‡Botanisches Institut, Biosynthesis Group, Postfach 111932, D-60054 Frankfurt, Germany

We have isolated, based on the knowledge of the complete genomic sequence of the cyanobacterium *Synechocystis* sp. PCC 6803, an open reading frame (slr0088) similar to known bacterial carotene desaturases and have analyzed the function of the encoded protein. Surprisingly, this protein has no detectable desaturase activity with phytoene, hydroxyneurosporene, or ζ -carotene as substrates, but is rather a β -carotene ketolase that acts asymmetrically introducing a keto group on only one of the two β -ionone rings of β -carotene to generate echinenone. This is in contrast to the so far characterized β -carotene ketolases that act symmetrically, producing the di-keto carotenoid canthaxanthin from β -carotene without significant accumulation of echinenone. We have designated this new gene *crtO*. The function of the *crtO* gene product has been demonstrated by 1) the biosynthesis of echinenone when the *crtO* gene is expressed in an *Escherichia coli* strain able to accumulate β -carotene, 2) the *in vitro* biosynthesis of echinenone from β -carotene with cell free extracts from *E. coli* cells that express the *crtO* gene, and 3) the absence of echinenone in a *Synechocystis* strain in which the *crtO* gene has been insertionally inactivated. The primary structure of the *Synechocystis* asymmetric ketolase bears no similarity with the known β -carotene ketolases. *crtO* is not required for normal growth under standard or high light conditions, neither is the photosynthetic activity of the *crtO*-deficient strain affected.

Carotenoids are pigments that are synthesized by all photosynthetic organisms as essential components of the photosynthetic apparatus (1). They are also synthesized in heterotrophic growing bacteria and fungi. They participate in light collection in photosynthetic organisms and play a protective role against oxidation damage induced by strong oxidants produced in the photosynthetic membranes upon illumination (2).

In plants and cyanobacteria, carotenoids are synthesized by a similar pathway, from the C_{40} precursor phytoene (Fig. 1). Phytoene is converted to ζ -carotene by two sequential desaturations catalyzed by the enzyme phytoene desaturase. ζ -Caro-

tene is further oxidized by ζ -carotene desaturase to give lycopene, which is converted to β -carotene by lycopene cyclase (reviewed in Refs. 3–5).

Phytoene desaturase from cyanobacteria and plants, which introduce only two double bonds to produce ζ -carotene, have no homology with the bacterial type phytoene desaturases, which introduce three or four double bonds, producing neurosporene or lycopene, respectively (6). Therefore a second enzyme (ζ -carotene desaturase) is required in cyanobacteria and plants for the production of lycopene from ζ -carotene. ζ -Carotene desaturase from plants is homologous to the plant and cyanobacterial type phytoene desaturases (7). Surprisingly, the only cyanobacterial ζ -carotene desaturase identified, from *Anabaena* sp. PCC 7120, is homologous to the bacterial type phytoene desaturases (8). It has been suggested that plant and bacterial desaturases have independent evolutionary origins and that in cyanobacteria the bacterial type desaturase has been restricted to the last two dehydrogenation steps, while the plant type desaturase took over the function for the first two desaturation steps of phytoene. In *Synechocystis* no ζ -carotene desaturase has been identified so far.

The carotenoid pattern of cyanobacteria is very diverse (4), but cyanobacteria are not able to synthesize α -carotene and its derivatives as well as epoxy carotenoids. In *Synechocystis* the major carotenoids accumulated are β -carotene, myxoxanthophyll, zeaxanthin, and echinenone (9, 10). The biosynthetic pathway of those carotenoids is outlined in Fig. 1. None of the enzymes involved in the biosynthesis of myxoxanthophyll, zeaxanthin, and echinenone from β -carotene has been characterized, nor have their genes been cloned in cyanobacteria. The biosynthesis of zeaxanthin requires an hydroxylase that has been already characterized in some bacteria (11, 12). The biosynthesis of myxoxanthophyll requires several unidentified enzymes, and some of them might be equivalent to the ones used in *Rhodobacter* for the synthesis of spheroidenone (13). *Synechocystis* is one of the very few species that accumulates echinenone in substantial amounts (9). The biosynthesis of echinenone requires a ketolase that attacks only one of the β -rings of β -carotene. Genes coding for β -carotene ketolases were cloned and sequenced from two different bacteria (14) and the green alga *Haematococcus* (15, 16). However, all these related enzymes catalyze the simultaneous introduction of a keto group into position 4 of every ionone ring at each end of the molecule, yielding the symmetrically di-keto carotenoid canthaxanthin. Echinenone, with only one keto group, is found as a minor intermediate of the reaction (17, 18).

In the *Synechocystis* genomic sequence (19) an ORF¹ (slr0088) has been found with significant homology to bacterial

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§ To whom correspondence should be addressed: Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Isla de la Cartuja, Americo Vespucio s/n, 41092 Sevilla, Spain. Tel.: 34-5-4489519; Fax: 34-5-4460065; E-mail: vioque@cica.es.

¹ The abbreviations used are: ORF, open reading frame; HPLC, high performance liquid chromatography; μ E, microeinstein.

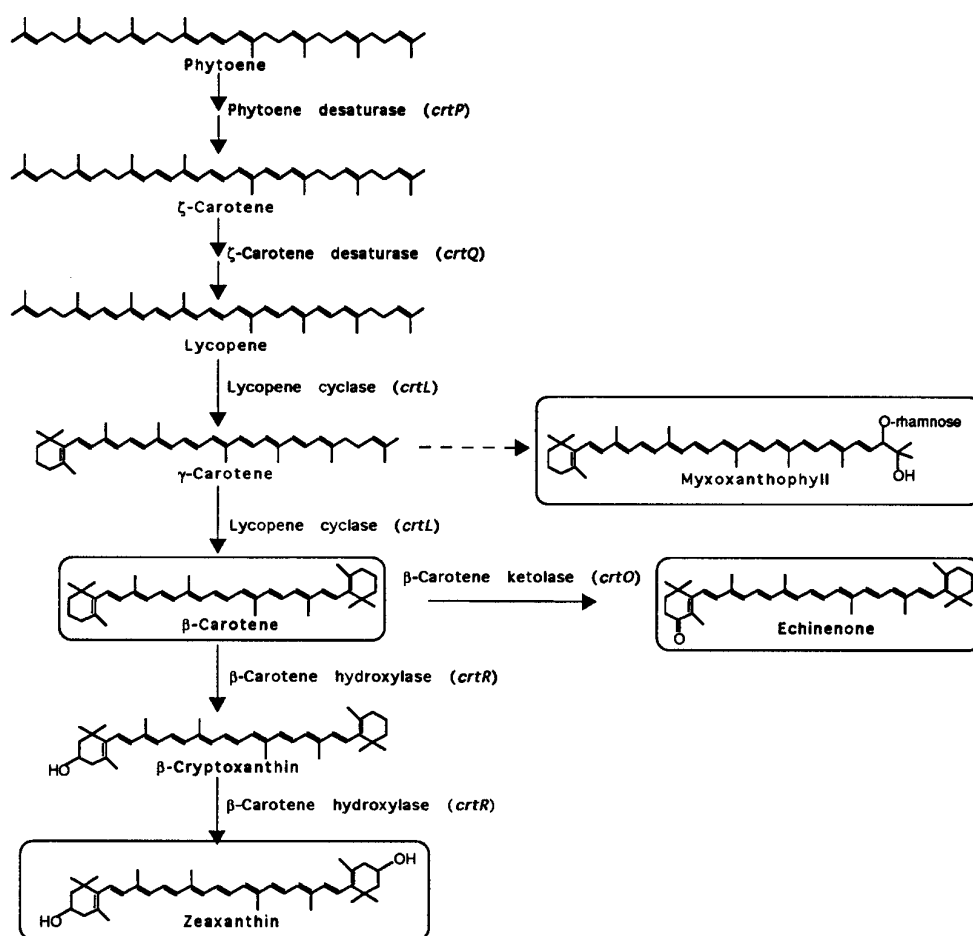


FIG. 1. **Carotenoid biosynthesis pathway.** The main carotenoids accumulated in *Synechocystis* 6803 are boxed. The pathway from γ -carotene to myxoxanthophyll requires several uncharacterized steps.

type carotene desaturases. Because it was possible that this protein was the *Synechocystis* ζ -carotene desaturase, we decided to undertake the cloning of the corresponding gene and the characterization of its function. Surprisingly slr0088 does not show any desaturase activity, but is rather a β -carotene ketolase that reacts asymmetrically on its substrate. We have demonstrated its function by functional complementation in *Escherichia coli*, by its *in vitro* activity, and by inactivation of the corresponding gene in *Synechocystis*.

This new β -carotene ketolase from *Synechocystis* is unrelated in structure to the symmetrically acting ketolases characterized in algae and bacteria and catalyzes a different reaction. Therefore we have named its gene *crtO* to differentiate it from the symmetrically acting ketolase genes (*crtW*).

EXPERIMENTAL PROCEDURES

Recombinant DNA Techniques—All manipulations were performed by standard methods (20) or as recommended by the manufacturers. *Synechocystis* cells were grown in BG11 medium (21) and DNA extracted as described (22). Southern blot was performed by standard procedures (20).

Cloning of β -Carotene Ketolase from *Synechocystis* 6803—ORF slr0088 was cloned by polymerase chain reaction based on the available *Synechocystis* genomic sequence (19). The forward primer (5'-AACAGAGAATTCATCACCACCGATGTTGTC-3') contains an *EcoRI* site (underlined) and overlaps the beginning of the coding sequence. The reverse primer (5'-AACAGAGGATCCTTACCAAAACGACGTTG-3') contains a *BamHI* site (underlined) and overlaps the 3'-end of the gene. A polymerase chain reaction product of the expected size was purified and treated with *EcoRI* and *BamHI* and cloned in the polylinker of pTrc99A (Pharmacia Biotech Inc.) to generate plasmid pTRCRT-O. In this plasmid expression of the *crtO* gene is controlled by the inducible pTrc promoter of the vector. The amino end of the expected recombinant

protein has two additional amino acids (MEFITTD... versus MITTD...).

Inactivation of *crtO*—To inactivate the *crtO* gene, a 1.3-kilobase pair *HincII* fragment containing a kanamycin resistance gene (*npt*) from Tn5 (23) was used to replace an internal *ClaI* fragment of the *crtO* gene. A plasmid with the *npt* gene inserted in the opposite orientation to the *crtO* ORF was used to transform *Synechocystis* sp. PCC 6803 wild type strain and cells were plated on kanamycin-containing plates. Transformants were grown on the same medium for several segregation rounds. Segregation was checked by Southern blot using as a probe an internal *BstXI* fragment from the *crtO* gene.

Complementation in *E. coli*—Plasmid pTRCRT-O was introduced in *E. coli* JM101 containing different plasmids depending on the function to be tested (see Table I). Plasmid pACCRT-EB (24) contains the genes *crtE* and *crtB* from *Erwinia*. Cells that carry this plasmid accumulate phytoene. Plasmid pACCRT-EBP (25) contains the genes *crtE* and *crtB* from *Erwinia* and the *crtP* gene from *Synechococcus*, and in plasmid pACCRT-EBI_{Re} the *crtP* gene is replaced by *crtI* from *Rhodobacter capsulatus* (26). Cells that carry these plasmids accumulate ζ -carotene and neurosporene, respectively. Plasmid pACCAR16 Δ crtX (14) contains the genes *crtE*, *crtB*, *crtI*, and *crtY* from *Erwinia*. Cells that carry this plasmid accumulate β -carotene. These four plasmids are all derivatives of vector pACYC184 carrying an origin of replication compatible with the origin of pTRCRT-O. Another vector used for complementation was pRKRT-C that is derived from pRK404 and carries the *crtC* gene from *R. capsulatus* coding for a neurosporene hydratase. The transformants were cultivated at 28 °C in LB medium containing ampicillin (100 μ g/ml), chloramphenicol (50 μ g/ml), and/or tetracycline (50 μ g/ml) according to the plasmids present. Cells were grown for 48 h in the presence of 2 mM isopropyl- β -D-thiogalactopyranoside before harvesting for carotenoid analysis.

Carotenoid Analysis—Carotenoids were extracted from freeze-dried *E. coli* or *Synechocystis* 6803 cells with methanol containing 6% KOH by heating for 15 min at 60 °C. After partitioning into 10% ether in petrol, the upper phase was collected, the solvent was evaporated, and the

TABLE I

Carotenoids accumulated in *E. coli* in the presence of pTRCART-O in combination with several plasmids that contain carotenoid biosynthesis genes

Carotenoids were extracted from *E. coli* cells carrying the plasmids indicated and analyzed as described under "Experimental Procedures."

Plasmid(s)	pTRCART-O	Carotenoids accumulated
pACCRT-EB	–	Phytoene
pACCRT-EB	–	Phytoene
pACCRT-EBP	–	ζ -Carotene
pACCRT-EBP	+	ζ -Carotene
pACCRT-EBIRc + pRKCR-C	–	Hydroxyneurosporene
pACCRT-EBIRc + pRKCR-C	+	Hydroxyneurosporene
pACCAR16 Δ crtX	–	β -Carotene
pACCAR16 Δ crtX	+	Echinenone, canthaxanthin, and β -carotene

residual carotenoids were resuspended in acetone. Separation and quantification of carotenoids were done by HPLC with a Nucleosil-C₁₈ 3 μ column and acetonitrile/methanol/2-propanol (85:10:5, v/v) as eluent at a flow rate of 1 ml/min. Spectra were recorded on-line at the elution peaks with a Kontron 440 photodiode array detector. For identification of carotenoids, authentic standards isolated and identified previously (9) were used.

In Vitro β -Carotene Ketolase Assay—Freshly harvested cells of JM101 carrying pCAR16 Δ crtX, which synthesize β -carotene, and of JM101 carrying pTRCART-O, in which the ketolase gene is expressed, were resuspended in 50 mM Tris-HCl, pH 7.5, and disrupted in a French pressure cell at 95 MPa. Both homogenates were treated with DNase (5 μ g/ml) for 15 min on ice. One served as a source of substrate and the other as a source of enzyme. The assay consisted of 250 μ l of each extract and 2 mM NADPH. Incubation was for 15 h at 30 °C. The equivalent to 600 ng of β -carotene was provided by the pCAR16 Δ crtX extract. The reaction was terminated by addition of 2.5 ml of methanol. After heating to 60 °C, the carotenoids were extracted and separated by HPLC as described above.

Determination of Photosynthetic Activity—*Synechocystis* 6803 wild type and *crtO*[–] strains were cultured at 30 °C in BG11 and bubbled with a continuous stream of 1.5% (v/v) CO₂ in air under constant illumination (50 μ E m^{–2} s^{–1}). For the *crtO*[–] strain, the medium was supplemented with kanamycin to a final concentration of 25 μ g/ml.

When the cultures reached a density of 0.2 absorbance unit at 580 nm, they were divided in two halves, one was maintained at 50 μ E m^{–2} s^{–1} and the other was illuminated at 250 μ E m^{–2} s^{–1}. Oxygen evolution was periodically determined at 30 °C upon irradiation with saturating white light by using a Clark-type oxygen electrode. Chlorophyll was determined as described previously (27).

RESULTS

Our interest in the biosynthetic pathway of carotenoids in cyanobacteria led us to identify the genes coding for the enzymes phytoene desaturase (*crtP*) and phytoene synthase from *Synechocystis* (28, 29) and ζ -carotene desaturase (*crtQ*) from *Anabaena* sp. PCC 7120 (25). In the *Synechocystis* genome data base an ORF (slr0088) with significant homology to bacterial type desaturase (*crtI*) genes has been identified. As the *Anabaena crtQ* gene is homologous to *crtI* rather than to the plant or cyanobacterial *crtP* genes (8), we decided to explore the possibility that slr0088 was the *Synechocystis* ζ -carotene desaturase.

Cloning and Functional Expression of *crtO*—The open reading frame identified as slr0088 in the *Synechocystis* genome was cloned by polymerase chain reaction and introduced in an expression vector for *E. coli* to generate plasmid pTRCART-O. *E. coli* cells carrying plasmid pTRCART-O synthesized a protein of the expected size (59 kDa) (not shown) upon induction with isopropyl- β -D-thiogalactopyranoside. To test the function of the expressed protein, plasmid pTRCART-O was introduced in *E. coli* in combination with several plasmids that allow the accumulation of different carotenoids (Table I). In *E. coli* cells that accumulate phytoene (containing pACCRT-EB), 1-hydroxyneurosporene (containing pACCRT-EBIRc and pRKCR-C), and

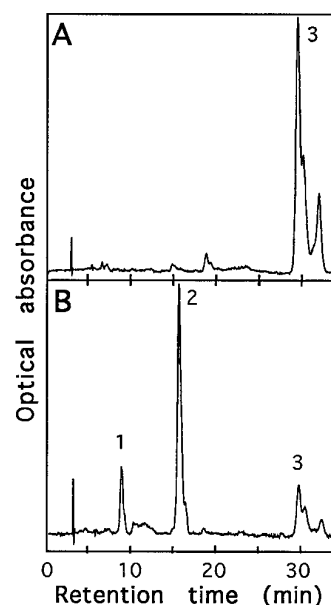


Fig. 2. Echinenone biosynthesis in *E. coli* by the *crtO* gene product. HPLC analysis of carotenoid pigments extracted from *E. coli* JM101 cells carrying plasmid pACCAR16 Δ crtX (A) and plasmids pACCAR16 Δ crtX + pTRCART-O (B). The carotenoids identified were canthaxanthin (1), echinenone (2), and β -carotene (3).

ζ -carotene (containing pACCRT-EBP), no desaturase activity could be detected when the *crtO* gene product was expressed. These results suggest that the *crtO* gene product is not a carotene desaturase, as are the enzymes encoded by *crtI* and *crtD*, nor is it related to the ζ -carotene desaturase gene from *Anabaena*, because no detectable desaturation products from phytoene, ζ -carotene, or 1-hydroxyneurosporene accumulated in *E. coli* cells that express *crtO*. Alternatively *crtO* could be a carotene desaturase in *Synechocystis* that cannot be functionally expressed in *E. coli*, even though the protein is synthesized or that requires additional cofactors not present in *E. coli*. However, when pTRCART-O was introduced in *E. coli* cells that accumulate β -carotene due to the presence of pACCRT16crtX, two additional carotenoids were observed, echinenone and canthaxanthin in lower amounts (Fig. 2). This result indicates that *crtO* is a β -carotene ketolase. Previously identified β -carotene ketolases produce the accumulation of canthaxanthin with little or none of echinenone. However, *crtO* produces the accumulation of much higher amounts of echinenone than it does of canthaxanthin. This correlates with the situation in *Synechocystis* that accumulates echinenone rather than canthaxanthin.

In Vitro Ketolase Activity of the *crtO* Gene Product—We have confirmed the *in vivo* complementation results by showing that the *crtO* gene product can synthesize echinenone from β -carotene *in vitro*. Cell homogenates from *E. coli* cells that express *crtO* can convert β -carotene to echinenone at a conversion rate of 12% in the presence of NADPH (Table II). Some formation of the di-keto derivative canthaxanthin (2% conversion) was also observed. This indicates that the specificity for β -carotene of the crude enzyme is not absolute. The reaction products echinenone and canthaxanthin were positively identified not only by co-chromatography with authentic standards but also by their absorbance spectra (Fig. 3). NADPH was required for activity.

Inactivation of the *crtO* Gene—To test the function of *crtO* in *Synechocystis*, we have inactivated the *crtO* gene by insertion of a kanamycin resistance cassette (Fig. 4A). The inactivated copy of *crtO* could be completely segregated as tested by Southern blot (Fig. 4B), and cells lacking a functional *crtO* gene seem to be viable. The carotenoid composition of the *crtO*-deficient

TABLE II

In vitro conversion of β -carotene by homogenates from *E. coli* cells expressing *crtO*

In vitro ketolase assays were carried out as described under "Experimental Procedures" with extracts from *E. coli* cells carrying plasmid pACCAR16 Δ crtX alone or with a mixture of the above with an extract of *E. coli* cells carrying plasmid pTRCrt-O.

Extracts	Carotenoid composition after reaction		
	β -Carotene	Echinenone	Canthaxanthin
pACCAR16crtX	510	0 ^{ng}	0
pACCAR16crtX + pTRCrt-O	429	62	9

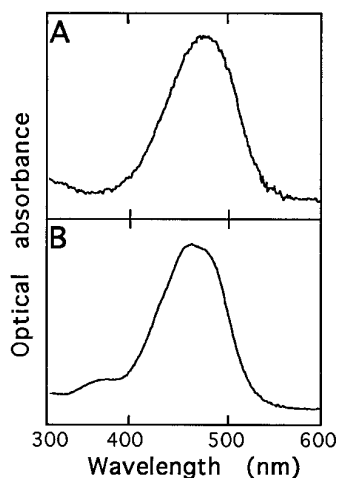


FIG. 3. Absorbance spectra of the reaction products formed *in vitro* by the *crtO* gene product from β -carotene. A, canthaxanthin; B, echinenone.

strain (*crtO*⁻) was compared with that of wild type *Synechocystis* (Fig. 5). Myxoxanthophyll, zeaxanthin, echinenone, and β -carotene were the main carotenoids identified in the wild type strain. In the mutant strain echinenone was absent, while there were wild type levels of the other carotenoids. No canthaxanthin was detected either in the wild type or in the *crtO*⁻ strain, even when a different HPLC system optimized for the separation of canthaxanthin from zeaxanthin (18) was used. This result indicates that *crtO* is required for echinenone biosynthesis in *Synechocystis* and, together with the previous results, clearly demonstrates that *crtO* is a β -carotene ketolase that acts asymmetrically to produce echinenone as the main product rather than that of canthaxanthin.

Characterization of the *crtO*⁻ Strain—The previously described results show that we have been able to completely segregate a strain lacking *crtO*, and that this strain is deficient in echinenone biosynthesis. To study what could be the functional role of echinenone, we have analyzed the behavior of the *crtO*⁻ strain when incubated under either low ($50 \mu\text{E m}^{-2} \text{s}^{-1}$) or high ($250 \mu\text{E m}^{-2} \text{s}^{-1}$) light intensity. Both wild type and *crtO*⁻ strains have similar growth rates at low and at high light intensity. The photosynthetic rate (as measured by O_2 evolution) of the *crtO*⁻ strain is also similar to that of wild type at either low or high light intensity. Therefore incubation at high light intensity does not reveal any significant differences between both strains regarding the measured parameters.

DISCUSSION

The cyanobacterium *Synechocystis* sp. PCC 6803 is the organism of choice for genetical studies. It is naturally transformable, and genes can be easily inactivated by targeting and homologous recombination. Furthermore, the complete se-

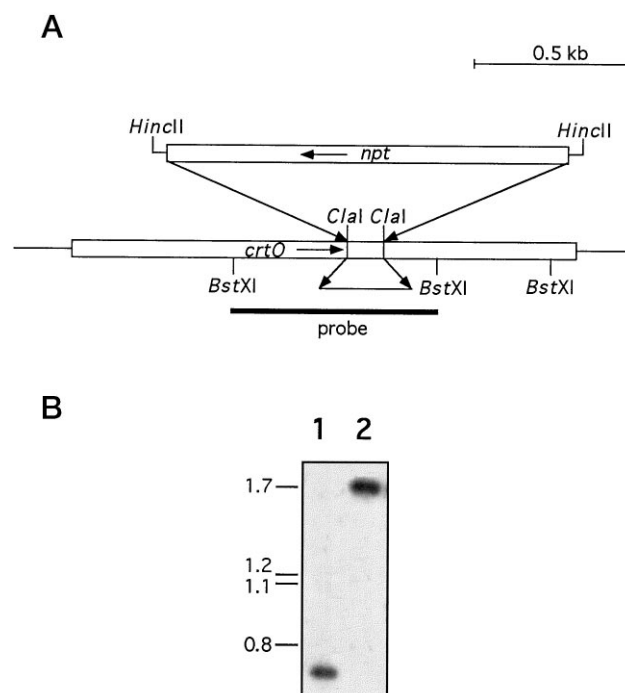


FIG. 4. Disruption of the *crtO* gene. A, strategy of disruption of *crtO* by insertion of the *npt* gene. B, Southern blot of *Synechocystis* genomic DNA from the wild type strain (lane 1) or the *crtO*⁻ strain (lane 2) digested with *Bst*XI and probed with the internal *Bst*XI fragment of *crtO*. Size markers (kilobase pairs) are indicated on the left.

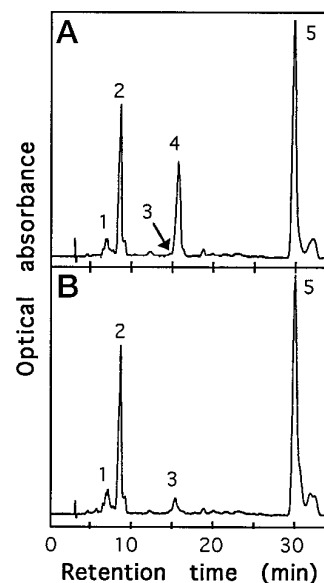


FIG. 5. The *crtO* gene is required for echinenone biosynthesis in *Synechocystis*. HPLC analysis of carotenoid pigments extracted from *Synechocystis* 6803 wild type strain (A) or *crtO*⁻ strain (B). The carotenoids identified were myxoxanthophyll (1), zeaxanthin (2), β -cryptoxanthin (3), echinenone (4), and β -carotene (5).

quence of the *Synechocystis* genome is available (30). Several genes of the carotenoid biosynthesis pathway have been identified in *Synechocystis*. The availability of the complete genome sequence gives us the opportunity to search for the so far unidentified genes of this pathway in a rational way. Among the open reading frames present in the *Synechocystis* genome, there is one with significant homology to bacterial type phytoene desaturases (*slr0088*). The only cyanobacterial ζ -carotene desaturase characterized so far is homologous to bacterial phy-

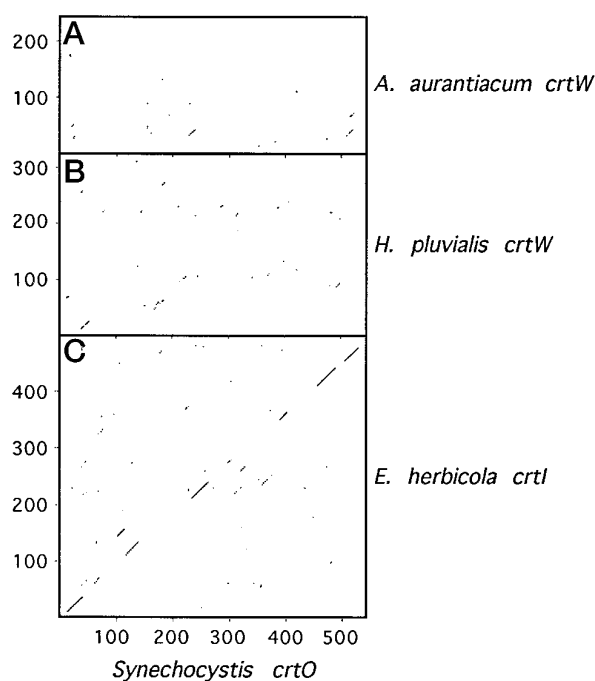


FIG. 6. Similarity plots of *Synechocystis* 6803 β -carotene ketolase (*crtO*) with other proteins. The sequence of *Synechocystis* 6803 β -carotene ketolase was compared with the sequences of *Haematococcus pluvialis* β -carotene ketolase (A), *Agrobacterium auranticum* β -carotene ketolase (B), and *Erwinia herbicola* phytoene desaturase (C). Similarity was calculated with a window of 21 amino acids and a stringency of 12 using the Compare program of the GCG package (33).

toene desaturases rather than to plant type phytoene desaturases, therefore it was reasonable to expect slr0088 to be the *Synechocystis* ζ -carotene desaturase. The results presented in this work clearly indicate that slr0088 is a β -carotene ketolase. This fact illustrates the risk of assuming functions for unknown ORFs based solely on weak homologies and indicates that functional studies are required to confirm the role of the expected protein product.

The *Synechocystis* mutant strain lacking *crtO* does not accumulate echinenone, but has normal levels of β -carotene. Therefore *crtO* is not required for the desaturation steps necessary to produce β -carotene, excluding that *crtO* is the functional ζ -carotene desaturase in *Synechocystis*. It should be noted that *crtO* is closely linked and in the same orientation as several other genes, possibly organized as an operon. Therefore inactivation of *crtO* could affect the expression of these other genes that could account for the phenotype observed. However, the function of those other genes is hypothetically unrelated to carotenoid biosynthesis (as deduced from sequence homologies), and some of them are expected to be essential. In any case a polar effect on genes placed downstream of *crtO* could not account for the *in vitro* activity of the *crtO* gene product and the complementation observed in *E. coli*.

As the plot in Fig. 6 clearly shows, *crtO* is homologous to bacterial type phytoene desaturases as exemplified by the *Erwinia herbicola* enzyme. The mechanism of carotene ketolases is not known in detail, but the symmetrically acting enzyme is most likely a dioxygenase.² Although the *in vitro* formation of echinenone by the *crtO*-derived ketolase was carried out in cell homogenates, a dependence of the reaction on NADPH was observed. This may be an indication for a different reaction mechanism responsible for the introduction of the keto group, involving a monooxygenase-type of hydroxylation followed by

dehydrogenation. *crtO* contains a nucleotide binding site at the amino end similar to the one in phytoene desaturases. This is most likely the site for the binding of the cofactor NADPH.

The three symmetrically acting β -carotene ketolases characterized so far, two from bacteria and one from a green algae, are homologous among themselves. They can be aligned, and four conserved regions have been identified (15). They do not contain a nucleotide binding site at their amino end. We have tried to identify in *crtO* regions of similarity with any of the four conserved domains in ketolases but there are no significant similarities (Fig. 6). Therefore *crtO* is a completely different enzyme, phylogenetically related to carotene desaturases rather than to ketolases. In addition *crtO* has also a different mechanism of substrate recognition, acting asymmetrically on β -carotene to introduce a keto group on only one of the β -ionone rings. In other words, *crtO* can use as substrate mainly β -carotene but echinenone very poorly. The previously identified β -carotene ketolases do not produce the accumulation of significant amounts of echinenone.

When *crtO* is expressed in *E. coli*, about 10% of the ketocarotenoid produced is canthaxanthin. Therefore *crtO* has not a strict specificity for β -carotene but can, at a low rate, introduce a keto group in the second ionone ring of echinenone to generate canthaxanthin. However, this effect is due to the high level expression of the ketolase and is restricted to the heterologous environment in *E. coli*, because no significant canthaxanthin accumulation is seen in *Synechocystis*.

The availability of a *Synechocystis* mutant strain deficient in the biosynthesis of echinenone will allow the study of the function of this carotenoid. It has been suggested that echinenone is located close to the reaction centers in the thylakoid membrane in a related cyanobacterium (31). However, our results indicate that the absence of echinenone has no effect on the growth rate or photosynthetic oxygen evolution at either low or high light. The specific role of echinenone in *Synechocystis* will require further investigation.

Carotene ketolases and their genes are of significant biotechnological interest for the production of carotenoids of commercial interest (32). The new β -carotene ketolase described here has a different substrate specificity of the previously known ketolases and therefore provides additional flexibility in the design of biotechnological procedures for the production of carotenoids of potential applied interest. The *Synechocystis* β -carotene ketolase could be useful for engineering of the biosynthesis of not only echinenone, but also other asymmetric carotenoids such as 3-hydroxyechinenone, 3'-hydroxyechinenone, and adonixanthin when expressed in combination with a carotenoid hydroxylase gene (*crtZ*).

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