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Sll0254 ($CrtL^{diox}$) Is a Bifunctional Lycopene Cyclase/Dioxygenase in Cyanobacteria Producing Myxoxanthophyll

Hatem E. Mohamed and Wim F. J. Vermaas*

School of Life Sciences, Arizona State University, P.O. Box 874501, Tempe, Arizona 85287-4501

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Upon depletion of Sll0254 in *Synechocystis* sp. strain PCC 6803, cyclized carotenoids were replaced by linear, relatively hydrophilic carotenoids, and the amount of the two photosystems decreased greatly. Full segregants of the sll0254 deletion in *Synechocystis* were not obtained, implying that this gene is essential for survival, most likely to allow normal cell division. The N-terminal half of Sll0254 has limited similarity to the family of lycopene cyclases, has an additional dehydrogenase motif near the N terminus, and is followed by a Rieske 2Fe-2S center sequence signature. To test whether Sll0254 serves as a lycopene cyclase in *Synechocystis*, the corresponding gene was expressed in *Escherichia coli* strains that can produce lycopene or neurosporene. In the presence of Sll0254 these linear carotenoids were converted into cyclized, relatively hydrophilic pigments, with masses consistent with the introduction of two hydroxyl groups and with spectra indicative of only small changes in the number of conjugated double bonds. This suggests that Sll0254 catalyzes formation of oxygenated, cyclized carotenoids. We interpret the appearance of the hydroxyl groups in the carotenoids to be due to dioxygenase activity involving the Rieske 2Fe-2S center and the additional dehydrogenase domain. This dioxygenase activity is required in the myxoxanthophyll biosynthesis pathway, after or concomitant with cyclization on the other end of the molecule. We interpret Sll0254 to be a dual-function enzyme with both lycopene cyclase and dioxygenase activity and have named it $CrtL^{diox}$.

Carotenoids play a central role in many organisms in quenching reactive oxygen species, preventing oxidation, and—in photosynthetic organisms—in harvesting light energy. Carotenoids are synthesized by plants and selected fungi and bacteria (3). Most of the naturally occurring carotenoids have a C_{40} methyl-branched hydrocarbon backbone derived from the successive condensation of eight C_5 isoprene units, initially resulting in a linear, methyl-branched hydrocarbon backbone. Toward the end of the biosynthetic pathway, carotenoids are commonly cyclized at one or both ends of the backbone. This reaction is catalyzed by lycopene cyclase, commonly present in bacteria, fungi, and plants (14). Thus far, several types of lycopene cyclase have been identified in different groups of organisms, and more types are likely to be present as there are organisms that contain cyclized carotenoids but that lack genes for any of the known types of lycopene cyclase.

One type of lycopene cyclase is the classical monomeric bacterial β-cyclase; this enzyme is encoded by *crtY*, which most likely is an ancestor of *crtL*, the β-cyclase gene in selected cyanobacteria (7), which, in turn, may have given rise to plant β- and ε-cyclase genes (6, 8) and to the gene for capsanthin/capsorubin synthase. A second type of lycopene cyclase is exemplified by the heterodimeric enzyme from the gram-positive bacterium *Brevibacterium linens* (15). The two parts of the heterodimer (CrtYc and CrtYd) may be fused, as they are in the lycopene β-cyclase from the thermoacidophilic archaeon *Sulfolobus solfataricus* (12). A third type of lycopene cyclase is a bifunctional lycopene cyclase/phytoene synthase that is found in selected fungi and was first identified in the heterobasidiomycete *Xanthophyllomyces dendrorhous* (28).

In addition to the cyclase groups listed above that all lead to the introduction of a ring at both ends of the carotenoid, several cyclases that lead to the introduction of a ring on only one end of the molecule have been identified as well. A lycopene monocyclase has been reported in a marine flavobacterium, P99-3 (26); this cyclase leads to the formation of the monocyclic carotenoid myxol. Although myxol is the carotenoid moiety of the glycosylated carotenoid myxoxanthophyll that is commonly found in cyanobacteria, no carotenoid glycoside was reported in the P99-3 strain. A second type of carotenoid monocyclase (CrtLm) has been isolated from two non-photosynthetic bacteria, *Rhodococcus erythropolis* AN12 and *Deinococcus radiodurans* R1 (25). These monocyclases display sequence similarity to plant CrtL-type lycopene β-cyclases but selectively cyclize only one end of the lycopene or neurosporene molecule to produce γ-carotene and β-zeacarotene, respectively. A third type with primarily monocyclase activity, CruA, was found in the photosynthetic green sulfur bacterium *Chlorobium tepidum* (16).

The only experimentally proven type of lycopene cyclase found in cyanobacteria thus far is CrtL. This cyclase is present in a subfraction of the cyanobacterial phylum including *Synechococcus* sp. strain PCC 7942 (7) as well as in several marine strains; interestingly, in the MED4 strain of *Prochlorococcus marinus*, a CrtL-type enzyme has been found that has both β- and ε-cyclase activity (24). However, although all cyanobacteria contain cyclic carotenoids, genes for CrtL are not found in a large number of sequenced cyanobacteria, including *Synechocystis* sp. strain PCC 6803, *Thermosynechococcus elongatus* BP-1, *Gloeobacter violaceous*, *Trichodesmium erythraeum*, *Nostoc punctiforme*, and *Nostoc* sp. strain PCC 7120. The 512-

* Corresponding author. Mailing address: School of Life Sciences, Arizona State University, P.O. Box 874501, Tempe, Arizona 85287-4501. Phone: (480) 965-6250. Fax: (480) 965-6899. E-mail: wim@asu.edu.

residue CruA from *C. tepidum* has reasonable similarity (35 to 38% identity) to predicted products (668 to 725 residues in length) of open reading frames (ORFs) in these cyanobacteria, but the cyanobacterial sequences do not have clear, conserved functional domains.

To probe for the possible existence of another type of lycopene cyclase in cyanobacteria, conserved cyanobacterial ORFs were scanned for domains that may be involved in lycopene cyclase activity. Sll0254 of *Synechocystis* sp. strain PCC 6803 (annotated in CyanoBase as a "probable phytoene dehydrogenase Rieske iron-sulfur component") was identified to be of potential interest as Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (21) recognized a significant CrtL-like lycopene cyclase domain in the dehydrogenase motif region 60 to 95 residues from the N terminus of this protein. To investigate the function of this protein, sll0254 was deleted from *Synechocystis* sp. strain PCC 6803 and was expressed in *Escherichia coli* strains producing neurosporene or lycopene. Based on these data, sll0254 is interpreted to encode a novel, combined carotenoid cyclase/dioxygenase in *Synechocystis* sp. strain PCC 6803.

MATERIALS AND METHODS

Strains, growth conditions, and visualization. *Synechocystis* sp. strain PCC 6803 was cultivated photomixotrophically on a rotary shaker at 30°C in BG-11 medium (22) buffered with 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–NaOH (pH 8.2) and supplemented with 5 mM glucose. For growth on plates, 1.5% (wt/vol) Difco agar and 0.3% (wt/vol) sodium thiosulfate were added. Unless indicated otherwise, cells were grown in continuous light at an intensity of 0.5 μmol of photons m^{-2} s^{-1} provided by cool-white fluorescent tubes. Growth was monitored by measuring the optical density of the cells at 730 nm using a Shimadzu UV-160 spectrophotometer.

Transmission electron microscopy was carried out as previously described (19).

Cloning sll0254 and construction of the pΔsll0254Sp and overexpression plasmids. The sll0254 ORF of *Synechocystis* sp. strain PCC 6803 was cloned by PCR using genomic DNA prepared as previously described (11). The forward primer was CGGGTTTTGG***G***CATG***C***GGTTAGATT (corresponding to base numbers 1507177 to 1507153 according to CyanoBase: www.kazusa.or.jp/cyano/cyano.html) with an engineered SphI restriction site (underlined; modified bases are in boldface and italicized). The reverse primer was CCAGGGGAA***TT***CCG CCCTGGAAT (base numbers 1504468 to 1504490) carrying an introduced EcoRI site (underlined; modified bases are in boldface and italicized). A PCR product of the expected size was purified and treated with restriction enzymes according to the restriction sites created on each primer. The sll0254 gene and its flanking regions were cloned into plasmid pUC19 using its EcoRI and SphI sites, creating the psll0254 plasmid. An internal fragment (1.8 kb) of the sll0254 gene was deleted by restriction at the NaeI (1506650) and HincII (1504854) sites near the beginning and end of the sll0254 ORF and replaced by a 1.4-kb spectinomycin resistance cassette digested with XmnI and SmaI. This creates the plasmid pΔsll0254Sp, which was used for transformation of the *Synechocystis* sp. strain PCC 6803 wild-type strain to generate the Δsll0254Sp mutant.

Plasmids pAC-LYC and pAC-NEUR (7) (kindly provided by F. X. Cunningham, University of Maryland) were used to mediate the formation of lycopene and neurosporene, respectively, in *E. coli* strains BL21, JM109, and DH5α. These strains were used for introduction of Sll0254 as follows. The ORF sll0254 was cloned from *Synechocystis* sp. strain PCC 6803 DNA by PCR using a forward primer with the sequence 5′-TTTTACCGTCTGCCTTT**CA**TATG**A**CTGAATT-3′, introducing an NdeI restriction site (underlined) at the sll0254 start codon (modified bases are in boldface), and a reverse primer with the sequence 5′-A CCGAAGGCTAAACTCG**A**GCTAAATTTGCAA-3′, carrying an XhoI restriction site (underlined) adjacent to the sll0254 stop codon (modified bases are in boldface). The engineered sites facilitate the cloning of sll0254 into the pET16b expression vector digested by NdeI and BamH I to create the pET16b-sll0254 plasmid. The cloned fragment was sequenced to confirm the correct sequence and orientation of the sll0254 gene. The pET16b-sll0254 plasmid was introduced into *E. coli* strains carrying either pAC-LYC or pAC-NEUR by electroporation, and transformants were selected for resistance to ampicillin and chloramphenicol.

TABLE 1. Distribution of the lycopene cyclase CrtL and Sll0254 orthologues among selected cyanobacteria and photosynthetic eukaryotes with a known genome sequence[a]

Organism	CrtL-type cyclase	Sll0254 orthologue
Anabaena variabilis ATCC 29413	−	+
Arabidopsis thaliana	+	−
Chlamydomonas reinhardtii	+	−
Crocosphaera watsonii WH 8501	−	+
Gloeobacter violaceus PCC 7421	−	+
Nostoc sp. strain PCC 7120	−	+
Nostoc punctiforme ATCC 29133	−	+
Prochlorococcus marinus, various ssp.	+	−
Synechococcus sp. strain PCC 6301/7942	+	−
Synechococcus sp. strain WH 8102	+	−
Thermosynechococcus elongatus BP-1	−	−
Trichodesmium erythraeum IMS101	−	+

[a] +, present; −, absent.

Carotenoid analysis. *Synechocystis* sp. strain PCC 6803 cells were harvested by centrifugation using cultures in exponential growth phase (optical density at 730 nm of ~0.5). Cell pellets were frozen in liquid nitrogen and freeze-dried. Pigments were extracted from freeze-dried cells by three successive extractions with methanol that contained 0.1% NH_4OH, and extracts were combined and evaporated under a stream of nitrogen until the samples were dry. Dried samples were redissolved in a small volume of NH_4OH-containing methanol and immediately subjected to high-performance liquid chromatography (HPLC) on an HP-1100 Chemstation with a Waters Spherisorb S5ODS2 (4.0 mm by 250 mm) column filled with C-18 reversed phase silica gel, using a linear 18-min gradient of ethyl acetate (0 to 95%) in acetonitrile-water-triethylamine (9:1:0.01, vol/vol/vol) at a flow rate of 1 ml/min. Absorption spectra of the eluted pigments were recorded continuously in the 360- to 665-nm range with an online photodiode array detector.

Mass spectroscopy. Collected carotenoid fractions were evaporated under nitrogen. Mass spectra were obtained by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Voyager DE STR Biospectrometry Work Station; Applied Biosystems, Foster City, CA). Before analysis, dried carotenoids were dissolved in 20 μl of methyl chloride, 10 μl of which was mixed with terthiophene (used as a matrix) dissolved in methyl chloride. Ions were generated by a pulsed 337-nm nitrogen laser and were accelerated to 20 kV. All spectra were obtained in the reflectron mode with delayed extraction (200 ns) and were the result of signal averaging from 200 to 300 laser shots.

RESULTS

Distribution of Sll0254 orthologues. The product of the sll0254 ORF has clear orthologues (55 to 65% identity) in only selected cyanobacterial genomes. As indicated in Table 1, currently sequenced cyanobacterial genomes carrying an sll0254 orthologue are the strains from the *Nostoc* cluster, as well as *G. violaceus* strain PCC 7421 and the marine strains *Crocosphaera watsonii* WH 8501 (previously *Synechocystis* sp. strain WH 8501) and *Trichodesmium erythraeum*. All these species lack a CrtL (plant-like lycopene cyclase) homologue. Sll0254 orthologues are absent in *Synechococcus* sp. strain PCC 6301/7942 and in marine *Synechococcus* and *Prochlorococcus* strains. Interestingly, these organisms have a CrtL orthologue (Table 1). The one cyanobacterium with a sequenced genome that lacks both *crtL* and sll0254 orthologues is the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (Table 1).

Various photosynthetic green sulfur bacteria (*C. tepidum* TLS, *Pelodictyon phaeoclathratiforme* BU-1, *Prosthecochloris aestuarii* DSM 271, and *Prosthecochloris vibrioformis* DSM 265)

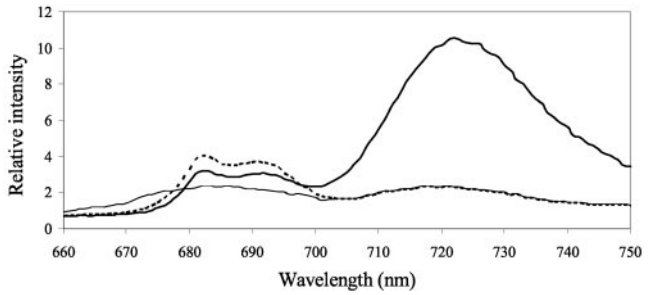

FIG. 1. The 77 K fluorescence emission spectra of the wild type (solid bold line) and the Δsll0254Sp transformant early after transformation (broken line) and after several more subcultures (solid thin line) in the presence of increasing concentrations of spectinomycin. Cells were grown at low-light intensity (0.5 μmol of photons m^{-2} s^{-1}). Each sample contained the same cell concentration. Fluorescence excitation was at 435 nm.

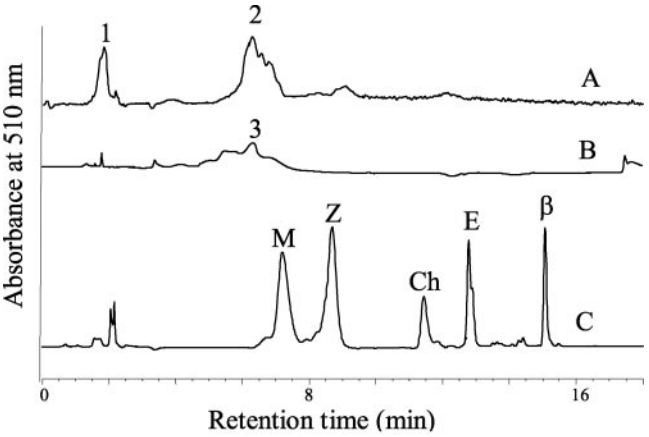

FIG. 2. HPLC fractionation of methanol/ammonia extracts from the Δsll0254Sp transformant grown in medium supplemented with 50 (A) or 200 (B) μg/ml spectinomycin and from wild-type *Synechocystis* sp. strain PCC 6803 (C). All cultures were grown in continuous light at a light intensity of 0.5 μmol of photons m^{-2} s^{-1}. The main pigments from the wild-type strain have been identified: M, myxoxanthophyll; Z, zeaxanthin; E, echinenone; β, β-carotene; and Ch, chlorophyll *a*. The absorption spectra of the pigments accumulated in peaks 1 to 3 are shown in Fig. 3.

carry a protein with a length (about 650 residues) similar to that of Sll0254 and with 32 to 34% identity relative to Sll0254. The corresponding *C. tepidum* protein (CrtU) was identified as γ-carotene desaturase (converting γ-carotene to chlorobactene), as knockout of the *crtU* gene led to γ-carotene accumulation and loss of chlorobactene (10). Desaturation catalyzed by this enzyme occurs in the ring of γ-carotene and also leads to a redistribution of one of the methyl groups on the ring. Cyanobacteria do not carry out chlorobactene synthesis or even dehydrogenation within a carotenoid ring, and therefore Sll0254 cannot simply have the same function as CrtU.

Attempted deletion of sll0254 in *Synechocystis* sp. strain PCC 6803. Upon transformation of *Synechocystis* with the pΔsll0254Sp plasmid that was described in Materials and Methods, an interesting phenotypic progression of the transformants was observed, presumably reflecting an Sll0254 dose response. Initially, after three to four restreakings at increasing spectinomycin concentrations (from 5 to 40 μg/ml), the transformant became bluish and light sensitive. The 77 K fluorescence emission spectrum of these cells showed that photosystem I (PS I) was depleted whereas PS II mostly remained: the 725-nm fluorescence emission maximum (due to PS I) was greatly decreased, whereas emission peaks around 683 and 693 nm (due to PS II) remained present (Fig. 1). This explains the bluish, light-sensitive phenotype (23) soon after transformation. Because of the light sensitivity of the transformants, selection for increased spectinomycin resistance was carried out at very-low-light intensity (0.5 μmol of photons m^{-2} s^{-1}) or under light-activated heterotrophic growth conditions (2). Upon further segregation at increased spectinomycin concentrations (50 to 400 μg/ml), both the chlorophyll and carotenoid levels decreased further, to result in very low chlorophyll fluorescence intensity at 77 K, with remnants of both PS I and PS II represented (Fig. 1). However, interestingly we were unable to obtain a complete segregant that lacked all wild-type sll0254 copies, regardless of the growth conditions: in all cases, PCR evidence of some remaining wild-type sll0254 could be obtained (data not shown). This suggests that at least some Sll0254 is required for growth of *Synechocystis*. Therefore, transformant analysis was carried out in the presence of high concentrations of spectinomycin. Growth of other spectinomycin-resistant transformants at similar concentrations of spectinomycin has not led to similar phenotypic characteristics as reported here, implying that the phenomena reported in the following section are a result of Sll0254 depletion.

Carotenoid analysis of the Δsll0254Sp transformant. Upon applying additional spectinomycin selection pressure, which caused a reduction in sll0254 gene copy number, the total carotenoid content was reduced in the Δsll0254Sp transformant, and the chlorophyll content of the cell dropped to essentially zero. Figure 2 presents the HPLC elution profile of pigments extracted from the transformant grown at two different levels of spectinomycin. In either case, chlorophyll and the usual cyanobacterial carotenoids had been depleted to barely detectable levels. Upon HPLC analysis, extracts from cells grown at the lower spectinomycin level showed a sharp peak (Fig. 2, peak 1) in 510-nm absorbance near the void volume and a broader peak (Fig. 2, peak 2) at a longer retention time but not coinciding with any of the four main *Synechocystis* carotenoids. According to their spectra and masses, the small and broad peaks at higher retention times in Fig. 2 (spectrum A) represent a mixture of different carotenoids that were atypical for wild-type *Synechocystis* (Fig. 2, spectrum C), presumably resulting from side reactions due to the accumulation of particular intermediates in the carotenoid biosynthetic pathway. When cells were grown at higher spectinomycin levels (further reducing the number of sll0254 gene copies), carotenoid levels were further depleted (Fig. 2, spectrum B). The absorption spectra of the carotenoids in peaks 1, 2, and 3 in Fig. 2 (spectra A and B) have been provided in Fig. 3. Spectrum 1 (maximum λ [λ_{max}], 474 and 499 nm) resembles that of a (pro)lycopene isomer, spectrum 2 (λ_{max}, 478 and 508 nm; III/II = 69%, where III/II refers to the ratio of the amplitude of the third and second peaks of the absorption) is close to that of lycopene, and spectrum 3 (λ_{max}, 476 and 510 nm; III/II = 133%) is most compatible with that of an asymmetric chro-

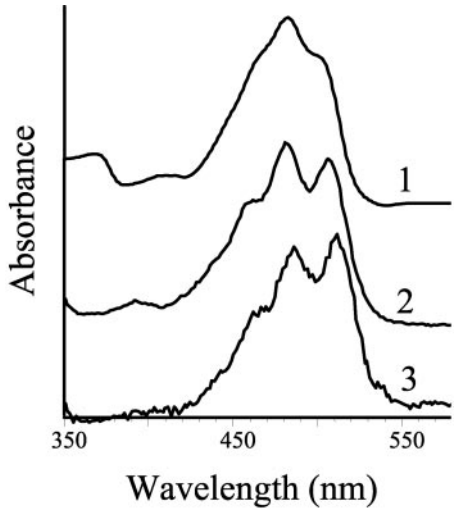

FIG. 3. Absorption spectra of pigments found in the Δsll0254Sp transformant. Spectrum numbers refer to pigment peaks identified in Fig. 2.

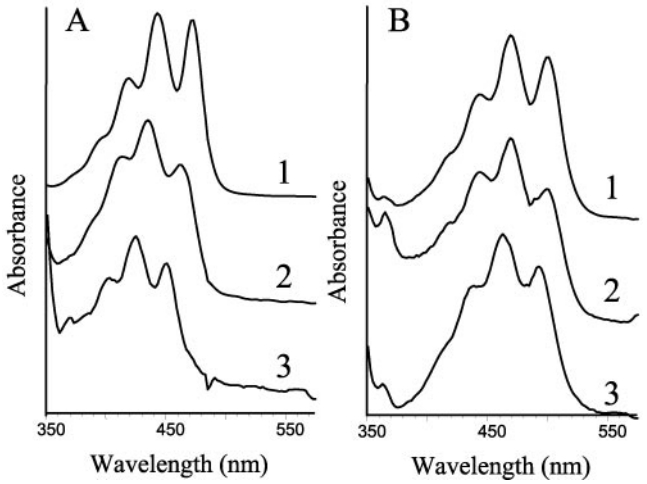

FIG. 5. Absorption spectra of carotenoids present in HPLC peaks 1, 2, and 3 (Fig. 4) that appear upon pET16b-sll0254 introduction in *E. coli* carrying pAC-NEUR (A) or pAC-LYC (B).

mophore such as 3,4-didehydroneurosporene. As the compounds detected by HPLC had a much shorter elution time than lycopene (compare Fig. 2 with Fig. 4), they likely are hydroxylated in a way that their conjugated double-bond system is not affected. Indeed, the compound giving rise to spectrum 1 was found to have a mass of 584, consistent with trihydroxy-lycopene. A compound with identical mass and HPLC mobility was also detected in other strains impaired in carotenoid biosynthesis (18, 19).

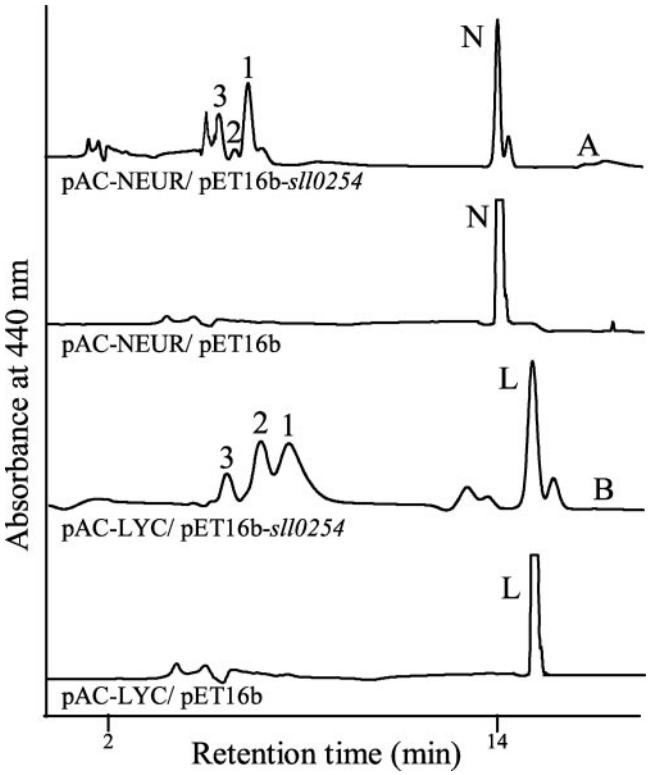

FIG. 4. HPLC chromatograms of methanol extracts from *E. coli* strain DH5α harboring pAC-NEUR (A) or pAC-LYC (B) and carrying pET16b-sll0254 (top) or the empty pET16b plasmid vector (bottom). N, neurosporene; L, lycopene. The absorption spectra of the pigments accumulated in the numbered peaks are shown in Fig. 5.

Sll0254 production in *E. coli*. As indicated in the previous section, an interesting feature of the Δsll0254Sp transformants was the lack of cyclized carotenoids and the accumulation of hydroxylated derivatives of lycopene and neurosporene, for example. This suggested that the virtual lack of Sll0254 led to a block behind lycopene or neurosporene but before a cyclized carotenoid. This would imply that Sll0254 might be involved with the cyclization reaction. To test this hypothesis, the sll0254 gene was cloned into the expression plasmid pET16b (Novagen, San Diego, CA), and the pET16b-sll0254 plasmid was introduced into *E. coli* strains carrying either pAC-LYC or pAC-NEUR and producing lycopene or neurosporene, respectively.

Figure 4 shows that expression of sll0254 in *E. coli* in the presence of lycopene or neurosporene resulted in the formation of new carotenoids. Spectra of peaks that are numbered in Fig. 4 are depicted in Fig. 5. In contrast to the results obtained upon overexpression of CrtL from *Synechococcus* sp. strain PCC 7942 (7), where resulting carotenoids were hydrophobic (β-carotene and β-zeacarotene), the majority of the newly formed pigments elute rapidly, indicating relatively hydrophilic pigments. However, peak 1 in the HPLC profile spectrally resembles neurosporene (III/II = 88%) (Fig. 5A) or lycopene (III/II = 68%) (Fig. 5B), indicating that Sll0254 activity in these cases affected hydrophilicity but did not greatly perturb the conjugated double-bond system. Interestingly, spectrum 3 in Fig. 5A resembles that of β-zeacarotene (III/II = 47%), a monocyclic carotenoid that can be derived from neurosporene by lycopene cyclase activity. Spectrum 3 in Fig. 5B is similar to that of γ-carotene (III/II = 41%). Therefore, the spectra of compound 3 in Fig. 4 are indicative of cyclized carotenoids (Fig. 5A and B), whereas their mobility on the HPLC column is much higher than expected for β-zeacarotene or γ-carotene. Upon matrix-assisted laser desorption ionization–time of flight

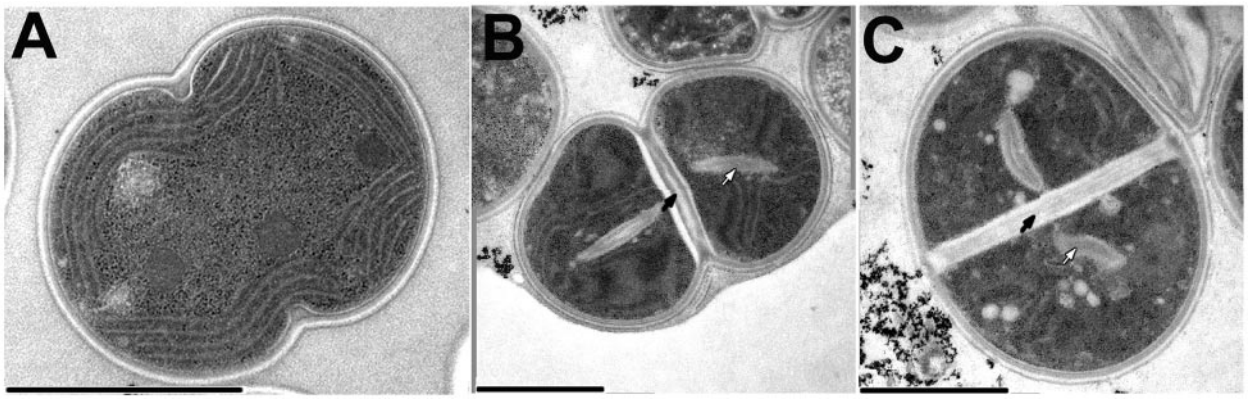

FIG. 6. Transmission electron micrographs of cells of wild-type *Synechocystis* sp. strain PCC 6803 (A) and the Δsll0254Sp transformant (B and C). The cell division plate (black arrow) and secondary cell division plates (white arrow) have been indicated in cells of the transformant. Scale bar, 1 μm.

analysis, the compounds shown in Fig. 5B gave rise to peaks at masses between 566 and 570, consistent with carotenoids carrying two hydroxyl groups. No evidence for the formation of β-carotene was observed; the small peaks appearing around lycopene and neurosporene appear to be isomers of these linear carotenoids, and the two HPLC peaks that are a little more hydrophilic than lycopene (Fig. 4B) are possibly composed of compounds with a single hydroxylation. The amount of these compounds was insufficient for a detailed characterization.

Therefore, expression of Sll0254 in *E. coli* gives rise to dihydroxylated carotenoids, some of which are cyclized. Based on Sll0254 sequence considerations, the masses of the products in *E. coli*, and the need of *Synechocystis* sp. strain PCC 6803 to synthesize myxoxanthophyll, we interpret our data to indicate that Sll0254 not only has lycopene cyclase activity but also displays dioxygenase activity; the rationale of this interpretation will be presented in more detail in the Discussion section.

Ultrastructure of Δsll0254Sp transformants. Now that the function of Sll0254 is apparent, the poor growth and lack of segregation of Δsll0254Sp transformants was unexpected as a *Synechocystis* mutant lacking *crtQ* (coding for ζ-carotene desaturase), and thus inhibited at an earlier step of the carotenoid biosynthesis pathway, can be segregated (4). For this reason, the ultrastructure of the transformants was investigated. As shown in Fig. 6, thylakoids were disorganized, and the cell wall was affected (resulting in cell clumping and a thicker peptidoglycan layer) as is commonly observed in mutants lacking myxoxanthophyll or carotenoids in general (19). However, the feature that was uncommon in the Δsll0254Sp transformants was the abnormal phenotype of cell division: in some cells, a cell wall was formed through the middle of the cell without consistent evidence of constriction, and new cell walls were in the process of being formed before cells had separated from the first division (Fig. 6). It is possible that these cells represent full segregants. Therefore, at least one of the reasons for the lack of segregation of the Δsll0254 strain may be the lack of normal cell division in the full segregant due to altered carotenoids in the cell wall rather than a strict requirement of cyclic carotenoids for photoprotection or related antioxidant functions.

DISCUSSION

Consequences of Sll0254 depletion. Even though sll0254 could not be deleted completely, partial segregants showed major differences relative to the wild type. One major effect of the partial deletion was a depletion of cyclic carotenoids and the formation of hydroxylated, linear carotenoids. Hydroxylation of carotenoids is generally observed in *Synechocystis* sp. strain PCC 6803 when timely utilization of linear carotenoids has been impaired due to mutations in downstream steps (18, 19). Therefore, the phenotype of Δsll0254Sp transformants is consistent with a blockage at the lycopene cyclization step and supports the interpretation of the *E. coli* expression data that Sll0254 is a lycopene cyclase in *Synechocystis* sp. strain PCC 6803.

A surprising observation was the unusual ultrastructure of the transformants that were depleted in Sll0254. Changes in the cell wall that result in cell aggregation, which is also obvious in the transformants depleted of Sll0254 (Fig. 6), already have been reported for strains impaired in biosynthesis of carotenoids and specifically of myxoxanthophyll (19), although the virtual formation of tetrads upon cell division was not observed before in myxoxanthophyll biosynthesis mutants. In *Staphylococcus haemolyticus*, formation of tetrads has been reported after protease treatment, which presumably leads to a lack of a protein with cell wall lytic activity and, thereby, a lack of separation of cells after division (30). In the case of *Synechocystis* cells that are depleted in cyclic carotenoids and carry hydroxylated linear carotenoids, changes in the cell wall and permeability may have led to a similar decrease in the separation of the cell wall between daughter cells, in line with our finding that myxoxanthophyll is very important for cell wall properties (19).

Comparison with strains impaired in earlier steps of carotenoid formation. Most cyanobacteria including *Synechocystis* sp. strain PCC 6803 produce lycopene from phytoene through the activity of two plant-type desaturases, phytoene desaturase (CrtP; Slr1254) (17) and ζ-carotene desaturase (CrtQ; Slr0940) (5). However, in *G. violaceus* strain PCC 7421, CrtP and CrtQ are replaced by a single-enzyme bacterial-type desaturase, CrtI (27). Upon deletion of either *crtP* or *crtQ* in *Synechocystis* sp. strain PCC 6803, PS II was lost (possibly due to the inability of

remaining carotenoids to stabilize the reaction center complexes), and about half of PS I remained, on a per-cell basis; PS I contained phytoene or ζ-carotene in the Δ*crtP* and Δ*crtQ* mutants, respectively (4). Upon depletion of sll0254, however, the amount of PS I was decreased first, followed by a major decline in both photosystems. As the remaining carotenoids in this strain are found to be rather hydrophilic (Fig. 2) and are likely to carry hydroxy groups, they may be unable to effectively bind in the hydrophobic pockets in the reaction center complexes that are usually occupied by β-carotene. This difference is unlikely to be limited to photosynthetic reaction centers. As the Δ*crtP* and Δ*crtQ* mutants are healthier and ultrastructurally more normal than the sll0254 transformants (see reference 19 for an electron micrograph of the Δ*crtQ* strain), the remaining carotenoids in the transformant with decreased Sll0254 levels do not appear to be able to substitute for other important cell functions carried out by the carotenoids found in the wild type, which most likely is the reason that Δsll0254 transformants, in contrast to Δ*crtQ* ones, do not segregate.

Sll0254: comparative sequence analysis. In Results, Sll0254 was shown to have lycopene/neurosporene cyclase activity and to lead to an unexpected array of oxygenated carotenoids. Excluding proteins within the cyanobacterial phylum, Sll0254 is most similar to CrtU (γ-carotene desaturase) of *C. tepidum* and other green sulfur bacteria. The obvious issue to be discussed first is the background of this apparent functional dichotomy between the two proteins.

As mentioned in the introduction, according to Motif Scan the Sll0254 sequence between residues 61 and 95 is recognized to belong in the lycopene cyclase protein family (accession number PF05834); a similar region of the *C. tepidum* CrtU protein is recognized to potentially belong to this family as well. In both Sll0254 and *C. tepidum* CrtU, this region contains a GXGXXG dehydrogenase motif in a hydrophobic region 65 residues from the N terminus; this motif and surrounding residues are reasonably well conserved in the lycopene cyclase family (Fig. 7A). Closer to the N terminus, Sll0254 and *C. tepidum* CrtU have another dehydrogenase motif that is missing in members of the traditional lycopene cyclase family (Fig. 7A). Whereas the similarity between Sll0254 and individual members of the lycopene cyclase family is insufficient to be convincing, an alignment with several members of the family presents a strong argument that Sll0254 and—to a lesser extent—CrtU share lycopene cyclase features (Fig. 7A). Limited similarity between Sll0254 and members of the lycopene cyclase family is present throughout the N-terminal half of Sll0254, up to the Rieske 2Fe-2S domain in this protein. As this part of the protein is about 300 residues in Sll0254 whereas CrtL- and CrtY-type cyclases are 400 to 500 residues, Sll0254 carries deletions relative to the lycopene cyclase family. Interestingly, some motifs that are highly conserved in the lycopene cyclase family are recognizable in Sll0254 but less so in *C. tepidum* CrtU; Fig. 7B presents as an example the TGY sequence that is highly conserved in lycopene cyclase members and that is TGF in Sll0254 but FRY in CrtU. In some regions of the N-terminal half of the protein, Sll0254 but not CrtU had interesting similarity to neoxanthin synthase (a lycopene cyclase family member) from potato (1) (Fig. 7C). The C-terminal half of Sll0254 contains the Rieske domain that is also conserved in CrtU of green sulfur bacteria but not in traditional lycopene cyclases, followed by a region that lacks clear functional identifiers.

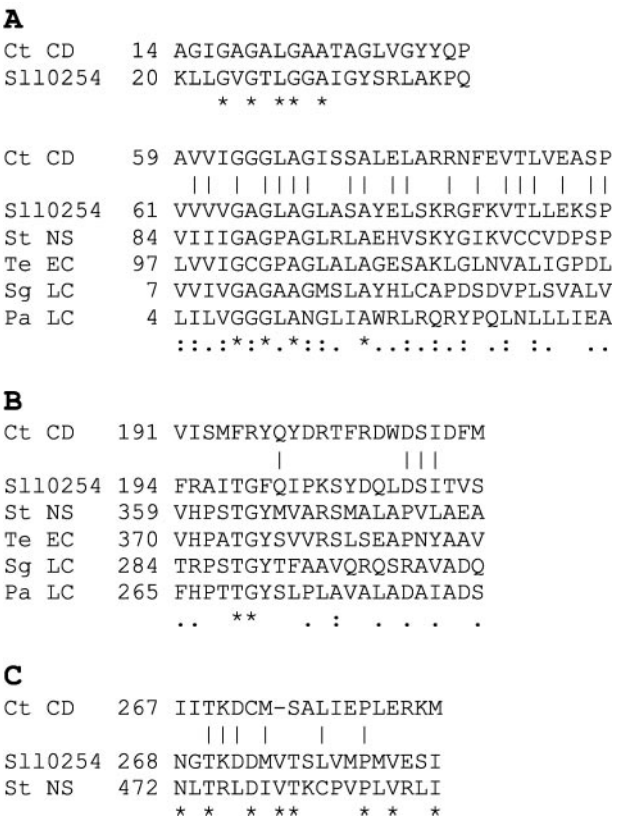

FIG. 7. Alignment of sections of Sll0254 with domains of members of the lycopene cyclase family and of γ-carotene desaturase (CrtU) from *C. tepidum*. (A) Shown are two desaturase motifs in the N-terminal region; the first motif is present in Sll0254 and in CrtU from *C. tepidum*, whereas the other is conserved in members of the lycopene cyclase family as well. (B) The TGY motif that is conserved in members of the lycopene cyclase family and, with a conserved modification, in Sll0254 but not in CrtU. (C) A region toward the C-terminal end of one of the lycopene cyclase family members, neoxanthin synthase from potato, that is reasonably conserved in a domain near the middle of Sll0254 but that is not found convincingly in *C. tepidum* CrtU. Asterisks indicate residues that are conserved between Sll0254 and four (A and B) or one (C) member of the lycopene cyclase family, colons signify conservation of residues between Sll0254 and more than one member of the lycopene cyclase family shown here, periods indicate conservation between Sll0254 and one member of the lycopene cyclase family shown here, and vertical lines indicate identity between Sll0254 and *C. tepidum* CrtU. Alignments were made using the DIALIGN program that is suitable for determining local similarities (20). St NS, neoxanthin synthase from *Solanum tuberosum* (potato); Te EC, ε-cyclase from *Tagetes erecta* (marigold); Sg LC, lycopene cyclase from *Streptomyces griseus*; Pa LC, lycopene cyclase (CrtY) from *Pantoea agglomerans*; and Ct CD, γ-carotene desaturase (CrtU) from *C. tepidum*.

We interpret our data to indicate that the traditional lycopene cyclase family and the N-terminal half of the Sll0254/CrtU group appear to be derived from a common ancestor, with Sll0254 but not CrtU displaying lycopene cyclase activity. Both Sll0254 and CrtU carry a sequence characteristic of a Rieske 2Fe-2S center near the center as well as two NAD(P)H dehydrogenase or FMN/FAD reductase motifs (GXGXXG) near the N terminus; of these dehydrogenase motifs only the

second one (residues 65 to 70 in Sll0254) is conserved in the traditional lycopene cyclase family (Fig. 7A). The Rieske consensus sequence and an FAD/FMN or NAD(P)H reductase/dehydrogenase domain, together with a mononuclear iron center that is characterized by two His residues and a Asp, are functional components of a Rieske non-heme iron dioxygenase that catalyzes oxygenation of a C=C double bond and results in a hydroxy group on both carbons and a saturation of the double bond (reviewed in references 9 and 29). Whereas Rieske-type dioxygenases generally have reductase (to provide electrons to the dioxygenase) and dioxygenase activities on different subunits (9, 29), in Sll0254 the activities appear to be combined according to the sequence and the activity of the protein in *E. coli*. The presence of a ferredoxin-like FeS cluster that is an electron transfer intermediate between the reductase and dioxygenase clusters in many Rieske-type dioxygenases (9, 29) is not apparent from the Sll0254 sequence, and so this electron transfer component may not be critical for this type of dioxygenase. The dioxygenase motif of Rieske-type dioxygenases consists of a Rieske domain that is readily recognizable in Sll0254 with characteristic Cys and His residues at residues 368 to 386 and a 2-His/1-carboxylate triad binding the mononuclear Fe(II) center. At present it is not certain which conserved His residues may be associated with the mononuclear center, but if the two His residues are closely clustered as in other Rieske non-heme dioxygenases (9), the His residues in the HGFH sequence at residues 113 to 116 of Sll0254 are good candidates as they are conserved in all known cyanobacterial Sll0254 homologues as well as in CrtU of green bacteria. However, in many dioxygenases the two His residues are far from each other in the primary sequence, and there are five other His residues that are conserved in all cyanobacterial Sll0254-like proteins. Therefore, an unequivocal assignment of the His residues contributing to the mononuclear Fe site cannot be made at this point.

A lycopene cyclase/oxygenase. Both Sll0254 from *Synechocystis* sp. strain PCC 6803 and CrtL from *Synechococcus* sp. strain PCC 7942 (7) recognize lycopene as well as neurosporene. However, whereas *crtL* expression in *E. coli* led to pure β-carotene or β-zeacarotene (7), expression of sll0254 led to a heterogeneous, much more hydrophilic array of products. Interestingly, the cyclic compounds observed upon production of Sll0254 in *E. coli* had a short retention time, and their mass was consistent with the presence of two hydroxyl groups, whereas the relatively small blue-shift in the absorption spectra indicated a reduction by no more than one in the number of conjugated double bonds in the carotenoid. This suggests oxygenation at one of the ends of the carotenoid. Sll0254 therefore catalyzes not only cyclization of lycopene or neurosporene but also oxygenation. This combination of cyclization and oxygenation is relevant for the reactions that lead from 3,4-didehydrolycopene to deoxymyxol in the myxoxanthophyll biosynthesis pathway (Fig. 8). The majority of cyanobacteria including *Synechocystis* sp. strain PCC 6803 and many *Nostocaceae* produce myxoxanthophyll (see table in reference 13), whereas in some other cyanobacteria such as *Synechococcus* sp. strain PCC 7942 (13) and *Synechococcus* sp. strain WH 8102 (H. Mohamed and W. Vermaas, unpublished data) this glycosylated carotenoid is not detectable under conditions of normal growth. For the myxoxanthophyll biosynthesis pathway,

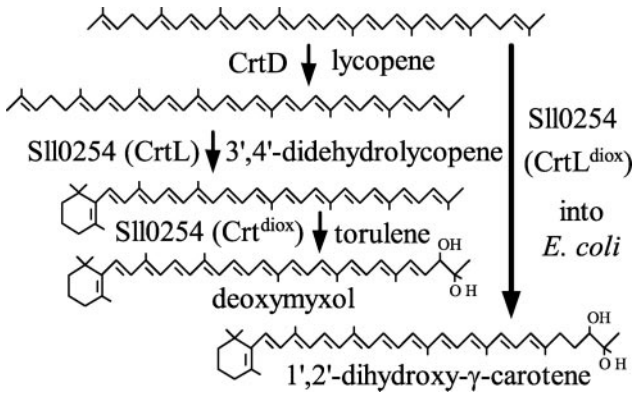

FIG. 8. Schematic representation of lycopene conversion to deoxymyxol by CrtD and Sll0254 in *Synechocystis* sp. strain PCC 6803 (left) and to 1′,2′-dihydroxy-γ-carotene by Sll0254 in an *E. coli* strain that produces lycopene (right).

3,4-didehydrolycopene (derived from lycopene via activity of CrtD, the C-3′,4′ desaturase) is expected to be the cyclase substrate, resulting in formation of the monocyclic torulene (Fig. 8). However, torulene has not been experimentally observed as an intermediate in *Synechocystis*, and instead the first detectable intermediate is deoxymyxol, carrying two hydroxy groups at the end of the molecule that was not cyclized (see reference 18). The enzyme responsible for the oxygenation reaction has not been identified previously, and—other than Sll0254—no strong contenders are apparent from the *Synechocystis* genome.

Our interpretation is that cyanobacteria such as *Synechocystis* sp. strain PCC 6803 with an Sll0254-like lycopene cyclase use this protein to cyclize 3,4-didehydrolycopene (produced by CrtD activity) on one end of the molecule and to oxygenate the other end, resulting in deoxymyxol. Well-studied cyanobacteria known to have an Sll0254-like lycopene cyclase can also synthesize myxoxanthophyll. It is not yet clear whether cyanobacteria with only a CrtL-like cyclase synthesize considerable amounts of myxoxanthophyll under particular conditions, but if so, they are expected to have a separate oxygenase.

At this stage, it is unclear how a similar reaction catalyzed by CrtU would aid in chlorobactene synthesis in *C. tepidum*, which requires a rearrangement of a methyl group in the γ-carotene ring in addition to desaturation of the ring (10). Hydroxylation of the end group of chlorobactene is accomplished through a bacterial hydratase, CrtC. However, CrtU might be involved in the synthesis of dihydroxylated chlorobactene that is not observed under routine conditions, or this dioxygenase function may have been lost in *C. tepidum*.

The results presented in this study clearly indicate that Sll0254 functions as a cyclase/oxygenase and that this activity is directly relevant for myxoxanthophyll biosynthesis, but we did not find evidence for Sll0254-catalyzed β-carotene formation in *E. coli*. Nonetheless, upon sll0254 depletion bicyclic carotenoids were no longer observed in *Synechocystis* sp. strain PCC 6803, suggesting that formation of β-carotene is dependent, directly or indirectly, on Sll0254. At least two scenarios may be considered. One is that there is a second cyclase in *Synechocystis* sp. strain PCC 6803 (for example, one of the *C. tepidum* CruA homologues) that specifically converts lycopene to β-car-

otene. However, unless the absence of Sll0254 also inactivates the cyclase that forms β-carotene, this scenario is not expected to lead to a virtual loss of all cyclic carotenoids upon depletion of sll0254. The second scenario, which we favor, is that due to heterologous expression of sll0254 in *E. coli* and the lack of potential regulatory proteins that may aid in cyclization on both sides of the molecule versus oxygenation on one of the sides, the amount of β-carotene formation in *E. coli* is very low relative to that of deoxymyxol-type components. Indeed, depletion of sll0254 affects synthesis of all cyclic carotenoids.

Based on the results reported here, we interpret Sll0254 in *Synechocystis* sp. strain PCC 6803 to be a lycopene cyclase/oxygenase, which to our knowledge is a novel combination of enzyme activities. We propose the name $CrtL^{diox}$ for Sll0254.

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REFERENCES

1. **Al-Babili, S., P. Hugueney, M. Schledz, R. Welsch, H. Frohnmeyer, O. Laule, and P. Beyer.** 2000. Identification of a novel gene coding for neoxanthin synthase from *Solanum tuberosum*. FEBS Lett. **485:**168–172.
2. **Anderson, S. L., and L. McIntosh.** 1991. Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. J. Bacteriol. **173:**2761–2767.
3. **Armstrong, G. A.** 1997. Genetics of eubacterial carotenoid biosynthesis: a colorful tale. Annu. Rev. Microbiol. **51:**629–659.
4. **Bautista, J. A., F. Rappaport, M. Guergova-Kuras, R. O. Cohen, J. H. Golbeck, J. Yehong Wang, D. Béal, and B. A. Diner.** 2005. Biochemical and biophysical characterization of Photosystem I from phytoene desaturase and ζ-carotene desaturase deletion mutants of *Synechocystis* sp. PCC 6803: evidence for PsaA- and PsaB-side electron transport in cyanobacteria. J. Biol. Chem. **280:**20030–20041.
5. **Breitenbach, J., B. Fernandez-Gonzalez, A. Vioque, and G. Sandmann.** 1998. A higher-plant type zeta-carotene desaturase in the cyanobacterium *Synechocystis* PCC 6803. Plant Mol. Biol. **36:**725–732.
6. **Cunningham, F. X., and E. Gantt.** 2001. One ring or two? Determination of ring number in carotenoids by lycopene epsilon-cyclases. Proc. Natl. Acad. Sci. USA **98:**2905–2910.
7. **Cunningham, F. X., Z. Sun, D. Chamovitz, J. Hirschberg, and E. Gantt.** 1994. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. strain PCC7942. Plant Cell **6:**1107–1121.
8. **Cunningham, F. X., B. Pogson, Z. Sun, K. A. McDonald, D. DellaPenna, and E. Gantt.** 1996. Functional analysis of the beta and epsilon lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell **8:**1613–1626.
9. **Ferraro, D. J., L. Gakhar, and S. Ramaswamy.** 2005. Rieske business: structure-function of Rieske non-heme oxygenases. Biochem. Biophys. Res. Commun. **338:**175–190.
10. **Frigaard, N.-U., J. A. Maresca, C. E. Yunker, A. D. Jones, and D. A. Bryant.** 2004. Genetic manipulation of carotenoid biosynthesis in the green sulfur bacterium *Chlorobium tepidum*. J. Bacteriol. **186:**5210–5220.
11. **He, Q., D. Brune, R. Nieman, and W. Vermaas.** 1998. Chlorophyll *a* synthesis upon interruption and deletion of *por* coding for the light-dependent NADPH. Eur. J. Biochem. **253:**161–172.
12. **Hemmi, H., S. Ikejiri, T. Nakayama, and T. Nishino.** 2003. Fusion-type lycopene β-cyclase from a thermoacidophilic archaeon *Sulfolobus solfataricus*. Biochem. Biophys. Res. Comm. **305:**586–591.
13. **Hirschberg, J., and D. Chamovitz.** 1994. Carotenoids in cyanobacteria, p. 559–579. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **Krubasik, P., and G. Sandmann.** 2000. Molecular evolution of lycopene cyclases involved in the formation of carotenoids with ionone end groups. Biochem. Soc. Trans. **28:**806–810.
15. **Krubasik, P., and G. Sandmann.** 2000. A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. Mol. Gen. Genet. **263:**423–432.
16. **Maresca, J. A., N.-U. Frigaard, and D. A. Bryant.** 2005. Identification of a novel class of lycopene cyclases in photosynthetic organisms, p. 884–886. *In* A. van der Est and D. Bruce (ed.), Photosynthesis: fundamental aspects to global perspectives. Allen Press, Lawrence, Kans.
17. **Martínez-Férez, I. M., and A. Vioque.** 1992. Nucleotide-sequence of the phytoene desaturase gene from *Synechocystis* sp. PCC 6803 and characterization of a new mutation which confers resistance to the herbicide norflurazon. Plant Mol. Biol. **18:**981–983.
18. **Mohamed, H. E., and W. Vermaas.** 2004. Slr1293 in *Synechocystis* sp. strain PCC 6803 is the C-3′,4′ desaturase (CrtD) involved in myxoxanthophyll biosynthesis. J. Bacteriol. **186:**5621–5628.
19. **Mohamed, H. E., A. M. L. van de Meene, R. W. Roberson, and W. F. J. Vermaas.** 2005. Myxoxanthophyll is required for normal cell wall structure and thylakoid organization in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J. Bacteriol. **187:**6883–6892.
20. **Morgenstern, B.** 2004. DIALIGN: Multiple DNA and protein sequence alignment at BiBiServ. Nucleic Acids Res. **32:**W33–W36.
21. **Pagni, M., V. Ioannidis, L. Cerutti, M. Zahn-Zabal, C. V. Jongeneel, and L. Falquet.** 2004. MyHits: a new interactive resource for protein annotation and domain identification. Nucleic Acids Res. **32:**W332–W335.
22. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
23. **Shen, G., S. Boussiba, and W. F. J. Vermaas.** 1993. *Synechocystis* sp. PCC 6803 strains lacking photosystem I and phycobilisome function. Plant Cell **5:**1853–1863.
24. **Stickforth, P., S. Steiger, W. R. Hess, and G. Sandmann.** 2003. A novel type of lycopene epsilon-cyclase in the marine cyanobacterium *Prochlorococcus marinus* MED4. Arch. Microbiol. **179:**409–415.
25. **Tao, L., S. Picataggio, P. E. Rouviere, and Q. Cheng.** 2004. Asymmetrically acting lycopene beta-cyclases (CrtLm) from non-photosynthetic bacteria. Mol. Genet. Genomics **271:**180–188.
26. **Teramoto, M., S. Takaichi, Y. Inomata, H. Ikenaga, and N. Misawa.** 2003. Structural and functional analysis of a lycopene beta-monocyclase gene isolated from a unique marine bacterium that produces myxol. FEBS Lett. **545:**120–126.
27. **Tsuchiya, T., S. Takaichi, N. Misawa, T. Maoka, H. Miyashita, and M. Mimoru.** 2005. The cyanobacterium *Gloeobacter violaceus* PCC 7421 uses bacterial-type phytoene desaturase in carotenoid biosynthesis. FEBS Lett. **579:**2125–2129.
28. **Verdoes, J. C., P. Krubasik, G. Sandmann, and A. J. J. van Ooyen.** 1999. Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. Mol. Gen. Genet. **262:**453–461.
29. **Wackett, L. P.** 2002. Mechanism and applications of Rieske non-heme iron dioxygenases. Enzyme Microb. Technol. **31:**577–587.
30. **Yabu, K., Y. Nishiyama, and T. Ochiai.** 1997. Protease-induced multicell formation in *Staphylococcus haemolyticus*. Microbiol. Immunol. **41:**799–803.