Carotenoid Biosynthesis in the Primitive Red Alga *Cyanidioschyzon merolae*<sup>7</sup>

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*Cyanidioschyzon merolae* is considered to be one of the most primitive of eukaryotic photosynthetic organisms. To obtain insights into the origin and evolution of the pathway of carotenoid biosynthesis in eukaryotic plants, the carotenoid content of *C. merolae* was ascertained, genes encoding enzymes of carotenoid biosynthesis in this unicellular red alga were identified, and the activities of two candidate pathway enzymes of particular interest, lycopene cyclase and β-carotene hydroxylase, were examined. *C. merolae* contains perhaps the simplest assortment of chlorophylls and carotenoids found in any eukaryotic photosynthetic organism: chlorophyll *a*, β-carotene, and zeaxanthin. Carotenoids with *ε*-rings (e.g., lutein), found in many other red algae and in green algae and land plants, were not detected, and the lycopene cyclase of *C. merolae* quite specifically produced only β-ringed carotenoids when provided with lycopene as the substrate in Escherichia coli. Lycopene β-ring cyclases from several bacteria, cyanobacteria, and land plants also proved to be high-fidelity enzymes, whereas the structurally related ε-ring cyclases from several plant species were found to be less specific, yielding products with β-rings as well as ε-rings. *C. merolae* lacks orthologs of genes that encode the two types of β-carotene hydroxylase found in land plants, one a nonheme diiron oxygenase and the other a cytochrome P450. A *C. merolae* chloroplast gene specifies a polypeptide similar to members of a third class of β-carotene hydroxylases, common in cyanobacteria, but this gene did not produce an active enzyme when expressed in *E. coli*. The identity of the *C. merolae* β-carotene hydroxylase therefore remains uncertain.

The unicellular red alga *Cyanidioschyzon merolae*, a resident of acidic hot springs, is considered to be one of the most primitive of photosynthetic eukaryotes (44, 57, 65). As such, the photosynthetic apparatus in this alga may provide the closest approximation to that of the prokaryotic ancestor of the modern-day chloroplast. We are especially interested in the ancestry and evolution of enzymes of the pathway that provides for the synthesis of the carotenoids, a family of isoprenoid pigments that are integral and essential constituents of the photosynthetic apparatus in all oxygenic photautotrophs. In this work, we exploit the recent availability of the nuclear (39), mitochondrial (47), and plastid (46) genome sequences of *C. merolae* to address the origin of carotenoid pathway genes in this alga.

The pathways of carotenoid biosynthesis in eukaryotic plants and in prokaryotic cyanobacteria, the latter of which are considered to be modern-day descendants of the ancestral plastid progenitor (40), are very much alike in their early stages. Reactions in plant chloroplasts that lead from the *C₅* isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate to the linear *C₅₀* carotenoid intermediate lycopene are catalyzed by enzymes similar in sequence to their cyanobacterial counterparts (53). Plant and cyanobacterial carotenoid pathways begin to diverge with the cyclization of lycopene to yield β-carotene, a carotenoid all but ubiquitous in oxygenic photosynthetic organisms (20), and subsequent pathway reactions, including the hydroxylation of each β-ring of β-carotene to produce zeaxanthin (3,3′-dihydroxy-β-carotene), a near omnipresent (20) photoprotective pigment in cyanobacteria (55), algae, and plants (24, 49), are catalyzed by distinctly different enzymes in cyanobacteria and plants.

An analysis of the carotenoid pathway as it now exists in the primitive alga *C. merolae* may provide clues as to when and how the pathways in eukaryotic plants diverged from that of the prokaryotic plastid ancestor and acquired new functionalities that enabled the synthesis of carotenoids not found in either the plastid progenitor or modern-day cyanobacteria. In this study, genes of *C. merolae* that encode polypeptides similar in sequence to known carotenoid pathway enzymes were identified; the major carotenoid pigments accumulated by *C. merolae* were ascertained; and the enzymatic activities of two gene products of particular interest, the putative lycopene cyclase and β-carotene hydroxylase enzymes, were examined.

**MATERIALS AND METHODS**

**Cell culture and harvest.** *Cyanidioschyzon merolae* strain 10D (NIES-1332) was obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Tsukuba, Japan. Cultures of *C. merolae* were grown at 40°C in one-liter batches of BG11 medium (51) adjusted to pH 2.5 with *H₂SO₄*. Cultures, in Corning 2.5-liter low-form culture flasks (Corning 4422-2XL), were bubbled with 5% CO₂ in air and shaken on a rotary platform shaker at 50 rpm. Illumination of ca. 30 μE·m⁻²·s⁻¹ was provided by daylight fluorescent tubes. In some cases, the cytochrome P450 inhibitors clotrimazole (Sigma C6019), miconazole (Sigma M3512), and aneymidol (Sigma A9431), solubilized or dispersed in ethanol (to sterilize), were added to growing cultures in the early log phase (optical density at 730 nm of ca. 0.2) to a final concentration of 300, 1,000, and 100 μM, respectively. The final concentration of ethanol was 0.1% (vol/vol) in these cultures and in control cultures lacking P450 inhibitor.

 Cultures in the mid-log phase of growth were harvested by centrifugation at room temperature. Growth of the *C. merolae* cultures was monitored by the optical density at 730 nm. Small-scale harvests were in 1.5-ml microcentrifuge tubes centrifuged at maximum speed for 30 seconds in an Eppendorf 5415 D microcentrifuge. Larger-scale harvests were done with a Sorvall GSA rotor centrifuged at 5,000 rpm (4,000 × *g*) for 10 min. Pellets were washed with 100 mM HEPES-KOH buffer at pH 8.0, and residual liquid was removed with a pipettor after centrifugation. Cell pellets were then immediately extracted for

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pigmentation analysis or were stored at −80°C in darkness, with extraction and analysis performed at a later time.

Nucleotide and protein sequence analyses. The GenBank nucleotide and protein sequence databases (http://www.ncbi.nlm.nih.gov/) were searched using the programsblast, blastp, and tblastn (1). Searches were carried out with, and sequences were obtained from, genomes available at websites of the *Cyandinoschyzon merolae* Genome Project (http://merolaio.biol.s.u-tokyo.ac.jp/), the Michigan State University *Galdieria sulphuraria* Genome Project (http://genomes.msu.edu/galdieria; see reference 3), the Phototrophic Prokaryotes Sequencing Project (http://genomes.tgen.org/Acaryochloris.html), and the Joint Genome Institute of the United States Department of Energy (http://www.jgi.doe.gov/)

Protein sequences were imported into ClustalX version 1.81 (63) and aligned using the default parameters except that the BLOSUM series protein weight matrix was specified. Minor adjustments to the alignments were made after a careful visual examination of the results. Neighboring-join trees were constructed with correction for multiple substitutions (30). Positions with gaps and regions of uncertain alignment were excluded from the analyses. A total of 342 positions were included in the analysis leading to the lycopene cyclase tree (see Fig. 3), and 230 positions were used to construct the CrtR tree (see Fig. 5). The alignments used to construct the neighboring-join trees are available upon request. Ten thousand bootstrap trials were conducted with the random number generator seed set to 111.

The programs ChloroP (18; available at http://www.cbs.dtu.dk/services/TargetP/) and TargetP (17; available at http://www.cbs.dtu.dk/services/TargetP/) were used to analyze various *C. merolae* gene products for the presence of chloroplast transit peptides and to predict their subcellular localization.

**PCR and gene cloning.** DNA from *C. merolae* was a gift of Tsuneyoshi Kuroiwa of Rikkyo University, Tokyo, Japan. Genomic DNA was purified from *Synechocystis sp. strain PCC6803* as previously described (64). A high-fidelity DNA polymerase (HF-2; BD Biosciences Clontech) was used to amplify the β-carotene hydrolase gene (*crtR; slr1168*) of *Synechocystis* sp. strain PCC6803, and the putative lycopene β-cyclase (LCyb) and β-carotene hydrolase genes of *C. merolae*. The paucity of introns in the genome of *C. merolae* (39) allows for PCR amplification of most open reading frames using genomic DNA, rather than cDNA, as the template. PCR products were sequenced after cloning to confirm that no errors were introduced by the PCR and that the cloned product was in the appropriate reading frame in the plasmid vector. PCR primers were designed with reference to the *Synechocystis* sp. strain PCC6803 genome sequence (28) and gene models (available at http://www.kazusa.or.jp/cyano/cyano.html), and to the *C. merolae* nuclear (39) and plastid (46) genome sequences and gene models (http://merolaio.biol.s.u-tokyo.ac.jp/). Primers were designed with the aid of the program Primer3 (52; http://frado.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

**OLIGONUCLEOTIDE PRIMERS TO AMPLIFY THE C. MEROLAE CRYPTEXANTHIN SYNTHESIS PATHWAY GENES**

| Primer                | Sequence                                      |
|----------------------|-----------------------------------------------|
| CmLCYN-PcII          | gagaagcagtGTTTTTTGGCTAACCTTGC                 |
| CmLCYC               | CAGGCAAAAGGGGAGCAATTC                       |
| SynCrtR-NcoI         | cacatacagaGCGAACCTCCACCAACCC                |
| SynCrtR-XhoI         | gagaagcagtACCGCACCACCTTCGGTCTG              |
| SynCrtRdel26N-BspHI  | cacatacagctGTTGGCTATTTGGGAGAAT              |
| SynCrtR-HindIII      | gagaagcagtTAGGTCGGAAAGGCAACCC               |
| CmRtR-BspHI          | gagaagcagtAGTTGAGTTTGTATTTTTTTGTAG          |
| SynCrtRdel26N-BspHI  | gagaagcagtTATTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| CmCrtR               | GGCCTCCTAAAATACATTACCTG                     |

* a. Bases complementary to *C. merolae* or *Synechocystis* sp. DNA are in uppercase. Reduced restriction sites are underlined. Initiation codons are in boldface.

A prospective *C. merolae* β-carotene hydrolase gene (*crtR*; CMV041C) was amplified by PCR using two different sets of oligonucleotide primers. In the first instance, using primers CmSynCrtRN-BspHI and CmSynCrtRC-HindIII (Table 1), the *C. merolae* crtR was amplified to give a product of 845 bp that was cloned in pSTBlue-1. After excision with PstI (in the multiple cloning site of pSTBlue-1) and HindIII (introduced by the PCR primer), the *C. merolae* crtR was inserted in the PstI and HindIII sites of pTrcHisB. Digestion of the resulting plasmid with NcoI, partial digestion with BspHI (several sites for this enzyme are present within the *C. merolae* crtR gene), and ligation of an agarose gel-purified fragment of the appropriate size yielded a plasmid, pCmCrtR, in which the full-length open reading frame of the *C. merolae* crtR had been placed under the control of the *E. coli*lac promoter.

In the second instance, using primers CmSynCrtRN-BstBI and CmSynCrtRC (Table 1), a BstBI site was introduced at the N terminus of the *C. merolae* crtR, and the agarose gel-purified 894-bp product was cloned in pSTBlue-1. The insert was excised as a Klenow-blunted BstBI-XhoI fragment (with the XhoI site provided by the multiple cloning site of the vector) and cloned in the MscI site (near the N terminus of the coding region of the *Synechocystis* crtR gene) and the XhoI site (downstream of the *Synechocystis* crtR gene in the multiple cloning site of the vector) of a plasmid (pSynCrtR; see above) that already contained the *crtR* gene of *Synechocystis* sp. strain PCC6803. The resulting plasmid, referred to as pSynCrtRdel26N, specified a fusion protein comprised of the first 29 amino acids of the *Synechocystis* CrtR fused to essentially the entire *C. merolae* crtR gene product (lacking only the initiation Met).

**Functional analysis of the lycopene β-cyclase and β-carotene hydrolase enzymes.** The candidate LCyb and β-carotene hydrolase (CrtR) enzymes of *C. merolae* were assayed by expressing the genes encoding them in strains of *Escherichia coli* (TOP10) engineered to accumulate their respective substrates, lycopene and β-carotene. In some experiments, the inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added (at a final concentration of 1 mM), either at the time of inoculation or as cultures reached mid-log phase. Cultures were grown in darkness at 30°C on a rotary shaker and plated on a minimal medium.
TABLE 2. Plasmids used in this study

| Plasmid        | Description and details of construction | Source or reference |
|----------------|-----------------------------------------|---------------------|
| pBBR122        | Broad host range, medium copy no. plasmid (2); compatible with p15A- and ColEl/ | MoBiTec             |
| pRARE          | Has tRNA genes for rarely used E. coli codons (41); Cap9, p15A ori | Novagen             |
| pSTBlue-1      | Blunt cloning vector, Amp9 and Kan9 | Novagen             |
| pT7Blue        | Blunt cloning vector, Amp9 | Novagen             |
| pTrcHisA       | Expression vector, ColEl ori; Amp9 | Invitrogen          |
| pTrcHisB       | Expression vector, ColEl ori; Amp9 | Invitrogen          |
| pAC-LYC        | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori | 15                   |
| pAC-LYCipi     | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori. Includes a gene encoding the Erwinia herbicola isopentenyl diphosphate isomerase for improved pigment yield. Extraneous sequence was removed from pAC-LYC by digestion with Sphi and Drd, blunting with mung bean nuclease, and recircularization. This 7.8-kb plasmid was then digested with HindIII and EcoRV to yield a 6.4-kb fragment that was ligated with a HindIII mung bean-blunted DraIII fragment of 2.55 kb obtained from pAC-EHER | This work |
| pAC-DELTA      | Produces δ-carotene (ε,ε-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pAC-EPISILON   | Produces ε-carotene (ε,ε-carotene) in E. coli; Cap9, p15A ori | 13                   |
| pAC-GAMMA      | Produces predominantly γ-carotene (β,β-carotene) in E. coli; Cap9, p15A ori | 13                   |
| pAC-BETAApi    | Produces β-carotene in E. coli; Cap9, p15A ori | 13                   |
| pBBR-BETA      | Produces β-carotene in E. coli; Kan9, compatible with p15A- and ColEl/ | This work |
| pAC-LYC        | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori. Includes a gene encoding the Erwinia herbicola isopentenyl diphosphate isomerase for improved pigment yield. Extraneous sequence was removed from pAC-LYC by digestion with Sphi and Drd, blunting with mung bean nuclease, and recircularization. This 7.8-kb plasmid was then digested with HindIII and EcoRV to yield a 6.4-kb fragment that was ligated with a HindIII mung bean-blunted DraIII fragment of 2.55 kb obtained from pAC-EHER | 15                   |
| pAC-LYCN       | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pAC-LYCipN     | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pHpKetoSK      | Haematococcus plavialis β-C-4-oxygenase cDNA in plBluescript SK- | 34                   |
| pCmLCYb        | C. merolae lycopene β-cyclase gene cloned in pTrcHisB | This work |
| pAtLCYe       | A. thaliana lycopene β-cyclase cDNA in plasmid pBluescript SK- | This work |
| pTRC-LCYb      | A. thaliana lycopene β-cyclase cDNA clone | This work |
| pAC-BETA-1      | Produces β-carotene in E. coli; Cap9, p15A ori | 11                   |
| pAC-DELTA      | Produces δ-carotene (ε,ε-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pAC-EPISILON   | Produces ε-carotene (ε,ε-carotene) in E. coli; Cap9, p15A ori | 13                   |
| pAC-GAMMA      | Produces predominantly γ-carotene (β,β-carotene) in E. coli; Cap9, p15A ori | 13                   |
| pAC-BETAApi    | Produces β-carotene in E. coli; Cap9, p15A ori | 13                   |
| pBBR-BETA      | Produces β-carotene in E. coli; Kan9, compatible with p15A- and ColEl/ | This work |
| pHpKetoSK      | Haematococcus plavialis β-C-4-oxygenase cDNA in plBluescript SK- | 34                   |
| pCmLCYb        | C. merolae lycopene β-cyclase gene cloned in pTrcHisB | This work |
| pAC-LYC        | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pAC-LYCipN     | Produces lycopene (β,β-carotene) in E. coli; Cap9, p15A ori | 61                   |
| pSyn7942Crl    | Synechococcus PCC7942 lycopene β-cyclase in pTrcHisA. Formerly referred to as pTrc-LCYKH | 15                   |
| pBS603dDOA     | Plasmid containing lycopene β-cyclase gene cty7m from the marine bacterium P99-3 | 62                   |
| pATLCYe        | A. thaliana lycopene ε-cyclase cDNA in plasmid pBluescript SK- | 14                   |
| pAalLCYe1      | Adonis aestivalis lycopene ε-cyclase 1 cDNA in plasmid pBluescript SK- | 12                   |
| pAalLCYe2      | Adonis aestivalis lycopene ε-cyclase 2 cDNA in plasmid pBluescript SK- | 12                   |
| pCmCrR         | C. merolae crR cloned in the expression vector pTrcHisB | This work |
| pSynCrR        | Synechocystis PCC6803 crR cloned in the expression vector pTrcHisB. The second codon specifies an E rather than a Q as a consequence of the cloning strategy. | This work |
| pSynCrRdel126N  | Synechocystis PCC6803 crR cloned in the expression vector pTrcHisB. Lacks codons for 26 N-terminal amino acids. The codon for the 27th amino acid was made an initiation codon. | This work |
| pSyn/CmCrR     | Chimeric Synechocystis PCC6803 and C. merolae crR cloned in the expression vector pTrcHisB. The entire open reading frame of the C. merolae crR, except the | This work |

*pamp*, ampicillin resistance; Cap9, chloramphenicol resistance; Kan9, kanamycin resistance; ori, origin of replication.

The synthesis of β-carotene from the isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate are present in the nuclear genome of C. merolae. Additionally, the plastid genome contains an open reading frame that specifies a polypeptide similar in


pigments was done using the formulae given by Hirschberg and Chamovitz (23) for dimethylformamide extracts of cell pellets.

**RESULTS**

**Candidate carotenoid pathway genes of C. merolae.** As a first step in defining the pathway of carotenoid biosynthesis in C. merolae, we searched the nuclear (39), mitochondrial (47), and plastid (46) genomes (available at [http://merolae.biol.s.u-tokyo.ac.jp/](http://merolae.biol.s.u-tokyo.ac.jp/)) of this unicellular red alga using amino acid sequences of known plant (Arabidopsis thaliana) and cyanobacterial (Synechocystis sp. strain PCC6803) carotenoid pathway enzymes as queries. A list of candidate carotenoid pathway genes identified in this way is given in Table 3. Single-copy genes orthologous to all those that are required for the synthesis of β-carotene from the isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate are present in the nuclear genome of C. merolae. Additionally, the plastid genome contains an open reading frame that specifies a polypeptide similar in
sequence to \(\beta\)-carotene hydroxylase enzymes of a type (CrtR type) found in cyanobacteria but not in land plants.

Two of the \textit{C. merolae} candidate carotenoid pathway enzymes, phytoene desaturase (PDS) and \(\xi\)-carotene desaturase (ZDS), are more similar in amino acid sequence to products of available cyanobacterial carotenoid pathway genes than to those specified by known plant genes. Two others, geranylgeranyl diphosphate synthase (GGPS) and phytoene synthase (PSY), are equidistant from extant cyanobacterial and green algal genes than to those of plants (cyanobacteria), the \(\epsilon\)-ring hydroxylase (which is needed to make lutein), zeaxanthin epoxidase (which converts zeaxanthin into violaxanthin), and violaxanthin de-epoxidase (which converts violaxanthin back into zeaxanthin).

\textbf{Carotenoids and chlorophylls of \textit{C. merolae}.} The chlorophyll and carotenoid pigments in cells of \textit{C. merolae} were analyzed by HPLC (Fig. 2). On the basis of their HPLC retention times, absorption spectra, and molecular masses, the major pigments were identified as chlorophyll \(a\), \(\beta\)-carotene (\(\beta,\beta\)-carotene), and zeaxanthin (3,3'-dihydroxy-\(\beta,\beta\)-carotene), with a smaller amount of \(\beta\)-cryptoxanthin (3-hydroxy-\(\beta,\beta\)-carotene), an intermediate in the biosynthesis of zeaxanthin from \(\beta\)-carotene, also observed (Table 4). The three major carotenoids of \textit{C. merolae} were present predominantly in the form of the all-\textit{trans} geometrical isomer, with lesser amounts of one or more cis-geometrical isomers. In addition to chlorophyll \(a\), several other peaks in HPLC elution profiles of \textit{C. merolae} pigment extracts displayed chlorophyll-like absorption spectra (cf. peaks 1, 5, 7, and 12 in Fig. 2A). The compounds represented by these quite minor peaks were not identified but likely include chlorophyll \(a\)' and phaeophytin \(a\), as identified in the closely related unicellular red alga \textit{Cyanidium caldarium} (66).
Functional analysis of the putative LCYb of *C. merolae*. Of all the prospective *C. merolae* carotenoid pathway enzymes, the candidate LCYb was the least well conserved in amino acid sequence when compared with known carotenoid pathway enzymes (Table 3). A neighbor-joining tree (Fig. 3) visually illustrates the similarity of the candidate LCYb of *C. merolae* to various lycopene β-cyclases, lycopene ε-cyclases (LCYe), and related polypeptides encoded by genes in other algae, plants, and cyanobacteria. Lycopene ε-cyclases catalyze a reaction very much like that catalyzed by lycopene β-cyclases, with the ε-ring introduced by LCYe differing from a β-ring only in the position of the double bond within the ring (Fig. 3, upper left; see reference 10). The putative LCYb of *C. merolae* is situated, together with several other algal polypeptides, near the base of a tree branch that leads to a compact cluster of plant LCYb (Fig. 3). With the exception of that of the LCY from the unicellular green alga *Haematococcus pluvialis* (shown to be an LCYb; 58), the enzymatic activities of the various algal polypeptides listed in Fig. 3 have not yet been ascertained.

The function of the candidate *C. merolae* LCYb was examined by producing the authentic, full-length gene product (504 amino acids in length; the presumptive plastid targeting sequence was not removed) in a strain of *E. coli* engineered to accumulate the substrate lycopene (this strain contained the plasmid pAC-LYCipi; Table 2). A near-to-complete conversion of lycopene into β-carotene (Fig. 4; compare panel B to panel A) was achieved with the introduction of plasmid pCmLYCb (Table 2), containing the *C. merolae* LCYb. The all-trans geometrical isomer of β-carotene was the major product (Fig. 4B, peak 1) and, as is typically observed with β-carotene-producing *E. coli* (13, 14, 54), two cis geometrical isomers (possibly the 9-cis and 15-cis; 54) also accumulated, becoming more prevalent in older cultures.

**Functional analysis of the putative CrtR of *C. merolae*.** The product of the candidate β-carotene hydroxylase gene of *C. merolae* resembles cyanobacterial β-carotene hydroxylase enzymes of a type referred to as CrtR (Fig. 5 and 6). The *C. merolae* crtR gene was amplified by PCR, and the product was cloned in an expression vector (to give plasmid pCmCrtR; Table 2) and then introduced into in a strain of *E. coli* engineered to accumulate the substrate β-carotene (this strain contained plasmid pAC-BETAipi; Table 2). No enzymatic activity

**TABLE 4. Identification of chlorophyll and carotenoid pigments in *C. merolae***

| HPLC peak no.| Absorption peaks | Molecular formula | Identification |
|--------------|-----------------|------------------|----------------|
| 2            | 425, 449, 476   | ND               | Zeaxanthin, cis isomer |
| 3            | 340, 425, 448, 474 | ND               | Zeaxanthin, cis isomer |
| 4            | 430, 455, 481   | C_{15}H_{20}O_{2} | Chl a          |
| 6            | 431, 663        | C_{20}H_{17}N_{2}O_{5}Mg | Chlorophyll a |
| 8            | 429, 455, 481   | C_{20}H_{17}O_{5} | β-Cryptoxanthin |
| 9            | 341, 425, 449, 475 | ND               | β-Cryptoxanthin, cis isomer |
| 10           | 430, 455, 482   | C_{20}H_{16}O_{5} | β-Carotene     |
| 11           | 425, 450, 476   | ND               | β-Carotene, cis isomer |
| 13           | 341, 425, 448, 474 | ND               | β-Carotene, cis isomer |

*a* Peak number in HPLC elution profile of Fig. 3A.  
*b* In HPLC mobile phase. Peaks in parentheses are “shoulders.”  
*c* Inferred from high resolution mass spectrometry.  
*d* ND, Not determined.
was detected (Fig. 7, compare panel B to panel A). In contrast, the introduction of the \(\text{crtR}\) gene of \(\text{Synechocystis}\) sp. strain PCC6803 (in plasmid pSynCrtR) into \(\text{E. coli}\) containing pAC-BETAipi resulted in a nearly complete conversion of \(\text{\(\delta9\)-carotene}\) into zeaxanthin (Fig. 7, compare panel C to panel A; also see reference 38).

Several strategies were employed in attempts to elicit enzymatic activity from the \(\text{C. merolae}\) \(\text{crtR}\) gene product. First, the inducer IPTG was added to \(\text{E. coli}\) cultures containing pAC-BETAipi and pCmCrtR in order to increase production of the \(\text{C. merolae}\) \(\text{crtR}\) gene product. However, the addition of IPTG greatly impaired the growth of these cultures, indicating that an overaccumulation of the membrane-integral \(\text{CrtR}\) polypeptide is in some way toxic to \(\text{E. coli}\). In any case, and whether IPTG was added to cultures at the time of inoculation or was not added until the mid-log phase of growth, no \(\beta\)-carotene

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**FIG. 3.** Neighbor-joining tree for polypeptides encoded by plant, algal, and cyanobacterial members of the lycopene cyclase (LCY) gene family. Structures and carbon numbering for \(\beta\)- and \(\epsilon\)-rings are illustrated at the upper left. The putative \(\text{C. merolae}\) LCYb is highlighted by placement within a rectangular box at the top of the figure. The encircled cluster designated “LCYb/e,” within the larger “Cyanobacterial LCYb” cluster, contains enzymes of mixed function that convert lycopene into products with both \(\beta\)- and \(\epsilon\)-rings (59). The cluster labeled “CCS/NSY,” within the “Plant LCYb” cluster, includes plant enzymes that function as capsanthin-capsorubin synthases (CCS) and/or neoxanthin synthases (NSY) but that also, in some cases at least, retain the ability to function as a lycopene \(\beta\)-ring cyclase. Bootstrap values for the major branches are indicated.
hydroxylase activity was detected in these experiments. A severe impairment of growth was also observed with the addition of IPTG to cultures containing plasmids pAC-BETAipi and pSynCrtR (with the Synechocystis crtR gene), and this strategy of increasing crtR gene expression actually proved counterproductive: the conversion of /H9252-carotene into zeaxanthin was less complete than for cultures lacking IPTG (data not shown).

Second, a plasmid containing tRNAs that are of low abundance in E. coli (pRARE; 43) was introduced into /H9252-carotene-accumulating E. coli (this strain contained plasmid pBBR-BETA rather than pAC-BETAipi to provide compatibility with the pRARE plasmid), together with the plasmid containing the C. merolae crtR (pCmCrtR). This was done in order to alleviate any limitations on translation that might result from the presence in the C. merolae crtR of a few codons that are used infrequently in E. coli. No activity was observed with the C. merolae crtR, but good activity was found, once again, for the product of the Synechocystis crtR gene (data not shown).

The sequence of the C. merolae CrtR is truncated at the N terminus by 22 amino acids or more relative to the sequences of all cyanobacterial CrtR (23 cyanobacterial sequences were available in GenBank as of 15 September 2006; that of the CrtR of Synechocystis sp. strain PCC6803 is displayed in Fig. 6; sequence alignments that include the other cyanobacterial CrtR are available upon request) and by 20 amino acids relative to CrtR-related sequences present in two other red algae (Fig. 6), with one or more deletions apparent near the C-terminal end as well. The Synechocystis crtR, when truncated at the N terminus to produce a polypeptide that commences at a point comparable to that of the CrtR of C. merolae (lacking codons for the 26 N-terminal amino acids; see plasmid pSynCrtRdel26N; the gene product begins with MNVAMF) (Table 2) was found to yield an enzyme significantly impaired in activity, albeit not completely inactivated. A chimeric gene (in plasmid pSyn/CmCrtR) created by appending the N-terminal portion of the Synechocystis crtR to the C. merolae crtR (the
gene product begins metaqplqtvqavpkeflqadggfnpnvaNSLLF
with residues in lowercase type provided by the gene product begins meatqplqtvqavpkeflqadggfnpnvaNSLLF
C. merolae crtR of the four sequences.

Examples of the C. merolae gene encodes a polypeptide with C. merolae gene encodes a polypeptide with
C. merolae CrtR relative to all other known CrtR, led us to con-
cider the possibility that the C. merolae CrtR might be a non-
functional pseudogene and, therefore, that some other C. merolae gene encodes a polypeptide with a beta-carotene hydroxylase activity. Examples of beta-carotene hydroxylases that are cytochrome P450 enzymes exist in bacteria (Thermus thermophila; 6) and in the flowering plant Arabidopsis thaliana (29). There are five putative P450-encoding genes in the nu-
clear genome of C. merolae (CMD096C, CMJ270C, CMJ284C, CMS319C, and CMR093C). The product of no one of these five bears any particular resemblance to the known P450 carotenoid beta-ring hydroxylases. Although two of the five (CMD096C and CMR093C) are predicted by ChloroP (18) to have a plastid transit sequence at the N terminus, no one of the five is predicted to be localized in the chloroplast by TargetP (17). The utility and reliability of these programs for predicting targeting and localization of red algal polypeptides is uncertain, and results obtained using the amino acid sequences of the five nuclear-encoded carotenoid pathway enzymes (PSY, PDS, ZDS, carotene isomerase [CrtISO], and LCYb; see Fig. 1) as queries do not inspire any confidence: only ZDS was predicted by ChloroP to have a chloroplast transit peptide, and only PSY and ZDS were indicated by TargetP as destined for the chloroplast.

To test the hypothesis that one of the five C. merolae P450 gene products might serve as the beta-carotene hydroxylase in C. merolae, we employed a strategy much like that used earlier by Schoefs et al. (56) to show that a cytochrome P450 served as the beta-carotene hydroxylase enzyme in a pathway leading to the carotenoid astaxanthin in the unicellular green alga Haematococcus pluvialis. Cultures of C. merolae were grown in the presence of three general inhibitors of P450 enzymes. Two of the inhibitors tested, clotrimazole and miconazole, at the relatively high concentrations used in these experiments (the concentrations used in this study were the same as used by Bede et al. [4] for cell suspension cultures of the sedge Cyperus iria, except that ancyclid was used in the present study at a 3.3-fold-higher concentration), very quickly (within one day) killed and bleached cultures of C. merolae. In the presence of the third inhibitor tested, ancyclid, cultures of C. merolae con-
tinued to grow and divide at the same rate as control cultures (as measured by the optical density at 730 nm) for about two generations before a relatively sudden arrest of growth. Anal-
yses of pigments extracted from cultures grown for about three days (and ca. 1 1/2 generations) in the presence of ancyclid revealed a modest decrease in the chlorophyll to carotenoid ratio (ca. 15%) relative to control cultures. The change appeared due largely to a reduction in cell content of chlorophyll rather than to an increase in carotenoids given that the amount of chlorophyll per unit of optical density at 730 nm for these C. merolae cultures diminished by approximately the same amount. Importantly, no significant change in the relative amounts of the various carotenoids, and specifically no reduc-
tion in the amount of zeaxanthin relative to C. merolae cultures diminished by approximately the same amount. Importantly, no significant change in the relative amounts of the various carotenoids, and specifically no reduc-
tion in the amount of zeaxanthin relative to C. merolae, was observed for the cultures grown in the presence of ancyclid.

DISCUSSION

Cyanidioschyzon merolae contains quite possibly the simplest assortment of chlorophylls and carotenoids to be found in any oxygenic photosynthetic organism, whether prokaryote or eu-
karyote: chlorophyll a, beta-carotene, and zeaxanthin (Fig. 4 and Table 4). The carotenoid pathway in C. merolae, proceeding from the C3 isoprenoid precursors isopentenyl diphosphate

FIG. 6. Alignment of the deduced amino acid sequence of the CrtR-type beta-carotene hydroxylase of the cyanobacterium Synechocystis sp. strain PCC6803 (Sy) and related polypeptides specified by genes in the red algae G. sulphuraria (Gs), C. caldarium (Cc), and C. merolae (Cm). Residues are in white text on a black background where identical for all four sequences and in black text on a gray background where identical for three of the four sequences.
and dimethylallyl diphosphate all the way to β-carotene, employs enzymes that are similar in amino acid sequence to the corresponding enzymes in green algae, in land plants, and in certain species of cyanobacteria. Of particular interest in regard to the origin and evolution of the enzymes of carotenoid pathways in eukaryotic plants and algae are not these similar-ities but rather the two distinct ways in which the pathway of *C. merolae* differs from those of land plants and green algae: the lack of carotenoids with ε-rings and the absence of a plant-type β-carotene hydroxylase enzyme.

### β-Rings, ε-rings, and origin of the lycopene ε-ring cyclase.

The relatively simple pigment composition of *C. merolae*, with chlorophyll *a*, β-carotene, and zeaxanthin as the primary pigments, has been observed for a few other red algal species (5, 11, 36, 60), but many more red algae have been found to accumulate carotenoids with ε-rings as well as β-rings (i.e., α-carotene and lutein; 5, 37, 60). Carotenoids with β-rings (i.e., β-carotene and zeaxanthin) are obligate components of the photosynthetic apparatus in virtually all oxygenic photosynthetic organisms that have been examined, whereas carotenoids with ε-rings (i.e., ε-carotene and luteoxanthin) or a mixture of β-rings and ε-rings (i.e., α-carotene and lutein) are less widely distributed (20). The universality of carotenoids with β-rings and the substantial amino acid sequence similarity between plant β-ring and ε-ring lycopene cyclase enzymes together make it clear that genes encoding lycopene ε-ring cyclase enzymes (LCYe) arose by duplication of pre-existing genes encoding lycopene β-ring cyclases. Interestingly, just such a gene duplication appears to have been “fossilized” in the genomes of two species in the prasinophyte genus *Ostreococcus* (*O. lucimarinus* and *O. tauri*), where putative LCYβ and LCYε-encoding genes are found in tandem and in the same reading frame without a termination codon between them (16; see http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html and http://genome.jgi-psf.org/Ostta4/Ostta4.home.html).

It has been argued that gene duplication does not commonly give rise to enzymes of new functionality but, rather, more generally allows for the “subfunctionalization” of an enzyme of multiple functionality (19, 25, 35, 50). In the context of the lycopene β-ring and ε-ring cyclase enzymes, this concept would predict that the ancestral lycopene β-ring cyclase evinced some degree of ε-ring cyclase activity prior to the gene duplication(s) that gave rise to the separate and distinct lycopene ε-cyclase-encoding genes now found in land plants and in green algae (lycopene cyclase-encoding genes or cDNAs are not yet available from any red alga that produces carotenoids with ε-rings). Most species of cyanobacteria for which the carotenoid composition has been determined do not accumulate carotenoids with ε-rings. There are, however, a few species of *Prochlorococcus* that accumulate such pigments. One of two LCY-encoding genes in the genome of *Prochlorococcus* sp. strain CCMP1986 (also known as *Prochlorococcus* sp. strain MED4) has been found to produce a lycopene cyclase enzyme of mixed function, able to add both ε-rings and β-rings when presented with lycopene as the substrate in *E. coli* (59; the second LCY from this organism yielded only β-rings).

When and how did the ability to produce carotenoids with ε-rings arise in red algae and other eukaryotic algae and in land plants? Did the prokaryotic progenitor of the chloroplast bring with it a gene for a bifunctional LCYβ/LCYε enzyme much like that of *Prochlorococcus* sp. strain CCMP1986, or did ε-cyclase activity arise only subsequent to this seminal event? Or a question more readily answered, does the product of the single lycopene cyclase gene in the primitive red alga *C. merolae*...
TABLE 5. HPLC retention time and absorption peaks for known carotenoids

| Carotenoida | HPLC retention time (min)b | Absorption peaksc |
|-------------|---------------------------|-------------------|
| Lycopene (β,β-carotene) | 12.4 | 448, 474, 506 |
| δ-Carotene (ε,β-carotene) | 14.6 | 434, 459, 490 |
| γ-Carotene (β,ε-carotene) | 15.5 | (440), 465, 495 |
| ζ-Carotene (ε,ε-carotene) | 17.4 | 418, 442, 472 |
| α-Carotene (β,ε-carotene) | 18.5 | (424), 449, 477 |
| β-Carotene (β,β-carotene) | 19.7 | 455, 482 |

a Produced in E. coli using the following plasmids (Table 2): pAC-LYCipi (lycopene), pAC-Delta (δ-carotene), pAC-Gamma (γ-carotene), pAC-Epsilon (ε-carotene), pAC-BetaAt + pAtLCYe (α-carotene), pAC-BetaApi (β-carotene).
b With isocratic mobile phase of 35% B; see Materials and Methods.
c In HPLC mobile phase. Peaks in parentheses are “shoulders.”

produce only β-ringed carotenoids or are carotenoids with ε-rings also formed?

Concurrent with the lack of ε-ring carotenoids in pigment extracts of cells of C. merolae (Fig. 2 and Table 4), HPLC elution profiles (Fig. 4, panel A) provided no evidence for the production of any carotenoids with ε-rings when lycopene was provided as the substrate for the C. merolae LCYb in E. coli. No peaks with retention times or absorption spectra (Table 5) comparable to those displayed by the mono- and bicyclic ε-ring carotenoids δ-carotene (one ε-ring; retention time, 14.6 min) and ε-carotene (two ε-rings; 17.4 min) or the ε/β-ringed carotenoid α-carotene (one β-ring and one ε-ring; 18.5 min; Fig. 4C) were detected, even when much larger amounts of sample (>10-fold) were analyzed. Carotenoids with ε-rings, if they are present at all, therefore comprise very much less than one percent of the total amount of cyclic products formed from lycopene through the action of the C. merolae LCYb in E. coli.

The high fidelity displayed by the C. merolae LCYb led us to reexamine the specificity of lycopene β-cyclase enzymes from a variety of bacteria, cyanobacteria, and plants. LCYb encoded by genes from three prokaryotic organisms that accumulate only carotenoids with β-rings, the phytopathogenic bacterium Pantoea agglomerans (plasmid pEhCryT; Table 2), the marine bacterium P99-3 (plasmid pBS603dDOA), and the cyanobacterium Synechococcus PCC7942 (plasmid pSym7942Ctrl), each were found to be high-fidelity β-ring cyclases (data not shown). LCYb from two flowering plants that accumulate substantial amounts of carotenoids with ε-rings, A. thaliana (plasmid pAtLCYbSK) and Adonis aestivalis (plasmid pAaLCYb), also were found to yield no trace of ε-ringed carotenoids when lycopene was provided as the substrate in E. coli (data not shown).

In contrast to the high fidelity of the various LCYb examined in this study, the structurally related lycopene ε-ring cyclase (LCYe) from the flowering plant Lactuca sativa (lettuce) was earlier found to be less than specific: two β-ringed carotenoids, γ-carotene (one β-ring) and α-carotene (one β-ring and one ε-ring), were produced as minor products when the enzyme was provided with lycopene as the substrate in E. coli (13). A reexamination of two LCYe from A. aestivalis (in plasmids pAaLCYe1 and pAaLCYe2; Table 2) and one from A. thaliana (plasmid pAtLCYe) revealed a comparable lack of specificity: β-rings, primarily in the form of the monocyclic γ-carotene, comprised ca. 3 to 8% of the total when these enzymes were presented with lycopene as the substrate in E. coli.

To give one example, Fig. 8A illustrates an HPLC elution profile of carotenoid pigments produced in E. coli from lycopene through the action of an A. thaliana LCYe (cells contained pAC-Delta, constructed by insertion of the A. thaliana lcyE into plasmid pAC-LYC; Table 2). The predominant product is δ-carotene (with one ε-ring; 14), but a minor peak (Fig. 8A, peak a1) that elutes immediately after δ-carotene exhibits a retention time (15.6 min) and an absorption spectrum (Fig. 8E) comparable to those of γ-carotene (one β-ring) produced in E. coli through the action of a lycopene β-ring monocyclase from the marine bacterium P99-3 (Fig. 8C and E; see reference 62). Confirmation that the carotenoid responsible, in large part, for this minor peak was indeed γ-carotene was obtained by an in vivo biochemical derivatization experiment, using an enzyme from the green alga Haematococcus pluvialis that adds a carbonyl to the number 4 carbon of β-rings (34) but not ε-rings (this carbon is unavailable in ε-rings because of the 4-5 double bond; see the lower right of Fig. 8 for structures of an ε-ring, a β-ring, and a 4-keto-β-ring). The introduction into δ-carotene-accumulating E. coli of a plasmid (pHPKetoSK) expressing the H. pluvialis β-C-4-oxidogenase resulted in a substantial diminution of the peak attributed provisionally to γ-carotene (Fig. 8A, peak a1), and a new peak (Fig. 8B, peak b1) appeared with a retention time (8.1 min) and an absorption spectrum (a broad ketocarotenoid-like spectrum with peaks at 472 and 493 nm; Fig. 8F) that were indistinguishable from those of 4-keto-γ-carotene produced in E. coli from γ-carotene (Fig. 8D and F). By virtue of their synthesis of carotenoids with β-rings as well as ε-rings, plant LCYe continue to betray their origin from LCYb, even as the LCYb that gave rise to them persist in maintaining a high degree of specificity.

Which gene encodes the β-carotene hydroxylase enzyme of C. merolae? Because C. merolae lacks orthologs of genes that encode the two types of β-carotene hydroxylase enzymes identified for land plants and green algae (CrtZ type and cytochrome P450 type), a C. merolae chloroplast gene that specifies a polypeptide similar in sequence to cyano bacterial CrtR-type β-carotene hydroxylase enzymes appeared, at first glance, a most likely candidate for the gene encoding this carotenoid pathway enzyme. Our inability to demonstrate any β-carotene hydroxylase activity for the product of this C. merolae gene, even as a cyano bacterial CrtR was found abundantly active under the same assay conditions, led us to consider the five cytochrome P450 genes in the nuclear genome of this alga as plausible alternative candidates. The lack of β-carotene hydroxylase activity for the C. merolae CrtR gene product in the heterologous E. coli assay system does not, of course, rule out such a function for this polypeptide in the chloroplasts of C. merolae. The C. merolae CrtR might require a specific cofactor, electron carrier, oxido reductase, or lipid environment that is available in plastids of C. merolae but not in E. coli. Similarly, the ineffectuality of ancymidol in inhibiting carotenoid hydroxylase in vivo (see above) cannot be considered as ruling out the products of these genes as β-carotene hydroxylase enzymes, since P450 enzymes vary widely in their sensitivity to inhibitors. A cessation of culture growth without any effect on inhibitors. A cessation of culture growth without any effect on
out by one of the P450s, one with a different function that is critical to cell growth, is more sensitive to the inhibitor. Assay of the individual C. merolae P450s in E. coli is problematic because E. coli likely lacks an appropriate P450 reductase.

Whether the C. merolae crtR, one of the five P450 genes in C. merolae, or some other gene in this alga specifies a β-carotene hydroxylase might more readily be ascertained using a genetic approach. The genetic tools for C. merolae are in their infancy, but gene replacement or inactivation by homologous recombination appears to be feasible, for nuclear genes at least.

FIG. 8. Analysis of products formed in E. coli from lycopene through the action of an Arabidopsis thaliana lycopene ε-ring monocyclase. Results obtained using a lycopene β-ring monocyclase from the marine bacterium P99-3 are shown for comparison. (A) HPLC elution profile for an extract of E. coli cells that contained pAC-DELTA, a plasmid constructed by insertion of an A. thaliana LCYe cDNA into plasmid pAC-LYC, the latter of which otherwise leads to the synthesis and accumulation of lycopene. (B) HPLC elution profile for an extract of E. coli cells that contained pAC-DELTA together with pHpKetoSK, a plasmid that contains and expresses a cDNA from Haematococcus pluvialis that encodes an enzyme that adds a carbonyl to the number 4 carbon of β-rings (34). (C) HPLC elution profile for an extract of E. coli cells that contained pAC-GAMMA, a plasmid constructed by insertion of a gene encoding the lycopene β-ring monocyclase of the marine bacterium P99-3 into plasmid pAC-LYC. (D) HPLC elution profile for an extract of E. coli cells that contained pAC-GAMMA together with pHpKetoSK. Panels E and F display absorption spectra for the indicated peaks in panels A to D. Mid-log-phase cultures were harvested for pigment extraction in order to minimize the relative amounts of cis-geometrical isomers, which increase in proportion in older cultures. HPLC separation was with an isocratic mobile phase of 35% B (ethyl acetate) in A (acetonitrile:water:triethylamine, 90:10:0.1) over 35 min. The detector was set to a wavelength of 510 nm in order to enhance the prominence of those peaks containing γ-carotene and 4-keto-γ-carotene.
(41). Whether this approach is applicable to genes of the plastid genome, and to crrR in particular, is not known. At present, we consider the identity of the β-carotene hydroxylase of C. merolae to be a matter of conjecture.

What is the origin of the two plant-type β-carotene hydroxylases? The widespread occurrence of crrR genes in cyanobacteria and the presence of homologs of this gene in the plastid genomes of three primitive red algae (C. merolae, G. sulphuraria, and C. caldarium) together indicate that a CrrR-encoding gene probably accompanied the ancestral plastid precursor. A gene related to crrR is not present, however, in the chloroplast genomes of three other red algal species (Porphyra yezoensis, Porphyra purpurea, and Gracilaria teniistipitata), nor is one apparent in the nuclear or plastid genomes of any plant or green alga for which sequence data are available. A crrR-related gene is absent as well from the nuclear and plastid genomes of the diatom Thalassiosira pseudonana, the chloroplast of which is considered to have been derived secondarily from a red algal endosymbiont (42). In both green and red algal plastid lineages, therefore, genes encoding the CrrR-type β-carotene hydroxylase appear to have been discarded and the function of the gene product has been replaced by other polypeptides (T. pseudonana contains a gene [ver2.0.genewise.35.111.1; http://genome.jgi-psf.org/thaps1/thaps1.home.html] for a CrtZ-type enzyme).

An interesting and open question is the origin of genes that encode the two types of β-carotene hydroxylase, one a P450 enzyme (P450 type; 29) and the other a nonheme diiron oxygenase of a class of enzymes that includes the membrane-integral fatty acid desaturases (CrtZ type; 61), that function in land plants and green algae. Orthologs of genes that encode these enzymes are not evident in the genomes of any of the so-far-sequenced cyanobacteria, nor are any such genes found in the genomes of any of the so-far-sequenced cyanobacteria, nor are any such genes found in the nuclear, plastid, or mitochondrial genomes of C. merolae. Genes for both types of enzyme may have been acquired from carotenoid-producing bacteria. Plant CrtZ-type β-carotene hydroxylases are similar in amino acid sequence to certain tene hydroxylases are similar in amino acid sequence to certain tene hydroxylases.

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