

Isorenieratene Biosynthesis in Green Sulfur Bacteria Requires the Cooperative Actions of Two Carotenoid Cyclases^{∇†}

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The cyclization of lycopene to γ - or β -carotene is a major branch point in the biosynthesis of carotenoids in photosynthetic bacteria. Four families of carotenoid cyclases are known, and each family includes both mono- and dicyclases, which catalyze the formation of γ - and β -carotene, respectively. Green sulfur bacteria (GSB) synthesize aromatic carotenoids, of which the most commonly occurring types are the monocyclic chlorobactene and the dicyclic isorenieratene. Recently, the *cruA* gene, encoding a conserved hypothetical protein found in the genomes of all GSB and some cyanobacteria, was identified as a lycopene cyclase. Further genomic analyses have found that all available fully sequenced genomes of GSB encode an ortholog of *cruA*. Additionally, the genomes of all isorenieratene-producing species of GSB encode a *cruA* paralog, now named *cruB*. The *cruA* gene from the chlorobactene-producing GSB species *Chlorobaculum tepidum* and both *cruA* and *cruB* from the brown-colored, isorenieratene-producing GSB species *Chlorobium phaeobacteroides* strain DSM 266^T were heterologously expressed in lycopene- and neurosporene-producing strains of *Escherichia coli*, and the *cruB* gene of *Chlorobium clathratiforme* strain DSM 5477^T was also heterologously expressed in *C. tepidum* by inserting the gene at the *bchU* locus. The results show that CruA is probably a lycopene monocyase in all GSB and that CruB is a γ -carotene cyclase in isorenieratene-producing species. Consequently, the branch point for the synthesis of mono- and dicyclic carotenoids in GSB seems to be the modification of γ -carotene, rather than the cyclization of lycopene as occurs in cyanobacteria.

Carotenoids are isoprenoid pigments produced in all known photosynthetic organisms, as well as in a variety of nonphotosynthetic fungi and bacteria, and over 700 different carotenoid compounds have been described (39). Although all have similar isoprenoid backbones and the majority are C₄₀ compounds, these basic structures can be modified in many ways, including cyclization, oxygenation, saturation or desaturation, or the addition of sugars, acyl groups, and other moieties. Carotenoids with cyclic end groups are associated with the photosynthetic reaction centers in green sulfur bacteria (GSB) (26) and are also integral components of photosystem I (PS I), PS II, and the cytochrome *b₆f* complexes in cyanobacteria (5, 28, 55, 56). Carotenoids quench singlet oxygen and chlorophyll triplets and are able to transfer light energy to chlorophyll via singlet states (18), thus participating in light harvesting, as well as photoprotection. These functions are very important in photosynthetic bacteria, and complete inactivation of the carotenogenic pathway in the cyanobacterium *Synechocystis* sp. strain PCC 6803 has been possible only in strains adapted to photoheterotrophic growth on glucose (3, 4).

GSB are obligately anaerobic chlorophototrophs, and because the light flux in anoxic environments tends to be very low, these bacteria have developed a large and highly efficient

antenna, the chlorosome, which is a membrane-bound sac in which bacteriochlorophyll (BChl) *c*, *d*, or *e* is self-aggregated into large oligomers. Although carotenoids account for less than 10% of the total pigment content in GSB, carotenoid-free strains and strains with only linear carotenoids exhibit significant growth impairment, which suggests that these compounds play important roles in cells (21, 38). The carotenoids produced in GSB are primarily carotenoids with cyclic, aromatic end groups (27, 48). The GSB species with BChl *c* or *d* as the primary antenna BChl mostly produce a monocyclic carotenoid, chlorobactene. Brown-colored species that synthesize BChl *e* as their main antenna BChl and that generally grow at greater depths in stratified lakes primarily make the dicyclic carotenoids isorenieratene and/or β -isorenieratene (51), as well as small quantities of OH-chlorobactene glucoside laurate (27). The actinobacteria are the only other lineage in which isorenieratene biosynthesis has been confirmed (30, 31, 32, 45), and the function of isorenieratene in these species is still unknown, although it may be related to responses to blue or UV light (31, 49). In actinobacteria, as in the GSB, CrtU oxidizes the β -ring and transfers the C-17 methyl group from the C-1 to the C-2 of the ring (21, 32). Aside from CrtB, which catalyzes the first committed step in carotenoid biosynthesis in all known C₄₀ carotenogenic pathways, CrtU is the only shared enzyme between the actinobacterial and GSB pathways for isorenieratene biosynthesis. In actinomycetes, phytoene is desaturated by CrtI, as in most other bacteria (31, 32, 45), and the resulting all-*trans*-lycopene is cyclized to β -carotene by the heterodimeric cyclase CrtYcYd in *Brevibacterium linens* (31) or by CrtY in *Streptomyces* spp. (32, 45) (Fig. 1A).

This pathway is completely different from the pathway of chlorobactene biosynthesis in GSB, which is much more simi-

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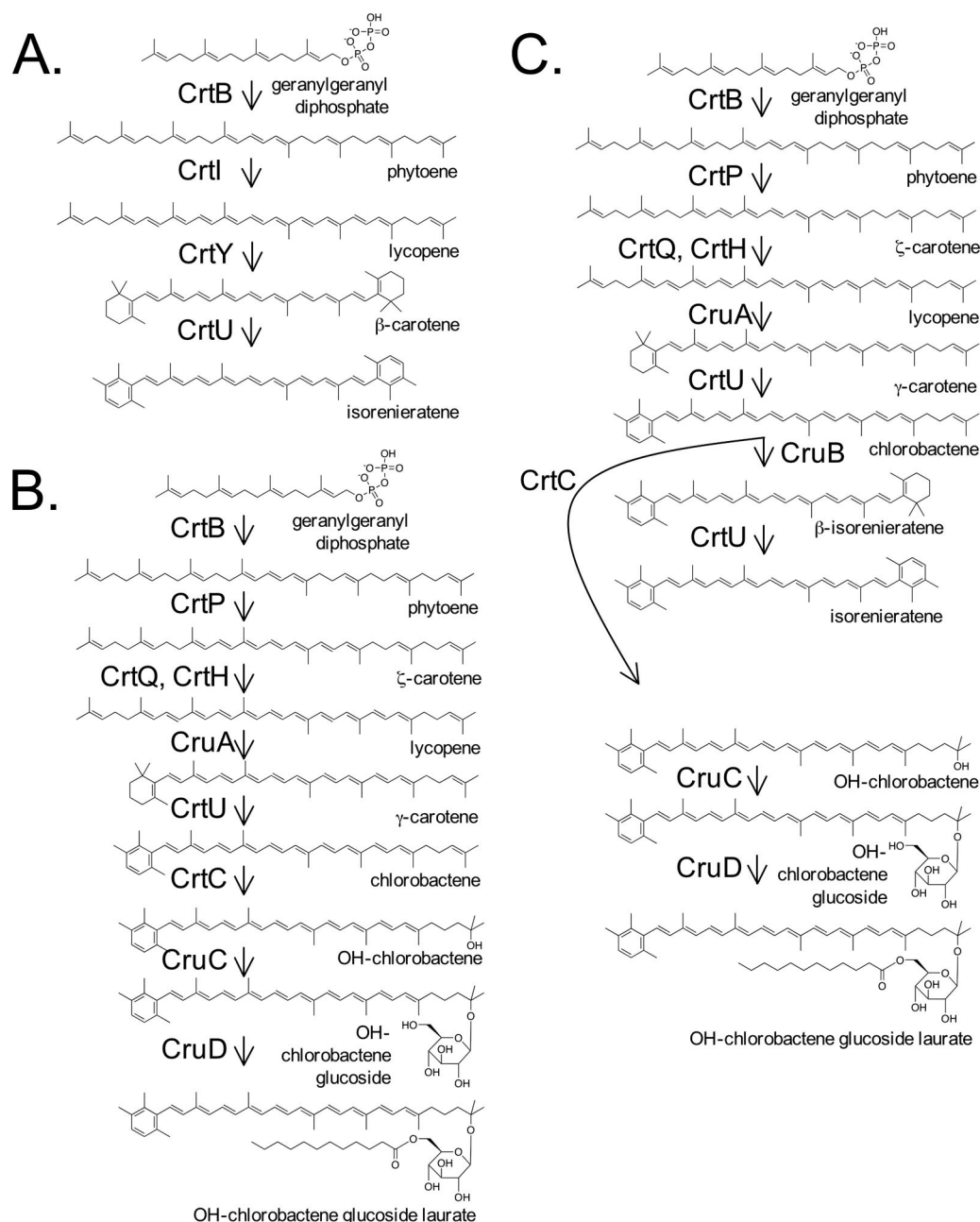


FIG. 1. Pathways of aromatic carotenoid biosynthesis. (A) Isorenieratene biosynthesis in *Actinobacteria*. (B) Chlorobactene biosynthesis in GSB. (C) Proposed pathway for isorenieratene biosynthesis in brown-colored GSB.

lar to the pathway for synechoxanthin biosynthesis in some cyanobacteria (39) (Fig. 1B) than it is to the pathway for isorenieratene biosynthesis in actinomycetes. In these photoautotrophs, phytoene is desaturated by CrtP and CrtQ (21), and the poly-*cis*-intermediates are isomerized by CrtH (21) and, possibly, by an isomerase that acts earlier in the pathway (33). Lycopene is then cyclized by a CruA-type cyclase (38). In cyanobacteria, CruA appears to be the major lycopene dicyclase and CruP the lycopene monocyclase, although CruP has a low level of lycopene dicyclase activity as well (38, 39).

When draft genomes became available for the isorenieratene-producing GSB species *Chlorobium phaeobacteroides*

strain DSM 266^T, *C. phaeobacteroides* strain BS-1, and *Chlorobium clathratiforme* DSM 5477^T (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>), these genomes were searched for genes encoding *cruA* homologs. Two homologs were found in each genome (see Table S1 in the supplemental material). In this paper, we describe the activities of the CruA orthologs, as well as those of the paralogous genes, designated CruB, from isorenieratene-producing species of GSB. The cyclization of γ -carotene by CruB to produce β -carotene is the first committed step in the biosynthesis of isorenieratene, an important biomarker compound (8, 9). In contrast to most other species with two independent carotenoid cyclases, which generally produce

molecules with differing numbers of rings or different types of rings (11–14, 47), GSB use CruA and CruB to produce a dicyclic carotenoid with identical rings.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type *Chlorobaculum tepidum* strain used in this study was the plating strain WT2321 (53), which was derived from strain TLS (ATCC 49652) (54). Total genomic DNA from *C. clathratiforme* strain DSM 5477^T and *C. phaeobacteroides* strains BS-1 and DSM 266^T (35) were obtained from Jörg Overmann (Ludwig Maximilians University, Munich, Germany). *Chlorobaculum limnaeum* strain 1549 is a brown-colored, BChl *c*-producing GSB species that is capable of using thiosulfate as an electron donor. This strain was obtained from John Ormerod, University of Oslo, Oslo, Sweden. *C. limnaeum* strain 1549 and *C. tepidum* were grown in CL medium (20) at 28 to 30°C and 40 to 46°C, respectively. The *C. tepidum* mutant strains were grown on solid CP medium (20) amended with 300 µg streptomycin ml⁻¹ and 150 µg spectinomycin ml⁻¹. *Escherichia coli* strain BL21(DE3) harboring plasmid pAC-LYC, which encodes the genes for lycopene synthesis (14), or pAC-NEUR, which encodes the genes for neurosporene synthesis (14), was used for complementation experiments. Cells were grown at 28 to 30°C on plates containing Luria-Bertani or M9 minimal medium supplemented with 25 µg chloramphenicol ml⁻¹, 100 µg ampicillin ml⁻¹, and/or 15 µg kanamycin ml⁻¹, as described in reference 38.

Genome analysis and phylogeny reconstructions. The *cruA* gene, encoding lycopene cyclase, was previously identified in a screen of a genomic library of *C. tepidum* (38). To identify the lycopene cyclases in other GSB, the predicted amino acid sequence of CruA was used as the query in tblastn searches against the genome sequences of 10 species of GSB. Because the amino acid sequences of these closely related, homologous proteins did not provide sufficient resolution in the tree, the nucleotide sequences of all *cruA* homologs were aligned using the ClustalX module within MacVector (Cary, NC). Based on this alignment, PAUP was used to draw a neighbor-joining phylogenetic tree, in which *cruA* from the cyanobacterium *Synechocystis* sp. strain PCC 6803 was chosen as the outgroup.

Homologs to other carotenoid biosynthetic genes in the genomes of GSB were also identified by using the predicted amino acid sequences of carotenoid biosynthesis genes from *C. tepidum* as queries in tblastn searches (21, 36). A gene was considered orthologous if the E value of the match between the *C. tepidum* query sequence and its best match in another genome was less than 10⁻⁸⁰. In the cases where no gene in the genome fell in this range, the E value of the best match was usually greater than 10⁻²⁰. The locus tags given in Fig. 2 and 3 and in Table S1 in the supplemental material are those given by the Integrated Microbial Genomes website (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>).

Constructs for the expression of *cruA* and *cruB* in *E. coli*. The *cruA* gene of *C. tepidum* (*cruA_{CT}*) was expressed from its own promoter in the pUC19-based plasmid pCTLY, as previously described (38). The *cruA* and *cruB* genes (*cruA_{CP}* and *cruB_{CP}*, respectively) were amplified from the genomic DNA of *C. phaeobacteroides* strain DSM 266^T using primer pairs CPL1-F3/CPL1-R1 and CPL2-FN/CP2404-KR (see Table S2 in the supplemental material). The PCR products were subcloned into vector pCR2.1-TOPO by using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) to produce plasmids pTA-CPL1 and pTA-KcruB. The fragments including *cruA* and *cruB* were then excised from those plasmids using the enzymes NdeI and BamHI for *cruA* and NcoI and KpnI for *cruB*. These DNA fragments were inserted into vectors pET16b (catalog no. 69662-3; Novagen, La Jolla, CA) and pCOLA-DUET1 (catalog no. 71406-3; Novagen, La Jolla, CA), respectively, generating plasmids pCPL1 and pCOLA-B (Fig. 2A and B).

Expression of *cruB* in *C. tepidum*. The *bchU* gene, which encodes the *C. tepidum* C-20 methyltransferase for BChl *c* biosynthesis (11, 37), was used as a platform for the heterologous expression of foreign genes in *C. tepidum*. An NcoI site was inserted at the start codon of *bchU* in the plasmid pCFT-X in which *bchU* is interrupted with the *aadA* cassette encoding streptomycin and spectinomycin resistance to produce plasmid pCFT-XN, and a 6.5-kb gene cluster from *C. clathratiforme* DSM 5477^T was inserted between the NcoI site and a downstream KpnI site, generating plasmid pXN-BEBj. The *aadA* cartridge from plasmid pSRA2 (20) was then reinserted at the KpnI site, generating plasmid pXN-BEBj-*aadA* (Fig. 2C). Plasmid pXN-BEBj-*aadA* was linearized with AhdII, and the linearized plasmid was used to transform wild-type *C. tepidum* as previously described (20).

Assays of CruA and CruB activity in *E. coli*. Combinations of plasmids pUC19, pET16b, pCOLA-DUET1, pCTLY, pCPL1, and pCOLA-B were transformed into electrocompetent *E. coli* BL21(DE3) cells harboring plasmid pAC-LYC or

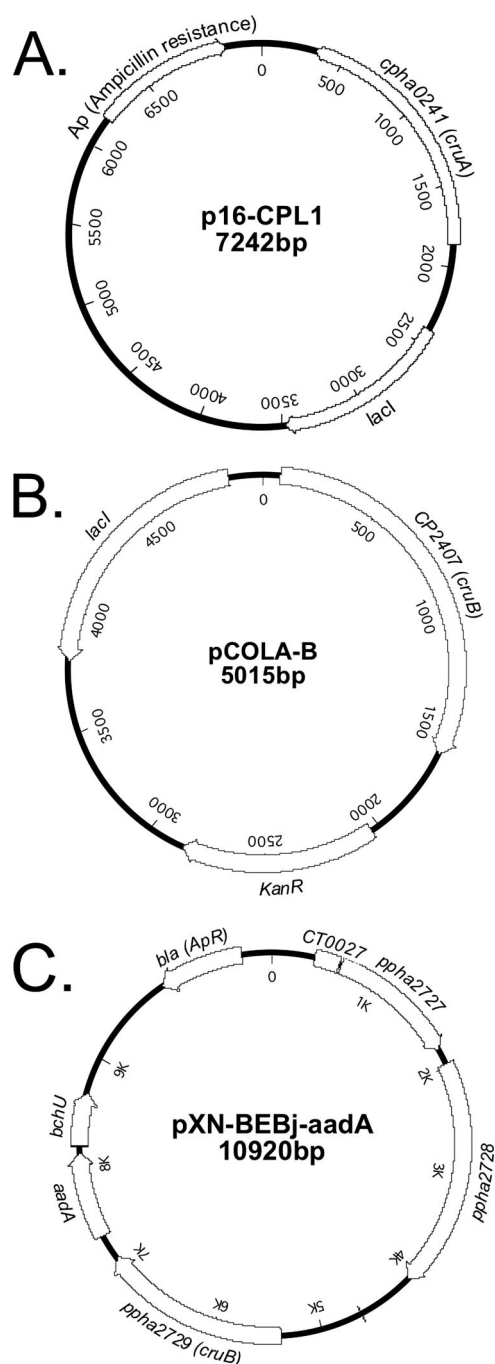


FIG. 2. Constructs for the expression of *cruA_{CP}* and *cruB_{CP}* in *E. coli* cells. (A) *cruA_{CP}* in vector pET16b. (B) *cruB_{CP}* in vector pCOLA. (C) Plasmid pXN-BEBj-*aadA* for the expression of *cruB_{CC}* in *C. tepidum* cells. The gene encoding the BChl *c* C-20 methyltransferase (CT0028; *bchU*) is replaced at the start codon of *bchU* with a gene cluster from *C. clathratiforme* and the *aadA* cassette conferring resistance to streptomycin and spectinomycin.

pAC-NEUR (see Table S3 in the supplemental material), and the cells were plated on Luria-Bertani medium amended with 25 µg chloramphenicol ml⁻¹, 15 µg kanamycin ml⁻¹, and 100 µg ampicillin ml⁻¹. Colonies became pigmented due to carotenoid production without induction after ~48 h at room temperature. For growth in liquid medium, colonies were picked and grown to stationary phase (approximately 24 h) in M9 medium supplemented with the same antibi-

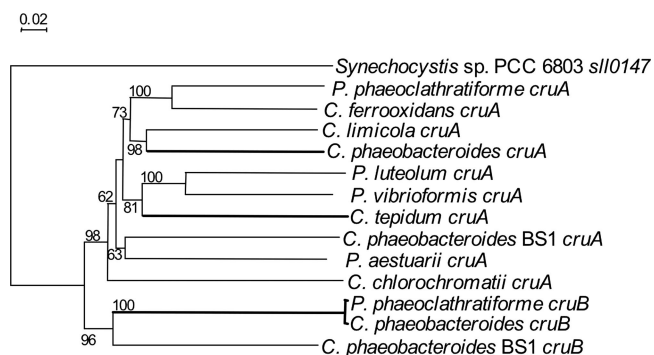


FIG. 3. Neighbor-joining phylogenetic tree of *cruA*-type lycopene cyclase-encoding genes. Nucleotide sequences were used because the similarity among the protein sequences is so high that it was not possible to determine the branching order. The *cruA* homolog (sll0147) from *Synechocystis* sp. strain PCC 6803 was used as the outgroup sequence. Bootstrap values for most nodes are indicated as percentages and were determined from 2,500 replicates. *C. ferrooxidans*, *Chlorobium ferrooxidans*; *C. limicola*, *Chlorobium limicola*; *P. luteolum*, *Pelodictyon luteolum*; *P. vibrioformis*, *Prosthecochloris vibrioformis*; *P. aestuarii*, *Prosthecochloris aestuarii*; “*C. chlorochromatii*,” “*Chlorobium chlorochromatii*.” Bar, 0.02 substitution per site.

otics. Genes expressed in the pCOLA vector were induced by the addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to the culture when the optical density at 600 nm had reached ~ 0.6 .

Pigment analysis. The analysis by high-performance liquid chromatography (HPLC) of pigments produced in *E. coli* and *C. tepidum* bacteria was as described in reference 38. For the analysis of carotenoids of brown-colored GSB strains, the HPLC protocol used the solvent system previously described (38). Solvent A is water/methanol/acetonitrile (62.5:21:16.5 by volume), and solvent B is methanol/ethyl acetate/acetonitrile (50:30:20 by volume). The protocol increases the solvent B concentration linearly from 70% to 94% over 10 min and then to 100% over the next 32 min. The solvent was held at 100% solvent B for 6 min and then returned to 70% solvent B over 3 min.

Carotenoid samples for mass analyses were purified by HPLC as described previously (38), using a Discovery 5- μ m C_{18} column (25 cm by 10 mm) (Supelco, Bellefonte, PA) and a gradient system starting with a concentration of 70% solvent B, increasing to 90% over 5 min and then increasing to 100% over 10 min. Solvent B was held at 100% for 15 min and then returned to 70%. The flow rate was maintained at 3.5 ml min⁻¹ for the entire procedure. Carotenoid masses were analyzed by using an atmospheric pressure chemical ionization source operating in the positive mode at the Proteomics and Mass Spectrometry Core Facility at The Pennsylvania State University.

RESULTS

***cruA* homologs in other GSB.** The predicted amino acid sequence of CruA_{Ct} was used as the query in a tblastn search (2) against each of the 10 available GSB genomes. Each of these genomes encodes one ortholog of CruA, which are, on average, 65% identical and 79% similar to CruA_{Ct}. Additionally, the genomes of the brown-colored, isorenieratene-producing species *C. clathratiforme* strain DSM 5477^T, *C. phaeobacteroides* strain DSM 266^T, and *C. phaeobacteroides* strain BS-1 each have *cruA* and a paralog of *cruA* which we have named *cruB* (see Table S1 in the supplemental material). On average, the CruB amino acid sequences are 56% identical and 74% similar to CruA_{Ct}, and the *cruB* genes group together as a distinct clade within the GSB *cruA* family of genes (Fig. 3). However, CruA_{Ct} and CruB are significantly more similar to each other than they are to either CruA or CruP from cyanobacteria, and the GSB-specific proteins CruA and CruB

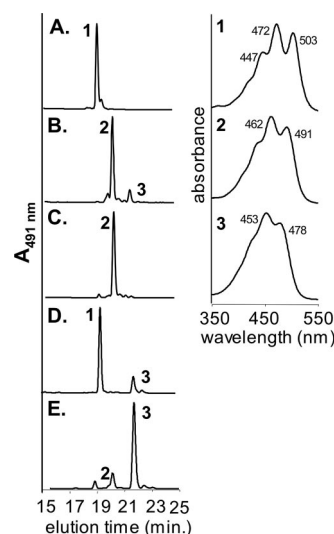


FIG. 4. HPLC elution profiles of pigments produced in *E. coli* strain BL21(DE3)::pAC-LYC, which synthesizes lycopene. Absorption spectra on the right correspond to the numbered peaks. (A) BL21(DE3)::pAC-LYC; peak 1 is lycopene. (B) BL21(DE3)::pAC-LYC transformed with pCTLY (encoding *cruA_{Ct}*). Peak 2 is γ -carotene and peak 3 is β -carotene. (C) BL21(DE3)::pAC-LYC transformed with pCPL1 (encoding *cruA_{Cp}*). (D) BL21(DE3)::pAC-LYC transformed with pCOLA-B (encoding *cruB_{Cp}*). (E) BL21(DE3)::pAC-LYC transformed with pCPL1 and pCOLA-B.

form a separate clade within the larger CruA-type carotenoid cyclase family. These observations suggested that the CruB enzymes were likely to possess a carotenoid cyclase activity.

Activity of CruA and CruB on lycopene in *E. coli*. CruA_{Ct} was previously shown to be a lycopene monocyase in *C. tepidum*, and this enzyme had only minimal dicyclase activity when expressed in a lycopene-producing strain of *E. coli* (38). However, since isorenieratene-producing GSB species produce some chlorobactene (27), two possible explanations existed for the occurrence of two cyclases in these organisms. If the pathways for chlorobactene and isorenieratene were to branch at the point of cyclization, CruA could be a lycopene monocyase, while CruB could be a dicyclase. Alternatively, the biosynthetic pathways could branch at the level of γ -carotene modification. CruA would again be a lycopene monocyase, but in this case, CruB would only have γ -carotene cyclase activity and thus would not be a lycopene dicyclase.

To distinguish between these two possibilities, *cruA_{Cp}* and *cruB_{Cp}*, from an isorenieratene-producing GSB species, were expressed singly and together in the lycopene-producing *E. coli* strain BL21(DE3) harboring the plasmid pAC-LYC. This *E. coli* strain was transformed with expression plasmid pCTLY, pCPL1, or pCOLA-B, encoding *cruA_{Ct}*, *cruA_{Cp}*, and *cruB_{Cp}*, respectively, or with empty vector pUC19, pET16b, or pCOLA (see Table S3 in the supplemental material). As expected, the control cells with plasmid pAC-LYC and the empty vectors pUC19 or pET16b and pCOLA-DUET1 produced only lycopene. Strains carrying plasmid pAC-LYC and expressing either *cruA_{Ct}* or *cruA_{Cp}* converted all available lycopene to γ -carotene (Fig. 4B and C, peak 2); unlike CruA_{Ct}, CruA_{Cp} had almost no detectable dicyclase activity (Fig. 4B, peak 3), and thus both are lycopene monocyases. When *cruB_{Cp}* was ex-

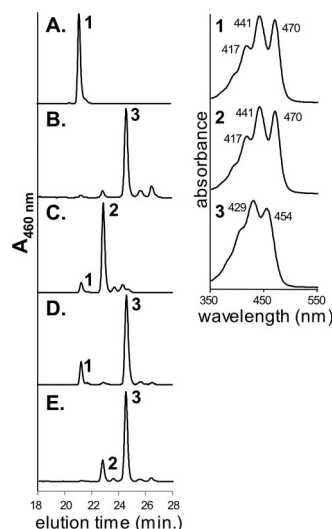


FIG. 5. HPLC elution profiles of pigments produced in *E. coli* strain BL21(DE3)::pAC-NEUR, which synthesizes neurosporene. Absorption spectra on the right correspond to the numbered peaks. (A) BL21(DE3)::pAC-NEUR; peak 1 is neurosporene. (B) BL21(DE3)::pAC-NEUR transformed with pCTLY (encoding *cruA_{Ct}*). Peak 3 is 7,8-dihydro- β -carotene. (C) BL21(DE3)::pAC-NEUR transformed with pCPL1 (encoding *cruA_{Cp}*). Peak 2 is 7,8-dihydro- γ -carotene. (D) BL21(DE3)::pAC-NEUR transformed with pCOLA-B (encoding *cruB_{Cp}*). (E) BL21(DE3)::pAC-NEUR transformed with pCPL1 and pCOLA-B.

pressed alone in lycopene-producing *E. coli* cells, the strain accumulated primarily lycopene and a small amount of β -carotene (<25% of the total pigment in the cells), but no γ -carotene was observed (Fig. 4D; see Table S3 in the supplemental material). This result indicated that lycopene is not the preferred substrate for *CruB_{Cp}*. However, when either the *cruA_{Ct}* or the *cruA_{Cp}* gene was expressed with *cruB_{Cp}*, almost all of the available lycopene was converted to β -carotene (Fig. 4E). Based on these results, the preferred substrate for *CruB_{Cp}* was clearly γ -carotene rather than lycopene, and thus, *CruB_{Cp}* was inferred to be a γ -carotene cyclase rather than a lycopene dicyclase. Because the masses of lycopene, γ -carotene, and β -carotene were previously determined in lycopene-producing *E. coli* strains expressing *cruA_{Ct}* (38) and because the compounds in the *E. coli* strains expressing *cruA_{Cp}* and *cruB_{Cp}* were chromatographically and spectroscopically identical to those products (Fig. 4), the masses of these products were not determined in this study.

Activity of *CruA* and *CruB* on neurosporene in *E. coli*. To analyze the substrate specificity of *CruA* and *CruB* further, *cruA_{Ct}*, *cruA_{Cp}*, and *cruB_{Cp}* were expressed singly and together in *E. coli* BL21(DE3)::pAC-NEUR (see Table S3 in the supplemental material), which produces neurosporene (Fig. 5, peak 1). *CruA_{Cp}* converted the available neurosporene to 7,8-dihydro- γ -carotene, which had an absorption spectrum identical to that of neurosporene but eluted $\sim$ 1.5 min later (Fig. 5C, peak 2; see Fig. S2 in the supplemental material). *CruA_{Cp}* appeared to modify only the ψ ends of carotenoids, and it exhibited only monocyclus activity. In contrast, *CruA_{Ct}* converted all of the neurosporene to dicyclic 7,8-dihydro- β -carotene (Fig. 5B, peak 3; see Fig. S2 in the supplemental mate-

rial). *CruA_{Ct}* acted only as a dicyclase with neurosporene as the substrate; the activity of *CruA_{Ct}* was not limited to the ψ ends, and in fact, *CruA_{Ct}* seemed to be equally active with ψ - and 7,8-dihydro- ψ -end groups (Fig. 5B; see Table S4 in the supplemental material). As it did when produced together with *CruA_{Ct}* or *CruA_{Cp}* (Fig. 5E), *CruB_{Cp}* alone produced primarily 7,8-dihydro- β -carotene (Fig. 5D); like *CruA_{Ct}*, *CruB_{Cp}* was a dicyclase when neurosporene was the substrate. The products of all of these reactions had molecular masses of 538 Da, which was consistent with the identification of these products as cyclized derivatives of neurosporene.

Expression of *cruB_{Cc}* in *C. tepidum*. No system for targeted inactivation of genes exists for any isorenieratene-producing strain of GSB. Instead, a method for heterologous gene expression was developed in the genetically amenable species *C. tepidum* by insertion of the foreign genes into the chromosome. To accomplish this heterologous expression, the *bchU* gene, which encodes the C-20 methyltransferase for BChl *c* synthesis in *C. tepidum* (23, 37), was replaced with a gene cluster that included *cruB* from *C. clathratiforme* (*cruB_{Cc}*). In addition to *cruB_{Cc}*, this gene cluster included genes encoding proteins with sequence similarity to a radical *S*-adenosylmethionine-type protein, a short-chain dehydrogenase, and *cruB*, as well as the *aadA* cassette from pSRA2 (Fig. 2C). After transformation, colonies resistant to streptomycin and spectinomycin appeared within a few days. After these colonies were streaked two times on solid medium, several transformant clones were selected for analysis, and the pigments of these strains were analyzed. Because *bchU* had been inactivated, the transformants produced BChl *d* rather than BChl *c*, and the absence of any detectable BChl *c* demonstrated that genomic alleles in the strain had segregated completely (Fig. 6, compare traces A and B with trace C). The primary carotenoid in the transformed strain was β -isorenieratene rather than chlorobactene, and only trace amounts of chlorobactene were detected (Fig. 7). All other carotenoids were dicyclic, and they included β -carotene and a small amount of isorenieratene. In this strain, *cruB_{Cc}* was expressed from its own promoter, and this level of expression was apparently sufficient to convert almost all of the carotenoids in the cell to dicyclic carotenoids. However, it was not sufficient for the synthesis of very much isorenieratene, which was a minor but nevertheless easily detected product.

DISCUSSION

The biosynthetic pathway for chlorobactene and its glycosylated and acylated derivatives in the GSB *C. tepidum* has previously been determined (Fig. 1B) (21, 36, 38, 39). The genomes of 10 other GSB encode orthologs of each of these genes, with only a few exceptions (see Table S1 in the supplemental material). Of note, however, is the fact that each of the three available genomes of brown-colored, isorenieratene-producing GSB encodes both an ortholog and a paralog of *cruA*. These paralogs, designated *cruB*, form a phylogenetically distinct group within the *cruA* clade (Fig. 3). Since *CruA* was previously shown to be strictly a lycopene monocyclus in GSB, and since only the genomes of isorenieratene-producing strains encode *cruB*, *CruB* was hypothesized to be a γ -carotene cyclase that is specifically required for the synthesis of isorenieratene.

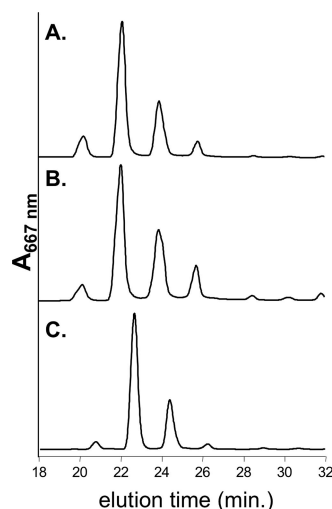


FIG. 6. HPLC analysis of pigments from three *C. tepidum* strains. (A) Analysis of the pigments in the *bchU* mutant of *C. tepidum* (37). The four peaks at 20, 22, 24, and 26 min are [8-ethyl,12-methyl]-BChl *d*, [8-ethyl,12-ethyl]-BChl *d*, [8-propyl,12-ethyl]-BChl *d*, and [8-isobutyl,12-ethyl]-BChl *d*, respectively. (B) Analysis of the pigments in a mutant in which a gene cluster, including *cruB*, from *C. clathratiforme* was inserted together with the *aadA* gene from pSRA2 into the *bchU* locus of wild-type *C. tepidum*. The identities of the peaks are the same as for trace A. The absence of BChl *c* demonstrates that complete segregation of the wild-type *bchU* allele and the modified *bchU* allele containing *cruB* from *C. clathratiforme* occurred. (C) Analysis of the pigments in wild-type *C. tepidum*, which produces BChl *c*. The four peaks at 21, 23, 25, and 26.5 min represent [8-ethyl,12-methyl] BChl *c*, [8-ethyl,12-ethyl] BChl *c*, [8-propyl,12-ethyl] BChl *c*, and [8-isobutyl,12-ethyl] BChl *c*, respectively.

When expressed in a lycopene-producing strain of *E. coli*, CruB_{CP} catalyzed the production of β -carotene at a very low level, and no γ -carotene accumulated. However, when expressed with CruA in a lycopene-producing strain of *E. coli*, all of the available lycopene was converted to β -carotene. Taken together, these results suggested that CruB_{CP} is a γ -carotene cyclase, but not a lycopene dicyclase, and that CruA produced the preferred substrate, γ -carotene, for CruB_{CP}.

When expressed in neurosporene-producing *E. coli*, CruB_{CP} efficiently cyclized both ends of neurosporene. It may be that the flexible 7',8'-dihydro- ψ end of neurosporene can assume a ring-shaped conformation and thus mimic the substrate γ -carotene. CruB_{CP} produced β -zeacarotene, which could in turn be recognized by CruB as a substrate and be cyclized again to yield 7,8-dihydro- β -carotene. The observation that CruB_{CP} had a very low activity on lycopene but high activity on the more-flexible neurosporene suggests that the enzyme recognizes a larger region of the molecule than just the ψ end that is cyclized. This differs from the situation for other characterized dicyclases, which only seem to recognize one end of the substrate molecule (11, 14, 41, 44).

CruA_{Ct} and CruA_{CP} are lycopene monocycluses which produce γ -carotene from lycopene. The function of CruA_{Ct} was confirmed both by inactivation of *cruA* in *C. tepidum* and by expression of the CruA_{Ct} gene in lycopene-producing *E. coli* cells, which produced γ -carotene instead of lycopene when *cruA_{Ct}* expression was induced (38). Like CruA_{Ct}, CruA_{CP} converted lycopene to γ -carotene. However, when neuros-

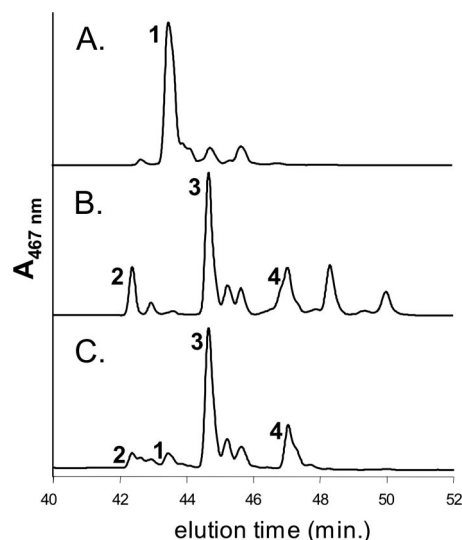


FIG. 7. HPLC analysis of carotenoids from GSB strains. (A) Elution profile of carotenoids from wild-type *C. tepidum*, which produces chlorobactene as the major carotenoid. (B) Elution profile of carotenoids from stationary-phase *C. phaeobacteroides* strain 1549, which produces β -isorenieratene, isorenieratene, and β -carotene as the major carotenoids. (C) Elution profile of carotenoids from *C. tepidum* strain CT0028::BEBj-*aadA*, which expresses *cruB* and which produces β -isorenieratene and β -carotene as the major carotenoids, together with a small amount of isorenieratene. Numbered peaks indicate chlorobactene (1), isorenieratene (2), β -isorenieratene (3), and β -carotene (4).

porene was provided as the substrate, CruA_{Ct} catalyzed the production of a dicyclic compound which was chromatographically and spectroscopically identical to that produced by CruB_{CP}, while CruA_{CP} produced only the monocyclic compound 7,8-dihydro- γ -carotene. This is in contrast to plant-type CrtL enzymes, which specifically modify the ψ end of neurosporene (11, 14, 41). These results show that, whether through a catalytic mechanism or substrate recognition, CruA_{CP} is more discriminating with respect to substrates than CruA_{Ct}.

To our best knowledge, the expression of the *cruB_{Cc}* gene in *C. tepidum* was the first time that a foreign gene other than a gene conferring antibiotic resistance was functionally expressed heterologously in GSB. Due to the use of the *bchU* locus as a platform for the expression of foreign genes, successful transformants carry an obvious change in pigmentation from BChl *c* to BChl *d* even if the inserted gene has no activity (37). Thus, this expression platform allows one to determine whether the strain is homozygous by examining the absorption spectrum of a pigment extract or by HPLC analysis. An alternative site that might also be useful for the expression of foreign genes is the *bchQ* locus, because the inactivation of *bchQ* causes a 15-nm blueshift in the Q_y absorption peak of aggregated BChl *c* in chlorosomes. This shift can also be observed easily by simply recording an absorption spectrum of the cells (24).

When expressed in *C. tepidum* from its own promoter, CruB_{Cc} catalyzed the cyclization of almost all available monocyclic carotenoids to dicyclic compounds. However, the primary carotenoids that accumulated in this strain were β -isorenieratene (ϕ , β -carotene) and β -carotene. In fact, very little

isorenieratene was observed in this strain. This observation suggested that CrtU might act before CruB_{Cc} on monocyclic carotenoids in this strain; although CruB_{Cc} requires a monocyclic substrate, the ring structure may not be important in substrate recognition. It also shows that although the addition of *cruB_{Cc}* alone is sufficient to make only dicyclic carotenoids, changes in the ring desaturase/methyltransferase CrtU might also be necessary in order for a strain to produce isorenieratene efficiently. Thus, although CruB is necessary for isorenieratene biosynthesis in GSB, multiple genetic changes would probably be necessary for the acquisition of an isorenieratene biosynthetic pathway. The characterization of CruB allows us to propose a biosynthetic pathway for isorenieratene in brown-colored GSB (Fig. 1C). The cyclization of γ -carotene to produce β -carotene is the first committed step in isorenieratene biosynthesis, which might also require a slightly different CrtU than that required for chlorobactene biosynthesis. Interestingly, although the CruB orthologs group together in a phylogenetic analysis, the CruA orthologs from isorenieratene-producing strains do not. Furthermore, the CrtU homologs from these same species do not cluster together; although they have similar substrates, they do not appear to be closely related to each other (see Figure S1 in the supplemental material).

In those plants and cyanobacteria that have been shown to have multiple, independent carotenoid cyclases, the different enzymes either make different numbers of rings or different types of rings, so cooperation between the different carotenoid cyclases generally results in carotenoids with chemically distinctive end groups (12, 13, 47). CruA and CruB, therefore, are unique in that the consecutive action of two different enzymes is required to make chemically identical end groups. The number of amino acid changes necessary to convert CruA into a lycopene dicyclase is probably very small, so the advantage in having two very similar proteins is not obvious. However, this level of enzymatic complexity is reminiscent of the complexity seen in converting phytoene to lycopene. Although a single enzyme, CrtI, can perform the same suite of reactions (22, 25, 34, 46, 50), two desaturases and at least one isomerase are required to convert phytoene into lycopene in GSB and most cyanobacteria (1, 3, 4, 6, 7, 19, 21, 34, 40, 43).

Little is known about how carotenoid composition varies chemically and quantitatively under different growth conditions in GSB. In other photosynthetic organisms, carotenoid composition can vary significantly for cultures grown under different environmental conditions, and the quantities and kinds of carotenoids present can affect the sensitivity of cells to oxidative stress (15, 29) and temperature stress (17, 52), as well as their fitness under different light intensities (21, 27). The multienzyme, multi-intermediate pathways found in GSB, cyanobacteria, and plants may allow these organisms to regulate the synthesis of carotenoids, as well as the flux through the different branches of the pathway, without resorting to transcriptional regulation. Because it can isomerize the *cis*-intermediates (40, 42), light itself may act as an important control signal. GSB, which have relatively small genomes that encode few transcriptional regulators (10, 16), might require such alternative regulatory mechanisms. However, the physiological significance of aromatization of the carotenoids is still unknown, as is the function of the glycosylated and acylated derivatives of these aromatic carotenoids. Although the *in vivo*

changes in carotenoid species and their quantities in response to varying environmental conditions have been noted (21, 27), they have not been systematically analyzed. When more-accurate information about the accumulation of the products under different conditions and their physiological functions is available, better experiments regarding the regulation points in this pathway will be possible, and greater understanding of the regulation of and flux through the carotenogenic pathway may explain the unusual complement of carotenogenic genes in GSB.

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